Cell Culture and Somatic Cell Genetics of Plants

INDRA K. VASIL
Editor-in-Chief

VOLUME 5

Phytochemicals in Plant Cell Cultures

FRIEDRICH CONSTABEL
INDRA K. VASIL
Editors

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Phytochemicals in Plant Cell Cultures

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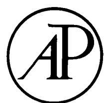

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General Preface

Recent advances in the techniques and applications of plant cell culture and plant molecular biology have created unprecedented opportunities for the genetic manipulation of plants. The potential impact of these novel and powerful biotechnologies on the genetic improvement of crop plants has generated considerable interest, enthusiasm, and optimism in the scientific community and is in part responsible for the rapidly expanding biotechnology industry.

The anticipated role of biotechnology in agriculture is based not on the actual production of any genetically superior plants, but on elegant demonstrations in model experimental systems that new hybrids, mutants, and genetically engineered plants can be obtained by these methods, and the presumption that the same procedures can be adapted successfully for important crop plants. However, serious problems exist in the transfer of this technology to crop species.

Most of the current strategies for the application of biotechnology to crop improvement envisage the regeneration of whole plants from single, genetically altered cells. In many instances this requires that specific agriculturally important genes be identified and characterized, that they be cloned, that their regulatory and functional controls be understood, and that plants be regenerated from single cells in which such gene material has been introduced and integrated in a stable manner.

Knowledge of the structure, function, and regulation of plant genes is scarce, and basic research in this area is still limited. On the other hand, a considerable body of knowledge has accumulated in the last fifty years on the isolation and culture of plant cells and tissues. For example, it is possible to regenerate plants from tissue cultures of many plant species, including several important agricultural crops. These procedures are now widely used in large-scale rapid clonal propagation of plants. Plant cell culture techniques also allow the isolation of mutant cell lines and plants, the generation of somatic hybrids by protoplast fusion, and the

regeneration of genetically engineered plants from single transformed cells.

Many national and international meetings have been the forums for discussion of the application of plant biotechnology to agriculture. Neither the basic techniques nor the biological principles of plant cell culture are generally included in these discussions or their published proceedings. Following the very enthusiastic reception accorded the two volumes entitled "Perspectives in Plant Cell and Tissue Culture" that were published as supplements to the *International Review of Cytology* in 1980, I was approached by Academic Press to consider the feasibility of publishing a treatise on plant cell culture. Because of the rapidly expanding interest in the subject both in academia and in industry, I was convinced that such a treatise was needed and would be useful. No comprehensive work of this nature is available or has been attempted previously.

The organization of the treatise is based on extensive discussions with colleagues, the advice of a distinguished editorial advisory board, and suggestions provided by anonymous reviewers to Academic Press. However, the responsibility for the final choice of subject matter included in the different volumes, and of inviting authors for various chapters, is mine. The basic premise on which this treatise is based is that knowledge of the principles of plant cell culture is critical to their potential use in biotechnology. Accordingly, descriptions and discussion of all aspects of modern plant cell culture techniques and research are included in the treatise. The first volume describes every major laboratory procedure used in plant cell culture and somatic cell genetics research, including many variations of a single procedure adapted for important crop plants. The second and third volumes are devoted to the nutrition and growth of plant cell cultures and to the important subject of generating and recovering variability from cell cultures. An entirely new approach is used in the treatment of this subject by including not only spontaneous variability arising during culture, but also variability created by protoplast fusion, genetic transformation, etc. Future volumes are envisioned to cover most other relevant and current areas of research in plant cell culture and its uses in biotechnology.

In addition to the very comprehensive treatment of the subject, the uniqueness of these volumes lies in the fact that all the chapters are prepared by distinguished scientists who have played a major role in the development and/or uses of specific laboratory procedures and in key fundamental as well as applied studies of plant cell and tissue culture. This allows a deep insight, as well as a broad perspective, based on

personal experience. The volumes are designed as key reference works to provide extensive as well as intensive information on all aspects of plant cell and tissue culture not only to those newly entering the field but also to experienced researchers.

Indra K. Vasil

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Preface

The previous volumes of this treatise have provided comprehensive coverage of the wide variety of laboratory procedures used in plant cell culture, the fundamental aspects of cell growth and nutrition, and plant regeneration and variability. The accumulation of phytochemicals (secondary metabolites) in plant cell cultures has been studied for more than thirty years. In recent years, however, there have been considerable interest and activity in the subject owing to the expectation of biotechnological application and industrial production. Inasmuch as this expectation became a problem, attention turned toward the analysis of the synthesis and accumulation of plant products. At present, two important events are taking shape: the realization of industrial plant cell culture for the production of phytochemicals, and a molecular biological approach to understanding the regulation of product synthesis. For the expeditious advancement of these two concepts and components, it appeared desirable to compile and review phytochemistry as studied by employing plant cell cultures. A comprehensive treatment of the subject in the tradition of the earlier volumes of this treatise required two volumes: *Cell Culture in Phytochemistry* (Volume 4) and *Phytochemicals in Cell Cultures* (Volume 5). Plant physiologists and biochemists will forgive our taking the liberty—for the sake of brevity—of using the term "phytochemistry" in a broad sense to cover their respective disciplines.

The timeliness of the proposed volumes must have been recognized worldwide, as the call for manuscripts was received with great enthusiasm. Reports at international conferences and workshops on phytochemistry and plant tissue culture had fallen far short of providing a comprehensive account of the remarkable progress made in the subject. Here, we gratefully acknowledge the cooperation of all of our colleagues who submitted up-to-date and thorough reviews of their fields of study. At one point we felt overwhelmed by the amount of material received, while at the same time we realized that a few groups of chemicals could not be included.

A science in flux is a fabric of differing thoughts, approaches, and interpretations, all in a state of evolution. A comprehensive treatise such as this should reflect this state, and thus we were anxious not to streamline the presentations. Some overlap in various chapters and some divergence of opinions should therefore be seen as helpful in a broad understanding of the subject. Students as well as colleagues in academia and industry will appreciate the overall effort and the diverse viewpoints presented.

We acknowledge the support of the Editorial Advisory Board in identifying this important area of plant cell culture research for these volumes. The assistance of our colleagues at the Plant Biotechnology Institute (PBI) in Saskatoon, particularly Drs. Balsevich, DeLuca, Eilert, Kurz, and Tyler, and the PBI secretarial staff, is gratefully acknowledged. Spouses of the editors deserve special thanks for enduring countless hours alone: Thank you, Christa and Vimla!

Friedrich (Fred) Constabel
Indra K. Vasil

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PART I

Phenylpropanoids, Naphthoquinones, and Anthraquinones

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CHAPTER **1**

Coumarins and Furanocoumarins

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I. INTRODUCTION

Cultured plant cells do not, as a rule, accumulate secondary metabolites as readily as their parent plants (Barz and Ellis, 1981; Dougall, 1981; Berlin, 1983; Ellis, 1984; Heinstein, 1985a,b). For example, whereas coumarins are produced in large quantities in various plants belonging to the families Rutaceae, Umbelliferae, and Solanaceae, cell cultures derived from these plants either lack coumarins, or their coumarin content is comparatively low (Murray *et al.*, 1982). Moreover, the amount of

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coumarin produced in cell cultures may vary from one transfer of cells to another. These differences cannot be attributed to genetic incompetence of the cultured cells, but result rather from abnormal gene expression (Muhitch and Fletcher, 1985). Growth media are adjusted primarily to sustain rapid growth of cells, and it has been suggested that selective genome expression corresponds closely with cell maturation (Yeoman *et al.*, 1980). For simplicity, coumarins isolated from cell cultures grown on standard growth media are referred to here as *constitutive coumarins*. This term need not imply, however, that their synthesis is independent of the growth-medium composition.

The level of secondary metabolites in cultured cells in general may be increased by several means. Individual high-producing cells can be selected for propagation from a heterogeneous cell population. Although this approach has been successful in some instances (Radwan and Kokate, 1980; Yamamoto *et al.*, 1982; Ohta and Yatazawa, 1982), Ellis (1984) showed that, under otherwise constant growth conditions, cell clones selected for high secondary metabolite contents eventually segregate into a heterogeneous population of low average yield. Basing their approach on the observation that polyamines extend the life span of mature nondividing cells, Muhitch and Fletcher (1985) investigated the effect of culture age and polyamine addition on the production of phenolics in Paul's scarlet rose suspension cultures. Addition of polyamines indeed induced changes in the type of phenolics in the culture as well as in their quantity. Neither one of these approaches, however, has been followed for production of coumarins. "Production media" have also been developed, based mainly on increased sucrose and reduced inorganic phosphate levels, as well as on a modified hormone regime (Phillips and Henshaw, 1977; Knobloch and Berlin, 1980; Sugano *et al.*, 1975). The effect of growth-medium composition on the production of coumarins has been extensively studied in tobacco suspension cultures (Okazaki *et al.*, 1982a,b).

Fungal-cell-wall-derived glucan fractions (elicitors) have been used to induce the accumulation of secondary metabolites with potential antimycotic activity (phytoalexins) in various cultured cells (Ebel *et al.*, 1976; Tietjen and Matern, 1984; Ellis, 1984; Heinstein, 1985a; see also Chapter 9, Volume 4, this treatise). Coumarins also accumulate in various diseased plants (for review, see Murray *et al.*, 1982), and this protocol has been successfully employed to induce coumarin synthesis in parsley (Tietjen *et al.*, 1983).

Most of this chapter is dedicated to a discussion of the induced accumulation of coumarins, the regulation of their biosynthesis, and their potential physiological function. Phenylcoumarins and isocoumarins,

Table I

Chemical Structures of Coumarins

No.	Compound	Substituents
1	Coumarin	$R_1 = R_2 = R_3 = H$
2	Umbelliferone	$R_1 = R_2 = H$; $R_3 = OH$
3	Skimmin	$R_1 = R_2 = H$; $R_3 =$ O-ß-D-glucosyl
4	Herniarin	$R_1 = R_2 = H$; $R_3 = OCH_3$
5	Demethylsuberosin	$R_1 = H$; $R_2 =$ [prenyl]; $R_3 = OH$
6	Umbelliprenin	$R_1 = R_2 = H$; $R_3 =$ O-[farnesyl]
7	Gravelliferone methyl ether	$R_1 =$ [1,1-dimethylallyl]; $R_2 =$ [prenyl]; $R_3 = OCH_3$
8	Esculetin	$R_1 = H$; $R_2 = R_3 = OH$
9	Esculin	$R_1 = H$; $R_2 =$ O-ß-D-glucosyl; $R_3 = OH$
10	Scopoletin	$R_1 = H$; $R_2 = OCH_3$; $R_3 = OH$
11	Scopolin	$R_1 = H$; $R_2 = OCH_3$; $R_3 =$ O-ß-D-glucosyl
12	Rutacultin	$R_1 =$ [1,1-dimethylallyl]; $R_2 = R_3 = OCH_3$

No.	Compound	Substituents
13	Daphnetin	$R = OH$
14	Hydrangetin	$R = OCH_3$

15 Xanthyletin

No.	Compound	Substituents
16	Marmesin	$R_1 = R_2 = R_3 = H$
17	Marmesinin	$R_1 = R_2 = H$; $R_3 =$ ß-D-glucosyl
18	Rutamarin	$R_1 =$ [1,1-dimethylallyl]; $R_2 = H$; $R_3 =$ [isobutyryl]
19	Rutaretin	$R_1 = R_3 = H$; $R_2 = OH$;
20	Rutarin	$R_1 = R_3 = H$; $R_2 =$ O-ß-D-glucosyl
21	Isorutarin	$R_1 = H$; $R_2 = OH$; $R_3 =$ ß-D-glucosyl

22 Nodakenetin

23 Ammirin

24 Graveolone

No.	Compound	Substituents
25	Psoralen	$R_1 = R_2 = H$
26	Bergaptol	$R_1 = OH$; $R_2 = H$
27	Bergapten	$R_1 = OCH_3$; $R_2 = H$
28	Isoimperatorin	$R_1 =$ O-[prenyl]; $R_2 = H$
29	Alloimperatorin methyl ether	$R_1 =$ [prenyl]; $R_2 = OCH_3$
30	Xanthotoxol	$R_1 = H$; $R_2 = OH$
31	Xanthotoxin	$R_1 = H$; $R_2 = OCH_3$
32	5-Hydroxyxanthotoxin	$R_1 = OH$; $R_2 = OCH_3$
33	Isopimpinellin	$R_1 = R_2 = OCH_3$

which originate from isoflavonoid (Brown, 1981) and polyketide intermediates (Stoessl and Stothers, 1978), respectively, are not considered, nor are angular furanocoumarins, which have never been isolated from cultured cells.

II. COUMARINS IN CROWN GALL TUMORS

Brown and Tenniswood (1974) reported that normal tobacco callus tissue contains bergapten (for chemical structures see Table I, **27**) and bound umbelliferone (**2**), esculetin (**8**), and scopoletin (**10**), whereas the corresponding crown gall tumor tissue cultures induced with *Agrobacterium tumefaciens* lack umbelliferone and bergapten. Instead, higher levels of esculetin and scopoletin were found, suggesting that, in tumors, the biosynthetic flow is diverted from the umbelliferone–furanocoumarin pathway in favor of esculetin and scopoletin (Fig. 1). Similarly, Reichling *et al.* (1979) reported that crown gall tumor tissues induced in *Matricaria chamomilla* produced neither the flavonoids nor the coumarins herniarin (**4**) and umbelliferone (**2**), typical for this plant.

Fig. 1. Patterns in coumarin biosynthesis. Coumarin numbers refer to Table I.

III. CONSTITUTIVE COUMARINS

Most of the information on coumarins produced in cultured cells has been summarized by Murray *et al.* (1982). Cultures of the rutaceous species *Ruta graveolens, R. graveolens* ssp. *hortensis,* and *Thamnosma montana* are particularly rich sources of coumarins (Table II). Rutacultin (**12**), which was originally thought to be produced only by cultured cells, was later also isolated from *Ruta* roots (Novak *et al.*, 1973). Umbelliprenin (**6**), on the other hand, which is one of the major coumarins in young *Thamnosma* seedlings, was not accumulated by the corresponding cell cultures (Kutney *et al.*, 1973). One may speculate that *Citrus* cultures at least should also produce coumarins under appropriate growth conditions, because the scopoletin (**10**) content of *Citrus* leaves, which had been originally proposed as a diagnostic marker for "young tree decline," was shown to be closely related to leaf age (Wheaton and Feldman, 1979).

Suspension cultures of *Hydrangea macrophylla* (Saxifragales) (Table II) accumulated appreciable amounts of umbelliferone (~1 mg per 5 g fresh weight of cells) and of the corresponding β-glucoside skimmin (~1 mg per 15 g fresh weight of cells) as well as two isocoumarinic derivatives and daphnetin 8-monomethyl ether (**14**). Callus cultures of *Swertia japonica* (Gentianaceae) (Table II) were shown to accumulate scopoletin and scopolin (**11**) at approximately 14 and 140 μg per gram dry weight of cells, respectively.

IV. INDUCED COUMARINS

The induced accumulation of scopoletin in cultured cells and its excretion from the cells has been investigated most thoroughly in several members of the Solanaceae. Early experiments employing *Atropa belladonna* root tissue culture (Mothes and Kala, 1955) revealed that, after feeding with L-phenylalanine, scopoletin was mostly excreted by the cells, whereas newly formed umbelliferone accumulated within the tissue. Later, *A. belladonna* callus culture was shown to contain scopoletin (Vakkari, 1980), and treatment of the culture with 0.05 m*M* L-methionine increased its amount from 0.7 to 1.1 mg per gram fresh weight of tissue.

Table II

Constitutive Coumarins from Cultured Plant Cells

Coumarin	Source	Reference
Herniarin (**4**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968
Gravelliferone methyl ether (**7**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968
Psoralen (**25**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968
Bergapten (**27**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968
Xanthotoxin (**31**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968
Umbelliferone (**2**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968; Varga *et al.*, 1975
Scopoletin (**10**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968; Varga *et al.*, 1975
Rutaretin (**19**)	*Ruta graveolens* L. callus	Reinhard *et al.*, 1968; Varga *et al.*, 1975
Rutarin (**20**)	*Ruta graveolens* L. callus	Varga *et al.*, 1975
Isorutarin (**21**)	*Ruta graveolens* L. callus	Varga *et al.*, 1975
Marmesin (**16**)	*Ruta graveolens* L. callus	Varga *et al.*, 1975
Xanthyletin (**15**)	*Ruta graveolens* L. callus	von Brocke *et al.*, 1971
Rutacultin (**12**)	*Ruta graveolens* L. suspension	Steck *et al.*, 1971
Rutamarin (**18**)	*Ruta graveolens* L. suspension	Steck *et al.*, 1971
Isopimpinellin (**33**)	*Ruta graveolens* L. suspension	Steck *et al.*, 1971
Umbelliferone (**2**)	*Ruta graveolens* L. ssp. *hortensis* callus	Varga *et al.*, 1978
Marmesinin (**17**)	*Ruta graveolens* L. ssp. *hortensis* callus	Varga *et al.*, 1978
Rutarin (**20**)	*Ruta graveolens* L. ssp. *hortensis* callus	Varga *et al.*, 1978
Isorutarin (**21**)	*Ruta graveolens* L. ssp. *hortensis* callus	Varga *et al.*, 1978
Alloimperatorin methyl ether (**29**)	*Thamnosma montana* Torr. & Frem. callus	Kutney *et al.*, 1973
Isopimpinellin (**33**)	*Thamnosma montana* Torr. & Frem. callus	Kutney *et al.*, 1973
Isoimperatorin (**28**)	*Thamnosma montana* Torr. & Frem. callus	Kutney *et al.*, 1973
Umbelliferone (**2**)	*Hydrangea macrophylla* Seringe var. Thunbergii Makino suspension	Suzuki *et al.*, 1977a
Skimmin (**3**)	*Hydrangea macrophylla* Seringe var. Thunbergii Makino suspension	Suzuki *et al.*, 1977b
Daphnetin 8-monomethyl ether (**14**)	*Hydrangea macrophylla* Seringe var. Thunbergii Makino suspension	Suzuki *et al.*, 1978
Scopoletin (**10**)	*Swertia japonica* Makino callus	Miura *et al.*, 1978
Scopolin (**11**)	*Swertia japonica* Makino callus	Miura *et al.*, 1978

Tobacco plants accumulate scopoletin after infection with various pathogens (for review, see Murray *et al.*, 1982). Fritig and Hirth (1971) reported that healthy tobacco tissue cultures already contain large quantities of scopoletin and that virus infection does not significantly change this amount. Okazaki *et al.* (1982a) later showed that accumulation of scopolin and scopoletin in tobacco tissue cultures strongly depends on the sucrose and phosphate levels as well as on the amino acid composition of the growth medium. Furthermore, scopoletin was mostly recovered from the culture filtrate while scopolin accumulated within the cells. Addition of several plant hormones increased the amount of both scopoletin and scopolin (Okazaki *et al.*, 1982b). Surprisingly, addition of 2,4-dichlorophenoxyacetic acid (2,4-D) enhanced the uptake of exogenously supplied scopoletin by the cells and its glucosylation to scopolin (**11**), whereas kinetin, indolyl-3-acetic acid (IAA), and naphthaleneacetic acid (NAA) adversely affected the quantity of scopoletin in the culture filtrate. In other cultured cells, an inhibitory effect of 2,4-D on the biosynthesis of phenolic acids had been observed (Sugano *et al.*, 1975).

Scopoletin accumulation on infection has been demonstrated in *Helianthus annuus* (Cohen and Ibrahim, 1975; Tal and Robeson, 1986) as well as in elicitor-treated *Gossypium arboreum* (Zeringue, 1984). This coumarin was, however, not reported from cotton suspension cultures induced with fungal conidia (Heinstein, 1985b).

Dark-grown parsley suspension cultures, lacking coumarins, accumulate fairly large quantities of isopimpinellin (**33**), bergapten (**27**), xanthotoxin (**31**), psoralen (**25**), and graveolone (**24**) in response to elicitor treatment (Tietjen *et al.*, 1983). Whereas an elicitor isolated from *Phytophthora megasperma* f.sp. *glycinea* predominantly induced the accumulation of psoralen and graveolone, bergapten and xanthotoxin were most abundant in cultures treated with an elicitor from *Alternaria carthami*. In both cases, most of the induced coumarins were recovered from the culture filtrate (Tietjen *et al.*, 1983). Similarly, dark-grown cell cultures of *Ammi majus* L. rapidly accumulate the coumarins ammirin (**23**), isopimpinellin (**33**), bergapten (**27**), marmesin (**16**), umbelliferone (**2**), and a compound tentatively identified as an isomer of marmesin in response to elicitor treatment (Hamerski *et al.*, 1987). The stereochemistry of ammirin, however, has not been confirmed. As in parsley, the bulk of these coumarins were recovered from the culture filtrate. For biosynthetic reasons (see Section V), induced *Ammi majus* cultures may represent an ideal system to study the flow of label from L-[^{14}C]phenylalanine into the coumarins, and possibly also to provide a clue to the conversion of 4-coumaric acid to umbelliferone (Fig. 1). Elicitor-induced coumarin ac-

cumulation has also been observed in dark-grown cultures of *Conium maculatum, Anethum graveolens* (D. Hamerski and U. Matern, unpublished) and *Arracacia esculenta* (K. Harter and U. Matern, unpublished). Unusually, induced *Arracacia* cultures accumulated demethylsuberosin (**5**) in their culture fluid besides umbelliferone, an umbelliferone ether, marmesin, bergapten and isopimpinellin. Within the Umbelliferae, carrot cultures appear to be exceptional. Despite contradictory reports on the presence of coumarins in healthy garden carrot (Ivie *et al.*, 1982; Ceska *et al.*, 1986), esculetin (**8**) (Khandobina *et al.*, 1982) and scopoletin (**10**) as well as various isocoumarins were isolated from diseased garden or mauve-coloured carrot (Coxon *et al.*, 1973; N. Saleh, National Research Center, Cairo, Egypt, personal communication). Nevertheless, accumulation of scopoletin has not been reported from induced carrot cultures (Kurosaki and Nishi, 1983). It appears possible that the 2,4-D concentration that sustained growth of cells in these experiments may have hindered the formation of scopoletin.

The rapid induction of coumarin accumulation in cultured cells leads to the question of the factors involved in the induction process. Because a direct interaction of elicitor with either enzyme proteins or nucleic acids appears unlikely, one must propose an intracellular signaling system. Although no conclusive information is available, a quick drop in cytoplasmic inorganic phosphate level concomitant with an inhibition of phosphate uptake occurs in parsley cells on addition of elicitor (Strasser *et al.*, 1983). Furthermore, expression of the full effect requires the presence of the elicitor for at least 20 min (Strasser and Matern, 1986). An involvement of polyphosphoinositides in signal transduction has been assumed. However, a careful analysis revealed no significant elicitor-induced changes in the relative labeling of phosphoinositides by either [2-^{3}H]inositol, [2-^{3}H]glycerol or [^{32}P]orthophosphate within 20 min following addition of the elicitor (Strasser *et al.*, 1986).

V. BIOSYNTHESIS

Coumarin (**1**) and umbelliferone originate from L-phenylalanine, most likely via formation of 2- and 2,4-dihydroxycinnamic acid, respectively (Fig. 1) (Murray *et al.*, 1982). Umbelliferone may be further converted by additional oxidation to, for example, esculetin (**8**) (Brown, 1985). Alternatively, umbelliferone may be prenylated, with subsequent formation either of a fused pyrone ring to form, for example, graveolone (**24**), or of

a fused furan ring and loss of a C_3 fragment to yield the various furanocoumarins (Fig. 1) (Murray *et al.*, 1982).

The formation of scopoletin is an exception to the scheme just outlined, because ferulic acid has been described as its immediate precursor in tobacco (Fig. 1) (Murray *et al.*, 1982). Nevertheless, enzymes isolated from tobacco tissue cultures methylate esculetin to scopoletin and isoscopoletin (Tsang and Ibrahim, 1979; Blume, 1982), although not with exclusive substrate specificity. A probably nonphysiological hydroxylation of coumarin to umbelliferone was accomplished with *Conium maculatum* and *Catharanthus roseus* but not with *Apocynum cannabinum* cell cultures (Carew and Bainbridge, 1976). On the other hand, labeled coumarin administered to Russet Burbank potato leaves was transported basipetally and recovered unchanged from roots and tubers (Gawronska *et al.*, 1982), even though potato naturally contains 7-oxygenated coumarins. Glycosylation, which has been observed in various cell cultures, is probably not required in biosynthesis (Fritig *et al.*, 1970), serving rather to facilitate vacuolar storage (Rataboul *et al.*, 1985; Werner and Matile, 1985). Ibrahim and Boulay (1980) partially purified a glucosyltransferase from tobacco cultures, which specifically glucosylates the 7-hydroxyl group in esculetin (**8**) and daphnetin (**13**), and to a lesser extent, that of umbelliferone (**2**), scopoletin (**10**), and hydrangetin (**14**). Tabata *et al.* (1984) fed esculetin to suspension cultures of *Lithospermum erythrorhizon*, *Gardenia jasminoides*, and *Nicotiana tabacum*. All three cultures formed the 6-*O*-β-glucoside esculin (**9**), but only *Gardenia* additionally synthesized some 7-*O*-β-glucoside. Unexpectedly, no scopolin (**11**) was reported from the experiments employing tobacco cultures, although such cultures reportedly contain esculetin 6-*O*-methyltransferase and scopoletin 7-*O*-glucosyltransferase activities (Blume, 1982). Esculin accumulated exclusively within the cells, and 2,4-D stimulated its formation from exogeneously supplied esculetin.

Cinnamic acid 4-hydroxylase, a microsomal enzyme, has been studied from parsley cultures (Scheel and Sandermann, 1975). This enzyme activity is induced on elicitor treatment and serves routinely for control of induction efficiency in our current research (see below). A crucial step in the biosynthesis of coumarins is the ortho-hydroxylation postulated to precede lactonization of either cinnamic acid (Gestetner and Conn, 1974; Ranjeva *et al.*, 1977) or 4-coumaric acid (Kindl, 1971), thus linking general phenylpropanoid metabolism with the coumarin-specific pathway. In all three reports, the hydroxylating activity was ascribed to chloroplast fractions. Despite continued efforts, however, ortho-hydroxylation of either cinnamic acid, 4-coumaric acid, 4-coumaroyl-CoA, or 5-coumaroyl shikimic acid ester could not be confirmed in extracts from various in-

duced cell cultures (H. Wendorff and U. Matern, unpublished). No attempts were made in these experiments to isolate plastids, because the parsley cells that accumulate coumarins efficiently on induction have been subcultured continuously in the dark for approximately 20 years and most likely lack normal plastids.

The prenylation of umbelliferone to yield demethylsuberosin (**5**) (Fig. 2) was accomplished *in vitro* by an enzyme isolated from *Ruta graveolens* cell cultures (Dhillon and Brown, 1976). The reaction is dependent on manganese and requires dimethylallyl diphosphate as cosubstrate. The enzyme was solubilized from isolated chloroplasts and partially purified. This observation has so far favored plastids as the sole site of coumarin synthesis. However, it is known that HMGCoA-reductase—an enzyme responsible for the biosynthesis of dimethylallyl diphosphate—is active in both plastids and microsomal preparations assigned to the endoplasmic reticulum (Suzuki and Uritani 1976). Only the microsomal enzyme activity appears to be induced upon elicitor treatment of potato (Oba *et al.*, 1985) and cultured parsley (Tietjen and Matern, 1983) or *Ammi majus* cells (D. Hamerski and U. Matern, unpublished). Moreover, furanocoumarin specific *O*-methyltransferases (see below) are not associated with chloroplasts (Brown, 1985).

The enzymatic cyclization of demethylsuberosin (**5**) to (+)marmesin (**16**) (Fig. 2) was demonstrated using microsomes from elicitor-induced *Ammi majus* cells in the presence of NADPH and oxygen (Hamerski and Matern, 1988). Inhibition studies showed this reaction to be catalyzed by a cytochrome P_{450}-monooxygenase, thus implying an oxidative cycliza-

+ DMAPP $\xrightarrow{Mn^{2+}}$ DMAPP. Umbelliferone Dimethylallyl Transferase

NADPH / O_2 Marmesin Synthase

NADPH / O_2 Psoralen Synthase

NADPH / O_2 Bergaptol Synthase

Fig. 2. Coumarin-specific enzyme reactions associated with the membranes of the endoplasmic reticulum in elicitor-induced *Ammi majus* cells. DMAPP = dimethylallyl diphosphate.

tion via the epoxide. However, no intermediate could be detected under any incubation condition. Although these results appear not to support the mechanism postulated for the formation of marmesin by Brown *et al.* (1970), a short-lived intermediate epoxide can not be ruled out completely due to the fact that enzymes attacking oxiranes are generally known to possess very high catalytic activities (Wistuba and Schurig, 1986). Marmesin synthase activity has also been found in microsomes from induced parsley (Wendorff, 1987) and *Arracacia* cells (K. Harter and U. Matern, unpublished) and is, in all cases, associated with the endoplasmic reticulum.

Recently, we could also demonstrate the NADPH-dependent conversion of synthetic (±)[3-^{14}C]marmesin into psoralen (**25**) (Fig. 2) by microsomes prepared from elicitor-induced parsley cells (Wendorff and Matern, 1986) (Fig. 2). Again, inhibition studies suggested a cytochrome P_{450}-dependent mechanism for the psoralen synthase reaction. Our results are in accord with the reaction sequence postulated previously for the biosynthesis of psoralen (Murray *et al.*, 1982). Microsomes derived from cells induced with *Phytophthora* elicitor, but not those from *Alternaria* elicitor-induced cells, catalyzed the NADPH-dependent formation of yet another product from the racemic marmesin mixture. Preliminary experiments suggest that this compound may be converted further to graveolone (**24**) by microsomes. Extensive dilution experiments employing either authentic (+)marmesin or authentic (−)marmesin (=nodakenetin) (**22**) revealed that only the former is converted to both psoralen and the product tentatively identified as a graveolone-intermediate. The mechanism of both of these reactions is unknown at present. However, assuming initial 3′-hydroxylation of (+)marmesin in both the cis- and trans-orientation, a subsequent break of the trans-vicinal bond must formally release water and acetone from the cis-hydroxylated substrate to yield psoralen (Fig. 3). Likewise, a 1-oxo-3-hydroxy-isopentyl-substituted umbelliferone anion would be the initial product from relocation of charge in trans-hydroxylated marmesin. This would then cyclize to graveolone (Fig. 3). The postulated trans-hydroxylated substrate for the latter reaction had been isolated from *Xanthoxylum arnottianum* Maxim. (Ishii *et al.*, 1973) and named xanthoarnol. This reaction scheme is still a hypothesis. Nevertheless, it is of great interest to see the previously reported differential induction of cells by the two elicitors (Tietjen *et al.*, 1983) reflected in the catalytic properties of isolated microsomes.

The formation of bergapten (**27**) has been suggested as proceeding from either 5-hydroxylated marmesin or via psoralen and bergaptol (**26**).

Fig. 3. Hypothetic sequence of reactions as suggested for psoralen and graveolone synthesis catalyzed by microsomal enzyme activities from elicitor-induced parsley cells. The letter B represents an enzyme base.

The endoplasmic membrane fractions from elicitor-induced *Ammi majus* cells catalyzed only the latter reaction (Fig. 2) (D. Hamerski and U. Matern, unpublished). Bergaptol synthase was also identified as a cytochrome P_{450}-dependent monooxygenase. The close spatial association of all the enzymes which sequentially catalyze the formation of bergaptol from umbelliferone (Fig. 2) and the fact that exogeneously supplied marmesin—in contrast to umbelliferone—is not readily incorporated into the psoralens make it likely that furanocoumarin synthesis occurs in the lumen of the endoplasmic reticulum and not as previously suggested in the plastids.

A 5-O-methyltransferase and an 8-O-methyltransferase accepting linear furanocoumarins (**26**, **30**, and **32**) as substrates were isolated from *Ruta graveolens* cell cultures and purified to homogeneity (Thompson *et al.*, 1978, Sharma *et al.*, 1979). Both enzymes exhibited the same molecular mass of between 85 and 110 kDa. The former enzyme specifically

methylated the hydroxyl group in the position ortho to the side chain in furanocoumarins, whereas the latter enzyme appeared to be less specific accepting 8-hydroxylated simple coumarins like daphnetin (**13**), also. Two methyltransferases with similar substrate specificities were recently described from elicitor-induced parsley cells (Hauffe *et al.*, 1986). 5-Hydroxyxanthotoxin (**32**) was a better substrate than bergaptol (**26**) for the 5-O-methyltransferase, which is in accord with the proposed biosynthesis of isopimpinellin (**33**) via xanthotoxin (**31**) (Murray *et al.*, 1982). The parsley methyltransferases possess molecular masses of 67 and 73 kDa, respectively, and possibly consist of two subunits.

VI. REGULATION OF BIOSYNTHESIS

Inducible cell cultures appear to be well suited for regulatory studies. Elicitor induction, however, usually triggers several rather than one particular pathway, including among others lignin biosynthesis. It thus remains difficult to evaluate the relative significance of, for example, changes in the general phenylpropanoid metabolism for coumarin synthesis, as long as no isoforms of individual enzymes can be exclusively assigned to coumarin biosynthesis.

The coordinated induction of phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, 4-coumarate:CoA ligase, and dimethylallyl diphosphate:umbelliferone dimethylallyltransferase in parsley cells in response to elicitor treatment has been reported (Hahlbrock *et al.*, 1981; Tietjen and Matern, 1983). The latter enzyme activity, specifically involved in the biosynthesis of coumarins, reached its maximum several hours later than that of the other enzymes and was assigned to a separate regulatory group of enzymes (Tietjen and Matern, 1983). Yet another transferase activity (dimethylallyl diphosphate:umbelliferone O-dimethylallyltransferase) found in the endoplasmic reticulum of elicitor-induced *Ammi majus* cells also showed such a delay in activation (Hamerski and Matern, 1988). A similar induction time course in response to elicitor was reported for the two coumarin-specific O-methyltransferase activities in cultured parsley cells (Hauffe *et al.*, 1986). In the cases of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase, *de novo* synthesis has been shown upon induction. This appears to be due to transient regulation of the respective translation and transcription rates (Kuhn *et al.*, 1984; Chappell and Hahlbrock, 1984; Schmelzer *et al.*, 1985).

VII. PHYSIOLOGICAL SIGNIFICANCE

The significance of coumarins is considered here only as it relates to infection. In differentiated plants, furanocoumarins are frequently excreted into schizolysigenous containers (Andon and Denisova, 1974) or into the waxy surface (Städler and Buser, 1984), exposing them to possible invaders. Similarly, cell cultures rapidly excrete most of the coumarins synthesized in response to elicitor treatment into the culture fluid (Tietjen *et al.*, 1983; Hamerski *et al.*, 1981).

Furanocoumarins are toxic to various organisms, and their potential use as phytoalexins has been reviewed (Murray *et al.*, 1982; Brown, 1981). On the other hand, a role for simple coumarins like scopoletin (**10**) in limiting spread of a pathogen is more difficult to define. Stoessl and Hohl (1981) argued that the direct antimycotic activity of scopoletin is negligible. However, it may contribute indirectly to the general defense reaction. Scopoletin activates the plant's hexose monophosphate pathway under stress (Hoover *et al.*, 1977). Furthermore, an inhibitory effect on the pectinolytic enzymes of pathogens has been reported (Ravise and Kirkiacharian, 1976). Scopoletin may also be oxidized by particular isoperoxidases (Reigh *et al.*, 1973), thus mediating the action of peroxidases (Wheatley and Schwabe, 1985) in a way similar to that postulated for the flavone apigenin (Yamauchi and Minamide, 1985).

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CHAPTER **2**

Flavonoids

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I. INTRODUCTION

Among the approximately 2000 flavonoids (Harborne *et al.*, 1975), some are accumulated by tissue and cell cultures. These compounds are

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listed in Table I. Since the 1960s, tissue cultures have become increasingly important as they have provided information on flavonoid biosynthesis and regulation of the corresponding pathways. It is these two aspects that are discussed here.

II. MAJOR CLASSES OF FLAVONOIDS

A. Classification of Flavonoids

The large number of flavonoids is conveniently divided into 12 classes (Harborne, 1980). In Fig. 1 chemical structures of these compounds are depicted; biflavonoids have been omitted and are discussed in Chapters 4 and 5, this volume. Dihydroflavonols are included, since these compounds are important intermediates, especially in anthocyanin biosynthesis. Anthocyanins also belong to the flavonoids (see Chapter 3, this volume). All structures described are based on the C_{15} skeleton of flavanone and are formed by the same pathways, that is, head-to-tail condensations of acetate units with phenylpropane precursors. Of the large number of flavonoid aglycones, only a few occur widely in nature. These aglycones have a common structure of the A ring (phloroglucinol type) (Harborne, 1980). Generally, the flavonoids occur as glycosylated and/or acylated conjugates. The observation of aurones and dihydrochalcones in tissue and cell cultures seems to be doubtful and is not referred to further.

B. Flavonoid Biosynthesis

Flavonoids are composed of two aromatic ring systems (A, B rings), which are formed by different biosynthetic pathways. The B ring is derived from the shikimate pathway via phenylalanine. The general phenylpropanoid pathway starts with the deamination of phenylalanine to *trans*-cinnamic acid. This reaction is catalyzed by phenylalanine ammonia-lyase (No. 1 in Fig. 2; PAL; EC 4.3.1.5), described by Koukol and Conn (1961). In the sequence of reactions leading to flavonoids, the next step is catalyzed by cinnamic acid 4-hydroxylase (No. 2 in Fig. 2; CAH; EC 1.14.13.11), a microsomal mixed functional cytochrome *P*-450–dependent monooxygenase catalyzing the parahydroxylation of *trans*-cinnamic acid to 4-coumaric acid (Potts *et al.*, 1974). The activation of hy-

Table I

Occurrence of Flavonoids in Cell and Tissue Cultures

Aglycone[a]	Conjugate[b]	Source[c]	Basal medium[d]	Reference
Chalcones				
Isoliquiritigenin	Not found	*Phaseolus aureus* (C, S)	PRL-4C	Berlin and Barz (1971)
Echinatin	Not found	*Glycyrrhiza echinata* (C)	W	Ayabe *et al.* (1980)
	Not found	*Glycyrrhiza uralensis* (C)	MS	Kobayashi *et al.* (1985)
Isoliquiritigenin	3′-*C*-Dimethylallyl	*Glycyrrhiza uralensis* (C)	MS	Kobayashi *et al.* (1985)
Flavanones				
Naringenin	Not found	*Picea excelsa* (C)	B5	Rolfs and Kindl (1984)
Liquiritigenin	Not found	*Glycyrrhiza uralensis* (C)	MS	Kobayashi *et al.* (1985)
Flavones				
Apigenin	7-*O*-Glucoside	*Petroselinum crispum*[g] (S)	B5	Kreuzaler and Hahlbrock (1973)
Chrysoeriol	7-*O*-Glucoside-6″-*O*-malonate			Matern *et al.* (1983)
	7-*O*-Apio-glucoside			
	7-*O*-Apiogluco-side malonate			
Luteolin	7-*O*-Glucoside			
Luteolin	6-C-Glucoside (Isoorientin)	*Linum usitatissimum* (C)	MS	Liau and Ibrahim (1973)
5,6,7,4′-Tetramethoxy-flavone	Not found	*Citrus aurantium* (C)	MS	Brunet and Ibrahim (1973)
3,6,7,8,4′-Pentamethoxy-flavone	Not found			
5,6,7,3′,4′-Pentameth-oxyflavone	Not found			

(continued)

Table I (*Continued*)

Aglycone[a]	Conjugate[b]	Source[c]	Basal medium[d]	Reference
5,6,7,8,3′,4′-Hexamethoxyflavone	Not found			
3,5,6,7,8,3′,4′-Heptamethoxyflavone	Not found			
Luteolin	Not found	*Trigonella foenum-graecum* (C)	MS	Uddin *et al.* (1977)
Apigenin	8-C,7-*O*-Diglucosid (vitexin 7-*O*-glucoside)			
5-Hydroxy-7,8,2′-trimethoxyflavone	Not found	*Andrographis paniculata* (C, differentiating)	W	Jalal *et al.* (1979)
5-Hydroxy-7,8-dimethoxyflavone	Not found			
5,2′-Dihydroxy-7,8-dimethoxyflavone	Not found			
7′,4′-Dihydroxy-flavone	6-C-Prenyl 8-C-Prenyl	*Glycyrrhiza echinata* (C)	W	Ayabe *et al.* (1980)
Apigenin	Not found	*Trigonella corniculata* (C)	MS	Khanna *et al.* (1980)
Aginenin, luteolin	Not found	*Dahlia pinnata* (C)	MS	Khanna *et al.* (1980)
Apigenin	Not found	*Solanum jasminoides* (C)	MS	Jain and Sahoo (1982)
Flavonols				
Quercetin	3-*O*-Rhamnoglucoside	*Camellia sinensis* (C)		Heller (1953) (Forrest, 1969)
Quercetin	3-*O*-Rhamnoglucoside 7-*O*-Glucoside	*Machaeranthera gracilis*[e] (C)	W/*f*	Stickland and Sunderland (1972)

Quercetin	3-*O*-Glucoside 3,7-Di-*O*-glucoside 3-*O*-Glucoside-malonate	*Petroselinum crispum*[g] (S)	B5	Kreuzaler and Hahlbrock (1973)
Isorhamnetin	3,7-Di-*O*-Glucoside malonate 3,7-Di-(*O*-glucoside malonate)			
Quercetin	3-*O*-Glucoside 3-*O*-Diglucoside	*Parthenocissus tricuspidata* (C)		Heller (1953) (Bleichert and Ibrahim, 1974)
Quercetin	Not found	*Crotalaria juncea* (C)	MS	Jain and Khanna (1974)
Quercetin	Not determined	*Impatiens balsamina* (S)	B5	Wellmann (1975)
Quercetin	Not found	*Trigonella foenum-graecum* (C)	MS	Uddin *et al.* (1977)
Quercetin	Not found	*Papaver rhoeas* (C)	MS	Khanna *et al.* (1980)
		Calendula officinalis (C)	MS	Khanna *et al.* (1980)
		Crotalaria burhia (C)	MS	Khanna *et al.* (1980)
Quercetin, kaempferol	Not found	*Lycopersicon esculentum* (C)	MS	Khanna *et al.* (1980)
		Agave wightii (C)	MS	Khanna *et al.* (1980)
Kaempferol	Not found	*Cheiranthus cheiri* (C)	MS	Khanna *et al.* (1980)
Isorhamnetin	Not found	*Argemone mexicana* (C)	MS	Khanna *et al.* (1980)
Quercetin	Not found	*Cassia torosa* (C)	MS	Takahashi *et al.* (1981)
Quercetin, kaempferol	3-*O*-Glucoside	*Solanum jasminoides* (C) *Solanum glaucophyllum* (C) *Solanum verbascifolium* (C)	MS	Jain and Sahoo (1982)
Quercetin	3-*O*-Glucuronide	*Anethum graveolens* (S)	B5	Möhle *et al.* (1985)

(continued)

Table I (*Continued*)

Aglycone[a]	Conjugate[b]	Source[c]	Basal medium[d]	Reference
Isoflavones				
Formononetin	Not found	*Glycyrrhiza glabra* ssp. *glandulifera* (C)	[f]	Furuya (1968)
Formononetin	Not determined	*Cicer arietinum* (C)	W	Sayagaver *et al.* (1969)
Daidzein	7(?)-*O*-(Acyl?)-glucoside	*Glycine max* (C)		Miller (1967) Miller (1969)
	7-*O*-Glucoside			
Genistein	7-*O*-Glucoside			
Daidzein	Not found	*Phaseolus aureus* (C, S)	PRL-4C	Berlin and Barz (1971)
Formononetin	Not found	*Glycyrrhiza echinata* (C)	W	Ayabe *et al.* (1980)
Daidzein	7-*O*-Glucoside	*Pueraria lobata* (C, S)	MS	Takeya and Itokawa (1982)
	8-C-Glucoside			
Genistein	Not found			
Formononetin, biochanin A	7-*O*-Glucoside	*Cicer arietinum* (S)	PRL-4C	Köster *et al.* (1983)
	7-*O*-Glucoside-6″-*O*-malonate			
Formononetin	Not found	*Glycyrrhiza uralensis* (C)	MS	Kobayashi *et al.* (1985)
3′-Hydroxyformononetin	Not found			
Daidzein	7-*O*-Glucoside	*Vigna angularis* (S)	MS	Hattori and Ohta (1985)
	7,4′-Di-*O*-glucoside			
2′-Hydroxydaidzein	7,4′-Di-*O*-glucoside			
Kievitone[h]	Contains a dimethylallyl moiety	*Phaseolus vulgaris* cv. Kievitsboon Koekoek	MS	Hargreaves and Selby (1978)
		Phaseolus vulgaris cv. Immuna	SH	Robbins *et al.* (1985)

Rotenoids				
Elliptone, deguelin, rotenone, tephrosin	Not found	*Tephrosia purpurea* (C)	MS	Sharma and Khanna (1975)
		Tephrosia vogelii (C)		
		Crotalaria buhria (C)	MS	Uddin and Khanna (1979)
Toxicarol, sumatrol	Not found	*Crotalaria buhria* (C)	MS	Uddin and Khanna (1979)
Deguelin, rotenone	Not found	*Derris elliptica* (C, differentiating)	MS	Kodama *et al.* (1980)
Pterocarpans				
Pterocarpin[h]	Not found	*Sophora angustifolia* (C)	W	Furuya and Ikuta (1968)
Maackiain[h]	Not found			
Pisatin[h]	Not found	*Pisum sativum* (C)	*f*	Bailey (1970)
Glyceollin[h] (isomers)	Contains a dimethylallyl moiety	*Glycine max* (C)	LS	Keen and Horsch (1972)
		Glycine max (S)	B5	Ebel *et al.* (1976)
Phaseollin[h]	Not found	*Phaseolus vulgaris* cv. Canadian wonder (S)	SH	Dixon and Fuller (1976)
				Dixon and Bendall (1978)
Medicarpin[h]	Not found	*Canavalia ensiformis* (C)		Miller (1967) (Gustine *et al.*, 1978)
Phaseollin[h]	Not found	*Phaseolus vulgaris*	MS	Hargreaves and Selby (1978)
Phaseollidin[h]	Contains a dimethylallyl moiety	*Phaseolus vulgaris* cv. Kievitsboon Koekoek (S)		
Medicarpin[h]	Not found	*Trifolium repens* (C)	B5	Gustine (1981)
Glyceollidin[h] (isomers)	Contains a dimethylallyl moiety	*Glycine max* (S)	B5	Zähringer *et al.* (1981)

(continued)

Table I (*Continued*)

Aglycone[a]	Conjugate[b]	Source[c]	Basal medium[d]	Reference
Phaseollin[h]	Not found	*Phaseolus vulgaris* cv. Immuna	SH	Robbins *et al.* (1985)
Medicarpin[h]	Not found	*Cicer arietinum* (S)	PRL-4C	Daniel *et al.* (1986)
Maackiain[h]	Not found			
Isoflavans				
Phaseollinisoflavan[h]	Not found	*Phaseolus vulgaris* cv. Kievitsboon Koekoek (S)	MS	Hargreaves and Selby (1978)
		Phaseolus vulgaris cv. Canadian Wonder (S)	SH	Dixon and Bendall (1978)
Coumestans				
Coumestrol	Not found	*Phaseolus aureus* (C, S)	PRL-4C	Berlin and Barz (1971)
Soyagol	Not found			
Coumestrol	Not found	*Phaseolus vulgaris* cv. Canadian wonder (S)	SH	Dixon and Bendall (1978)
Coumestrol	Not found	*Pueraria lobata* (C, S)	MS	Takeya and Itokawa (1982)

[a]Common trivial names used; for structures, see Harborne and Mabry (1982) or Ingham (1983).
[b]No trivial names used (rutinoside ≡ rhamnoglucoside).
[c]C, Callus culture; S, suspension culture.
[d]Modification of basal media; see reference in last column. Abbreviations used: PRL-4C, Gamborg (1966); MS, Murashige and Skoog (1962); W, White (1943); B5, Gamborg *et al.* (1968); LS, Linsmaier and Skoog (1965); SH, Schenk and Hildebrandt (1972).
[e]Previous name, *Haplopappus gracilis*.
[f]Medium; see reference in last column.
[g]Previous name, *Petroselinum hortense*.
[h]Phytoalexins; may occur constitutively in some cultures.

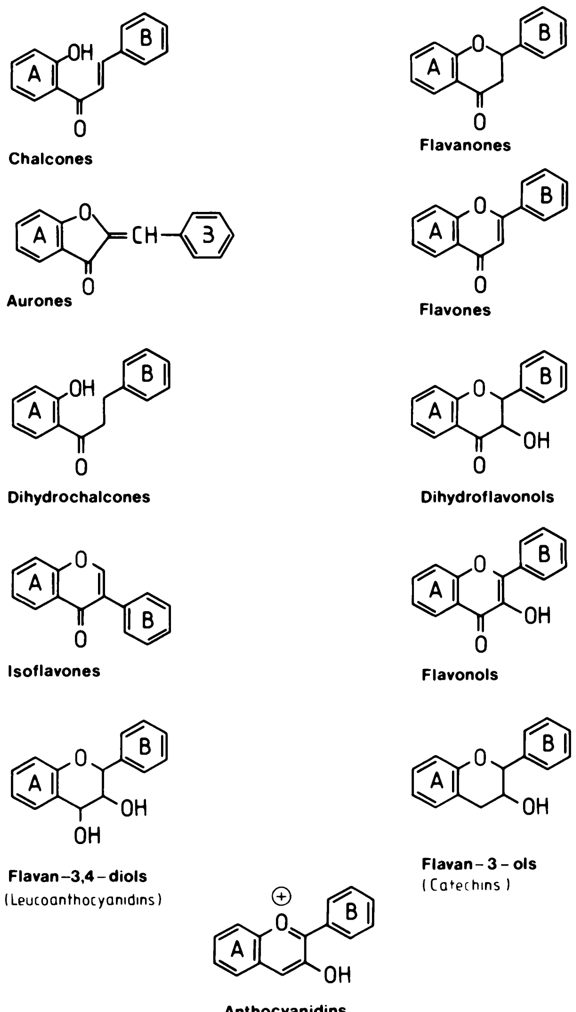

Fig. 1. The major classes of flavonoids.

droxycinnamic acids resulting in thioesters of coenzyme A, an ATP-dependent step, is catalyzed by 4-coumarate:CoA ligase (No. 3 in Fig. 2; 4CL; EC 6.2.1.12), described by Gross and Zenk (1974). Ring A is formed by successive head-to-tail condensations of three acetate units to 4-coumaroyl-CoA, resulting in the flavonoid C_{15} skeleton. The acetate units are derived from malonyl-CoA. This reaction is catalyzed by chalcone synthase (No. 4 in Fig. 2; CHS; EC 2.3.1.74), leading to chalcones, which are central intermediates in flavonoid biosynthesis. The activity of this

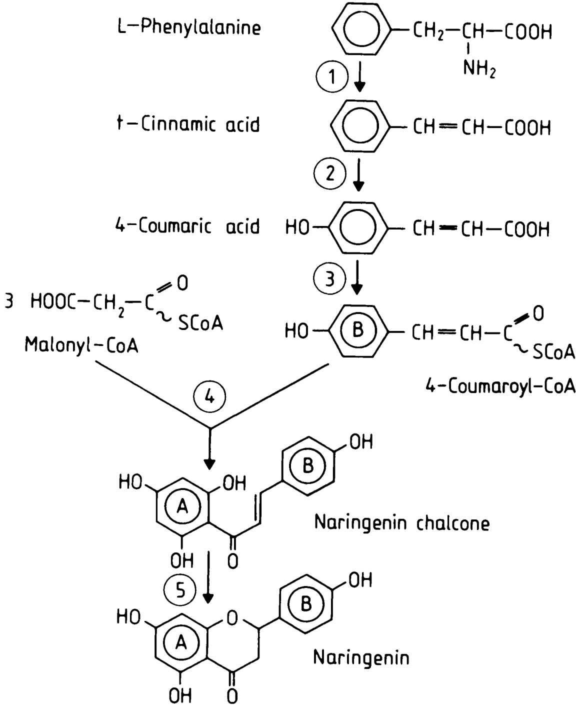

Fig. 2. Pathway leading to the phloroglucinol-type chalcone–flavanone isomers. For enzyme reactions, see text.

enzyme was first described by Kreuzaler and Hahlbrock (1972), using cell cultures of parsley. All substrates of CHS are coenzyme-A thioesters. The primary product from this enzymatic step is a chalcone (Heller and Hahlbrock, 1980). In a subsequent step the chalcone is cyclized to the corresponding flavanone by the activity of chalcone isomerase (No. 5 in Fig. 2; CHI; EC 5.5.1.6), first described by Moustafa and Wong (1967). The CHS-driven reaction leads to phloroglucinol-type (5-hydroxy) flavonoids, whereas an enzyme catalyzing a reaction leading to compounds of the recinol type (5-deoxy), which is widespread among chalcones, flavanones, and especially, isoflavonoids, has not been isolated. For details of properties of chalcone synthase and chalcone biosynthesis see the review by Ebel and Hahlbrock (1982).

All other flavonoids are derived from the isomers chalcone and flavanone. The biogenetic relationship of the aglycones of flavonoids is shown in a simplified scheme in Fig. 3. Oxidation of flavanones with oxygen leads to flavones. In this context two types of enzymes have

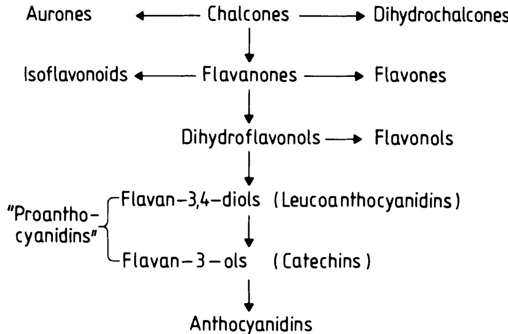

Fig. 3. Biogenetic relationship of the flavonoid classes.

been described. A soluble enzyme from parsley cell cultures needs ascorbate, Fe^{2+}, and 2-oxoglutarate as cosubstrate (Sutter *et al.*, 1975; Britsch *et al.*, 1981), whereas an oxidase from intact plants of *Antirrhinum majus* is membrane-bound (microsomal) and NADPH-dependent (Stotz and Forkmann, 1981). The 3-hydroxylation of flavanones to dihydroflavonols is catalyzed by a soluble 2-oxoglutarate-dependent dioxygenase that requires Fe^{2+} and ascorbate as cofactors (Forkmann *et al.*, 1980). The enzymatic oxidation of dihydroflavonols to flavonols was demonstrated by Britsch *et al.* (1981) with extracts from cell cultures of parsley. Similar to the flavanone 3-hydroxylase, this soluble enzyme is a 2-oxoglutarate-dependent dioxygenase, working with Fe^{2+} and ascorbate as cofactors. Therefore, in this reaction a 2,3-dihydroxyflavanone has been postulated as an intermediate (Britsch *et al.*, 1981).

Isoflavonoids are formed by intramolecular aryl migration of the B ring from position 2 to 3. This step has been demonstrated on an enzymatic basis with extracts from soybean cell cultures (Hagmann and Grisebach, 1984). During this reaction a double bond is introduced, so the flavanone is converted into an isoflavone. This isoflavone synthase is also a microsomal cytochrome *P*-450–dependent monooxygenase with NADPH and oxygen as cosubstrates. The isoflavones are the precursors of all other isoflavonoids. Their biogenetic relationship has been reviewed by Dewick (1982).

C. Substitution Reactions

The flavonoid skeleton without any substituents is shown in Fig. 4. Substitution mainly means hydroxylation and/or *O*-methylation.

Flavonoid skeleton

Fig. 4. The flavonoid skeleton and the positions of substituents.

1. Hydroxylation

All known chalcone synthases from various sources form a phloroglucinol structure at ring A, with hydroxyl groups in positions 5 and 7. Enzymes catalyzing a reaction leading to 5-deoxy flavanones are unknown. The 4′ OH of the B ring is also part of the basic structure of all flavonoids. This group has been introduced at the level of cinnamic acids (4-coumaric acid). Further substitution reactions mostly take place at the flavonoid stage. Substitution reactions at the level of cinnamic acids are the exception. Kamsteeg *et al.* (1981) described a 4-coumaroyl-CoA-3-hydroxylase from *Silene dioica*. Hydroxylations mainly occur at 3′ and 5′ positions of the B ring. The flavonoid 3′-hydroxylase is, like cinnamic acid 4-hydroxylase, a microsomal NADPH- and oxygen-dependent heme-containing monooxygenase. The first report of this enzyme was given by Fritsch and Grisebach (1975); it was extracted from anthocyanin-containing cell cultures of *Machaeranthera gracilis*. Preparations from plants of *Verbena hybrida* contain activities catalyzing both 5′- and 3′-hydroxylation (Stotz and Forkmann, 1982). Because these hydroxylases accept flavanones as well as dihydroflavonols, they have been called flavonoid hydroxylases.

2. *O*-Methylation

Formation of methoxy groups is carried out by position-specific soluble *S*-adenosyl-L-methionine:*O*-methyltransferases (OMTs). Among various *O*-methyltransferases, two representative examples should be described here. The flavonoid 3′-*O*-methyltransferase from parsley cell cultures methylates luteolin (a flavone) and quercetin (a flavonol) in 3′ position (Ebel *et al.*, 1972). Another interesting example is the isoflavone 4′-*O*-methyltransferase from cell cultures of chickpea (*Cicer arietinum*), which methylates the isoflavones daidzein (5-deoxy) and genistein (5-hydroxy) in 4′ position (Wengenmayer *et al.*, 1974).

D. Conjugation Reactions

Flavonoids rarely occur as aglycones. Within the cells they are normally present in conjugated forms, which means the aglycones are bound to other compounds provided by primary and secondary metabolism. A list of the most important groups of such conjugation partners has been given by Barz and Köster (1981).

1. Glycosylation

Glycosylation can take place at oxygen or carbon atoms of the aglycone. C-Glycosides of flavonoids should be considered as a separate group by reason of their chemical properties (Chopin *et al.*, 1982). The formation of *O*-glycosides has been well investigated on an enzymic level. Glycosyltransferases are soluble enzymes with high position and donor specificity and lower specificity for the acceptors. The sugar components are activated by nucleotide binding, normally UDP. Two different glucosyltransferases have been found in parsley cell cultures. One glucosylates flavanones, flavones, and flavonols as well in the 7 position. Besides this 7-*O*-glucosyltransferase, a 3-*O*-glucosyltransferase has been isolated that glucosylates flavonols and flavonol-7-*O*-glucosides in the 3 position (Sutter and Grisebach, 1973). A 7-*O*-glucosyltransferase from chickpea shows an absolute donor specificity to UDP-glucose and a high acceptor specificity for 4′-methoxyisoflavones (formononetin, biochanin A), as shown by Köster and Barz (1981). Monosaccharides other than glucose are transferred to flavonoids by analogous mechanisms catalyzed by corresponding donor- and position-specific glycosyltransferases. A detailed review has been given by Ebel and Hahlbrock (1982).

2. Acylation

Acylation of flavonoid glycosides is a frequently occurring step at the end of the biosynthetic pathway of flavonoid conjugates. Transfer of acyl groups can take place to the sugar moieties and to the aglycones themselves. The occurrence of acylated flavonoid aglycones has been summarized by Wollenweber (1985). But more widespread are acylated flavonoid *O*-glycosides. A list of these glycosides has been given by Harborne and Williams (1982). Among the acids provided for acyl transfer, malonic acid is very common (for a review, see Barz *et al.*, 1985). In parsley (Kreuzaler and Hahlbrock, 1973; Matern *et al.*, 1983) as well as

Cicer arietinum (Köster *et al.*, 1983), flavonols/flavones and isoflavones, respectively, are accumulated as 6-*O*-malonylglucosides. The chemical structure of these compounds is depicted in Fig. 5. The corresponding malonyltransferases have been extensively characterized. These enzymes are soluble proteins using malonyl-CoA as an acyl donor. From cell cultures of parsley two different malonyltransferases have been isolated. One enzyme is a flavone/flavonol 7-*O*-glucoside malonyltransferase, and the other one transfers malonyl residues to flavonol 3-*O*-glucosides (Matern *et al.*, 1981). In chickpea cells an isoflavone 7-*O*-glucoside-malonyltransferase has been described (Köster *et al.*, 1984). In parsley as well as chickpea, malonyl transfer to the sugar moiety is the last step in flavonoid biosynthesis. It has been suggested by Matern *et al.*

Isorhamnetin 3,7-di-(O-glucoside-malonate)

Chrysoeriol 7-O-apioglucoside-malonate

Formononetin 7-O-glucoside-6''-malonate (R=H)

Biochanin A 7-O-glucoside-6''-malonate (R=OH)

Fig. 5. Malonylglucosides of flavonoids accumulated by cell cultures: flavonol–flavone conjugates in *Petroselinum hortense* (Kreuzaler and Hahlbrock, 1973) and isoflavone conjugates in *Cicer arietinum* (Köster *et al.*, 1983).

(1983, 1986) that malonylation is a prerequisite for transport of flavonoids into the central vacuole of the cell (see also Chapter 3, this volume).

3. Prenylation

Many flavonoids contain prenyl side chains bound to different carbon atoms. Because the isoprenyl residue is transferred as a whole, it should be interpreted as a conjugation reaction. A corresponding prenyltransferase participates in glyceollin biosynthesis. Such an enzyme has been described in soybean cell cultures. It is a membrane-bound enzyme and prenylates 3,6*a*,9-trihydroxypterocarpan in positions 2 and 4. The donor substrate is dimethylallylpyrophosphate (Zähringer *et al.*, 1981). It should be mentioned that in biosynthesis of prenylated flavonoids, phenylpropanoid as well as terpenoid metabolism is involved.

E. Polymers

Polymers derived from flavonoids are not discussed in this chapter in detail. They are referred to in Chapters 4 and 5, this volume, where proanthocyanins, catechins, and tannins are treated. The major types of phenolic polymers occurring in plants are lignins and tannins. Like conjugation, oxidative polymerization is one form of metabolic turnover (see Section V). Among the flavonoid polymers, biflavonoids possess an exceptional position with respect to their biosynthetic pathway. For a review of this class of flavonoids see Geiger and Quinn (1982).

III. ADVANTAGES OF CELL CULTURES IN STUDIES OF FLAVONOID BIOSYNTHESIS

Plant cells cultured in suspension have successfully been used for studies of flavonoid biosynthesis and for identification of intermediates of this pathway (for a review, see Ebel and Hahlbrock, 1982). Cell cultures are also very useful sources for the isolation of enzymes of flavonoid pathways. They can provide biochemists with aseptic and nearly homogeneous cell material, which can be propagated under very defined conditions. Suspension cultures have also been employed for feeding experiments with radioactive precursors and for supplementa-

tion of the flavonoid pathway by feeding appropriate intermediates subsequent to a block in the corresponding pathway. Furthermore, cell cultures are suitable systems for the study of turnover and degradation. In phytopathology, model systems with cell cultures are of value for studies of phytoalexin biosynthesis and the regulation of its induction. Also, regulation of flavonoid biosynthesis at the transcriptional and/or translational level has been demonstrated using freely suspended cells.

Some of the disadvantages should be mentioned also. Often, accumulation of secondary products is restricted to differentiated cells or specialized tissues. During subculturing a selection of fast-growing, nonproducing cells may take place. Related to this is the problem of maintaining the capacity of cell lines to accumulate secondary products during serial passages. But in several cases the yield of secondary products is higher in cell cultures than in the source plant (for a review, see Dougall, 1981; also Chapter 6, Volume 4, this treatise). Cryopreservation and other storage methods may be used to maintain high-yielding cell lines (for a review, see Chapter 11, Volume 4, this treatise).

IV. FLAVONOIDS IN TISSUE AND CELL CULTURES

A. Occurrence

Table I is a compilation of the flavonoids that have been reported to occur in cell and tissue cultures. A large number of the compounds are isoflavonoids. This class, therefore, has been subdivided into isoflavones, isoflavanones, rotenoids, pterocarpans, isoflavans, and coumestans. Their structures, biosynthesis, and occurrence have been reviewed by Dewick (1982) and Ingham (1983).

Most of the flavonoids accumulated by cultures are flavones, flavonols, isoflavones, rotenoids, and pterocarpans. In contrast, chalcones, flavanones, isoflavans, and coumestans are rarely observed. We found no examples where dihydrochalcones, aurones, or dihydroflavonols had been found to accumulate. Two *C*-glucosylflavones have been identified and have been listed under flavones.

Often only aglycones have been identified. The accumulation of free aglycones within the classes of flavones, flavonols, and isoflavones, however, seems to be questionable, in many cases the result of insufficient extraction procedures. A complete structural elucidation of conju-

gates has been performed for flavones and flavonols from parsley (Kreuzaler and Hahlbrock, 1973; Matern *et al.*, 1983) as well as for the isoflavones of chickpea (Köster *et al.*, 1983). These 6-*O*-malonylglucosides (Fig. 5) accumulate in plants and cell cultures. These malonic acid hemiesters are very unstable and may undergo rapid hydrolysis during extraction (Matern, 1983; Hinderer *et al.*, 1986a). Nevertheless, the potential for accumulation of free flavonoid aglycones is present and widespread, especially within the classes of pterocarpans, rotenoids, and coumestans, which are generally not glycosylated or acylated. Most cultures are characterized with regard to how the yield of flavonoids is affected by medium constituents or how the induction of flavonoid biosynthesis is affected by other environmental conditions. Some of these aspects are discussed in the following paragraphs.

B. Inducibitility of Flavonoid Biosynthesis

1. Light

The best-known system is the flavonoid-accumulating cell culture of *Petroselinum hortense*. Flavonoid accumulation depends on light treatment. In dill cultures a UV- (ultraviolet-) induced accumulation of quercetin 3-*O*-glucuronide has also been described (Möhle *et al.*, 1985). In parsley a sequence of well-known events lead to flavonoid biosynthesis. During this induction process, enzymes are regulated coordinately within two sequences, designated as group I and group II (for review, see Ebel and Hahlbrock, 1982). Group I includes the enzymes of general phenylpropanoid metabolism, of which phenylalanine ammonia-lyase is probably the rate-limiting enzyme. The enzymes of the flavonoid glycoside pathway belong to group II, beginning with chalcone synthase as a key enzyme. The light-induced transient activity changes of about 16 enzymes are closely related to flavone and flavonol biosynthesis. The maxima of flavonoid biosynthesis and the *de novo* synthesis of both groups of enzymes are different. Maximum flavonoid accumulation is achieved in 7 to 10 hr, and the enzymes of group I reach their maximum enzyme protein synthesis 7 hr after the beginning of illumination (Schröder *et al.*, 1979; Ragg *et al.*, 1981). The enzymes of group II are synthesized at a maximum rate at 10 to 12 hr after the beginning of illumination (Schröder *et al.*, 1979; Gardiner *et al.*, 1980). These changes in enzyme synthesis depend on regulation of mRNA transcription, as shown by RNA blot hybridization experiments using cDNA of PAL, hydroxycinnamate:CoA ligase (Kuhn *et al.*, 1984), and CHS (Kreuzaler *et al.*, 1983). Usually, light-induced phenylpropanoid synthesis is medi-

ated by phytochrome (for a review, see Schopfer, 1977), whereas with parsley cell cultures it has been suggested that phytochrome is only active subsequent to UV irradiation (Wellmann, 1971). The synergistic effect of UV and red and far red has been shown by Duell-Pfaff and Wellmann (1982). Considering these results, a blue-light receptor has been postulated (Duell-Pfaff and Wellmann, 1982). As claimed by Hrazdina (1982), it cannot be excluded that at least in anthocyanin accumulation the response to UV treatment is a stress effect. Nevertheless, phytochrome is involved in this induction, as shown by Wellmann and Baron (1974). The dose response of group I and II enzymes to UV radiation seems to be linear within a certain range. The synthesis of PAL depends on the amount of P_{fr} present in the cells (Wellmann and Schopfer, 1975). As shown more recently, the whole sequence of events leading to increased transcription of the respective mRNA depends on the activation by UV (Kuhn *et al.*, 1984; Kreuzaler *et al.*, 1983). Almost nothing is known about the signal sequence during light-induced mRNA transcription.

2. Elicitors and Phytoalexins*

Elicitors have been defined as molecules that induce phytoalexin accumulation. The phytoalexins are low-molecular-weight substances with antimicrobial effects, produced by plants as a response to microbial infection (Darvill and Albersheim, 1984). Within the flavonoids, with some exceptions, phytoalexins belong to the isoflavonoid class, mainly to the 5-deoxypterocarpans. Their occurrence and metabolism have been extensively reviewed by Dixon *et al.* (1983), Ingham (1983), and Dewick (1982). As shown by Dixon (1980), cell cultures are ideal systems for the investigation on phytoalexin induction and biosynthesis. With respect to axenic conditions, cell cultures are very useful systems for phytopathologists. Elicitors can be applied without wounding, and rapid responses can be measured very easily, for example, phytoalexins in ether extracts of the culture medium (H. Kessmann and W. Barz, personal communication). Cell-culture systems can be employed for experiments on host–pathogen interactions as well as for biosynthetic studies. In Table I, flavonoid phytoalexins occurring in various cell cultures are marked with a superscript *h*. These compounds are exclusively found in legumes. All of them are pterocarpans except the isoflavanone kievitone, and phaseollinisoflavan. Moreover, kievitone is

*See also Chapter 9, Volume 4, this treatise.

Fig. 6. Structures of some isoflavonoid phytoalexins occurring in cell cultures.

the only 5-hydroxyflavonoid phytoalexin occurring in cell cultures. Structures of some of these phytoalexins are depicted in Fig. 6.

V. TURNOVER AND DEGRADATION

Flavonoid catabolism in cell cultures has been reviewed by Barz (1977) and Hösel *et al.* (1977). Synthesis and metabolism of aromatic constituents occur simultaneously. When natural products are completely degraded, the reactions taking place are referred to as catabolism. In the absence of specific knowledge of the reactions occurring, this kind of metabolism should be called turnover (Barz *et al.*, 1985). Turnover of flavonoids might be determined by pulse labeling with appropriate radioactive precursors. For this kind of feeding experiment, cell cultures are well suited. In principle, turnover of a compound can take place by different reactions: interconversion, conjugation, polymerization, and degradation (Barz *et al.*, 1985). Interconversion reactions occur within a biosynthetic sequence, for example, turnover of flavanones during biosynthesis of flavonols, isoflavonoids, and anthocyanins (see Fig. 3).

Conjugation reactions (glycosylation, acylation, prenylation; see Section II,D) also lead to a turnover of flavonoid aglycones. But conjugation does not have to be a "one way" reaction, although conjugates from flavonoids are end products of the respective pathway and are accumulated in the central vacuole, as has been demonstrated for flavone/ flavonol malonylglucosides in parsley cell cultures (Matern *et al.*, 1983). However, in chickpea roots it has been shown that isoflavone malonylglucosides (see Fig. 5) are unequivocally turned over (Jaques *et al.*, 1985). Investigations of the role of esterases during deacylation of malonyl conjugates revealed one highly specific malonylesterase, which is only active with the naturally occurring malonylglucosides, but not with the often used synthetic substrates (Hinderer *et al.*, 1986a). Different kinds of soluble esterases that hydrolyze malonylhemiesters have been detected in parsley. These enzymes do not possess high specificity for malonates and have therefore been designated as aryl- or acetylesterases (Matern, 1983). In contrast, the specific malonylesterase of chickpea is membrane-bound (Hinderer *et al.*, 1986b) and has also been detected in cell cultures (S. Daniel and W. Barz, unpublished results). Its occurrence is in good agreement with the occurrence of the specific isoflavone-7-*O*-glucoside β-glucosidases (Hösel *et al.*, 1977). By the consecutive action of esterase and glucosidase, isoflavone aglycones can be released from the corresponding conjugates in chickpea.

For chalcones, flavones, aurones, and flavanols it has been shown that the aglycones are degraded by a peroxidative mechanism (for a review, see Barz and Köster, 1981). The resulting compounds might be aromates, especially benzoic acid derivatives. Further degradation can take place by ring-fission reactions catalyzed by dioxygenases (for a review, see Barz *et al.*, 1985). Besides these catabolic routes, flavonoids may undergo polymerization, also catalyzed by peroxidases or phenolases. The synthesis of these ethanol-insoluble polymers mainly occurs with compounds of a certain substitution pattern. Compounds such as 4'-hydroxy or 3',4'-dihydroxy isoflavones are preferentially polymerized (Barz, 1975). Regulation, cellular compartmentation, and the physiological role of degradation and turnover in plants are not yet understood (Barz *et al.*, 1985).

VI. CONCLUDING REMARKS

Since the mid-1970s, cell cultures have proven very useful systems for elucidating flavonoid biosynthesis and its enzymic control. In the fu-

ture, new enzymes will be isolated and characterized; this fundamental research should concentrate on pterocarpan biosynthesis. It can be confidently expected that marked progress will arise in the elucidation of regulation of key enzymes on a transcriptional level. As far as isoflavonoids are concerned, our knowledge of elicitor-induced phytoalexin biosynthesis will increase. Using cell cultures, new phytochemicals may also be found. Endeavor should concentrate on studies of factors that increase the yield of secondary products and that improve the stability of cell cultures with regard to secondary metabolism.

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CHAPTER 3

Anthocyanins

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I. INTRODUCTION

Anthocyanins occur widely in gymnosperms, monocotyledons, and dicotyledons. They are of chemotaxonomic value and also play a role as

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genetic markers (for a review, see Hrazdina, 1982). The main function of anthocyanins is pigmentation of flowers and fruits. Anthocyanins are also indicators of stress. Techniques for isolation and identification of anthocyanins have been reviewed by Harborne *et al.* (1975) and Harborne and Mabry (1982).

In this chapter the occurrence of anthocyanins in tissue (callus) and cell (suspension) cultures is reviewed. Attention is focused on special aspects of their biosynthesis and regulation of biosynthesis and accumulation. Some aspects common to the biosynthesis of flavonoids and anthocyanins are described in Chapter 2, this volume. The general phenylpropanoid pathway is also described in Chapter 2, this volume. The review comprises literature from 1970 to 1985 (computer search, BIOSIS).

II. STRUCTURE OF ANTHOCYANINS

A. Anthocyanidins

The majority of anthocyanidins (aglycones) differ in the hydroxylation and methylation pattern of the B ring while having a common basic structure of rings A and C, as given in Fig. 1. In Table I the substitution pattern of the six most common anthocyanidins derived from this structure is specified. The most common *O*-methyl ethers are peonidin, petunidin, and malvidin, derived from cyanidin or delphinidin. Very rarely has alteration of the basic structure (Fig. 1) been observed. 3-Deoxy-, 6-hydroxy-, or 3-methoxyanthocyanidins have not been detected in cell and tissue cultures. 5-Methyl ethers of delphinidin, petunidin, and malvidin are exclusively found in Plumbaginaceae (Harborne, 1980). The 7-methyl ether of malvidin, called hirsutidin, has been detected in tissue cultures of *Catharanthus roseus* (Carew and Krueger, 1976).

Fig. 1. Basic structure of the most common anthocyanidins (see also Table I).

Table I

B-Ring Substitution Pattern of Anthocyanidins[a]

Anthocyanidin	R_1	R_2	R_3
Pelargonidin	—OH	—H	—H
Cyanidin	—OH	—OH	—H
Delphinidin	—OH	—OH	—OH
Peonidin	—OH	—OCH_3	—H
Petunidin	—OH	—OH	—OCH_3
Malvidin	—OH	—OCH_3	—OCH_3

[a]See Fig. 1.

B. Anthocyanidin Conjugates

In general, anthocyanidins are present in conjugated forms, bound to sugars. The resulting glycosides become water soluble and are stored in the vacuolar sap of mature cells. Glycosidic variation leads to about 200 sugar derivatives of anthocyanidins. The most common anthocyanidin, cyanidin, exists in about 40 different glycosidic structures (Harborne, 1980). Further variation of these molecules arises from acylation of the glycosides.

1. Glycosylation

Glycosylation of anthocyanidins commonly takes place in the 3 or 5 positions. Rarely, positions 7 and 4′ are glycosylated. Anthocyanins are all *O*-glycosides. The flavylium cation of the anthocyanidin renders them unstable. It can be assumed that the first glycosylation occurs at the 3-*O* position of the aglycone, imparting a stabilizing effect on the molecule and thus allowing anthocyanin accumulation (Ebel and Hahlbrock, 1982). It is most likely that glycosylation as well as acylation occur subsequent to other modifications of the flavonoid skeleton (Ebel and Hahlbrock, 1982). The bulk of the glycosides are glucosides, but other sugars, such as rhamnose, galactose, xylose, and arabinose, are also used for glycosylation.

2. Acylation

Data have become available on acylated flavonoids and anthocyanins. Harborne and Boardley (1985) examined 81 species in 27 families for the occurrence of anthocyanins as zwitterions, which indicates an acylation

Fig. 2. Main anthocyanin of "black carrot" (*Daucus carota* ssp. *sativa*) plants and tissue cultures. Structure according to Harborne *et al.* (1983).

with a dicarboxylic acid (e.g., malonic acid). More than half of the anthocyanins were found to be such zwitterions. Determination of the acyl moiety in a few samples showed malonic acid attached to glucose. In parsley cell cultures malonylation of the glucose moieties is assumed to be the last step in biosynthesis of flavonoid glycosides (Ebel and Hahlbrock, 1977).

Malonylation or, in general, acylation might have been overlooked in the past, because the isolation procedures normally applied often resulted in deacylated products. Mild extraction conditions have to be used (Köster *et al.*, 1983) in order to obtain a genuine composition of plant constituents. Besides malonic acid, hydroxycinnamic acids often seem to occur as the acyl residues of anthocyanins. Carrot cell cultures (*Daucus carota* ssp. *sativa*) accumulate large amounts of one main anthocyanin, its structure (Fig. 2) having been determined (Harborne *et al.*, 1983; Hopp and Seitz, 1987). The cyanidin triglycoside is acylated at the xylosyl residue by sinapic acid. The first sugar, bound to cyanidin in position 3, is galactose. This anthocyanin also occurs in the intact plant (Harborne *et al.*, 1983), and its structure is discussed with respect to transport into the central vacuole (see Section VI). This carrot pigment, together with a malvidin 3-(*p*-coumaroylrutinoside)-5-glucoside found in calli of *Solanum tuberosum* (Harborne and Simmonds, 1962) and *Petunia hybrida* (Colijn *et al.*, 1981), are the only acylated anthocyanins of cell and tissue cultures, the acyl moiety of which has been determined.

III. OCCURRENCE OF ANTHOCYANINS IN TISSUE AND CELL CULTURES

Anthocyanins occurring in plant culture systems have been listed by Butcher (1977) and Harborne (1980). Their citations, today, are in-

Table II

Occurrence of Anthocyanins in Tissue and Cell Cultures

Source	Culture	Anthocyanins	Basal medium[a]	Reference
Vitis vinifera	Callus	Not identified	[b]	Slabecka-Szweykowska (1952)
Vitis (hybrids)	Suspension	(Cyanidin 3-glucoside)?	MS	Yamakawa *et al.* (1983)
Zea mays cv. Black Mexican Sweet	Callus	Cyanidin 3-glucoside, pelargonidin 3-glucoside	[b]	Straus (1959)
Solanum tuberosum cv. Congo	Callus	Malvidin 3-(*para*-coumaroylrutinoside) 5-glucoside		Harborne and Simmonds (1962)
Solanum jasminoides	Callus	Cyanidin, petunidin[c]	MS	Jain and Sahoo (1982)
Parthenocissus tricuspidata	Callus	Cyanidin, delphinidin, malvidin 3-glycosides and 3,5-diglycosides	Heller (1953)	Stanko and Bardinskaya (1963)
		Cyanidin 3,5-diglucoside	Heller (1953)	Bleichert and Ibrahim (1974)
Machaeranthera gracilis[d]	Callus	Cyanidin 3-glucoside and 3,5-diglucoside	W	von Ardenne (1965)
	Callus	Cyanidin 3-glucoside and 3-rutinoside	W[b]	Stickland and Sunderland (1972)
Daucus carota cv. Kintoki	Callus	Cyanidin glycosides	W	Sugano and Haysahi (1967)
Daucus carota	Callus	Malvidin 3,5-diglucoside	MS	Ibrahim *et al.* (1971)
Daucus carota spp. *sativa*	Callus	Cyanidin[c]	Blakely and Steward (1961)	Alfermann and Reinhard (1971)

(*continued*)

Table II (*Continued*)

Source	Culture	Anthocyanins	Basal medium[a]	Reference
	Callus	Cyanidin xylo-glucoside	I2a (Seitz and Richter, 1970)	Schmitz and Seitz (1972)
	Suspension	Cyanidin xylo-glulcoside	I2a	Noé *et al.* (1980)
	Suspension	Cyanidin 3-(sinapoylxylosyl-glucosylgalactoside)	B5	Harborne *et al.* (1983)
Daucus carota	Suspension (chem-ostat)	Cyanidin[c]	[b]	Dougall and Weyrauch (1980)
Daucus carota cv. Kurodagosun	Suspension	Cyanidin[c]	Lin and Staba (1961)	Ozeki and Komamine (1981)
Daucus carota	Suspension	Cyanidin 3-glucogalactoside, 3,5-digalactoside, 3-glucoside, and 3-galactoside	[b]	Hemingson and Collins (1982)
Dimorphotheca auriculata	Callus	Cyanidin 3-glucoside, delphinidin 3-glucoside	Not mentioned	Harborne *et al.* (1970)
Dimorphotheca sinuata	Callus	Cyanidin 3-glucoside, delphindin 3-glucoside	MS	Ball *et al.* (1972)
Helianthus tuberosus	Callus	Cyanidin 3-glucoside and 3,5-diglucoside	MS	Ibrahim *et al.* (1971)

Linum usitatissimum	Callus	Cyanidin 3,5-diglucoside	MS	Ibrahim *et al.* (1971)
Rosa multiflora	Callus	Cyanidin 3,5-diglucoside, delphinidin 3,5-diglucoside	MS	Ibrahim *et al.* (1971)
Rosa sp.	Suspension	Not identified	MX1 (Nash and Davies, 1972)	Davies (1972)
Malus pumila	Callus	Cyanidin 3,5-diglucoside	MS	Ibrahim *et al.* (1971)
Malus pumila spp *domestica*	Callus	Not identified	W	Oota *et al.* (1983)
Eucalyptus citriodora	Callus	Pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside	[b]	Ram *et al.* (1971)
Populus (hybrids)	Suspension	Cyanidin 3-glucoside	Linsmaier and Skoog (1965)	Matsumoto *et al.* (1973)
Impatiens balsamina	Suspension	Not identified	B5	Wellmann (1975)
Urginea maritima	Callus	Not identified	Staba (1969)	Shyr and Staba (1976)
Catharanthus roseus	Callus	Hirsutidin, malvidin, petunidin (glucosides)	PRL-1 (Gamborg, 1970)	Carew and Krueger (1976)
	Suspension	Hirsutidin, malvidin petunidin[c]	MS	Knobloch *et al.* (1982)
Pyrus communis spp. *communis* and spp. *sativa*	Callus	Not identified	W/MS	Mehra and Daidka (1979)
Euphorbia millii	Callus	Cyanidin[c]	MS	Yamamoto *et al.* (1981)

(*continued*)

Table II (*Continued*)

Source	Culture	Anthocyanins	Basal medium[a]	Reference
Petunia hybrida	Suspension /callus	Petunidin, malvidin[c], malvidin 3-(*para*-coumaroyl rutinoside) 5-glucoside	MS	Colijn *et al.* (1981)
Strobilanthes dyeriana	Callus	Cyanidin 3,5-diglucoside, peonidin 3,5-diglucoside	B5	Smith *et al.* (1981)
Matthiola incana (different mutants)	Callus	Cyanidin 3-glucoside, 3,5-diglucoside, 3-sambubioside (differently acylated), and 3-sambubioside-5-glucoside (differently acylated)	I2a (Seitz and Richter, 1970)	Leweke and Forkmann (1982)
Forsythia suspensa	Callus	Not identified	[b]	Bader *et al.* (1984)
Oryza sativa	Callus	Not identified	[b]	Niizeki *et al.* (1985)
Ipomoea batatas	Suspension	Not identified	PRL-4c (Gamborg, 1966)	Nozue and Yasuda (1985)

[a]Abbreviations for media: MS, Murashige and Skoog (1962); W, White (1943); B5, Gamborg *et al.* (1968).
[b]Medium; see reference column.
[c]Determination of anthocyanidins only.
[d]Previous name, *Haplopappus gracilis* (Nutt.) Gray.

complete and may lead to the conclusion that anthocyanin-producing cultures are uncommon. An updated list is presented in Table II. The table comprises all tissue and cell cultures except those systems in which anthocyanin accumulation is correlated with redifferentiation from the undifferentiated callus.

Many cultures (27 different species) were found to be capable of anthocyanin production. Seven different anthocyanidins were detected: cyanidin (17 species), delphinidin (5 species), malvidin (4 species), petunidin (3 species), pelargonidin (2 species), and one example for both peonidin and hirsutidin. With regard to acyl moieties, most of the data seem to be unreliable due to inappropriate extraction procedures (see Section II,B,2). In general, it can be assumed that plant cells cultivated *in vitro* retain a capacity for producing the same anthocyanins as *in vivo*.

Much information is available on the anthocyanins of various carrot cultures, in particular those of *Daucus carota* ssp. *sativa* ("black carrot") (Alfermann and Reinhard, 1971; Schmitz and Seitz, 1972; Harborne *et al.*, 1983). Other cultivars were studied by Sugano and Hayashi (1967; i.e., cv. Kintoki) and Ozeki and Komamine (1981; i.e., cv. Kuroda-gosan). Dougall and Weyrauch (1980) as well as Hemingson and Collins (1982) used a culture derived from wild carrot. Most likely, these carrot cultures contain anthocyanins of similar structures: cyanidin 3-di- or triglycosides, probably acylated (see Section II,B and Fig. 2), which are the characteristic pigments in the Umbelliferae (Harborne, 1976). The detection of a malvidin 3,5-diglycoside in a carrot culture (Ibrahim *et al.*, 1971) is certainly erroneous. Likewise, reports on cyanidin 3,5-digalactoside (Hemingson and Collins, 1982) are questionable. A second important system for biosynthetic and kinetic studies is *Machaeranthera gracilis* (≡ *Haplopappus gracilis*) (von Ardenne, 1965; Stickland and Sunderland, 1972; Fritsch and Grisebach, 1975; Wellmann *et al.*, 1976).

IV. BIOSYNTHESIS OF ANTHOCYANINS

The cooccurrence of anthocyanins and flavonols in many plant species suggests that these two classes of flavonoids are closely related with respect to their biosynthetic pathways (Ebel and Hahlbrock, 1982). The primary flavonoid precursors are chalcones. One prerequisite for chalcone synthesis is the general phenylpropanoid pathway, leading to hydroxycinnamoyl:CoA thioesters. This pathway is described in Chapter 2, this volume. The pathway leading to cyanidin, starting with the biosynthesis of chalcones, is shown in Fig. 3.

Chalcone

Flavanone

Dihydrokaempferol

Eriodictyol

Dihydroquercetin

3.4-Leucocyanidin (Flavan-3.4-diol)

Cyanidin

Fig. 3. Pathway leading to cyanidin. For enzyme reactions 5–8, see text.

Cell cultures are suitable systems for studying biosynthetic pathways. In the case of flavonoids, including anthocyanins, three approaches were made: (1) tracer experiments with radioactive precursors, (2) isolation and characterization of the enzymes involved in this pathway, and (3) feeding experiments with putative biosynthetic intermediates subsequent to a genetic or physiological block. Genetically defined mutants from intact plants with respect to anthocyanin biosynthesis exist for many angiosperms: *Antirrhinum majus* (Harrison and Stickland, 1974;

Stickland and Harrison, 1974), *Petunia hybrida* (Kho *et al.*, 1975, 1977), *Zea mays* (McCormick, 1978), *Matthiola incana, Callistephus chinensis, Dianthus caryophyllus* (see review by Seyffert, 1982). Physiological blocks can be produced for the general phenylpropanoid pathway by use of inhibitors and phytohormones (see Section V,C). In addition to the chemogenetic studies with mutants, enzymatic investigations using cell cultures mainly contributed to an understanding of the sequence of enzyme reactions involved in anthocyanin synthesis, as shown in Fig. 3. Enzymes and reactions of the general phenylpropanoid pathway as well as the biosynthesis of chalcones (chalcone synthase), flavanones (chalcone isomerase), and dihydroflavonols (flavonoid 3'- and flavanone 3-hydroxylases) are described in Chapter 2, this volume.

Here, some information on enzymes from anthocyanin-producing systems should be added, although, in general, they do not differ from common enzymes of flavonol biosynthesis. Chalcone synthase (CHS, EC 2.3.1.74), the first enzyme of the flavonoid pathway, has been characterized extensively with parsley cell cultures (for reviews, see Ebel and Hahlbrock, 1982; Dixon *et al.*, 1983). This enzyme has also been found in cyanidin-synthesizing suspension cultures; the synthase from *Machaeranthera gracilis* showed behavior with respect to pH optima and substrate specificity similar to that of the parsley enzyme (Saleh *et al.*, 1978). This culture coaccumulated cyanidin and quercetin glycosides (Stickland and Sunderland, 1972). CHS from carrot cell cultures has been investigated. In this system cyanidin is the only flavonoidal aglycone (H. U. Seitz, unpublished). The CHS of carrot likewise did not show any significant difference from the parsley enzyme with respect to pH dependence, substrate specificity, end-product inhibition, and structure (Hinderer and Seitz, 1985; Ozeki *et al.*, 1985). Investigations with carrot suggested that *in vivo* and *in vitro* CHS is rate-limiting for anthocyanin formation and the key enzyme for the regulation of anthocyanin biosynthesis (Hinderer *et al.*, 1984; Hinderer and Seitz, 1985, 1986).

Contrary to earlier reports, the primary products of the CHS reaction are chalcones and not flavanones, as shown with highly purified parsley enzyme (Heller and Hahlbrock, 1980) and in assays with lowered pH (Sütfeld and Wiermann, 1980). Chalcone formation *in vitro* has also been demonstrated with purified CHS from carrot (Ozeki *et al.*, 1985) and with extracts from a mutant of *Dianthus caryophyllus* that lacks chalcone isomerase (No. 5 in Fig. 3; CHI; EC 5.5.1.6) (Spribille and Forkmann, 1982). CHI activity was detected in various anthocyanin-containing flowers (Seyffert, 1982; Chmiel *et al.*, 1983; Van Weely *et al.*, 1983; Hinderer *et al.*, 1983) and in cell cultures of *Machaeranthera gracilis* (Wellmann *et al.*, 1976) and *Daucus carota* (Hinderer *et al.*, 1984).

As mentioned above, the natural starter substrate of CHS is believed to be 4-coumaroyl-CoA in carrot (Hinderer and Seitz, 1985) as well as in parsley (Schütz *et al.*, 1983). Therefore, naringenin should be a common precursor of all anthocyanins (and most other flavonoids; see Chapter 2, this volume) and substitution of ring B occurs at the flavonoid stage. Nevertheless, it cannot be said with certainty that hydroxyl groups of ring B are not introduced at an earlier stage in some species.

Formation of eriodictyol (3′-hydroxynaringenin) using caffeoyl-CoA instead of 4-coumaroyl-CoA was demonstrated *in vitro* with CHS from different sources, for example, from cell cultures of parsley, *Machaeranthera gracilis* (Saleh *et al.*, 1978), and carrot (Hinderer and Seitz, 1985). The CHS from carrot had about one order of magnitude higher affinity for 4-coumaroyl-CoA than for caffeoyl-CoA, and it has been suggested that the catalyzed reaction leading to eriodictyol *in vitro* does not play a role *in vivo* (Hinderer and Seitz, 1985). Feeding experiments with successful incorporation of naringenin in cyanidin had been performed with cell cultures of *Machaeranthera gracilis* (Fritsch and Grisebach, 1975) and *Daucus carota* (Hinderer *et al.*, 1984) and with flowers of *Matthiola incana* (Seyffert, 1982). The hydroxylating enzymes (Nos. 6 and 7 in Fig. 3; flavonoid 3′-, flavonoid 3′,5′-, and flavanone 3-hydroxylase) leading to eriodictyol and dihydroflavonols are mentioned in Chapter 2, this volume. Feeding experiments using cell cultures and flowers (mutants) supported evidence that dihydroflavonols (dihydrokaempferol, dihydroquercetin) are precursors of anthocyanin biosynthesis (Fritsch *et al.*, 1971; Fritsch and Grisebach, 1975; Seyffert, 1982; Hinderer *et al.*, 1984; Ozeki and Komamine, 1985a). It was suggested that dihydroflavonols are the last stage during anthocyanin biosynthesis where the 3′-hydroxy group can be introduced (Heller *et al.*, 1985a).

Little has been known about the reactions leading from the dihydroflavonol to the corresponding anthocyanidin. Flavan-3,4-diols and flav-3-enes were supposed to be intermediates in the anthocyanin pathway (Hrazdina, 1982). The former ones are the leucoanthocyanidins, which occur as di- or oligomeric forms in proanthocyanidins (see Chapter 4, this volume). Studies with acyanic mutants of *Matthiola incana* gave the evidence that leucoanthocyanidins are indeed precursors of anthocyanins. Successful supplementation of defined lines, blocked in anthocyanin biosynthesis, with leucopelargonidin and leucocyanidin were in correlation with the corresponding genotype (Heller *et al.*, 1985a). In addition, the enzyme for the conversion of dihydroflavonols into flavan 3,4-*cis*-diols has been demonstrated with flower extracts from *M. incana* (Heller *et al.*, 1985b). This dihydroflavonol 4-reductase (No. 8 in Fig. 3) catalyzes a NADPH-dependent, stereospecific reduction of (+)-di-

hydroflavonols to the corresponding cis isomers of leucoanthocyanidins. The best substrate was dihydrokaempferol, but dihydroquercetin and dihydromyricetin were also reduced. The occurrence of this enzyme activity among the mutants of *M. incana* could be correlated with the particular genotype. This new enzyme has also been found in cell cultures of *Pseudotsuga menziesii* and *Ginkgo biloba* and has been related to the synthesis of proanthocyanidins (Stafford and Lester, 1985). This shows a close correlation between anthocyanidin and proanthocyanidin biosynthesis.

Anthocyanins are all *O*-glycosides, and it is most likely that a first glycosylation step in the 3 position stabilizes the flavylium cation. Enzymes catalyzing glycosylations of flavonoids are well characterized and have been listed by Ebel and Hahlbrock (1982) (see also Chapter 2, this volume). These glycosyltransferases use mainly UDP sugars as donor substrates and show remarkable specificities for the position of glycosylation, whereas the specificity for the acceptor substrate is, in general, relatively low. So, the anthocyanidin 3-*O*-glucosyltransferases from red cabbage (Saleh *et al.*, 1976a) and cell cultures of *Machaeranthera gracilis* (Saleh *et al.*, 1976b) and carrot (Petersen and Seitz, 1986) glucosylated both anthocyanidins and flavonols. Interestingly, the anthocyanin accumulating *in vivo* in carrot cell cultures possesses a galactosyl residue as the first sugar attached to cyanidin (see Fig. 2). Therefore, UDPgalactose was also offered, which was a better donor substrate than UDPglucose (W. Hopp, unpublished). An anthocyanidin-specific 3-*O*-glucosyltransferase was described from *Silene dioica* petals (Kamsteeg *et al.*, 1978a).

The glycosylation of the aglycone is accepted to be a final step in anthocyanin biosynthesis. Frequently, the 3-monoglycosides are not the end products accumulating in the cells, and subsequent glycosylations, acylations, and methylations take place *in vivo*. Enzymes for further glycosylations of anthocyanidin 3-monoglycosides have been found in *Silene dioica*: a 3-*O*-glucoside rhamnosyltransferase and a 3-rhamnosylglucoside 5-*O*-glucosyltransferase (Kamsteeg *et al.*, 1980a,b). The consecutive action of these three different glycosyltransferases can readily explain the occurrence of 3-rutinoside-5-glucosides of pelargonidin and cyanidin *in vivo* (Kamsteeg *et al.*, 1978b).

As mentioned in Section II,B, the accumulation of acylated anthocyanins is widespread within the angiosperms. Among the acyl moieties used for acylation of anthocyanins, cinnamic acids and malonic acid are very common. Enzymes for malonyl transfer have been characterized frequently in parsley and chickpea (see Chapter 2, this volume) that are related to the biosynthesis of 6-*O*-malonylglucosides of flavonols, flavones, and isoflavones (Matern *et al.*, 1981; Köster *et al.*, 1984). Mal-

onyltransferases for anthocyanins have not been reported, but it seems likely that malonyl-CoA is the donor substrate.

Petals of *Silene diocia* offer a good system for studying conjugation of anthocyanins. A cinnamoyltransferase, which catalyzes the acylation of anthocyanidin 3-rhamnosylglucoside or 3-rhamnosylglucoside-5-glucoside in the 4 position of rhamnose, was described by Kamsteeg *et al.* (1980c). This enzyme used 4-coumaroyl-CoA and caffeoyl-CoA as donor substrates. In general, the transferases require activated donor substrates, such as UDP sugars (glycosyltransferases) and acyl-CoAs (acyltransferases).

Besides hydroxylation, *O*-methylation often occurs as a substitution reaction in anthocyanin biosynthesis. Methyltransferases (OMTs) for the methylation of flavones, flavonols, and isoflavones are well characterized and are discussed in Chapter 2, this volume. These enzymes methylate flavonoid aglycones. Petunidin, malvidin, and peonidin are methylated in positions 3′ and 5′ (see Table I and Fig. 1). Very rarely are A ring hydroxyls methylated (Hrazdina, 1982). The *O*-methylation of anthocyanins has been demonstrated with extracts from flowers of *Petunia hybrida*. The donor substrate was *S*-adenosyl-L-methionine (SAM), and 3-(*p*-coumaroyl)-rutinoside-5-glucosides of cyanidin, delphinidin, or petunidin acted as acceptor substrates, which were methylated in the 3′ and 5′ positions. No methylation was observed with anthocyanidins, anthocyanidin 3-glucosides, or 3-rutinosides (Jonsson *et al.*, 1982). Four isoenzymes from *P. hybrida* could be separated due to their different pI's. They possessed similar properties, except for their affinity to the substrates and the mechanism of inhibition by demethylated SAM (Jonsson *et al.*, 1984).

In summary, at least in *Petunia hybrida*, methylation is the last step in anthocyanin biosynthesis, occurring after glycosylation and acylation, contrary to what has been observed for the pathways to flavones, flavonols, and isoflavones, where methylation together with hydroxylation occurs at the stage of the aglycone. It remains to be seen if this holds true for anthocyanin synthesis in general.

V. REGULATION OF ANTHOCYANIN BIOSYNTHESIS IN TISSUE AND CELL CULTURES

Regulation of anthocyanin biosynthesis is closely connected with regulation of flavonoid biosynthesis, in general. The pathway leading to

flavonoids or anthocyanins can be subdivided into two parts: (1) the general phenylpropan pathway, starting with phenylalanine and leading to hydroxycinnamoyl-CoA thioesters, and (2) the flavonoid-(acyl)-glycoside pathway, starting with chalcone formation and leading to the actual flavonoid conjugates. The enzymes of parsley cell cultures have been divided into two groups according to their coordinated regulation within each induction group (for a review, see Hahlbrock and Grisebach, 1979; see also Chapter 2, this volume). Considering reports on regulation of enzyme activities responsible for flavonoid biosynthesis, two have been thought to be key enzymes: phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), both being located at branching points of the pathway, with PAL leading to the general phenylpropan and CHS to the flavonoid pathway.

For anthocyanins most of the enzymatic studies on the regulation of biosynthesis have involved PAL, which can be assayed in most sources without major problems. In recent years data on the action of CHS in anthocyanin-synthesizing systems has become available. In general, this enzyme has been found at activity levels one to two orders of magnitude lower than that of PAL. It is believed to play a more important role in the regulation of anthocyanin biosynthesis (Hinderer *et al.*, 1983, 1984; Ozeki *et al.*, 1985; Ozeki and Komamine, 1985a).

A. Accumulation in Relation to Cytodifferentiation

Most of the culture systems mentioned in Table I are reported to be heterogeneous with regard to anthocyanin pigmentation. Colored and uncolored cells occur in one and the same callus or cell culture. The frequency of anthocyanin-containing cells can be very low, as, for example, in *Petunia hybridia* callus (Colijn *et al.*, 1981).

Anthocyanin pigmentation is a cytodifferentiation process, and therefore, it is not surprising that it is strongly effected by phytohormones (see Section V,C). The close relationship between anthocyanin formation and morphological differentiation (embryogenesis) was shown with a carrot suspension culture (Ozeki and Komamine, 1981).

Many investigators were able to increase the anthocyanin content considerably by selecting cell lines with high potency for anthocyanin production in, for example, *Daucus carota* (Alfermann and Reinhard, 1971), *Dimorphotheca sinuata* (Ball *et al.*, 1972), *Petunia hybrida* (Colijn *et al.*, 1981), and *Vitis hybrida* (Yamakawa *et al.*, 1983). The feasibility of selecting cell lines was demonstrated by Yamamoto *et al.* (1982), who for the selection of anthocyanin-producing *Euphorbia milli* strains analyzed cultures over 29 passages using cell pedigree and statistics. The result

was a strain capable of a sevenfold increase in anthocyanin production. The pigment level was found to be stable after 24 subcultures. Another method for selection of high-pigment-producing strains was described for the carrot suspension cells. Ozeki and Komamine (1981) reported that the ability for anthocyanin synthesis depended on the size of the cell aggregates. They subsequently used sieving procedures (nylon nets) and density-gradient centrifugation (Ficoll) to select anthocyanin-producing cells. Furthermore, clonal studies with carrot cell cultures indicated that the increase in the ability of cells to accumulate anthocyanin is not due to mutations (Dougall *et al.*, 1980). Finally, the potential to accumulate anthocyanins can be maintained during cryopreservation in liquid nitrogen (Seitz *et al.*, 1985).

B. Role of Light

In general, anthocyanin accumulation in cell or tissue cultures strictly depends on or at least is strongly stimulated by light. There are some exceptions, however, which should be mentioned. The accumulation of anthocyanins in the dark has been reported in *Strobilanthes dyeriana* (Smith *et al.*, 1981), *Vitis hybrida* (Yamakawa *et al.*, 1983), and several carrot cultures (Alfermann and Reinhard, 1971; Schmitz and Seitz, 1972; Noé *et al.*, 1980; Ozeki and Komamine, 1981; Harborne *et al.*, 1983). The latter may have derived this ability from the black carrot (*Daucus carota* ssp. *sativa*), which can accumulate large amounts of anthocyanins in the root.

The cultures of *Machaeranthera gracilis* are good examples for light-induced anthocyanin accumulation. Dark-grown callus cultures were completely devoid of anthocyanins (Reinert *et al.*, 1964; von Ardenne, 1965). Anthocyanin formation was effected by blue light with high energy but not by red light (Reinert *et al.*, 1964). Lackmann (1971) determined 372 and 438 nm as maxima of the action spectrum. With *Populus hybrida* cell cultures, blue light was also found to be the most effective (Matsumoto *et al.*, 1973). More advanced studies using suspension cultures of *Machaeranthera gracilis* revealed UV below 345 nm as the only light frequency inducing anthocyanin formation. Contrary to parsley cell cultures (see Chapter 2, this volume), only high doses of continuous UV were effective (Wellmann *et al.*, 1976). Under these conditions drastic increases in activities of PAL, CHS, and CHI prior to anthocyanin accumulation had been observed. Here, it may not be excluded that anthocyanin production on ultraviolet (UV) irradiation is a photoindependent stress response (Hrazdina, 1982). For reports on the involve-

ment of phytochrome in anthocyanin biosynthesis see the review by Hrazdina (1982).

C. Influence of Growth Substances

As mentioned in Section V,A, anthocyanin accumulation as part of cytodifferentiation may be closely related to morphological differentiation even in cell cultures. Differentiation processes are regulated by phytohormones. The composition of growth substances in the medium, therefore, strongly influences anthocyanin accumulation. Chemicals promoting or inhibiting anthocyanin synthesis, including growth substances, are summarized in tables by Hrazdina (1982) and Dougall (1979).

Dark-grown carrot tissue cultures require the presence of an auxin (2,4-D, NAA, or IAA) for anthocyanin accumulation; auxins may substitute for light (Alfermann and Reinhard, 1971). Comparative studies on the effect of auxins in light-dependent and -independent anthocyanin formation in different carrot clones suggest that anthocyanin is not regulated by PAL, but by an enzyme in a later part of the pathway (Stärk *et al.*, 1976).

Contrary to this, Sugano and Hayashi (1967) found inhibition of anthocyanin accumulation in light-grown carrot callus cultures when 2,4-D was used as an auxin. Ozeki and Komamine (1981, 1982) induced carrot cell suspensions for anthocyanin synthesis by withdrawing 2,4-D. Subsequent addition of auxins inhibited anthocyanin synthesis completely. Studies on the enzymatic control of this induction and suppression suggest that CHS may be the key enzyme in anthocyanin formation regulated by 2,4-D in this system, and its activity changes are reflected in changes of the level of transcription (Ozeki and Komamine, 1985b). Inhibition of anthocyanin accumulation by auxins was also observed with cell cultures of *Machaeranthera gracilis* (Constabel *et al.*, 1971; Stickland and Sunderland, 1972), *Petunia hybrida* (Colijn *et al.*, 1981), *Vitis hybrida* (Yamakawa *et al.*, 1983), and *Ipomoea batatas* (Nozue and Yasuda, 1985), whereas cultures of *Dimorphotheca auriculata* (Harborne *et al.*, 1970) and *Rosa* sp. (Davies, 1972) showed enhanced pigment formation by auxin treatment.

Unlike auxins, exogenous gibberellins are not necessary for growth of plant culture systems. Nevertheless, gibberellic acid (GA_3) showed strong inhibitory effects on anthocyanin accumulation in several cultures. Arditti and Ball (1971) briefly reported the inhibitory effect of GA_3 on anthocyanin formation in *Dimorphotheca sinuata* callus. Using

carrot callus cultures, a broad dose dependence between 10^{-12} *M* (no inhibition) to 10^{-4} *M* (full inhibition) was found (Schmitz and Seitz, 1972). Continuous cultivation in the presence of GA_3 (3×10^{-7} *M*) resulted in anthocyanin-free cell lines. These cells were also devoid of any other flavonoids as well as CHS and CHI activities (H. U. Seitz and W. Hinderer, unpublished). Enzymes of the general phenylpropan pathway are still present, however, but with reduced activities (Heinzmann and Seitz, 1974; Heinzmann *et al.*, 1977). Similarly, in cell cultures of *Machaeranthera gracilis*, light-induced anthocyanin accumulation could be totally inhibited by GA_3, whereas PAL was not (Gregor, 1974). Comparative enzymatic studies and feeding experiments performed with callus and suspension cultures of *Daucus carota* clearly demonstrated that GA_3 blocked anthocyanin synthesis at the level of CHS (Hinderer *et al.*, 1984). This enzyme activity rapidly declined and showed a strict correlation to both GA_3 and anthocyanin concentration. The inhibitory effect of GA_3 on anthocyanin synthesis was also reported for two other carrot cell cultures (Ozeki and Komamine, 1982; Cheng *et al.*, 1985).

Like auxins, cytokinins are essential growth substances in the field of plant tissue culture. Few data are available for the action of cytokinins on the accumulation of anthocyanins. In *Populus* cell suspension cultures, kinetin showed a marked inhibitory effect on anthocyanin production (Matsumoto *et al.*, 1973). Ozeki and Komamine (1982) reported a promotive effect of various cytokinins in carrot cell cultures, induced for anthocyanin synthesis by media lacking auxins. Cell cultures of *Machaeranthera gracilis* showed, likewise, increased pigment content with increased cytokinin [6-benzylaminopurine (BAP), kinetin] concentrations in media with low auxin concentrations. This effect was reversed in presence of high auxin levels (Constabel *et al.*, 1971). The counteraction of auxins and cytokinins with respect to anthocyanin accumulation was also demonstrated with cell cultures of grape vine (Yamakawa *et al.*, 1983).

The action of abscisic acid (ABA) in anthocyanin-producing cell cultures was reported only once. Like GA_3, ABA suppressed induced anthocyanin synthesis in carrot cell cultures (Ozeki and Komamine, 1982).

D. Composition of Nutrients

The composition of the medium with respect to salts and carbon source can influence accumulation of secondary products in cell cultures (Zenk *et al.*, 1977). It is suggested that the culture conditions for maximal

growth differs from those for maximal accumulation of a specific secondary product. The media most used for plant tissue cultures are growth media. In order to develop a "production medium" three components have to be considered: (1) the carbon source, mostly sucrose or glucose, and its concentration, (2) the concentration of nitrogen-containing salts, mostly nitrate and ammonium, and (3) the concentration of phosphate. Further, compounds as well as environmental conditions, such as pH, temperature, and aeration rate, have to be taken into consideration. Each constituent of the culture medium may have a different effect on growth and anthocyanin accumulation.

High sucrose (e.g., 8%) and low phosphate and nitrate concentrations induced anthocyanin formation, whereas addition of phosphate or nitrogen-containing mineral salts suppressed anthocyanin synthesis, in cell cultures of *Catharanthus roseus* (Knobloch *et al.*, 1982) and *Vitis* (Yamakawa *et al.*, 1983). High sucrose concentration also stimulated anthocyanin accumulation in *Populus* cell cultures (Matsumoto *et al.*, 1973), but in cultures of *Dimorphotheca sinuata* (Ball and Arditti, 1974) and *Petunia hybrida* (Colijn *et al.*, 1981) this condition led to inhibition of anthocyanin formation. The sugar source may also affect anthocyanin production. The best sugar for supporting anthocyanin formation was found to be sucrose in maize (Straus, 1959) and *Populus* cell cultures (Matsumoto *et al.*, 1973), and glucose in *Parthenocissus tricuspidata* (Bleichert and Ibrahim, 1974).

Using chemostat-grown carrot cells, Dougall *et al.* (1983a,b) demonstrated that limiting the growth by reducing phosphate or ammonium resulted in increased anthocyanin yields.

VI. ACCUMULATION OF ANTHOCYANINS IN THE CENTRAL VACUOLE

Fritsch and Grisebach (1975) suggested a hypothesis for anthocyanin biosynthesis, specifying that transport into the central vacuole parallels glycosylation by a glycosyltransferase, which should be located in the tonoplast membrane. In view of this interesting hypothesis, several attempts were made to prove the subcellular localization of these enzymes in the vacuolar membrane. Hrazdina *et al.* (1978), using vacuoles from petals of *Hippeastrum* and *Tulipa*, showed that CHS, CHI, and a UDPglucose:anthocyanidin glucosyltransferase were neither present in isolated vacuoles nor in a pellet representing tonoplast membranes.

Similar results were reported with respect to a methyltransferase in *Petunia hybrida* (Jonsson *et al.*, 1983). There are strong indications that the endoplasmic reticulum is the site of phenylpropane and flavonoid biosynthesis (Wagner and Hrazdina, 1984). Hopp *et al.* (1985) showed that among the enzymes tested, only CHS was partially associated with tonoplast membranes. This might be an indication that chalcone synthesis is carried out at the cytosolic face of the tonoplast. Sasse *et al.* (1979) and Hopp *et al.* (1985) established isolation and purification procedures for anthocyanin-containing vacuoles from cell cultures of *Daucus carota.* The stability of these anthocyanin-containing carrot vacuoles was very high. No anthocyanin efflux occurred within at least 2 hr (Hopp *et al.*, 1985). These vacuolar preparations are an ideal material for studying anthocyanin uptake. Radioactive anthocyanin labeled *in vivo* was isolated from anthocyanin-containing cell cultures and used for transport studies with isolated vacuoles. The structure of the anthocyanin from carrot cell cultures was proven to be a cyanidin-3-(sinapoylxylosylglucosylgalactoside) by Harborne *et al.* (1983); a reinvestigation by Hopp and Seitz (1987) came to the same result. This anthocyanin is transported into isolated vacuoles by a pH-dependent mechanism with an optimum at pH 7.5. The transport can be impaired by a protonophore like carbonyl cyanide *m*-chlorophenylhydrazone. A direct involvement of ATP·Mg has not been proven. The anthocyanin transport seems to be specific, because vacuoles isolated from other species do not accumulate this cyanidin glycoside. Glycosylation and, especially, acylation with sinapic acid are absolutely essential prerequisites for the transport; deacylated anthocyanin is not taken up by isolated vacuoles. Anthocyanin transport is thought to function as an ion-trap mechanism (Hopp and Seitz, 1987). In this context it is of great interest to understand the molecular mechanism of anthocyanins in aqueous acid media similar to those of the vacuolar sap. Under these conditions equilibrium between the flavylium cation and a quinoidal base does exist. At pH values ranging from 6 to 8, deprotonation leads to resonance-stabilized quinoidal anions and also to a neutral quinoidal base. It was suggested that anthocyanin is transported in the neutral form (Matile, 1984). At low pH values in aqueous media, water addition in position 2 and, to a less extent, in position 4 leads to colorless compounds, so-called carbinol pseudobases and chalcone pseudobases. Brouillard (1981), therefore, suggested a molecular mechanism preventing hydration. The value of acylation might be stacking of the aromatic part of the acid and the pyrylium ring of the anthocyanidin. Best protection would be achieved with diacylated anthocyanins, but monoacylated ones may protect themselves by self-association of anthocyanin molecules (Asen *et al.*, 1972). It was also suggested by Brouillard (1983) that the

glycosidic part of the molecule functions as a spacer bearing the acyl residue, which may interact with the pyrylium ring. With regard to these data, a general principle for the uptake of anthocyanins might be proposed. Acylation is required for transport by preventing hydration, with consequent loss of color in the vacuolar sap of intact vacuoles (Hopp and Seitz, 1987). Alternatively anthocyanins might be synthesized within provacuoles, which may fuse with other provacuoles or the central vacuole. Such a mechanism was proposed for transport of tannins (Baur and Walkinshaw, 1974; Parham and Kaustinen, 1977).

With respect to the molecular organization of anthocyanins in the vacuolar sap, interesting cytological observations were made. Pecket and Small (1980) and Small and Pecket (1982) described intensively colored bodies within the vacuolar sap of more than 70 species. These anthocyanoplasts have been known for some time (Bünning, 1949). More recently, Hemleben (1981), Neumann (1983) and Nozue and Yasuda (1985) also observed these vacuolar structures. It has been suggested that these anthocyanoplasts are associated with membranes and that these structures are the site of anthocyanin biosynthesis (Nozue and Yasuda, 1985; Yasuda and Shinoda, 1985).

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CHAPTER 4

Proanthocyanidins and Catechins

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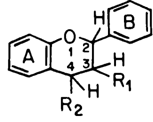
I. OCCURRENCE

Catechins, leucoanthocyanidins, and proanthocyanidins are flavan derivatives:



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Catechins are flavan-3-ol compounds (R_1 = OH; R_2 = H), and monomeric leucoanthocyanidins are flavan-3,4-diols (R_1 = R_2 = OH). The oligomers and polymers resulting from the condensation of catechins and leucoanthocyanidins are commonly referred to as proanthocyanidins (Haslam, 1979). Because leucoanthocyanidins (particularly those with 5,7-dihydroxy substitution in the A ring) are unstable and are rapidly involved in subsequent metabolism, investigators deal as a rule with catechins and proanthocyanidins. In cell cultures, formation of proanthocyanidins is also usually accompanied by the formation of catechins. Therefore, in this chapter, both flavonoid classes will be considered simultaneously.

As early as in 1962, the callus tissues of *Acer pseudoplatanus* cultured on media with 2,4-dichlorophenoxyacetic acid (2,4-D) were found to produce several proanthocyanidins (Goldstein *et al.*, 1962). Nearly at the same time, it was shown that the callus tissues of *Juniperus communis* accumulate proanthocyanidins as well, but their content is eight times lesser than that in the corresponding intact plant (Constabel, 1963).

Later on, the formation of proanthocyanidins was investigated in more detail with callus tissues initiated from woody stems of the tea plant (*Camellia senensis*) (Forrest, 1969) and cambial tissues of several *Prunus* species (Feucht, 1975), as well as with the cell suspension cultures of Paul's scarlet rose (Davies, 1972a), and *Acer pseudoplatanus* (Westcott and Henshaw, 1976). In all of these cases, in the analyzed cells and tissues together with proanthocyanidins, simple catechins [(−)-epicatechin and (+)-catechin] were also detected. (−)-Epicatechin and (+)-catechin were also found to accompany proanthocyanidins in cultured callus tissues derived from young stems and leaves of the tea plant (Koretzkaya and Zaprometov, 1975a), in callus tissues from young shoots and cell-suspension cultures of *Crataegus monogyna*, *C. oxyacantha*, and *Ginkgo biloba* (Schrall and Becker, 1977), in callus tissues from cotyledons and cell-suspension cultures of *Pseudotsuga menziesii* (Stafford and Cheng, 1980), and in callus tissues from young shoots and cell suspension cultures of *Cryptomeria japonica* (Ishikura and Teramoto, 1983).

At the same time, in callus tissues derived from *Theobroma cacao* cotyledons, one catechin only, namely, (−)-epicatechin, was detected besides proanthocyanidins (Jalal and Collin, 1977), and in the cell-suspension culture of *Phaseolus vulgaris* only a small amount of proanthocyanidins was found (Dixon and Bendall, 1978).

The structures of (+)-catechin, (−)-epicatechin, and one of the common dimeric proanthocyanidins, B-1 proanthocyanidin, are given on page 79:

(+)-Catechin (−)-Epicatechin B-1 Proanthocyanidin

Acid-catalyzed hydrolysis of B-1 proanthocyanidin yields cyanidin from the upper part of the molecule, and (+)-catechin from the lower part. Modern terminology also designates B-1 proanthocyanidin as the dimer (−)-epicatechin–(+)-catechin (Haslam, 1979). The most common dimeric proanthocyanidins are B-2 [(−)-epicatechin-(−)-epicatechin], and B-4 [(+)-catechin-(−)-epicatechin].

The cultivation of flavan-synthesizing tissues and cells was mostly performed on Heller's and Murashige–Skoog's nutrient media with addition of auxins [usually 2,4-D, sometimes naphthaleneacetic acid (NAA)] and frequently, cytokinins [kinetin; 6-benzylaminopurine (BAP); zeatin].

Rapid synthesis of proanthocyanidins and catechins, as well as of other phenolic compounds, usually starts after nitrate depletion of the nutrient medium (Nash and Davies, 1972) and reaches a maximum at the end of the linear growth phase (Schrall and Becker, 1977; Zaprometov *et al.*, 1979).

As a rule, cultured cells and tissues accumulate less flavan derivatives than do the corresponding tissues of intact plants. For example, the total amount of proanthocyanidins and catechins in the callus tissues of *Prunus* was about 25 times lower than in the intact plant tissues (Feucht, 1975); the callus tissues of *Theobroma cacao* synthesized 6 times less proanthocyanidins and 100 times less (−)-epicatechin than did the cotyledons (Jalal and Collin, 1977); the cell-suspension cultures of *Cryptomeria japonica* contained 100–200 times less flavan compounds than the leaves of this plant (Teramoto and Ishikura, 1985); and the callus tissues initiated from tea-plant leaves and stems contained 20–50 times less flavan compounds than the corresponding intact plant tissues (Zaprometov *et al.*, 1979).

As an exception, the cell-suspension culture of *Pseudotsuga menziesii* should be mentioned. In this case, the level of proanthocyanidins reached 40%, while in cotyledons it was only 2–3% (Stafford and Cheng, 1980).

It is well known that the ability of plant tissues and cells cultivated *in vitro* to synthesize various secondary compounds depends to a great degree on the composition of the nutrient medium (the sources and amounts of carbon and nitrogen), hormones and their analogs, as well as illumination.

II. INFLUENCE OF PHYTOHORMONES

A. Auxins

Although indolyl-3-acetic acid (IAA) itself is not used for culturing flavan-synthesizing cells and tissues, its closely related analog, indolylbutyric acid, has been included at a concentration of 1 mg/liter in nutrient media for the tissue cultures of *Cryptomeria japonica* (Ishikura and Teramoto, 1983) and *Theobroma cacao* (Jalal and Collin, 1977). However, in most investigations the synthetic growth regulators 2,4-D and NAA were used.

In the callus tissues of the tea plant derived both from young stems and leaves, flavan formation was stimulated by 2,4-D in the range 5×10^{-6} to 2×10^{-5} *M* (Koretzkaya and Zaprometov, 1975a,b; Zaprometov *et al.*, 1979). Further increase in 2,4-D concentration resulted in progressive lowering of the biosynthesis of both catechins and proanthocyanidins.

In cell suspension cultures of Paul's scarlet rose, suppression of the flavan synthesis began at lower 2,4-D concentrations, starting from 7.5×10^{-7} *M* (Davies, 1972b). The optimal 2,4-D concentration for the synthesis of flavan compounds in the cell culture of *Acer pseudoplatanus* was 9×10^{-7} *M* (Westcott and Henshaw, 1976). Nearly the same 2,4-D concentration (10^{-6}–10^{-5} *M*) was optimal for flavan synthesis in the callus tissues of *Eucalyptus robusta* and *Cryptomeria japonica* (Samejima *et al.*, 1982). Contrary to these data, complete suppression of proanthocyanidin synthesis by 2,4-D (2×10^{-7} to 2×10^{-5} *M*) was observed in callus cultures of *Crataegus monogyna* (Schrall and Becker, 1977).

NAA was used in the cultivation of *Crataegus monogyna* callus tissues (Schrall and Becker, 1977). In this case, increase in the NAA concentration from 2×10^{-7} to 2×10^{-5} *M* resulted in sharp stimulation of tissue growth, but significant reduction of the biosynthetic potential of proanthocyanidin formation. NAA at a concentration of 5×10^{-5} *M* was used in cell culture of *Pseudotsuga menziesii* (Stafford and Cheng, 1980).

In callus tissues of *Cryptomeria japonica* and *Eucalyptus robusta*, the effect of NAA (10^{-7}–10^{-6} *M*) in promoting the formation of flavan compounds was somewhat inferior to that of 2,4-D (Samejima *et al.*, 1982).

The investigation of the influence of NAA on the formation of flavan compounds in tea-plant callus tissues revealed that substitution of NAA (3×10^{-7} *M*) for 2.4-D (2×10^{-5} *M*) led to considerable increase in the synthesis of flavans (Zagoskina and Zaprometov, 1979). With 2×10^{-5} *M* NAA the content of flavan compounds increased more than 10 times as compared to the control (2×10^{-5} *M* 2,4-D).

In subsequent subculturing the formation of flavans can still be increased up to two times, though tissue growth decreases in this case. Thus in tea-plant callus cultures 2,4-D is the best auxin for tissue growth, and NAA for the formation of flavans. In cell suspension cultures of the tea plant, NAA also stimulated the formation of flavans but without lowering cell growth (Bagratishvili *et al.*, 1980).

B. Cytokinins

Zeatin (0.05 mg/liter) was used as a component of the nutrient media for cultivation of flavan-synthesizing tissues and cells of *Theobroma cacao*, though the specificity of its effect was not studied (Jalal and Collin, 1977). The nutrient medium for the cultivation of callus tissues derived from the tip section of a young *Cryptomeria japonica* shoot contained 0.1 mg/liter kinetin; for the subsequent cultivation of the cell suspension the kinetin concentration was lowered to 0.01 mg/liter (Ishikura and Teramoto, 1983). BAP at the same concentration (0.01 mg/liter = 0.5 μM) was used for cell cultures of *Pseudotsuga menziesii* (Stafford and Cheng, 1980).

The investigation of the effect of three kinetin and BAP concentrations (0.01, 0.1, and 1.0 mg/liter) on cell growth and formation of catechins and proanthocyanidins in callus tissues of *Cryptomeria japonica* showed that the greatest effect on the accumulation of both classes of flavan compounds was reached with 1.0 mg/liter kinetin (Samejima *et al.*, 1982). BAP was less efficient and at the concentration of 1.0 mg/liter suppressed the synthesis of flavan compounds.

An investigation of the effect of different kinetin concentrations (2×10^{-7} to 4×10^{-5} *M*) on the formation of phenolic compounds in callus tissues initiated from tea-plant stems and leaves showed that although with 2×10^{-6} *M* kinetin the formation of the total amount of phenolic compounds and lignin increased significantly, the accumulation of flavans remained almost constant (Zagoskina and Zaprometov, 1983). At

the same time in the cell suspension culture derived from a tea-plant stem callus, 5×10^{-6} to 2.5×10^{-5} *M* kinetin promoted the synthesis of both all soluble phenolic compounds as well as of flavans (Bagratishvili *et al.*, 1980).

In callus tissues of *Crataegus monogyna*, kinetin (2×10^{-7} to 4×10^{-5} *M*) suppressed the formation of monomeric phenolic compounds as well as proanthocyanidins (Schrall and Becker, 1977).

C. Gibberellins

Gibberellic acid (GA_3) at a concentration of 1.0 mg/liter and particularly at 10 mg/liter, enhanced significantly the formation of flavans in the callus tissues of *Cryptomeria japonica* (Samejima *et al.*, 1982) but did not affect the formation of monomeric polyphenols and of proanthocyanidins in *Crataegus monogyna* callus tissues at 2×10^{-7} to 2×10^{-5} *M* (Schrall and Becker, 1977).

D. Abscisic Acid and Ethylene

Abscisic acid (5×10^{-6} *M*) reduced the formation of flavans by 15 to 20% in a cell-suspension culture of the tea plant (Bagratishvili *et al.*, 1984). Ethylene in the form of ethrel (2-chloroethylphosphonic acid) enhanced markedly the formation of flavans but reduced cell growth by nearly 20% (Bagratishvili *et al.*, 1984).

III. NUTRIENTS AND PRECURSORS

Glucose and sucrose at a concentration of 2 to 3.5% have been used as carbon sources by many authors (Ishikura and Teramoto, 1983; Koretzkaya and Zaprometov, 1975a; Phillips and Henshaw, 1977; Amorim *et al.*, 1977). In tea-plant callus tissues, increase in glucose concentration from 2.5 to 10% or substitution of glucose by sucrose did not affect the formation of flavans (Zaprometov, 1981). However, the cell suspension of Paul's scarlet rose exhibited a decrease in the synthesis of proanthocyanidins when the glucose concentration was increased from 3.6% (0.2 *M*) to 7.2% (0.4 *M*) (Amorim *et al.*, 1977).

In the cell-suspension culture of the tea plant, a twofold increase in nitrate concentration in the nutrient medium reduced the formation of both the total soluble phenolic compounds and flavans, whereas a decrease in nitrate content by four times enhanced the formation of flavans and of total soluble phenolic compounds almost by two times without reducing cell growth (Bagratishvili *et al.*, 1980). Only in the cell culture of *Pseudotsuga menziesii* did synthesis of proanthocyanidins not decrease with increasing nitrate concentration (Stafford and Cheng, 1980). Attempts were unsuccessful to intensify the formation of flavans in callus cultures of the tea plant by addition to the nutrient medium of their distant (quinic and shikimic acids, acetate, L-phenylalanine, *trans*-cinnamic acid) or close (naringenin, dihydroquercetin) precursors (Zaprometov *et al.*, 1976).

IV. EFFECT OF ILLUMINATION

The favorable effect of illumination on the synthesis and accumulation of phenolic compounds in both intact plants and cultured cells and tissues is well known. This effect is accounted for both by augmentation of the pool of precursors and substrates in photosynthetic tissues and by the light-induced activity of some enzymes in the biosynthesis of phenolic compounds. The latter possibility has been studied in detail (Hahlbrock *et al.*, 1978).

Although the illumination of cultured cells and tissues was in many cases the precondition of their ability to synthesize anthocyanins, flavones, and flavonols (see, e.g., Zaprometov, 1978), only one publication reported that light is required for the synthesis of catechins and proanthocyanidins (Schrall and Becker, 1977). In this investigation, the callus tissues and cell suspensions of two hawthorn species (*Crataegus monogyna* and *C. oxyacantha*) and *Ginkgo biloba* were cultured in the presence of NAA and kinetin in the dark or under continuous illumination. In the dark the content of soluble phenolic compounds remained low for 10 weeks; phenolcarboxylic acids only were identified (*para*-coumaric, vanillic, ferulic, chlorogenic acids, etc.). Under illumination, inhibition of the growth of cells and significant increase in the formation of the total soluble phenolics (by nearly four times with the *C. monogyna* callus) were observed. In addition to phenolcarboxylic acids, (+)-catechin, (−)-epicatechin, and several proanthocyanidins were synthesized.

In all of the other cases, light was not a necessary factor in the biosynthesis of flavans, although illumination usually intensified (after an induction period) the formation of flavan derivatives. Such a stimulation was shown, for example, with the cell suspension culture of Paul's scarlet rose in synthesizing catechins and proanthocyanidins (Davies, 1972a), and with callus tissues (Forrest, 1969; Koretzkaya and Zaprometov, 1975b) and cell suspension cultures (Bagratishvili and Zaprometov, 1982) of the tea plant.

As indicated above, the activity of some enzymes involved in the biosynthesis of phenolic compounds can be induced by illumination. In this respect, one of the most studied enzymes is L-phenylalanine ammonia-lyase (PAL). It was shown that in case of the flavan-synthesizing cell suspension culture of *Acer pseudoplatanus*, PAL activity increased three to four times at the beginning of intense formation of phenolic compounds and subsequently decreased sharply (Westcott and Henshaw, 1976). In rose cell cultures not all developmental steps exhibited correlation between PAL activity and the synthesis of polyphenols (Davies, 1972b).

In the tea-plant callus tissue, PAL activity increased rapidly, simultaneously with the formation of flavans up to the fiftieth day, then decreased sharply, though the flavan concentration continued to increase (Shipilova *et al.*, 1978). In this case, illumination (16 hr light and 8 hr dark) significantly enhanced PAL activity. In *Cryptomeria japonica* cell cultures grown under continuous illumination, PAL exhibited two activity maxima: after 15 hr (basic) and after 12 days (additional) (Teramoto and Ishikura, 1985). The inhibitor of PAL, L-1-aminohydroxyphenyl-2-propionic acid, at a concentration of 0.3 m*M* suppressed the first maximum only but significantly reduced the formation of flavans.

V. EFFECT OF POLYAMINES

In order to hinder aging of rose cells in culture, spermidine (25 μM) and sucrose were added to the nutrient medium during the stationary growth phase. As a result, both the amount and diversity of flavans increased (Muhitch and Fletcher, 1985). After such treatment the synthesis of (−)-epicatechin and (−)-epigallocatechingallat started in cells, along with the synthesis of gallic acid and dimeric B-1 proanthocyanidin.

This seems to be the first time that formation of galloylated catechin could be initiated in cell cultures.

VI. BIOSYNTHESIS

According to Haslam's hypothesis (Haslam, 1979), the common precursor of catechins and proanthocyanidins is flav-3-en-3-ol, the synthesis of the latter proceeding through the intermediate dihydroquercetin and flavan-3,4-diol steps. In this case, the amount of NADPH controls the final nonenzymatic steps of the biosynthesis of flavans. High NADPH content mainly leads to formation of catechins, whereas deficiency in NADPH favors the synthesis of proanthocyanidins.

With L-[^{14}C]phenylalanine as precursor of flavans in the tea-plant cell suspension culture, however, the synthesis of (−)-epicatechin occurred at even a greater rate than that of dimeric proanthocyanidins, notwithstanding that NADPH synthesis in the cells was lower than that in the intact plant tissue (Nikolaeva *et al.*, 1982). In the cell suspension culture of *Pseudotsuga menziesii*, L-[^{14}C]phenylalanine also proved to be more efficient in promoting the formation of (−)-epicatechin and (+)-catechin than that of proanthocyanidins (Stafford *et al.*, 1982). Thus, these data do not support Haslam's nonenzymatic hypothesis of flavan biosynthesis.

It has been established with flavan-producing cell cultures of *Ginkgo biloba* and *Pseudotsuga menziesii* that (+)-catechin and (+)-gallocatechin are synthesized, respectively, from (+)-dihydroquercetin and (+)-dihydromyricetin, with intermediate formation of flavan-3,4-*cis*-diols (leucocyanidin and leucodelphinidin) (Stafford and Lester, 1984, 1985). Both reactions are catalyzed by soluble enzymes of the reductase type, with NADPH as reducing agent:

(+)-Dihydroquercetin —NADP·H$_2$→ Leucocyanidin —NADP·H$_2$→ (+)-Catechin

VII. CONCLUSIONS

The data presented here indicate that tissues and cells cultured both in the dark and light preserve their ability to synthesize flavan compounds.

The formation of flavans in cell cultures can be increased significantly by appropriate selection of the nutrient medium (carbon and nitrogen amounts, composition and amount of phytohormones or their synthetic analogs) and illumination conditions. Nevertheless, as a rule, cell cultures synthesize flavan compounds in smaller amounts and diversity than do the parent plants. This applies particularly to catechins. Thus, the tissue and cell cultures of tea plant lose the ability to synthesize (−)-epigallocatechin, (+)-gallocatechin, (−)-epicatechingallat, and (−)-epigallocatechingallat, although in leaves these catechins account for 90% of the total amount of catechins.

Similar behavior was observed with the culture of rose cells, although young cultures still preserved some ability for the synthesis of galloylated catechins (Muhitch and Fletcher, 1984). In some cases, the use of polyamines might open an approach to the restoration of lost ability (Muhitch and Fletcher, 1985).

In general, the proanthocyanidin/catechin ratio is much greater in cell cultures than in original plant tissues. This could be due to a particularly strong deceleration of the conversion of flavan-3,4-diol to catechins (the second reductase reaction; see above).

Also, in the dark, green tissues lose their photoautotrophic property, and the high differentiation level is destroyed in such cultures. As a result, in many cases, the flavan composition of cultured tissues (whatever their origin—stem, leaf, bud, cotyledon) becomes rapidly similar to that of underground organs (i.e., roots). In this respect, the tea plant, which synthesizes a particularly great variety of flavans, offers a prime example.

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CHAPTER 5

Tannins, Lignans, and Lignins

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I. INTRODUCTION

Early studies of the formation of tannins and lignins in cultured plant tissues and cells have been summarized in comprehensive reviews (Teuscher, 1973; Butcher, 1977). Therefore, in the present chapter, attention will be devoted to more recent publications.

II. TANNINS

Tannins can be classified into two groups: hydrolyzable and condensed tannins (Haslam, 1966). This classification reflects both the phys-

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icochemical properties and the biogenesis of tannins. Hydrolyzable tannins usually result from binding to sugar fragments (mainly to the D-glucose moiety) of gallic, meta-digallic, or hexahydroxydiphenic acid residues. The direct precursors of these phenolcarboxylic acids are shikimic or dehydroshikimic acids.

Condensed tannins are formed by oxidative condensation of flavonoids (mainly flavan-3,4-diol monomers, less frequently catechins, stilbenes, and dihydrochalcones). The precursors of flavonoids and, accordingly, condensed tannins are malonyl-CoA and *para*-hydroxycinnamoyl-CoA (possibly also caffeoyl-CoA).

Condensed tannins are usually stored in heartwood and bark, and hydrolyzable tannins in leaves, fruit pods, and galls (Haslam, 1966).

A. Condensed Tannins

Because precursors of condensed tannins are mainly flavans (flavan-3,4-diols and flavan-3-ols), it is in many cases difficult to draw a clear distinction between oligomeric proanthocyanidins and true condensed tannins. Therefore, a great part of the information given in Chapter 4, this volume, applies directly to the present chapter.

The results of cytochemical studies of the formation and accumulation of condensed tannins has proven to be very valuable. As early as 1963, tannins of proanthocyanidin origin were shown to accumulate in some cells of *Juniperus communis* callus tissues, with predominant localization in the vacuoles (Constabel, 1963). Subsequent electron-microscopic examinations suggested that in cells of *J. communis* callus tissues, condensed tannins are probably synthesized in the dictyosomes (Golgi apparatus) and then accumulate in the vacuoles (Constabel, 1969). In cell suspension cultures of *Picea glauca*, the most probable site of synthesis of tannins was presumed to be the membrane system of the endoplasmic reticulum (ER) (Chafe and Durzan, 1973). The authors envisioned that small tannin vesicles are eventually torn off the ER and are gradually transformed into small vacuoles. These move then to the central vacuole to merge with it. In such a way, tannins are transported to the central vacuole, the main compartment for accumulation of tannins in the protoplast. It should be mentioned that with the cell suspension culture of *P. glauca*, continuous illumination led to significant decrease in the formation of tannins, as compared to normal physiological conditions (14 hr light plus 10 hr darkness) (Durzan *et al.*, 1973). Electron-microscopic examination of *Pinus elliottii* callus tissues showed that tannins are syn-

thesized in the membranes of the smooth ER, then transported by vesicles to the central vacuole (Baur and Walkinshaw, 1974). An intermediate involvement of the Golgi apparatus as the site of tannin precursor accumulation was assumed. These authors also found that formation and accumulation of tannins takes place in metabolically active cells. Although electron microscopy is not suitable for distinguishing between condensed and hydrolyzable tannins, it may be assumed that in cultured cells of *Picea glauca* and *Pinus elliottii* condensed tannins were formed, because the latter are typical for the original plants.

Leaf-explant-derived callus tissues of *Onobrychis viciifolia* contain numerous condensed-tannin-filled cells (Lees, 1986). Tissues (cotyledons and roots) of *O. viciifolia* that normally do not contain condensed tannins form callus with tannin-filled cells. In this case, however, the presence of cytokinins (benzylaminopurine or kinetin) in the culture medium is necessary. Specialized cells filled with flavan derivatives (mainly proanthocyanidins) were detected previously in leaf- and stem-derived tea-plant callus tissues (Strekova *et al.*, 1980).

As well as with other secondary compounds, the formation of condensed tannins in cultured cells and tissues depends on nutrition and illumination. Increase in glucose concentration in the nutrient medium from 1 to 6% led to significant increase in the content of condensed tannins in *Juniperus communis* callus tissues (Constabel, 1968). In cell-suspension cultures of *Acer pseudoplatanus*, depletion of nitrogen led to accumulation of great amounts of condensed tannins (Westcott, 1976). In this case, L-phenylalanine ammonia-lyase (PAL) activity initially correlated with the formation of tannins and then decreased, although the synthesis of tannins went on (Westcott and Henshaw, 1976).

Ethylene produced from 2-(chloroethyl)phosphonic acid (CEPA) at a concentration of $1.0 \times 10^{-5} + 1.0 \times 10^{-4}$ *M* inhibited the formation of condensed tannins in the cell suspension culture of *Acer pseudoplatanus* (Westcott, 1976).

Callus and cell-suspension cultures derived from young shoots of *Cryptomeria japonica* synthesized, together with two catechins [(+)-catechin and (−)-epicatechin] and one or two dimeric proanthocyanidins, a great amount of polymeric proanthocyanidins, that is, typical condensed tannins (Ishikura and Teramoto, 1983). In this case, the content of tannins (in cyanidin equivalents) in dark-green callus was 26.6% of dry weight, and in light-cultured cells, only 5.2%.

Unusually high amounts of proanthocyanidin-derived tannins—up to 60% of dry weight—were found in the light-grown callus tissues of *Pseudotsuga menziesii* (Monroe and Johnson, 1984).

B. Hydrolyzable Tannins

There are but few data on the formation of hydrolyzable tannins in cultured cells and tissues. Thus, in cell suspension cultures of Paul's scarlet rose, together with proanthocyanidins, glucogallin (monogalloyl-D-glucose) and one or two gallotannins were identified (Davies, 1972). The callus tissues of *Quercus rubur* synthesize only small amounts of gallic acid and β-penta-*O*-galloyl-D-glucose, whereas in the parent plant, *meta*-digallic and hexahydroxydiphenic acid derivatives (e.g., vescalagin and castalagin) are synthesized in great amounts and diversity (Haslam and Lilley, 1985). The loss of the high level of morphological differentiation in cultured cells and tissues seem to affect particularly their ability to synthesize hydrolyzable tannins.

III. LIGNANS

The term *lignans* usually designates the optically active dimers of C_6–C_3 units (hydroxycinnamic acids or alcohols). Many of these compounds possess hepatotropic and antihepatotoxic (hepatoprotecting) activity (Wagner, 1985). Although there are many reports about the occurence of lignans in plants, still no data are available about their isolation from cultured cells and tissues.

Attempts to use callus tissues and cell suspension cultures of *Silybum marianum* as sources of flavonolignans (silybin, silydianin, silychristin) proved to be of little success, although the intact plants accumulate these compounds. The cotyledon-derived callus of *S. marianum* initially possessed the ability to synthesize flavonolignans, but this ability completely disappeared later (Schrall and Becker, 1977). However, the authors could achieve silybin synthesis by adding to the cell suspension culture the precursors of silybin, namely, the dihydroflavonol taxifolin (dihydrokaempferol), and coniferyl alcohol. In this case the formation of silybin proceeded rapidly (after several hours). If the flavone luteolin was added to the cultured cells instead of taxifolin, another flavonolignan was synthesized, hydnocarpin (a compound typical for *Hydnocarpus wightiana*). Similar results were obtained with the cell-free extract of *S. marianum* suspension-cultured cells and a horseradish peroxidase preparation (Schrall and Becker, 1977). In spite of the inability of the *S. marianum* cell suspension culture to synthesize flavonolignans, the addition of some flavonolignans (silybin, silydianin) to such cultures at con-

centrations between 10^{-6} and 10^{-4} *M* stimulated cell growth, although the differentiation of cells remained unaffected (Becker and Schrall, 1977).

IV. LIGNINS

The formation of lignins occurs usually in the tracheid-like elements and parenchyma-like cells, as well as in intercellular spaces (Butcher, 1977). Cultured cells generally preserve their ability to synthesize lignin. Cell suspensions of *Petroselinum crispum* (previously called *P. hortense*) seem to be one of the exceptions. This culture does not synthesize lignin and does not contain the isoenzymes specific for lignin biosynthesis, those of 4-coumarate-CoA ligase and *O*-methyltransferase (Hahlbrock, 1977). The reason for such behavior is still unclear. The cell-suspension culture of *Glycine max* grown in the dark on B5 medium also does not synthesize lignin (Farmer, 1985). With lowering of the NO_3^- and PO_4^{3-} content (LS, B5 medium), however, the cells begin to synthesize significant amounts of extracellular lignin.

The lignin of cultured soybean cells is closely related in composition to that of woody gymnosperms (Nimz *et al.*, 1975). Guaiacol, vanillin, syringaldehyde, coniferyl, and sinapic aldehydes were identified among the pyrolysis products of this lignin.

In cultured soybean cells two isozymes of cinnamoyl-alcohol dehydrogenase (an enzyme involved in lignin biosynthesis) were detected. The substrate specificity of one of the isozymes corresponded to the composition of soybean lignin (Wyrambik and Grisebach, 1975).

Of hormones influencing the formation of lignin, cytokinins seem to be the most active. Thus, in cultured callus tissues and cells of *Nicotiana tabacum*, kinetin increased the content of Klason's lignin from 3–5 to 22% of dry weight (Bergmann, 1964). In this case the differentiation level (i.e., the amount of tracheid-like elements) in the cultures increased. Other authors (Yamada and Kuboi, 1976) reported that kinetin (10^{-5} *M*) in cultured *N. tabacum* cells stimulated differentiation (appearance of organized xylem elements) and also caused an increase in the percentage of lignified cells from 0.5 to 2.8%. In cell suspension cultures of *Acer pseudoplatanus*, kinetin stimulated in a similar way the formation of lignin (Carceller *et al.*, 1971). In callus cultures derived from young tea-plant stems, kinetin (5×10^{-6} *M*) caused an increase in the amount of tracheid-like elements, and almost twofold intensification of lignin syn-

thesis (Zaprometov *et al.*, 1986). Concomitantly, the activity of the covalently linked peroxydase form increased sharply.

In order to stimulate lignification in cell suspension cultures of various plants, combinations of cytokinin (benzylaminopurine, 5 μM) with auxin (naphthalene acetic acid, 5 μM) were also used. In most of the cases such combinations induced together with lignin formation the activity of coniferin-β-glucosidase also (Hösel *et al.*, 1982).

In *Nicotiana tabacum* callus tissues, kinetin (1 mg/liter) stimulated incorporation of L-[^{14}C]phenylalanine into coumarins (scopoletin and scopolin) and the formation *de novo* of PAL, without affecting the labeling of lignin (Hino *et al.*, 1982). The reason for such anomalous behavior (as concerns the lignin formation) is not clear.

The data about the influence of different auxin concentrations on lignin formation in cultured cells and tissues are contradictory. Thus, in cultured *Acer pseudoplatanus* cells, a change in 2,4-dichlorophenoxyacetic acid (2,4-D) concentration from 1 to 10 mg/liter caused marked increase in lignin formation (Carceller *et al.*, 1971), whereas cultured carrot cells exhibited an opposite trend: lignin content increased with twofold decrease in 2,4-D concentration (from 1.0 to 0.05 ppm) (Sugano *et al.*, 1975). The optimal 2,4-D concentration for the growth of tea-plant callus tissues was 2×10^{-5} *M*. Substitution of naphthaleneacetic acid (3×10^{-7} to 2×10^{-5} *M*) for 2,4-D led to significant increases in lignin formation (three- to fourfold) (Zagoskina and Zaprometov, 1979).

In *Nicotiana tabacum* callus tissues, lignin formation was inhibited by addition of abscisic acid (0.01–5.0 mg/liter) to the nutrient medium (Li *et al.*, 1970). However, the strongest inhibitor of lignin formation proved to be L-aminohydroxy-3-phenylpropionic acid (AOPP). It supresses PAL activity and thus deprives the lignification process of the necessary substrates (Amrhein and Gödeke, 1977). AOPP at a concentration of 0.3 m*M* almost completely inhibited the formation of lignin and lignin-like compounds in cultured *Lonicera prolifera* cells (Amrhein *et al.*, 1983).

Inhibition of extracellular lignin formation in soybean cell-suspension cultures was observed with addition to the medium of a fungal elicitor from *Phytophthora megasperma* f.sp. *glycinea* (Farmer, 1985). This was accompanied by decrease in the activity of two enzymes involved in lignin biosynthesis, namely, PAL and 4-coumarate-CoA ligase.

The cell suspension cultures of *Triticum aestivum* and *Glycine max* were used for studying the metabolism of xenobiotics, including herbicides (Sandermann *et al.*, 1984). It was found that 2,4-D and pentachlorophenol copolymerize with phenylpropan monomers, and that they are consequently incorporated into true lignin. In this case lignin formation is one of the pathways for detoxification of xenobiotics.

The data accumulated since the late 1970s indicate that lignin composition even in the same plant is not always uniform. For example, guaiacyl lignin mainly may be formed in parenchyma cells, and syringyl lignin in xylem (Wiermann, 1981).

In tea-plant callus tissues, lignin is not detected in tracheid-like elements but is found within specialized cells and in their walls (as follows from the reaction with phloroglucinol and hydrochloric acid) (Zaprometov *et al.*, 1979). A similar pattern of lignin localization was found in the cell-suspension culture of *Acer pseudoplatanus* (Carceller *et al.*, 1971).

The influence of long-term culturing on formation of lignin and soluble phenolic compounds was studied with stem-derived tea-plant callus tissues. As an example, the tissues of the tenth and forty-seventh subcultures (passages) were compared (each subculture lasted 5 weeks) (Strekova *et al.*, 1980). After the thirty-seventh subculture, the content of flavans decreased 10 times, the content of total soluble phenolic compounds 5 times, and the amount of lignin only 2 times. Thus, lignin biosynthesis, at least in some plant tissue cultures, seems to be a more conservative process than the formation of flavan derivatives.

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CHAPTER 6

Naphthoquinones

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I. INTRODUCTION

Naththoquinones are found sporadically in about 20 families of higher plants, including Ebenaceae, Droseraceae, Balsaminaceae, Juglandaceae, Plumbaginaceae, Bignoniaceae, and Boraginaceae. Phylloquinone (vitamin K_1), however, occurs universally in green plants. Some of the natural naphthoquinones (lawsone, juglone, shikonin) have been used as dyes since ancient times. Furthermore, several compounds are known to have interesting physiological properties, such as anti-

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microbial (plumbagin, shikonin), antitumor (lapachol), antiinflammatory (shikonin), and phytotoxic (juglone) activities. According to Inoue and Inouye (1983), the naphthoquinones of higher plants are biosynthesized through the following five routes: (1) *ortho*-succinylbenzoic acid (OSB) pathway (e.g., lawsone, juglone, vitamins K_1 and K_2), (2) *para*-hydroxybenzoic acid–mevalonic acid (MVA) pathway (e.g., shikonin), (3) homogentisic acid–MVA pathway (e.g., chimaphilin), (4) acetic acid–MVA pathway (e.g., plumbagin), and (5) MVA pathway (e.g., hemigossypolon). In recent years, cell cultures of several naphthoquinone-containing plants, including *Lithospermum, Echium, Plumbago, Juglans, Catalpa, Galium,* and *Streptocarpus,* have been established by several workers to elucidate the biosynthetic pathways as well as the regulatory mechanism of naphthoquinone production.

II. NAPHTHOQUINONES IN CELL CULTURES

A. Shikonin Derivatives

Callus cultures of *Lithospermum erythrorhizon,* which were derived from the seedlings and grown on Linsmaier–Skoog (LS) medium containing indole-3-acetic acid (IAA) and kinetin in the dark, are capable of producing the same 1,4-naphthoquinones as those found in the root bark of the intact plant (Tabata *et al.,* 1974; Konoshima *et al.,* 1974; Mizukami *et al.,* 1978). These red pigments, which accumulate on the outside of cell walls of cultured cells, consist of shikonin (**1**) [an optical isomer of alkannin (**2**)] and its fatty acid esters (Fig. 1). Cell suspension cultures grown in M9 production medium (Fujita *et al.,* 1981b) also produce the same shikonin derivatives, whose relative proportions are similar to those in the root (Fujita *et al.,* 1982). The content of shikonin derivatives in cultured cells was remarkably increased by the repeated selection of subcultured callus tissues in successive transfer generations (Mizukami *et al.,* 1978) or the selection of single-cell clones (Tabata *et al.,* 1978). As a result, the total shikonin content of a selected cell strain M18 was as high as 1.2 mg per gram fresh weight, an eightfold increase over the content of the original callus culture.

Callus cultures of *Echium lycopsis,* another boraginaceous plant containing red pigments in the root bark, were also found to produce shikonin derivatives when grown on LS agar medium containing IAA and kinetin in the dark (Inouye *et al.,* 1981). The total content of pigments (12.3% of dry weight), which consisted of β,β-dimethylacryl, β-

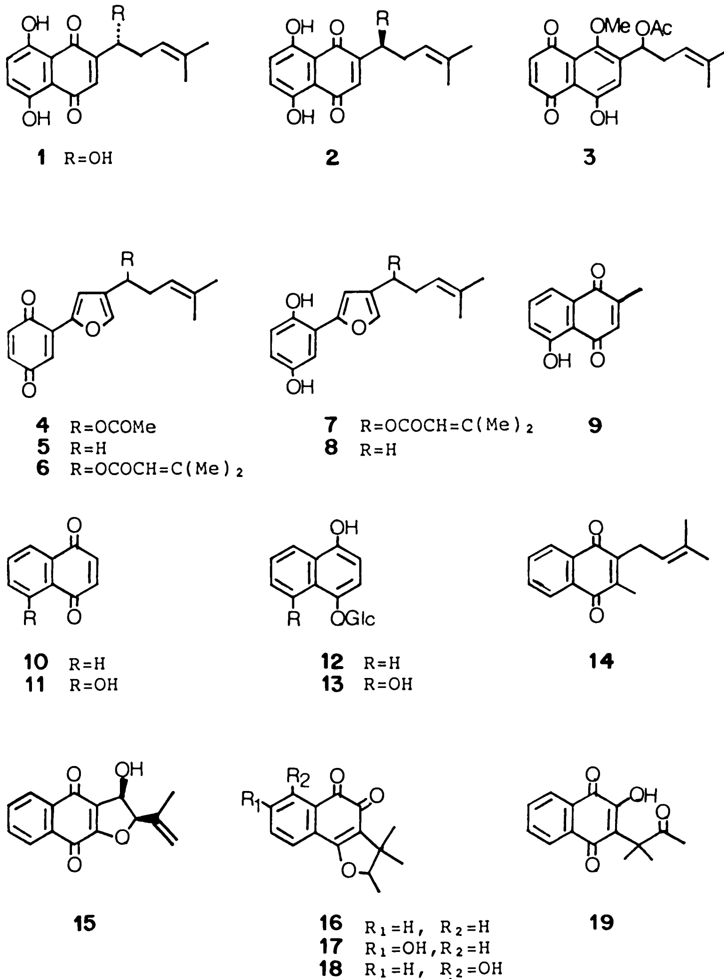

Fig. 1. Naphthoquinones and related compounds produced by various plant cell cultures.

hydroxyisovaleryl, acetyl, isobutyl, and isovaleryl derivatives of shikonin, was 350 times higher than that of the root. In addition to shikonin derivatives, two new quinones, echinone (**3**) (red-orange oil, 24.5 mg) and echinofuran (**4**) (orange-colored oil, 36.6 mg), were isolated from fresh callus cultures (375 g) of *E. lycopsis*. Echinone, which showed a strong antibacterial activity (Tabata *et al.*, 1982), is structurally related to acetylshikonin, whereas echinofuran appears to be an unusual metabolite arising from a key intermediate, geranylhydroquinone (**22**), in the biosynthesis of shikonin. Two congeners of echinofuran, echinofuran B (**5**) and C (**6**), were also isolated from the cell cultures as well as the roots

of *Lithospermum erythrorhizon* (Fukui *et al.*, 1984a). Interestingly, the new compound echinofuran B (yield, 3.8 mg per gram dry weight) was formed *de novo* only when activated carbon was added to the suspension culture.

Yazaki *et al.* (1986) have succeeded in isolating two supposed intermediates of shikonin biosynthesis (Inouye *et al.*, 1979), that is, *meta*-geranyl-*para*-hydroxybenzoic acid (**21**) and geranylhydroquinone (**22**), from shikonin-producing *Lithospermum* cell-suspension cultures. The yields of these colorless, oily compounds were 71 and 73 mg per 400 g dry weight, respectively.

Fukui *et al.* (1983a) examined the absolute configuration of naphthoquinone derivatives and found that not only the roots but also the callus tissues of *Lithospermum erythrorhizon* and *Echium lycopsis* produced both the *R* form (shikonin) (**1**) and the *S* form [alkannin (**2**)] in various ratios, depending on the esterified derivatives. Although the derivatives produced by *Lithospermum* were primarily of the *R* form, those isolated from *Echium* consisted largely of the *S* form. It was suggested that the absolute configuration of a particular derivative may be liable to be changed from the *R* form to the *S* form, or vice versa by the genetic factors and physiological conditions of the cells. However, pharmacological tests showed that no significant difference in the antiinflammatory activity is found between a pair of enantiomers, shikonin and alkannin (Tanaka *et al.*, 1986).

B. Plumbagin

Heble *et al.* (1974) detected the presence of plumbagin (**9**) in callus cultures derived from the stem segments of *Plumbago zeylanicum* (Plumbaginaceae) and cultured on Lin–Staba medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin under continuous illumination. The plumbagin content varied from 0.11 to 3.30 mg/100 g fresh weight, depending on cell strains and growth hormones. The plumbagin content of callus tissues was comparable to that of the leaves (1.0 mg per gram fresh weight), but much smaller than that of the roots of the intact plant (6.50 mg per gram fresh weight).

C. Glycosides of Oxygenated Naphthalenes

Naphthoquinones usually do not occur as glycosides in higher plants. However, Müller and Leistner (1978) isolated not only 1,4-naphtho-

quinone (**10**) and juglone (**11**) but also new glucosides of the corresponding hydroquinones (**12, 13**) from mesocarp callus and cell-suspension cultures of *Juglans major* and *J. microcarpa* (Juglandaceae). These compounds were formed in 20-day-old callus cultures in significant amounts only when grown on modified B5 medium containing naphthalene-1-acetic acid (NAA) as the sole hormone. The major component in the *J. major* callus was the glucoside of juglone (6.59 mg per gram dry weight). The content of the same glucoside in the *J. microcarpa* callus (26.73 mg per gram dry weight) was higher than that of its aglucone, juglone (0.08 mg per gram dry weight).

Inoue *et al.* (1984a) also reported the isolation of a new glucoside, 2-carbomethoxy-3-prenyl-1,4-naphthoquinone diglucoside (**35**), from cell suspension cultures of *Galium mollugo* (Rubiaceae) that were incubated in Gamborg B5 medium supplemented with 2,4-D, NAA, IAA, and kinetin for a period of 7 days. The yield of the new compound was increased to 7.1 g per 7 liters of medium by administration of the precursor OSB to the medium.

D. Lapachone Derivatives

In callus cultures derived from seedlings of *Catalpa ovata* (Bignoniaceae), Ueda *et al.* (1980) detected the presence of 11 naphthoquinone congeners, including menaquinone-1 (**14**), catalpalactone, and derivatives of α-lapachone (**27**), which are known constituents previously obtained from the wood of the original plant. Of these constituents, 8-hydroxydehydro-iso-α-lapachone, 3,8-dihydroxydehydro-iso-α-lapachone, dehydro-α-lapachone, and 8-hydroxy-2-isoprenylfuranonaphthoquinone were isolated from fresh callus tissues as orange-red or red crystals. The callus also produced a specific naphthoquinone, 3-hydroxydehydro-iso-α-lapachone (**15**). However, none of the naphthoquinones was detected in the callus cultures grown on LS agar medium containing 2,4-D in place of IAA and kinetin.

E. Dunnione Derivatives

In cell suspension cultures of *Streptocarpus dunnii* (Gesneriaceae), Inoue *et al.* (1983) isolated six naphthoquinones from a mixture of free cells and poorly differentiated plantlets grown in LS medium containing IAA and kinetin in the dark. These substances were identified as a red pigment, dunnione (**16**), and a yellow isomer, α-dunnione (**32**), which are

known constituents of the leaves of *S. dunnii*, and new related substances, dehydrodunnione, 7-hydroxydunnione (**17**), 8-hydroxydunnione (**18**), and streptocarpone (**19**). These unusually prenylated naphthoquinones could also be isolated from the leaves of the intact plant. Interestingly, the major component of the suspension cultures was found to be α-dunnione (10 mg per 870 ml of medium), although that of the mature leaves was dunnione (300 mg per 57 g of leaves).

Examination of the optical properties showed that dunnione isolated from mature plants was absolutely optically pure, but the same quinone from the cultures consisted of both *d* and *l* enantiomers in a ratio 53 : 47. On the other hand, α-dunnione from the mature plant was a mixture of *d* and *l* enantiomers in the ratio 45 : 55, whereas the same compound from the cell cultures was a mixture of both enantiomers in the ratio 55 : 45. These results demonstrated that even the same compounds show different optical rotations depending on their origin.

III. BIOSYNTHETIC PATHWAYS OF NAPHTHOQUINONES

A. Biosynthesis of Shikonin

The biosynthetic pathway of shikonin was investigated by Inouye *et al.* (1979), who administered labeled precursors, *para*-[3-^{3}H]hydroxybenzoic acid and [2-^{14}C]mevalonic acid, to *Lithospermum erythrorhizon* callus cultures (strains M18 and M231a) grown on LS agar medium containing IAA and kinetin, in the dark. The results of experiments indicated that *para*-hydroxybenzoic acid (**20**) was incorporated into the hydroquinone portion of shikonin, whereas two molecules of [2-^{14}C]MVA were incorporated into shikonin, labeling C-1′ and C-5′ positions of the side chain. Furthermore, the chemically synthesized labeled substances *meta*-geranyl-*para*-[8′-^{3}H]hydroxybenzoic acid and [8′-^{3}H]geranylhydroquinone were almost specifically incorporated into shikonin. Tracer experiments showed that both *meta*-geranyl-*para*-hydroxybenzoic acid (**21**) and geranylhydroquinone (**22**) are the biosynthetic intermediates of shikonin (Scheme 1). The biosynthetic pathway of shikonin proposed by Inouye's group has been supported by the isolation of these intermediates from shikonin-producing cells of the strain M18 (Yazaki *et al.*, 1986).

Scheme 1. Biosynthetic pathway leading to the formation of shikonin (1) in *Lithospermum erythrorhizon* callus cultures (Adapted from Inouye *et al.*, 1979, by permission.)

B. Biosynthesis of Lapachone Derivatives

Inouye *et al.* (1978) studied the biosynthesis of lapachone derivatives by administering [2'-^{14}C]carboxyl-OSB to *Catalpa ovata* callus cultures. From comparisons of incorporation rates of OSB into the metabolites, they proposed that several naphthoquinone congeners of lapachol are biosynthesized from OSB (**23**) via 2-carboxy-4-oxo-α-tetralone (COT) (**24**), 2-prenyl-COT (**25**), and catalponone (**26**) (Inoue *et al.*, 1981) (Scheme 2). Furthermore, dilution analysis after administration of the labeled OSB to callus cultures demonstrated that both prenylation and decarboxylation in the main biosynthetic route from OSB to quinonoids proceed stereospecifically (Inoue *et al.*, 1980).

C. Biosynthesis of Dunnione

Inoue *et al.* (1984b) elucidated the biosynthetic pathway of dunnione and its congeners by studying the incorporation of stable labeled precur-

Scheme 2. Biosynthetic pathway leading to the formation of α-lapachone (27) in *Catalpa ovata* callus cultures (Adapted from Inouye *et al.*, 1978, by permission.)

sors into quinones produced by cell suspension cultures of *Streptocarpus dunnii*. The results of experiments indicated that dunnione (**16**), α-dunnione (**32**), and 8-hydroxydunnione (**18**) are biosynthesized from OSB (**23**) via 1,4-dihydroxy-2-naphthoic acid (**28**), lawsone (**29**), lawsone 2-prenyl ether (**30**), and the Claisen-type rearrangement product of the latter, 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone (**31**), which proved to be the key intermediate for all the naphthoquinones in this plant (Scheme 3).

D. Biosynthesis of Naphthoquinone Diglucoside

The biosynthetic pathway of a unique metabolite, 2-carbomethoxy-3-prenyl-1,4-naphthoquinone diglucoside (**35**) produced by *Galium mollugo* cell-suspension cultures was investigated by Inoue *et al.* (1984a). In the administration experiments, the quinone content of the cultured cells had been reduced through steady-state continuous culturing in a chemostat under phosphate-limiting conditions, so that the specific incorporation of the labeled precursor, [2′-carboxy-^{13}C]OSB, was as high as 93.3%. The analyses showed that the diglucoside is biosynthesized through key intermediates, 1,4-dihydroxynaphthoic acid (**33**) and 1,4-dihydroxy-3-prenyl-2-naphthoic acid (**34**) (Scheme 4).

Scheme 3. Biosynthetic pathways leading to the formation of dunnione (**16**) and α-dunnione (**32**) in *Streptocarpus dunnii* cell suspension cultures (Adapted from Inoue *et al.*, 1984, by permission.)

Shikimate → → 23 → 33 → 34 → 35

Scheme 4. Biosynthetic pathway leading to 2-carbomethoxy-3-prenyl-1,4-naphthoquinone di-β-glucoside (**35**) in *Galium mollugo* cell suspension cultures (Adapted from Inoue *et al.*, 1984a, by permission.)

IV. INTRACELLULAR LOCALIZATION OF NAPHTHOQUINONES

Submicroscopic studies of naphthoquinone-producing cells have so far been made only for cultured cells of *Lithospermum erythrorhizon* in which the biosynthesis of shikonin derivatives could readily be induced by transferring the cells from LS liquid medium to M9 production medium. Electron-microscopic observations of shikonin-producing cells suggested that the pigments accumulate in "secretion vesicles" (0.1–0.2 μm in diameter), which originate from electron-dense, spherical swellings formed in highly elongated, rough endoplasmic reticulum (Tsukada and Tabata, 1984). Most of these vesicles appeared to fuse with the plasma membrane to secrete the contents, consisting mainly of shikonin derivatives (27.2%), lipids (28.6%), and proteins (21.5%), to the outside of the cell wall.

Although the results of this study indicated a close relationship between the metabolic differentiation and morphological changes in cell structure, biochemical aspects of naphthoquinone synthesis remain to be elucidated in detail.

V. REGULATION OF NAPHTHOQUINONE PRODUCTION

Studies on the regulation of naphthoquinone biosynthesis have largely been concentrated on the elucidation of physical and chemical factors controlling shikonin production in *Lithospermum* cell cultures. The shikonin synthesis in both *Lithospermum* (Tabata *et al.*, 1974) and *Echium* cultures (Fukui *et al.*, 1983a) was found to be strongly repressed by either white or blue light but not by red or green. Experiments have

shown that lumiflavine arising from the blue-light-induced photodegradation of flavine mononucleotide inhibits the biosynthesis of shikonin in *Lithospermum* cells (M. Tabata and K. Yazaki, unpublished data).

Plant growth hormones are also known as important factors controlling the biosynthesis of naphthoquinones. Tabata *et al.* (1974) found that the synthetic auxin 2,4-D completely inhibits the synthesis of shikonin derivatives, whereas the natural auxin IAA was rather stimulatory. In this connection, Inouye *et al.* (1979) showed that [5-^{3}H]shikimic acid administered to *Lithospermum* callus cultures grown on a medium containing 2,4-D instead of IAA was incorporated into *meta*-geranyl-*para*-hydroxybenzoic acid (**21**) but was hardly incorporated into geranylhydroquinone (**22**). It is likely, therefore, that 2,4-D inhibits the decarboxylation of the former intermediate into the latter.

Gibberellin A_3 also strongly inhibits shikonin synthesis in *Lithospermum* callus cultures, at a concentration as low as 10^{-7} *M*, without affecting cell growth (Yoshikawa *et al.*, 1986). The amount of endogenous GA-like substances in the shikonin-producing strain M18 was shown to be much smaller than that of the nonproducing strain B17, suggesting that GA could be one of the important endogenous regulators in the biosynthesis of shikonin.

Among several chemical substances known to affect shikonin biosynthesis, the effect of certain acidic polysaccharides is of special interest. Fukui *et al.* (1983b) found that the addition of either agaropectin or pectic acid to LS liquid medium induced shikonin production in suspension cultures of the cell strain M18. The experiments suggested that this strain lacks the ability to synthesize an acidic polysaccharide that is necessary, directly or indirectly, for the initiation of shikonin biosynthesis, although it is capable of producing large amounts of shikonin derivatives on LS agar medium.

As regards the nutritional factors affecting the biosynthesis of shikonin derivatives in *Lithospermum* cultures, Mizukami *et al.* (1977) observed that sucrose at high concentrations (5–10%), addition of ascorbic acid, or L-phenylalanine increased the shikonin content. In contrast to these substances, high concentrations of nitrogen sources (NH_4NO_3 and urea) as well as Ca^{2+} and Fe^{2+} inhibited shikonin production. On the other hand, in an attempt to find a liquid medium in which *Lithospermum* cells (strain M18) would produce shikonin in the absence of agar or pectic acid, Fujita *et al.* (1981a) found that the cell suspension cultures did produce shikonin pigments in White's medium that contained nitrate as the sole nitrogen source. In fact, the shikonin synthesis was repressed by the addition of even a small amount of ammonium (3% of the total

nitrogen) to the medium. It was also found that an increase in the concentration of Cu^{2+} causes a threefold increase in the yield of shikonin derivatives (Fujita *et al., 1981b).*

On the basis of these findings and a thorough examination of all the medium components for their effects on shikonin production, Fujita *et al.* (1981b, 1982) devised a production medium named M9, which proved to be most suitable for shikonin production. The development of the production medium and the establishment of the two-stage culture system, employing a set of connected fermenters, have realized the industrial production of shikonin derivatives (Fujita *et al.*, 1982). The yield of shikonin in the large-scale setup has been increased to 4 g per liter of medium (~20% of dry weight) in a 2-week culture. The details of the production system have been described elsewhere (Tabata and Fujita, 1985).

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CHAPTER 7

Anthraquinones

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I. INTRODUCTION

Anthraquinones (AQs) are derived from anthracenes and have two keto groups, mostly in positions 9 and 10 (Fig. 1). The basal compound, anthraquinone (9,10-dioxoanthracene), can be substituted in various ways, resulting in a great diversity of structures. AQs are widely distributed in the plant kingdom (Table I; cf. Zenk and Leistner, 1968). AQs appear in oxidized and reduced (anthrones) form as well as in a free or a combined (glycosidic) state. They are isolated from various plant parts, that is, leaf, stem, root (the principal site of accumulation), pod, seed, coat and embryo, but not endosperm. Naturally occurring AQs possess dyeing and/or pharmacological (purgative) properties.

AQs and reduced AQs and their glycosides are produced and accumulated in varying proportions; from spring to fall reduced AQs prevail, during winter reduced AQs only are present. During storage of plants

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Anthraquinone Anthrone Anthranol

Fig. 1.

part of the anthrones and anthranols are oxidized to AQs. Thus, the content of AQs gradually increases, and that of reduced AQs diminishes.

Since the late 1960s, callus and cell suspension cultures of AQ-producing plants have been established and investigated with regard to AQ quality, quantity, and biosynthetic pathways. In some cases AQs were found to be accumulated in rather high amounts, contrary to observations with many other plant cell-culture systems, where formation of secondary metabolites was strongly repressed. The pattern of the AQs formed, however, differed more or less from that of the intact plant; novel substances appeared that previously had never been found in the intact plant. Thus, there may exist an opportunity to produce substances by way of cell cultures not obtainable in entire plants.

Table I

Angiosperm Plant Families with Species Producing Anthraquinones[a]

Dicotyledoneae	Dicotyledoneae (continued)
Anacardiaceae	Rhizophoraceae
Apocynaceae	Rubiaceae*
Asclepiadaceae	Rutaceae[b]
Bignoniaceae	Saxifragaceae
Caesalpiniaceae*	Scrophulariaceae
Caryophyllaceae	Solanaceae[c]
Compositae	Verbenaceae
Euphorbiaceae	
Gesneriaceae	Monocotyledoneae
Lythraceae	Liliaceae
Polygonaceae*	Xanthorroeaceae
Rhamnaceae*	Xyridaceae[d]

[a]The most important families are marked by an asterisk.
[b]Chakraborty *et al.* (1978).
[c]Knapp *et al.* (1972).
[d]Fournier *et al.* (1975).

II. QUALITY OF ANTHRAQUINONES IN CALLUS AND CELL SUSPENSION CULTURES

AQs and AQ glycosides detected and/or isolated from plant cells cultured *in vitro* are listed in Table II.

Table II

Anthraquinones and Anthraquinone Glycosides in Plant Cell Cultures[a]

Compound	Species	Reference
Monohydroxyanthraquinones		
1-Hydroxy-2-methyl-AQ[b]	*Streptocarpus dunnii*	Inoue *et al.*, 1984b
	Cinchona ledgeriana	Robins *et al.*, 1986; Wijnsma *et al.*, 1984a
1-Hydroxy-3-methyl-AQ (pachybasin)	*Digitalis lanata*	Furaya *et al.*, 1972
5-Hydroxy-2-methyl-AQ	*Cinchona ledgeriana*	Robins *et al.*, 1986
1-Hydroxy-2-hydroxymethyl-AQ	*Streptocarpus dunnii*	Inoue *et al.*, 1984b
	Cinchona pubescens	Mulder-Krieger *et al.*, 1984; Wijnsma *et al.*, 1986a
	Cinchona ledgeriana	Mulder-Krieger *et al.*, 1982; Robins *et al.*, 1986; Wijnsma *et al.*, 1984a
Dihydroxyanthraquinones		
1,2-Dihydroxy-AQ (alizarin)	*Rubia cordifolia*	Leistner and Zenk, 1967
	Morinda citrifolia	Leistner, 1973, 1975
	Galium mollugo	Bauch and Leistner, 1978a
	Cinchona pubescens	Mulder-Krieger *et al.*, 1984
	Cinchona ledgeriana	Mulder-Krieger *et al.*, 1982
glucoside	*Cinchona succirubra*	Khouri *et al.*, 1986
-*O*-glucosylxylosyl (alizarin-2-primveroside)	*Rubia cordifolia*	Suzuki *et al.*, 1982
1,4-Dihydroxy-AQ glucoside	*Cinchona succirubra*	Khouri *et al.*, 1986
1,8-Dihydroxy-AQ	*Cinchona pubescens*	Mulder-Krieger *et al.*, 1984
	Cinchona ledgeriana	Mulder-Krieger *et al.*, 1982
2,6-Dihydroxy-AQ glucoside	*Cinchona succirubra*	Khouri *et al.*, 1986
1-Methoxy-2-hydroxy-AQ	*Cinchona pubescens*	Mulder-Krieger *et al.*, 1984
	Cinchona ledgeriana	Mulder-Krieger *et al.*, 1982
1-Hydroxy-2-methoxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
1,2-Hydroxy-3-methyl-AQ*	*Digitalis lanata*	Furuya *et al.*, 1972
1,3-Dihydroxy-2-methyl-AQ (rubiadin)	*Morinda citrifolia*	Inoue *et al.*, 1981; Leistner, 1975

(continued)

Table II (*Continued*)

Compound	Species	Reference
	Cinchona pubescens	Mulder-Krieger *et al.*, 1984
	Cinchona ledgeriana	Mulder-Krieger *et al.*, 1982; Wijnsma *et al.*, 1984a
	Digitalis lanata	Furuya and Kojima, 1971
1,6-Dihydroxy-2-methyl-AQ* (soranjidiol)	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
-*O*-glucosylxylosyl	*Morinda lucida*	Igbavboa *et al.*, 1985
1,7-Dihydroxy-2-methyl-AQ*	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
1,4-Dihydroxy-3-methyl-AQ*	*Digitalis lanata*	Furuya *et al.*, 1972
1,8-Dihydroxy-3-methyl-AQ (chrysophanol)	*Cassia angustifolia*	Baier and Friedrich, 1978; Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia obtusifolia	Takahashi *et al.*, 1978
	Cassia tora	Tabata *et al.*, 1975; Takahashi *et al.*, 1978
	Cassia occidentalis	Kitanaka *et al.*, 1985; Rai and Shok, 1982
	Cassia nodosa	Rai and Shok, 1982
	Cassia alata	Rai and Shok, 1982
	Cassia podocarpa	Rai and Shok, 1982
	Rheum palmatum	Rai, 1978a; Rai and Turner, 1974
	Rumex patientia	Suchi *et al.*, 1973
	Rumex alpinus	Van den Berg and Labadie, 1981
	Rhamnus frangula	Van den Berg and Labadie, 1984; Hoefle *et al.*, 1982
	Rhamnus purshiana	Van den Berg and Labadie, 1984
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia occidentalis	Rai and Shok, 1982
	Cassia nodosa	Rai and Shok, 1982
	Cassia alata	Rai and Shok, 1982
	Cassia podocarpa	Rai and Shok, 1982
	Rumex alpinus	Van den Berg and Labadie, 1981
	Rhamnus frangula	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
1-Methoxy-2-hydroxy-3-methyl-AQ (digitolutein)	*Digitalis lanata*	Furuya and Kojima, 1971
1,8-Dihydroxy-2-hydroxymethyl-AQ (aloe-emodin)	*Cassia angustifolia*	Baier and Friedrich, 1970; Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia obtusifolia	Takahashi *et al.*, 1978
	Cassia occidentalis	Rai and Shok, 1982
	Cassia alata	Rai and Shok, 1982

(*continued*)

Table II (*Continued*)

Compound	Species	Reference
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia occidentalis	Rai *et al.*, 1974
	Cassia alata	Rai *et al.*, 1974
	Rhamnus frangula	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
2,4-Dihydroxy-3-hydroxymethyl-AQ (lucidin)	*Morinda citrifolia*	Inoue *et al.*, 1981; Leistner, 1973
	Galium mollugo	Bauch and Leistner, 1978a
-*O*-glucosylxylosyl	*Morinda citrifolia*	Inoue *et al.*, 1981
	Morinda lucida	Igbavboa *et al.*, 1985
	Galium mollugo	Bauch and Leistner, 1978a; Inoue *et al.*, 1984a
2-Hydroxy-4-methoxy-3-hydroxymethyl-AQ	*Rubia cordifolia*	Suzuki *et al.*, 1982
1,3-Dihydroxy-2-oxy-AQ (nordamnacanthal)	*Morinda citrifolia*	Leistner, 1975
1,8-Dihydroxy-3-carboxy-AQ (rhein)	*Cassia angustifolia*	Baier and Friedrich, 1970; Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia alata	Rai and Shok, 1982
	Cassia podocarpa	Rai and Shok, 1982
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia nodosa	Rai and Shok, 1982
	Cassia alata	Rai and Shok, 1982
	Cassia podocarpa	Rai and Shok, 1982
Trihydroxyanthraquinones		
1,2,4-Trihydroxy-AQ (purpurin)	*Rubia cordifolia*	Suzuki *et al.*, 1982
	Galium mollugo	Bauch and Leistner, 1978a; Inoue *et al.*, 1979
	Cinchona pubescens	Wijnsma *et al.*, 1986a
	Cinchona ledgeriana	Robins *et al.*, 1986; Wijnsma *et al.*, 1984a
glucoside	*Cinchona succirubra*	Khouri *et al.*, 1986
1,2,7-Trihydroxy-AQ glucoside	*Cinchona succirubara*	Khouri *et al.*, 1986
1-Methoxy-2,4-dihydroxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
1,3-Dihydroxy-4-methoxy-AQ*	*Cinchona ledgeriana*	Robins *et al.*, 1986; Wijnsma *et al.*, 1984a
1,2-Dimethoxy-3-hydroxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
	Cinchona ledgriana	Wijnsma *et al.*, 1984a
1,3-Dimethoxy-2-hydroxy-AQ	*Cinchona ledgeriana*	Robins *et al.*, 1986; Wijnsma *et al.*, 1984a

(*continued*)

Table II (*Continued*)

Compound	Species	Reference
1,2,3-Trimethoxy-AQ	*Cinchona ledgeriana*	Robins *et al.*, 1986
5,6-Dimethoxy-1-hydroxy-2-hydroxymethyl-AQ*	*Cinchona ledgeriana*	Wijnsma *et al.*, 1984a
1,5,6-Trihydroxy-2-methyl-AQ (morindone)	*Morinda citrifolia*	Inoue *et al.*, 1981; Leistner, 1975; Leistner and Zenk, 1967
-*O*-glucosylxylosyl	*Morinda citrifolia*	Inoue *et al.*, 1984a
	Morinda lucida	Igbavboa *et al.*, 1985
1,6-Dihydroxy-8-methoxy-3-methyl-AQ (questin)	*Cassia occidentalis*	Kitanaka *et al.*, 1985
1,6-Dihydroxy-5-methoxy-2-methyl-AQ	*Cinchona ledgeriana*	Robins *et al.*, 1986
2,8-Dihydroxy-1-methoxy-3-methyl-AQ (obtusifolin)	*Cassia obtusifolia*	Takahashi *et al.*, 1978
6,7-Dihydroxy-1-methoxy-2-methyl-AQ*	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
1,6-Dihydroxy-5-methoxy-2-methyl-AQ	*Cinchona ledgeriana*	Wijnsma *et al.*, 1984a
1,8-Dihydroxy-6-methoxy-3-methyl-AQ (physcion)	*Cassia angustifolia*	Baier and Friedrich, 1970; Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia obtusifolia	Takahashi *et al.*, 1978
	Cassia tora	Tabata *et al.*, 1975; Takahashi *et al.*, 1978
	Cassia occidentalis	Kitanaka *et al.*, 1985
	Rumex alpinus	Van den Berg and Labadie, 1981
	Rhamnus frangula	Van den Berg and Labadie, 1984; Hoefle *et al.*, 1982
	Rhamnus purshiana	Van den Berg and Labadie, 1984
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Rhamnus frangula	Van den Berg and Labadie, 1984
1,8-Dihydroxy-6-methoxy-3,7-dimethyl-AQ*	*Cassia occidentalis*	Kitanaka *et al.*, 1985
1,2,8-Trihydroxy-3-methyl-AQ*	*Rhamnus frangula*	Hoefle *et al.*, 1982
1,4,8-Trihydroxy-3-methyl-AQ (islandicin)	*Cassia obtusifolia*	Takahashi *et al.*, 1978
	Cassia occidentalis	Kitanaka *et al.*, 1985
1,4,5-Trihydroxy-2-methyl-AQ	*Cinchona ledgeriana*	Robins *et al.*, 1986
1,2,4-Trihydroxy-3-methyl-AQ*	*Digitalis lanata*	Furuya *et al.*, 1972
1-Methoxy-2,5-dihydroxy-3-methyl-AQ*	*Tectona grandis*	Dhruva *et al.*, 1972
1-Methoxy-2,4-dihydroxy-3-methyl-AQ	*Digitalis lanata*	Furuya and Kojima, 1971
1,6,8-Trihydroxy-3-methyl-AQ (emodin)	*Cassia angustifolia*	Baier and Friedrich, 1970; Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974

(*continued*)

Table II (*Continued*)

Compound	Species	Reference
	Cassia obtusifolia	Takahashi *et al.*, 1978
	Cassia tora	Tabata *et al.*, 1975
	Cassia occidentalis	Kitanaka *et al.*, 1985; Rai and Shok, 1982
	Cassia podocarpa	Rai and Shok, 1982
	Rheum palmatum	Rai, 1978a; Rai *et al.*, 1974
	Rumex patientia	Suchy *et al.*, 1973
	Rumex alpinus	Van den Berg and Labadie, 1981
	Rhamnus frangula	Van den Berg and Labadie, 1984; Hoefle *et al.*, 1982
	Rhamnus purshiana	Van den Berg and Labadie, 1984
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Rhamnus purshiana	Van den Berg and Labadie, 1984
	Cinchona succirubra	Khouri *et al.*, 1986
3,5,6-Trihydroxy-2-methyl-AQ*	*Morinda citrifolia*	Inoue *et al.*, 1981
-*O*-glucosylxylosyl*	*Morinda citrifolia*	Inoue *et al.*, 1981
1,6,8-Trihydroxy-3-methyl-AQ glucoside	*Cinchona succirubra*	Khouri *et al.*, 1986
1,2,4-Trihydroxy-3-carboxy-AQ	*Rubia cordifolia*	Suzuki *et al.*, 1982
	Galium mollugo	Bauch and Leistner, 1978a
-*O*-glucosyl	*Galium mollugo*	Bauch and Leistner, 1978a
-*O*-glucosylxylosyl (galiosin)	*Galium Mollugo*	Bauch and Leistner, 1978a
Tetrahydroxyanthraquinones		
1,2,5,8-Tetrahydroxy-AQ glucoside	*Cinchona succirubra*	Khouri *et al.*, 1986
2,4,5-Trihydroxy-1-methoxy-AQ*	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
1,3,5-Trihydroxy-4-methoxy-AQ*	*Cinchona ledgeriana*	Wijnsma *et al.*, 1984a
1,4,6-Trihydroxy-5-methoxy-2-methyl-AQ	*Cinchona ledgeriana*	Wijnsma *et al.*, 1984a
1,3-Dihydroxy-2,5-dimethoxy-AQ	*Cinchona ledgeriana*	Robins *et al.*, 1986; Wijnsma *et al.*, 1984a
4,6-Dihydroxy-2,7-dimethoxy-AQ*	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
2-Hydroxy-1,3,4-trimethoxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
	Cinchona ledgeriana	Robins *et al.*, 1986; Wijnsma *et al.*, 1984a
1,5,8-Trihydroxy-6-methoxy-3-methyl-AQ (xanthorin)	*Cassia obtusifolia*	Takahashi *et al.*, 1978
1,2,5,6-Tetramethoxy-AQ*	*Cinchona ledgeriana*	Robins *et al.*, 1986
1,2,6,8-Tetrahydroxy-3-methyl-AQ*	*Rhamnus frangula*	Hoefle *et al.*, 1982
1,3,5,6-Tetrahydroxy-2-methyl-AQ*	*Morinda citrifolia*	Inoue *et al.*, 1981
-*O*-glucosyl-xylosyl*	*Morinda citrifolia*	Inoue *et al.*, 1981
1,3,6-Trihydroxy-5-methoxy-2-methyl-AQ*	*Cinchona ledgeriana*	Wijnsma *et al.*, 1984a

(*continued*)

Table II (*Continued*)

Compound	Species	Reference
1,4,6-Trihydroxy-5-methoxy-3-methyl-AQ*	*Cinchona ledgeriana*	Wijnsma *et al.*, 1984a
1,2,8-Trihydroxy-5-methoxy-3-methyl-AQ*	*Rhamnus frangula*	Hoefle *et al.*, 1982
2,4,5,6-Tetrahydroxy-3-hydroxy-methyl-AQ*	*Morinda citrifolia*	Inoue *et al.*, 1981
-*O*-glucosylxylosyl*	*Morinda citrifolia*	Inoue *et al.*, 1981
1,4-Dimethoxy-2,3-methylenedioxy-AQ*	*Cinchona ledgeriana*	Robins *et al.*, 1986; Wijnsma *et al.*, 1984a
1,5-Dimethoxy-2,3-methylenedioxy-AQ*	*Cinchona ledgeriana*	Robins *et al.*, 1986
Pentahydroxyanthraquinones		
2,5-Dihydroxy-1,3,4-trimethoxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
3,5-Dihydroxy-1,2,4-trimethoxy-AQ	*Cinchona ledgeriana*	Wijnsma *et al.*, 1984a
2-Hydroxy-1,3,4,6-tetramethoxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
2-Hydroxy-1,3,4,7-tetramethoxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
2-Hydroxy-1,3,5,6-tetramethoxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
2-Hydroxy-1,3,4,7-tetramethoxy-AQ	*Cinchona pubescens*	Wijnsma *et al.*, 1986a
2,4,6-Trihydroxy-1,3-dimethoxy-AQ	*Cinchona ledgeriana*	Robins *et al.*, 1986
2,6,8-Trihydroxy-1,7-dimethoxy-3-methyl-AQ (aurantioobtusin)	*Cassia obtusifolia*	Takahashi *et al.*, 1978

[a] Asterisk indicates novel compound.
[b] AQ ≡ anthraquinone.

A. Anthraquinones of the Acetate–Polymalonate Biosynthetic Pathway

As early as 1970, anthracene derivatives were detected in callus cultures derived from cotyledons of *Cassia angustifolia* (Baier and Friedrich, 1970), irrespective of the growth hormones [2,4-dichlorophenoxyacetic acid (2,4-D) or indolyl-3-acetic acid (IAA)] and environmental conditions employed. In a further study the occurrence of oxidized AQs was confirmed, and reduced AQs were detected also (Friedrich and Baier, 1973) (Tables III and IV). The chrysophanolanthrone found may have been a fission product of the dianthrone and, therefore, not native. All oxidized and reduced AQs occurred in the form of glucosides also. Callus cultures established similarly from cotyledons and hypocotyls of *Cassia senna* (synonym, *C. acutifolia*) and cultured for 7 to 10 months also produced AQs and free anthrones as well as the corresponding *o*-glucosides, except physcion glucoside. Contrary to callus cultures of *C.*

Table III

Anthraquinones of the Acetate–Polymalonate Biosynthetic Pathway Found in Cell Cultures[a]

1,8-Dihydroxy-3-methyl-AQ[b]	Chrysophanol
1,2,8-Trihydroxy-3-methyl-AQ	2-Hydroxy chrysophanol
1,8-Dihydroxy-6-methoxy-3-methyl-AQ	Physcion
1,2,8-Trihydroxy-5-methoxy-3-methyl-AQ	2-Hydroxy physcion
1,8-Dihydroxy-6-methoxy-3,7-dimethyl-AQ	7-Methyl physcion
1,6,8-Trihydroxy-3-methyl-AQ	Emodin
1,8-Dihydroxy-3-carboxy-AQ	Rhein
1,8-Dihydroxy-2-hydroxymethyl-AQ	Aloe emodin
1,4,8-Trihydroxy-3-methyl-AQ	Islandicin
1,5,8-Trihydroxy-6-methoxy-3-methyl-AQ	Xanthorin
2,8-Dihydroxy-1-methoxy-3-methyl-AQ	Obtusifolin
2,6,8-Trihydroxy-1,7-dimethoxy-3-methyl AQ	Aurantiobtusin

[a]For references, see Table II.
[b]AQ ≡ anthraquinone.

angustifolia, sennosides were not found in this plant (Rai *et al.,* 1974). Callus derived from hypocotyl or stem of *C. obtusifolia* and cultured in light produced AQs with aloe-emodin as the principal AQ [medium with 8.6 μM IAA and 8.9 μM benzyladenine (BA)]. When grown in the presence of 0.6 μM IAA and 0.09 μM BA and producing rootlets, the aloe-emodin content in the callus was reduced in favor of emodin and obtusifolin (Takahashi *et al.,* 1978). Calli derived from seedlings of *C. tora* were cultured in the dark in media containing various growth hormones in different concentrations. After a growth period of 55 days all cultures contained known AQs and one compound not identified. AQs (chrysophanol and physcion) were present also in illuminated callus cultures of *C. tora* (Tabata *et al.,* 1975). AQs (Fig. 2), including the newly detected 7-methylphyscion, the dianthrone chrysophanol-10,10′-dianthrone, the tetrahydroanthracenes germichrysone (**1**), methylgermitorosone (**2**), and 7-methyltorosachrysone (**3**) [novel substance; witness also the occurrence of torosachrysone (**4**), novel substance, in cell cultures of *Rhamnus frangula* (Burlager *et al.,* 1984)], and the xanthone pinselin (**5**), were isolated from callus cultures of *C. occidentalis* (Kitanaka *et al.,* 1985). Furthermore, chrysophanol, emodin, and aloe-emodin were detected. In cell cultures of *C. nodosa, C. alata,* and *C. podocarpa,* the AQs and AQ glucosides indicated in Table III were found (Rai and Shok, 1982). Also, in callus and cell suspension cultures of *C. torosa,* germichrysone (**1**) could be detected, but this reduced anthracene was converted into pinselin after a culture period of 6 weeks, a conversion that could be inhibited

Table IV

Anthrones and Their Glucosides of the Acetate–Polymalonate Biosynthetic Pathway Found in Cell Cultures

Anthrone	Species	Reference
Chrysophanolanthrone	*Cassia angustifolia*	Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Rhamnus frangula	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Rhamnus frangula	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
Chrysophanol dianthrone	*Cassia angustifolia*	Friedrich and Baier, 1973
	Cassia senna	Rai *et al.*, 1974
	Cassia occidentalis	Kitanaka *et al.*, 1985
	Rumex alpinus	Van den Berg and Labadie, 1981
	Rhamnus frangula	Van den Berg and Labadie, 1984
glucoside	*Cassia augustifolia*	Friedrich and Baier, 1973
	Rhamnus frangula	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
Palmidin A, B, C, D	*Cassia angustifolia*	Friedrich and Baier, 1973
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Cassia angustifolia	Friedrich and Baier, 1973
Sennedin A, B, C, D	*Cassia angustifolia*	Friedrich and Baier, 1973
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
Emodinanthrone	*Rhamnus frangula*	Van den Berg and Labadie, 1984
glucoside	*Rhamnus frangula*	Van den Berg and Labadie, 1984
Emodindianthrone	*Cassia angustifolia*	Friedrich and Baier, 1973
	Rhamnus frangula	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
	Rhamnus frangula	Van den Berg and Labadie, 1984
Aloe-emodin anthrone	*Cassia angustifolia*	Friedrich and Baier, 1973
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973
Physcionanthrone	*Rhamnus frangula*	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
glucoside	*Rhamnus frangula*	Van den Berg and Labadie, 1984
	Rhamnus purshiana	Van den Berg and Labadie, 1984
Physciondianthrone	*Rumex alpinus*	Van den Berg and Labadie, 1981
glucoside	*Rhamnus frangula*	Van den Berg and Labadie, 1984
Chrysophanol physicion-dianthrone	*Rumnex alpinus*	Van den Berg and Labadie, 1981
Rheidin A, B	*Cassia angustifolia*	Friedrich and Baier, 1973
glucoside	*Cassia angustifolia*	Friedrich and Baier, 1973

1 Germichrysone

2 Methylgermitorosone
$R_1 = CH_3$
$R_2 = OCH_3$
$R_3 = OH$

3 7-Methyltorosachrysone
$R_1 = CH_3$
$R_2 = OH$
$R_3 = H$

4 Torosachrysone
$R_1 = H$
$R_2 = OH$
$R_3 = H$

5 Pinselin

Fig. 2.

by addition of ascorbic acid to the medium (Noguchi and Sankawa, 1982; Takahashi *et al.*, 1978, 1981). Callus cultures of *Rumex patientia* have been shown to contain chrysophanol and emodin (Suchy *et al.*, 1973); and those of *Rumex alpinus*, physcion, some anthrones, and one AQ glucoside (van den Berg and Labadie, 1981). From callus tissues of *Rhamnus frangula* and *R. purshiana* cultured in a medium containing 4.5 μM 2,4-D and 0.5 μM kinetin at 25°C in the dark, AQs, anthrones, and their glucosides were isolated (van den Berg and Labadie, 1981). In a similar investigation of callus of *Rhamnus frangula* (Höfle *et al.*, 1982) in a medium containing 2 μM 2,4-D only, free AQs were detected, among them four novel AQ species unknown for the plant [2-hydroxyphyscion, 2-hydroxychrysophanol, 5-hydroxyemodin, and torosachrysone (**4**)].

B. Anthraquinones of the Shikimate–*o*-Succinylbenzoate Biosynthetic Pathway

Six AQ derivatives were isolated from callus tissues of *Digitalis lanata* and identified, among them digitolutein and 4-hydroxydigitolutein (synonym, 3-methylpurpurin-1-methyl ether; novel compound) (Furuya and Kojima, 1971). The other AQs were present in trace amounts only;

they were identified as 3-methylquinizarin, pachybasin, 3-methylpurpurin (novel compound), and 3-methylalizarin (Furuya *et al.*, 1972). In cell cultures of *Tectona grandis* a novel compound, 5-hydroxydigitolutein, has been found (Dhruva *et al.*, 1972).

The majority of investigations of AQs in callus and cell suspension cultures have been performed with members of the Rubiaceae, particularly with the genera *Morinda* and *Galium*, but also with *Rubia* and *Cinchona*. In 1975 a stable cell line of *M. citrifolia* was shown to produce high amounts of AQs when naphthaleneacetic acid (NAA) was used as growth-regulating substance. The cell line failed to form any AQ when NAA was substituted by 2,4-D (Zenk *et al.*, 1975). Alizarin, rubiadin, nordamnacanthal, and morindone have been isolated from these cell suspension cultures. Moreover, lucidin, an AQ not known to be a constituent of the intact *Morinda* plant at that time, was shown to occur in this cell culture (Leistner, 1975).

A cell suspension culture derived from *Morinda lucida* was shown to contain the glycoside lucidinprimveroside as the principal pigment when cultured in a heterotrophic state in the dark (medium containing sucrose), whereas morindoneprimveroside prevailed in the intact root (Igbavboa *et al.*, 1985). Over time, relatively high illumination intensities (5000–6000 lux) resulted in the formation of green cell cultures able to grow in a medium lacking sucrose. In this state no AQs were formed in the cells, but lipoquinones [phylloquinone (synonym, vitamin K_1), plastoquinone, tocopherol, and ubiquinone] were produced. The same behavior, however, was shown to also occur in the presence of sucrose. When such a culture was transferred to fresh medium containing sucrose and cultured in the dark, lipoquinones and chlorophyll disappeared and AQs were produced. From this behavior it can be concluded that the biosynthetic pathway proceeds from the same compounds, chorismate via isochorismate and *O*-succinylbenzoate to 1,4-dihydroxy-2-naphthoate (see also Section IV), an intermediary compound representing the branching point, leading to either phylloquinones (in the photosynthetically active cells) or to AQs (in the nonphotosynthetically active cells) (Fig. 3).

In a more recent ultra structural study Yamamoto *et al.* (1987) reported on differences in subcellular structures between AQ producing and nonproducing cells of *M. lucida*. Irregular or distorted plastids containing starch grains were observed in the AQ producing cells, together with a highly elongated rough endoplasmatic reticulum. The possibilities have been discussed whether the AQs are actually formed in the distorted plastids or whether a common precursor of phylloquinone and AQ, which is probably synthesized in the cytosol, could not be taken up by

O

CH_3

Prenyl

O

Phylloquinones

OH

COOH

Isochorismate

+

α-Oxoglutarate

3-Prenylation

OH

1,4-Dihydroxy-2-
naphthoic acid
(DHNA)

O

O

Anthraquinones

Fig. 3.

the distorted plastids for phylloquinone synthesis, but was used for AQ synthesis by different enzymes located in the cytosol.

Cell suspension cultures of *Galium mollugo* were shown to contain lucidinprimveroside as the principal pigment besides purpurincarboxy-1-glucoside and galiosin, and the AQs alizarin, purpurin, purpurincarboxylic acid, and lucidin (Bauch and Leistner, 1978a,b). In a medium containing 9 μM 2,4-D, 2.7 μM NAA, and 0.9 μM kinetin, the appearance of the aglycones in comparison to the glycosides was delayed; in the presence of 15 μM NAA, as the sole hormone, the yields of aglycones and glycosides increased at about equal rates. Whereas purpurincarboxylic acid glucoside was detectable in cells cultured in various media, galiosin occurred only in B5 medium (Gamborg *et al.*, 1968) with 0.06 M sucrose, 2 g/liter NZ-amine, and 15 μM NAA. Since the analysis of factors affecting growth and AQ production in a batch culture system is rather difficult to perform because of changes in growth rate and medium composition, chemostat cultures with phosphate as limiting factor have been established (Wilson and Marron, 1978). As a result, cell proliferation and AQ production have shown to be parallel.

Cell suspension cultures of *Streptocarpus dunnii* generating plantlets were shown to produce 1-hydroxy-2-hydroxymethyl AQ and 1-hydroxy-2-methyl AQ (Inoue *et al.*, 1984b). Callus and cell suspension cultures of *Cinchona* species produced AQs more readily than alkaloids. The possibility of a competition for a common precursor (e.g., mevalonate) as a cause for low alkaloid levels in the cell cultures may explain this situation (see Wijnsma *et al.*, 1984a). As AQs are not present in healthy intact plants but are produced readily in cell cultures, the possibility of production due to stress conditions was considered. Thus, they were expected to be elicited by fungal elicitors. This hypothesis was substantiated by treatment of cell cultures of *C. ledgeriana* and also *Rubia tinctoria* and *Morinda citrifolia* with an autoclaved suspension of *Aspergillus niger* mycelium, leading to an increased AQ production (Wijnsma *et al.*, 1986c). Moreover, it could be demonstrated that plant parts of *C. ledgeriana* infected with pathogens contain AQs. In studies with cell cultures of *Cinchona* species not only callus cultures [*C. ledgeriana* (Mulder-Krieger *et al.*, 1982; Wijnsma *et al.*, 1984a,b, 1986b), *C. pubescens* (Mulder-Krieger *et al.*, 1984; Wijnsma *et al.*, 1986a)] but also cell suspension cultures [*C. ledgeriana* (Robins *et al.*, 1986; Wijnsma *et al.*, 1986b)], *C. succirubra* (synonym, *C. pubescens*) (Khouri *et al.*, 1986)] have been investigated. In all these studies the AQs have been found to exist in a free form, not as glycosides. Investigations by Khouri *et al.* (1986), however, have shown all AQs to be glucosides. The majority of them were glucosides of alizarin and emodin, both of which amounted to 80% of total AQs. The remaining

20% consisted of the glucosides of 1,4-dihydroxy-, 2,6-dihydroxy-, 1,2,7-trihydroxy-, 1,2,4-trihydroxy- and 1,2,5,8-tetrahydroxy-AQs.

In experiments with cell suspension cultures of *Cinchona ledgeriana* (Robins *et al.*, 1986) L-tryptophan [as in the case of *Morinda citrifolia* (El Shagi *et al.*, 1984)] and glyphosate [as in the case of *Galium mollugo* (Amrhein *et al.*, 1980)] were found to be inhibitory for the production of AQs. AQs detected in cell cultures of *Cinchona* species are listed in Table II.

III. QUANTITATIVE ASPECTS OF ANTHRAQUINONE OCCURRENCE

Contents (in percentages or micromoles per gram fresh or dry weight of cell material) and yields (in milligrams or micromoles per vessel or liter) of AQs in callus and cell suspension cultures are affected by diverse chemical (minerals, carbohydrates, growth regulating substances, vitamins, amino acids) and physical (light, temperature) factors. For quantification, mostly spectrophotometric methods are used. Because in this case the total of AQs, AQ glycosides, anthrones, and anthrone glycosides is estimated and because the structures of both aglycones and carbohydrate moieties vary substantially, resulting in rather different molecular weights, it is not easy to find a reference substance for comparative purposes, a matter that compounds the dilemma of any calculation of AQ yields. It has been pointed out (Zenk *et al.*, 1975) that in the case of cell cultures of *Morinda citrifolia,* however, 90% of the absorption at 434 nm is due to about four main AQ glycosides, whereas only 10% of the absorption is due to AQ aglycones. As in these cell cultures AQ glycosides exceed AQs and as the molar extinction coefficients of several glycosides and aglycones at this wavelength do not differ appreciably, alizarin has been selected as a reference substance (molar extinction coefficient $\epsilon = 5.500$ at 434 nm in 80% aqueous ethanol), and as a basis a $M_r \approx 400$ of the AQ glycosides is used for the calculations. In this way an estimation of contents and yields on weight basis is possible even in cases where only micromoles per gram or per liter are indicated, and even when numeric values are not expressed explicitly in the original paper. Contents and yields of AQs and AQ glycosides following the acetate–polymalonate pathway are listed in Table V, those following the shikimate–*o*-succinylbenzoate pathway in Table VI.

In one of the most cited papers in the field of quantitative aspects of

Table V

Quantitative Analytical Data of Cell Cultures Containing Anthraquinones Following the

Species	Anthraquinone determination mode and reference substance	Cell mass contents (fresh weight, mg per tube)	Total contents	
			Percentage fresh weight	Percentage dry weight
Cassia angustifolia	Spectrophotometry; dihydroxy-AQ[b] monoglucoside, 525 nm			0.4
				1.6
Cassia tora	Spectrophotometry; chrysophanol, 502 nm	**288**		0.107
		24		**0.282**
		124		0.156
Cassia senna	Spectrophotometry; alizarin, 510 nm			1.2
				1.215
				1.285
				1.042
				1.225
				1.56
Cassia occidentalis				0.62
Cassia nodosa				0.50
Cassia alata				0.75
Cassia podocarpa				1.30
Rheum palmatum	Spectrophotometry; 1,8-dihydroxy-AQ, 510 nm			0.30
Rumex alpinus	Densitometry; individually, 430 nm		0.0053	
			0.0006	
Rhamnus frangula	Spectrophotometry; 1,8-dihydroxy-AQ, 500 nm		0.11	
			0.05	
			0.80	
Rhamnus purshiana	Spectrophotometry; 1,8-dihydroxy-AQ, 500 nm		0.14	

[a]Maximum values in each category are **boldfaced**.

[b]AQ ≡ anthraquinone.

[c]Composed of chrysophanol, 0.450; physcion, 0.050; emodin, 0.055; rhein, 0.045; Aloe-emodin, 0.055; chrysophanolanthrone, 0.048; and chrysophanoldianthrone, 0.032.

[d]Composed of the glucosides (%) of chrysophanol, 0.252; emodin, 0.057; rhein, 0.040; and aloe-emodin, 0.051.

[e]Composed of chrysophanol, emodin, and aloe-emodin.

[f]Composed of the glycosides of chrysophanol, emodin, and aloe-emodin.

[g]Chrysophanol.

[h]Composed of the glycosides of chrysophanol and rhein.

[i]Composed of chrysophanol, aloe-emodin, and rhein.

[j] Composed of the glycosides of chrysophanol, aloe-emodin, and rhein.

[k]Composed of chrysophanol, emodin, and rhein.

[l]Composed of the glycosides of chrysophanol and rhein.

Acetate–Polymalonate Biosynthetic Pathway[a]

Anthraquinone yields (μg per tube)	Contents (% dry weight) Anthraquinone aglycones	Anthraquinone glycosides	Reference
			Friedrich and Baier, 1973
			Friedrich and Baier, 1973
308			Tabata *et al.*, 1975
68			Tabata *et al.*, 1975
350			Tabata *et al.*, 1975
	0.8[c]	0.4[d]	Rai *et al.*, 1974
	0.815	0.4	Rai, 1978b
	1.1	0.185	Rai, 1978b
	0.727	0.315	Rai, 1978b
	0.820	0.405	Rai, 1978b
	1.1	0.46	Rai, 1978b
	0.32[e]	0.30[f]	Rai and Shok, 1982
	0.10[g]	0.40[h]	Rai and Shok, 1982
	0.30[i]	0.45[j]	Rai and Shok, 1982
	0.80[k]	0.50[l]	Rai and Shok, 1982
	0.05	0.25	Rai, 1978a
			Van den Berg and Labadie, 1981
	Traces	0.11	Van den Berg and Labadie, 1984
			Höfle *et al.*, 1982
			Höfle *et al.*, 1982
	Traces	0.14	Van den Berg and Labadie, 1984

AQ formation in plant cell cultures, Zenk *et al.* (1975), using cell suspension cultures of *Morinda citrifolia*, investigated the influence of 146 different synthetic compounds with auxin activity. Only 19 substances were able to support growth; only a few of them affected the formation of AQs, among them NAA. 2,4-D was shown to be rather ineffective. Thus, in the standard nutrient medium for all further studies, NAA (10 μM) was used as an auxin. The inhibitory effect of 2,4-D on AQ formation was not alleviated by simultaneous addition of equimolar concentrations of NAA, but could be reverted by subculture using NAA for 2,4-D in the medium, even after several years. An addition of IAA to the

Table VI

Quantitative Analytical Data of Cell Cultures Containing Anthraquinones Following the Shikimate–*o*-Succinylbenzoate Biosynthetic Pathway[a]

Species	Anthraquinone determination mode and reference substance	Cell mass contents (g/liter)		Anthraquinones					Reference
				Contents			Yields		
		Fresh weight	Dry weight	μM gm fresh weight	Micromoles per gram dry weight	Percentage dry weight	μmol/liter	mg/liter	
Morinda citrifolia	Spectrophotometry; alizarin, 434 nm		5.5				0	0	Zenk *et al.*, 1975
			5.5				1200		Zenk *et al.*, 1975
			3.5				2100		Zenk *et al.*, 1975
		351	7	17.9	900		**6300**	**2500**	Zenk *et al.*, 1975
				15.6	110				Zenk *et al.*, 1975
Galium mollugo	Spectrophotometry; lucidin, 420 nm	**302**	21.0	1.5	21.0		440	250	Bauch and Leistner, 1978a
		270	12.6	0.4	7.8		98	60	Bauch and Leistner, 1978a
		302	**37.8**	11.9	95.2		**3600**	**2030**	Bauch and Leistner, 1978a
		106	11.8	0.4	3.1		37	20	Bauch and Leistner, 1978a
	Spectrophotometry; alizarin, 434 nm		15					180	Wilson and Marron, 1978
			4.9					3	Wilson and Marron, 1978
Rubia cordifolia	Spectrophotometry; purpurin, 516 nm		30		72		2200		Suzuki *et al.*, 1984
Cinchona succirubra	Spectrophotometry; alizarin, 435 nm	**130.9**	4.5		1.8		8.1		Khouri *et al.*, 1986
		96.7	**4.8**		1.8		8.6		Khouri *et al.*, 1986
		64.1	2.8		**7.0**		**19.7**		
Cinchona ledgeriana	Spectrophotometry; alizarin, 500 nm		**24.1**			0.107		**23.5**	Wijnsma *et al.*, 1986a
			5.3			**0.144**		5.8	Wijnsma *et al.*, 1986a
	Spectrophotometry; rubiadin, 490 nm					0.096			Harkes *et al.*, 1985

[a]Culture mode in all cases was the cell suspension procedure. Maximum values in each category are **boldfaced**.

medium containing 10 μM NAA resulted in a decrease of AQ accumulation. All other growth hormones (gibberellic acid, kinetin, abscisic acid) were shown to be effective or inhibitory to AQ accumulation. Of all carbohydrates tested for growth and AQ accumulation, sucrose proved to be optimal, and an enormous increase of product yield was obtained by raising the sucrose concentration in the standard medium to 0.2 *M*. The presence of pancreatic casein hydrolysate (NZ-amine) in the medium was a definite requirement for growth and AQ formation, but it could be replaced by asparagine as sole source of organic reduced nitrogen. Doubling the nitrate concentration in the medium did not stimulate or reduce AQ synthesis; increasing the phosphate concentration to 400 m*M* enhanced AQ accumulation to about 50%. Iron and calcium ions were absolutely necessary for growth and pigment formation. Also, vitamins and cofactors (myoinositol, thiamine, nicotinic acid, and to a certain extent, pyridoxin) were indispensable for satisfactory growth and metabolite production. Initial optimum pH values for growth and pigment formation were between 4.0 and 7.5; optimum temperature for both parameters was 30°C.

In further studies Zenk *et al.* (1984) used 40 different synthetic phenoxyacetic acids substituted in para position with halogen. All supported growth, and product formation increased with the character of the halogen atom: F < Cl < Br < I. Highest yields were obtained in the presence of 4-methyl-, 2,3-dimethyl-, or 2-bromophenoxyacetic acids, producing amounts exceeding those by NAA by more than 50%. But when an oxygen atom was inserted between the *para*-methyl group and the aromatic ring (i.e., 4-methoxy), AQ accumulation was drastically reduced, similar to the level with 2,4-D. Also, L-tryptophan and its precursors were potent inhibitors of AQ formation in *Morinda citrifolia* cell suspension cultures [El Shagi *et al.*, 1984; witness also the inhibition of AQ formation by L-tryptophan in cell suspension cultures of *Cinchona ledgeriana* (Robins *et al.*, 1986)]. In feeding experiments with either shikimate or *O*-succinylbenzoate together with L-tryptophan, the ability to form pigments could not be restored. Thus, it was concluded that the repression site was not due to its interference with the pathway of aromatic amino acid biosynthesis, but must be located further down the specific AQ pathway.

Good growth and high production of one cell line of *Morinda citrifolia* led to experiments using biotechnological methods (Vogelmann *et al.*, 1976; Wagner and Vogelmann, 1977). In experiments with different types of bioreactors, flat-blade turbine, perforated-disk impeller, draft-tube reactor, and airlift reactor, it was demonstrated that the phase of cell growth was separated from the phase of AQ formation. At first,

glucose accumulated due to early consumption of the fructose moiety of sucrose. This phase ended when maximum raw cell dry weight was reached. Ten days later, at the end of the second phase, maximum AQ yield was obtained. The AQ yield was strictly dependent on the rate of aeration. Reducing this parameter resulted in reduced AQ yield, whereas the yield of cell mass remained the same. The AQ yield in an airlift fermenter was about 30% higher than the yield in a shake flask. Favorable results lasted only until the cell density reached 20 g cell dry weight per liter, then shear stresses due to high viscosity and swollen cells became the limiting factor. For this reason a reactor type with a Kaplan turbine was used. But despite a low speed of 350 rpm, which is the minimum necessary for sufficient macromixing, the yield of AQs was only about one-third of that reached in the airlift reactor. Thus, it was concluded that the airlift reactor might be the system of choice for further optimization of the production of metabolites by plant cell cultures.

Immobilization of cells to improve the productivity of plant cell cultures has been performed with cultures of *Morinda citrifolia* (Brodelius *et al.*, 1979). Entrapping cells in alginate beads and culturing them in a nutrient medium without growth hormones resulted in higher AQ production than with cells freely suspended in media containing growth hormones. With immobilization the increase of cell number was small, and it seemed that the biochemical potential was diverted from growth by cell division to secondary product formation. Plasmolysis and respiration proved that the immobilized cells were viable after a culture period of 22 days at 23°C. Because such immobilization experiments were performed successfully also with other product-forming cell cultures, the opportunity to entrap cells in gels in order to increase and stabilize the biosynthetical potential should be investigated further.

Determination of content and yield of AQs in cell suspension cultures of *Galium mollugo* (Bauch and Leistner, 1973b) was carried out using lucidin as a reference substance for quantifying the aglycones (absorption at 415 nm), and lucidinprimveroside for quantifying the glycosides (absorption at 408 nm) and total pigment (absorption at 420 nm). Maximum cell yield and AQ content and yield (maximum values are listed in Table VI) were very dependent on the composition of the nutrient medium. In contrast to the results with *Morinda citrifolia* cell suspension cultures, the AQ production in *G. mollugo* cells was realized in media with 2,4-D. The entire AQ pathway in *G. mollugo* cell cultures was blocked by addition of the nonselective herbicide glyphosate to the nutrient medium, an inhibition that was alleviated by chorismate and *O*-succinylbenzoate. This observation suggested that glyphosate inhibits a step in the biosynthetic sequence from shikimate to chorismate, and shikimate is enriched in the cultured cells (Amrhein *et al.*, 1980; witness

also the inhibition of the AQ formation by glyphosate in cell suspension cultures of *Cinchona ledgeriana*, Robins *et al.*, 1986).

Studies similar to those with *Morinda citrifolia* (see above) have been performed with cell suspension cultures of *Rubia cordifolia* (Suzuki *et al.*, 1982, 1984). Contrary to the *Morinda* cell cultures, sucrose as carbon source was inferior to glucose; optimum AQ yields were obtained with 0.27 *M* glucose and 0.11–0.83 m*M* myoinositol. The best inorganic nitrogen source was a 1 : proportion of $NH_4^+ : NO_3^-$, total amount of nitrogen as in the basal Murashige–Skoog (MS) medium (i.e., 60 mmol/liter). These conditions were fulfilled with 30 m*M* NH_4NO_3 and by substituting the KNO_3 with the corresponding amount (18.8 m*M*) of KCl. NAA as an auxin was optimal at 2.25 μ*M*. A revised medium accounting for all optimization results raised the AQ yield about twofold. Illumination of the cell cultures reduced the AQ yields irrespective of the light quality (white, blue, or red light), and a lower ratio between culture medium volume and culture vessel volume favored AQ production (150 ml medium in a 500-ml Erlenmeyer flask proved to be more effective than 50 ml) (Suzuki *et al.*, 1985).

In broad-spectrum cell-culture experiments with cell suspension cultures of *Cinchona ledgeriana* using three different concentration variants in the dark or with illumination highest AQ yields were found in the dark with media containing a medium concentration of minerals, low auxin, high cytokinin, and high concentration of organic constituents (Harkes *et al.*, 1985). Highest AQ contents in suspension-cultured cells of the same material were obtained in a medium containing 0.06 *M* sucrose, but a fourfold concentration of inorganic nitrogen (Wijnsma et al., 1986b, 1987). In cell suspension cultures of *C. succirubra* (synonym, *C. pubescens*), maximum AQ yields occurred with IAA as an auxin at 143 μ*M*; in cultures of *C. ledgeriana*, the corresponding maximum values occurred in the presence of 0.3 μ*M* zeatin riboside and 2.5 μM indolyl-3-butyric acid (IBA) (Robins *et al.*, 1986).

The complexity of conditions necessary to produce high AQ content has been demonstrated by Schulte *et al.* (1984), using cell suspension cultures of plant species belonging to the family Rubiaceae. The results clearly show our lack of knowledge of the regulatory principles that govern secondary metabolism in cell suspension cultures.

IV. BIOSYNTHESIS

As biosynthetic pathways leading to naphthoquinones and AQs frequently are very similar, this chapter may in part overlap with Chapter

6, this volume. Experiments with intact plants often fail to produce the secondary products in question during the time of the experiment. This dilemma can be overcome by using cell cultures, because time and conditions of production of the secondary metabolites can be controlled.

Principally, there are two ways leading to AQ formation: the acetate–polymalonate and the shikimate–*o*-succinylbenzoate pathways. The former is characterized by an intermediary polyketide formed from activated acetate and malonate (Fig. 4). AQs formed in this way generally are substituted in the two aromatic rings, contrary to the AQs following the latter biosynthetic route with substitutions only in ring C. The acetate–polymalonate pathway is realized particularly in species of the Rhamnaceae (*Rhamnus* spp.) and Polygonaceae (*Rumex* spp. and *Rheum palmatum*) as well as Caesalpiniaceae (*Cassia* spp.), whereas the shikimate–*O*-succinylbenzoate route was found to occur especially in the Rubiaceae (*Rubia, Morinda, Galium,* and *Cinchona* spp.). As excellent presentations of the shikimate–*o*-succinylbenzoate biosynthetic pathway have been published (Leistner, 1985a,b), only a short excerpt is given here.

Feeding [2-^{14}C]acetate to the intact plant *Rubia tinctorum* resulted in the incorporation of activity only in ring C of alizarin and purpurin, and partially in the keto groups of the quinone ring. In contrast, the radioactivity of [1,2-^{14}C]shikimate could be localized only in ring A. But [U-^{14}C]shikimate was incorporated into the whole AQ, the shikimate ring being transformed into ring A and the carboxyl group into one of the keto groups of ring B of the AQ. This was the first indication of the existence of a biosynthetic pathway quite different from the acetate–polymalonate route (Leistner and Zenk, 1967).

In feeding experiments using labeled shikimate with cell suspension cultures of *Morinda citrifolia*, alizarin (1,2-dihydroxy-AQ) was found to be radioactive, as expected (Leistner, 1975). Further tracer studies suggested that besides shikimate, glutamate and mevalonate provide the carbon skeleton of alizarin, with *o*-succinylbenzoate as intermediary product formed from shikimate via chorismate with α-oxoglutarate. Not

Fig. 4.

only alizarin (i.e., an AQ substituted solely in ring C), but also morindone (1,5,6-trihydroxy-2-methyl-AQ) was produced. The biosynthesis of this AQ has also been proven to be via the shikimate–*o*-succinylbenzoate route, and it could be demonstrated that the hydroxy groups attached to ring A are not derived from the hydroxy groups of shikimate. These hydroxy groups must be introduced at a later stage of the biosynthetic pathway. This is an exception to the general rule that requires that AQs substituted in the rings A and C be synthesized via the acetate–polymalonate pathway.

According to an earlier concept, AQ biosynthesis following the shikimate–*o*-succinylbenzoate pathway begins with the nucleophilic attachment of succinic semialdehyde thiamine pyrophosphate to chorismate (Heide *et al.*, 1982a,b). But Weische and Leistner (1985) supported the suggestion that neither shikimate nor chorismate but isochorismate is the immediate precursor and that chorismate is converted to isochorismate prior to incorporation into quinones. Thus, direct combination of *o*-succinylbenzoate with chorismate is blocked; previous positive results are due to minor contamination with isochorismate of the chorismate samples.

In the next step, *o*-succinylbenzoate has to be activated. This was shown to take place in cell-free extracts of cell suspension cultures of *Galium mollugo*, where coenzyme A acts as activator (Heide *et al.*, 1982a,b). The site of activation is the residual carboxylic group of the succinyl chain, and only this structure is biologically active and leads to the ring closure, giving 1,4-dihydroxy-2-naphthoic acid (DHNA) by bacterial enzyme preparations (Kolkmann and Leistner, 1985). Previous results (Heide *et al.*, 1982a,b; Kolkmann *et al.*, 1982) showing that the aromatic carboxylic group is activated have been proven to be wrong. After formation of DHNA the site of prenylation of this intermediary compound has to be determined. This problem could be solved by comparison of the relative position of the carbon atom derived from the 2′ carbon atom of 4-[2′-^{13}C]carboxyphenyl-4-oxobutanoic acid and the β carbon (i.e., CH_2OH) in lucidin, and it was found that prenylation of DHNA by mevalonate occurs in position 3 of DHNA (Inoue *et al.*, 1979, 1984a). These experiments have been performed with chemostat cultures (of *Galium mollugo*), and incorporation rates of 83.8% of *o*-succinylbenzoate into lucidinprimveroside were obtained, contrary to 1% or less in feeding experiments with intact plants. Further confirmation of these results was obtained by Heide and Leistner (1981, 1983) and Inouye *et al.* (1982). Ring closure of the dimethylallyl side chain leads eventually to ring C, and thus, the entire AQ molecule with the rings A, B, and C is completed.

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PART **II**

Mevalonates

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CHAPTER 8

Monoterpenes and Sesquiterpenes

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I. GENERAL

As befits both their industrial importance as perfumery and flavoring compounds and their widespread occurrence in higher plants, there has been considerable interest in the formation of the lower terpenes by plant tissue cultures. About 180 relevant publications involving some 60 plant species have appeared; most merely record more or less successful attempts to induce cultures to accumulate such compounds, but more fundamental studies on control mechanisms, storage structures, and enzymology have been carried out (Charlwood and Charlwood, 1986). In this chapter, questions of priority and completeness are largely ignored, and often, citations are given to work containing leading references.

Most callus and suspension cultures derived from explants of those species of herbaceous plants and trees that produce mono- and sesquiterpenes do not accumulate such products under conventional condi-

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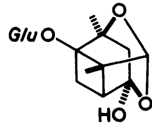
tions. However, assay of plants regenerated from such cultures has shown that this is not due to a deletion or permanent modification of the genetic information. Indeed, the chemical totipotency of callus and the interrelationship between organogenesis and terpene accumulation is well demonstrated by the ability of 2-year-old cultures of *Rosmarinus officinalis* and *Lavandula angustifolia* that were devoid of secondary metabolites to accumulate some of the monoterpenes characteristic of the parents (at ~12% of their levels) in regenerated shoots (Webb *et al.*, 1984). And similarly, the C_{10} moiety of pyrethrins, and also citronellol together with citronellal, could be detected in shoots induced from callus of *Chrysanthemum cinerariifolium* and *Eucalyptus citriodora*, respectively, that had contained no terpenoids (Cashgap *et al.*, 1978; Gupta and Mascarenhas, 1983). Similar results were obtained with *Pelargonium* species (Brown and Charlwood, 1986a).

Much of the literature on terpenoid metabolism in tissue cultures is difficult to assess. Often no proof of *de novo* synthesis [e.g., by use of ^{14}C-labeled precursors or by rigorous gas chromatography–mass spectrometry (GC–MS) analysis], rather than carryover from the explant, is presented. Sometimes products are quite inadequately characterized [e.g., only one GC or thin-layer chromatography (TLC) trace is recorded]. Frequently it is impossible to elucidate the yield from the presented information, and many claims are solely recorded in brief conference reports. In addition, many proposed efficacious variations of medium and culture conditions have been found in our hands, and no doubt in the unpublished experience of others, to be of doubtful or at least of very limited value. Nevertheless, many excellent studies do exist, and almost always callus, rather than suspension cultures, have been found to accumulate the lower terpenes under the (often admittedly limited) conditions studied. Numerous media and environmental regimes have been screened, but best results seem to have been achieved using surprisingly few systems: typically, the Murashige and Skoog, Nash and Davies, or White media, sometimes with diurnal variation of temperature and photoperiod. As for the production of alkaloids, the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) to the medium has frequently been stated to have deleterious effects on terpene accumulation, although there appears to be little real evidence on this point.

Alkaloid synthesis and accumulation does not seem to be associated with any particular glands or structures in the tissues of higher plants, but that of monoterpenes, and presumably also of their sesqui-homologs, does reside in variously identified secretory cells, resin or oil ducts, hairs, glandular epithelial cells, and trichomes. Consequently, it has

often been considered that some degree of differentiation of callus tissue is necessary to enable the lower terpenes to accumulate. Unfortunately, it is not at all clear what inter- or intracellular structures are required, as most cultures that have been found to accumulate terpenes have been inadequately characterized morphologically, and electron-microscopic evidence is lacking. It is probably best to define differentiation in the required sense as movement away from meristematic activity (synthesis of secondary metabolites is thought not to occur in meristematic cells of the intact plant), towards cellular maturation at the biochemical level (Constabel *et al.*, 1974; Brown and Charlwood, 1986b). On this view, fine, fast-growing suspensions and friable callus, although yielding a rapid accumulation of biomass, would be extremely poor sources of secondary metabolites. It is often such material that has been used in studies of terpenoid synthesis with the typically poor success mentioned above. Aside from problems of differentiation, or lack of it, the rapidly dividing cells of fast-growing cultures would channel their material and energy resources into primary metabolism rather than into the production of secondary products. The latter could thus be regarded as the luxuries achieved by slow-growing or even old-age cultures in the stationary phase. A survey of the literature supports this view of the importance of differentiation in the above sense (Brown and Charlwood, 1986b) and suggests that most (not all—see below) of the established culture lines that successfully sustain terpenoid synthesis are a consequence of the fortuitous choice of age and type of explant and of conditions that achieve this end. An early observation was that callus of *Pimpinella anisum* and *Ruta graveolens* only produced essential oils (with the components in the approximate proportions as in the field-grown plants) after the phytohormone levels in the media had been adjusted to induce differentiation (Becker, 1970) or when (in the latter species) specialized storage cells had been induced by an appropriate photoperiod (Nagel and Reinhard, 1975). These oils were unusual in that the lower terpenes were minor components compared with polyketide derivatives, but a more typical example was that callus of *Mentha piperita* grown on media supplemented with glucose and benzylaminopurine produced mainly pulegone and menthofuran (whereas the intact plant accumulated mainly menthone and menthol in similar yield), and these monoterpenes were associated with rudimentary buds endowed with secretory cells characteristic of the species. Illumination of the cultures was essential for oil production, and increase of the intensity of light increased the biosynthetic activity but not the number of secretory cells (Bricout and Paupardin, 1975). Higher levels of glucose in the medium

depressed monoterpene biosynthesis (Paupardin *et al.*, 1980). Only "highly organized" culture lines could achieve the synthesis of menthone and menthol, and callus of *Citrus limonia* produced the characteristic limonene-derived compounds in similar secretory cells, but this ability for synthesis was lost after several subcultures (Paupardin, 1976). More recently, detailed correlations between the formation of the valepotriate iridoids and differentiation levels in cultures of Valerianaceae have been discussed (Violon *et al.*, 1984), and differentiated root cultures of *Paeonia lactiflora* produced quantities of the unusual glucoside paeoniflorin (1) in yields (~1.7% dry weight) akin to the levels in the intact plant (Yamamoto *et al.*, 1985).



I

Differentiation has been demonstrated to be necessary for many of the other successful culture lines cited in the following sections. However, it does not seem to be an invariable prerequisite for terpenoid accumulation in callus of *Ocimum basilicum* (Lang and Hörster, 1977) or *Citrus limonia* (de Billy and Paupardin, 1971); and cultures of *Mentha piperita* that produced pulegone and piperitone (up to 2.2% dry weight—distributed between cells and medium) carried out the synthesis not in specialized glandular structures but in giant dedifferentiated cells of which the culture was mainly composed (Kireeva *et al.*, 1978). Undifferentiated fine cell suspensions of *Pelargonium fragrans* also produced geranoil and nerol at levels about 3% of that in the parent plant when the photoperiod was suitably adjusted (Brown and Charlwood, 1986c). It was proposed that a phytochrome-mediated regulation of HMG-CoA reductase was involved in switching on terpenoid synthesis, but that only low accumulations could be attained in the absence of the storage facilities provided by differentiation, or by the presence of a two-phase "sink" (see Section V). Geraniol, farnesol, and probably many other mono- and sesquiterpenes are toxic at low concentrations to cell cultures, and those cells of the population that accumulated such compounds in the absence of specific storage glands or even of suitable vacuoules, and that could not excrete them into the medium, would presumably soon die. Another possibility is that in the absence of such storage or excretion, the cells could detoxify their own products using

enzyme systems that are usually compartmented away from the sites of terpene synthesis and accumulation; then they would survive but would apparently not possess the ability to synthesize terpenes. Such detoxification could utilize novel terpene epoxidases and epoxide hydratases that have been extracted from callus of *Jasminum officinale* and that accept isopentenol, geraniol, and nerol and their epoxides as substrates. These enzymes, which can be extracted from cultures at levels of activity up to 100-fold greater than can be obtained from leaves of the parent plant, may catalyze the first steps of a degradation process that yields C_2 and C_5 fragments utilizable in other metabolic pathways (Banthorpe and Osborne, 1984). A light-dependent callus line of *Pinus radiata* has also been established that synthesizes α- and β-pinenes under illumination, but degrades these *via* α-terpineol and 2-methyl-4-methylstyrene to toluene and acetone when kept in complete darkness (Banthorpe and Njar, 1984). It has not been generally appreciated that the accumulations in cultures of a terpenoid or other secondary metabolite depend on the predominance of synthetic over degradative processes. The presence of uncompartmented and active enzymes of the latter type could wipe out nascent endogenous products. This possibly is supported by observations that undifferentiated callus of *Rosa* cultivars (Banthorpe and Barrow, 1983; Banthorpe *et al.*, 1986b) and of a variety of herbaceous plants of *Jasminum, Rosmarinus, Lavandula, Anethum, Ocimum,* and *Tanacetum* species did not accumulate detectable quantities of the mono- and sesquiterpenes characteristic of the intact plants but nevertheless appeared to possess the full enzymatic machinery for the formation of the parents of these classes—geraniol, nerol, and 2(*E*)-farnesol—as well as for some of the derived products, such as sabinene, α-pinene, and citronellol and caryophyllene (Banthorpe *et al.*, 1986a). These enzymes (e.g., farnesol synthetase) could be extracted from the cultures with activities up to 600-fold the levels obtainable under the same conditions from leaves of the parent species. These results may not reflect an intrinsically higher synthetic ability of the cultures but may merely be the result of the ease of extraction owing to the more fragile cell walls and the relative lack of phenolic compounds in callus as compared with its parent. Of course it may be that the enzymes *are* derepressed in the cultures, but whatever the explanation, the findings suggest that callus should provide an excellent source of biomass for studies on the purification of the enzymes of the terpenoid pathway, and perhaps, of other pathways of secondary metabolism.

Whether the explanation of the general lack of terpene accumulation in callus cultures is due to autonecrosis or to the presence of uncompartmented degradative enzymes (or both), the solution is to induce suffi-

cient differentiation to allow for storage structures to be formed, either as specialized glands and ducts or intracellularly as vacuoles, without pushing on to the stage of plantlet regeneration. For suspension cultures, intracellular storage vacuoles must be available, or the terpenes must be excreted and if toxic be removed from the system. These and other approaches are discussed in Section V.

II. MONOTERPENE BIOSYNTHESIS

Monoterpene biosynthesis has been reviewed and an essentially complete list of references compiled (Charlwood and Charlwood, 1986). Several key examples are discussed in Section I.

A. Conventional Monoterpenes

One of the earliest successful cultures was a callus line from *Tanacetum vulgare* that was maintained for 2 years. This accumulated high (0.1% wet weight; 50% of yield from foliage) levels of oil, comprising 22 of the 26 monoterpenes present in the parent tissue, but the main difference in composition was that sabinene constituted 80 and 2% of the oil from callus versus leaf extract, respectively, in comparison with 3 and 79% for isothujone. This suggested that the hydrocarbon sabinene was the immediate precursor of isothujone and accumulated in the cultures because of oxygen deficiency (Banthorpe and Wirz-Justice, 1972), a proposal confirmed by later tracer experiments on whole plants.

Foliage of *Thuja occidentalis* yielded an oil containing thujone (the epimer of isothujone) as the main component, but cell suspensions did not form any [3.1.0.]-bicyclohexane derivatives but rather the minor compounds of the natural oil such as α-terpineol and camphor; these were not stored in the cells but were excreted. The unusual irregular monoterpene β-thujaplicin (**2**) was also partially excreted and complexed with

HO O

2

Fe^{3+} in the medium (Witte *et al.*, 1983). Callus lines from *Pinus radiata,* which were stable for a least 1 year, accumulated up to 40% (wet weight) of the monoterpenes found in the needles and stem of the parent, but the main component was α-pinene (87–100%) rather than β-pinene. Cell-free extracts of the cultures converted isopentenyl pyrophosphate into geraniol and nerol, and α- and β-pinenes in up to 46% yield; these are probably the most active crude extracts that sustain monoterpene synthesis that have been obtained from any plant source (Banthorpe and Njar, 1984). Despite several early failures, cultures of many *Mentha* species that are very biosynthetically active have been established. In one study, *M. piperita* and *M. rotundifolia* callus yielded 60% wet weight of the oil of the intact plants, but the monoterpenes produced were generally more oxidized (C=O, C=C, predominantly) than in the latter, the reduction of the $\Delta^{4(8)}$ double bond of precursors being blocked in the cultured cells. Several other species produced yields of oil with the same composition as that from the parents, but in much reduced yields (Bricout *et al.*, 1978b). Production of monoterpenes in cell cultures of many other *Mentha* species was very variable between both taxa and cell lines; several only synthesized the early precursors of the menthane compounds characteristic of the genus, but some hybrid lines (e.g., *M. spicata* × *suaveolens*) yielded the major components of the intact plant. There was a distinct correlation between levels of differentiation and accumulations of products (Charlwood and Charlwood, 1983).

In contrast to the lack of success with *Rosa* cultures outlined in Section I, callus of *R. damascena* produced linalool, geraniol, and citronellol, and their glucosides, as in the parent tissue, but at levels much lower than in petals. The biosynthetic ability of the callus was maximal after lengthy periods in culture (60 days; stationary phase?) but fell off on subculturing (Kireeva *et al.*, 1977). Callus and suspension cultures of *Perilla frutescens* produced the monoterpenes [linalool, limonene, perilla ketone (**3**) and isoegomaketone (**4**)] typical of the species, in the natural propor-

3 **4**

tions and in good (0.1% wet weight) yields (Sugisawa and Ohnishi, 1976; Nabeta *et al.*, 1983). Similar qualitative and quantitative replication

occurred for pericarp callus of *Citrus limonia* (α-pinene, limonene, linalool, citrals, etc.) and *Artemisia genipi* (α-pinene, sabinene, thujone) (Bricout and Paupardin, 1974; Leddet *et al.*, 1984). The majority of calluses derived from a variety of genetically diverse specimens of *Chrysanthemum cinerariifolium* produced low concentrations of pyrethrins, and formation of the insecticides correlated well with the degree of differentiation (Zieg *et al.*, 1983). In contrast, others found that only chrysanthemic acid (the monoterpene moiety of the pyrethrins) was feebly produced by the same type of culture (Kueh *et al.*, 1985). Adjustment of the photoperiod for cell suspensions of *Pelargonium fragrans* led to the detection of monoterpenes in undifferentiated cultures (Brown and Charlwood, 1986a,b), but a similar stratagem did not ameliorate the situation for suspensions of *Apium graveolens* (Watts *et al.* 1984). The levels of limonene and other monoterpenes increased as chlorophyll was induced in the latter cultures by the addition of 3,5-dichlorophenoxyacetic acid (3,5-D) to the medium, however, and after three subcultures the levels were similar to those in the intact plant. Despite this greening, there was neither chloroplast formation nor differentiation in the suspensions (Watts *et al.*, 1985). Previously, it has been shown that greening did not influence monoterpene synthesis in callus of *Tanacetum vulgare* (Banthorpe and Wirz-Justice, 1972).

B. Iridoids

Iridoids, which possess the iridane skeleton (**5**) and comprise the monoterpene moiety of many terpene alkaloids, were produced in cell

5

suspensions of *Rauwolfia*, *Gardenia*, and other species, and such cultures have been used in elegant tracer studies to elucidate the biosynthetic pathways to loganin, secologanin, and related compounds (Kobayashi *et al.*, 1985; Uesato *et al.*, 1986). The valepotriates, a subclass of intense pharmacological interest, were produced in both differentiated and un-

differentiated callus of Valerianaceae, often at levels higher than in the parent plants (Becker *et al.*, 1984).

III. SESQUITERPENE BIOSYNTHESIS

The most impressive work on sesquiterpene biosynthesis involves callus and suspensions of *Andrographis paniculata*, which yielded three new paniculides: bisabolenoid lactones with the skeleton (**6**), found nei-

6

ther in the parent nor elsewhere. Tracer studies on the cultures and using derived cell-free extracts led to very detailed mechanistic information about the biosynthesis of the parent 2-(*E*)- and 2-(*Z*)-farnesols and (*Z*)-γ-bisabolene (Allison *et al.*, 1968; Anastasis *et al.*, 1984). These studies illuminate the advantages of the use of culture extracts, (see Section I) which give (1) high incorporation of exogenously added precursors and thus (2) allow the use of carbon-13 tracer and NMR techniques to detect the labeling pattern. Other important work concerns phytoalexins. Ipomeamarone was accumulated on transfer of *Solanum berthauldi* callus to suspension (Oba and Uritani, 1979), and formation of lubimin, rishitin, and solvetivone was elicited by inoculation of suspensions of *S. tuberosum* with pathogenic bacteria (Brindle *et al.*, 1983). Subsequently, several C_{15} phytoalexins—hemigossypol, phytotuberin, phytotuberol, epirishitin, debneyol, and capsidiol—have been elicited from suspensions or, less frequently, callus, by bacteria or enzymatic (e.g., cellulase) treatment (Watson *et al.*, 1985; Heinstein, 1985; Brooks *et al.*, 1986).

Sesquiterpenes are also formed by callus of *Matricaria chamomilla* (caryophyllene, chamomillol), *Pimpinella anisum* (β-bisabolene), *Perilla* species (cuparene), and *Lindera strychnifolia* (caryophyllene) (Reichling *et al.*, 1984, 1985; Nabeta *et al.*, 1984; Tomita *et al.*, 1969), by cultures of liver-

worts (germacrenes, azulenes) (Takeda and Katoh, 1983), and by suspensions of *Nigella damascena* (abscisic acid) (Lehmann *et al.*, 1983).

IV. BIOTRANSFORMATIONS

Although synthesis and storage of monoterpenes rarely occurs in fine cell suspensions, such cultures often possess the ability to transform exogenous substrates. This has attracted much attention in view of possible industrial applications due to the ease of batch culture and extraction of products, and the subject has been reviewed (Charlwood and Charlwood, 1986). Usually incubation periods of 8 hr to 8 days are employed, but rarely have the time courses of metabolism been followed, and little consideration appears to have been given to possible secondary (nonenzymatic) reactions. Most studies have used cell suspensions of *Nicotiana* or *Mentha* species (Aviv *et al.*, 1983) with acyclic (geraniol, nerol, citral) or menthane-type (menthone, pulegone, α-terpineol) monoterpenes as substrates. Almost no attention has been paid to the other five classes of monoterpenes or sesquiterpenes, and immense scope for investigation exists. The transformations are usually of a very simple chemical type (e.g., oxidation, reduction, or hydroxylation) and lead to very predictable products, although there is often no correlation between the biotransformational ability and the monoterpene content of the parent tissues (Aviv *et al.*, 1981). Some reactions are enantioselective, for example, in the hydroxylation of α-terpineol (Suga *et al.*, 1982), and completely foreign substrates (e.g., carvoximes) can be metabolized (Suga *et al.*, 1984).

V. SPECIAL TECHNIQUES

Several techniques have been developed to generate or improve terpenoid biosynthesis in cell cultures. The simplest is the use of submerged morphogenic cultures derived from callus cultures (see Section I). Such cultures of *Pelargonium* species yielded up to 10% the levels of monoterpenes in the parent plant and up to 100-fold the levels of the parent callus (Brown and Charlwood, 1986a). Another approach is to induce

polyploidy by colchicine treatment (Bricout *et al.*, 1978a); although this causes a 70-fold increase in valepotriate accumulation in suspensions of *Valeriana wallichii*, the stimulation may have been caused by gene amplification or by selection of high-producing cells (Becker and Chavadej, 1985) and the generality of the effect is not known. The use of a two-phase system for suspension cultures has been advocated whereby addition of a lipophilic inert phase (Miglyol; RP-8) prevents the loss by volatilization of excreted products. Using this technique, increases in yields of α-pinene, limonene, and β-thujaplicin were achieved from cultures of *Thuja occidentalis* (Berlin *et al.*, 1984), and apparently non-yielding suspensions of *Matricaria chamomilla* and *V. wallichii* gave compounds characteristic of the parents (Becker and Herold, 1983; Bisson *et al.*, 1983). Similar results were obtained when an absorbing resin was added to the medium (Forche *et al.*, 1984). These additives may well be traps for volatiles, but they could also stabilize labile products or be sinks to remove toxic compounds and so enhance and maintain the viability of the cultures. A fourth approach utilizes the presumption that the majority of cultures (as well as meristematic cells) have regulatory genes that suppress secondary metabolism. Isolation of artificially produced disregulatory mutants, or of the presumed very small natural population of cells that are sufficiently differentiated to sustain terpene production, could lead to the establishment of very desirable clones. The problem is of selection, but this may be soluble using radioimmunoassay. The latter technique has been devised for a monoterpene (loganin) and used to study the formation of secologanin in cell cultures (Tanahashi *et al.*, 1984).

Biotransformation may be revitalized by the use of plant cells immoblized in matrices such as calcium alginate or polyacrylamide. Such engulfed cell lines of *Mentha* species maintained their synthetic abilities, (e.g., pulegone → menthol), and the products were more easily released from the bonded cells than from freely suspended cultures (Galun *et al.*, 1983). Cell division in such matrices was largely inhibited by gamma irradiation without, however, affecting the biosynthetic abilities of the imprisoned inhabitants (Galun *et al.*, 1985).

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CHAPTER 9

Diterpenes

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I. INTRODUCTION

Although the area of plant cell culture has been the subject of rapidly increasing interest and investigation in times, the large majority of such studies has focused on phytochemicals of pharmaceutical interest and within such families as alkaloids and steroids. Reviews that summarize these investigations are available (Constabel and Kurz, 1979; Staba, 1980: Kutney, 1984; Misawa, 1985). Studies within the diterpene area have been limited to several laboratories, and the present chapter concentrates on results from an extensive program in our laboratory and is

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concerned with the plant cell production of the cytotoxic agents tripdiolide (**1**) and triptolide (**2**), natural products isolated from the Chinese plant, *Tripterygium wilfordii*.

Our research, involving the propagation of plant cell cultures of *Tripterygium wilfordii,* was stimulated by the research of the late S. M.

1 R = OH
2 R = H

3

17

18

19

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21

22

HO 23

Kupchan (see Kupchan *et al.*, 1972) in which it was demonstrated that tripdiolide and triptolide reveal significant activity *in vivo* against L-1210 and P-388 leukemias in the mouse and *in vitro* against cells derived from human carcinoma of the nasopharynx (see Section III). Because the extracts of *T. wilfordii* are also used in Chinese herbal medicine, there have been extensive investigations on the chemistry (Zhou *et al.*, 1982) and pharmacology (Research Group of Lei-Gong-Teng, 1982; Zheng *et al.*, 1983a,b; Ngan *et al.*, 1984) of such extracts.

Our tissue culture program (Kutney *et al.*, 1980, 1981a, 1983) has involved a detailed series of investigations directed at (a) development of satisfactory growth conditions, (b) optimization of growth parameters for the production of the target compound tripdiolide (**1**), and (c) detailed chemical analyses of the cell-produced secondary metabolites in addition to the target compounds. The discussion that follows provides a description of our results within these categories.

II. CULTURE TECHNIQUES

Stem and leaf explants were obtained from *Tripterygium wilfordii* plants maintained under normal greenhouse conditions. Explants were placed on B5 and PRL-4 media (Eveleigh and Gamborg, 1968) solidified with Bacto-agar (Difco) (8 g/liter) and supplemented with numerous combinations of the following compounds: 2,4-dichlorophenoxyacetic acid (D) (2 mg/liter), kinetin (K) (0.1 mg/liter), 1-naphthaleneacetic acid (NA) (0.15 mg/liter), indole-3-acetic acid (I) (2 mg/liter), 6-benzylaminopurine (B) (2 mg/liter), 4-aminobenzoic acid (P) (2 mg/liter), and coconut milk (Co) (100 ml/liter). The explants and resulting calli were incubated at room temperature (25 ± 3°C) in darkness. Many calli grew and were transferred to fresh media of the same or different composition. Subculture occurred about every 4 weeks. Preliminary selection of promising cell lines was based on growth vigor as well as qualitative thin-layer

chromatography (TLC) and cytotoxic activity analyses, as discussed below.

The cell line designated TRP 4a was selected for further investigation after these initial screenings. This cell line was initiated as a leaf explant on PRI_2Co_{100} agar [i.e., PRL-4 medium supplemented with indole-3-acetic acid (2 mg/liter) and coconut milk (100 ml/liter)], transferred to PRD_2Co_{100} agar, and maintained on the latter medium.

Suspension cultures of TRP 4a were generated in PRD_2Co_{100} broth and were maintained as stock cultures by regular subculture using 10% inoculum at 3-week intervals. All suspension cultures grown in conical flasks were incubated without illumination at 27 ± 1°C on a rotary shaker with a $\frac{7}{8}$-in. throw and run at 140 rpm.

III. CHEMICAL ANALYSIS

In order to ascertain the levels of tripdiolide (Td) in both the callus and cell suspension cultures, a method involving a TLC assay and using fluorimetric detection was developed (Kutney *et al.*, 1981b). After the initial small-scale experiments were optimized in terms of growth parameters (see below), scale-up of the fermentation (up to 60 liters) in a Chemapec bioreactor was achieved and detailed chemical analyses of the metabolites produced were performed. The latter studies, described below, involved extraction of cells and broth with ethyl acetate and subsequent chromatographic separation to obtain the pure chemical components. The latter were then submitted for the usual spectroscopic (ultraviolet, infrared, nuclear magnetic resonance, mass spectrometry) determinations to complete the structure elucidations.

IV. CYTOTOXIC ANALYSIS

Evaluation of the cytotoxicity present in the fractions obtained from the cell fermentations was performed at Arthur D. Little, Cambridge, Massachusetts. The assays were done using KB cells (human epidermoid carcinoma of the nasopharynx type 9 KB-5) as developed under a program sponsored by the National Cancer Institute, National Institutes

of Health, Bethesda, Maryland. In general, ED_{50} values expressed in micrograms per milliliter were obtained. This is the calculated effective dose that inhibits growth of 50% of the control growth.

A. Initial Medium Studies on the Production of Tripdiolide

The influence of hormones and hormone-like compounds on the production of Td by TRP 4a cell suspension cultures was examined. The PRL-4 medium of Eveleigh and Gamborg (1968) (without casein hydrolysate) was used as the basic medium. This was supplemented with various levels of Co, K, D, and NA, as listed in Table I. Cultures were harvested and analyzed after 5 and 6 weeks of incubation. These times were selected because preliminary screening experiments carried out using PRD_2Co_{100} indicated that high cytotoxic activities were generally associated with samples extracted from 4- to 6-week-old cultures. At this point of the project only qualitative TLC and cytotoxicity (KB) analyses were available to monitor the level of tripdiolide. These results together with the growth assessment of the cultures are shown in Table I. Cultures grown in medium with $Co_0K_{1.0}NA_2$ gave the strongest indication of Td on TLC, but copious root formation in the culture made it less desirable for future use in large-scale fermentations. Instead, the medium supplemented with kinetin (0.5 mg/liter) and naphthaleneacetic acid (0.5 mg/liter) ($PRNA_{0.5}K_{0.5}$) was selected as it produced a positive TLC analysis for Td plus good KB assay results.

B. Effect of Inoculum Size

Parallel time-course (42 days) experiments using TRP 4a in $PRNA_{0.5}K_{0.5}$ broth were set up with three different inoculum sizes: 10% (standard), 50%, and 100%. Duplicate samples were harvested and analyzed at 7-day intervals starting at day 21 of incubation. Positive TLC analyses for Td were shown by all the broth extracts. However, a significant difference in Td concentration could not be determined. Likewise, KB assays did not discriminate among the cultures because all samples gave ED_{50} values of less than 1. The results, overall, showed that inoculum sizes larger than 10% did not prevent production of Td. However, there was no apparent indication of higher yield. For practical purposes, subsequent experiments employed the standard inoculum size of 10%.

Table I

Effect of Various Medium Supplements on the Growth and Tripdiolide Production by *Tripterggium wilfordii* (TRP 4a) Cell Suspension Cultures

PRL-4 medium supplemented with[a]				Time of growth					
				5 weeks			6 weeks		
Co	K	D	NA	Growth assessment	TLC assay[b]	KB assay[c]	Growth assessment	TLC assay[b]	KB assay[c]
0	0.5	2		+	?	1.7	+	?	7.5
0	1	2		+	−	3.0	+	−	43
0	1.5	2		+	−	0.5	+	−	9
0	0.5		0.15	+	−	1.3	+	−	1.3
0	0.5		0.5	++	+	1.01	++	−	<1
0	0.5		1	++	−	1.1	+++	?	21.5
0	0.5		2	++	?	4.5	++ (a few roots)	+	9
0	0.5		2.5	++	−	1.25	++++	−	28.5
0	1		0.15	+	−	<1	+	−	27
0	1		0.5	++	−	1.45	++	?	12.5
0	1		1	++	−	1.45	+++ (a few roots)	−	26.5
0	1		2	++	−	1.8	++	−	26
0	1		2.5	++	++	<1	+++ (many roots)	++	23.5
10	0	2		++	−	43.5	++	−	29
10	0.5	2		+++	+	28	++	−	—
10	1	2		+++	?	2.6	+++	−	22
10	1.5	2		+++	?	10.05	++	?	14.5
30	0	2		++++	+?	49	+++	+?	1.8
30	0.5	2		++++	−	14	++++	−	6.6
30	1	2		++++	−	10.5	+++	−	6.2
30	1.5	2		+++	−	42	+++	−	30.5
60	0	2		++++	−	12	+++	−	20.5
60	0.5	2		++++	−	12	++++	−	12
60	1	2		++++	+?	12	++++	−	25
60	1.5	2		++++	−	34	+++	−	37

[a]Units for the concentration of supplements are as follows: coconut milk (Co), milliliters per liter of broth; kinetin (K), 2,4-dichlorophenoxyacetic acid (D), and 1-napthaleneacetic acid (NA), milligrams per liter of broth.

[b]Thin-layer chromatographic assays were qualitative.

[c]KB assays are expressed as ED_{50} values in micrograms per milliliter.

C. Time-Course Study in $PRNA_{0.5}K_{0.5}$ Medium

At this point, we developed a rapid TLC assay of Td using fluorimetric detection (Kutney *et al.*, 1981b) that was accurate for Td concentrations of 0.2 to 3.6 μg. Therefore, a detailed time-course study, including Td measurement, using cell suspension cultures was carried out in $PRNA_{0.5}K_{0.5}$ medium. Triplicate samples (500 ml) were harvested at weekly intervals and analyzed individually. Other culture parameters monitored at the same time included cell dry weight [cells were weighed after filtration through Miracloth (Calbiochem), and lyophilization], pH, and refractive index. These results are shown in Fig. 1.

Significant Td formation occurred after 14 days. A maximal concentration of 2.3 mg/liter of culture broth was attained around day 35. Gener-

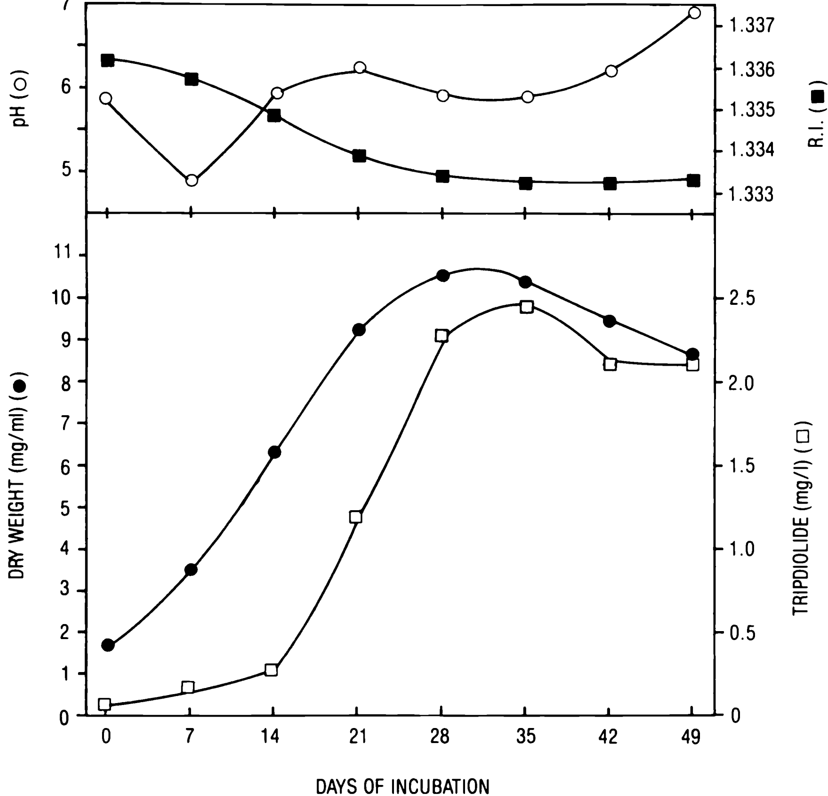

Fig. 1. TRP 4a culture growth and Td production in $PRNA_{0.5}K_{0.5}$ (no casein hydrolysate) broth, using 21-day-old inoculum grown in PRD_2Co_{100} broth.

ally, Td was found to be present in both the cell extracts as well as the broth. Because of interference by the cooccurrence of several quinonemethide compounds (see below) in the cell extracts, however, only broth extracts were analyzed by the fluorimetric method for the concentration of Td. Biomass, in terms of cell dry weight, increased from 1.7 mg/ml at day 0 to the peak of 10.5 mg/ml at day 28. After this time the cells appeared to enter a stationary phase.

D. Effect of Younger Inoculum

In all previous experiments inocula were from 18- to 22-day-old stock cultures (PRD_2Co_{100}) that had reached early stationary phase, as assessed by biomass measurement. One idea for shortening the time for Td production was to use a younger inoculum for the production phase. To test this approach, a time-course experiment was performed using the $PRNA_{0.5}K_{0.5}$ medium and 11-day-old inocula. Cell suspension cultures (3 × 500 ml) were harvested at weekly intervals and analyzed for Td. Cell dry weight, pH, and refractive index of each sample were also recorded. Results are shown in Fig. 2.

Growth was rapid after a short lag period so that a maximal dry weight of 11.9 mg/ml was reached by day 21. Use of inocula still in growth phase and containing residual sucrose likely accounts for these differences in growth rate and cell yields. The peak concentration of Td again occurred after about 35 days of incubation. However, this level (1 mg/liter) was less than half that obtained from cultures using older inocula. Therefore, subsequent experiments employed inocula about 3 weeks old.

E. Influence of Medium Composition on Tripdiolide Production

Although we had established that formation of Td by TRP 4a cells in $PRNA_{0.5}K_{0.5}$ medium can be achieved with a peak level of more than 2 mg/liter, it was of interest to examine different production media in the continuing effort to improve the yield of the desired compounds. The effects of two other basal media [those of Murashige and Skoog (1962) (MS) and Hildebrandt and Schenk (1972) (SH)] on growth and Td production of TRP 4a cells were compared with $PRNA_{0.5}K_{0.5}$ medium in a series of parallel experiments. The media were prepared as $MSNA_{0.5}K_{0.5}$ (2% sucrose) and $SHNA_{0.5}$. Suspension cultures (250 ml each) were harvested

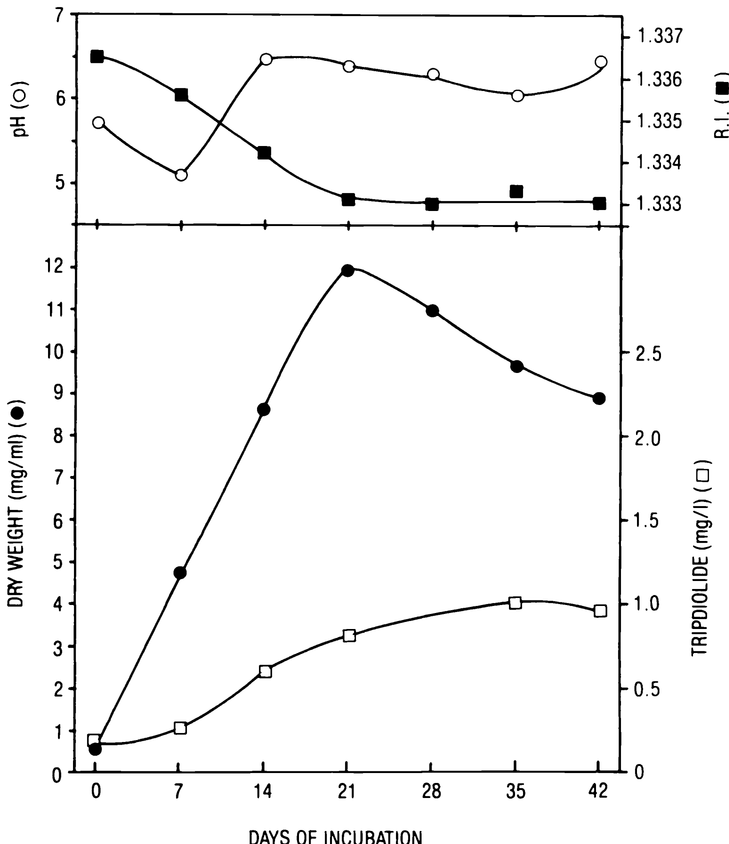

Fig. 2. TRP 4a culture growth and Td production in $PRNA_{0.5}K_{0.5}$ (no casein hydrolysate) broth, using 11-day-old inoculum grown in PRD_2Co_{100} broth.

at appropriate times over a 45-day incubation period and analyzed for Td concentration and cell dry weight. Results are shown in Fig. 3.

A more detailed time-course experiment in $MSNA_{0.5}K_{0.5}$ (2% sucrose) medium was carried out and afforded a Td concentration of greater than 3.0 mg/liter after 37 days of incubation. Good biomass production, in terms of cell dry weight, was also obtained. These results strongly indicated that $MSNA_{0.5}K_{0.5}$ (2% sucrose) is a more effective Td production medium than similarly supplemented PRL-4 or SH media for cultivation of TRP 4a cell suspension cultures in shake flasks.

Comparison of the three basal media (PRL-4, MS, SH) reveal several major differences in their compositions. Some possible key components are the following: (a) concentration of available nitrogen in the forms of

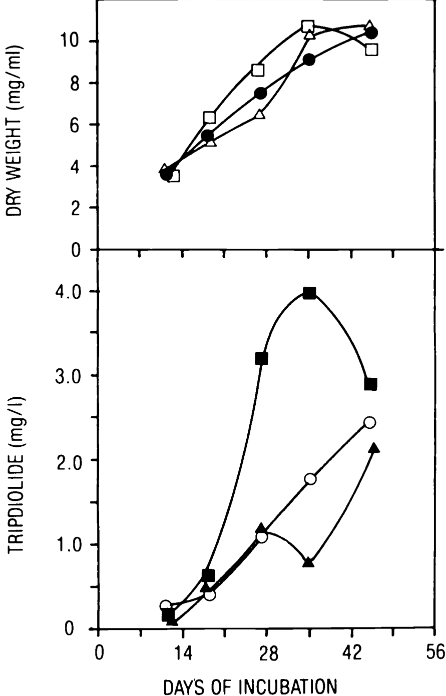

Fig. 3. Comparison of three different basal media for growth and tripdiolide production by TRP 4a: ●,○,$PRNA_{0.5}K_{0.5}$; □,■, $MSNA_{0.5}K_{0.5}$ (2% sucrose); △,▲, $SHNA_{0.5}K_{0.5}$. Inocula were grown for 20 days in PRD_2Co_{100}.

NH_4^+ or NO_3^-; (both ions are present in much higher concentrations in the MS medium); (b) concentration of calcium chloride ($CaCl_2 \cdot 2H_2O$) is also higher in MS (440 mg/liter); (c) concentration of thiamine is much lower in MS (0.1 mg/liter) than in the other two media (10 and 5 mg/liter); (d) glycine (2 mg/liter) is only present in MS. In addition, there are other differences in the micronutrients (e.g., Mn^{2+}, Zn^{2+}, Cu^{2+}, and Co^{2+}).

The effect of different levels of some of these components was studied using $MSNA_{0.5}K_{0.5}$ medium as the basal one. Results of some of these preliminary experiments with TRP4a cultures are described below.

1. Effect of Ammonium Nitrate Concentration

Three different levels of ammonium nitrate [850, 1650, (standard), and 2450 mg/liter] were evaluated. Cultures (2 × 250 ml) were harvested at appropriate times after 28 days of incubation. Dry biomass yield and Td

concentration were monitored (Fig. 4A). Tripdiolide production was highest in medium with 1650 mg/liter of ammonium nitrate. Biomass yield was greatest when ammonium nitrate at 850 mg/liter was used.

2. Effect of Sucrose Concentration

Three sucrose concentrations were tested, namely, 1% (10 g/liter), 2% (20 g/liter) (standard) and 4% (40 g/liter). TRP 4a cultures (2 × 125 ml) were procured and analyzed at appropriate time intervals after 28 days of incubation. Results, as shown in Fig. 4B, indicated Td production reached the same level (2.3 mg/liter) at day 43 in extracts from cultures with both 2 and 4% sucrose, whereas 1% sucrose afforded a lower Td concentration (1.8 mg/liter) after the same time period. Cultures grown in 4% sucrose showed the best biomass yield, whereas the 1% sucrose samples afforded the lowest yield.

3. Effect of Calcium Chloride Concentration

Three different calcium chloride concentrations were used [220, 440 (standard) and 880 mg/liter]. Cultures (2 × 125 ml) were harvested and analyzed at appropriate times after 28 days of incubation. Biomass yield

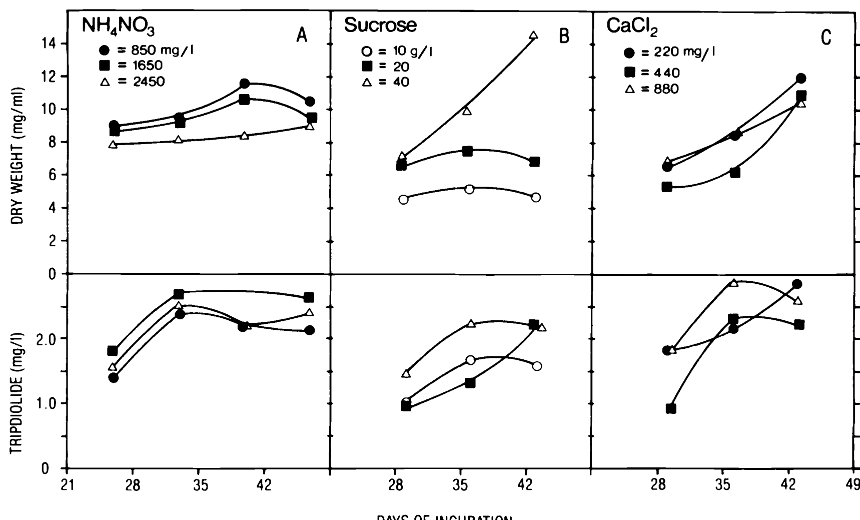

Fig. 4. Effects on Td and dry biomass production of different medium concentrations of NH_4NO_3 (A), sucrose (B), and $CaCl_2$ (C). $MSNA_{0.5}K_{0.5}$ was the basic medium, and inocula were grown for 20 days in PRD_2Co_{100}.

was very similar for all three calcium chloride concentrations (Fig. 4C), whereas Td production was maximal with 880 mg/liter of calcium chloride at day 43 (2.6 mg/liter).

V. ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES

Having established the various growth parameters for the cell cultures, it was appropriate to consider scale-up experiments and obtain definitive information about the chemical structures of the products formed during the fermentation process. Of primary interest, and in relationship to production of Td, was the question as to whether the cooccurring secondary metabolites produced in addition to Td possessed chemical structures related to Td and whether they might represent structural templates biosynthetically related to the target compounds. The occurrence of such compounds may shed important information on the biosynthesis of Td and, in turn, provide possible avenues to improve its production further.

Organic solvent extraction (ethyl acetate) of the cells and broth followed by conventional silica gel chromatography afforded, in addition to Td, two other compounds of the diterpene family. Spectroscopic data and comparison with an authentic sample identified one of these compounds as dehydroabietic acid (**3**). The other metabolite was clearly a novel compound, and more extensive research was required to settle its structure. Spectroscopic data revealed carboxylic acid and alcohol functions situated on a typical diterpene skeleton of the dehydroabietic acid family but with a C_3,C_4-disubstituted ring-A system. Such a structure was highly interesting because tripdiolide is clearly a diterpene with the lactone ring attached to ring A and requiring the C_3,C_4-substitution pattern. Furthermore, the genesis of the novel triepoxide system, present in ring C of Td, could plausibly arise biosynthetically from an aromatic ring C characteristic of the dehydroabietic acid system. In summary, it appeared that this novel compound may indeed bear a biosynthetic relationship to Td.

Chemical synthesis developed from dehydroabietic acid (**3**), along the route **3** → **4** → **5** → **6** (Scheme 1), proved the required ketone **6**, which could then be converted to the unsaturated hydroxyester **17** (Scheme 2).

3 → 4: $Pb(OAc)_4$; ϕH, pyridine, reflux, 3h

4 (67%)

4 → 5: 1) BF_3–LAH, r.t. 2) NaOH, H_2O_2

5 → 6: pyridine-chlorochromate, CH_2Cl_2, r.t.

4→5→6 (12%)

6 → 7: TOSNH-NH_2, ϕH, BF_3, r.t.

7 (>90%)

7 → 8: 1) BuLi, TMEDA, -78°-0°C 2) $(CH_2O)_n$, 0°C

6→7→8 (70%)

8 → 6 + 10 + 9: 1) BH_3·THF, r.t. 2) NaOH, H_2O_2, r.t.

6 (30%) + 10 (10%) + 9 (20%)

Scheme 1. Synthesis of diterpene intermediates from dehydroabietic acid (3).

6

KCN

18-Crown-6 in DMSO/DMF

NC

HO H

$SOCl_2$/Py

NC

11

+

NC

12

(2:1) NaOEt EtOH

(5:1) (60% from 6)

11 + 12 (2:1)

DIBAL-H(1.2 eq.)

Hexane, 0°C

OHC H

13

+

OHC H

14

(2:1) 88%

14

$NaClO_2$ + (OH, OH)

–H_2O – 0.1 *N* HCl

O=C, OH, H

15

CH_2N_2

O=C, OMe, H

16 (72% from 14)

$h\nu$ + O_2

OH

O=C, OMe, H

17

Scheme 2. Synthetic route to isolated diterpene 17.

The latter compound proved to be identical with the ester derived from diazomethane treatment of the novel carboxylic acid obtained in the cell fermentation process. Thus the novel metabolite clearly bears the structure **24** (R=OH).

Based on the cooccurrence of **3** and **24** (R=OH), it was attractive to consider that the biosynthetic pathway leading to tripdiolide may involve the intermediates **3** and **24** (R=OH) shown in Scheme 3.

The remaining metabolites isolated from the tissue culture media were identified, by comparison with authentic samples, to be celastrol

CH_2OH

Geranyl-geraniol

Labdane-type

Pimarane-type

COOH

3

Abietane

R

HOOC

24

O

O

O

HO

O

OH

O

H

O

Tripdiolide

Scheme 3. Postulated biosynthetic pathway leading to tripdiolide.

Scheme 4. Synthesis of diterpene intermediates from ketone **6**. Yields: **25** → **26** (63%); Overall (85%). Ratio **26**:**27** = 3:5.

(**18**), an isomeric triterpene quinone methide **19**, a structure (**20**) closely related to tingenone, oleanolic acid (**21**), polpunonic acid (**22**) and β-sitosterol (**23**).

VI. BIOTRANSFORMATION EXPERIMENTS

In order to derive additional information about the biosynthesis and/or cell production of tripdiolide, a number of biotransformation experiments are under consideration. Such experiments involve substrates possessing the diterpene system, at a lower oxidation level, and that can be derived by chemical conversions of the readily available dehydroabietic acid (**3**). For this purpose, the synthetic routes summarized in Schemes 1 and 4 have been developed, and incubation experiments with substrates such as **9, 10, 26,** and **27** and growing cultures of *Tripterygium wilfordii* will be pursued in order to evaluate their role, if any, in cell production of tripdiolide.

VII. CONCLUSIONS

The production of the potentially important antineoplastic agent tripdiolide (Td) in tissue cultures of *Tripterygium wilfordii* has been demonstrated. Although Td production, at a level of 4.0 mg/liter, has not yet

been fully optimized, this yield is about 36 times greater than that reported for the plant by Kupchan *et al.* (1972). Future yield improvements may be obtained through increased understanding of the Td biosynthetic pathway and its regulation or, alternatively, through the biotransformation mentioned in Section VI.

VIII. STUDIES IN OTHER LABORATORIES

Misawa (1985) and colleagues have also reported studies on plant tissue culture of *Tripterygium wilfordii*. The level of Td in the culture was very low (95 μg/liter). Similarly, a report by Dujack *et al.* (1980) provides a description of their results with *T. wilfordii*, but no characterization of metabolites produced was reported.

One report (Miyasaka *et al.*, 1985) on production of the diterpene ferruginol by cell suspension cultures of *Salvia miltiorrhiza* has appeared. These authors studied the time-course production of ferruginol and the effects of auxins and light on ferruginol production and on cell growth.

Studies with cell cultures of *Thuja occidentalis* by Witte *et al.* (1983) have also reported the presence of the diterpenes, dehydroabietane, 2-dehydroferruginol, and ferruginol. The latter compounds were recognized by the technique of gas liquid chromatography combined with mass spectrometry (GC–MS) so, again, no information is available on the levels of these diterpenes produced in the culture media. The cell suspension culture of *Cryptomeria japonica* studied by Ishikura *et al.* (1984) has been reported to contain two diterpenes: abietatriene and ferruginol.

Diterpene production by callus cultures of some plants belonging to the family Cupressaceae has been reported by Ohgaku *et al.* (1984). Here again, GC and GC–MS analyses were performed to identify such diterpenes as abietatriene, totarol, ferruginol, hinokiol, so levels of production of the specific compounds have not been well established.

Dehydroabietane and another unidentified diterpene hydrocarbon has been found in the steam distillates of callus cultures of *Melissa officinalis* (Koch-Heitzmann *et al.*, 1985). Gas liquid chromatographic analysis was employed to evaluate the diterpene components, which varied in relative proportions depending on the age of the culture. Older cultures tended to reveal a higher content of the dehydroabietane system.

In conclusion, and as already noted at the outset, studies on plant

tissue cultures for the production of phytochemicals within the diterpene family have been limited. This situation is in marked contrast to that involving alkaloid and steroid production where, more definitive clinical and pharmaceutical interests are established.

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CHAPTER 10

Bufadienolides*

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I. INTRODUCTION

Cardiac glycosides are naturally occurring steroids with a powerful stimulating action on the cardiac muscle. These compounds are composed of an aglycone, which is either a cardenolide or a bufadienolide, and a hydrophilic carbohydrate moiety linked at the C-3 position. The cardenolides are C_{23} steroids containing an α,β-unsaturated γ-lactone ring, and the bufadienolides are C_{24} homologs of cardenolides containing a δ-lactone ring with a conjugated diene system. Many different sugars have been isolated from cardiac glycosides, and all except glucose are deoxyhexoses and methyl ethers. The glycoside linkage for a D sugar is β, and α for an L sugar.

*To Prof. Dr. Sumitra Sen with best wishes on her fiftieth birth anniversary.

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II. NATURAL OCCURRENCE OF BUFADIENOLIDES

In plants, cardiac glycosides occur only in angiosperms. The cardenolides are more commonly and abundantly found than the bufadienolides (Hegnauer, 1970). Bufadienolides have been found to occur in some members of Melianthaceae, Ranunculaceae, Crassulaceae, and Liliaceae (Table I). Structures are shown in Fig. 1.

Table I

Natural Occurence of Bufadienolides

Family	Plant species	Bufadienolide (Fig. 1—structural formula no.)	Reference
Ranunculaceae	*Helleborus*	Hellebrin (**1**)	Reznichenko *et al.* (1964), Hegnauer (1970)
Crassulaceae	*Kalanchoe lanceolata*	Lanceotoxin (**2**)	Anderson *et al.* (1984)
	Kalanchoe diagremontiana	Diagremontianin (**3**)	Wagner *et al.* (1985)
	Tylecodon wallichii	Cotyledoside (**4**)	Steyn *et al.* (1984)
Melianthaceae	*Bersama*	Bersaldigenin (**5**)	Vanhaelen and Baudin (1967), Hegnauer (1970)
	Melianthus comosus	Melianthugenin (**6**)	Koekemoer *et al.* (1971)
Liliaceae	*Bowiea volubilis*	Bovoside A	Reznichenko *et al.* (1965)
	Urginea maritima	Scillaren A (**7**), proscillaridin A (**8**), and minor glycosides, i.e., scilliphaeoside, scilliroside, scillirubroside, scilliglaucoside, glucoscillaren A, scillicyanoside	Stoll *et al.* (1933), Stoll and Kreis (1951), Wartburg *et al.* (1968)
	Urginea indica	Scillaren A, proscillaridin A, scilliphaeoside	Rangaswami and Subramanian (1956), Jha and Sen (1981)

III. TISSUE CULTURE FOR PRODUCTION OF BUFADIENOLIDES

Although plant tissue culture has been a subject of much interest for the production, biotransformation, and discovery of new biochemicals, as exemplified by a number of excellent reviews (Böhm, 1980; Dougall, 1979; Furuya, 1982; Jones, 1983; Kurz and Constabel, 1979; Reinhard and Alfermann, 1980; Staba, 1985), very little work has so far been done on bufadienolide production in tissue cultures. As a matter of fact, greater emphasis is evidently given to the production of a few types of high-value, plant-specific compounds. Only few therapeutically important bufadienolides are known as compared to cardenolides (Engel, 1984). It is not surprising, therefore, that work on tissue culture systems in this area has been mainly concentrated on the production and biotransformation of cardenolides (Alfermann *et al.*, 1977, 1983). Moreover, cell cultures of the species investigated so far have been found to be incapable of synthesizing the bufadienolides characteristic of the parent plant (Shyr and Staba, 1976). The isolation and development of newer and therapeutically better bufadienolides remain to be explored. It may be pointed out that proscillaridin A, obtained from squill bulbs, is a valuable cardiac drug (Engel, 1984) that can be administered to patients who cannot withstand digitalin.

The present chapter covers the literature up to mid-1986 on the tissue culture studies on bufadienolide-yielding plants, including the Indian squill, which is being investigated in the author's laboratory.

IV. PRESENT STATUS OF TISSUE CULTURE OF PLANTS YIELDING BUFADIENOLIDES

A. *Helleborus*

Helleborus yields hellebrin. There is a single report available on anther culture of *H. foetidus* (Zenkteler *et al.*, 1975). The authors reported the development of embryoids from pollen. No report is available on hellebrin production in tissue culture.

1 Hellebrin

2 Lanceotoxin

3 Daigremontianin

4 Cotyledoside

5 Bersaldigenin-(1,3,5'-orthoacetate)

6 Epoxymelianthugenin

7 R = Scillabiose = Scillaren A

8 R = Rhamnose = Proscillaridin A

B. *Kalanchoe*

In *Kalanchoe daigremontiana,* budding from epidermal cells has been reported (Bigot, 1976). No reports are available on bufadienolide production in this species or in other species of *Kalanchoe in vitro* (Doreswamy, 1965; Mclaren and Thomas, 1967; Robbins and Harvey, 1971).

C. *Bowiea volubilis*

The bulb of *Bowiea volubilis* has long been known to be a source of cardiac glycosides (bufadienolides) of scillaren type (Reznichenko *et al.*, 1965; Watt and Breyer-Brandwizk, 1962). Vegetative reproduction via divisions of the mother bulb is very slow, and seed propagation leads to variability. The most important reason of propagating this plant is to maximize high-drug-yielding clones. Tissue culture studies on plant regeneration and rapid multiplication were carried out in our laboratory (Jha and Sen, 1985). Inflorescence segments were used as explants, as even after several trials, bulbs could not be successfully sterilized. Callus was induced on Murashige and Skoog's (MS) (1962) medium supplemented with 1 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 15% (v/v) coconut milk. Shoot regeneration occurred after two or three subcultures in the same medium with low concentration of 2,4-D. Complete rooted plants were obtained in MS medium without any hormone. To propagate *B. volubilis* rapidly, bulblets produced *in vitro* were used as secondary explants. Two types of secondary explants were used: (a) split shoots, and (b) individual scale leaves. Although each split shoot produced two to four daughter shoots in the presence of 6-benzylaminopurine (BAP, 2–5 mg/liter), the response of individual scales was different. Numerous small globular shoot primordia developed from the adaxial surface of outer and inner scales within 4 to 6 weeks. The shoot primordia developed into bulbous shoots in presence of 2 mg/liter BAP and 0.05 mg/liter 2,4-D. These shoots multiplied rapidly in shake culture using liquid MS medium. From each scale, 400–600 bulblets could be produced in 16 to 20 weeks. Eighty percent of the plants survived on transfer to potted soil. The bufadienolide content of these transplanted bulblets is under investigation.

Fig. 1. Structural formulas of naturally occurring bufadienolides.

D. *Urginea maritima*

Squill is one of the most ancient of medicinal plants. It closely resembles *Digitalis* in increasing the vigor and diminishing the frequency of cardiac action. The principal bufadienolide is scillaren A, which on hydrolysis by the enzyme present (scillarenase) gives proscillaridin A and glucose. Complete hydrolysis yields the aglycone scillaridin A, and scillabiose (rhamnose and glucose residues). Since Stoll *et al.* (1933) isolated and crystallized scillaren A, a dozen bufadienolides have been reported from bulbs of squill (Stoll and Kreis, 1951; von Wartburg *et al.*, 1968).

1. Tissue Culture

Kaul *et al.* (1967) examined semipurified extracts of *Urginea maritima* tissue cultures for their effects on respiration, heart rate, and blood pressure in anesthesized rabbits. They reported that the semipurified extracts of squill tissue cultures established from bulbs produced a pronounced vasolidation and bradycardia in anesthesized rabbits. This was the first report of tissue culture study on white squill. Lutz (1970) carried out detailed studies on induction of callus and establishment of tissue culture and on production of bufadienolides in callus culture. The only other report available is that by Shyr and Staba (1976), who carried out studies with an aim to detect bufadienolides, anthocyanins, and other compounds.

a. Explant. For establishing tissue cultures, bulbs were used as explant. Lutz (1970) used several populations of *Urginea maritima* for establishing cultures and reported that age of bulb used as explant affects the content of total glycosides in calli derived from them. Lutz used 10–15% sodium hypochlorite for 45 min for sterilization of bulbs.

b. Culture Media. Lutz (1970) induced calli on bulb explants on MS medium supplemented with 2,4-D, indole-3-acetic acid (IAA), and kinetin (Kn). The calli were subcultured at 4-week intervals and maintained at 21 to 25°C. Calli were used after the first and second subculture for chemical analysis.

Shyr and Staba (1976) used callus tissues established from bulbs for chemical analysis and maintained them in darkness in revised tobacco medium (Staba, 1969) with 1 mg/liter 2,4-D. These cultures were subdivided and grown on the same medium, containing 5, 1, or 0.1 mg/liter

2,4-D or 1 mg/liter Kn, and subcultured at 6-week intervals. They established suspension cultures by transferring callus cultures into 500-ml flasks containing 100 ml of revised tobacco liquid medium with either 5 or 0.1 ppm 2,4-D, 0.1 ppm Kn, or without growth regulators. They were grown under light or in the dark and subcultured at 3-week intervals. All cultures were grown at 21°C and harvested for chemical analysis at the end of the third passage.

c. Differentiation. Squill tissue differentiation was reported to be affected by both 2,4-D and Kn (Shyr and Staba, 1976). Tissues maintained and grown on medium containing 2,4-D remained highly differentiated (Carew and Staba, 1965). Tissues remained undifferentiated in high–2,4-D medium (5 ppm). Rhizogenesis was induced in tissues growing on medium containing 0.1 ppm 2,4-D or without growth regulators, or 0.1 ppm Kn. Roots were also formed from tissues growing in the presence of 1 ppm Kn, but toxic effects were observed as the tissues darkened (Shyr and Staba, 1976).

2. Bufadienolide Analysis

Lutz (1970) carried out detailed qualitative analysis of bufadienolides in callus cultures by paper chromatography using chloroform–methanol (1 : 1) as solvent and antimony trichloride in sulfuric acid as detection reagent. Depending on similarity of R_f values with standard squill bufadienolides, it was concluded that glycoside extracts of calli from the first and second passages showed the presence of the same glycosides as reported in bulbs of white squill, that is, scillaren A, proscillaridin A, scilliglaucoside, scillicyanoside, and scilliroside. However, the ultraviolet (UV) spectrum of the glycoside mixture from newly formed calli and that of native bulbs revealed that whereas bufadienolides show a characteristic absorption maxima at 300 nm, the glycoside mixture obtained from calli showed absorption maxima at 293, 285, and 278 nm. Quantitative estimation of total glycosides of the first- and second-passage calli showed a decrease to about one-fourth the content normally found in the tissues of a fresh bulb.

Shyr and Staba (1976) isolated three major compounds by thin-layer chromatography (TLC) from the previously reported cardioactive semipurified extracts of *Urginea maritima* (Kaul *et al.*, 1967). The extracts were done from suspension cultures of callus tissues established in the 1970s (Carew and Staba, 1965; Shyr and Staba, 1976). Scillaren A and pros-

cillaridin A were not detected in the glycoside extracts. The UV absorption maximum of 300 nm characteristic of the six-membered lactone ring was not obtained with the compounds isolated. The three compounds isolated showed absorption maxima of 293, 292, and 289 nm. The infrared (IR) absorption bands for C=O stretching (1720 cm^{-1}) and C=C stretching (1639 and 1540 cm^{-1}) were observed for the doubly unsaturated δ-lactone ring in the standards whereas one compound showed absorption band for C=O stretching at 1735 cm^{-1} and other two compounds exhibited bands at 1700 cm^{-1}. Absorption bands for C=C stretching (1650 and 1580 cm^{-1}) were obtained for the three compounds isolated. The IR spectra indicated absence of a six-membered lactone ring in the compounds isolated. The UV, IR, and mass spectra indicated that the three compounds were not identical or similar to standard squill bufadienolides (Shyr and Staba, 1976).

E. *Urginea indica*

The principal bufadienolides of Indian squill are the same as those of European (white) squill, *Urginea maritima* (i.e., proscillaridin A and scillaren A). A large population of plants was screened for bufadienolide content in the bulbs (Jha, 1983; Jha and Sen, 1983). After screening, tissue cultures from selected strains were established in order to compare the bufadienolide content of the plant with that in the derived callus cultures. Tissue cultures have been established from high-yielding diploid, triploid, and tetraploid cytotypes of *U. indica*. Plant regeneration through organogenesis (Jha *et al.*, 1984) and somatic embryogenesis (Jha and Sen, 1986) has been reported. A number of chromosomal variants have also been obtained through tissue culture (Jha, 1986; Jha and Sen, 1987).

1. Tissue Culture

a. Explant. Bulbs were used as explants for establishing callus cultures from diploid and triploid plants. Bulbs of tetraploid plants did not respond to any culture conditions tried, and young inflorescence axes were used for establishing cultures from tetraploid plants. Bulbs were sterilized with 0.1% mercuric chloride for 25 min, and segments of inflorescence for 8 min. The explants were thoroughly washed with sterile water (five times).

b. Culture Media. MS medium was used for all of the cytotypes; however, the hormone and vitamin requirements were different. Callus was induced on diploid bulb explants on MS medium supplemented with 10 mg/liter thiamine–HCl, 5 mg/liter nicotinic acid, 1 mg/liter pyridoxine–HCl, along with either a combination of 4 mg/liter 2,4-D, 2 mg/liter α-naphthaleneacetic acid (NAA), 2 mg/liter Kn, and 1 g/liter yeast extract or a combination of 2 mg/liter 2,4-D and 15% (v/v) coconut milk (Jha *et al.*, 1984). Callus was established from triploid bulb explants on MS medium supplemented with 4 mg/liter 2,4-D, 2 mg/liter NAA, and 2 mg/liter Kn, and in tetraploid inflorescence explants on MS medium with 2 mg/liter 2,4-D and 2 mg/liter Kn (S. Jha, unpublished work). All cultures were grown at 22 to 25°C, a maximum relative humidity of 55 to 60%, and under Philips fluorescent daylight tubes emitting 3200 lux for a 16-hr-light and 8-hr-dark period.

c. Organogenesis. Shoot regeneration occurred in callus cultures established from diploid and triploid plants but not from tetraploid plants. Shoot primordia developed after two or three subcultures in the callus induction medium, but increased growth of shoot primordia was obtained in media containing lesser amounts of auxins and vitamins. Rooted bulbous plants were obtained in MS medium without any hormones. By using regenerated bulbs as secondary explants, nearly 300–400 bulblets were produced from each scale leaf in MS medium supplemented with low amounts of auxins and cytokinins (Jha *et al.*, 1984).

Plants derived from callus cultures of diploid *Urginea indica* show chromosomal variations, both numerical and structural (Jha and Sen, 1984, 1987) whereas plants regenerated from callus cultures of triploid *U. indica* are very stable as far as chromosome number and morphology is concerned.

d. Somatic Embryogenesis. Somatic embryogenesis was noted in callus tissues obtained from bulb explants of diploid *Urginea indica* (Jha and Sen, 1986) and from inflorescence explants of tetraploid *U. indica*. In both cases, embryogenic callus was formed when 1-year-old friable calli were allowed to remain on the high–2,4-D medium for a prolonged period. Globular embryoids were induced to develop to complete bulbous plants following transfer to MS medium containing 0.05–0.1 mg/liter BAP (or 15% coconut milk or BAP 0.1 mg/liter and 10% coconut milk) for 4 to 6 weeks and then to MS medium with 0.01 mg/liter NAA and 0.05 mg/liter Kn for 8 weeks. A final period in liquid MS medium stimulated shoot and root growth to the point where successful trans-

planting of plants to soil was assured (Jha and Sen, 1986). The bulbs derived from somatic embryos were highly polyploid (Jha, 1986).

2. Bufadienolide Analysis

Chemical analysis of calli and regenerated plants was done following the methods reported earlier (Jha and Sen, 1981, 1983). Analysis of callus at different stages of morphogenesis were performed by TLC. The detection reagent used was a 3% aqueous solution of chloramine T and 25% ethanolic solution of trichloroacetic acid (TCA) (1 : 4), which was sensitive to nearly 0.01 μg per spot as detected by color in UV (Jha and Sen, 1981).

a. Undifferentiated Calli. Young (1, 2, and 6 months) and old (2, 4, and 6 years) calli, growing in the presence of 1 to 2, 0.5, mg/liter 2,4-D and 10 mg/1 Kn, or 0.5 mg/liter 2,4-D and 2 mg/liter BAP, were examined. No bufadienolide was detected by TLC in calli growing in any of the hormone combinations at any stage.

b. Differentiating Calli. Callus showing rhizogenesis growing in presence of 0.5 mg/liter 2,4-D or 1–2 mg/liter NAA did not show presence of bufadienolides. Roots differentiating from calli also did not show any trace of glycosides.

Callus showing shoot differentiation showed the presence of proscillaridin A, as detected by faint yellow TLC spots under UV using chloramine T–TCA. No quantification could be carried out as only very small amounts of glycosides were isolated. Embryogenic calli from diploid and tetraploid plants also did not show the presence of bufadienolides.

c. Regenerated Bulbs. Regenerated bulbs after 1 year of transplantation were analyzed. All regenerated bulbs (derived from diploid, triploid, or tetraploid parents through organogenesis and/or somatic embryogenesis) showed the presence of bufadienolides characteristic of the parent plants. Proscillaridin A and scillaren A have been isolated and identified from regenerated bulbs (S. Jha, unpublished work).

The bulbs derived from tissue culture differed in their bufadienolide content also. The bulbs derived from somatic embryos were highly polyploid and showed nearly a threefold increase in bufadienolide content as compared to diploid parents (Jha, 1986).

V. CONCLUSION

Many plant cell cultures produce the secondary substances expected from them. Besides these systems, however, there exist many cell cultures that continually fail to form compounds characteristic of the parent plants. As Böhm (1982) has pointed out, "positive results have repeatedly been summarized, negative findings, if they do not remain unpublished, are scattered through literature." The results obtained so far from the limited work in the area carried out in the author's laboratory seems to be encouraging. Rapid propagation could be achieved in slowly propagating species. Plants derived from tissue culture have the potential of containing higher amounts of metabolites. It seems the potential to biosynthesize bufadienolides strictly correlates with shoot differentiation. In *Urginea indica,* plants regenerated from unproductive cell cultures synthesize the bufadienolides characteristic of the plant. No attempt has been made to utilize the cell-culture system for biosynthesis and biotransformation studies on bufadienolides, as has been done for *Digitalis* (Alferman *et al.*, 1983). More investigation would help in understanding and solving the problems regarding synthesis of bufadienolides in cell and tissue culture.

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CHAPTER **11**

Cardenolides

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I. CARDENOLIDES: STRUCTURE, BIOSYNTHESIS, AND SIGNIFICANCE

Cardenolides are steroids with the following unique chemical characteristics: (a) substitution of the steroid ring system at position 17β with an unsaturated five- or six-membered lactone ring (butenolide or bufa-

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dienolide ring) and at position 14β with a hydroxy group, which causes the unusual cis connection of the rings C and D, and (b) linkage of the aglycones to unusual sugars (deoxy and methoxy sugars) or to acids at the hydroxy group at position 3β. Cardenolides are formed in several families of higher plants and some animals but have not been detected in microbial cultures (Luckner, 1984). They are derived from cholesterol via pregnenolone and progesterone (Grunwald, 1980; Nahrstedt, 1982). Tissue cultures forming cardenolides have been obtained from several species of *Digitalis*. This chapter therefore concentrates on work with *Digitalis* cell, tissue, and organ cultures.

II. ESTABLISHMENT OF *DIGITALIS* TISSUE CULTURES

Since the pioneering work of Staba (1962), cell, tissue, and organ cultures have been established from different types of explants of a rather long list of *Digitalis* species:

D. ambigua: seedlings (Hagimori *et al.*, 1980)
D. cariensis: hypocotyl (Nover *et al.*, 1980; Tewes *et al.*, 1982)
D. ferruginea: hypocotyl, seedlings (Hagimori *et al.*, 1980; Tewes *et al.*, 1982)
D. grandiflora: hypocotyl (Nover *et al.*, 1980; Tewes *et al.*, 1982)
D. heywoodii: hypocotyl (Tewes *et al.*, 1982)
D. laevigata: hypocotyl (Tewes *et al.*, 1982)
D. lanata: anthers, corolla, cotyledons, anther filament, hypocotyl, leaves, pistil, roots, seedlings, sepals, stem (Büchner and Staba, 1964; Diettrich *et al.*, 1986; Garve *et al.*, 1980; Hagimori *et al.*, 1980; Lui and Staba, 1979; Nickel and Staba, 1977; Nover *et al.*, 1980; Reinhard *et al.*, 1975; Staba, 1962; Tewes *et al.*, 1982)
D. lutea: hypocotyl, seedlings (Hagimori *et al.*, 1980; Nover *et al.*, 1980; Tewes *et al.*, 1982)
D. mertonensis: hypocotyl, seedlings (Hagimori *et al.*, 1980; Medora *et al.*, 1967; Nover *et al.*, 1980; Tewes *et al.*, 1982)
D. obscura: anthers, cotyledons, hypocotyl, leaves, roots (Perez-Bermudez *et al.*, 1983, 1984, 1987)
D. purpurea: anthers, cotyledons, hypocotyl, leaves, roots, seedlings, stem (Büchner and Staba, 1964; Corduan and Spix, 1975; Diettrich *et*

al., 1980; Gurny *et al.*, 1980; Hagimori *et al.*, 1980, 1982a; Hirotani and Furuya, 1977; Kartnig and Kobosil, 1977; Kartnig *et al.*, 1976; Nover *et al.*, 1980; Pétiard *et al.*, 1971; Pilgrim, 1972; Rücker *et al.*, 1976, 1981; Staba, 1962; Tewes *et al.*, 1982; Wichtl *et al.*, 1978).

Only a few cell types of the explants, however, were able to differentiate and form a primary callus: the xylem parenchyma cells, for example, in anther filament and leaf explants of *D. lanata* (Diettrich *et al.*, 1986), the pollen cells, for example, in anthers of *D. purpurea* and *D. obscura*, forming haploid cell strains (Corduan and Spix, 1975; Perez-Bermudez *et al.*, 1987), and protoplasts obtained from mesophyll cells of *D. lanata* (Li, 1981).

A large variety of cell strains with diverse properties were formed from these cell types (see Diettrich *et al.*, 1986, for strains derived from xylem parenchyma cells). Stable cardenolide formation was rarely observed. Several authors described considerable cardenolide content in newly established callus (Hagimori *et al.*, 1980; Kartnig, 1977; Kartnig *et al.*, 1983; Rücker *et al.*, 1976, 1981; Wichtl *et al.*, 1978), but the cardenolides disappeared during repeated subculture (Hagimori *et al.*, 1980; Kartnig, 1977; Kartnig *et al.*, 1983; Wichtl *et al.*, 1978). Thus in long-term cultures without organogenesis, either no cardenolides could be detected (Graves and Smith, 1967; Gurny *et al.*, 1981; Hagimori *et al.*, 1980; Helmbold *et al.*, 1978; Hirotani and Furuya, 1977; Kartnig, 1977; Kartnig *et al.*, 1983; Nickel and Staba, 1977; Pilgrim, 1972; Reinhard *et al.*, 1975; Stohs and Rosenberg, 1975; Stohs and Staba, 1965) or with sensitive methods, for example, specific radioimmunoassays, only traces of cardenolides were measurable (Garve *et al.*, 1980; Lui and Staba, 1979; Nover *et al.*, 1980; Diettrich *et al.*, 1987c). The positive results of Büchner and Staba (1964), Kaul *et al.* (1967), Medora *et al.* (1967), Pétiard and Demarly (1972), and Pétiard *et al.* (1971, 1972a,b) could not be verified in other laboratories. They probably are due to shortcomings in analysis.

The lack of cardenolide biosynthesis in the morphologically unorganized cultures was caused by the absence of the enzymes catalyzing cardenolide formation rather than by the deficiency of the respective precursors. Feeding of cholesterol, pregnenolone, and progesterone known to be incorporated into cardenolides (see Section I) did not trigger cardenolide biosynthesis (Elze *et al.*, 1974; Graves and Smith, 1967). Also, the enzyme system forming pregnenolone from cholesterol could not be substantiated in *D. purpurea* cell cultures unable to form cardenolides (Pilgrim, 1972).

Stable cardenolide biosynthesis was shown to proceed in cultures regenerating shoots (Section III) or forming somatic embryos (Section

IV). Cultures consisting of nonembryogenic brown or green tissues (Reinhard *et al.*, 1975; Diettrich *et al.*, 1986) or regenerating roots (Diettrich *et al.*, 1986; Hagimori *et al.*, 1980, 1984a; Hirotani and Furuya, 1977; Lui and Staba, 1979) contained either no or only small amounts of cardenolides. In contrast, the primary roots formed directly on leaf explants of *D. purpurea* had a considerable cardenolide content (Rücker *et al.*, 1976, 1981, 1983). It decreased, however, when the roots were isolated and grown as separated entities (Rücker *et al.*, 1983).

III. BIOSYNTHESIS OF CARDENOLIDES IN *DIGITALIS* SHOOT CULTURES

A. Establishment of Shoot Cultures from Shoot Tip Meristems and Nonmeristematic Tissues

Sterile shoots suitable for cultivation *in vitro* were obtained (a) from shoot tip meristems of *D. lanata* that were part of the shoot tips of adult plants (Breuel *et al.*, 1984; Diettrich *et al.*, 1987b; Dobos *et al.*, 1982; Erdei *et al.*, 1981; Luckner *et al.*, 1984; Schöner and Reinhard, 1982; Springer *et al.*, 1986) or seedlings (Lui and Staba, 1979), and (b) from cells of other tissues (see Section II) after dedifferentiation and formation of new meristemoids, for example, directly within leaf fragments of *D. purpurea* (Rücker, 1982; Rücker *et al.*, 1981) or fragments of cotyledons, hypocotyls, leaves, and roots of *D. obscura* (Perez-Bermudez *et al.*, 1983, 1984) as well as in callus of *D. ambigua, D. cariensis, D. ferruginea, D. grandiflora, D. lanata, D. lutea, D. mertonensis, D. obscura*, and *D. purpurea* (Corduan and Spix, 1975; Diettrich *et al.*, 1986; Hagimori *et al.*, 1980; Hirotani and Furuya, 1977; Nover *et al.*, 1980; Perez-Bermudez *et al.*, 1987; Tewes *et al.*, 1982). Most convenient was the establishment of shoot cultures from shoot tip meristems. This method is used routinely in the micropropagation of plants, a procedure established also for *D. lanata* (Breuel *et al.*, 1984; Diettrich *et al.*, 1987b; Dobos *et al.*, 1982; Erdei *et al.*, 1981; Luckner *et al.*, 1984; Schöner and Reinhard, 1982; Springer *et al.*, 1986).

Shoot multiplication was brought about by depressing the dominance of the apical meristem by addition of cytokinins, which caused shoot formation from the axillary meristems of the mother shoots. The daughter shoots formed were used for further propagation (Breuel *et al.*, 1984; Diettrich *et al.*, 1987b; Dobos *et al.*, 1982; Erdei *et al.*, 1981; Luckner *et*

al., 1984; Schöner and Reinhard, 1982; Springer *et al.*, 1986). They were grown for multiplication either on solidified or in liquid nutrient media. In routine experiments the increase in dry weight of *D. lanata* shoots grown on solidified medium containing 10 μmol of benzyladenine (BA) per liter was about 8-fold within 1 month (Luckner and Diettrich, 1985). It was much higher (about 20- to 40-fold) when *D. lanata* and *D. purpurea* shoots were cultivated under submerged conditions (Hagimori *et al.*, 1984c; Lui and Staba, 1979).

B. Cardenolide Formation in Shoots Grown *in Vitro*

In vitro–cultivated shoots of *D. lanata* (Luckner and Diettrich, 1985; Lui and Staba, 1979, 1981) and *D. purpurea* (Hagimori *et al.*, 1980, 1982a,b,c, 1983, 1984a,b,c; Hirotani and Furuya, 1977) were able to form and accumulate cardenolides. Shoot cultures of *D. purpurea* contained digitoxin and purpureaglycoside A (Hirotani and Furuya, 1977). In shoot cultures of *D. lanata* the lanatosides A, B, and C, digitoxigenin, gitoxigenin, digoxigenin, and probably glucodigifucoside and glucoverodoxin were identified (Luckner and Diettrich, 1985; Lui and Staba, 1981). These results demonstrated that the cardenolide pattern of the *in vitro*–cultivated shoots, in contrast to that of the somatic embryos (Section IV,B), resembled the pattern of the leaves of the mother plants. The optimum cardenolide content of the *D. lanata* shoots was about 0.6 μmol g^{-1} dry weight (Lui and Staba, 1981), that of the *D. purpurea* shoots about 0.15 μmol g^{-1} dry weight (Hagimori *et al.*, 1984c), that is, it was much smaller than the cardenolide content of leaves from plants grown in the field [*D. lanata:* ~3 μmol g^{-1} dry weight (Lui and Staba, 1979); *D. purpurea:* ~2.7 μmol g^{-1} dry weight (Hagimori *et al.*, 1984a)].

The addition of potential cardenolide precursors, for example, cholesteryl acetate and progesterone, increased the cardenolide content of *D. lanata* shoot cultures about threefold (Lui and Staba, 1979); progesterone, that of *D. purpurea* shoot cultures about two-fold (Hagimori *et al.*, 1982c, 1983). Radioactively labeled progesterone was incorporated into the cardenolides formed (Hagimori *et al.*, 1984a).

Removal of BA from the medium (Lui and Staba, 1981) as well as addition of gibberellic acid (Hagimori *et al.*, 1982b; Lui and Staba, 1981) or abscisic acid (Hagimori *et al.*, 1982b) caused an increase in the cardenolide content of the shoots. In addition, growth and cardenolide concentration depended on the carbon and nitrogen sources, phosphate

level, as well as the presence of myoinositol, thiamine, EDTA, and several inorganic salts in the medium (Hagimori *et al.*, 1982b,c; Luckner and Diettrich, 1985).

IV. BIOSYNTHESIS OF CARDENOLIDES IN CULTURES OF SOMATIC *DIGITALIS* EMBRYOS

A. Development of Somatic Embryos

Somatic embryos were obtained (a) directly with suitable explants, for example, anthers of *D. obscura* (Perez-Bermudez *et al.*, 1987), with or without the formation of a primary callus, or (b) from the parenchyma-like cells of long-term cultures of embryogenic cell strains derived from filaments of *D. lanata* and hypocotyls of *D. lutea* (Diettrich *et al.*, 1986; Nover *et al.*, 1980; Tewes *et al.*, 1982).

The formation of somatic embryos was most thoroughly investigated with cultures of the embryogenic *D. lanata* strains V and VII (Garve *et al.*, 1980; Diettrich *et al.*, 1986). In cultures of these strains, somatic embryos were formed from the parenchyma-like cells grown in media with a high auxin/cytokinin ratio. Embryo development was elicited by lowering this ratio. It included the following stages:

1. Formation of small clusters of meristematic cells within the wall of individual parenchyma-like cells, occuring either in cell colonies or separately
2. Development of the clusters of meristematic cells to separate entities containing a core of small, polyedric, plasma-rich cells surrounded by a layer of larger vacuolated cells, separating the cluster from nearby parenchyma-like cells
3. Development of more or less round, smooth globular embryoids (diameter, about 0.5–1 mm) that at the beginning were white, ultraviolet- (UV) sensitive, and unable to build chloroplasts (stage-I embryoids), but later turned yellow and formed chloroplasts on illumination (stage-II embryoids)
4. Formation of bipolar, heart-shaped and torpedo-shaped embryos that developed to plantlets with roots and leaves; many of these plantlets, however, were anomalous and fewer than 1% developed into normal plants during further cultivation

Somatic embryogenesis was optimum when *D. lanata* cell cultures were grown successively in the following media (Diettrich *et al.*, 1986; Garve *et al.*, 1980; Kuberski *et al.*, 1984):

Nutrient medium I [high auxin/cytokinin ratio, containing 5 μmol 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 μmol kinetin (Kin) per liter]: rapid growth of small colonies of parenchyma-like cells
Nutrient medium II [reduced auxin activity, containing 5 μmol naphthaleneacetic acid (NAA) and 0.1 μmol Kin per liter]: formation of meristematic cell colonies
Nutrient medium V (low auxin/cytokinin ratio, containing 0.05 μmol NAA and 5 μmol BA per liter): formation of globular embryos
Nutrient medium VII (containing 5 μmol BA per liter): formation of bipolar embryos and plantlets

Stage-I embryoids were able to form secondary embryoids that separated from the former and grew as distinct entities. Development to stage-II embryoids was inhibited by growth in high density. Hence embryogenic strains of *D. lanata* may be cultivated in the form of stage-I embryoids by high-density growth in nutrient medium V. These cultures consisted exclusively of stage-I embryoids. Their dilution resulted in the more or less synchronous development of the stage-I embryoids into stage-II embryoids and, later, to bipolar embryos (Luckner and Diettrich, 1985; Scheibner *et al.*, 1988).

Embryo formation and development were influenced by the carbon source of the medium (most suitable was maltose, which was slowly degraded in *D. lanata* cell cultures) and by the nitrogen source (optimum NO_3^-/NH_4^+ ratio was 5–10 : 1, that is, a reduced level of NH_4^+; Kuberski *et al.*, 1984). This corresponded with the fact that embryogenesis was triggered by starvation (Kranz and Nover, 1983).

B. Integration of Cardenolide Formation in the Developmental Program of Somatic Embryogenesis

Analysis of somatic embryos at different stages demonstrated an increase of the cardenolide level during development (Luckner and Diettrich, 1985; Scheibner *et al.*, 1988):

1. Rapidly growing colonies of parenchyma-like cells and clusters of meristematic cells contained only very small amounts of cardenolide (<0.001 μmol g^{-1} dry weight).
2. Nonilluminated embryoids had a cardenolide content that was at least 10 times higher (~ 0.01 μmol g^{-1} dry weight).
3. Stage-II embryoids illuminated occasionally with low doses of white light (low enough not to cause chlorophyll accumulation) contained cardenolide levels of about 0.1 μmol g^{-1} dry weight (this value was in the magnitude of the maximum cardenolide content of the somatic embryos of *D. lanata* strain S 2, which were unable to form chloroplasts, Ohlsson *et al.*, 1983).
4. Stage-II embryoids and bipolar embryos illuminated with optimum light intensities and light periods accumulated more than 1 μmol of cardenolides per gram dry weight. These structures contained normally developed chloroplasts (Diettrich *et al.*, 1986) with considerable quantities of chlorophyll.

Though the highest cardenolide contents were found in the illuminated, green, chloroplast-containing embryos, no direct relation existed between the cardenolide and chlorophyll amounts (Scheibner *et al.*, 1987). Both processes showed the following:

1. Different time courses during the development of the stage-II embryoids
2. No parallel increase on illumination with different light intensities, light qualities, and photoperiods
3. Different reduction after inhibition of chloroplast development with low doses of antibiotics and herbicides (e.g., chloramphenicol, 3-aminotriazol, and SAN 9789)

The increased cardenolide base level in the nonilluminated stage-II embryoids and bipolar embryos demonstrated their competence to form and accumulate larger amounts of cardenolide. Illumination caused the step-by-step transformation of the amyloplasts present in the nonilluminated embryos to chloroplasts (Diettrich *et al.*, 1986), which may be involved in cardenolide biosynthesis. The formation of chloroplasts had, however, no influence on cardenolide biosynthesis without prior development of competence for embryo development (or for the formation of shoots; see Section III). Cell strains unable to form shoots or somatic embryos were found to be free of major amounts of cardenolides even

though turning green on illumination and containing fully developed chloroplasts (Diettrich *et al.*, 1986; Hagimori *et al.*, 1982a, 1984a,b; Reinhard *et al.*, 1975).

Embryoids treated with light of different wavelengths showed highest cardenolide accumulation on irradiation with blue and near-red light, in contrast to low cardenolide accumulation on irradiation with far-red light (Scheibner *et al.*, 1987). This indicated that a blue-light photoreceptor and phytochrome or protochlorophyllide-holochrome may participate in the regulation of cardenolide biosynthesis and accumulation. Irradiation with blue light also caused high cardenolide amounts in cultures of *D. lanata* strain S 1 (Ohlsson *et al.*, 1983). Embryoids irradiated each day with the same amounts of white light (equal daily energy flux) contained the highest cardenolide contents if the irradiation period (photoperiod) was more than or equal to 12 hr day^{-1}, that is, long-day conditions (Scheibner *et al.*, 1987).

The cardenolides formed in the embryoids differed from those found in adult plants. Stage-II embryoids of *D. lanata* strain VII contained digitoxigenin derivatives (probably glucodigifucoside and odorobioside G) as main cardenolides (Hering *et al.*, 1987), though strain VII was derived from a plant with a high content of digoxigenin derivatives in the leaves of the rosette. The predominance of digitoxin derivatives agreed with results demonstrating that zygotic *D. lanata* seedlings contained either no digoxigenin derivatives or only small amounts, in contrast to the adult plants (Aldrich *et al.*, 1956; Balbaa *et al.*, 1970; Kartnig and Hiermann, 1980; Weiler and Westekemper, 1979; Wichtl, 1972; Wichtl and Freier, 1978). The occurrence of digitoxigenin derivatives thus is characteristic of embryos and young seedlings irrespective of whether they are of zygotic or of somatic origin. The reported formation of digitoxin, lanatoside A, gitoxin, digitoxigenin, and digoxin in somatic embryos of *D. lanata* strain S 1 (Markkanen *et al.*, 1985) may be questionable.

The maximum cardenolide content of the embryoid globules was more than 1 $\mu mol\ g^{-1}$ dry weight (~8 μmol per liter of culture; Table I). So far, the selection of higher-yielding cell lines from *D. lanata* strain VII has failed. Though individual embryoids showed striking differences in the cardenolide content (Luckner *et al.*, 1981), subcultures derived from embryoids with maximum cardenolide content contained the same mean cardenolide amount and the same heterogeneity as the parent cultures. Hence the different cardenolide contents of the individual embryoids were caused either by different epigenetic (developmental) states or, if originating genetically, were not stable enough to be preserved in the derived cultures (Luckner and Diettrich, 1985).

Table I

Maximum Cardenolide Content in Different Types of *Digitalis* Cultures[a]

Species/strain	Material cultivated	Cardenolides: Micromoles per gram dry weight	Cardenolides: Micromoles per liter of culture	References
D. grandiflora	Somatic embryos	0.07		Nover *et al.* (1980)
D. purpurea	Shoots	0.15	3	Hagimori *et al.* (1984c)
	Shoots[b]	0.02		Hagimori *et al.* (1984a)
	Roots[b]	0.001		Hagimori *et al.* (1984a)
	Parenchyma-like cells[c]	0.0001		Hagimori *et al.* (1982a, 1984a,b)
D. lanata				
Strain V	Somatic embryos	0.1	1	Garve *et al.* (1980); Nover *et al.* (1980)
Strain S-1	Somatic embryos	0.4	3	Ohlsson *et al.* (1983)
Strain VII	Somatic embryos	>1	8	Scheibner *et al.* (1988)
Strain S-2	Somatic embryos[b]	0.06		Ohlsson *et al.* (1983)
D. lanata	Shoots	0.6	30	Lui and Staba (1981)
	Shoots[d]	0.1		Luckner and Diettrich (1985)
	Parenchyma-like cells[c]	0.001		Scheibner *et al.* (1988)

[a]Unless otherwise indicated the cultures were grown submerged and irradiated with white light.
[b]Grown in the dark.
[c]Cultivated with or without irradiation.
[d]Cultivated on solidified medium.

V. TRANSFORMATION OF CARDENOLIDES IN PARENCHYMA-LIKE *DIGITALIS* CELLS

Cardenolides were modified structurally by cell cultures of *Digitalis* even if these did not synthesize cardenolides *de novo*. The reactions found most widespread were glucosylation, hydroxylation at different positions, acetylation of glycosidically bound sugars, deglucosylation, and deacetylation (for summaries, see Alfermann and Reinhard, 1980; Furuya, 1978; Reinhard, 1974; Reinhard and Alfermann, 1980). Transfor-

mation of added cardenolides was also carried out by cell cultures derived from plants (e.g., *Daucus carota*) not able to form cardenolides under any conditions (Jones *et al.*, 1978; Jones and Veliky, 1981; Veliky *et al.*, 1980). The enzymes catalyzing the transformations therefore either did not belong to cardenolide metabolism and reacted with the cardenolides due to limited substrate specificity or, in cell cultures with competence for cardenolide formation, probably were part of cardenolide biosynthesis but were expressed independently from the enzymes forming the cardenolide skeleton *de novo*. Most thoroughly investigated were glucosylation and 12β-hydroxylation in cell strains of *D. lanata*.

A. Glucosylation of Cardenolides in *Digitalis lanata* Cell Cultures

Digitalis lanata cell cultures glucosylated cardenolides devoid of a terminal glucose residue, for example, aglycones, mono-, di-, and tri-digitoxosides. Cardenolides already containing a terminal glucose moiety were not glucosylated (Diettrich *et al.*, 1987a; Reinhard, 1974; Reinhard and Alfermann, 1980; Reinhard *et al.*, 1975). In leaves and cell cultures of *D. purpurea*, the existence of a membrane-associated sterol:UDPG glucosyltransferase was demonstrated (Yoshikawa and Furuya, 1979). This enzyme glucosylated digitoxigenin and digitoxin. It reacted, however, much faster with sterols, like stigmasterol and cholesterol, and therefore probably did not belong to cardenolide metabolism *sensu stricto*.

Kinetic experiments with living cells showed that the glucosylation of digitoxin may reach 0.4 nmol sec^{-1} g^{-1} dry weight in parenchyma-like cells of *D. lanata* strain VII (Diettrich *et al.*, 1987a). The purpureaglycoside A formed was accumulated in the vacuoles (Kreis and Reinhard, 1985a; Pfeiffer *et al.*, 1982), which could be isolated via protoplasts (Kreis and Reinhard, 1985b; Pfeiffer *et al.*, 1982). Glucosylation was a prerequisite for the translocation to and accumulation of cardenolides in these organelles (Diettrich *et al.*, 1987a; Kreis and Reinhard. 1985a).

Nonglucosylated cardenolides were taken up rapidly by *D. lanata* cells and protoplasts, displaying apparent free-space kinetics. They were again easily washed out, which indicated their location in the cytoplasm. The formation and accumulation of glucosylated cardenolides depended on the metabolic state of the cells (Diettrich *et al.*, 1987a). Optimum accumulation of purpureaglycoside A reached about 50 μmol

g^{-1} dry weight (Kreis and Reinhard, 1985a). In experiments with *D. lanata* strain VII, which accumulated about 3 μmol purpureaglycoside A per gram dry weight (~0.3 μmol g^{-1} fresh weight) the purpureaglycoside A was enriched in the cells > 60-times (Diettrich *et al.*, 1987a). The accumulation of purpureaglycoside A was shown to be an energy-driven process and was inhibited reversibly by low temperature (4°C), cyanide (1 mmol $liter^{-1}$), vanadate (0.1 mmol $liter^{-1}$), or nonglucosylated cardenolides (Kreis and Reinhard, 1985a).

B. 12β-Hydroxylation of Cardenolides in *Digitalis lanata* Cell Cultures (Formation of Digoxigenin Derivatives)

Certain cell strains of *D. lanata* unable to synthesize cardenolides *de novo* hydroxylated added digitoxin derivatives in position 12β (Heins, 1978; Reinhard, 1974; Reinhard *et al.*, 1975). This reaction was catalyzed by an endoplasmic-reticulum-bound monooxygenase system (digitoxin 12β-hydroxylase) (Petersen and Seitz, 1985). The enzyme hydroxylated several digitoxin derivatives, including β-methyldigitoxin, a semisynthetic compound preferably used in experiments with living cells (the methyl group prevented glucosylation of the terminal digitoxose residue; see Section V,A). Digitoxin 12β-hydroxylase needed O_2 and $NADPH_2$ as cosubstrates and was inhibited by CO. The CO inhibition was reversed by radiation with blue light (λ = 450 nm), indicating the participation of cytochrome *P*-450.

Digitalis lanata cell strains obtained from plants with a high content of digoxin derivatives showed large differences in 12β-hydroxylating capacity (Alfermann *et al.*, 1977; Heins, 1978; Reinhard and Alfermann, 1980). Maximum hydroxylation rates were about 0.15 nmol sec^{-1} g^{-1} dry weight with β-methyldigitoxin as substrate (Alfermann *et al.*, 1985). This 12β-hydroxylation paralled the growth of the *D. lanata* cell cultures. Optimum results were obtained with high glucose, phosphate, and O_2 levels as well as with methanol as solvent for the cardenolides added (Alfermann *et al.*, 1985; Spieler *et al.*, 1985).

Digitalis lanata cells immobilized by entrapment into gel beads hydroxylated β-methyldigitoxin for 170 days without fading in activity, that is, much longer than suspended cells (Alfermann, 1983; Alfermann *et al.*, 1983). The velocity of hydroxylation was the same in suspended and entrapped cells (Moritz *et al.*, 1982; see, however, Alfermann *et al.*, 1980).

VI. IS THERE A FUTURE FOR THE BIOTECHNOLOGICAL PRODUCTION AND TRANSFORMATION OF CARDENOLIDES BY *DIGITALIS* CELL AND ORGAN CULTURES?

Cardenolides interact with the Na^+,K^+-dependent ATP phosphorylase (Na^+,K^+-ATPase) of human beings and other animals (Schwartz and Collins, 1982). Several cardenolide glycosides are used as drugs that "normalize" heart activity by inhibiting the Na^+,K^+-ATPase of heart muscle cells. In Europe and North America, cardenolide glycosides of *D. lanata* and *D. purpurea,* for example, digitoxin, digoxin, deacetyllanatoside C, and lanatoside C have found use in medicine.

The most important raw material for these compounds are leaves of cultivated *D. lanata* (Luckner and Diettrich, 1979; Mastenbroek, 1980, 1985; Neczypor *et al.*, 1980). Because cardenolides are expensive, used in rather large amounts, and can neither be produced by microorganisms nor economically by chemical synthesis, their formation by biotechnological methods is of economic interest. The described cultivation of tissues and organs of *Digitalis* species *in vitro* may be a base for biotechnological cardenolide production in the future. However, further research is necessary before any biotechnological production can be established. Though *D. purpurea* shoots (Hagimori *et al.*, 1984c) and *D. lanata* embryos (M. Luckner and B. Diettrich, unpublished results) were successfully grown in fermenters, both entities were rather difficult to handle. Their growth was too slow and the amounts of cardenolides formed were too small for any economical biosynthesis. Furthermore, the embryos contained cardenolides that differed in their sugar moieties from those used in medicine (see Section IV,B). It might be expected that their pharmacological activity is similar to that of the cardenolides used in therapy (see Chen, 1970), but this has not been substantiated.

Of prime importance for further progress will therefore be the selection of new more suitable cell lines. Research activities should be concentrated on the establishment of cell strains from as yet unused cell types, *Digitalis* species, and varieties as well as on the production and the selection of mutants. Of importance in this respect will be the establishment of haploid strains. Haploid cell strains have been obtained from anthers of *D. purpurea* (Corduan and Spix, 1975) and *D. obscura* (Perez-Bermudez *et al.*, 1987), but in spite of considerable effort they could not be derived from anthers and ovules of *D. lanata* (H. Böhm, personal communication; M. Luckner and B. Diettrich, unpublished re-

sults). In addition, it may be of importance to select new cell lines from the heterogeneous pool of cell strains cultivated for long periods *in vitro*. Based on this heterogeneity, lines able to form cardenolides in parenchyma-like cells, that is, without the formation of embryos, have been isolated from the embryogenic *D. lanata* cell strain VII (Luckner and Diettrich, 1987). It will be the aim of further experiments to develop from these lines stable, cardenolide-producing strains, which, it is hoped, will be more suitable for the biotechnological production of cardenolides than those cell strains described above.

In contrast to the *de novo* biosynthesis of cardenolides, the 12β-hydroxylation of β-methyldigitoxin by cell cultures of *D. lanata* seemed to become economically significant and has therefore been developed to semiindustrial scale. With strains transforming added β-methyldigitoxin almost quantitatively to β-methyldigoxin in airlift fermenters (working volume, 200 liters), about 100 g of β-methyldigoxin was obtained within an incubation period of 14 days. Reduction of cost was brought about using part of the foregoing culture for inoculation of the next batch (repeated batch cultivation). By this method six runs were possible in a 3-month period without reduction of productivity, forming a total of 500 g of β-methyldigoxin (Alfermann *et al.*, 1985). At Boehringer Mannheim GmbH. the procedure was tested on the industrial scale. The process must compete, however, with 12β-hydroxylation of digitoxigenin derivatives by Streptomycetes (Karoly *et al.*, 1981; Natonek *et al.*, 1980; Nozaki *et al.*, 1965), a method used at Gedeon Richter Ltd., Budapest. This competition and the decreasing demands in digoxin derivatives were the reason that the 12β-hydroxylation of digitoxin derivatives by *D. lanata* cell cultures has not been introduced at the industrial scale.

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CHAPTER 12

Saponins (Ginseng Saponins)

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I. INTRODUCTION

Saponins are glycosidic compounds occurring abundantly in the plant kingdom. A simple test for saponins is to shake an aqueous alcoholic

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plant extract in a test tube and note if a persistent fine foam is formed above the liquid surface. Some saponins also have the ability to hemolyze blood cells and to form unsoluble complexes with cholesterol.

Being glycosidic plant products, the saponins are composed of a parent compound (called genin or sapogenin) and a variable sugar component. Saponins generally are classified as follows:

1. Triterpene glycosides: ginsenosides (ginseng), glycyrrhizin (licorice), saikosides (Bupleurum root), etc.
2. Spirostanol glycosides (synonym, steroidal glycosides)
3. Steroidal alkaloid glycosides.

Research profiles until 1982 of saponin production by plant cell cultures have been described in detail by Barz *et al.* (1977), Fujiwara (1982), Reinert and Bajaj (1977), and Staba (1980), and there have been only a few publications on saponins since 1983. The objective of this chapter is, therefore, to present progress in the saponin production by Korean ginseng cell cultures since 1982.

Panax ginseng C. A. Meyer is a herbaceous plant belonging to the Araliaceae. Its root, called ginseng or Korean ginseng, is a favorite tonic and health food worldwide. Chemical and pharmacological studies on ginseng confirmed that among ginseng saponins, ginsenosides Rb_1 and Rg_1 are the most active principles. The isolation of panaxadiol (Furuya *et al.*, 1973; Jhang *et al.*, 1974), panaxatriol (Furuya *et al.*, 1970), oleanolic acid, and ginsenosides Rb_1 and Rg_1 (Furuya *et al.*, 1973) from ginseng callus, the effects of auxins on growth and saponin production (Furuya *et al.*, 1983a,c, 1984), and the regulation of saponin production by biosynthetic precursors and some bioregulators (Furuya *et al.*, 1983b) in ginseng cell suspension cultures have been investigated. The relationship between saponin production and growth in various cell cultures (Furuya, 1981) and differentiated tissues (Furuya *et al.*, 1986) of *P. ginseng* are described in this chapter.

II. CALLUS INDUCTION AND SELECTION

A. Pg-1 Callus

Callus was derived from the petiole of 2-year-old ginseng in 1967 (Furuya *et al.*, 1970, 1973). The stock culture has been maintained on MS [Murashige and Skoog's (1962)] agar medium containing 0.1 ppm of

2,4-D (2,4-dichlorophenoxyacetic acid), at 25°C in the dark by subculture at 4-week intervals (Pg-1 callus).

Pg-1 callus was subcultured also with K1 (kinetin, 0.1 ppm; see Table I for media), but without 2,4-D, under illumination (2500–4000 lux, 16hr/day) with warm fluorescent light in a phytotron cabinet. This Pg-1 K1 callus gradually generated roots and shoots. The roots were selected and subcultured with IBA1 (indole-3-butyric acid, 1 ppm) instead of K1 in the dark, and continued to form roots in the dark only (Pg-1 IBA1 callus).

Calli of the two cell lines (1–2 g) were transferred onto 40 ml of medium in a 100-ml Erlenmeyer flask and subcultured statically at 3- to 4-week intervals.

B. Habituated Callus

Non-auxin-requiring (habituated) callus was derived from the Pg-1 callus as follows. The callus was transferred to medium containing various amounts of 2,4-D (the first passage). Calli grown on medium with low concentrations of 2,4-D, such as 0.01, 0.001, and 0.0001 ppm, were transferred again to the corresponding medium containing the same amounts of 2,4-D (the second passage). Growth of each callus, however, was increasingly difficult to measure after 5 weeks. For the fourth passage, calli were transferred to the basal medium (2,4-D omitted) but generally did not grow at all. During the fifth passage, however, a small increase in the rate of growth was observed. From the tenth passage on, calli began to grow well on the basal medium. The growth rate relative to that of the Pg-1 callus was approximately 60–70%.

C. Pg-3 Callus

Slices of 5-year-old Korean ginseng root were placed on MS agar medium supplemented with 2,4-D (1 ppm) and K (0.1 ppm) in 1978 (Furuya *et al.*, 1984). The developing callus (Pg-3) was transplanted onto the same medium, maintained at 25°C in the dark, and subcultured at intervals of 4 weeks (Pg-3 DK callus). After a third subculture, the callus was transferred onto MS medium containing IBA (2 ppm) and K (0.1 ppm). The callus was kept at 25°C in the dark and subcultured at intervals of 4 weeks for about a year, and eventually vigorous growth was achieved (Pg-3 B2K callus).

III. DETERMINATION OF GROWTH RATIO

A. Pg-1 Callus

Callus (1–3 g) was transferred to the test agar medium placed in a flask. After 4 weeks, the average fresh weight of the calli in 6 to 12 flasks was determined. In the case of suspension culture, 15 g of callus was transferred to 250 ml of medium in a 1-liter Erlenmeyer flask in reciprocal culture, and 30 g of callus to 500 ml in a similar flask in rotary culture. After 4 weeks, the average fresh weight of the calli in 4 flasks was determined. After extraction of 50 g of these callus tissues with methanol, the residual material was dried in an oven at 80°C and weighed. All experiments were carried out two or three times.

B. Habituated Callus

Three pieces of calli (~0.3 g of fresh weight) were transferred to the test medium, the growth rate measured, and the increase in fresh weight after 4 weeks averaged.

C. Pg-3 Callus

Medium (250 ml) in a 1-liter Erlenmeyer flask was inoculated with callus and cultured on a reciprocal shaker (80 strokes per minute, each stroke 8 cm in length) at 25°C in the dark. After 4 weeks of culture the callus was harvested, and the growth ratio determined. Two flasks were used in each experiment.

IV. SEPARATION OF SAPONINS

The *n*BuOH-soluble layer separated from the MeOH extract of Pg-1 callus (10 kg fresh weight) was evaporated to obtain crude saponins. The saponins (Fig. 1) were separated on a column of Sephadex LH-20 using $CHCl_3$–MeOH (1 : 2), and then purified by silica gel column chro-

20(S)-Protopanaxadiol
R_1=R_3=H

Rb_1; R_1=glu$\overset{2\;1}{-}$glu, R_3=glu$\overset{6\;1}{-}$glu
Rb_2; R_1=glu$\overset{2\;1}{-}$glu, R_3=glu$\overset{6\;1}{-}$ara
Rb_3; R_1=glu$\overset{2\;1}{-}$glu, R_3=glu$\overset{6\;1}{-}$xyl
Rc ; R_1=glu$\overset{2\;1}{-}$glu, R_3=glu$\overset{6\;1}{-}$ara
Rd ; R_1=glu$\overset{2\;1}{-}$glu, R_3=glu

20(S)-Protopanaxatriol
R_2=R_3=H

Re ; R_2=glu$\overset{2\;1}{-}$rham, R_3=glu
Rf ; R_2=glu$\overset{2\;1}{-}$glu, R_3=H
Rg_1; R_2=glu, R_3=glu
Rg_2; R_2=glu$\overset{2\;1}{-}$rham, R_3=H
Rh_1; R_2=glu, R_3=H

Fig. 1. Saponins of ginseng.

matography using $CHCl_3$–MeOH. Ginsenosides Rg_1, Re, Rb_1, and Ro were isolated from each fraction, yielding 1750, 220, 295, and 260 mg, respectively. Ginsenoside Rg_1 isolated as its acetate gave colorless leaflets (m.p. 242.5–243°C) Rb_1 as white powder (m.p. 197–198°C), Re as colorless needles, crystallized from 50% MeOH (m.p. 201–203°C), and Ro as colorless needles from MeOH (m.p. 239–241°C). Four ginsenosides were confirmed by melting point (m.p.), infrared (IR), nuclear magnetic resonance (NMR), and mass spectra in comparison with authentic ginsenosides. The isolation of ginsenosides Re and Ro from ginseng callus was demonstrated for the first time. Furthermore, the presence of all ginsenosides, Ro, Ra, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, and Rh, was detected by TLC, as well as HPLC, and determined by densitometry (Furuya *et al.*, 1973, 1983c).

V. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SAPONINS

Waters HPLC equipment (Model ALC/GPC 244) was used, with Shodex OH Pak-804; column, 0.8 × 50 cm; CH_3CN–H_2O (85:15); flow rate, 1.5 ml/min; chart speed 0.25 cm/min; pressure, 20 kg/cm^3; detector

RI. The *R*t values (in minutes) for the following ginsenosides were found: Rg_1, 19.8; Re, 22.4; Rb_1, 33.8; Ra, 36.4; Ro, 47.2 (Sticher and Soldati, 1979; Soldati and Sticher, 1980). Identification by comparison with authentic saponins was performed, as shown in Fig. 2.

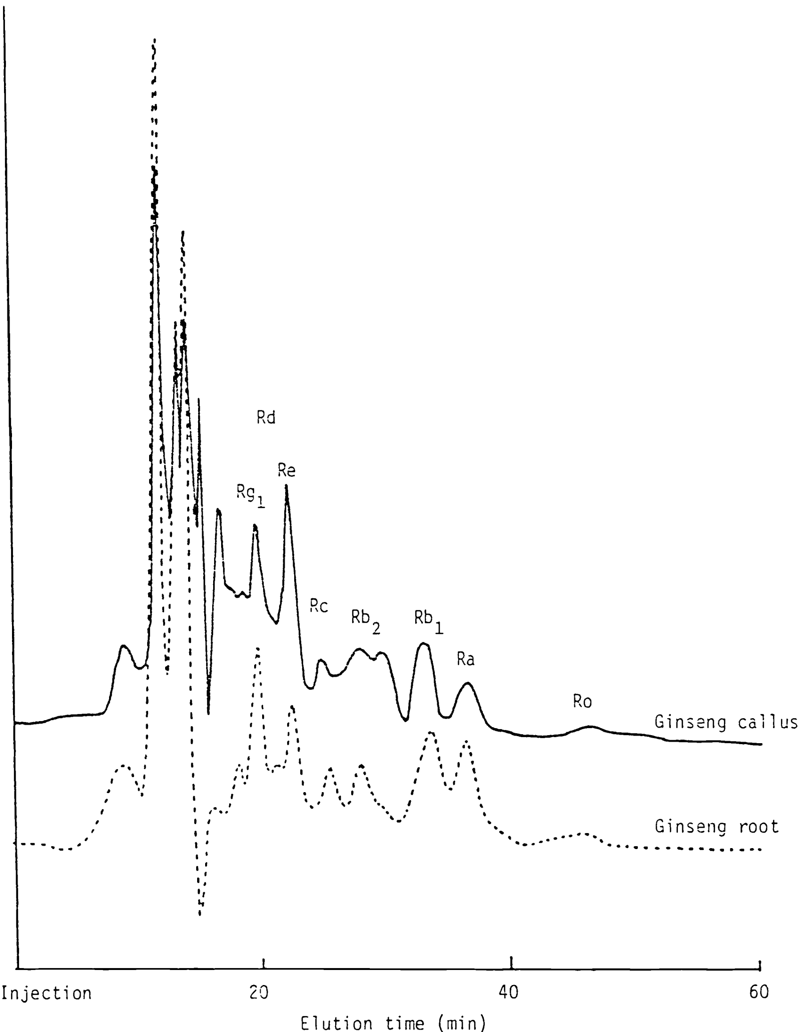

Fig. 2. High-performance liquid chromatograms of crude saponins in ginseng callus and ginseng root.

VI. DETERMINATION OF SAPONINS

The crude saponins obtained from K1 and IBA1 calli were spotted together with the standard samples of ginsenosides Rb_1 and Rg_1 on Merck silica gel TLC plate 60 F254 and were developed with an upper layer of *n*BuOH–AcOH–H_2O (4:1:5). The spots of saponins were detected by spraying with 10% H_2SO_4 followed by heating at 105°C for 10 min and determined by densitometry using Shimadzu Model CS-910 dual-wavelength TLC scanner (λ_s = 530 nm, λ_r = 700 nm) (Sanada *et al.*, 1978), as shown in Fig. 3.

The R_f values for the following ginsenosides were found: Ro, 0.04; Ra, 0.08; Rb_1, 0.15; Rb_2, 0.19; Rc, 0.25; Rd, 0.34; Re, 0.41; Rf, 0.44; Rg_1, 0.50; Rg_2, 0.52; Rh_1, 0.64. The amount of the Rb group was calculated as the total of ginsenosides Ra, Rb_1, Rb_2, Rc, and Rd, with protopanaxadiol as the sapogenin, and the Rg group was calculated as the total of ginsenosides Re, Rf, Rg_1, Rg_2 and Rh_1, with protopanaxatriol.

Total saponin in each culture shows the content of pure ginsenosides, determined by TLC densitometry. All data are the average value of duplicate estimations in each of two or three different cultures, and especially in five cultures in the IBA series.

VII. EFFECTS OF PLANT GROWTH REGULATORS AND LIGHT ON SAPONIN PRODUCTION AND GROWTH IN STATIC CULTURES

A. Pg-1 Callus

The effects of auxins and cytokinins on saponin content and growth in static cultures using IBA1 callus were examined, and a better growth ratio and increased amount of saponin were observed in the combination of IBA and K (Table I). Although 2,4-D produced the best growth in the 2,4-D callus, it inhibited the growth of the IBA1 callus, and in 5 ppm 2,4-D the growth completely stopped.

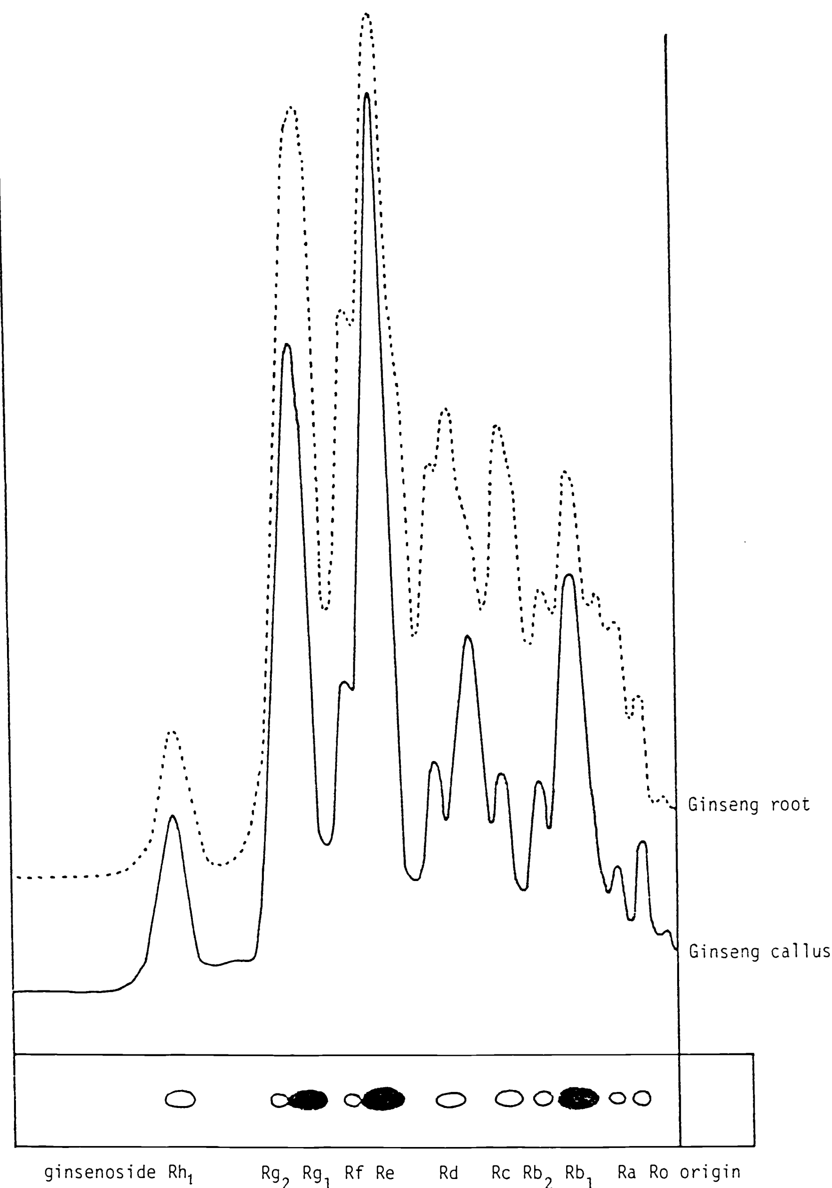

Fig. 3. Thin-layer chromatogram and dual-wave length spectra of crude saponins in ginseng callus and ginseng root.

Table I

Effects of Plant Growth Regulators on Saponin Production and Growth in Static Cultures of IBA1 Callus

Medium[a]	Growth ratio	Dry weight (g) per 100 g fresh weight	Saponin[b] content (mg) per 100 g fresh weight			Rb group / Rg group
			Rb group	Rg group	Total	
IAA1	2.68	3.85	19.0	27.7	46.7	0.69
IAA1 K0.1	3.04	3.87	19.6	17.4	37.0	1.13
IAA5	3.43	3.71	13.1	12.8	25.9	1.02
IAA5 K0.1	3.67	3.80	17.6	29.4	47.0	0.60
NAA1	2.98	3.48	23.7	19.1	42.8	1.24
NAA1 K0.1	2.52	3.40	20.5	18.6	39.1	1.10
NAA5	3.22	3.32	21.7	17.0	38.7	1.28
NAA5 K0.1	3.36	3.16	21.8	15.3	37.1	1.42
2,4-D1	2.69	3.40	21.4	19.9	41.3	1.08
2,4-D1 K0.1	2.88	2.02	44.2	14.4	58.6	3.07
IBA1	3.40	2.80	22.0	19.8	41.8	1.11
IBA1 K0.1	3.62	3.00	15.6	15.1	30.7	1.03
IBA5	3.61	2.80	14.2	9.4	23.6	1.51
IBA5 K0.1	3.18	2.60	31.2	25.6	56.8	1.22
IBA1 K1	4.33	2.60	22.6	16.4	39.0	1.38
IBA1 K5	3.89	2.00	16.8	13.2	30.0	1.27
IBA1 P0.1	4.22	4.76	23.1	18.9	42.0	1.22
IBA5 K1	5.14	3.04	6.2	11.4	17.6	0.54
K1	4.34	2.60	24.4	26.3	50.7	0.93
K5	3.93	2.61	15.6	11.4	27.0	1.37
P1	4.80	3.03	15.7	13.9	29.6	1.13

[a]Abbreviations: IAA, indole-3-acetic acid; NAA, 1-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; K, kinetin; P, *N*-phenyl-*N*′-(4-pyridyl)urea.

[b]Rb group indicates ginsenosides Ra, Rb_1, Rb_2, Rc, and Rd, with protopanaxadiol as the sapogenin, and Rg group indicates ginsenosides Re, Rf, Rg_1, Rg_2, and Rh_1, with protopanaxatriol.

The effects of illumination (2500–4000 lux) on the saponin content and growth of K1 callus, which generated shoots under the light, are shown in Table II. The use of P [*N*-phenyl-*N*′-(4-pyridyl)urea] in light produced the best growth ratio, but saponin content was lowest. This was due to the decrease in amounts of Rb-group saponins (Furuya *et al.*, 1983c).

B. Habituated Callus

The effect of 2,4-D and IAA (indole-3-acetic acid) on saponin production in habitutated callus as compared with Pg-1 callus was examined.

Table II

Effects of Light on Saponin Production and Growth in Static Cultures of K1 Callus

Medium	Growth ratio	Dry weight (g) per 100 g fresh weight	Saponin content (mg) per 100 g fresh weight			Rb group / Rg group
			Rb group	Rg group	Total	
Dark						
K1	3.32	2.13	23.9	10.8	34.7	2.21
K5	1.50	2.09	22.6	11.5	34.1	1.97
P1	3.70	2.71	22.3	21.6	43.9	1.03
P5	4.17	2.19	31.2	21.9	53.1	1.42
IBA1	2.75	2.98	26.0	26.4	52.4	0.98
IBA5	2.95	2.70	21.4	12.7	34.1	1.69
Light						
K1	3.70	2.49	27.5	17.8	45.3	1.54
K5	1.97	2.25	24.5	18.5	43.0	1.32
P1	4.23	2.96	14.9	22.2	37.1	0.67
P5	4.51	2.18	15.3	14.0	29.3	1.09
IBA1	3.20	2.79	30.6	12.5	43.1	2.45
IBA5	3.87	2.59	30.3	10.2	40.5	2.97

The saponin content (total saponin) in Pg-1 callus was 0.82% on a dry-weight basis when the callus was cultured on the medium containing 0.1 ppm 2,4-D (i.e., the normal condition in the 2,4-D callus). The ratio of the Rg group to the Rb group was about 3 to 2. The production of saponin was maximum at 0.1 ppm 2,4-D and gradually decreased with an increase of 2,4-D, up to 0.22% in the presence of 5 ppm 2,4-D. The growth corresponded fairly well to the production of saponin. The saponin content of the habituated callus derived from the Pg-1 callus was only 0.09% of dry weight on the basal medium (not containing any auxins), however, although the growth rate (6.8) was almost the same as in the Pg-1 callus (7.2 in 0.1 ppm 2,4-D). The saponin content slightly increased with additional amounts of 2,4-D, but at 5.0 ppm 2,4-D the growth was suppressed to 1.8 (Table III).

The effects of IAA on the production of saponin in both calli were investigated. Saponin content and growth rate were not significantly affected by IAA. Endogenous IAA is present at a concentration of around 10 and 45 $\times 10^{-9}$ gram per gram fresh weight in 2,4-D–requiring and habituated calli, respectively (Nishio *et al.*, 1976). Remarkably, the

Table III

Effects of 2,4-D on Saponin Production in 2,4-D and Habituated Calli

Callus	2,4-D (ppm)	Growth rate	Saponin content (% dry weight) Rg group	Rb group	Total
2,4-D–requiring	0.00	4.2	0.19	0.16	0.35
	0.01	4.7	0.24	0.22	0.46
	0.10	7.2	0.48	0.34	0.82
	0.50	5.8	0.33	0.25	0.58
	1.00	5.0	0.32	0.22	0.54
	5.00	2.7	0.16	0.06	0.22
Habituated	0.00	6.8	0.05	0.04	0.09
	0.01	6.2	0.05	0.05	0.10
	0.10	2.8	0.09	0.07	0.16
	0.50	2.4	0.08	0.07	0.15
	1.00	2.5	0.08	0.07	0.15
	5.00	1.8	0.06	0.06	0.12

content of the Rb group, especially in Pg-1 callus, decreased by the removal of 2,4-D from the medium, for example, from 42% total saponin in the presence of 2,4-D (0.1 ppm) to 33% in cultures without or with 1 to 20 ppm IAA (for effects of auxins on saponin production during successive cultures see Furuya *et al.*, 1983a).

VIII. EFFECT OF VARIOUS CULTURE CONDITIONS ON SAPONIN PRODUCTION AND GROWTH IN SUSPENSION CULTURES

A. Pg-1 Callus

Comparison of saponin production and growth of IBA1 callus, which generated roots in the dark, was made under various conditions. In suspension cultures on a gyratory shaker, growth was excellent compared to cultures on a reciprocal shaker, especially when IBA was used as a hormone, but saponin production was higher in reciprocal than in gyratory cultures, except when 2,4-D was used. It was observed that a

combination of IBA and K resulted in a lower growth ratio but a much higher saponin production than when using IBA with P. A combination of IBA (2 ppm) with K (0.1 ppm) gave the best production index (growth and saponin production), and IBA (5 ppm) with P (0.1 ppm) gave the best growth ratio (8.19) (Furuya *et al.*, 1983c).

The gyratory suspension cultures produced soft, brownish cell aggregates generating many roots and showed 1.8 times the growth rate and increased production index compared with reciprocal suspension cultures. Also, cell suspension cultures produced higher amounts of saponins than the static cultures.

The best condition for callus cultures, the combination of IBA (2 ppm) and K (0.1 ppm), produced almost the same saponin content, especially in protopanaxatriol-group saponins, as that of the crude drug. In protopanaxadiol-group saponins, the content of Rb_1 was higher than that of the crude drug, but Rc and Rd were lower.

B. Pg-3 Callus

Pg-3 callus cultured on agar was transferred to a 5-liter jar fermenter containing 4 liters of test medium. The aeration ratio was 1 volume (aeration) per volume (medium) per minute (VVM) at 100 rpm.

Pg-3 callus grown on agar also was transferred to 500 ml of medium in a 1-liter Erlenmeyer flask and cultured on a reciprocal shaker for 4 weeks. Cells cultured in this manner were then used as inocula for a 30-liter jar fermenter containing 25 liters of test medium. The inoculum size was 24 or 48 g/liter. The aeration ratio was 0.25 VVM. Three turbine types (disk, angled disk, and anchor type; Fig. 4) and two speeds (100 and 150 rpm) were tested. The results are summarized in Table IV (Furuya *et al.*, 1984). MS medium without NH_4NO_3 was similar to regular MS medium for growth and saponin content. Use of MS medium without NH_4NO_3, but with 0.5% glucose and 2% sucrose, and another 2% sucrose added after 2 weeks of culture, resulted in a higher growth ratio and higher dry weight (g/liter) than regular MS medium containing 3% sucrose; the relative amount of saponins (mg/liter) decreased only slightly.

Among three turbine types (Fig. 4), the angled-disk turbine provided the best growth ratio and dry weight increase (g/liter) but the lowest saponin content. When the agitation was accelerated to 150 rpm, the growth ratio and dry weight decreased, but the saponin content increased, giving a saponin production (mg/liter) similar to that at 100 rpm.

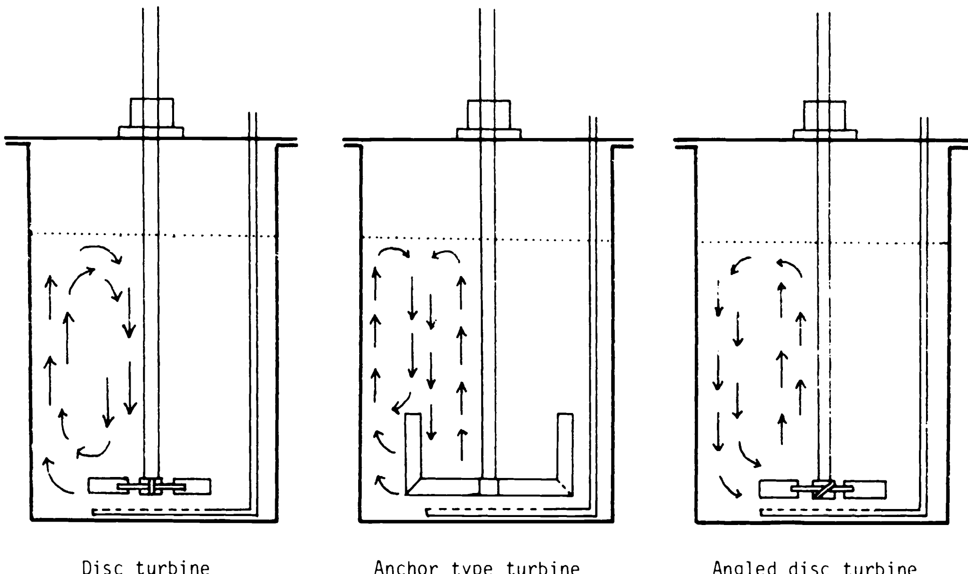

Fig. 4. Three turbine types used in 30-liter jar fermenter culture.

In a 30-liter jar fermenter culture, the increase of the growth ratio and dry weight were not accompanied by an increase of the saponin content. This observation indicated that the saponin production per culture was about equal. A jar fermenter culture is not comparable to a shake-flask suspension culture. Therefore, it is necessary to further examine the culture conditions for cells in jar fermenters. Repeated selection of cell lines from Pg-3 callus and the engineering of a new device for mass culture in a ton-scale tank are now in progress.

IX. EFFECT OF INHIBITORS AND PRECURSORS ON SAPONIN PRODUCTION

The effect of semicarbazide in the presence of several precursors was studied with Pg-1 callus (Furuya *et al.*, 1983b). The highest saponin content was obtained in the presence of mevalonate in addition to semicarbazide, the amount being more than 2 and 2.5 times that in the absence of both mevalonate and semicarbazide, and in the presence of mevalonate only, respectively. On the contrary, no recognizable change

Table IV

Effect of Medium Conditions and Turbine Types in 30-Liter Jar Fermenter Culture of Pg-3 B2K Callus[a]

Medium	Turbine (rpm)	Inoculum size (g/liter)	Growth ratio	Dry weight: g/100 g (fresh weight)	Dry weight: g/liter	Total saponin content: mg/100 g (fresh weight)	Total saponin content: mg/100 g (dry weight)	Total saponin content: mg/liter
MS Sucrose 3%	Disk (100)	24	4.45	5.71	6.1	41.1	720	43.9
	Disk[b] (50)	48	4.33	5.64	11.7	23.1	410	48.0
MS–NH_4NO_3 Sucrose 3%	Disk (100)	24	5.00	8.86	10.6	41.3	466	49.4
		48	4.10	5.56	10.9	18.6	335	36.5
MS-NH_4NO_3 Sucrose 2% + glucose 0.5%	Disk (100)	24	4.33	7.54	7.8	55.3	733	57.2
		48	6.12	3.17	9.3	18.4	580	53.9
MS–NH_4NO_3 Sucrose 2% + glucose 0.5%; add sucrose 2% after 2 weeks	Disk (100)	48	5.04	5.95	14.4	20.7	348	50.1
	Anchor (100)	48	4.66	6.86	15.3	19.5	284	43.5
	Angled (100)	48	6.45	5.48	17.0	9.7	177	30.1
	Disk (150)	48	3.86	5.82	10.8	26.7	459	49.6
	Angled (150)	48	3.81	7.74	14.2	24.2	313	44.4

[a]Aeration ratio, 0.25 VVM; culture period, 28 days; disk, disk turbine; anchor, anchor-type turbine; angled, angled-disk turbine.
[b]0.5 VVM, two-disk turbines.

was detected in the content of phytosterols (Table V). It seems, therefore, that the synthetic pathway of saponins in callus tissues may be competitively inhibited by semicarbazide against precursors such as mevalonate. Moreover, it was clarified that the simultaneous addition of mevalonate and semicarbazide enhances saponin production more than that of mevalonate only in both Pg-1 and habituated calli, the increase in quantity attaining approximately a factor of 2.

In the presence of mevalonate, thiosemicarbazide also promoted the production of saponins, whereas hydroxylamine and 2,4-dinitrophenylhydrazine were ineffective. In contrast, phytosterol production was somewhat inhibited by semicarbazide and thiosemicarbazide even in the presence of mevalonate, particularly in normal callus. The results are summarized in Table V. From these data, it is suggested that some intermediary steps in the phytosterol biosynthesis in callus tissue are inhibited by carbazides, and consequently, the mevalonate pathway is pushed toward saponin biosynthesis [for the regulation of saponin and phytosterol biosynthesis by end-product (β-sitosterol and ginsenoside Rg_1) inhibition see Furuya *et al.*, 1983b].

Table V

Effects of Several Inhibitors, with or without Mevalonate, on Saponin and Phytosterol Production in Calli[a]

Inhibitors (25 ppm)	Mevalonate (50 ppm)	Saponin content (% dry weight)	Phytosterol content (% dry weight)
2,4-D callus			
Semicarbazide	+	2.27	0.31
Thiosemicarbazide	+	2.09	0.18
Hydroxylamine	+	1.36	0.34
2,4-Dinitrophenylhydrazine	+	1.12	0.42
None—1	+	1.49	0.56
None—2	−	0.85	0.44
Habituated callus			
Semicarbazide	+	0.19	0.37
Thiosemicarbazide	+	0.28	0.20
Hydroxylamine	+	0.11	0.30
2,4-Dinitrophenylhydrazine	+	0.08	0.29
None—1	+	0.13	0.38
None—2	−	0.06	0.30

[a]Cultured for 21 days; inhibitors and mevalonate were added at tenth day of culture.

X. DIFFERENTIATION AND SAPONIN PRODUCTION

From Pg-3 callus cultures of Korean ginseng, shoots and roots were formed at a high rate (in all flasks) under optimal conditions (Fig. 5). The saponin production and the morphological structures of the differentiated plantlets closely resembled those of the native plants (Furuya *et al.*, 1986).

The saponin content in various calli and differentiated tissues were determined according to the TLC method. The values are shown in Table VI and are compared to the saponin content of the source plant. As a result, it was demonstrated that the shoots (K1) and the roots (IBA1) produced larger amounts of saponins than the original callus (DK callus): 3.5 times more in K1 and 4.9 times more in IBA1 tissues. The saponin content in suspension culture (IBA2, K0.1 medium) was comparable to those of the aerial part and the root of the plant on a fresh-weight basis. On the other hand, the saponin content of the cultured roots on a dry-weight basis was 1.71% in static culture and 1.27% in suspension, and those were 3–4 times higher than that in the plant root, 0.40%. Moreover, the ratio of the ginsenoside Rb group to the Rg group was calculated for a quality evaluation of the ginseng saponin. The ratios in DK callus and K1 shoot resembled those in the aerial part of the native plant, whereas in the root cultures (i.e., IBA1 root and IBA2 K0.1 suspension) they resembled those in the plant root.

XI. HAIRY ROOT CULTURE AND SAPONIN PRODUCTION

Hairy root culture of ginseng was established after roots were induced on Pg-4 callus following infection with *Agrobacterium rhizogenes* (Yoshikawa and Furuya, 1987). The transformed cultures of ginseng could be subcultured as an axenic root culture in the absence of phytohormones, and grew with extensive lateral branches more rapidly than the ordinary cultured roots induced by hormonal control from Pg-1 callus (Fig. 6). It was also demonstrated that the hairy roots contain the same saponins (ginsenosides) as those of the native root, up to about 2.4 times in the quantity, and up to about 2 times in comparison

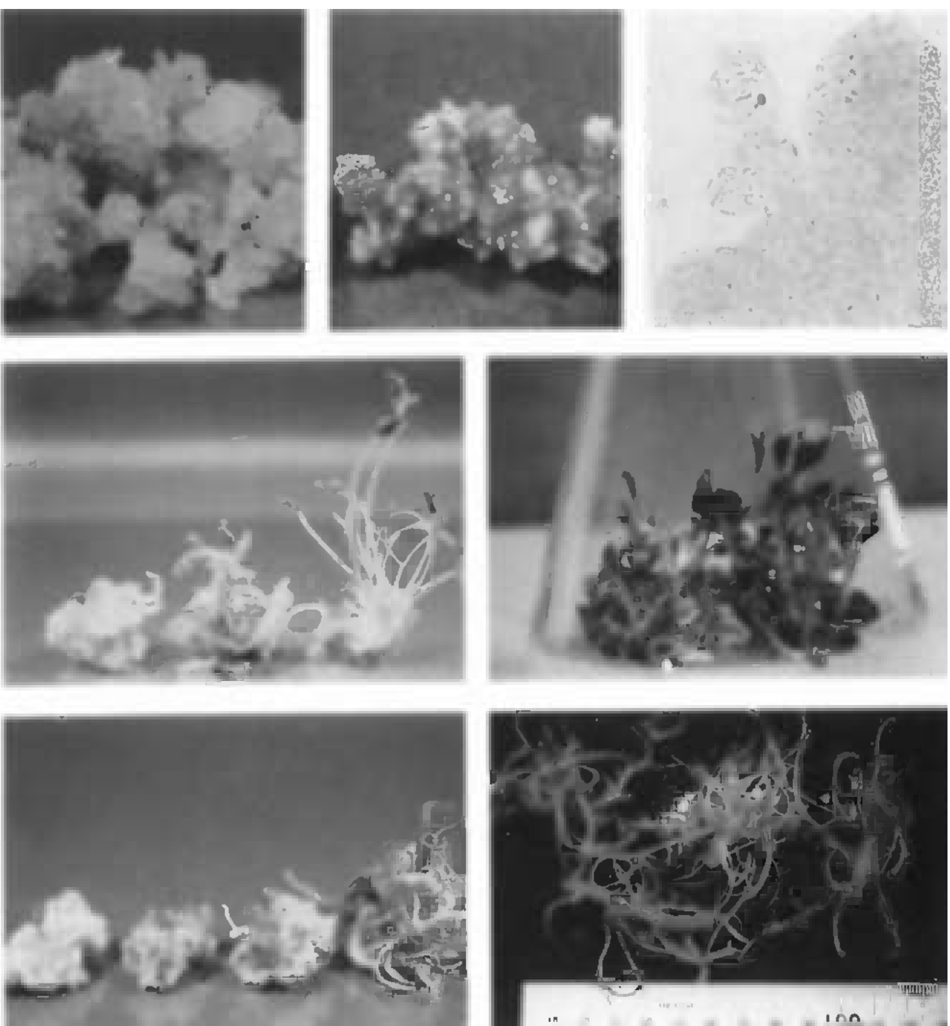

Fig. 5. Systematic formation of shoots, roots and plantlets from callus cultures of *Panax ginseng*. a; Ginseng callus derived from native ginseng root on MS medium containing 2,4-D (2,4-dichlorophenoxyacetic acid) 1 ppm and kinetin 0.1 ppm (DK callus). b; Meristemoids induced from the DK callus (a) on the meaium without 2,4-D. c; A cross section of the meristemoids (b), × 20. d; Shoots formed from the meristemoids (b) on the medium containing kinetin 1 ppm, stepwise from left to right. e; Cloning plantlets developed from shoots (d) on the K 1 medium under illumination. f; Roots formed from meristemoids (b) on the medium containing IBA (indole-3-butyric acid) 1 ppm, stepwise from left to right. g; Roots cultured in the liquid medium containing IBA 2 ppm and kinetin 0.1 ppm in the dark.

Table VI

Comparison of Saponin Production between Various Ginseng Cultured Tissues and Original Plant

Callus or tissue	Growth ratio[a]	Dry weight (g) per 100 g fresh weight	Saponin content (mg) per 100 g fresh weight			Rb group / Rg group	Total saponin per dry mass (% weight)
			Rb group	Rg group[b]	Total		
Static							
DK callus (Fig. 1a)	4.30	2.48	2.4	8.4	10.8	0.29	0.44
K1 shoot (Fig. 1d,e)	5.81	2.96	15.1	22.3	37.4	0.67	1.26
IBA1 root (Fig. 1f)	3.40	3.09	27.6	25.1	52.7	1.10	1.71
Suspension							
IBA2 K 0.1 root (Fig. 1g)	6.22	6.31	41.6	38.8	80.4	1.07	1.27
Plant							
Aerial part (stem and leaf)		9.53	21.7	62.1	83.8	0.35	0.88
Root		23.91	59.0	37.3	96.3	1.58	0.40

[a]Growth ratio was determined by increase of fresh weight after 4 weeks of culture. The values are the quotient of the fresh weight after 4 weeks of culture and the fresh weight of the inoculum.

[b]The amount of Rb group was calculated as the total of ginsenosides Ra, Rb_1, Rb_2, Rc, and Rd, with protopanaxadiol as the sapogenin, and that of Rg group was calculated as the total of ginsenosides Re, Rf, Rg_1, Rg_2, and Rh, with protopanaxatriol. Each value in the cultured tissues shows the average of duplicate estimations in four flasks of three different cultures. Each value for the original plant is the average of duplicate estimations in three different samples.

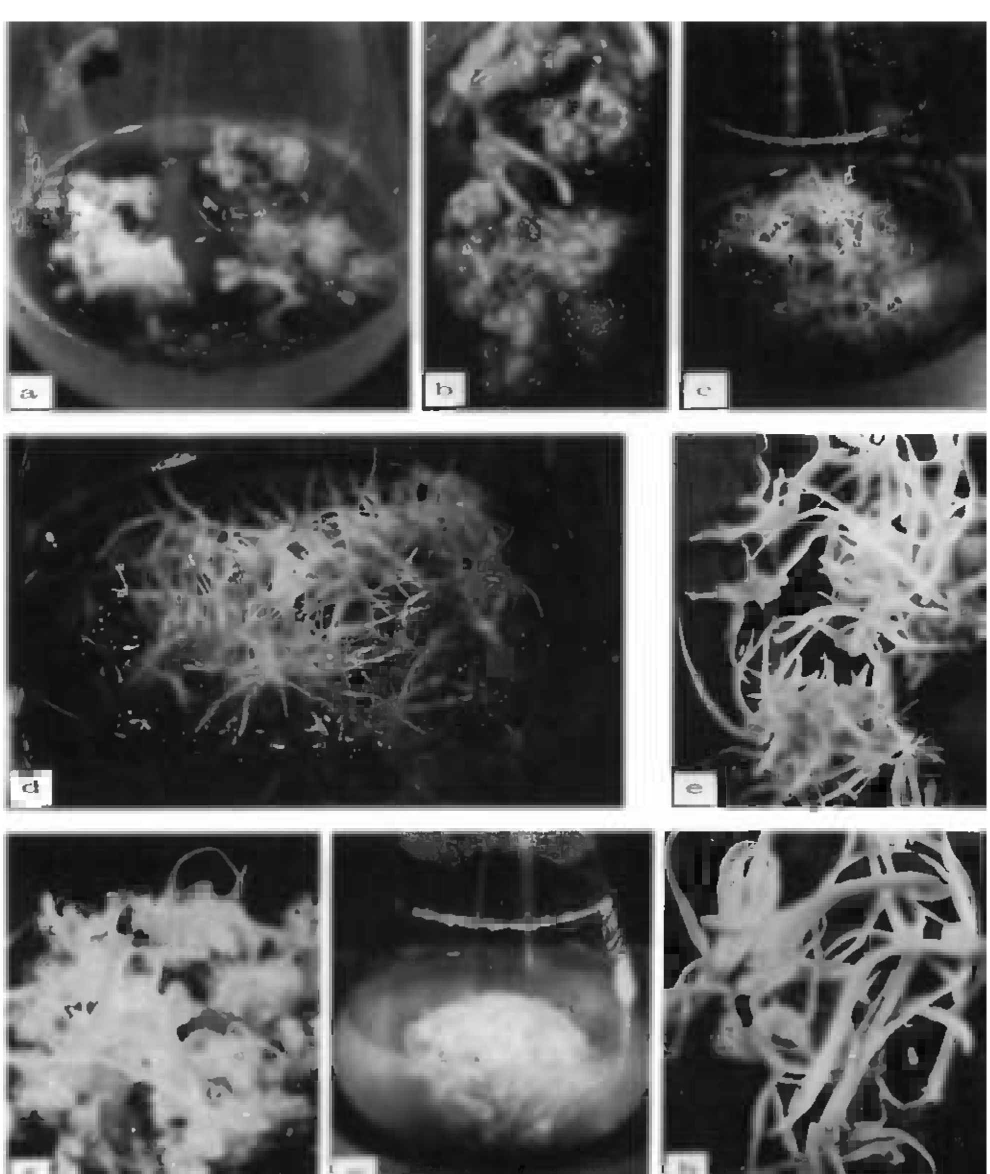

Fig. 6. a; *Panax ginseng* hairy roots induced from callus after infection of *Agrobacterium rhizogenes*. b; The enlarged photo of a. c–e; Hairy roots cultured in MS medium without hormone. f; Nontransformed Pg-1 IBA 1 tissues cultured on agar medium supplemented with 2 ppm IBA and 0.1 ppm kinetin. g–h; Nontransformed ordinary roots cultured in the same liquid medium as above.

Table VII

Growth and Saponin Contents of Callus, Ordinary Cultured Roots, Hairy Roots, and Native Root of *Panax Ginseng*

Tissue	Medium[a]	Growth[b] Ratio	Dry wt(g) per 100g fr.wt	Saponin content (mg) per 100g fresh wt			Rb group / Rg group	Total saponin per dry mass (wt %)
				Rb group	Rg group[c]	Total		
Pg-4 callus	B2K0.1	2.85	5.97	28.25	10.59	38.84	2.67	0.65
Pg-4								
hairy roots	—	3.07	10.09	24.78	11.08	35.86	2.24	0.36
hairy roots	K0.1	2.25	10.29	21.74	14.64	36.38	1.48	0.35
hairy roots	B2	5.11	9.62	39.57	26.64	66.21	1.49	0.69
hairy roots	B0.5K0.1	4.30	10.45	56.31	39.97	96.28	1.41	0.92
hairy roots	B2K0.1	6.20	10.58	55.70	44.54	100.24	1.25	0.95
Pg-1								
ordinary roots	B2K0.1	3.96	5.57	25.65	25.01	50.66	1.03	0.91
ordinary roots	—	1.26	5.31	15.32	4.87	20.19	3.15	0.38
Native root			23.91	59.03	37.28	96.31	1.58	0.40

[a]Each tissue (duplicate flasks) was cultured in Murashige and Skoog's basal medium containing the following hormones: —, no supplement; B, IBA (ppm); K, kinetin (ppm).

[b]Growth ratio was determined by increase of fresh weight after 3-week culture. The values are the quotient of the fresh weight after 3-week culture and the fresh weight of the inoculum.

[c]The amount of the Rb group was calculated as the total of ginsenosides Ra, Rb_1, Rb_2, Rc and Rd, with protopanaxadiol as the sapogenin; the Rg goup was calculated as the total ginsenosides Re, Rf, Rg_1, Rg_2 and Rh, with protopanaxatriol. Each value represents the average of duplicate estimations in 2 flasks.

with that of the ordinary cultured roots, on dry weight basis, as shown in the Table VII.

Pg-4 callus was derived on MS medium containing 2,4-D (1 ppm) and K (0.1 ppm) with a 2-year-old ginseng root cultivated in Shimane, Japan, in October 1980. The callus was maintained on the same medium and subcultured at 25°C in the dark at 3-weeks intervals. After 3 years of subculture, the callus was transferred onto MS medium containing IBA (2 ppm) and K (0.1 ppm), named B2K0.1 medium.

XII. CONCLUSION

On the basis of the studies described here, the large-scale production of ginseng root was investigated in 30-liter, 2000-liter, and 20-ton fermenters by Ushiyama *et al.* (1986). It was shown that the productivity of the cultures in a 20-ton tank was more than 500 mg/liter/day as dry material. The saponin (ginsenosides) content and composition of the products were almost the same as that of cultivated ginseng root.

The development of industrial ginseng cell cultures is now complete. These results demonstrates one more successful method for the production of useful secondary plant products.

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PART III

Alkaloids

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CHAPTER 13

Phenylalkylamines (*Ephedra* Alkaloids)

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I. INTRODUCTION

Several species of *Ephedra* (Gnetaceae) contain the alkaloidal amine "ephedrine." The ephedrines are L-ephedrine ($C_{10}H_{15}ON$); *d*-pseudoephedrine ($C_{10}H_{15}ON$); *para-N*-methylephedrine ($C_{11}H_{17}ON$); *p*-norephedrine ($C_9H_{13}ON$); *d*-norpseudoephedrine ($C_9H_{13}ON$), and *d-N*-methylpseudoephedrine ($C_{11}H_{17}ON$) (Cromwell, 1955).

The alkaloids ephedrine (Fig. 1) and pseudoephedrine are largely used as antispasmodic and circulatory stimulants. Ephedrine is extensively used as a substitute for epinephrine against bronchial asthma of allergic and reflex types. It is also used orally and locally in patients

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Fig. 1. Structure of ephedrine.

suffering from hay fever, urticaria, and other allergic reactions (Chopra *et al.*, 1956).

II. DISTRIBUTION

The genus is scattered all over the world and is found in the Mediterranean, the Himalayas (Afghanistan, Leh, western Tibet, Sikkim), the Andes, and the Rocky Mountains, from Chile to California. A number of species grow abundantly in the drier regions of the Himalayas. Three species of *Ephedra* occur in northern and northwestern China. Distribution of various species of *Ephedra* in the world and their alkaloid content are presented in Table I. A few other species known to contain eph-

Table I

Alkaloid Content of Various *Ephedra* Species and Other Species Containing Ephedrine

Species	Origin	Percentage alkaloid on dry-weight basis	Reference
Ephedra			
E. gerardiana	Leh, India	1.70	Ramawat and Arya, 1979a
		2.15–2.79	Chopra *et al.*, 1956
E. nebrodensis	Kagan, Pakistan	1.3	Shah and Shah, 1966
E. foliata	Thar Desert, India	0–trace	Chopra *et al.*, 1956
		Trace–0.01	Ramawat and Arya, 1979a
E. sinica	Shansi, China; Mongolia	1–2.5	Hu, 1969
E. equisetina	Shansi, China; Mongolia		
E. intermedae	Shansi, China; Mongolia		
E. distachyta	Europe	Positive test	Cromwell, 1955
Catha edulis		D-norephedrine	Cromwell, 1955
Taxus baccata		Ephedrine	Cromwell, 1955

edrine are also presented. It is clear that Indian *Ephedra, E. gerardiana* Wall. (synonyms, *E. nebrodensis* Tineo, *E. major* Host., *E. vulgaris* Rich.), *E. intermedia* Shrenik & Meyer (synonym, *E. pachyclada* Boiss.) (Chopra *et al.*, 1956; Satyvati *et al.*, 1976), and Chinese *Ephedra, E. sinica* Stapf. and *E. equisetina* Bunge (Nadkarni, 1954; Hu, 1969), are rich sources of alkaloid.

III. TISSUE CULTURE

A. Review

Ephedra foliata, a widely scattered and available species in India, has been cultured *in vitro* for various types of study, that is, to demonstrate regenerative potentialities of the female gametophyte (Sankhla *et al.*, 1967), culture of the male (Konar, 1963) and female gametophytes (Konar and Singh, 1979; Singh *et al.*, 1981), and to determine the amino acid content (Uddin, 1977). We have studied carbohydrate nutrition and metabolism (Ramawat and Arya, 1977), nitrogen nutrition and its effect on protein content (Ramawat and Arya, 1980), morphogenesis in callus (Ramawat and Arya, 1976), and ephedrine production (Ramawat and Arya, 1979a,b,c) in *E. foliata* and *E. gerardiana*. Straus and Gerding (1963) used *Ephedra* tissues to study the indoleacetic acid oxidase enzyme activity.

B. Callus Culture

Seeds of *E. gerardiana* were procured from the Divisional Forest Officer, Leh (Jammu and Kashmir, India), and seeds of *E. foliata* Boiss. were collected from the Botanical Garden, University of Jodhpur. The seeds were surface sterilized with 90% ethanol for 1 min followed by 5% sodium hypochlorite solution for 10 min. Seeds were finally rinsed with sterilized distilled water three times before transferring them onto the surface of static Murashige and Skoog's (MS; Murashige and Skoog, 1962) medium in Erlenmeyer flasks. The pH of the medium was adjusted to 5.8 to 6.0 before autoclaving at 15 psi for 20 min.

Seeds of both species germinated within 3 to 5 days. Callus was initiated within 7 days but developed slowly. In the case of *E. foliata*, cotyledons and hypocotyls were excised and transplanted onto fresh MS

medium. In the case of *E. gerardiana* seedlings (except root), callus became fragile and turned into loose, pale yellow callus tissue in 3 to 4 weeks. Excised segments of *E. foliata* were transferred three or four times at 15-day intervals to obtain homogenous callus. Cultures were maintained on MS medium supplemented with kinetin (0.1–0.5 mg/liter), α-naphthaleneacetic acid (NAA, 10 mg/liter), sucrose 30 g/liter, and agar (8 g/liter).

C. Culture Conditions

Cultures were grown on the surface of 40 ml of static MS medium in 100-ml Erlenmeyer flasks kept at 26 ± 2°C under fluorescent and incandescent (3:1 ratio) light (1000 lux, 16 hr/day).

D. Ephedrine Production

All *Ephedra* species do not contain the same amount of alkaloid. In India, one species, *E. foliata*, grows abundantly in the Thar desert in western India and contains traces of alkaloid, whereas another species, *E. gerardiana*, is rich in alkaloid but is not easily accessable due to its high-altitude habitat and snow.

The alkaloid content was determined by the method of Yamasaki *et al.* (1973). In some cases purified ephedrine·HCl was obtained. Usually, pseudoephedrine was detected in traces only. The alkaloid content in callus of both species is shown in Table II. Tissues of *E. foliata* were found to be devoid of ephedrine, whereas in *E. gerardiana*, alkaloid was detected after 6 weeks of callus growth. Light stimulated the production of alkaloid in *E. gerardiana* callus (Ramawat and Arya, 1979a).

1. Effect of Growth Regulators

The maximum amount of ephedrine (0.3%) was obtained in tissues grown on medium containing kinetin and indolebutyric acid (IBA, 10 mg/liter). Increased kinetin (1 mg/liter) or added morphactin (1 mg/liter) had a moderate effect on ephedrine yield. 2,4-Dichlorophenoxyacetic acid (2,4-D) was found to be inhibitory to ephedrine production (Ramawat and Arya, 1979b).

Table II

Total Alkaloid Content of Callus Tissues from Two Species of *Ephedra*

Medium	Factor	Percentage of total alkaloid at 8 weeks of growth	
		E. gerardiana	*E. foliata*
MS	Darkness	0.150	−NT[a]
	Light	0.175	−NT
Revised tobacco[b]	Darkness	0.090	NT
	Light	0.110	NT

[a] NT ≡ not traceable.
[b] Khanna and Stabs (1968).

2. Effect of Amino Acids

The maximum yield of ephedrine was recorded in callus tissues grown on MS medium supplemented with 0.1 g/liter L-phenylalanine. Moderately high ephedrine content was recorded with phenylalanine (0.4 g/liter), DL-methionine (0.1 and 0.4 g/liter), and glycine (0.1 g/liter). Tissues grew well with such treatments, and growth of tissues did not correlate with ephedrine production (Ramawat and Arya, 1979c).

3. Synergistic Effect of Indolebutyric Acid and Amino Acids

On the basis of earlier results, IBA (10 mg/liter) was used in place of NAA (10 mg/liter), and precursor amino acids were incorporated in the medium. A synergistic effect of IBA and L-phenylalanine and DL-methionine was observed on ephedrine yield (Ramawat and Arya, 1979c).

E. Differentiation of High-Yielding Strains

Attempts were made to differentiate the high-yielding strains. Shoot and root formation was observed in both species of *Ephedra*. In *E. gerardiana*, shoots were developed in tissues grown on MS medium supplemented with kinetin or 6-benzylaminopurine (1.0 mg/liter) and without auxin (Ramawat and Arya, 1976). In *E. foliata*, shoots of various shapes and sizes were observed in tissues grown on media containing kinetin

(0.1–0.5 mg/liter) and IBA or NAA (0.01–1.0 mg/liter) (H. C. Arya and K. G. Ramawat, unpublished results). Root formation was found not to be a regular feature. Morphactin and gibberellic acid failed to induce any organogenesis.

Furthermore, shoot and root formation from female gametophytes, excised from young ovules, was reported in *E. foliata* grown on MS medium supplemented with coconut milk (10%), kinetin, and 2,4-D. For further development of shoot buds, neither auxin nor cytokinin was needed (Konar and Singh, 1979; Singh *et al.*, 1981).

IV. CONCLUSION

Ephedrine and pseudoephedrine are among the most commonly used naturally occurring drugs. But commercially, they are obtained by synthesis only. Although limited success was achieved with cultured tissues, much more work is needed to develop high-yielding clones and to hybridize the desert species.

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CHAPTER 14

Pyrrolidines, Piperidines, and Pyridines

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I. INTRODUCTION

Alkaloids described here are derivatives of pyrrolidine, piperidine, and pyridine (Fig. 1) and have mostly simple structures. The alkaloids belonging to these groups are known to have divergent physiological activities. However, most plant tissue culture studies on these alkaloids are concerned with tobacco alkaloids and nicotinic acid derivatives. Consequently, the present chapter focuses on those restricted secondary metabolites in cultured plant cells.

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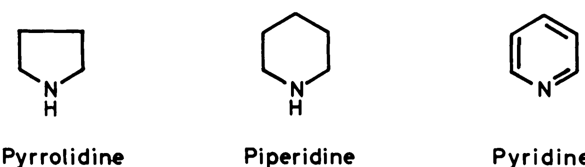

Fig. 1. Structures of pyrrolidine, piperidine, and pyridine.

II. PYRROLIDINE AND PIPERIDINE ALKALOIDS

A few studies on pyrrolidines and piperidines by plant tissue culture can be found in the literature. Nétien and Combet (1970) failed to detect such piperidine alkaloids as γ-coniceine, coniine, and *N*-methylconiine in *Conium maculatum* callus cultures. Lobeline and other piperidine alkaloids were produced by *Lobelia inflata* tissue cultures (Wysokinska, 1977). The alkaloid contents were lower in the cultures than in the intact plant. Tobacco alkaloids are described in Section IV.

III. PYRIDINE ALKALOIDS

Apart from tobacco alkaloids, nicotinic acid and its metabolites are major compounds covered by the studies reporting pyridine alkaloids in cultured plant cells.

The production of trigonelline (*N*-methylnicotinic acid) in plant tissue culture was described for the first time by Khanna and Jain (1972). The 8-week-old callus tissues of *Trigonella foenum-graecum* grown on Murashige–Skoog (1962) medium (MS) with 1 mg liter^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) showed 4.5% (percentages based on dry weight of cells throughout this chapter) trigonelline, whereas cultures supplied with 0.5 g liter^{-1} nicotinic acid showed 5.25%. Radwan and Kokate (1980) also studied trigonelline production by cell culture of the same species. The content was 2.12% in this case. This compound is the first natural hormone that has been chemically characterized. It regulates cell proliferation by cell arrest in either G_1 or G_2 of a mitotic cell cycle (Lynn *et al.*, 1978).

Metabolism of nicotinic acid and related compounds in cell suspension cultures was extensively studied by Barz and colleagues (Leienbach *et al.*, 1975, 1976; Heeger *et al.*, 1976; Leienbach and Barz, 1976; Neuhann *et al.*, 1979; Willeke *et al.*, 1979) and Antony *et al.* (1975). Nicotinic acid and nicotinamide adenine dinucleotide were metabolized to other compounds of the pyridine nucleotide cycle, and either trigonelline or nicotinic acid *N*-α-L-arabinoside. In turn, the latter two compounds were metabolized to nicotinic acid and other compounds of the cycle (Fig. 2). These two types of nicotinic acid conjugates are believed to be a storage form of nicotinic acid. Their formation was strictly alternative in 50 cell suspension cultures of wide taxonomic origin (Willeke *et al.*, 1979).

Suzuki *et al.* (1986) found glucosylation products of pyridoxine in

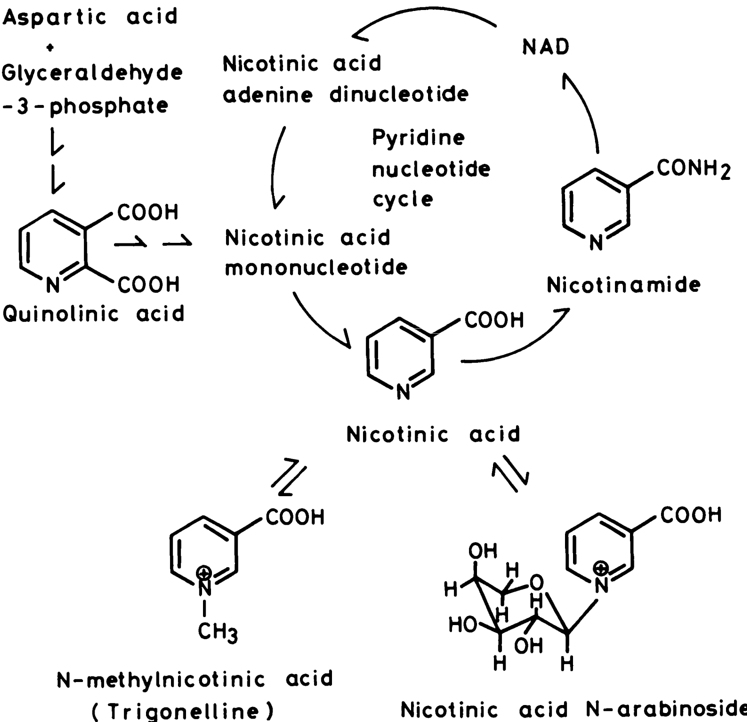

Fig. 2. Biosynthesis of nicotinic acid and its derivatives.

soybean and rice tissue cultures grown on a sucrose medium supplemented with 10 m*M* pyridoxine.

IV. TOBACCO ALKALOIDS

Since Speake *et al.* (1964) isolated nicotine from *Nicotiana tabacum* cell cultures, tobacco tissue cultures have provided useful experimental materials for investigating the formation of tobacco alkaloids and its regulation by various ways. Other minor alkaloids were also detected in cultured cells of *Nicotiana* spp. (Fig. 3): anatabine (Furuya *et al.*, 1966; Lockwood and Essa, 1984), anabasine, anatalline, myosmine, and nicotelline (Lockwood and Essa, 1984). Nicotine has been a main target of study among them. In one case, however, an abnormally high level of nornicotine was accumulated in *N. tabacum* cv. Wisconsin-38 callus cultures (Tiburcio *et al.*, 1985a).

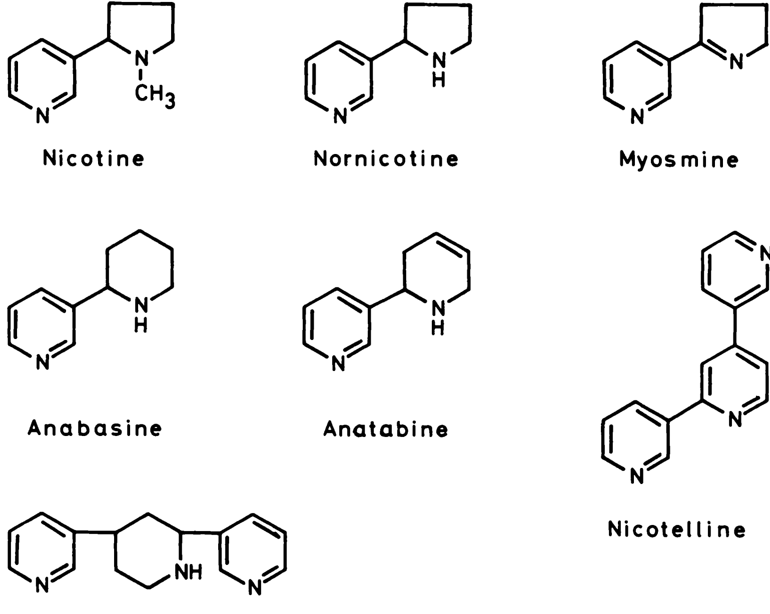

Fig. 3. Structures of the alkaloids detected in tobacco tissue cultures.

A. Biological Control

1. Genotypes and Explants

Using high- and low-alkaloid lines of burley tobacco (*Nicotiana tabacum*), which are isogenic except for the two loci for alkaloid accumulation, Kinnersley and Dougall (1980) and Miller *et al.* (1983) found that the genotype influences strongly the nicotine content of callus cultures. Sabour *et al.* (1986) observed a very high variation of *in vitro* growth rate and nicotine content within each cultured cell line from parental, sexual, and somatic hybrids of several *Nicotiana* species.

Cell suspensions derived from embryos of *N. rustica* were better able to synthesize alkaloid than cells derived from the stem pith (Krikorian and Steward, 1969). Explants from apical pith tissue of *N. tabacum* cv. Burley 21 gave calli having far more nicotine than cultures derived from basal pith explants. Furthermore, the stem pith calli showed greater nicotine productivity than the leaf callus (Kinnersley and Dougall, 1982). On the contrary, Speake *et al.* (1964), Tabata and Hiraoka (1976), and Röper *et al.* (1985) found no differences in the nicotine content of callus cultures derived from different organs of tobacco.

2. Selection of Cell Lines

With a few exceptions (e.g., Ohta and Yatazawa, 1980), alkaloid concentrations in tobacco tissue cultures generally decrease with prolonged periods of subculture (Dawson, 1960; Tabata and Hiraoka, 1976), as is observed in most plant tissue cultures. Therefore, selection is usually inevitable to establish a cell line with high alkaloid productivity. For the success of such selection, a wide variation in metabolite concentrations of individual cells, cell aggregates, or calli must be present. The variation in nicotine content was observed in *Nicotiana tabacum* cv. Delcrest × cv. McNair 133 callus (Röper *et al.*, 1985). It was also observed in *N. rustica* var. *brasilia* cell clones from single cells and/or two- to eight-celled aggregates, ranging from 0.0035 to 0.0866% (Tabata and Hiraoka, 1976). One clone had a stable nicotine-producing capability even at the fifty-fourth passage after cloning (0.291%). Several cell lines of *N. tabacum* cv. Bright Yellow with much higher nicotine contents were obtained by the "cell squash method" applied to single-cell clones (Ogino *et al.*, 1978). The nicotine content of these cell lines ranged from 1.0 to 3.4%. Using the same cultivar, Ohta *et al.* (1978a) induced more than 100 callus tissues

from tops or roots of sterile seedlings, and selected one callus line having high nicotine-producing capacity (2.14%).

Robins *et al.* (1987) used nicotinic acid as a selective agent for the isolation of high nicotine-producing lines of *N. rustica* 'hairy root' cultures. The treatment increased the levels of nicotine and anatabine by a factor of 2–3 and up to 10, respectively, over the corresponding controls.

3. Organogenesis

It is a fact that the culture conditions inducing organogenesis stimulate alkaloid accumulation in tobacco tissue cultures (Neumann and Müller, 1971; Waller and Nowacki, 1978; Tabata *et al.*, 1971; Tabata and Hiraoka, 1976; Piñol *et al.*, 1985). The alkaloid production, however, is not necessarily directly coupled to bud or root formation in spite of the apparent parallelism between them (Tabata *et al.*, 1971). Because nicotine is accumulated in nonorganized callus in many cases, organogenesis is not prerequisite for the alkaloid production in cultured tobacco cells (Takahashi and Yamada, 1973; Tabata and Hiraoka, 1976; Piñol *et al.*, 1984). Piñol *et al.* (1984, 1985) claimed that cellular differentiation causes the loss of meristematic areas along with the inhibition of alkaloid synthesis.

The rhizogenic activity of nicotine (50 mg liter^{-1}) was reported for tissue cultures of *Nicotiana tabacum* var. *humilis* (Peters *et al.*, 1974) and *Phaseolus vulgaris* (Peters *et al.*, 1976). But Sefcovic and Hricova (1972), Tabata and Hiraoka (1976), and Saunders *et al.* (1981) were unable to observe such an effect of nicotine with *N. tabacum* or *N. rustica* var. *brasilia* callus cultures.

Hamill *et al.* (1986), Rhodes *et al.* (1986), and Robins *et al.* (1987) studied tobacco alkaloid formation by "hairy root" cultures of *Nicotiana rustica* cv. V12 transformed with *Agrobacterium rhizogenes.* The amounts of major alkaloids, nicotine, anatabine, nornicotine, and anabasine in those cultures were comparable with those of true roots. They considered this type of *in vitro* culture a potential system for the production of useful plant secondary metabolites.

Tobacco plants regenerated from calli were shown to have the ability to synthesize alkaloids (Tabata *et al.*, 1968; Verzar-Petri and Kovacs, 1968; Sefcovic *et al.*, 1973). The high nicotine-producing ability of selected cell lines of *Nicotiana tabacum* cv. Bright Yellow was retained through the redifferentiation and dedifferentiation process (Tabata *et al.*, 1978).

B. Chemical Control

1. Plant Growth Regulators

Many reports illustrate that plant growth regulators, especially auxins, have striking effects on nicotine accumulation in tobacco tissue cultures. A synthetic auxin, 2,4-D, was inhibitory to nicotine production in *Nicotiana tabacum* callus cultures at concentrations higher than 10^{-6} or 10^{-5} *M*, depending on callus lines (Furuya *et al.*, 1966, 1967, 1971; Tabata *et al.*, 1971; Shiio and Ohta, 1973a; Takahashi and Yamada, 1973; Lockwood and Essa, 1984). On the other hand, indolyl-3-acetic acid (IAA) did not inhibit it at a concentration range between 10^{-6} and 10^{-5} *M* (Furuya *et al.*, 1966, 1967, 1971; Takahashi and Yamada, 1973; Ogino *et al.*, 1978). Takahashi and Yamada (1973) found that 2,4-D at a lower concentration (10^{-8} *M*) stimulated nicotine production, and IAA at higher concentrations (10^{-4}, 10^{-3} *M*) inhibited it in *N. tabacum* cv. BrightYellow callus cultures. These findings suggest that 2,4-D does not differ from IAA in its effect on nicotine production. Another auxin, naphthaleneacetic acid (NAA), was shown to have a narrow concentration range (0.15–0.2 ppm) optimal to nicotine production in *N. tabacum* cv. Bright Yellow callus (Ohta *et al.*, 1978a). Nicotine productivity under auxin conditions that are stimulatory or inhibitory to nicotine production is reversible, provided that a callus line has the potential to synthesize the compound (Furuya *et al.*, 1971; Shiio and Ohta, 1973a).

There are a few reports on the effects of cytokinins on the alkaloid production of tobacco tissue culture. Kinetin stimulated nicotine production in shoot-forming callus of *Nicotiana tabacum* cv. Bright Yellow at concentrations up to 2 ppm (Tabata *et al.*, 1971), whereas it inhibited the nicotine production of nonorganogenic callus cultures of the same cultivar (Shiio and Ohta, 1973a).

2. Nutrient Factors

The basal medium most frequently used for studies on alkaloids in tobacco tissue cultures is MS (or its modifications), which was developed for better growth of tobacco cells cultured *in vitro* (Murashige and Skoog, 1962). However, it is another question whether the medium best for growth is also best for alkaloid production. Heller's medium stimulated alkaloid synthesis but was not optimal for growth (Neumann and Müller, 1971). Furuya *et al.* (1971) isolated and identified nicotine in

tobacco callus cultures on White's medium. Röper *et al.* (1985) recorded the highest nicotine content (5.3%, 920 mg liter^{-1}) among the published data on tobacco tissue culture by using mixotrophic green cell suspensions of *Nicotiana tabacum* cv. Delcrest × cv. McNair 133 cultured in SH–M medium (Mitchell and Gildow, 1975) containing 0.2 mg liter^{-1} NAA, 0.2 mg liter^{-1} benzylaminopurine, and 2% glucose.

Slightly higher concentrations (3–5%) of sucrose than the usually employed ones (2–3%) have been found to be optimal for nicotine production in *Nicotiana tabacum* callus (Ohta *et al.*, 1978a) or cell suspension cultures (Mantell *et al.*, 1983; Röper *et al.*, 1985). Nicotine was not detected in *N. tabacum* cv. Delcrest × cv. McNair 133 when glucose was substituted for sucrose in Linsmaier–Skoog (1965) medium (LS), whereas the former was very effective for nicotine production in tobacco green cells cultured in LS liquid medium, as mentioned above. Organic acids (pyruvic, citric, malic, and fumaric acids) increased the total alkaloid content of tobacco callus to 3.75% (Tiburcio *et al.*, 1985a).

The original nitrogen concentration (840 mg liter^{-1}) in MS medium was optimal to both the growth and nicotine production in *Nicotiana tabacum* cv. Bright Yellow (Ohta *et al.*, 1978a). In this case, the NH_4/NO_3 nitrogen ratio was fixed to 1:1.91. Röper *et al.* (1985) cultured callus tissues derived from two cultivars of *N. tabacum* on LS agar medium supplemented with 300 mg liter^{-1} glutamine. The use of nitrate as a sole inorganic nitrogen source increased or decreased the amount of nicotine, depending on callus line and incubation period. Substitution of 4 g liter^{-1} ammonium sulfate for ammonium nitrate reduced nicotine production of the callus.

The callus of *Nicotiana tabacum* cv. Anand-2 was successfully grown on modified MS medium containing 10 m*M* urea as sole nitrogen source (Ravishankar and Mehta, 1981). Nicotine content of the callus tissue was 3.5 times higher (0.783%) than that of the control.

On the basis of the observation that nicotine accumulation is first detected in *Nicotiana tabacum* cv. NC2512 cell suspension cultures when medium phosphate is completely depleted, Mantell *et al.* (1983) succeeded in accelerating nicotine accumulation by reducing medium phosphate to one-tenth the level normally employed.

Pyridoxine, nicotinic acid, and glycine, which are included in MS but not in LS medium, seem not to be essential for nicotine production (e.g., Röper *et al.*, 1985). The other components in various recipes for culture media remain to be studied for their effects on the alkaloid production of tobacco tissue cultures.

3. Precursor Effect

Generally, the addition of the tobacco alkaloid precursors to the culture medium lowered the nicotine concentration in tobacco tissue cultures, compared with the control culture (Ohta *et al.*, 1978b; Neumann and Müller, 1971; Miller *et al.*, 1983; Lockwood and Essa, 1984).

4. Others

Poorly growing cultures incubated in a medium containing growth inhibitors generally showed increased alkaloid synthesis (Neumann and Müller, 1971).

C. Physical Control

Illumination was inhibitory to nicotine production of *Nicotiana tabacum* cv. Bright Yellow callus cultures. Its effect was probably caused by the inhibition of biosynthesis rather than by the stimulation of nicotine catabolism (Ohta and Yatazawa, 1978). In contrast, illumination enhanced nicotine production in bud-forming callus derived from the same tobacco cultivar and kept on medium containing no auxin but various concentrations of kinetin (Tabata *et al.*, 1971). Röper *et al.* (1985) reported that illumination promoted nicotine production in green cell suspensions of *N. tabacum* without organ differentiation. The cause of this discrepancy is not yet known.

The effect of temperature on nicotine production in tobacco tissue cultures was studied with *Nicotiana tabacum* cv. Bright Yellow by Ohta and Yatazawa (1978). The optimum temperature for both callus growth and nicotine production was 25°C. Nicotine content in callus tissues grown at 19 and 30°C decreased to 14.2 and 38.3%, respectively, of that grown at 25°C. The refrigerated storage of *N. tabacum* cv. Bright Yellow callus cultures around 4°C for 2 months affected a relative amount of alkaloids, depending on cell line, when recultured under normal conditions (Hiraoka and Kodama, 1984).

D. Nicotine Production in Liquid Medium

Although there have been numerous reports on tobacco cell suspension cultures, including continuous cultivation in 20-kiloliter fermenters

(Hashimoto *et al.*, 1982; Kato, 1982; Azechi, 1984), studies on nicotine production in cell suspensions are rather scarce. Neumann and Müller (1971) found that suspension cultures grown in MS medium formed alkaloids, whereas callus cultures did not. On the contrary, nicotine content in suspension culture was lower than that in corresponding callus cultures of *Nicotiana tabacum* cv. Bright Yellow (Tabata *et al.*, 1978). Notably, there was a positive correlation between the nicotine content of the two culture systems. A batch culture of tobacco (*N. tabacum* cv. NC2512) accumulated up to 2% nicotine (Mantell *et al.*, 1983). Nicotine production was repressed in 20-liter fermenters, compared to shaking flasks (Röper *et al.*, 1985).

Although nicotine production by cell culture systems does not seem to be profitable practically (Misawa, 1985), some patents have been filed describing it (e.g., Shiio and Ohta, 1973b; Smith and Pearson, 1978).

E. Metabolism and Regulation

1. Biosynthesis of Tobacco Alkaloids and Enzymes Involved

The routes of nicotine biosynthesis have been elucidated by labeling and enzymatic studies, mainly with intact tobacco plants (Leete, 1983). The outline of pathways leading to nicotine is shown in Fig. 4. The key intermediate in biosynthesis of the pyrrolidine ring of nicotine is putrescine, which is derived from either ornithine or arginine (Slocum *et al.*, 1984). Putrescine is metabolized further into aromatic amides or pyrrolidine alkaloids, depending on the external and internal conditions in which plants, organs, or cells grow. Enzymology of nicotine metabolism in tobacco plant was reviewed by Smith (1981) and Waller and Dermer (1981).

The different importance of arginine and ornithine as precursors of putrescine in cultured tobacco cells can be seen in the literature. Mizusaki *et al.* (1973) found that ornithine decarboxylase (ODC) activity in callus was higher than that in roots of decapitated tobacco (*Nicotiana tabacum* cv. Bright Yellow). Heimer *et al.* (1979) reported that the value of ODC activity was 4- to 10-fold as high as that of arginine decarboxylase (ADC) activity in tobacco suspension cultures. Ravishankar and Mehta (1982) observed increased ODC activity and decreased activity of ornithine carbamoyltransferase along with enhanced nicotine accumulation in floral bud callus of *N. tabacum* cv. Anand-2 grown on MS medium

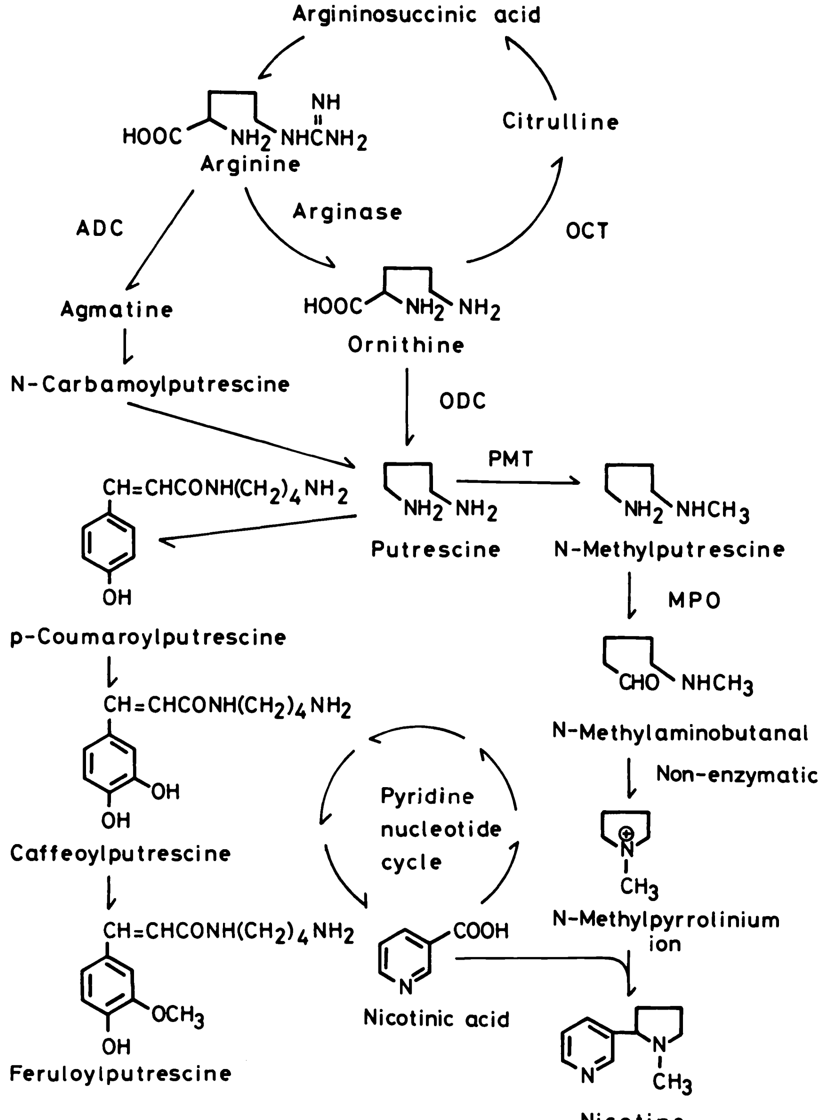

Fig. 4. Biosynthesis of nicotine and related metabolism in tobacco. ADC, arginine decarboxylase; MPO, *N*-methylputrescine oxidase; ODC, ornithine decarboxylase; OCT, ornithine carbamoyltransferase; PMT, putrescine *N*-methyltransferase.

with 10 m*M* urea as sole nitrogen source. Palazón *et al.* (1987) cultured *N. tabacum* cv. Burley 21 callus tissues on MS medium containing NAA at concentrations optimal (1 μM) or supraoptimal (11.5 μM) for nicotine biosynthesis. The former increased free putrescine and nicotine contents and ODC activity compared with the latter. They deduced ornithine and ODC as a key intermediate and an enzyme involved in nicotine pathway. On the contrary, Tiburcio *et al.* (1985b), Tiburcio and Galston (1986) and Feth *et al.* (1986) proposed an important role for ADC in the biosynthesis of pyrrolidine alkaloids from findings based on simultaneous analysis of polyamines and pyrrolidine alkaloids, inhibitor experiments on ADC and ODC, and tracer experiments with *N. tabacum* cv. Wisconsin-38 callus cultures.

Tobacco cell suspension cultures grown in LS medium supplemented with 0.2 mg liter^{-1} 2,4-D had high ODC activity but no putrescine *N*-methyltransferase (PMT) and *N*-methylputrescine oxidase activity (Mizusaki *et al.*, 1972, 1973). It suggests that the biosynthetic pathway leading to nicotine is blocked at, or both at and after, methylation of putrescine under these culture conditions. Putrescine is metabolized to form such aromatic amides as *p*-coumaroyl-, caffeoyl-, and feruloylputrescine in those cultures where the potential to synthesize nicotine is repressed (Mizusaki *et al.*, 1971). Their formation is known to be connected with the onset of reproductive organs (Cabanne *et al.*, 1981) and virus resistance in the intact plant (Martin-Tanguy *et al.*, 1976; Slocum *et al.*, 1984). Takahashi and Yamada (1973) also failed to detect any PMT activity in *Nicotiana tabacum* cv. Bright Yellow callus tissue cultured on LS medium containing 10^{-3} *M* IAA, where nicotine production is completely restrained in spite of vigorous callus growth. Ohta and Yatazawa (1980) found PMT activity in a nicotine-productive (3.38%) callus line of *N. tabacum* cv. Bright Yellow but none in a less productive (0.05%) one, both of which were cultivated on modified MS medium supplemented with 0.15 mg liter^{-1} NAA. Feth *et al.* (1986) and Wagner *et al.* (1986b) also confirmed that PMT is the enzyme under rigid control for nicotine biosynthesis in *N. tabacum* cv. Samsun calli cultured on nicotine-induction medium consisting of MS, 0.15 mg liter^{-1} NAA, and 0.02 mg liter^{-1} kinetin. The findings mentioned above suggest that auxin levels regulate putrescine metabolism: its lower levels favor the formation of tobacco alkaloids, and higher ones the formation of aromatic amides by suppressing PMT (and *N*-methylputrescine oxidase) activity.

The activities of several enzymes involved in or related to pyridine nucleotide cycle were determined in connection with the regulation of nicotine accumulation in tobacco roots and calli (Wagner *et al.*, 1986a, 1986b). They confirmed that nicotinic acid is replenished through two

routes: directly from nicotinic acid mononucleotide and via the synthesis and degradation of NAD.

2. Accumulation, Excretion, and Metabolism of Alkaloids

Exogenously supplied nicotine was demethylated to form nornicotine by cell suspension cultures derived from *Nicotiana glauca* and three strains of *N. tabacum* (Barz *et al.*, 1978). The NAA levels in the culture medium affected the balance of nicotine and nornicotine in *N. tabacum* cv. Burley 21 callus, suggesting the stimulation of *N*-demethylation of nicotine by a higher concentration of NAA (Piñol *et al.*, 1985). The activation of nicotine demethylation also occurred in *N. tabacum* cv. Wisconsin-38 callus (Tiburcio *et al.*, 1985a). The callus tissues derived from the same cultivar accumulated nicotine (as much as 6.2%) when they were grown on MS medium with 0.03 mg $liter^{-1}$ kinetin, 2 mg $liter^{-1}$ IAA, and 32 m*M* nicotine. Demethylation of accumulated nicotine was not observed.

Tobacco tissue cultures excrete varying amounts of alkaloids into the medium (Ohta *et al.*, 1978a; Ohta and Yatazawa, 1980; Tiburcio *et al.*, 1985a,b), up to 34% of total alkaloids accumulated (Ohta *et al.*, 1978b).

V. CONCLUSIONS AND PERSPECTIVES

Tobacco alkaloids have attracted much interest in tissue culture studies dealing with pyrrolidine, piperidine, and pyridine alkaloids. The interest is based not on the practical production of tobacco alkaloids but on the fundamental knowledge of alkaloid formation and its regulation. The use of *Nicotiana* spp. as experimental material, and the study of alkaloid formation as a subject, has brought better understandings of metabolism of these alkaloids and its regulation. Various factors affecting alkaloid production have been recognized. Studies of regulatory mechanisms of alkaloid production, including some enzymes, revealed key steps in biosynthetic pathways. The important roles or interesting physiological activities of intermediates of tobacco alkaloid biosynthesis, such as nicotinic acid and its derivatives, and polyamines and their conjugates, are well known or have been uncovered. Tobacco tissue culture provides one of the most useful experimental systems to study

the relationship between primary and secondary (alkaloid) metabolism and its regulation.

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CHAPTER 15

Tropanes

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I. INTRODUCTION

Alkaloids have assumed prominence among secondary metabolites. In *Datura* more than 30 alkaloids have been found (Verzár-Petri, 1971). A few decades ago *D. stramonium* was the main species supplying hyoscyamine. Since the 1970s, interest has increasingly shifted toward *D. innoxia*, a possible source of scopolamine.

According to Chan and Staba (1965), the alkaloid content of *Datura stramonium* cultures (5 months old) varied between 0.004 and 0.056%. Romeike and Koblitz (1970) reported that the alkaloid content of a callus of *D. stramonium* (5 years old) and of the stem callus of *D. metel* (19 months old) amounted to 0.0026 and 0.00185%, respectively. Krikorian

*Former name, Gizella Verzár-Petri.

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Table I

Tissue and Cell Cultures Accumulating Tropane Alkaloids

Reference	Method	Plant	Results
Griffin (1979)	Callus culture	*Duboisia* hybrid	Callus tissue transferred to media containing 1 g/liter scopolamine; uptake of alkaloid; no metabolism to other *Datura* alkaloids
Smorodin *et al*. (1979)	Suspension culture	*Datura innoxia*	Nitrogen and phosphorus source affected alkaloid content
Kibler and Neumann (1979)	Fermenter batch culture, MS medium 1500 lux	*Datura innoxia*	Scopolamine major alkaloid; no correlation between scopolamine and hyoscyamine in various strains (diploid, haploid)
Yankulov *et al*. (1979)	Another culture, White's medium	*Datura innoxia*	Total alkaloids in haploid higher than in diploid callus
Atanassov *et al*. (1980)	Halperin's medium	*Datura innoxia*	Bud formation; variability of chromosome number
Yamada and Hashimoto (1982)	Cell culture, LS medium	*Hyoscyamus niger*	Hyoscyamine and scopolamine produced
Koul *et al*. (1983)	Cell suspensions, producing roots and shoots	*Hyoscyamus muticus*	Alkaloid accummulation higher during stationary phase, independent of morphogenesis
Hiraoka and Tabata (1983)	LS medium	*Datura innoxia*	Scopine, scopoline, pseudotropine, tropine converted into corresponding acetates
Hashimoto and Yamada (1983)	2-year-old suspension culture	*Hyoscyamus niger*	Alkaloids in medium
Kitamura *et al*. (1985)	MS medium, light	*Duboisia myoporoides*	Alkaloid distribution in regenerated plants
Endo and Yamada (1985)	B5 medium	*Duboisia leichhardtii*, *D. myoporoides*, *D. hopwoodi*	Cell cultures from roots attaining 1.16% scopolanine (dry weight)
Oksman-Caldentey and Strauss (1986)	Liquid NT medium, dark incubation	*Hyoscyamus muticus*	Scopolamine content in protoplast-derived cell cultures

and Steward (1969) did not detect tropane alkaloids in tissue cultures of various *Datura* species.

The effect of light on young callus tissues of root and leaf origin and their alkaloid production was studied by Verzár-Petri *et al.* (1978). They concluded that callus tissues of root origin synthesize a greater amount of alkaloids in the dark, and those of leaf origin, in light. The requirement for dark for the production of alkaloids such as atropine and scopolamine, thought to be mainly produced in roots, has not been established (Bhandary, 1969; Hiraoka and Tabata, 1974; Hashimoto and Yamada, 1983). It seems, however, that biosynthesis of these alkaloids is correlated with the organization of roots.

It has been demonstrated that the Ri plasmid present in *Agrobacterium rhizogenes* causes transformed plant cells to proliferate rapidly and exhibit extensive lateral branching as massive roots, so-called hairy roots, on a hormone-free medium (White and Nester, 1980; Chilton *et al.*, 1982; Tepfer, 1984). In the transformed plant cells, genes with integrated T-DNA are transcribed and translated (Huffman *et al.*, 1984; White *et al.*, 1985), and the transformed plant cells proliferate on a hormone-free medium as hairy roots even after *Agrobacterium* is eliminated. Kamada *et al.* (1986) induced hairy roots by the inoculation of *Agrobacterium rhizogenes* on sterile plants of *Atropa belladonna*. Axenic cultures were obtained by culturing segments of hairy roots on hormone-free Murashige and Skoog (MS) agar medium (1962) with carbenicillin (1 mg/liter), subculturing on MS medium without antibiotic. The axenic culture of the hairy roots proliferated 60-fold, based on the initial fresh weight after 1 month of culture. The presence of atropine and scopolamine in hairy roots was examined by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), and contents were analyzed by gas liquid chromatography (GLC). The results showed two alkaloids, hyoscyamine and scopolamine, and contents were the same as or even higher than those of plants grown in the field. Some details of tropane alkaloid producing tissue and cell cultures are presented in Table I.

II. MATERIALS AND METHODS

A. Tissue Culture

For the tissue culture of tropane alkaloid plants a modified culture medium after Murashige–Skoog (Maróti, 1976) solidified with agar was

used. Kinetin and 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/liter each), agar (8 g/liter), nicotinic acid (0.5 mg/liter) and pyridoxine–HCl (0.1 mg/liter) were added; the pH was adjusted to 6. For callus induction sterilized pieces of leaf or root of *Datura innoxia* Mill. were used. The organs were washed with detergents, sterilized with 70% ethanol for 1 min, then with a solution of diacid [ethanol mercury chloride and methylpyridine chloride (Butenko, 1984)], and rinsed with sterile distilled water. Explants and callus tissues were grown in test tubes containing 40 ml of culture medium, and were transferred to fresh culture medium at 6-week intervals.

B. Extraction and Purification of Alkaloids

The calli were separated from the culture media, then both the media and the calli were lyophilized. The extraction was performed with MeOH : 28% NH_4OH (9 : 1) overnight, then with chloroform for 6 hr in a Soxhlet apparatus. The chloroform extract was evaporated under vacuum. The residue was treated with 2 × 20 ml of H_2SO_4, then adjusted to pH 9 with 20% NH_4OH. The alkaloids were separated with 30 ml of chloroform; this was repeated three times. The solution was then filtered through anhydrous sodium sulfate, and the solvent was evaporated. The remaining material was diluted in 10 ml of $CHCl_3$, and this solution was used for various analyses. Occasionally, fresh tissues were also processed, homogenized with quartz sand, and extracted as described above.

C. Quantitative and Qualitative Determination of Alkaloids

For quantitative determination of tropane alkaloids we used (1) the method that has been described in the Sixth Hungarian Pharmacopoeia, i.e., titration in water-free medium, and (2) the more sensitive method of using a tropeoline amphiindicator (Lörincz and Szász, 1961).

For qualitative determination the following methods were used:

1. Thin-layer chromatography (Verzár-Petri *et al.*, 1974)
2. Gas chromatography (Verzár-Petri and Haggag, 1976)
3. Autoradiography (Verzár-Petri, 1969)

D. Isotopic Experiments

Two-year-old root callus cultures were separated from the culture media and put into solutions containing radioactive compounds: sodium [2-^{14}C]acetate (specific activity, 28.62 μCi/mg) and [3-^{14}C]phenylalanine (specific activity, 13.64 μCi/mg). The callus was kept in a thermostat at 25°C in the dark or under a fluorescent lamp (2500 lux). At certain times (1–48 hr) part of the incubated callus was removed, washed, and extracted as described above.

III. RESULTS AND DISCUSSION

A. Alkaloid Content and Composition

Generally, the total alkaloid content of the intact plant was higher than that of the tissue cultures, whether these originated from leaf, stem, or root. The alkaloid content in tissues used in our experiments is presented in Table II.

In leaf callus cultures the quantity of scopolamine was smaller than that of hyoscyamine, whereas the plant accumulates scopolamine as the main alkaloid. Hyoscyamine 6-OH, a reaction product of epoxidation

Table II

Alkaloid Content of 4-Year-Old *Datura innoxia* Callus Cultures[a]

Material and methods	Alkaloid content of cultures (% dry weight)	Alkaloid content of intact organs (% dry weight)	Alkaloid production of tissue culture in relation to that of intact organ
Root culture cultivated in light	0.0390	0.33	10 times lower
Leaf culture cultivated in light	0.0226	0.25	10 times lower
Root culture cultivated in dark	0.015	0.33	20 times lower
Leaf culture cultivated in dark	0.0075	0.25	30 times lower

[a] Culture media showed a positive alkaloid reaction with Dragendorff regent, but the quantity could not be determined. (See also Figs. 1–13).

that occurs in leaves, was also present. As well, norscopolamine was detected. The same components were found in very small quantity in the culture media.

In callus derived from leaves of *Datura innoxia* cultivated in light, eight alkaloids were determined: cuscohygrine, meteloidine, teloidine, hyoscyamine, 3–6 ditigloiloxytropane, scopolamine, 6-OH-hyoscyamine, and norscopolamine. Also, a great quantity of tropine and tropic acid was found in free, nonesterified form, together with two unknown alkaloids that appeared on the gas chromatogram at 220 and 226°C (Table III).

In root callus cultures of *Datura innoxia* cultivated in the dark, tropine and tropic acid occurred in low, 6-OH-hyoscyamine and cuscohygrine, the intermediates of tropane alkaloid biosynthesis, in large amounts. The concentration of scopolamine was higher than that of hyoscyamine, a ratio, as for leaf callus, similar to that of the intact root (see Fig. 3). In our opinion, based on earlier isotopic examinations (Verzár-Petri *et al.*, 1974), the appearance of 6-OH-hyoscyamine is a sign of scopolamine biosynthesis because it is a compound always present in the interconversion of hyoscyamine to scopolamine, whereas cuscohygrine is a characteristic alkaloid of the root, which, according to our own observations,

Table III

Level of Alkaloids in *Datura innoxia* Tissue Cultures, on the Basis of Gas Chromatography

	Alkaloid component[a]									
Tissue culture type	A	B	C	D	E	F	G	H	J	K
Leaf tissue culture (cultivated in light)	5	2	3	5	3	2	3	1	2	2
Culture-medium of leaf tissue culture (cultivated in light)	1	2	2	2	1	2	2	3	1	2
Leaf tissue culture (cultivated in dark)	—	1	3	2	2	3	2	4	2	—
Root tissue culture (cultivated in light)	—	2	3	1	3	5	4	4	5	4
Root tissue culture (cultivated in dark)	3	4	2	3	1	3	—	2	4	2
Culture-medium of root tissue culture (cultivated in dark)	1	2	2	1	—	1	2	1	2	2

[a] A, tropine; B, cuscohygrine; C, meteloidine; D, tropic acid; E, teloidine; F, hyoscyamine; G, ditigloyloxytropane; H, scopolamine; J, 6-OH-hyoscyamine; K, norscopolamine.

is the first to appear on germination (Verzár-Petri and Kiet, 1977). The quantity of the alkaloids present in the culture medium was extremely small (see Tables I and II).

B. Alkaloid Precursors

Incorporation of Sodium [2-^{14}C]Acetate and [3-^{14}C]Phenylalanine

The aim of the investigation was to detect the influence of sodium [2-^{14}C]acetate and L-[3-^{14}C]phenylalanine on changes of the alkaloid content in root and leaf callus cultures of *Datura innoxia,* and to determine their incorporation over 24 and 48 hr (Table IV). In comparison

Table IV

Changes of Alkaloid Content of *Datura innoxia* Tissue Cultures during Incubation with Various Radioactive Precursors

	Type of alkaloid									
Experimental material	A[a]	B	C	D	E	F	G	H	J	K
Control	4	2	3	5	3	3	3	2	3	2
Leaf tissue culture cultivated in light; 24 hr of incubation, with sodium [2-^{14}C]acetate	2	2	4	3	2	4	2	4	2	5
Leaf tissue culture cultivated in light; 48 hr of incubation, with sodium [2-^{14}C]acetate	—	1	2	2	2	3	2	4	5	4
Root tissue culture cultivated in light; 1 hr of incubation, with [3-^{14}C]phenylalanine	2	3	1	3	1	2	4	—	2	2
Root tissue culture cultivated in light; 24 hr of incubation, with [3-^{14}C]phenylalanine	—	1	1	2	1	—	4	—	3	3

[a] For A–K, see Table III footnote.

with the control in experiments carried out with [3-^{14}C]phenylalanine, the total alkaloid content of the culture decreased during an incubation time of 24 hr and, over time, became undetectable. The quantity of tropine (tropanol) was rather high in the control tissue culture. On incubation for 1 hr it decreased, and after 24 hr it had disappeared. Tropic acid also decreased rapidly after a 24-hr incubation. Specific radioactivity was very small, and specific incorporation was 0.0002%. This result would suggest that no new tropic acid was formed and that the presence of phenylalanine is an obstacle in the biosynthesis of tropic acid. Synthesis of hyoscyamine and scopolamine did not take place either. Scopolamine and hyoscyamine disappeared following 24 hr of incubation. In such cases, according to Hiraoka (1976) and Romeike and Koblitz (1970), the tissue culture used up the tropine for the biosynthesis of acetyltropine. Meteloidine and, especially, the ditigloyloxytropane can be found and are well detectable in the cultures incubated for 1 and 24 hr. It seems that their biosynthesis is not hindered in the presence of phenylalanine, because the acid part of these alkaloids is not tropic acid, but tiglic acid.

Sodium acetate increases alkaloid formation in *Datura innoxia* tissue cultures. Total alkaloid content during the incubation was as follows (percentage of dry weight):

Control	0.0226%
24 hr of incubation, leaf culture	0.0483%
48 hr of incubation, leaf callus culture	0.025 %

The individual alkaloids varied during the incubation. On incubation for 24 hr the quantity of almost all the alkaloids, with the exception of tropine and tropic acid, increased. On incubation for 48 hr the quantity of all the alkaloids decreased, with the exception of 6-OH-hyoscyamine. Levels of hyoscyamine and scopolamine also decreased, but compared to the control they were still higher. Their specific radioactivity (dpm/mmol) was as follows:

	24 hr of incubation	48 hr of incubation
Ditigloyloxytropane	766.341.29	792.372.20
Scopolamine	501.314.64	346.295.44
Hyoscyamine	230.047.30	159.868.91

The specific radioactivity of ditigloyloxytropane was highest. This observation is in agreement with the pattern of alkaloid content of *Datura*

innoxia root when supplied with sodium [2-^{14}C]acetate (Verzár-Petri *et al.*, 1974, 1978), that is, the radioactive sodium acetate was incorporated first of all into the tigloylester alkaloids, which then remained either in this form or transformed into another product.

By summing up the results of the two experiments with adult plants and tissue cultures derived from them, one may suggest that from sodium acetate leucine, isoleucine was formed first, and in the following step, tiglic acid formed from isoleucine; the original tiglic acid formed an ester with the tropine and resulted in the tigloylester alkaloid. Furthermore, the effect of growth regulators (kinetin and 2,4-D) on the growth of callus from flowers (petal, ovary) of 3-year-old *Datura innoxia* was examined (Dung *et al.*, 1981; Szöke *et al.*, 1982). The growth dynamics of the tissues grown in light and dark was examined in a modified MS medium containing 1 mg/liter each of kinetin and 2,4-D. During an incubation period of 6 weeks the fresh and dry weight, the daily growth rate, and growth values of the callus tissues were measured. With callus of both petal and ovary origin the growth value based on fresh and dry weight was considerably higher in the dark than in the light. The growth of the callus cultures, except those from petals and grown in light, slowed down in the sixth week (Figs. 1–3). A high kinetin concentration impeded alkaloid production. Under the influence of 5 mg/liter 2,4-D the alkaloid contents increased. In the case of callus tissues of root origin

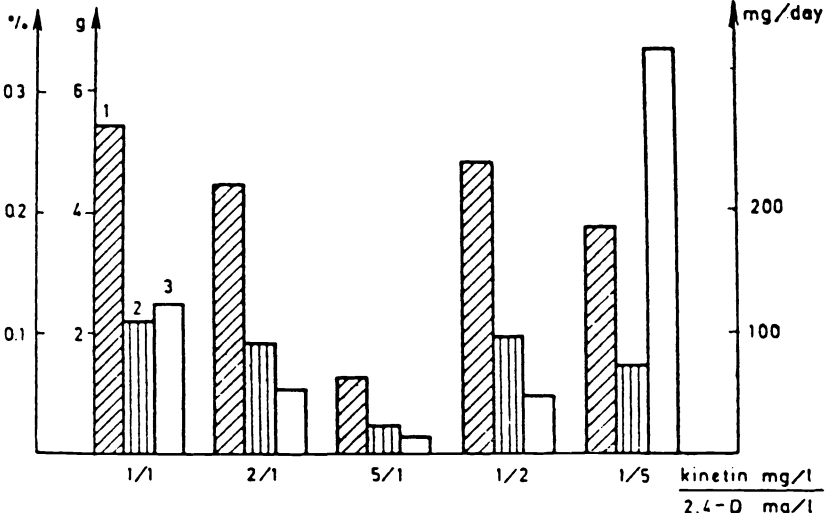

Fig. 1. Effect of growth hormones on callus of leaf origin of *Datura innoxia:* 1, fresh weight (g); 2, growth rate (mg/day); 3, alkaloid content (% dry weight).

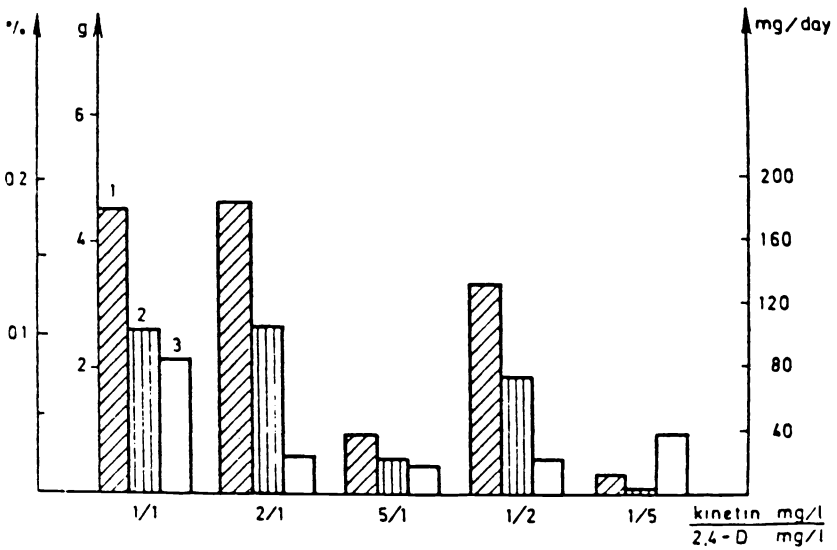

Fig. 2. Effect of growth hormones on the alkaloid content of callus of corolla origin of *Datura innoxia* (for symbols, see Fig. 1 legend).

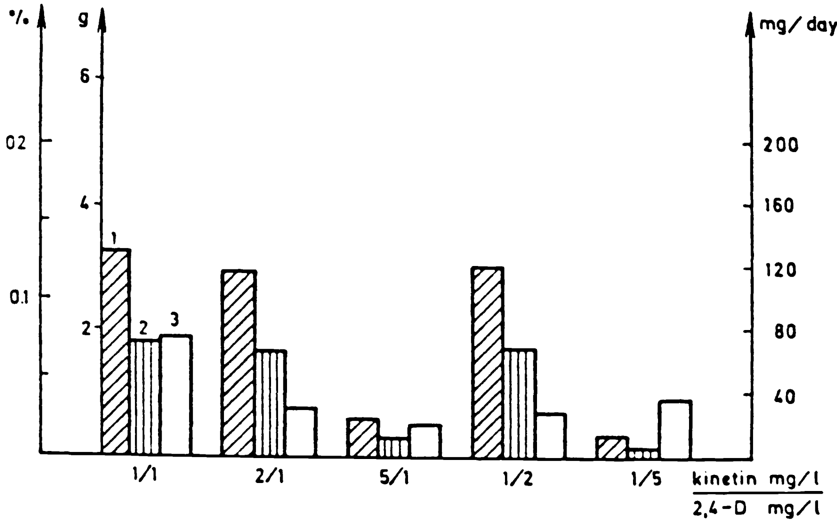

Fig. 3. Effect of growth hormones on the alkaloid content of callus tissues of gynoeceum origin of *Datura innoxia* (for symbols, see Fig. 1 legend).

it attained the alkaloid level formed in the intact root. Under the influence of illumination the alkaloid content of the callus tissues of root and leaf origin increased twofold over levels in cultures grown in the dark.

IV. SUMMARY

Root, stem, and corolla callus was cultured on Murashige–Skoog media, under 2500 lux and in the dark. It was found that the alkaloid content of callus grown in light was higher. The amount of alkaloid was essentially smaller in all callus cultures (4 years old) than that in the explants. The nonesterified and norcompounds were found in relatively high quantities.

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CHAPTER 16

Quinolizidines and Pyrrolizidines

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I. INTRODUCTION

Quinolizidine alkaloids are common natural products of many Fabaceae (synonym, Leguminosae) (Mears and Mabry, 1971). Frequently they are called lupin alkaloids because they are present in all species of the large genus *Lupinus*. Lupanine, a typical tetracyclic quinolizidine alkaloid, is shown in Fig. 1. At present about 70 related structures are known, including hydroxylated, dehydrogenated, and partially degraded compounds as well as simple esters.

Pyrrolizidine alkaloids are characteristic secondary compounds found particularly in several genera of the Asteraceae, Boraginaceae, and Fabaceae (Smith and Culvenor, 1981; Robins, 1982). They are generally

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present as ester alkaloids. The respective aminoalcohol (necine base), which represents the bicyclic pyrrolizidine nucleus, is esterified with the necine acid moiety. The ester alkaloids may occur as monoesters, diesters, or, as exemplified in Fig. 1, macrocyclic diesters. More than 200 pyrrolizidines have been isolated from natural sources.

On account of circumstantial and experimental evidence the main functions of the two groups of alkaloids is chemical defense. Quinolizidine alkaloids deter the feeding of herbivores such as mammals, mollusks, and insects (Waller and Nowacki, 1978; Wink, 1984b, 1985a). They inhibit the growth of microorganisms (Wink, 1984a) and even possess allelopathic properties (Wink, 1983). For pyrrolizidine alkaloids the protective function is even more obvious. Their role as powerful repellents, especially for insects, is well documented (Rothschild, 1973). Some insects even store pyrrolizidine alkaloids for their own chemical defense, or use them as pheromone precursors (Boppré, 1986). Many pyrrolizidine alkaloids are cytotoxic, and these are often responsible for poisoning of livestock and people (Mattocks, 1986).

II. QUINOLIZIDINE ALKALOIDS

A. Accumulation in Cell Suspension Cultures

Like many other plant cell culture systems, callus and cell suspension cultures isolated from quinolizidine-producing plant species lack the ability to accumulate substantial amounts of alkaloids (Wink *et al.*, 1980, 1981, 1983). A total of 10 species from 6 genera of Fabaceae have been studied as cell suspension cultures (Table I). The alkaloid levels found in these cultures are in the range of about 0.01 to 10 μg per gram fresh weight. Thus the alkaloid concentrations in cell cultures are three to five orders of magnitude lower than in the respective intact plants. The alkaloid patterns are simple and uniform. Lupanine (Fig. 1) always figures as the main alkaloid. It is frequently accompanied by minor alkaloids, which can be identified in trace amounts by capillary gas liquid chromatography in combination with mass spectroscopy (GLC–MS), a method that has proved very valuable for the separation, detection, and unequivocal identification of quinolizidine alkaloids (Wink *et al.*, 1980). In *Lupinus polyphyllus* cell cultures the minor alkaloids comprise sparteine, tetrahydrorhombifoline, 17-oxosparteine, 17-oxolupanine, 4-hydroxylupanine, and trace amounts of alkaloid esters (Wink *et al.*, 1982).

Table I

Quinolizidine Alkaloids Produced by Cell Suspension Cultures and the Respective Intact Plants[a]

Species	Cell culture, lupanine (% of total alkaloids)	Intact plant (leaves)	
		Main alkaloids	Percentage of total alkaloids
Lupinus			
L. polyphyllus	70–90 (10)[b]	Lupanine	40
L. luteus	95 (2)	Sparteine	66
L. hartwegii	80 (5)	Aphylline, epiaphylline	72
Cytisus			
C. scoparius	50–70 (1)	Sparteine	62
C. purpureus	95 (1)	*N*-Methylcytisine	64
C. canariensis	95 (2)	Cytisine, anagyrine	79
Laburnum alpinum	95 (0)	Ammodendrine, *N*-methylcytisine	84
Baptisia australis	98 (0)	*N*-Methylcytisine	74
Genista pilosa	95 (1)	Sparteine	70
Sophora japonica	95 (0)	Unindentified	

[a] According to Wink *et al.* (1983).
[b] In parentheses, number of identified minor alkaloids.

The respective intact plants are characterized by quite different alkaloid patterns (Table I). Only in *Lupinus polyphyllus* does lupanine figure as the major alkaloid in both intact plants and cultured cells. All other species accumulate different major alkaloids. As the various structures may derive biosynthetically from lupanine, the uniform expression

Lupanine
ca. 70 structures
Leguminosce (Lupinus)

Senecionine
> 200 structures
Asteraceae (Senecio)
Boraginaceae (Heliotropium)
Leguminosae (Crotalaria)

Fig. 1. Lupanine and senecionine, typical representatives of the tetracyclic quinolizidine alkaloids and the macrocyclic pyrrolizidine alkaloids, respectively.

of lupanine accumulation in cell cultures from different sources supports the assumption that lupanine synthesis may be regarded as the common basic pathway of quinolizidine alkaloid formation (Wink and Hartmann, 1980a, 1985). Only this pathway seems to be expressed in undifferentiated cell suspension cultures.

In comparison to heterotrophic cell suspension cultures of *Lupinus polyphyllus*, photomixotrophic cultures with developed chloroplasts accumulate up to 10 times more lupanine (Wink and Hartmann, 1980a). A positive correlation was found to exist between chlorophyll and alkaloid contents. This is in agreement with the finding that lupanine synthesis is localized in lupin leaf chloroplasts (Wink and Hartmann, 1980b, 1982a).

B. Biosynthetic Studies

From *Lupinus polyphyllus* cell suspension cultures an enzyme preparation was obtained that incorporated isotopically labeled cadaverine into a tetracyclic quinolizidine alkaloid. This alkaloid could be identified as 17-oxosparteine (Wink and Hartmann, 1979; Wink *et al.*, 1979). The enzyme system catalyzes the overall reaction summarized in Fig. 2. It converts three cadaverine units to 17-oxosparteine without the occur-

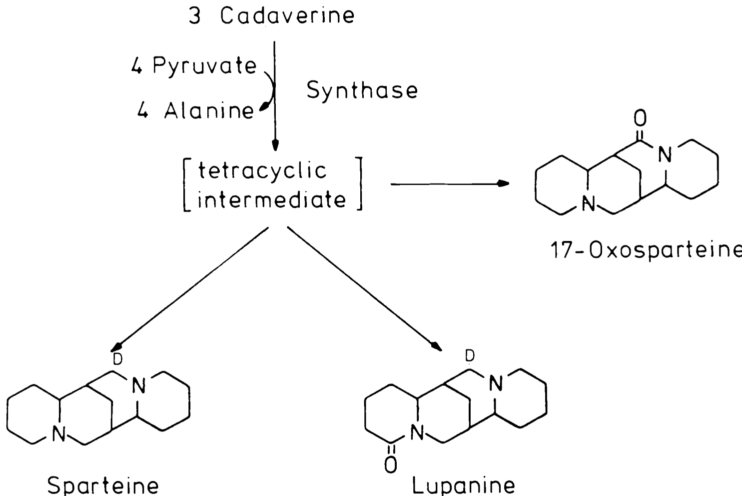

Fig. 2. Overall reaction of the enzymatic synthesis of tetracyclic quinolizidine alkaloids. In enzyme assays, 17-oxosparteine is observed as reaction product. *In vivo*, lupanine and sparteine are formed without the intermediacy of 17-oxosparteine.

rence of free intermediates. The necessary removal of four amino groups of the three cadaverine units occurs via transamination, with pyruvate as specific amino acceptor. Subsequently, this enzyme system was detected in intact plants, too. Within the plant it was found to be restricted to the leaves (Wink and Hartmann, 1981) and localized in the chloroplasts (Wink and Hartmann, 1980b, 1982a). Isolated intact chloroplasts synthesize lupanine on feeding of labeled cadaverine. Lysine decarboxylase, the first enzyme of the alkaloid-specific pathway, was also detected in leaf chloroplasts and cell cultures (Hartmann *et al.*, 1980; Schoofs *et al.*, 1983). Figure 3 summarizes the subcellular localization of quinolizidine alkaloid biosynthesis within the chloroplast. It is notable that the biosynthesis of the precursor lysine shares the same compartment (Mazelis *et al.*, 1976; Wallsgrove and Mazelis, 1980). Both pathways are localized in the chloroplast stroma.

In vivo tracer studies with chirally labeled cadaverines revealed that 17-oxosparteine cannot be an intermediate in the biosynthesis of lupanine and sparteine (Fraser and Robins, 1984; Golebiewski and Spenser, 1984; Spenser, 1985). Cadaverine deuterated at the carbon 1 is incorporated into lupanine and sparteine with retention of label at the carbon 17 (Fig. 2). However, in *in vitro* enzyme assays, 17-oxosparteine is formed stoichiometrically. One explanation of this discrepancy would be that the undisturbed biosynthetic sequence proceeds via an early tetracyclic intermediate directly to lupanine or sparteine, whereas in enzyme preparations this intermediate is released (or stabilized) as 17-oxosparteine. Studies with isolated lupin chloroplasts add some support in favor of this idea. Intact chloroplasts produce exclusively lupanine on

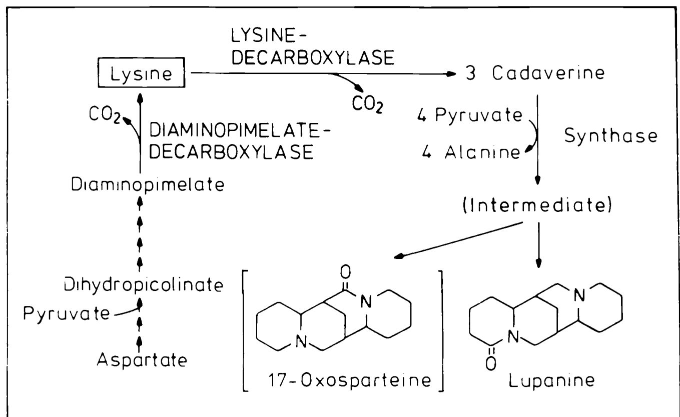

Fig. 3. Localization of lupanine biosynthesis in *Lupinus* leaf chloroplasts.

feeding of cadaverine, but as soon as the integrity of the chloroplast is disturbed, sparteine or 17-oxosparteine is formed. Further work with purified enzymes is required to establish the detailed biosynthethic sequence from cadaverine to the tetracyclic alkaloids. Assuming lupanine synthesis as the basic route of this pathway, cell suspension cultures that possess the ability to synthesize lupanine are valuable experimental systems for future studies.

Laburnum anagyroides accumulates as major components alkaloids of the α-pyridone type, that is, cytisine and *N*-methylcytisine. The enzyme that specifically catalyzes the *N*-methylation of cytisine, an *S*-adenosyl-L -methionine:cytisine *N*-methyltransferase, could be demonstrated in various tissues of the plant (Wink, 1984c). Relatively high activities of the enzyme could also be detected in cell suspension cultures of two related species, that is, *L. alpinum* and *Cytisus canariensis*, which accumulate α-pyridone alkaloids, too (Table I). This is remarkable because α-pyridone alkaloids are not detectable in the cultures and the biosynthetic route leading from lupanine to cytisine does not seem to be expressed in these cell cultures (Wink *et al.*, 1983).

C. Induction of Alkaloid Accumulation in Cell Suspension Cultures

The failure of cell suspension cultures to accumulate substantial amounts of quinolizidine alkaloids does not seem to be due to an insufficient expression of the genes responsible for alkaloid biosynthesis. The activities of the enzymes of lupanine biosynthesis are similar to or one order of magnitude lower than in intact plants (Wink and Hartmann, 1982b). Similar to the leaves of intact plants, cell suspension cultures of *Lupinus* and *Cytisus scoparius* kept under a day–night regime show a diurnal fluctuation of alkaloid accumulation and alkaloid excretion into the culture medium (Wink and Hartmann, 1982c). Thus quinolizidine alkaloid accumulation seems to be a rather dynamic process. In order to modulate experimentally the quinolizidine accumulation, various compounds known to trigger gene regulation or elicit phytoalexin production were tested (Wink and Witte, 1983; Wink, 1985b). A variety of alkaloids not related to lupine alkaloids (e.g., coniine and papaverine), some polyamines, and metabolic inhibitors were found to induce a substantial increase of the quinolizidine alkaloid levels in cell cultures. The activating compounds do not seem to induce synthesis of biosynthetic enzymes but modulate the dynamic balance between alkaloid synthesis and degradation (Wink, 1985b).

III. PYRROLIZIDINE ALKALOIDS

A. Accumulation and Synthesis in Plant *in Vitro* Systems

To our knowledge there exists not a single positive reference about accumulation of pyrrolizidine in plant cell cultures. In our laboratory we have established cell cultures from *Senecio vulgaris, S. vernalis, S. viscosus, S. carniolicus, S. rupester,* and *Symphytum officinale*. Except trace amounts of senecionine *N*-oxide found transiently in a suspension culture of *S. rupester*, none of the cultures synthesized pyrrolizidine alkaloids in callus or in suspension culture (K. von Borstel, A. Ehmke, and T. Hartmann, unpublished). All efforts to induce alkaloid synthesis by variation of the culture conditions or treatment with compounds known to elicit phytoalexin formation failed. On the other hand, root cultures of *S. vulgaris* were shown to synthesize and accumulate alkaloids (Hartmann and Toppel, 1987). The alkaloid pattern was the same as found in the intact plant (Hartmann and Zimmer, 1986).

The biosynthesis of pyrrolizidine alkaloids has been intensively studied. From *in vivo* tracer experiments it is well established that the necine base is derived from arginine or ornithine via two symmetrical intermediates, putrescine and homospermidine (Khan and Robins, 1981, 1985; Robins and Sweeney, 1983; Rana and Robins, 1983; Grue-Sorensen and Spenser, 1982, 1983). The necine acid moiety is derived from isoleucine (Crout *et al.*, 1966; Cahill *et al.*, 1980). Thus the whole carbon skeleton of senecionine originates from two amino acids: 2 mol ornithine/arginine and 2 mol isoleucine (Fig. 4). *Senecio* root cultures offer an excellent system to study the biochemistry of pyrrolizidine alkaloid biosynthesis. Potential precursors are incorporated into the alkaloids with high efficiency. With ^{14}C-labeled putrescine and spermidine, incorporation rates of 20 to 30% were obtained (Hartmann and Toppel, 1987). *Senecio* root cultures provide a simple system for the synthesis of labeled pyrrolizidine alkaloids that are not easily accessible by other methods (Hartmann and Toppel, 1987).

B. Selective Accumulation of Alkaloid *N*-Oxides by Cell Suspension Cultures

It has long been known that in plants, pyrrolizidine alkaloids occur as mixtures of the tertiary alkaloids and the respective alkaloid *N*-oxides

Arginine
Ornithine

Isoleucine

Putrescine

Homospermidine

Senecionine

Fig. 4. Biosynthetic derivation of senecionine. The necine base originates from arginine/ornithine via the symmetrical intermediates putrescine and homospermidine. Isoleucine is the precursor of the necine acid.

(Fig. 5) (Phillipson, 1971; Phillipson and Handa, 1978). In *Senecio* species these *N*-oxides are not only the dominating alkaloid form found in the various plant tissues (Hartmann and Zimmer, 1986), they are also detectable as primary products of biosynthesis and accumulation in root cultures (Hartmann and Toppel, 1987). Cell suspension cultures obtained from pyrrolizidine-producing plants, although incapable of synthesizing alkaloids (see above), are able to take up and accumulate pyrrolizidine alkaloid *N*-oxides selectively (von Borstel and Hartmann, 1986). Cell cultures of non-pyrrolizidine-alkaloid-producing plants do not take up the *N*-oxides. Uptake studies with ^{14}C-labeled senecionine *N*-oxide proved that the *N*-oxides are actively taken up by and stored in the vacuoles prepared from *S. vulgaris* cell cultures (Ehmke *et al.*, 1988). It is assumed that the very polar saltlike *N*-oxides are molecular species that can be translocated and stored in a much safer way than the respective tertiary alkaloids. Regarding the function of the pyrrolizidine alkaloids as chemical protectives, maintenance of appropriate concentrations and safe storage of the alkaloids must be a prerequisite to fulfill this function in the plant.

Fig. 5. Senecionine *N*-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*, and the respective tertiary alkaloid.

IV. CONCLUSION

Cell cultures established from plants that produce quinolizidine or pyrrolizidine alkaloids are certainly unimportant if the intention is production of substantial amounts of alkaloids. Quinolizidines are produced in trace amounts (usually less than 5 μg/g fresh weight), and pyrrolizidines, if at all, in undetectable amounts. But as emphasized by Wink (1985a), besides biosynthesis, many more processes, such as transport, accumulation, and degradation, are of crucial importance as to whether a cell culture actually produces secondary products or not. In this respect cell cultures have been found to be very valuable in studying the complex and dynamic physiology of quinolizidine alkaloids. In the case of the pyrrolizidines, cell cultures are valuable tools to study the mechanisms of selective transport and accumulation of alkaloid *N*-oxides, which appear to be fully expressed in contrast to biosynthesis.

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CHAPTER 17

Isoquinolines

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I. INTRODUCTION

Isoquinoline-type alkaloids show biological activities like those of morphinane-, protoberberine-, and benzophenanthridine-type alkaloids, and they are widely distributed in the plant kingdom, mainly in

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Papaveraceae, Berberidaceae, Ranunculaceae, and Menispermaceae. The production of some pharmaceutically interesting compounds from these plants by means of plant cell culture has been extensively studied.

II. PRODUCTION OF ISOQUINOLINES

A. Papaveraceae

Furuya *et al.* (1972, 1976) (Fig. 1) investigated callus tissue of *Papaver somniferum* and isolated a new alkaloid, norsanguinarine (**1**), as well as the following alkaloids: sanguinarine (**2**), chelirubine (**3**), dihydrosanguinarine (**6**), oxysanguinarine (**5**), protopine (**12**) (Fig. 3), cryptopine (**10**), magnoflorine (**15**), and choline. Moreover, 11 representative species [*Eschscholzia californica, Chelidonium japonica, Macleaya cordata, Papaver setigerum* and *P. bracteatum* (which contain morphinane alkaloids), *P. orientale, P. rhoeas, Dicentra peregrina, Corydalis incisa,* and *C. pallida*] have been subject to comparison to intact plant and callus tissues. All callus tissues were similar to each other, and the alkaloids found were benzophenanthridine-, protopine- and aporphine-type alkaloids, which are

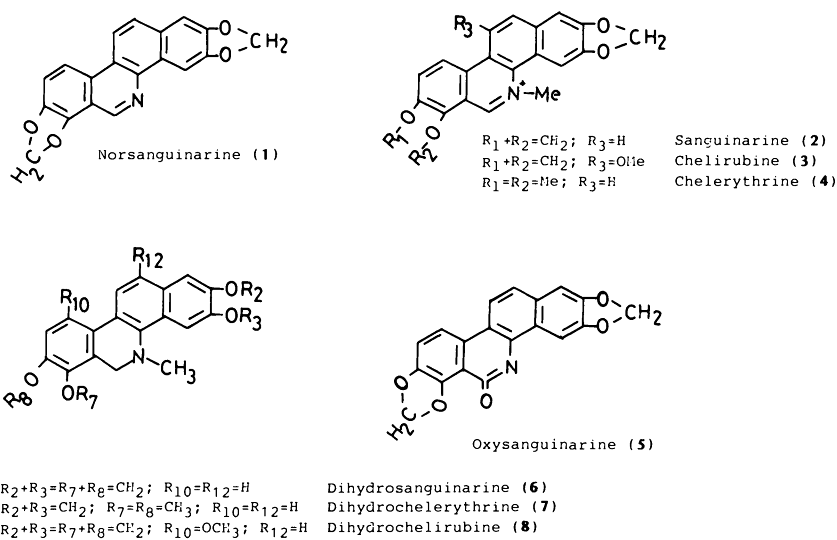

Fig. 1. Alkaloids from callus tissue.

		R_1	R_2	R_3	R_4	R_5
Berberine	(16)	O–CH$_2$–	O	O Me	O Me	H
Palmatine	(17)	O Me	O Me	O Me	O Me	H
Coptisine	(18)	O–CH$_2$–	O	O–CH$_2$–	O	H
Jatrorrhizine	(19)	O H	O Me	O Me	O Me	H
Columbamine	(20)	O Me	O H	O Me	O Me	H
Thalifendine	(21)	O–CH$_2$–	O	O Me	O H	H
Thalidastine	(22)	O–CH$_2$–	O	O Me	O H	OH
Epiberberine	(23)	O Me	O Me	O–CH$_2$–	O	H
Berberastine	(25)	O–CH$_2$–	O	O Me	O Me	OH
Dehydrochei-lanthifoline	(26)	O Me	OH	O–CH$_2$–	O	H
Groenlandicine	(27)	O H	O Me	O–CH$_2$–	O	H
Dehydro-discretamine	(29)	O H	O Me	O Me	O H	H
Desoxy-thalidastine	(24)	O–CH$_2$–	O	O Me	O H	H
Scoulerine	(31)	O Me	O H	O H	O Me	
Styropine	(32)	O–CH$_2$–	O	O–CH$_2$–	O	

Fig. 2. Alkaloids from callus tissue.

simpler and more widely distributed than the morphinane-type alkaloids characteristic of parent plants (Ikuta *et al.*, 1974). The benzophenanthridine alkaloids found in callus cells of poppy are not present in the original plants; plant tissue culture indicates considerable differences between the alkaloids produced by cultures and by original

plants. L-Stylopine (**32**) (Fig. 2) and **12** were identified as the main alkaloids from callus cells derived from seedlings of *Papaver bracteatum*, and also, a small amount of thebaine was identified (Kamimura and Nishikawa, 1976). In addition to these results, orientalidine (**30**), isothebaine (**46**) (Fig. 3) and sanguinarine (**2**) were isolated from callus cultures of *P. bracteatum* on Murashige–Skoog (MS) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin and 1% polyvinylpyrrolidone (Lockwood, 1981). Cell suspension cultures derived from *P. somniferum*, *P. setigerum*, and *P. nudicaule* were found to contain **12, 2, 46,** and **30** (Lockwood, 1981). Tissue cultures of *Macleaya microcarpa* produced allocryptopine (**13**), (**12**), and (**2**) (Koblitz *et al.*, 1975).

Berlin *et al.* (1983) reported that suspension cultures of *Eschscholzia californica* derived from seedlings accumulated the dihydro forms of the benzophenanthridine alkaloids dihydrosanguinarine (**6**), dihydrochelirubine (**8**), dihydromacarpine (**9**), and dihydrochelerythrine (**7**), all of which are known to be constituents of *Eschscholzia*. Dihydrochelirubine (**8**) was found to be the main constituent of the cultured cells (Table I). The yields of alkaloids depended on the conditions of the media. Moreover, the callus tissues derived from *Corydalis* species produced sanguinarine (**2**), protopine (**12**), and a new, simple isoquinoline alkaloid; the callus tissues derived from *C. ophiocarpa* had a capacity to convert tetrahydroprotoberberine type compounds via protopine-type compounds to benzophenanthridine-type alkaloids (Iwasa and Takao, 1982).

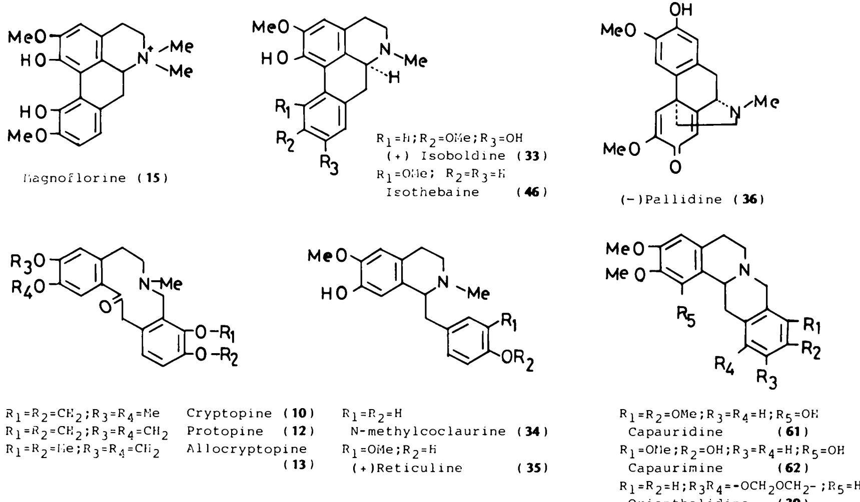

Fig. 3. Alkaloids from callus tissue.

Table I

Tissue Cultures with Isoquinoline Alkaloids

Plant	Organs	Media[a] (mg/liter)	Reference
Eschscholzia californica	Root, stem, seedling	MS: D, 0.1; K, 0.1	Ikuta *et al.* (1974) Berlin *et al.* (1983)
Chelidonium japonica	Hypocotyl	MS; D, 1; K, 0.1; CM	Ikuta *et al.* (1974)
Macleaya cordata	Stem	MS; D, 0.1; K, 0.1	Ikuta *et al.* (1974)
	Stem	MS; D, 1; K, 0.2; GA, 0.1	Koblitz *et al.* (1975)
Papaver somniferum	Capsule Stem	MS; D, 0.1; K, 0.1	Furuya *et al.* (1972b)
	Seedling	MS; D, 10; K, 0.1	Lockwood (1981)
P. setigerum	Seedling	MS: D, 1; K, 0.1	Ikuta *et al.* (1974)
	Seedling	MS; D, 10; K, 0.1	Lockwood (1981)
P. bracteatum	Seedling	MS; D, 1; K, 0.1	Ikuta *et al.* (1974)
	Seedling, petiole, root	MS; D, 1; K, 0.1	Kamimura and Akutsu (1976); Kamimura and Hishikowa (1976)
	Seedling	MS, D, 1; K, 0.1; 1% polyvinylpyrrolidine	Lockwood (1981)
P. orientale	Seedling	MS; D, 1; K, 0.1	Ikuta *et al.* (1974)
P. rhoeas	Seedling	MS; D, 1; K, 0.1	Ikuta *et al.* (1974)
P. nudicaule	Seedling	MS; D, 1; K, 0.1	Lockwood (1981)
Dicentra peregrina	Stem	MS; D, 1; K, 0.1	Ikuta *et al.* (1974)
Fumaria capreolata	Seedling	LS	Tanahashi and Zenk (1985)
Corydalis incisa	Petiole	MS; D, 1; K, 0.1	Ikuta *et al.* (1974)
C. pallida	Stem	MS; D, 1; K, 0.1	Ikuta *et al.* (1974)
C. ophiocarpa	Stem	MS; D, 1; K, 0.1	Iwasa and Takao (1982)
Nandina domestica	Stem	MS; D, 1; K, 0.1	Ikuta and Itokawa (1982b)
Mahonia japonica	Stem	MS; D, 1; K, 0.1	Ikuta and Itokawa (1982b)
Berberis stolonifera		LS	Hinz and Zenk (1981)
Berberis wilsonae		MS; IAA, 0.2; D, 0.2, K, 2	Breuling *et al.* (1985)
Plagiorhegma dubium (synonym, *Jeffersonia dubia*)	Leaf	B5; D, 1	Arens *et al.* (1985)
Stephania cepharantha	Tuber	MS; D, 1; K, 0.1; IAA, 1–5; K, 0.1–1	Akasu *et al.* (1976)
Dioscorephlum cumminsii	Stem	MS; D, 1; K, 0.1	Furuya *et al.* (1983)
Coptis japonica	Petiole	MS; D, 1; K, 0.1	Furuya *et al.* (1972a)
	Flower bud	LS; D, 5×10^{-6} μM; K, 5×10^{-7} μM	Yamamoto *et al.* (1981)
	Flower bud	LS; NAA, 100 μM; BA, 1 μM	Fukui *et al.* (1982)
	Root	LS; NAA, 10 μM, BA, 0.01 μM	Sato and Yamada (1984)
	Petiole	LS ($CuSO_4$, 1 μM)	Morimoto *et al.* (1986)
	Leaf	NAA; 10^{-5} μM; BA, 10^{-8} μM	
Thalictrum minus	Stem	MS; D, 5, 1, 0.1; K, 0.1	Ikuta and Itokawa (1982a)
	Leaf	LS; NAA, 100 μM; BA, 1 μM	Nakagawa *et al.* (1984)

[a] Abbrevation: MS; Murashige–Skoog; LS, Linsmair–Skoog; D, 2,4-dichlorophenoxyacetic acid; K, kinetin; NAA, naphthaleneacetic acid; BA, 6-benzyladenine; GA, gibberellic acid; CM, coconut milk.

In callus cultures derived from seedlings of *Fumaria capreolata* a total of 10 known isoquinoline alkaloids were found along with many structurally different i.e., sanguinarine (**2**), coptisine (**18**), dehydrocheilanthifoline (**26**), magnoflorine (**15**), protopine (**12**), scoulerine (**31**), isoboldine (**33**), *N*-methylcoclaurine (**34**), reticuline (**35**), and a simple isoquinoline as well as a rather complicated structure like pallidine (**36**), a morphinane-dienone-type alkaloid (Tanahashi and Zenk, 1985). The culture yielded a biomass of about 12 g dry weight per liter of medium; the dried cells contained about 0.1% alkaloids.

B. Ranunculaceae

Berberine alkaloids have been used for Chinese medicine or folk medicine in Japan and East Asia and have a big market in these countries. Therefore, production of berberine from callus cultures has been investigated by Japanese researchers.

Callus cultures of *Coptis japonica* produced mainly berberine (**16**) and jatrorrhizine (**19**), characteristic of the rhizome, but in relatively small amounts (Furuya *et al.*, 1972a; Ikuta *et al.*, 1974). Moreover, palmatine (**17**), coptisine (**18**), columbamine (**20**), berberastine (**25**), epiberberine (**23**), groenlandicine (**27**), and **15** were identified (Ikuta *et al.*, 1974; Ikuta and Itokawa, 1982b). Callus cultures of *Thalictrum minus*, which is used as folk medicine in Japan and East Asia, accumulated a large amount of **16** and nine other protoberberine alkaloids: **17–20**, thalifendine (**21**), thalidastine (**22**), desoxythalidastine (**24**), and **15**. It is interesting that the alkaloid berberine was present in callus tissues in much greater amounts (0.67% dry weight) than in the stem and leaves of the parent plant (0.0019%) (Ikuta and Itokawa, 1982a).

C. Berberidaceae

Plants of the family Berberidaceae contain protoberberine-type alkaloids. Callus cultures of *Nandina domestica* produced 11 different protoberberine alkaloids, dehydrodiscretamine (**29**), **16–22, 24, 25, 27,** and an aporphine-type alkaloid, **15;** furthermore, **19** was the main component (Ikuta and Itokawa, 1982b, 1987). Callus cultures of *Mahonia japonica* produced five different protoberberines, **15–20,**and accumulated a large amount of **19** (Ikuta and Itokawa, 1982b). Hinz and Zenk (1981) isolated **19** as a major alkaloid in addition to three minor protoberberine

alkaloids, **16–17**, from cell suspension cultures of *Berberis stolonifera*. *Berberis wilsonae* callus cultures produced four protoberberine-type alkaloids, **16**, **17**, **20**, and **19**, the latter as the main product (Breuling *et al.*, 1985). Jatrorrhizine (**19**) and lignane glucosides were isolated from a crude methanolic extract of cultured *Plagiorhegma dubium* (*Jeffersonia dubia*) cells (Arens *et al.*, 1985). All tissue cultures of berberidaceous plants accumulated jatrorrhizine (**19**) as the main alkaloid.

D. Menispermaceae

Callus tissue derived from the tuber of *Stephania cepharantha* produced biscoclaurine alkaloids aromoline (**37**) and berbamine (**38**), but cepharanthine and isotetrandrine, main alkaloids of the intact plant, did not occur. The enzymes of methylation and methylenedioxy-group formation in the final steps of the biosynthetic route leading to the biscoclaurine alkaloids cepharanthine and isotetrandrine were absent from the callus. Moreover, from the neutral fraction of the MeOH extract of the callus, cepharanone I (**39**) and II (**40**) and cepharadione A (**41**) and B (**42**), norcepharadione (**43**), liriodenine (**44**), and lysicamine (**45**) were isolated.

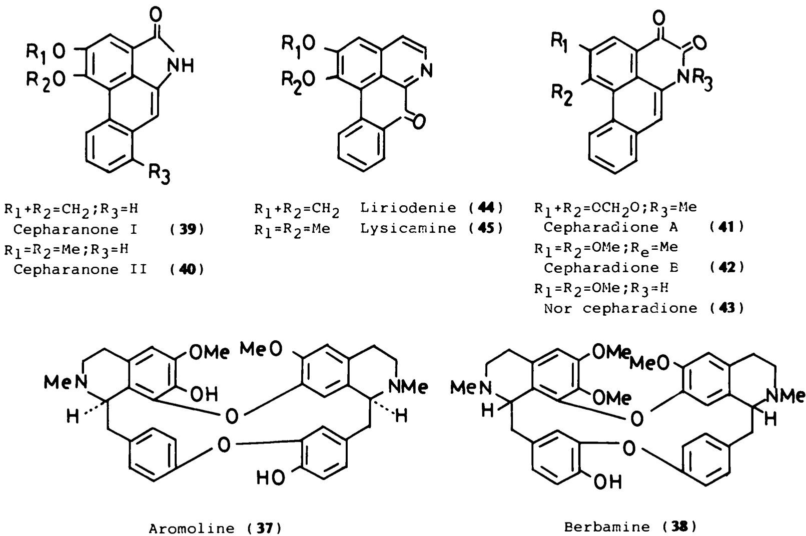

Fig. 4. Alkaloids from callus tissue.

These compounds were observed in the intact plant in trace amounts (Akasu *et al.*, 1974, 1975, 1976). Palmatine (**17**), **19**, and **15** were isolated from cultured cells of *Dioscoreophyllum cumminsii*. The **19** content in cultured cells was 40–100 times higher than that of the intact plant, but **20**, which is a minor component in the original plant, was not detected. Moreover, it was observed that the addition of indolylacetic acid (IAA) or naphthaleneacetic acid (NAA) to the growth medium increased the alkaloid content as compared with 2,4-D (Furuya *et al.*, 1983).

III. CHEMOTAXONOMIC APPROACH

Eleven representative species of Papaveraceae and Fumariaceae were investigated for their alkaloid content in callus cells by Ikuta *et al.* (1974). All callus cells contained norsanguinarine (**1**), sanguinarine (**2**), dehydrosanguinarine (**6**), oxysanguinarine (**5**), chelirubine (**3**), protopine (**12**), and magnoflorine (**15**), even though benzophenanthridine alkaloids may not have been present in the original plants. Morphinanes were not produced in any of the callus cells derived from the *Papaver* species investigated. All were similar to each other even though the redifferentiated plantlets derived from the callus tissues again showed the typical varying alkaloid content of the original plants (Ikuta *et al.*, 1974). The alkaloid types found were benzophenanthridine, protopine, and aporphine, but quaternary protoberberine, normally found in the original plants, was not detected in the callus cells. In addition to the 11 species, 5 others, which have been reported, are summarized in Table II and were also compared with the alkaloid content from the callus cells of Papaveraceae and Fumariaceae. The alkaloid types were similar to those given above, except for callus of *Fumaria*, which produced coptisine (**18**) and pallidine (**36**). It is very interesting from a phylogenetic point of view that the alkaloid types produced from callus cells are simpler than those from the original plants; protopine, aporphine, protoberberine, benzophenanthridine, phthalidoisoquinoline, and morphine types are widely distributed in Papaveraceae and Fumariaceae (Preininger, 1985).

From the results of investigation of the callus cells it might be possible to discuss the chemotaxonomic significance of the distribution of the alkaloids of Papaveraceae and Fumariaceae. On the other hand, Bandoni *et al.* (1975) have tried to differentiate *Argemone* species on the basis of sanguinarine, but benzophenanthridine alkaloids may not be signifi-

Table II

Alkaloids of Papveraceous Callus Tissues

Original plant of callus tissues	Type of alkaloid: Benzophenanthridine (1)	(5)	(6)	(2)	(3)	Protopine (12)	Aporphine (15)	Reference
Papaveraceae								
Eschscholziae								
Eschscholzia californica	+	+	+	+	+	+	+	
Chelidoniae								
Chelidonium japonicum	+	+	+	−	−	+	−	
Ch. majus				+		+		Böhm and Frank (1980)
Macleaya cordata	+	+	+	+	+	+	−	
M. microcarpa				+		+		Böhm and Frank (1982)
Papavereae								
Papaver somniferum	+	+	+	+	−	+	−	
P. setigerum	+	+	+	+	−	+	+	
P. bracteatum	+	+	+	+	+	+	+	
P. orientale	+	+	+	+	−	+	+	
P. rhoeas	+	+	+	+	−	+	+	
P. nudicaule				+		+		Lockwood (1981)
Fumariaceae								
Dicentra peregrina	+	+	+	+	−	++	+	
Corydalis incisa	+	+	+	+	−	+	+	
C. pallida	+	+	+	+	−	+	+	
C. ophiocarpa						+		Iwasa and Takao (1982)
Fumaria capreolata				+		+	+	Tanahashi and Zenk (1985)

Table III

Alkaloids of Callus Tissues

Original plant of callus tissues	Type of alkaloid: Protoberberine (16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	Aporphine (15)	Reference
Berberidaceae										
Nandina domestica	+	++	+	++	+	+	+	+	+	Ikuta and Itokawa (1982b)
Mahonia japonica	+	+	+	++	+	–	–	–	+	Ikuta and Itokawa (1982b)
Berberis stolonifera	+	+		++	+					Hinz and Zenk (1981)
B. wilsonae	+	+		++	+					Breuling *et al.* (1985)
Jeffersonia dubia				+						Arens *et al.* (1985)
Menispermaceae										
Dioscoreophyllum comminsii	–	+		++	–			+	+	Furuya *et al.* (1983)
Tinospora caffra		+		+	+				+	Rueffer (1985)
T. crispa		+		+						Minoda *et al.* (1982)
Chasmanthera dependens		+		+					+	Rueffer (1985)
Stephania japonica		+		+					+	Rueffer (1985)
Ranunculaceae										
Thalictrum minus	++	+	+	+	+	+	+	+	+	Ikuta and Itokawa (1982a)
Coptis japonica	++	+	+	++	+	–	–	+	+	Ikuta *et al.* (1975)

cant chemotaxonomic markers in the original plants, because although alkaloids are formed in callus tissues, they may not be present in the original plants. On the contrary, these compounds are significant chemotaxonomic markers in studies utilizing callus tissues. The isolation of protoberberine- and aporphine-type alkaloids from berberidaceous, ranunculaceous, and menispermaceous callus cultures reported by others workers are summarised in Table III. Jatrorrhizine has been isolated from all of the species and was the main product, except in *Thalictrum* tissue cultures. The accumulation of jatrorrhizine is very interesting from the chemotaxonomic point of view and also in relation to Rueffer's jatrorrhizine biosynthesis results using *Berberis* spp. tissue cultures (Rueffer and Zenk, 1986).

Considering these results, the chemical investigation of callus cultures may be applicable to chemotaxonomic studies. The structures of the alkaloid types and postulated biosynthetic relationships in these plant tissue cultures are given in Scheme 1.

protopine type
Papaveraceae
Fumariaceae

tetrahydroberberine type

benzophenanthridine type
Papaveraceae
Fumariaceae

benzyltetrahydro
isoquinoline type

aporphine type
Papaveraceae
Fumariaceae
Ranunculaceae
Berberidaceae
Menispermaceae

protoberberine type
Ranunculaceae
Berberidaceae
Menispermaceae

Scheme 1. The fundamental biosynthesis route.

IV. BIOTRANSFORMATION AND BIOSYNTHESIS

A. Tetrahydroprotoberberines

Bioconversion of both *trans*- and *cis*-13-methyltetrahydroprotoberberine *N*-quaternary salts (**47** and **48**) to a corycavine analog **50** in callus cultures derived from the stems of *Corydalis* spp. has been demonstrated. Feeding experiments using both *trans*- and *cis*-13-hydroxytetrahydroprotoberberine *N*-methyl salts (**49** and **55**) have established the biotransformation sequence (**49**) and (**55**) → 13-hydroxyallocryptopine (**51**) → 13-oxoallocryptopine (**52**) → a spirobenzylisoquinoline (**53**) + a benzindanoazepine (**54**) in *Corydalis* spp. callus cultures. Transfer of the methyl from nitrogen to oxygen occurs during conversion of **52** to **53.** Incorporation of carbon-13 label of the *N*-methyl group of the protoberberine precursor **49*** (labeled compound) into the *O*-methyl group in the spirobenzylisoquinoline **53** was demonstrated. Therefore, the *O*-methyl group at C-8 of **53** arises from the *N*-methyl group of the protopine skeleton, that is, migration of the methyl group from nitrogen to oxygen occurs during the ring rearrangement. The biosynthetic pathway to the corycavine analog and the spirobenzylisoquinoline- and benzindanoazepine-type alkaloid are summarized in Scheme 2 (Iwasa *et al.*, 1985). Corycavine analog (**50**) and **51–54** were obtained from feeding experiments in *Corydalis* spp. callus cultures, as metabolites not normally detected in the callus and the original plant (Iwasa *et al.*, 1984a). Furthermore, the structure and stereochemistry of base **53** have been determined by X-ray analysis (Iwasa *et al.*, 1984b). This observation demonstrated that it is possible to produce new compounds in plant cell cultures using biotransformation of special substrates.

B. Benzophenanthridines

Takao *et al.* (1983) established callus tissues of *Macleaya cordata* from the stem and the root and studied the stereospecifity of the pathway for the biosynthesis of chelerythrine (**4**), sanguinarine (**2**), chelirubine (**3**), and macarpine (**11**) from tetrahydroberberine precursors. Predominantly, (−)-*S* enantiomers and *cis*-*N*-methyl derivatives of the tetrahydroprotoberberines could be stereospecifically metabolized to the benzophenanthridine skeleton. The incorporation experiments defined the following biosynthetic pathway: (−)-(*S*)-7,8,13,13*a*-tetrahydroberberine (**56**) → (−)-*cis*-*N*-methyl-7,8,13,13*a*-tetrahydroberberinium salt (**57**) →

(48) R=Me
(55) R=OH
(47) R=Me
(49) R=OH
(48) → (50)
(55) → (52)
(47) → (50)
(49) → (51)
(50) R=Me
(51) R=OH
(51) → (52)
(52)
(52) → (53)
(52) → (54)
(53)
(54)

Scheme 2. Biotransformation of the 13-Methyltetrahydroprotoberberines (47 and 48) and the 13-hydroxytetrahydroprotoberberines (49 and 55). (From Iwasa *et al.*, 1984).

allocryptopine (**13**) → chelerythrine (**4**) + (−)-*cis*-*N*-methyl-7, 8, 13, 13*a*-tetrahydrocoptisinium (**58**) → protopine (**12**) → sanguinarine (**2**) → chelirubine (**3**) → macarpine (**11**). Administration of (−)-(*S*)-7,8,13, 13*a*-tetrahydroberberine (**56**) led to incorporation into berberine (**16**), whereas (+)-(*R*)-7,8,13,13*a*-tetrahydroberberine (**59**) yielded only dehydroberberine (**60**) and berberine (**16**). Compound **60** probably is the

Scheme 3. Biosynthetic sequence for the alkaloids of *Macleaya cordata* as obtained by incorporation experiments (*: indicates the result obtained by the use of intact plants). (From Takao *et al.*, 1983.)

intermediate between tetrahydroberberine (**59**) and berberine (**16**). The conversion shown in Scheme 3 also takes place in the parent plant. The studies with intact plant material would not have allowed determination of such a complete biosynthesis sequence. Various intermediates were isolated and, in some cases, structurally elucidated for the first time.

C. Protoberberines

The biosynthetic sequence for berberine, starting with (*S*)-reticuline, is shown in the Scheme 4. The enzymology of (*S*)-reticuline formation is known in detail (Zenk *et al.*, 1985). Four additional enzymes are involved in the biosynthesis of berberine, starting from (*S*)-reticuline. Enzyme 1 is specific for the substrates with *S* configuration and not for those with *R* configuration. Neither (*S*)- nor (*R*)-reticuline *N*-oxide was transformed by the crude or by the homogeneous enzyme. The enzyme activity was conveniently monitored using *N*-CT_3-(*S*)-reticuline as substrate and following the release of tritium in the aqueous phase of the

Scheme 4. The biosynthetic pathway to protoberberine alkaloids by *Berberis* callus cultures (Rueffer and Zenk, 1986).

incubation mixture; exactly one-third of the radioactivity contained in the *N*-methyl group was lost during the cyclization of (*S*)-reticuline to the tetrahydroprotoberberine molecule (Steffens *et al.*, 1984). A cell-free system of *Macleaya microcarpa* cell cultures was previously shown to catalyze the conversion of (*S*)-reticuline (**35**) to (*S*)-scoulerine (**31**), and the enzyme involved was named the berberine bridge enzyme (Rink and Böhm, 1975).

Enzyme 2, (*S*)-scoulerine 9-*O*-methyltransferase, is a highly stereo- and regiospecific metyltransferase (SAM). A variety of closely related tetrahydroprotoberberines were tested, and none of the naturally occurring substrates served as methyl-group acceptor except (*S*)-scoulerine. The *R* enatiomers of scoulerine and dehydroscoulerine were not used in the enzyme reaction. It has been unequivocally established that only one of the hydroxyl groups of (*S*)-scoulerine, namely, the one at position 9, is methylated, the product being (*S*)-tetrahydrocolumbamine. In the course of biosynthesis of berberine-type alkaloids, (*S*)-scoulerine is expected to undergo various methylation steps as well as the oxidation of ring C (Muemmler *et al.*, 1985).

Enzyme 3 catalyzes the dehydrogenation of the tetrahydroprotoberberine molecule at carbon 14 and nitrogen 7, and the 7,14-dehydroberberinium intermediate aromatizes further in ring C to yield the protoberberine molecule, giving rise to the last intermediate columbamine. Enzyme 3 catalyzes the dehyrogenation of more than 20 different

terahydroberberines to the corresponding protoberberine alkaloids. The enzyme is absolutely specific for substrates with *S* configuration. The enzyme was found to occur in a number of plant cell cultures, especially in those strains of *Berberis* that produce considerable amounts of protoberberines (Amann *et al.*, 1984).

Enzyme 4 catalyzes the formation of the methylenedioxy group in the A ring of the protoberberine molecule from columbamine, the immediate precursor of berberine. The enzyme was assayed for its catalytic activity by using 3-*O*-CT_3-columbamine as substrate. The enzyme reaction was followed by monitoring the release of tritium into the aqueous phase of the incubation mixture. Exactly one-third of the radioactivity of the 3-*O*-methyl group was lost during the transformation of columbamine to berberine (Rueffer and Zenk, 1985).

Enzyme 5 specifically transfers the methyl group from (*S*)-adenosyl-L-methionine to the 2-OH position of columbamine, producing palmatine. Enzyme 5 in *Berberis* cell and callus cultures specifically transfers the methyl group of SAM exclusively to the quaternary acceptor columbamine to yield palmatine, a dead-end metabolite in the protoberberine pathway due to the methylation of all its functional hydroxyl groups (Rueffer *et al.*, 1986). The enzyme acts only on the quaternary alkaloid as substrate, not on its tetrahydro derivative.

Tetrahydrocolumbamine does not reside at a triple branch point leading to tetrahydroberberine and tetrahydropalmatine, as has been previously suggested (Beecher and Kelleher, 1983). Therefore, the previously observed methylation of tetrahydrocolumbamine in *Berberis aggregata* must be incorrect (Rueffer and Zenk, 1986). Jatrorrhizine, which contains an unusual 2-*O*-methylation pattern, has one of three possibilities: (a) demethylation of palmatine, (b) a reopening of the methylenedioxy group of berberine, and (c) precursor of reticuline rather than protoshinomenine, which already possesses the methyl group of the A ring in the correct position. Beecher and Kelleher (1983) proved that berberine is a biosynthetic precursor of jatrorrhizine by using chemically synthesized [9-^{14}C]berberine and callus cultures of *B. aggregata*. They also proposed the mechanism shown in Scheme 5 for the one-side opening of the methylenedioxy group. Hydride attack at the methylenedioxy carbon of berberine promotes an electron shift to the quaternary nitrogen to give the stable quinone–tertiary amine. This then picks up a proton and undergoes a reversal of the electron flow to give the quaternary salt of jatrorrhizine. These results were confirmed enzymatically (enzyme 6) by Rueffer *et al.* (1983). A minor route can be envisaged through (*S*)-protosinomenine, which already carries the methoxy group in the correct 7 position.

Berberine

Jatrorrhizine

Scheme 5. Postulated mechanism for the biosynthetic conversion of berberine to jatrorrhizine. (From Beecher and Kelleher, 1983.)

V. ACCUMULATION OF PROTOBERBERINE ALKALOIDS

A. *Thalictrum minus*

Cells of *Thalictrum minus* produced large amounts of berberine in static culture, even without selection (Ikuta and Itokawa, 1982a). Cultured cells showed a level that was about 350 times higher than that in the original plant. Cell suspension cultures of the callus produced up to 0.8 g berberine per liter of medium and released most of it into the liquid medium; a considerable amount of berberine crystallized as the nitrate or chloride, depending on the kind of major anion present in the medium. This is the first observation that alkaloids accumulate as crystals in the culture medium of plant cells (Nakagawa *et al.*, 1984).

B. *Coptis japonica*

High berberine-producing cell lines were established by repeated cloning and selection (Yamamoto *et al.*, 1981). Fukui *et al.* (1982) reported

that berberine productivity was stable during 3 years of continuous subcultivation and reached a maximal alkaloid content of 1.67 g per liter of medium (11.4% dry weight) in one culture period (21 days). Sato and Yamada (1984) established a high berberine-producing culture of *Coptis* cells, and the highest productivity of one selected cell line showed 13.2% (dry weight) and 1.39 g per liter of medium over 21 days. Morimoto *et al.* (1986) demonstrated that gibberellic acid (GA_3) treatment showed a remarkable change of incorporation of nitrogen and sucrose into *Coptis* cells and enhanced berberine production to 15.7% (dry weight) with GA_3 (10^{-6} *M*) within 14 days on modified Linsmaier–Skoog (LS) medium ($CuSO_4$, 1 μ*M*) supplemented with 10^{-5} *M* NAA and 10^{-8} *M* 6-benzyladenine (BA).

It takes more than 5 to 6 years under cultivation to harvest one small rhizome weighing about 1–2 g (dry weight), and the content of berberine is about 8% dry weight (~160 mg per rhizome). The berberine obtained from callus of 1 liter of medium in one culture period (14–21 days) is approximately equal to the value of one original rhizome culti-

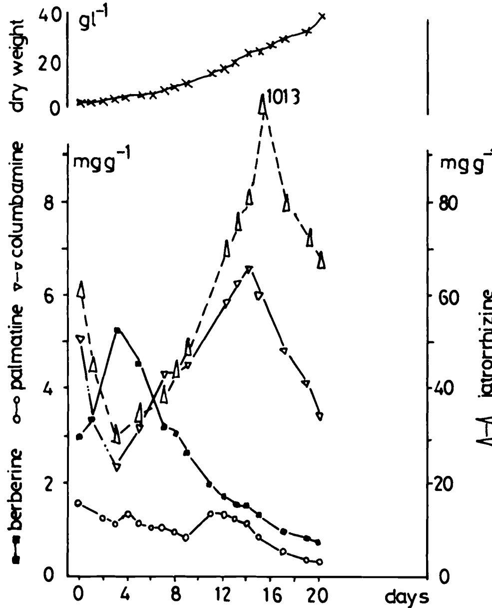

Fig. 5. The kinetics of alkaloid accumulation during the growth cycle and the yield in dry weight at PO_2 = 50% in the culture broth. (From Breuling *et al.*, 1985.)

vated during 5 to 6 years. The need for the production of berberine from callus cultures is thus obvious and important.

C. *Berberis* Species

Suspension culture cells of *Berberis stolonifera* produced one major and three minor protoberberine alkaloids. The major compound was identified as jatrorrhizine, and the minor ones as berberine, columbamine, and palmatine. The maximum amount of alkaloid production was 10% (dry weight), and 1.7 g per liter of medium within 14 days in culture. Jatrorrhizine is the major alkaloid of *Berberis* cell cultures (Hinz and Zenk, 1981). Suspension cultures of *B. wilsonae* produced berberine, palmatine, columbamine, and jatrorrhizine (Breuling *et al.*, 1985); their formation depended on the concentration of dissolved oxygen and on the aeration rate (palmatine was less affected). Figure 5 shows the kinetics of accumulation of the four alkaloids during the growth cycle. Berberine accumulated at a very early stage and decreased, whereas jatrorrhizine and columbamine accumulated. Maximum jatrorrhizine accumulation (10% dry weight) was observed on day 15. The results suggested the intermediate function of berberine in columbamine and jatrorrhizin biosynthesis (Beecher and Kelleher, 1983; Rueffer and Zenk, 1986).

VI. IMMOBILIZED CELLS

Immobilized microbial cell systems have rapidly been developed. The superiority of immobilized cells over free cells was demonstrated by Brodelius and Mosbach (1982). These systems, therefore, have been applied to the industrial production of useful compounds, such as food and pharmaceuticals. Furuya *et al.* (1984) reported that calcium alginate was superior to other entrapping materials for a variety of plant cells.

Papaver somniferum cells were immobilized in calcium alginate, where they continued to live for 6 months. The immobilized cells converted codeinone to codeine (70% yield), and 88% of the codeine converted was excreted into the medium.

Immobilized cells of *Coptis japonica* produced berberine and minor protoberberine alkaloids and excreted them into the medium over a period of 60 days (Yoshikawa *et al.*, 1985). The immobilization of plant cells seems to show potential for the production of natural compounds.

VII. REDIFFERENTIATED PLANTLETS AND ALKALOIDS

A. *Macleaya cordata*

Both morphology and alkaloid pattern of redifferentiated plantlets and original plants were found to be almost the same in *Macleya cordata*. Norsanguinarine was the main alkaloid in the callus tissues, but only a trace was present in plantlets. On the other hand, the benzophenanthridine-type alkaloid chelerythrine (**4**) was not found in the callus tissues but occurred in the redifferentiated plantlets and original plants. The amount of protopine and allocryptopine (**13**) in the callus tissues was lower than that in the redifferentiated plantlets (Ikuta *et al.*, 1974) (Table IV).

B. *Corydalis pallida*

Plantlets regenerated from callus tissues of *Corydalis pallida* were observed to contain all alkaloids that were present in the callus. Norsanguinarine was mainly found in the callus tissues cultured on a medium containing 2,4-D, and also in trace amounts in both the plantlets and original plants.

Furthermore, the protoberberine-type alkaloids capauridine (**61**) and capaurimine (**62**), which were isolated from *Corydalis pallida*, were detected in the plantlets but not in callus tissues; capaurimine was present in a small amount in callus tissues only, not in plantlets. Both alkaloids were absent from the callus tissues cultured on a medium containing 2,4-D (Table IV) (Ikuta *et al.*, 1974).

C. *Papaver bracteatum*

Buds were initiated in 3-year-old callus subcultured on MS medium containing IAA (1 mg/liter), kinetin (0.1 mg/liter), and coconut milk (CM) (7%). The buds did not develop further than 1 to 1.5 cm in height and did not form shoots and roots. Buds with callus on medium containing IAA under light and the callus subcultured on medium containing IAA in the dark, as well as callus tissues subcultured on medium containing 2,4-D, had similar patterns of alkaloids (Table IV). The buds with

Table IV

Alkaloids of Callus and Redifferentiated Plantlets[a]

Plant	Alkaloid											Total alkaloids (g) percentage fr.wt.
	Norsanguinarine	Oxysanguinarine	Dihydrosanguinarine	Sanguinarine	Chelirubine	Chelerythrine	Protopine	Allocryptopine	Magnoflorine	Capaurimine	Capauridine	
Macleaya cordata												
Callus	+	+	+	+	+	−	+	+	−	−	−	0.009
Redifferentiated plantlet	±	−	±	+	+	+	++	++	−	−	−	0.50
Original plant	±	−	±	+	+	+	++	++	−	−	−	0.61
Corydalis pallida												
Callus	+	+	+	+	−	−	+	+	+	−	−	0.01
Redifferentiated plantlet	±	−	±	+	−	−	++	++	+	+	+	0.17
Papaver bracteatum												
Callus (dark)	+	+	+	+	−	−	+	+	+	−	−	0.028
Callus (light)	+	−	+	+	−	−	+	+	+	−	−	0.024

[a] From Ikuta *et al.* (1974).

callus subcultured on medium containing IAA had higher alkaloid levels than callus subcultured on medium containing 2,4-D (Ikuta *et al.*, 1974).

Kutchan *et al.* (1983) reported that cultured *Papaver bracteatum* cells from seedlings accumulate large amounts of dopamine (0.1–4 mg per gram fresh weight), and small amounts of thebaine (0–6 μg per gram fresh weight) and sanguinarine (10–50 μg per gram fresh weight). In static cultures, thebaine was detected in small amounts, mainly in shoots and meristemoids grown in the presence of 5 mg/liter BA or 2 mg/liter indolylbutyric acid (IBA) and 2 mg/liter BA. The transfer of cells to media without 2,4-D, BA, or IBA induced plant regeneration, and thebaine accumulation was enhanced considerably in shoots and meristemoides. Moreover, when static cultures were transferred to hormone-free cell suspension medium, time-dependent change in dopamine content and elevation of thebaine content occurred. A direct correlation between thebaine content and the extent of organogenesis was observed.

Table V

Alkaloid Content of Callus Tissues and Rhizome of the Original and Regenerated *Coptis* Plant[a]

Culture periods (weeks)	Tissue	Growth regulators in culture medium	Alkaloid: Berberine (μg/g fr. wt)	Alkaloid: Berberine (μg/g d. wt)	Alkaloid: Jatrorrhizine (μg/g fr. wt)	Alkaloid: Jatrorrhizine (μg/g d. wt)	Growth[b] (mg fr. wt/flask)
6	Callus	None	432	—	456	—	250
6	Callus	2,4-D(1 mg/l)	584	—	912	—	375
3	Callus	2,4-D(1 mg/l) + ki-	425	—	434	—	325
6	Callus	netin(0.1 mg/l)	774	9.0	816	10.0	500
	Rhizome of the regenerated plant[c]		—	42.6	—	19.9	—
	Rhizome of the original plant		—	50.4	—	11.5	—

[a] From Ikuta *et al.* (1975).
[b] Initial fr. wt of callus tissues per flask was 160 mg.
[c] Alkaloid content was estimated 5 years after the regeneration from callus cultures.

D. *Coptis japonica*

Friable yellow callus derived from the petiole of *Coptis japonica* plants was subcultured on MS medium containing 1 mg/liter 2,4-D and 0.1 mg/liter kinetin. All the main alkaloids in the rhizome of this plant were found to be present in callus cultures; berberine and jatrorrhizine were the main alkaloids. The restoration of the parental alkaloid content was observed in the rhizome of plants regenerated from callus cultures (Table V). The results indicate that plantlets regenerated from callus cultures were normal in both morphology and biosythetic activity (Ikuta *et al.*, 1974).

VIII. CONCLUSIONS

Several previous attempts have been made to produce useful medicinal alkaloids by cell culture. Some callus tissues have, indeed, produced many kinds of isoquinoline alkaloids, particularly callus tissues of *Papaver somniferum, Thalictrum minus, Coptis japonica,* and *Fumaria capreolata*. These results show that callus tissues have sufficient biosynthetic ability, and the production of berberine from callus cultures seems to carry hopes of success. On the other hand, tissue cultures of *Corydalis* species have produced the new compound **53** by way of a biotransformation experiment. This fact suggested that it is possible to produce new useful compounds from plant tissue cultures using the biotransformation of special substrates. Moreover, as many results of plant chemistry may enhance the development of plant chemotaxonomy, so the results of analyses of secondary product formation with callus tissues may help to distinguish one group of original plants from another.

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CHAPTER 18

Isoquinolines (*Papaver* Alkaloids)

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I. INTRODUCTION

The opium poppy, *Papaver somniferum,* is one of man's oldest cultivated plants, its cultivation predating recorded history (De Candolle, 1886). The therapeutic use of poppy capsule latex was recorded by Theophrastus in the third century B.C. (Hort, 1916), and Dioscorides in A.D. 77 discussed the curative properties of the opium poppy and described the different uses for both latex and extracts of whole plants

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(Gunther, 1959). Today the constituents of the opium poppy still have a role in modern medicine for the treatment of severe and moderate pain, coughs, and diarrhea.

Opium is the dried cytoplasm of a specialized internal secretory system, the laticifer. When the unripe capsule is cut, cream-colored latex oozes to the surface, where it dries to form a dark brown sticky material that is harvested as raw opium. More than 40 alkaloids have been identified in *P. somniferum* (Bentley, 1971; Santavy, 1970), at least 25 of which occur in the latex (Osol and Pratt, 1973). However, of prime importance from a medicinal viewpoint are the benzylisoquinolines, papaverine and noscapine (narcotine), and the phenanthrenes (morphinans), codeine and morphine.

The opiates are industrial commodities of plant origin for which there is still considerable demand. The 1986 demand for legal opium is estimated to be in excess of 1,000,000 kg (Anonymous, 1985). The requirements for the isolated alkaloids are 663,462 kg for codeine and 197,862 kg for morphine. At this time, supplies from the legal cultivation of the opium poppy are adequate, although legal production and exportation has been limited since 1953 by the United Nations Opium Conference Protocol. The world requirements and the limited availability of codeine per se from the poppy plant has made codeine production by stable cell cultures of the genus *Papaver* an obvious target for exploitation. *Papaver* species produce a wide range of isoquinolines, sometimes with very high yields, and within the individual species there is considerable intraspecific variation in alkaloid content (Phillipson, 1983). The major producers of the morphinans are *P. somniferum* L. and *P. bracteatum* Lindl., but this group of alkaloids has also been reported in low yield from *P. fugax* L., *P. setigerum* D.C., *P. orientale* L., and *P. rhoeas* L. (Phillipson, 1983), and from herbarium material of *P. acrochaetum* Borm., *P. caucasicum* Bieb., *P. cylindricum* Cullen, *P. gracile* Boiss., and *P. persicum* Lind. (Wieczorek *et al.*, 1986).

The industrial production of opiates from tissue culture is dependent on the large accumulation of alkaloids in cells or culture medium. While there are some major successes in plant cell culture in terms of cells with high yields of isoquinolines, the most important members of this group, from a commercial and pharmaceutical viewpoint, the morphinans, have proved difficult to produce in plant cell cultures.

Most cultured *Papaver* cells, either as callus or cell suspensions, readily produce sanguinarine, dihydrosanguinarine, norsanguinarine, and oxysanguinarine (Ikuta *et al.*, 1974; Kozovkina and Rabinovich, 1981; Kutchan *et al.*, 1985). Isolations of magnoflorine (Ikuta *et al.*, 1974), styl-

opine (Kamimura *et al.*, 1976), cryptopine (Furuya *et al.*, 1972; Anderson *et al.*, 1983), chelirubine (Ikuta *et al.*, 1974), noscapine (Khanna and Sharma, 1977), protopine (Forche and Frautz, 1981), orientalidine, and isothebaine (Lockwood, 1981) have also been reported (Fig. 1 and Table I). Numerous reports of the production of morphinans, thebaine, co-

(**1**) Stylopine

(**2**) orientalidine

(**3**) sanguinarine $R^1 = R^2 = CH_2$; $R^3 = H$

(**7**) chelerubine $R^1 = R^2 = CH_2$; $R^3 = OCH_3$

(**8**) protopine $R^1 = R^2 = CH_2$

(**9**) cryptopine $R^1 = R^2 = CH_3$

(**4**) oxysanguinarine R = O

(**5**) dihydrosanguinarine R = H

(**10**) noscapine

(**6**) norsanguinarine

(**11**) Isothebaine

(**12**) magnoflorine

Fig. 1. Nonmorphinan alkaloids from *Papaver* species.

Table I

Alkaloids[a] of *Papaver* Cell Cultures

<table>
<tr><th rowspan="2"></th><th colspan="7">Benzophenanthridines</th><th colspan="2">Protopines</th><th colspan="2">Aporphines</th><th rowspan="2">Phthalide isoquinoline (10)</th><th colspan="3">Morphinan</th></tr>
<tr><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>11</th><th>12</th><th>13</th><th>14</th><th>15</th></tr>
<tr><td>P. somniferum</td><td>+</td><td>−</td><td>+</td><td>+</td><td>+</td><td>+</td><td>−</td><td>+</td><td>+</td><td colspan="2">+</td><td>−</td><td>+</td><td>+</td><td>+</td></tr>
<tr><td>P. setigerum</td><td>−</td><td>−</td><td>+</td><td>+</td><td>+</td><td>+</td><td>−</td><td>+</td><td>−</td><td colspan="2">+</td><td>−</td><td>−</td><td>−</td><td>−</td></tr>
<tr><td>P. bracteatum</td><td>−</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>−</td><td colspan="2">+</td><td>−</td><td>+</td><td>−</td><td>−</td></tr>
<tr><td>P. orientale</td><td>−</td><td>−</td><td>+</td><td>+</td><td>+</td><td>+</td><td>−</td><td>−</td><td>−</td><td colspan="2">−</td><td>−</td><td>−</td><td>−</td><td>−</td></tr>
<tr><td>P. rhoeas</td><td>−</td><td>−</td><td>+</td><td>+</td><td>+</td><td>+</td><td>−</td><td>−</td><td>−</td><td colspan="2">−</td><td>+</td><td>+</td><td>−</td><td>+</td></tr>
<tr><td>P. nudicule</td><td>−</td><td>−</td><td>+</td><td>−</td><td>−</td><td>−</td><td>−</td><td>+</td><td>−</td><td colspan="2">−</td><td>−</td><td>−</td><td>−</td><td>−</td></tr>
</table>

[a] See Fig. 1 for structures.

Fig. 2. Morphinan alkaloids and precursors.

deine, and morphine (Fig. 2), from cell cultures of *P. somniferum* and *P. bracteatum* occur in the literature, although yields are low compared with the high yields of plants. Research suggests that culture conditions can be manipulated to promote morphinan alkaloid production (Constabel, 1985).

II. FACTORS THAT PROMOTE THE PRODUCTION OF MORPHINAN ALKALOIDS IN TISSUE CULTURE

A. Occurrence

Alkaloids were first reported from extracts of *P. somniferum* callus cultures by Ranganathan *et al.* (1963) but without precise chemical identification. Since then the numerous reports on the production of morphinans in tissue cultures of *Papaver* have shown the occurrence of these alkaloids, in significant amounts, to be a rare phenomenon.

The availability of radioimmunoassay and other improved analytical systems may, in part, explain the surge in reports of the accumulation of morphinan alkaloids that has occurred since 1980. To date, the most promising results have been obtained with some cell cultures developed from *P. bracteatum*, which produces thebaine, and *P. somniferum*, which has been shown to produce thebaine, codeine, and morphine. However, close inspection of recent analyses show concentrations of morphinans in cell cultures that do not exceed that of leaf tissue (0.14% dry weight) and that are much lower than levels of alkaloid normally found in the dried latex (opium) (≃20% dry weight).

B. Genetic Factors

Material of *P. bracteatum* employed in cell-culture work has usually originated from cv. Arya II (Constabel, 1985), whereas *P. somniferum* material has generally been ill defined given the large number of cultivars that occur. Nyman and Hall (1976) stated, "Very little of the inheritance of the opium alkaloids is known today, although varietal studies on this were started 35 years ago." Since then this Swedish group has described a number of chemovarieties of *P. somniferum* and thus has contributed to the genetic control of some steps of the biosynthetic pathways and dominance relationships of the morphinan alkaloids (Nyman, 1978, 1980; Nyman and Hansson, 1978). More recently the inheritance of the five major alkaloids, morphine, codeine, thebaine, noscapine, and papaverine, has been studied in the interspecific cross *P. somniferum* × *P. setigerum;* increases in codeine and thebaine were found in different F_1 plants, whereas in some F_2 plants the content of all these alkaloids, except that of codeine, exceeded the content of the parental and F_1 generations. The absence of noscapine was generally dominant

over its presence (Khanna and Shukla, 1986). The multitude of genotypes of *P. somniferum* and its hybrids would suggest that variation in response to the *in vitro* culture conditions may be exploited to produce high-yielding cell lines for a particular alkaloid or group of alkaloids. However, Constabel (1985) surveyed 46 different samples of seeds obtained through the Gene Resources Center, Ottawa, from several botanic gardens and failed to develop morphine-producing cell cultures. Developments in the production of radioimmunoassay (RIA) for the morphinan alkaloids (Hodges and Rapoport, 1982a; Hsu *et al.*, 1983a) have greatly facilitated monitoring cell culture lines. Hodges and Rapoport (1982a) showed, through RIA, that many of their cultures of *P. somniferum* contained morphinan alkaloids. In contrast to the alkaloid distribution found in whole plants, five of the cultures accumulated a predominance of codeine or thebaine. Since in *P. bracteatum* the biosynthetic route to morphine stops at thebaine, whereas in *P. lasiotrix* the breakoff point is salutaridine (Sariyar and Phillipson, 1977), it seems likely that there exists, or can be developed, plants or cell cultures with high codeine yields. Studies on these latter stages in morphinan alkaloid biosynthesis with the location and isolation of the enzymes would seem of prime importance. Work in this area commenced in the 1970s (Roberts and Antoun, 1978; Antoun and Roberts, 1975), using the whole plant, and is being actively investigated, using cell cultures, by Zenk's group. The isolation and purification of many of the enzymes of the biosynthetic pathway are now well documented (Zenk, 1985; Zenk *et al.*, 1985). It is expected that current work on the enzymes of the biosynthetic pathway will stimulate research on the inheritance of the enzymes required for the production of the morphinans. Wieczorek *et al.* (1986) have developed RIA for subnanomole quantities for six of the opium alkaloids, (*S*)- and (*R*)-reticuline, salutaridine, thebaine, codeine, and morphine. These assays were used to screen herbarium material of 100 *Papaver* species and to analyze *P. somniferum* plant populations for breeding purposes. This particular group of RIAs should prove invaluable for developing cell culture lines high in particular morphinan alkaloids.

C. Effect of Cultural Conditions and Plant Regeneration on Morphinan Production

Plant cell cultures may be considered a useful means of studying cell differentiation and morphogenesis by varying nutritional factors, environmental conditions, and hormone regimes. The production of sec-

ondary metabolites has been associated with these factors; consequently, the concept has arisen that the development of appropriate nutritional and hormone regimes would promote the production of morphinans in the plant cell culture to levels similar to those observed in the whole plant. An attempt to evaluate the nutrient media that have been used has been made in Tables II through V. From the data available in the literature, no one medium and hormone combination would appear to guarantee successful alkaloid production.

Media after Gamborg *et al.* (1968) and Murashige and Skoog (1962) are most commonly used for *Papaver* cultures and have supported successful alkaloid production. High levels of the hormone 2,4-dichlorophenoxyacetic acid (2,4-D) (>1 mg liter^{-1}) often prevented alkaloid production, although Tam *et al.* (1980) successfully isolated codeine from cultures grown on a medium high in 2,4-D. The presence of cytokinins, that is, benzyladenine and kinetin, appears to be beneficial for morphinan alkaloid production and permitted codeine formation, according to Staba *et al.* (1982) and Hodges and Rapoport (1982a). Yoshikawa and Furuya (1985) have reported the production of codeine and thebaine in green callus with relatively high levels of kinetin or *N*-phenyl-*N'*-(4-pyridyl)urea. The addition of tyrosine and ascorbic acid as media supplements was also found to promote morphinan accumula-

Table II

Thebaine Accumulation in *Papaver bracteatum* Cell Cultures

Thebaine (mg g^{-1})	Morphogenic response	Analytical method[a]	Reference
0.060[b]	Callus	TLC, GC	Kamimura *et al.* (1976)
0.0013[b]	1-year-old strain, and cv. Arya II cell suspension	TLC	Shafiee *et al.* (1976, 1978)
0.070[b]	cv. Arya II callus with shoots (several-year-old strain)	TLC, HPLC	Staba *et al.* (1982)
0.00087[c]	cv. Arya II callus with shoots and meristemoids (2-year-old strain)	HPLC	Kutchan *et al.* (1983)

[a] GC, Gas chromatography; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.
[b] Dry-weight value.
[c] Fresh-weight value.

Table III

Media Used for *Papaver bracteatum* Cell Cultures

Thebaine	Medium[a]	Auxin[b] (mg liter^{-1})	Cytokinin[c] (mg liter^{-1})	Supplement[d]	Reference
+	MS	2,4-D (1.0)	KIN (0.1)	——	Kamimura *et al.* (1976)
			KIN (1.0)	CM (10%)	
+	RT	2,4-D (0.1)	——	——	Shafiee *et al.* (1976)
+	MS	IBA (1.0)	BA (3.0)	——	Staba *et al.* (1982)
+	RT	——	——	——	Zito and Staba (1982)
+	RT	IAA	——	——	Zito and Staba (1982)
+	MS	——	BA (5.0)	——	Kutchan *et al.* (1983)
−	MS	2,4-D (2.0)	——	——	Kutchan *et al.* (1983)

[a] MS, Murashige and Skoog (1962); RT, Vincent *et al.* (1977).
[b] 2,4-D, 2,4-dichlorophenoxyacetic acid, IAA, indolyl-3-acetic acid; IBA, indolebutyric acid.
[c] BA, 6-benzyladenine; KIN, kinetin.
[d] CM, coconut milk.

tion (Kamimura *et al.*, 1976; Khanna *et al.*, 1978; Hsu, 1981), as have low levels of the inhibitors of protein formation, such as cycloheximide, puromycin, and actinomycin (Hsu, 1981). Although alkaloid precursor supplement has been used successfully to enhance the production of other alkaloids (Krueger and Carew, 1978; Anderson *et al.*, 1986; Deus and Zenk, 1982), confirmation of the effect of precursor feeding with *Papaver* cell cultures is lacking. There is evidence to suggest that media without hormones, while promoting cell differentiation, have also been successful in supporting morphinan alkaloid production. These observations have led to the suggestion that alkaloid production may be closely dependent on cell differentiation. The transfer of *P. somniferum* callus to solid or liquid media without hormones led to the formation of embryoids that physically resembled normal seed embryos, that is, were characterized by two stubby cotyledons and a short, thick hypocotyl–root axis (Nessler and Mahlberg, 1979). Unlike seed embryos, however, the cell-culture embryoids were found to accumulate thebaine (0.2% dry weight). At the same time a correlation was found between meristemoid development and high lipid content (Schuchmann and Wellmann, 1983; Yoshikawa and Furuya, 1985). A time-course study on the induction and maturation of somatic embryos confirmed the onset of thebaine production, which was developmentally regulated by gradual removal of auxin from the culture medium (Galewsky and Nessler, 1986). Under these conditions neither morphine nor codeine was produced in detectable quantity. It appears, therefore, that alkaloid synthesis in somatic em-

Table IV

Accumulation of Morphinan Alkaloids in *Papaver somniferum* Tissue Cultures

Alkaloid[a] ($mg\ g^{-1}$)					
Thebaine	Codeine	Morphine	Morphogenic response	Analytical methods[b] used for alkaloid identification	Reference
+	+	+	Callus and suspensions.	TLC, IR	Khanna and Khanna (1976)
14.9[c]	3.4[c]	13.1[c]	Suspensions with laticifer cells		Khanna *et al.* (1978)
–	1.5[a]	–	Cell suspensions (6- to 12-month-old strain)	TLC, GC	Tam *et al.* (1980)
0.012[d]	0.034[d]	0.001[d]	New callus	HPLC, RIA	Hodges and Rapoport (1982a)
0.0001[c]	0.004[c] 0.033[c]	–	Callus	HPLC, GC, MS	Kamo *et al.* (1982)
0.013[c]		–	Meristemoids		
+	+	–	Callus and root structure (several-year-old strain)	TLC, HPLC, GC, MS	Staba *et al.* (1982)
+	+	+	Cell suspensions; shoot buds	TLC, GC	Yoshikawa and Furuya (1982)
–	+	+	Callus	TLC, HPLC, GC, MS, NMR, UV, IR	Hutin *et al.* (1983)
2.0[c]	+	+	Embryoids; regenerated plantlets	TLC, GC	Schuchmann and Wellmann (1983)
0.18[d]	–	–	Suspensions (media only)	TLC, GC, MS	Lockwood (1984)
+	+	–	Green callus	TLC, GC	Yoshikawa and Furuya (1985)
0.015[c]	–	–	Embryoids	TLC, HPLC, GC, MS	Galewsky and Nessler (1986)

[a] +, Alkaloid detected but not quantified; –, alkaloid not detected.

[b] GC, gas chromatography; HPLC, high-pressure liquid chromatography, IR, infrared spectrography; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; RIA, radioimmunoassay; TLC, thin-layer chromatography; UV, ultraviolet spectroscopy.

[c] Dry-weight value.

[d] Fresh-weight value.

bryos requires a specific level of differentiation. Further, spontaneous loss of embryogenic potential in some culture lines disrupted alkaloid formation (Galewsky and Nessler, 1986). A requirement for specific types of tissue differentiation may partially explain the conflicting reports in the literature of morphinan alkaloid biosynthesis in tissue

Table V

Media Used for *Papaver somniferum* Cell Cultures

Morphinan alkaloids	Medium[a]	Auxin[b] (mg liter^{-1})	Cytokinin[c] (mg liter^{-1})	Supplement[d] (mg liter^{-1})	Reference
+	MS	2,4-D (0.1)	—	—	Khanna and Khanna (1976)
+	MS	—	—	Tyr. (12.5–1000)	Khanna *et al.* (1978)
+	MS	—	—	AA (500–1000)	Khanna *et al.* (1978)
−	MI/MS	NAA (0.2)	KIN (0.1)	CW (10%)	Nessler and Mahlberg (1979)
−	MS	2,4-D (0.1)	ZEA (0.025)	—	Morris and Fowler (1980)
−	MS	2,4-D (1.0)	KIN (0.1)	—	Morris and Fowler (1980)
+	B5	2,4-D (1.0)	KIN (0.1)	CH (1 g)	Tam *et al.* (1980)
+	MS	2,4-D (0.5)	KIN (0.1)	—	Hsu (1981)
+	MS	2,4-D (0.02)	—	CM (10%)	Hsu (1981)
+	MS	2,4-D (0.2)	2iP (0.03)	—	Hodges and Rapoport (1982a)
+	MS	NAA (0.2)	—	—	Hodges and Rapoport (1982a)
+	MS	—	KIN (0.03)	—	Hodges and Rapoport (1982a)
+	MS	2,4-D (0.1)	KIN (0.1)	—	Kamo *et al.* (1982)
+	MS	NAA (0.2)	KIN (0.4)	—	Kamo *et al.* (1982)
−	MS	2,4-D (2.0)	KIN (0.25)	—	Nessler (1982)
−	MS	NAA (2.0)	KIN (0.25)	—	Nessler (1982)
+	MS	2,4-D (1.0)	—	—	Staba *et al.* (1982)
+	MS	2,4-D (0.1)	—	—	Staba *et al.* (1982)
+	HE	2,4-D (0.1)	KIN (1.0)	—	Hutin *et al.* (1983)
+	B5	2,4-D (2.0)	—	—	Schuchmann and Wellmann (1983)
+	B5	—	—	—	Schuchmann and Wellmann (1983)
−	MS	2,4-D (10.0)	KIN (1.0)	—	Lockwood (1984)
−	MS	2,4-D (10.0)	KIN (0.1)	AA (10 g)	Lockwood (1984)
+	MS	2,4-D (1.0)	KIN (0.1)	CW (10%)	Yoshikawa and Furuya (1982, 1985)
+	MS	2,4-D (0.25)	—	—	Galewsky and Nessler (1986)
+	MS	—	—	—	Galewsky and Nessler (1986)

[a] B5, Gamborg *et al.* (1963); HE, Heller (Gautheret, 1959); MI, Miller (Gamborg *et al.*, 1968); MS, Murashige and Skoog (1962).
[b] 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid.
[c] 2iP, 2-isopentenyladenine; KIN, kinetin; ZEA, zeatin.
[d] AA, ascorbic acid; CH, casein hydrolysate; CW, coconut water; Tyr., L-tyrosine.

culture. *Papaver somniferum* cell cultures grown on media designed specifically to promote roots and shoots yielded significant levels of thebaine, codeine, and morphine, while regenerated plantlets had alkaloid levels at least quantitatively similar to those of normally grown seedlings. Indeed, the level of alkaloids, particularly thebaine, was higher than in normally produced seedlings of a similar developmental stage (Kamo *et al.*, 1982; Schuchmann and Wellmann, 1983; Yoshikawa and Furuya, 1985). Although in these particular experiments alkaloid levels were commensurate with levels found at the appropriate developmental stage of the normal plant, they do not compare favorably on a dry-weight basis for the levels found in the mature plant. In an analogous situation, it has been shown in *P. bracteatum* (Day *et al.*, 1986) that a large yield of thebaine can be produced in regenerated plants that have been derived from the embryogenic callus cultures.

D. Correlation of Morphinan Production with Laticifer Formation

In *P. somniferum* and *P. bracteatum* the morphinan alkaloids accumulate in the latex, which is contained in structurally and physiologically specialized cells, the laticifers. Roberts *et al.* (1983, and references therein) demonstrated that both the 1000 *g* vacuoles and the supernatant fraction of the latex are required for alkaloid biosynthesis, and that a subpopulation of dense vacuoles of the 1000 *g* pellet functions as a storage compartment for alkaloids. Because morphine may constitute as much as 10 to 20% of the latex, the alkaloid content of these vacuoles reaches very high levels. Homeyer and Roberts (1984) showed that [^{14}C]morphine readily moved across the vacuolar membrane and was not displaced from the vacuole in the short term, although in the long term some turnover of alkaloid in these vacuoles has been observed (Fairbairn and Steele, 1981, and references therein). The ability of these vacuoles to store the morphinan alkaloids without significant metabolic degradation determines the high levels of alkaloid that accumulate in these plants, and this suggests that to obtain commercially viable levels of the morphinan alkaloids in tissue culture, the development of laticifer-like cells, or something equivalent, may be essential.

The data given in Tables II through V suggest that the appearance of tracheids in cell cultures may be of importance in identifying differentiation that may lead to alkaloid accumulation. The extent to which laticiferous cells may be present in cell cultures that produce morphinans is not available for some of the earlier work. Details on the development of laticifers and laticifer-like cells in young seedlings and plantlet re-

generants is now well documented (Thureson-Klein, 1970; Nessler and Mahlberg, 1977, 1979). A correlation in *P. bracteatum* seedlings between the onset of thebaine formation and the development of laticifers exists (Rush *et al.*, 1985). The formation of laticifers in cell cultures of *P. bracteatum* has been demonstrated (Kutchan *et al.*, 1985, 1986). In this latter instance, thebaine was apparently found almost exclusively in the vacuoles isolated from the laticiferous cells (Kutchan *et al.*, 1985, 1986).

Investigation by Nessler *et al.* (1985) has shown the occurrence of latex-specific proteins. Polyclonal antibodies prepared against these major latex proteins (MLPs) and secondarily labeled with fluorescein isothiocyanate indicated that MLPs are only found in poppy latex, specifically in the latex cytosol, and not the alkaloid-containing vacuoles. Nessler *et al.* (1985) speculated that these MLPs may represent some of the enzymes associated with alkaloid biosynthesis and that are known to be cytosolic enzymes (Roberts *et al.*, 1983, and references therein; Zenk, 1985). The development of the MLP antibodies should make the detection of laticifers in tissue culture easier, and should the MLPs prove to represent certain key enzymes of biosynthesis, they will be a useful tool for probing the sites of alkaloid synthesis in both higher plants and in cell culture. Two membrane-associated enzymes of berberine synthesis, berberine bridge enzyme (BBE) and (*S*)-tetrahydroprotoberberine oxidase (STOX), have been found exclusively in vesicles of dictyosomal origin with a density of 1.14 g cm^{-3}, as shown by direct enzymatic assay as well as immunoelectrophoresis. Freeze–thaw methods easily released both enzymes (Amann *et al.*, 1986). Because the STOX enzyme is membrane bound and is also known to convert (*S*)-reticuline to 1,2-dehydroreticuline (Zenk, 1985), it is interesting to speculate on the localization of the enzyme that converts 1,2-dehydroreticuline to (*R*)-reticuline and the controversial enzyme that promotes the conversion of (*R*)-reticuline to salutaridine (Hodges and Rapoport, 1982b; Zenk, 1985; Fig. 2). These enzymes may also be bound to the membrane of a similarly specialized vesicle and hence may be responsible for the difficulties that occur in obtaining and maintaining cell cultures rich in the morphinans.

The whole problem of concurrent cytodifferentiation and morphinan alkaloid accumulation appears further compounded by the detection of cells that resemble the early stages in laticifer formation, that is, cells rich in vesiculating endoplasmic reticulum that, however, do not accumulate alkaloids (Nessler and Mahlberg, 1977). In many instances cell cultures are reported to lose the ability to produce alkaloids with time (Hodges and Rapoport, 1982a; Kamimura *et al.*, 1976; Kamimura and Nishikawa, 1976; Constabel, 1985). One may question whether this results from repression or loss of the enzymes of synthesis, or through stimulation of the further metabolism of the alkaloids formed.

E. Role of Stress in Alkaloid Production

It has been suggested by Constabel (1985) that the occurrence of codeine reported by Tam *et al.* (1980) may have been the result of stress because attempts to duplicate the work were not successful. While the occurrence of codeine, as shown in Table IV, may today be explained by assuming an undetected cytodifferentiation, it may also, or instead, relate to an unregistered temporary stress.

Stress has been identified as a factor that may increase morphinan production; Lockwood (1984) reported exposure of cells to 5°C for a period of 3 days prior to harvesting as beneficial to thebaine accumulation. Temperature stress is still a factor to be thoroughly evaluated. Other possibilities for producing stress certainly exist. Laughlin and Munro (1983) observed a 75% increase in morphine concentration of leaves and stems subsequent to infection of plants with *Sclerotinia sclerotiorum*. These observations have prompted further investigations with cell cultures. Elicitors derived from pathogenic microorganisms, that is, autoclaved broad-spectrum wilt fungi conidia and homogenates, have been used with *P. somniferum* to increase yields of morphinans (Heinstein, 1985). Constabel's group, while not able to repeat the elicitation of increased levels of morphinans, were able to demonstrate considerably enhanced yields (2.5% dry weight) of sanguinarine (Eilert *et al.*, 1985). The cells also responded with browning but did not show lysis (Eilert and Constabel, 1985). Other pathogens elicited a somewhat weaker response. These cell cultures appeared to be principally parenchyma cells, and the only changes observed were the occurrence of electron-dense droplets dotting the tonoplast, stacking of the endoplasmic reticulum, and dilation of cisternae. Sanguinarine occurrence appears to require no cell differentiation. The extent to which alkaloid production in cell culture results from stress factors merits further investigation, because such a reaction may help to elucidate the factors that initiate enzyme formation and activation, and subsequently, alkaloid biosynthesis.

III. BIOTRANSFORMATIONS

Biotransformation experiments with cell cultures may shed light on the variability of cell-culture synthesis of the morphinan alkaloids. Hodges *et al.* (1977) found *P. bracteatum* not only contained the enzyme sequence to thebaine but also that which will reduce codeinone to co-

deine; however, enzymes for the demethylation to produce either codeinone or morphine were not present. Thebaine in these experiments was also metabolized, but not to either oripavine or northebaine via direct demethylation. Latex from *P. somniferum* may convert codeine to morphine; the formation of codeine and morphine *N*-oxides has also been observed (Fairbairn *et al.*, 1978). Demethylation has been established as an active metabolic process (Miller *et al.*, 1973), and normorphine has been found in raw opium (Phillipson *et al.*, 1976). The degradative pathway from morphine most likely involves initial demethylation to normorphine, which is subsequently degraded to nonmorphine metabolites. Vágújfalvi and Petz-Stifter (1982) showed that oxidation is a major degradation process in whole-plant latex, with the formation of *N*-oxides and pseudomorphine from morphine in the presence of H_2O_2 and peroxidase.

Papaver cell cultures with poor performance as producers of the morphinan alkaloids would appear to be ideal candidates for investigations of alkaloid formation and degradation. For the production of the morphinan alkaloids there is an absolute requirement for (*R*)-reticuline. Although in whole plants (*S*)-reticuline is formed from (*S*)-norlaudanosoline, it is readily converted to the *R* isomer in *P. somniferum* (Zenk, 1985) (Fig. 2). The isomer ratio *S*:*R* is about 3:1 in the latex (Wieczorek *et al.*, 1986). In plant cell cultures (*R*,*S*)-reticuline was stereospecifically converted into (*S*)-scoulerine and (*S*)-cheilanthifoline, but no apparent utilization was made of the (*R*)-reticuline (Furuya *et al.*, 1978). This group also presented evidence for the conversion of (−)-codeinone to (−)-codeine and showed that their cell cultures would not further metabolize thebaine, codeine, or morphine. Cell cultures of *P. somniferum* cv. Marianne were investigated (Tam *et al.*, 1982) and found to convert thebaine to neopine (3%) and codeinone to codeine (1.5%) but could not metabolize codeine, neopine, or DL-norlaudanosoline. Further progress in this area has been made with the immobilization of *P. somniferum* cells on calcium alginate, where they continue to demonstrate biological activity for up to 6 months. The immobilized living cells performed the biotransformation of (−)-codeinone to (−)-codeine in both shake flasks and a column bioreactor. The biotransformation ratio in the shake flask (70.4%) for immobilized cells was higher than for cell suspensions (60.8%). Furthermore, 88% of the codeine produced was excreted into the medium. The column bioreactor was functional for 30 days under optimal conditions and had a conversion ratio of 41.9% (Furuya *et al.*, 1984). Investigations of biotransformations with cell cultures thus highlight some of the problem steps in the biosynthesis of morphinans and at the same time show that operative enzymes under ideal conditions can produce potentially commercially useful levels of a given product.

IV. CONCLUSIONS

Since the elegant review of morphinan alkaloid production in *Papaver* cell cultures by Constabel (1985), little real progress appears to have been made that would increase the probability of commercial production of these alkaloids from cell culture. Although there are more data to suggest that cell differentiation is vital for morphinan production, the overall picture remains unclear. It is possible that alkaloid synthesis may not require a laticiferous system, but that large-scale accumulation of the alkaloids may require the development of specialized sequestratory cells, the use of the media as a lytic compartment, as suggested by Wink (1984), or the development of an artifactual equivalent.

The presence of laticifers in *P. somniferum* somatic embryos (Nessler, 1982) and the presence of morphinan alkaloids in those embryos derived from *P. somniferum* cell cultures (Schuchmann and Wellmann, 1983) make the commercial production of morphinan alkaloids a distinct possibility, provided, of course, that somatic embryogenesis can be accomplished on a scale large enough. The fact that most cell cultures producing morphinan alkaloids produce thebaine and codeine rather than morphine is a favorable aspect in that the requirement for the synthesis of codeine from morphine (Hodges and Rappoport, 1982a) is obviated.

The interest in the use of fungal elicitors to improve alkaloid yield may have far-reaching consequences. Not only are they expected to play an important role in the elucidation of the induction of enzymes catalyzing reactions that lead to natural products (Darvill and Albersheim, 1984), but as a technique it may be amenable to commercial application.

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CHAPTER **19**

Quinoline Alkaloids of *Cinchona*

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I. INTRODUCTION

The genus *Cinchona* (Rubiaceae) has been of great therapeutic value for many years. The antimalaria activity of extracts of *Cinchona* bark has been known for centuries, and this has led to its widespread use. The compound found to be responsible for this activity is quinine. The importance of quinine as an antimalaria drug has decreased considerably due to the advent of synthetic drugs that are more effective. As the

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malaria parasites have developed resistance against most of the synthetic drugs, however, there is a renewed interest in the usè of quinine for the treatment of malaria (Overbosch *et al.*, 1984; van der Kaay, 1986). Quinidine, another major *Cinchona* alkaloid, is used for the treatment of cardiac arrhythmias and has been shown to also be an effective antimalaria agent (Warhurst, 1981). Besides their pharmaceutical use the *Cinchona* alkaloids are used extensively in the food and soft drink industry because of their bitter taste. It is estimated that the total annual trade in *Cinchona* alkaloids amounts to 50,000,000 U.S. dollars (UNCTAD/GATT Report, 1982).

Cinchona alkaloids are still extracted from the bark of *Cinchona* species, the best known of which are *C. ledgeriana* Moens and *C. pubescens* Vahl (synonym, *C. succirubra* Pavon ex Klotsch). The taxonomy of the genus, however, is very complex due to extensive crossing among different species, and hence it is very difficult to identify a given plant. Some authors argue that *C. ledgeriana* is not a distinct species (Ridsdale *et al.*, 1985).

Cinchona trees have been cultivated in plantations for more than 130 years for production of *Cinchona* bark, the raw material for the extraction of the alkaloids. After about 7 to 12 years of growth, the bark of the trees is harvested, at which time the alkaloid content can be as high as 18%, but generally the alkaloid content is about 12% (Smit, 1984). Not only quinine and quinidine, but some 35 other alkaloids have also been found in *Cinchona*. Besides quinine and quinidine and their dihydroderivatives, cinchonine and cinchonidine, with their corresponding dihydroderivatives, are major alkaloids in *Cinchona* bark. In Scheme 1 the structures and biosynthetic routes are given. In the leaves of *Cinchona*, indole alkaloids constitute the major components (Zèches *et al.*, 1980; Keene *et al.*, 1983), especially the semidimeric cinchophyllines. Also the monomeric indole alkaloids aricine, cinchonamine, 10-methoxycinchonamine, and quinamine are more abundant in the leaves than in the bark.

Much research has been directed at the production of useful compounds in plant tissue culture systems. First reports on the cell and tissue culture of *Cinchona* concerned micropropagation of trees, with special emphasis on the propagation of high alkaloid-yielding clones (Chatterjee, 1974; Hunter, 1979; Krikorian *et al.*, 1982; Koblitz *et al.*, 1983a, 1984). Other reports showed the use of *Cinchona* cell cultures for studies of the biosynthesis of alkaloids. For several reasons *Cinchona* seems an interesting model system. First of all the biosynthetic routes (Scheme 1) are rather straightforward. Second, in the plants the biosynthesis of the alkaloids takes place in the leaves, according to Klein

Secologanin + Tryptamine

Strictosidine

Corynantheal

Cinchophyllines

Cinchonaminal

Cinchonamine

Quinamine

R = H Cinchonidinone
R = OCH_3

R = H
R = OCH_3 Quinidinone

R = H Cinchonidine
R = OCH_3 Quinine

R = H Cinchonine
R = OCH_3 Quinidine

Scheme 1. Biosynthesis of *Cinchona* alkaloids.

Horsman-Relijk (1960). After their synthesis, the alkaloids are transported to the bark, where they are stored. So one would expect that in suspension cultures of *Cinchona*, different types of cells will exist, namely, cells that synthesize the alkaloids and excrete them, and cells that absorb the alkaloids and subsequently store them. The *Cinchona* cell culture system would thus offer opportunities to study the mechanisms involved in these processes. In fact, there exist at least two types of cells in *Cinchona* suspension cultures, as can be visualized using fluorescence microscopy. Some of the cells contain blue-fluorescing compounds, maybe the quinoline alkaloids (see Neumann *et al.*, 1983). Other cells contain orange- or red-fluorescing compounds that are probably anthraquinones (R. Wijnsma, unpublished results).

That *Cinchona* cell cultures produce two kinds of secondary metabolites at the same time makes it an interesting system to study competitive effects for mutual common precursors in corresponding pathways. Competition for a mutual precursor has been described also for *Galium mollugo* cell cultures (Leistner, 1985).

In the following paragraphs the various aspects of cell and tissue culture of *Cinchona*, cell culture methods, analytical methods, alkaloid production, and biosynthetic studies using *Cinchona* tissue culture systems are dealt with in more detail.

II. CELL CULTURE METHODS

First reports on callus and cell suspension cultures of *Cinchona* were presented by Staba and Chung (1981). The media used by them and various other authors are listed in Tables I and II. Optimization of the growth media for *C. pubescens* callus was reported by Mulder-Krieger *et al.* (1982a). The influence of the mineral salt composition as well as the influence of the quantity and quality of growth hormones was systematically investigated. Murashige–Skoog (MS) medium with half-strength mineral salts in combination with naphthaleneacetic acid (NAA) (2–3 ppm) and kinetin (0.1–0.2 ppm) gave the best growth. Another study aimed at optimization of the medium for growth and secondary metabolite production by Harkes *et al.* (1985), who applied the method described by De Fossard *et al.* (1974), showed that good growth of *C. ledgeriana* tissue cultures can be obtained with media containing high concentrations of mineral salts in combination with high levels of auxins and high levels of organic nutrients. Wijnsma *et al.* (1986a) investigated

Table I

Media Used for *in Vitro* Culture of *Cinchona ledgeriana* and Maximum Alkaloid Contents Found

Culture type	Basal medium[a]	Auxin: Type[b]	Auxin: Concentration (ppm)	Cytokinin: Type[c]	Cytokinin: Concentration (ppm)	Additives[d]	Alkaloid content (μg/g)	Analysis method[e]	Reference
Callus, suspension, root	B5	NAA	2				Unknown	–	Whitten and Dougall (1981)
Suspension, root, shoot	MS	2,4-D	1	Kin.	0.1	PVP	400 250 25	HPLC DW	Anderson *et al.* (1982)
Suspension	MS	2,4-D	0.5	BA	3		—	HPLC	Staba and Chung (1981)
Root		IBA	3	—			—	DW	
Shoot		—		BA	5		4500		
Callus	MS	2,4-D	0.5	BA	3		170	HPLC	Chung and Staba (1984)
Root		IBA	3	—			260	DW	
Shoot		—		BA	5		2840		
Shoot	MS[f]	IBA	1	—		GA, phloro.	3	RIA, FW	Robins *et al.* (1984)
Shoot	MS	IBA IAA	1 0.2	BA	1	GA, phloro.	—	—	Koblitz *et al.* (1983a)
Callus	[g]	Mixture		Mixture			130	HPLC, FW	Harkes *et al.* (1985)
Shoot	MS[f]	IBA	1	BA	1	GA, phloro.	—	—	Hunter (1979)
Callus	MS	2,4-D	0.22	Zea.	0.22	Cysteine	1100	Weight, DW	Mulder-Krieger *et al.* (1982c)

(*continued*)

Table I (*Continued*)

Culture type	Basal medium[a]	Auxin: Type[b]	Auxin: Concentration (ppm)	Cytokinin: Type[c]	Cytokinin: Concentration (ppm)	Additives[d]	Alkaloid content (μg/g)	Analysis method[e]	Reference
Suspension	B5	2,4-D	1	Kin.	0.2		43	HPLC, DW	Wijnsma *et al.* (1986a)
Callus	N&N	NAA	7.5	Kin.	0.5–1		Unknown	Unknown	Noerhadi (1982)
Suspension	B5	2,4-D	1 or 2	BA	0.5		170	HPLC	Hunter *et al.*
				Kin.	1	L-Tryp.	520	DW	(1982)
Shoot	MS	IBA	Variable	BA	Variable	GA, phloro.	—	—	Krikorian *et al.* (1982)
Suspension	MS	2,4-D	2.23	—		GA, phloro.	—	—	Krikorian *et al.* (1982)
Suspension	B5	2,4-D	0.5	BA	0.1		10	FW	Parr *et al.* (1984a,b)
Root	MS	2,4-D	1	Kin.	0.1		220[h]	HPLC	Hay *et al.*
						L-Tryp.	1150		(1986)
Suspension	B5	2,4-D	1	Kin.	0.1	PVP	—	—	Allan and Scragg (1985)
Shoot	—	IBA, IAA, NAA							Chatterjee (1974)

[a] B5, Gamborg B5; MS, Murashige and Skoog; N&N, Nitsch and Nitsch.
[b] 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indoleacetic acid; IBA, indolebutyric acid; NAA, naphthaleneacetic acid.
[c] BA, benzyladenine; kin., kinetin; zea., zeatin.
[d] GA, gibberelic acid; L-tryp., L-tryptophan; phloro., phloroglucinol; PVP, polyvinyl pyrrolidone.
[e] DW, dry weight; FW, fresh weight; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay.
[f] MS medium modified after Jones *et al.* (1977).
[g] Basal medium was varied.
[h] Only alkaloid yields are given.

Table II

Media Used for *in Vitro* Culture of *Cinchona pubescens* and Maximum Alkaloid Contents Found[a]

Culture type	Basal medium[a]	Auxin: Type	Auxin: Concentration (ppm)	Cytokinin: Type	Cytokinin: Concentration (ppm)	Additive	Alkaloid content (μg/g)	Analysis method	Reference
Suspension, root, shoot	MS	2,4-D IBA ——	0.5 3	BA —— BA	3 5	——	0 0 3800	HPLC DW	Staba and Chung (1981)
Callus, suspension,	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	1196 74	HPLC DW	Creche *et al.* (1985)
Shoot	MS	IBA	1	BA	1	GA, phloro.	——	——	Koblitz *et al.* (1983a)
Suspension	MS	2,4-D	1	Kin.	0.2	GA, L-tryp.	100 9200	TLC/fluor.[b], DW	Koblitz *et al.* (1983b)
Suspension	MS	2,4-D	1	Kin.	0.2	GA, L-tryp.	25 857	TLC/fluor., DW	Schmauder *et al.* (1985)
Callus	MS	2,4-D	0.22	Zea.	0.22	Cysteine	1000	Weight, DW	Mulder-Krieger *et al.* (1984)
Callus	MS	Variable		Variable		Cysteine	212	TLC/fluor., DW	Mulder-Krieger *et al.* (1982b)
Callus	MS	2,4-D IBA	1 5 or 8	BA	1 or 2	Cysteine	——	——	Mulder-Krieger *et al.* (1982a)

[a] See Table I for explanation of abbreviations.
[b] TLC/fluor., thin-layer chromatography and fluoroscence.

the influence of varying sucrose and nitrate levels (carbon/nitrogen ratio) on the growth and secondary metabolite formation in *C. ledgeriana* cell suspension cultures. It was found that best growth occurred in a medium containing the level of nitrate as in B5 medium and 4% sucrose.

From data available in the literature it can be concluded that the growth of *Cinchona* callus and suspension cultures is generally very slow. No specific growth rates or doubling times have been reported, but based on results by Koblitz *et al.* (1983b) it can be calculated that the doubling time of a cell suspension culture of *C. pubescens* is about 100 hr. This agrees very well with the doubling time reported for a *C. ledgeriana* culture (Wijnsma *et al.*, 1986a).

Several authors observed browning and necrosis of *Cinchona* cultures. To prevent this, several antioxidants were added to the culture media. Cysteine was found to be effective at concentrations of 50 mg/liter (Mulder-Krieger *et al.*, 1982a,b). Phloroglucinol also was found to be effective in concentrations of 0.5 to 1.0 m*M* (Hunter, 1979; Koblitz *et al.*, 1983b; Robins *et al.*, 1984).

In addition to unorganized tissue cultures of *Cinchona*, several authors have described morphologically differentiated cultures. Staba and Chung (1981) showed that by varying the hormone concentration, it was possible to obtain either shoot or root or callus cultures. Anderson *et al.* (1982) and Robins *et al.* (1984) described the use of differentiated cultures, and Hay *et al.* (1986) reported the use of a root suspension culture of *C. ledgeriana* in studies of the biosynthesis of the quinoline alkaloids.

III. ANALYTICAL METHODS

A. Extraction

Cinchona callus and cell suspension cultures accumulate considerable amounts of anthraquinones (Harkes *et al.*, 1985; Wijnsma *et al.*, 1986a; Chung and Staba, 1984; Mulder-Krieger *et al.*, 1982c, 1984) of the type normally found in Rubiaceae (Wijnsma *et al.*, 1984, 1986c; Wijnsma and Verpoorte, 1986). Addition of biotic elicitors to suspension cultures of *C. ledgeriana* caused a considerable increase in anthraquinone content (Wijnsma *et al.*, 1985), and the authors were able to demonstrate the presence of anthraquinones in bark samples of *C. ledgeriana* infected with *Phytophthora cinnamomi*, whereas no anthraquinones were present

in healthy bark samples (Wijnsma *et al.*, 1986c). These studies confirmed that anthraquinones in *Cinchona* are phytoalexins.

The considerable amount of anthraquinones present in *Cinchona* tissue culture material poses a severe problem for the quantitative analysis of alkaloids. It was found that anthraquinone aglucones interfere with the high-performance liquid chromatography (HPLC) determination of quinoline alkaloids using both the HPLC systems described by McCalley (1983a), Smith (1984), and Wijnsma *et al.* (1987). Also, the presence of anthraquinones in the alkaloid extracts gives rise to unacceptably long run times, for example, in excess of 2 hr using the HPLC system described by Smith (1984).

Most authors use liquid–liquid extraction procedures for the extraction of alkaloids from *Cinchona* tissue culture material, starting with an extraction step under acidic conditions to remove most of the interfering compounds. Then, after alkalinization of the aqueous layer, the alkaloids are extracted; this extract is used for analysis (Koblitz *et al.*, 1983b; Hunter *et al.*, 1982). In our laboratory a more elaborate procedure for the extraction of alkaloids from *Cinchona* tissue culture material is employed. Full details of the procedure are published elsewhere (Wijnsma *et al.*, 1987). In short, the procedure is as follows. The cell homogenate or the medium to be extracted is made acidic (pH = 2) and extracted twice with chloroform. After alkalinization of the aqueous layer the alkaloids are extracted with chloroform, then the chloroform fraction is extracted with an acidic aqueous solution. From this solution the alkaloids can be extracted after alkalization to give an alkaloid extract free of interfering substances.

An alternative to liquid–liquid extraction methods can be found in the use of small preconcentration columns like kieselguhr (Extrelut for example) or reversed-phase C_{18} or C_8 columns, as for the extraction and subsequent purification of alkaloids from tissue cultures of *Catharanthus roseus* (Kohl *et al.*, 1983; Renaudin, 1985). Hay *et al.* (1986) used kieselguhr columns for the purification of the alkaloids from *Cinchona ledgeriana* root suspension cultures after feeding of L-tryptophan. The authors investigated the possibility of employing reversed-phase preconcentration columns for the purification of extracts from *C. ledgeriana* cell suspension cultures. E. J. Allan and A. H. Scragg (personal communication) reported the use of C_{18} precolumns, but in our hands the use of C_{18} columns did not result in acceptable results, because of the relatively low and erratic recovery of indole alkaloids such as cinchonamine (R. Wijnsma and R. Verpoorte, unpublished results). The same was observed for the recovery of other indole alkaloids like vincamine using

C_{18} columns (Michotte and Massart, 1985). However, the use of C_8 reversed-phase preconcentration columns seems to lead to better results. It is possible to obtain an alkaloid extract that is free of anthraquinones, having a small elution volume, and to obtain a recovery better than 90% for both the indole alkaloids and the quinoline alkaloids (R. Wijnsma and R. Verpoorte, unpublished results).

B. Chromatographic Methods for Separation and Quantification of *Cinchona* Alkaloids

Quantification of individual alkaloids produced in cell and tissue culture systems has been achieved by several chromatographic methods. Thin-layer chromatography (TLC) has been used frequently. After elution from TLC plates, alkaloids were determined by ultraviolet (UV) spectrometry (Mulder Krieger *et al.*, 1984). Also, TLC combined with fluorodensitometry has been used (Mulder-Krieger *et al.*, 1982c). Koblitz *et al.* (1983b) and Schmauder *et al.* (1985) eluted the alkaloids from TLC plates and used fluorescence spectroscopy for quantification. Because none of the TLC systems described is capable of separating all eight quinoline alkaloids (Verpoorte *et al.*, 1980), HPLC is most widely applied for quantification of alkaloids (Table III). Hunter *et al.* (1982) described the use of a straight-phase HPLC system developed by McCalley (1983a). This system has also been used in studies by Harkes *et al.* (1985) and Wijnsma *et al.* (1986a). Although a good separation can be obtained, the reproducibility of retention times and the relative high cost of the eluent (66% hexane) make this system less suited for routine analysis. McCalley (1983b) also described a reversed-phase HPLC system capable of separating the four parent alkaloids and their dihydroanalogs in less than 30 min. The two systems were considered complementary in the analysis and identification of the alkaloids of *Cinchona* tissue culture extracts (McCalley, 1983b). Verpoorte *et al.* (1984) developed a reversed-phase ion-pairing HPLC system. This system permitted obtaining a baseline separation of the four parent alkaloids, their corresponding dihydroanalogs, and the indole alkaloids cinchonamine, 10-methoxycinchonamine, and quinamine, using dodecylsulfonic acid and cetrimide to mask the remaining free silanol groups in the stationary phase, which resulted in an excellent peak shape and good separation. The supplier of the stationary-phase material has changed the method of preparation and with recent batches of stationary-phase material, the method does not work any more. In need of a good HPLC system, Hobson-Frohock and Edwards (1982) developed a cyanopropyl column

Table III

High-Performance Liquid Chromatography Systems in Use for Separation and Quantification of *Cinchona* Alkaloids Extracted from Tissue Culture Systems

Stationary phase	Mobile phase[a]	Alkaloids[b] separated	Reference
Hypersil 5 μm, 250 × 4.6 mm	Hexane/dichloromethane/methanol/DEA = 66/31/2.0/0.65; flow, 2.0 ml/min	Qd, C, HQd, Cd, Q, HCd, HQ	Harkes *et al.* (1985); Wijnsma *et al.* (1986a); McCalley (1983a)
Hypersil ODS 5 μm, 250 × 4.6 mm	0.1 *M* KH_2PO_4, pH = 3.0, containing 0.05 *M* hexylamine with 4.0 or 5.6% CH_3CN; flow, 1.0 ml/min	C, Cd, HC, HCd, Qd, Q, HQd, HQ	McCalley (1983b)
μBondapak Phenyl, 300 × 3.9 mm	0.05 *M* NaH_2PO_4/2-methoxyethanol/ CH_3CN = 60/15/15, pH = 4.5 (adopted from Smith, 1984); flow, 0.5 ml/min	Q, Qd	Hay *et al.* (1986)
μBondapak Phenyl, 300 × 3.9 mm	0.05 *M* NaH_2PO_4/2-methoxyethanol/ CH_3CN = 80/5/15, pH = 4.5; flow, 2.0 ml/min	Cd, C, HCd, HC, Q, Qd, HQ, HQd	Smith (1984); Wijnsma *et al.* (1986b)
Lichrosorb RP 18, loaded with dodecylsulfonic acid and cetrimide, 250 × 4.6mm	0.02 *M* methanesulfonic acid in water/dioxane/sulfuric acid = 98.5/1.0/0.5, pH = 3.5; flow, 1.5 ml/min	C, Cd, HC, HCd, Qd, Q, HQd, HQ, CA, QA, 10-methoxyCA	Verpoorte *et al.* (1984)
Lichrosorb Si60, 5 μm, 250 × 4.6 mm	Chloroform/i-prop./DEA/water = 940/57/ 2/1	Q, Qd, C, Cd	Anderson *et al.* (1982)
Spherisorb CN 5 μm, 250 × 4.6 mm	6.8 mM NaH_2PO_4/CH_3CN/methanol/THF = 50/17/28.7/3.3; flow: 1.5 ml/min	Q, Qd, Cd, C	Hobson-Frohock and Edwards (1982)
Ultrasphere Si 5 μm, 250 × 4.6 mm; Partisil PXS 10/25, 250 × 4.6 mm	THF/*n*-butylchloride/NH_3 = 60/40/0.25; flow, 2.0 ml/min	C, Cd, Qd, HCd, Q, HQd, HQ	Chung and Staba (1984)

[a] DEA, diethylamine; CH_3CN, acetonitrile; i-prop., isopropanol; THF, tetrahydrofuran.

[b] C, cinchonine; CA, cinchonamine; Cd, cinchonidine; HC, dihydrocinchonine; HCd, dihydrocinchonidine; HQ, dihydroquinine; HQd, dihydroquinidine; 10-methoxyCA, 10-methoxycinchonamine; Q, quinine, QA, quinamine; Qd, quinidine.

in the reversed-phase mode. Chung and Staba (1984) reported a dual-column HPLC system for analyses of tissue cultures of *Cinchona*. Their system consisted of an Ultrasphere-Si column combined with a Partisil PXS 10/25 column. They were able to achieve a good separation, but analysis times were extremely long (>70 min). The HPLC system now adopted by the authors is that of Smith (1984). Hay *et al.* (1986) adopted this system in a modified version. Now a baseline separation of the four parent alkaloids and their dihydroanalogs can be obtained in less than 30 min with reproducible retention times using a cheap eluent. This system is well suited for routine analysis of large numbers of samples.

For the detection of *Cinchona* alkaloids after chromatographic separation, UV monitoring at 280 nm seems to be most appropriate. At 313 nm the absorbances of both the methoxylated and the nonmethoxylated alkaloids are almost equal, but at this wavelength, detection of the indole alkaloids is much less sensitive. Ultraviolet detection at 254 nm or less may enhance the sensitivity of the detection of the quinoline alkaloids but decreases the selectivity of detection. Also, the detection of the indole alkaloids is much less sensitive at this wavelength. It can be advantageous to combine UV detection with fluorescence detection. Due to their strong fluorescence under acidic conditions (most eluents used in reversed-phase HPLC contain phosphate buffer at low pH), the quinoline alkaloids can be detected with high sensitivity and high specificity.

C. Immunoassay Procedures

Another method for the analysis of secondary metabolites in plants or cell cultures is the application of immunoassay procedures (Weiler, 1978, 1982: see Chapter 15, Volume 4, this treatise). Verpoorte *et al.* (1985) reported the use of commercially available immunoassays for the analysis of quinidine in *Cinchona* tissue culture material. They have compared an enzyme-multiplied immunoassay technique (EMIT) and a fluorescence immunoassay (FIA) for cross-reactivity of other *Cinchona* alkaloids and for sensitivity. They concluded that the sensitivity was not better than HPLC employing 280-nm UV detection. Better selectivity of the EMIT assay made them conclude that the EMIT method was the method of choice. Robins *et al.* (1984) developed a radioimmunoassay (RIA) for the quantitative determination of quinine in *Cinchona* tissue cultures. Due to scintillation counting, the RIA described by Robins *et al.*

Table IV

Cross-reactivity of *Cinchona* Alkaloids in Immunoassays[a]

Alkaloid	RIA_Q	RIA_{Qd}	$ELISA_Q$	$ELISA_{Qd}$	$EMIT_{Qd}$	FIA_{Qd}
Quinine	100%	—	100%	0	0	0
Dihydroquinine	35%	—	2.7%	0	0	0
Cinchonidine	14%	—	1.2%	0	0	0
Cupreine	7.3%	—	0.75%	0	—	—
Dihydrocupreine	3.2%	—	0.70%	0	—	—
Epiquinine	—	—	—	—	5%	9%
Quini(di)none	0.6%	—	1.60%	4%	0	4%
Cinchoni(di)none	0	—	0	0.1%	0	8%
Quinidine	0	—	0	100%	100%	100%
Dihydroquinidine	0	—	0	0	46%	91%
Cinchonine	0	—	0	0	22%	93%
Cupreidine	0	—	0	0.15%	20%	74%
Dihydrocupreidine	0	—	0	0	—	—
Epiquinidine	—	—	—	—	9%	0
Cinchonamine	—	—	—	—	0	3%
Sensitivity	50 pg	1 ng	10 pg	100 pg	70 ng	40 ng

[a] Data for the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA) procedures are from Morgan *et al.* (1985); data for the commercially available enzyme-multiplied immunoassay technique (EMIT) (SYVA) and the fluorescence immunoassay (FIA) (AMES TDA) are from Verpoorte *et al.* (1985).

was much more sensitive than both the FIA and EMIT (50 pg for the RIA compared to 40 ng for the FIA; see also Table IV). Morgan *et al.* (1985) described the development of RIA for quinidine and enzyme-linked immunosorbent assay (ELISA) for quinine and quinidine. The results of the four immunoassays are summarized in Table IV.

D. Indole Alkaloids

For the indole alkaloids isolated from *Cinchona* (cinchonamine, 10-methoxycinchonamine, quinamine, and the semidimeric cinchophyllines) TLC seems the method of choice. Thin-layer chromatography in combination with a ferric chloride spray reagent followed by heating is a very effective method for the detection of these alkaloids (Mulder-Krieger *et al.*, 1982c).

IV. ALKALOID PRODUCTION IN *CINCHONA* TISSUE CULTURES

Only a few systematic studies directed at stimulation of the alkaloid production in *Cinchona* tissue cultures, either by medium optimization or other methods, have been published. For *Catharanthus roseus*, for example, a number of papers have appeared dealing with the design of production media (e.g., Zenk *et al.*, 1977; Knobloch and Berlin, 1980). For *Cinchona* no special production media have been designed. Mulder-Krieger *et al.* (1982b) found that optimum alkaloid production (quinine and quinidine) occurred when callus of *C. pubescens* was cultured on media containing zeatin (0.22 ppm) and 2,4-dichlophenoxyacetic acid (2,4-D) (0.22 ppm) or indolebutyric acid (IBA) (0.20 ppm). All other combinations of growth hormones tested were inferior with respect to alkaloid production. Harkes *et al.* (1985), reporting on the optimization of media for the production of both alkaloids and anthraquinones in tissue cultures of *C. ledgeriana*, observed that a plated cell suspension grown in the dark produced more alkaloids than those grown in the light, that high concentrations of mineral salts improved alkaloid yield, that the concentrations of auxins should be rather low and that organic nutrients should be present in the medium at high concentrations. The influence of some of the major medium components on the alkaloid and anthraquinone production by *C. ledgeriana* suspension cultures has been studied by Wijnsma *et al.* (1986a). A maximum specific productivity (MSP; milligrams of product per gram dry weight) curve for alkaloid production was constructed, from which it became clear that optimum alkaloid production occurred at normal levels of nitrate in combination with 4% sucrose. All other reports dealing with alkaloid production in *Cinchona* tissue cultures contain only isolated data on alkaloid levels.

Staba and Chung (1981) published alkaloid contents of shoot, root, and undifferentiated cultures of both *Cinchona pubescens* and *C. ledgeriana*. They concluded that only leaf organ (shoot) cultures contained detectable amounts of quinoline alkaloids, whereas cell suspension cultures and root organ cultures contained no detectable amounts of alkaloids. The nonmethoxylated quinoline alkaloids constituted the major part of the alkaloid extract. Anderson *et al.* (1982), in contrast to the findings by Staba and Chung, found that the fine suspension cultures of root cells contained the highest levels of quinoline alkaloids, with quinidine being the major component. The alkaloid concentration in the root cells was about 500 mg per gram dry weight. Whitten and Dougall (1981) described the presence of fluorescing compounds in sus-

pension cultures of *C. ledgeriana*, and they stated that an inverse relationship existed between the accumulation of the fluorescing compounds and the NAA concentration in the medium. Looking at the data available on alkaloid production in *Cinchona* cell and tissue cultures, it emerges that the organized cultures like shoot cultures contain the highest amounts of alkaloids. This might point to a correlation between morphological and biochemical differentiation.

V. BIOSYNTHETIC STUDIES USING *CINCHONA* TISSUE CULTURES

The biosynthetic pathway leading to the quinoline alkaloids starts with the same steps as the general biosynthetic pathway of all terpenoid indole alkaloids. L-Tryptophan is decarboxylated by tryptophan decarboxylase (TDC) to yield tryptamine. Tryptamine in turn is coupled to secologanin by strictosidine synthase to yield strictosidine (see Scheme 1). The further steps in the pathway leading to the quinoline alkaloids are still unclear, but it is generally believed that corynantheal is one of the intermediates. Using plants, Battersby and Parry (1971) were able to demonstrate the incorporation of tritium-labeled corynantheal in the quinoline alkaloids; however, recovery of the radiolabel in the alkaloid fraction was very low (0.17%). Tryptophan decarboxylase is considered to be one of the key enzymes in the biosynthesis of the indole alkaloids, and it has been demonstrated that stimulation of TDC activity can lead to increased alkaloid contents, both in *Peganum harmala* (Sasse *et al.*, 1982) and *Catharanthus roseus* (Sasse *et al.*, 1982; Knobloch and Berlin, 1983) cell suspension cultures. Schmauder *et al.* (1985) showed that TDC activity in cell suspension cultures of *Cinchona pubescens* is present and that the activity can be increased about 18-fold when the cells are cultured in the presence of L-tryptophan. They were also able to demonstrate that the addition of L-tryptophan to the cultures resulted in an increase in the alkaloid content of the cells. Furthermore, they tested the activity of a number of enzymes in primary metabolism. An increase in alkaloid content of *Cinchona* cultures after L-tryptophan feeding has also been reported by Hunter *et al.* (1982), Koblitz *et al.* (1983b), and Hay *et al.* (1986). Growth of the cultures in the presence of L-tryptophan is, however, severely inhibited (Koblitz *et al.*, 1983b; Hay *et al.*, 1986). Hunter reported an increase in alkaloid content of the cultures after feeding of L-tryptophan but did not present any data on the growth of the cultures.

An improvement of the production of *Cinchona* alkaloids by means of L-tryptophan feeding to suspension cultures has been claimed (Koblitz *et al.*, 1983c). Hay *et al.* (1986) showed by feeding experiments with L-[methylene-^{14}C]tryptophan, using root suspension cultures of *C. ledgeriana*, that L-tryptophan is incorporated into quinine and quinidine. However, the recovery of radiolabel in the alkaloids was low, about 0.25% of the administered labeled L-tryptophan. In our laboratories experiments were conducted in which L-tryptophan and tryptamine were fed to *C. ledgeriana* cell cultures that under the growth conditions employed do not produce alkaloids (R. Wijnsma, T. van der Leer and R. Verpoorte, unpublished results). L-Tryptophan feeding resulted in severe growth inhibition, but alkaloids could not be detected. It was found, however, that rapid uptake of the administered L-tryptophan took place. Tryptamine feeding also did not result in the production of alkaloids, but tryptamine had no toxic effects on the cells. From these results it may be concluded that not only TDC but also other enzyme systems can become the limiting step in the biosynthesis of *Cinchona* alkaloids.

Corynantheal feeding experiments have also been performed. Corynantheal is the putative intermediate after strictosidine formation (Battersby and Parry, 1971). The typical pathway leading to the quinoline alkaloids begins with corynantheal. In this case also the feeding of a precursor did not result in the formation of quinoline alkaloids (R. Wijnsma, T. van der Leer and R. Verpoorte, unpublished results). Corynantheal was rapidly taken up by the cells and extensively metabolized. At present, some of the metabolites formed are being isolated and identified. From these experiments one may conclude that either corynantheal is not an intermediate, or the biosynthesis of the quinoline alkaloids is blocked at the level of the corynantheal-converting enzyme. Robins and Rhodes (1986) demonstrated that in alkaloid-accumulating cultures of *Cinchona ledgeriana*, the key enzymes TDC and strictosidine synthase were present. Furthermore, they characterized an enzyme catalyzing a late step in the biosynthetic route, the reduction of cinchoninone or quinidinone to cinchonine/ cinchonidine or quinine/quinidine in the presence of NADPH cinchoninone:NADPH oxidoreductase (Isaac *et al.*, 1986).

In conclusion, it may be stated that a number of steps in the biosynthesis of the *Cinchona* alkaloids still remain to be investigated in more detail. For example, the validity of the intermediacy of corynantheal has not been proven unequivocably. If it is an intermediate one may ask which step(s) follow in the sequence leading to cinchoninone and quinidinone. Also, an intriguing question remains as to at which point the methoxylation of the quinoline nucleus takes place. From the pres-

ence of 10-methoxycinchonamine in *Cinchona* plants and calli (Mulder-Krieger *et al.*, 1982c, 1984), it could be concluded that methoxylation takes place during the early steps of the biosynthesis.

VI. PROSPECTS AND STATUS

Concerning the agrobiotechnological aspects of the cell and tissue culture of *Cinchona*, the prospects are very encouraging. The vegetative propagation of high-yielding clones is possible, and in the future even the possibilities of crop improvement by *in vitro* techniques might come into focus. Concerning the industrial biotechnological aspects of the cell and tissue culture of *Cinchona*, the production of the alkaloids by means of a biotechnological exploitation of the genus, the results obtained so far are far less encouraging. The highest value reported is about 4 mg per gram dry weight (Staba and Chung, 1981). Even in the case of precursor feeding, the content of alkaloids in the cells is extremely low. A consequence of the low product content is that the process of production of the alkaloids by means of *Cinchona* plant cell fermentation seems far from economically feasible. In fact, *Cinchona* seems to be a very good example of the "inability of plant cells to produce secondary substances," the title of the lecture presented by Böhm at the International Association for Plant Tissue Culture congress in Tokyo 1982 (Böhm, 1982). Considering the progress that has been made, however, especially in the field of biosynthesis of the alkaloids and regulation of the biosynthesis of anthraquinones, a better understanding of the mechanisms underlying the regulation of the biosynthetic pathways can probably be realized in the near future. The unraveling of the enzymes involved in the various biosynthetic steps will also help to bring about a better understanding and characterization of these enzymes, and might possibly open the way for genetic manipulation of the cells in order to improve alkaloid yields.

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CHAPTER **20**

β-Carbolines and Indole Alkylamines

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I. INTRODUCTION

The simplest "secondary" metabolites derived from tryptophan are indole alkylamines and β-carboline alkaloids. The indole alkylamines are substituted tryptamines. Their biosynthesis, metabolism, and taxonomic distribution has been reviewed in detail (Smith, 1977a,b). Simple β-carboline alkaloids comprise compounds containing the tricyclic pyrido[3,4-*b*]indole ring system with alkyl substitution at C_1. The pyridyl ring occurs in three different states of oxidation. So far more than 60 alkaloids of this group have been detected in 26 often taxonomically unrelated plant families (Allen and Holmstedt, 1980). Because of their pharmacological properties, for example, psychotropic activities, detailed studies of the organic and biosynthesis of several indole alkalymines and β-carboline alkaloids have been performed (for references, see Allen and Holmstedt, 1980). Although the origin of the indolyl part of the tricyclic ring system from tryptophan has clearly been

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proven, the origin of the "nontryptophan" biosynthetic condensation adduct and the *in vivo* sequence of the biosynthetic intermediates are as yet unclear. Feeding experiments with different plants suggest the possibility of various pathways, but enzymatic confirmation is lacking. The question arises whether suitable plant cell culture systems can be established to clarify this point, as has been shown for flavonoid, isoquinoline, and monoterpenoid indole alkaloid biosynthesis (Hahlbrock and Grisebach, 1979; Zenk, 1980; Zenk *et al.*, 1985). The purpose of this chapter is to review the plant cell cultures that have been shown to produce indole alkylamines and β-carboline alkaloids, and to analyze whether these systems might be rendered suitable for biochemical and biotechnological studies.

II. CELL CULTURE SYSTEMS

Of the many plants known to produce and accumulate β-carboline alkaloids, only reports on cell cultures of *Peganum harmala* deal with these secondary metabolites. It will be shown later that the pathway to β-carbolines seems to be well expressed only in freshly intiated, rather slowly growing and differentiated cell cultures of *P. harmala*. Rapidly growing suspension cultures contain no or low levels of β-carbolines. It is of course premature to conclude from the cultures of one plant species that the β-carboline pathway is generally poorly expressed under cell culture conditions. On the other hand, fine suspension cultures of *Passiflora caerulea*, established in our laboratory, also failed to accumulate β-carboline alkaloids. As β-carbolines can easily be detected by their strong and typical fluorescence (Sasse *et al.*, 1980), it is hardly possible to overlook their presence. Cell cultures of other plants listed as β-carboline alkaloid producers (*Apocynum cannabinum, Papaver rhoeas*) also did not show this typical fluorescence. In view of the fact that product levels of tryptophan-derived secondary metabolites are often low under cell culture conditions, it is likely that high accumulation of β-carbolines may be difficult to achieve. Whether the special conditions we employed to improve productivity of *Peganum harmala* cells for β-carboline alkaloids show better results also in other systems will have to be seen.

After feeding of L-tryptophan the formation of the β-carbolines norharman and harman were found in cell cultures of *Phaseolus vulgaris* and *Cinchona* species (Veliky and Barber, 1975; Wijnsma *et al.*, 1986). These two genera are not known to biosynthesize β-carboline alkaloids. Such

findings demonstrate the capacity of plant cell cultures to form "foreign" compounds by biotransformation. It also shows that enzymes probably not involved in the natural pathway of β-carboline biosynthesis may metabolize fed tryptophan or tryptamine to β-carbolines. For example, the ever-present enzyme serine hydroxymethyltransferase catalyzes the formation of tetrahydronorharman from serine and tryptamine (Pearson and Turner, 1979). The action of such enzymes can easily complicate interpretations of feeding experiments even with β-carboline-containing plant species. Nevertheless, only β-carboline-producing plant species should be considered for biosynthetic studies, and consequently, *Peganum harmala* cell cultures remain the only system available.

The same situation is also true for the indole alkylamines. Besides tryptamine, which seems to be present in many cell cultures (e.g., monoterpenoid indole alkaloid–producing plants such as *Catharanthus roseus;* Merillon *et al.*, 1986), only the presence of the indole alkylamines serotonin (5-hydroxytryptamine) and 6-hydroxytryptamine (the latter in traces) in *Peganum harmala* cell cultures has been reported. Thus, this chapter is indeed a summary of the formation of harman alkaloids, that is, β-carbolines with a methyl group at C_1, and serotonin in cell culture.

III. FORMATION OF HARMAN ALKALOIDS AND SEROTONIN

The first report on the formation of harmine by callus cultures of *Peganum harmala* was presented by Reinhard *et al.* (1968). In more-detailed studies by Nettleship and Slaytor (1971, 1974a,b) and McKenzie *et al.* (1975), further β-carboline alkaloids and indole alkylamines were detected in callus cultures (Fig. 1). Except for the glycosides, this alkaloid spectrum was also found in cell suspension cultures (Barz *et al.*, 1980, Sasse *et al.*, 1982a). Thus, all β-carbolines of the plant may be found in heterotrophic cell cultures, too. However, the various lines may differ in their alkaloid composition (methylated versus hydroxylated, fully aromatic versus hydro-β-carbolines). Photoautotrophic and green photomixotrophic cultures did not form these alkaloids (Barz *et al.*, 1980). Leaves of the plant also do not contain β-carboline alkaloids (Gröger, 1960). Most cell lines accumulated serotonin and small amounts of 6-hydroxytryptamine (Nettleship and Slaytor, 1974b; McKenzie *et al.*, 1975; Barz *et al.*, 1980; Sasse *et al.*, 1982a). The presence of higher levels of

Fig. 1. Alkaloids detected in cell cultures of *P. harmala*. A, B, harman alkaloids: C, indole alkylamines.

R_1	R_2	A	B	R_1	R_2	C
OH	H	Harmol	Harmalol	OH	H	Serotonin
OCH_3	H	Harmine	Harmaline	H	OH	6-Hydroxytryptamine
OCH_3	OGlc	Ruine	Dihydroruine			

serotonin in the cultures is especially noteworthy, as this compound has not been found in any part of the source plant.

In general, alkaloid levels of 0.05 to 0.1% of the dry mass were found in callus and suspension cultures. It seems possible to maintain this low level of productivity over years, provided the cell lines are not maintained at maximum growth rates. High-yielding cell lines with up to 2.3% β-carbolines have been described (Sasse *et al.*, 1982a). The general problem of qualitative and quantitative instability of the productivity of cell lines exists especially for high-yielding cell lines, but also for lower-yielding ones being maintained under somewhat growth-limiting conditions. When product formation is adversely affected by growth, the problem of losing initial productivity increases. This also applies to the *Peganum* system, as was first indicated by Nettleship and Slaytor (1974a), when they noted gradual alterations of their stock lines. Without selection the alkaloid content of most newly established callus cultures readily decreased by a factor of 10 during the first 15 passages on the growth medium, while calli became whiter, smoother, and showed less morphological differentiation (Sasse *et al.*, 1982a). This is also true for liquid cultures. The fate of the highest-yielding cell line 57, with a maximum specific content at the beginning of 2.3% harman alkaloids (Sasse *et al.*, 1982a), is given in Fig. 2. During subcultivation the alkaloid content decreased gradually. One year after selection, 1% serotonin and 1% β-carbolines (mainly harmalol and harmine) were still accumulated. Three years after initiation, however, alkaloids and serotonin were no longer formed by the cells. The culture had also changed its appearance. In the beginning the culture had a tendency to

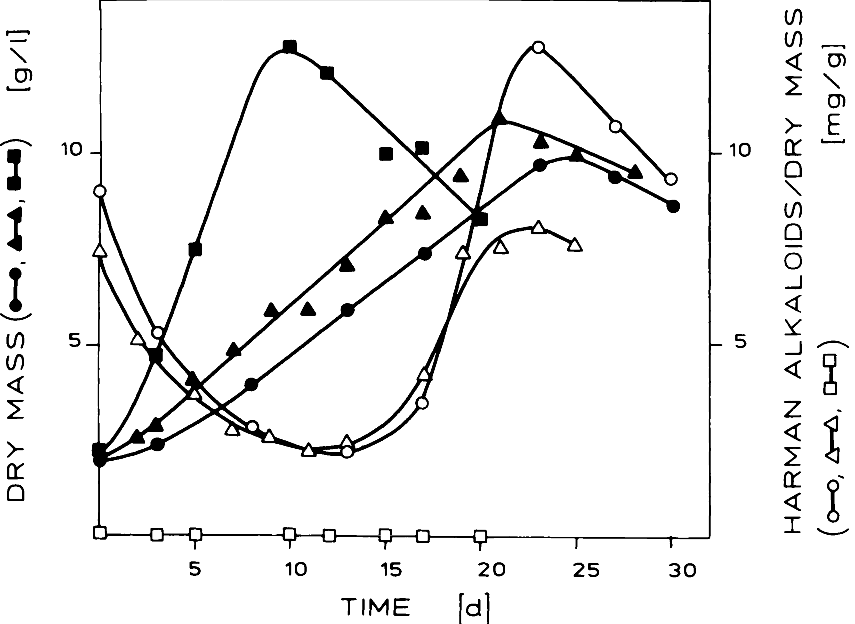

Fig. 2. Growth and harman alkaloid production of a high-producing cell line of *P. harmala* in suspension culture 4 months (●,○), 1 year (▲,△), and 7 years (■,□) after selection.

form aggregates and undergo morphological differentiation, especially in the late growth phase. With decreasing tendency to differentiate, the culture lost its ability to synthesize β-carbolines and serotonin. Without selection even the best-producing cell line had changed to a rapidly growing, finely dispersed suspension culture devoid of alkaloids. By reducing the 2,4-dichlorophenoxyacetic acid (2,4-D) concentration of the growth medium at the beginning to 1 μ*M*, we have established new lines, again accumulating up to 2% β-carbolines and 0.8% serotonin. These slowly growing suspension cultures exist as small, rootlike structures up to 4 mm in length and 0.2 mm in diameter and show many fluorescent cells under the microscope. These cultures have now retained their differentiated state for 2 years and may be used as stock cultures for further studies. However, the unsatisfactory situation remains that high productivity occurs only during slow growth and morphological differentiation. The question consequently arises as to whether screening, selection, media variation, or elicitation would be possible alternatives for establishing productive systems.

As the β-carbolines show a typical fluorescence, calli can be screened

visually for fluorescent areas, and selected and subcultured. We have done this for roughly 1 year (10 subcultures per callus clone). With continuous selection the high initial alkaloid level could be maintained during this period. Without further selection, however, the highly productive but slowly growing and morphologically differentiated calli rapidly lost their synthetic capacity (Sasse *et al.*, 1982a). Evidently, the screening did not result in true variants, as the fluorescent areas of the calli may have only represented a different physiological state rather than a genetic or epigenetic alteration (Berlin and Sasse, 1985).

Media variation or the development of induction–production media (Sasse *et al.*, 1982c) has often been quite successful in stimulating secondary product formation in cultured cells. In particular, lowering or depletion of 2,4-D and phosphate increased the alkaloid formation in *Peganum harmala* cells (Nettleship and Slaytor, 1974a; Sasse *et al.*, 1982c). Thus, serotonin and β-carboline formation of the above-mentioned line 57 was distinctly increased in such a medium. Growth was reduced, and the cells tended to differentiate (Fig. 3). But after the line had changed into a rapidly growing, finely dispersed cell suspension culture, the

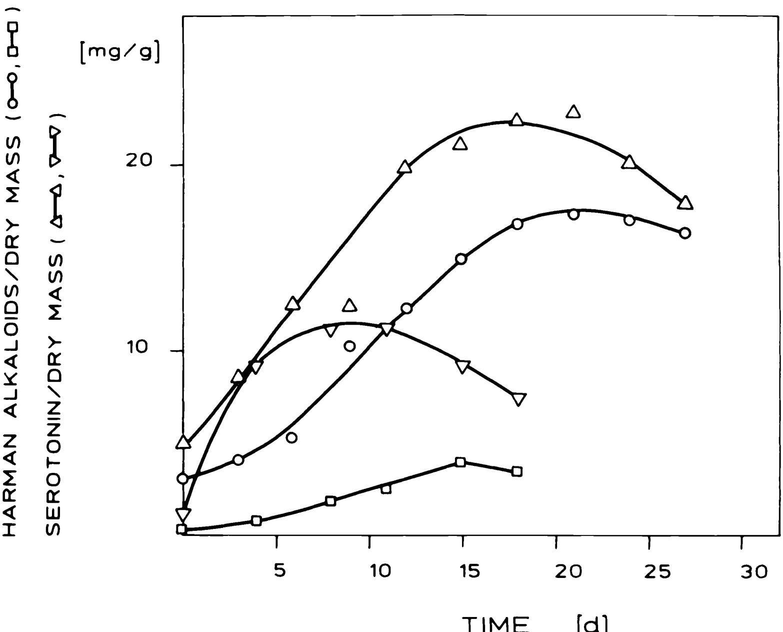

Fig. 3. Alkaloid accumulation of a lumpy, high-producing (○,△) and a fine, nonproducing but still inducible (□,▽) culture of *P. harmala* after transfer to a production medium.

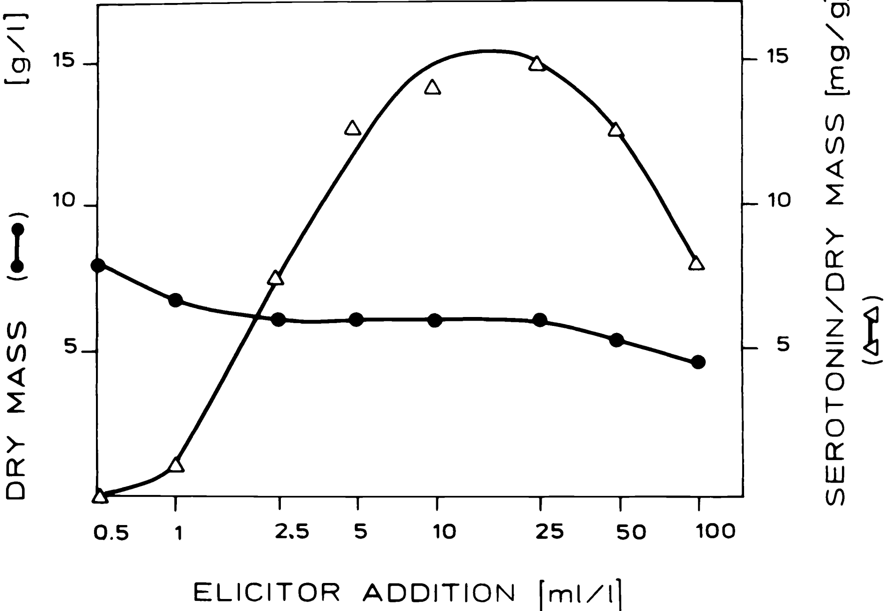

Fig. 4. Serotonin accumulation of a fine suspension culture of *P. harmala* 21 days after transfer of the cells to the growth media, to which different amounts of a fungus culture supernatant were added. Harman alkaloids were <0.05 mg/g. Control: growth medium without elicitor addition (13 g dry mass per liter, 0.2 mg serotonin per gram dry mass).

alkaloid production was not resumed after transfer of the cells to the production medium. Thus, the proposed production medium seems only to be useful for cultures that have retained the competence for alkaloid biosynthesis, recognized by the accumulation of low alkaloid levels on the growth medium. When 14-day-old cells of the 8-year-old line 57 are transferred to a full medium devoid of 2,4-D, growth ceases completely, and only traces of serotonin are formed.

Sometimes, formation of secondary metabolites can be induced by biotic and abiotic elicitors (DiCosmo and Misawa, 1985). Screenings with media and cell extracts of known phytopathogenic and newly isolated microorganisms (F. Sasse, E. Forche, and H. Reichenbach, unpublished results) showed that a great number of cell extracts or culture supernatants were able to induce serotonin and even β-carboline alkaloid formation in line 57. Suspensions with 50 mg of cells were incubated with different concentrations of the various microbial extracts or culture supernatants and screened for fluorescence. Quantitative analyses yielded harmalol levels of up to 0.15% and serotonin of up to 1.5%. Figure 4

shows an example of serotonin accumulation in relation to the amount of elicitor added. Distinctly increased levels of serotonin and β-carbolines were only achieved at growth-inhibitory concentrations of the elicitors. There were no indications that the induction of serotonin or β-carboline biosynthesis resulted from specific effects of a distinct biotic or abiotic compound. Given suitable doses most microorganisms had an eliciting effect, but extracts of soya flour or peptone, toxic concentrations of heavy-metal salts such as zinc or copper sulfate, to mention only a few, also had the same effect. Thus, elicitation of serotonin and β-carbolines in cell cultures of *Peganum harmala* should rather be seen as an unspecific growth-inhibitory stress induction.

IV. STUDIES OF BIOSYNTHESIS AND REGULATION

The biosynthetic steps to β-carbolines as shown in Fig. 5 have been proposed from feeding experiments with intact plants (Gröger, 1985). Up to now plant cell cultures have not provided further insights. Nettleship and Slaytor (1974b) showed that feeding and trapping experiments with callus cultures were severely hampered by compartmentation and side reactions; 5- and 6-hydroxytryptophan and -tryptamine were not metabolized. The main problem of feeding experiments with cell cultures is that nearly all tryptamine is converted to serotonin (Nettleship and Slaytor, 1974a; Sasse *et al.*, 1982b), a compound that is not present in the differentiated plant. During our studies significant incorporation of anthranilic acid, tryptophan, and tryptamine into β-carbolines was only observed with high-yielding, morphologically differentiated cell cultures. Such differentiated cultures may be useful for further investigations of β-carboline biosynthesis on the enzyme level.

The biosynthesis of serotonin in higher plants (*Peganum, Juglans*) starts with the decarboxylation of tryptophan followed by 5-hydroxylation (Sasse *et al.*, 1982b; Grosse *et al.*, 1983). This is in contrast to animal cells, where tryptophan is hydroxylated before decarboxylation.

When one compares low- and high-producing cell lines or transfers of *Peganum* cells from lowly to highly productive culture conditions, tryptophan decarboxylase (TDC) activity is greatly increased in the highly productive cells (Sasse *et al.*, 1982b). Thus, it was concluded that TDC

Fig. 5. Proposed biosynthetic pathways from tryptamine to 1-methyl-β-carbolines. (I) Acetaldehyde as reaction partner gives tetrahydrocompounds as first tricyclic condensation products. (II) Direct condensation with pyruvate to 1-methyl-1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid as intermediate. (III) *N*-Acetylation by acetyl-CoA and cyclic dehydration of *N*-acetyltryptamine gives dihydro-β-carbolines as first tricyclic compounds (Gröger, 1985).

exerts a regulatory role by controlling the flow of the primary metabolite tryptophan into secondary pathways. Indeed, rapidly growing *Peganum* cells, having lost their ability to produce β-carbolines or serotonin, did not contain measurable TDC activity. Thus, the absence of β-carbolines and especially serotonin may be accounted for by the lack of TDC. The interesting fact is that *Peganum* cells unable to synthesize serotonin *de novo* convert large amounts of fed tryptamine to serotonin (Fig. 6) (Sasse *et al.*. 1982b). The second step of serotonin biosynthesis remains well expressed even in nonproducing cell cultures. According to the literature and our experience with many independently established cell lines, this activity was high in all *Peganum* cell cultures independent of their serotonin levels synthesized *de novo*. Tryptamine feeding did not increase β-carboline levels in nonproducing cells. This may be due to the immediate competitive hydroxylation of tryptamine when taken up, but could also be due to the absence of other biosynthetic enzymes.

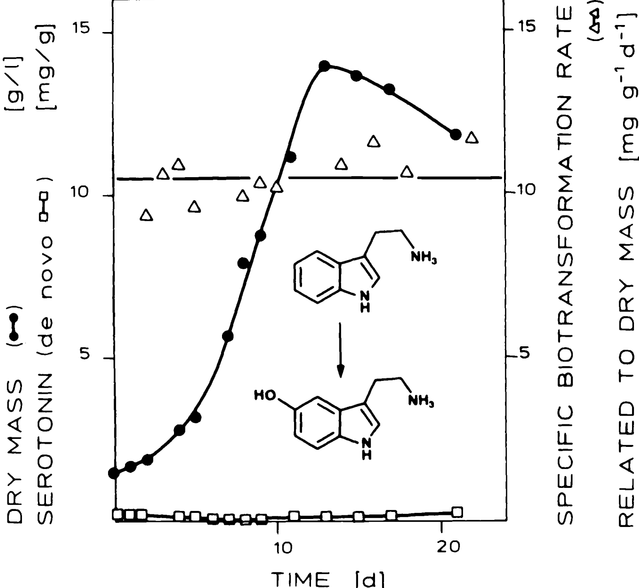

Fig. 6. Specific biotransformation rate of a fine, suspended, nonproducing cell culture of *P. harmala* (5-hydroxylation of fed tryptamine to serotonin).

V. A BIOCHEMICAL SELECTION SYSTEM

Based on the knowledge that cessation of serotonin formation by rapidly growing *Peganum* cells was merely due to the loss of TDC activity, we tried to select for cells having expressed TDC. It has been shown that 4-fluoro- and 4-methyltryptophan are toxic tryptophan analogs, which can be detoxified by decarboxylation (Sasse *et al.*, 1983a). Therefore, we selected for cell lines tolerant to these analogs and expected to find among these, as in the case of *Catharanthus* cells (Sasse *et al.*, 1983b), lines that were at least partially resistant because they were endowed with higher TDC activity and were thus superior in detoxifying the amino acid analogs. Several resistant lines tolerating up to 100-fold higher levels of the analog (0.5–1 m*M*) were characterized. Serotonin levels in the range of 0 to 2.0% of dry mass were found. A comparison of growth, serotonin accumulation, and TDC activity of a low- and a high-producing 4-fluorotryptophan-resistant cell line is shown in Fig. 7. The usefulness of this

selection is evident. For the first time high production of serotonin occurred in rapidly proliferating cell lines on the growth medium in the absence of the analog. Long-term stability of the lines was easily maintained in the presence of the analog.

The above selection was, however, only effective in repairing the two-step biosynthesis of serotonin. Despite the increased TDC activities, the levels of β-carboline alkaloids remained rather low (0.01% and less). Unlike the wild-type cells, however, many fluorescent cells in the call-uses were detected. Therefore, we cultured fluorescent areas of the resistant clones separately. So far, however, we could only increase the specific alkaloid levels by 10- to 100-fold. The more highly fluorescent the clones, the poorer their growth, and productivity was lost without screening. Thus, the analytically screened resistant cells behaved as the wild-type cells. High TDC activity may be required for high alkaloid production but is evidently not sufficient.

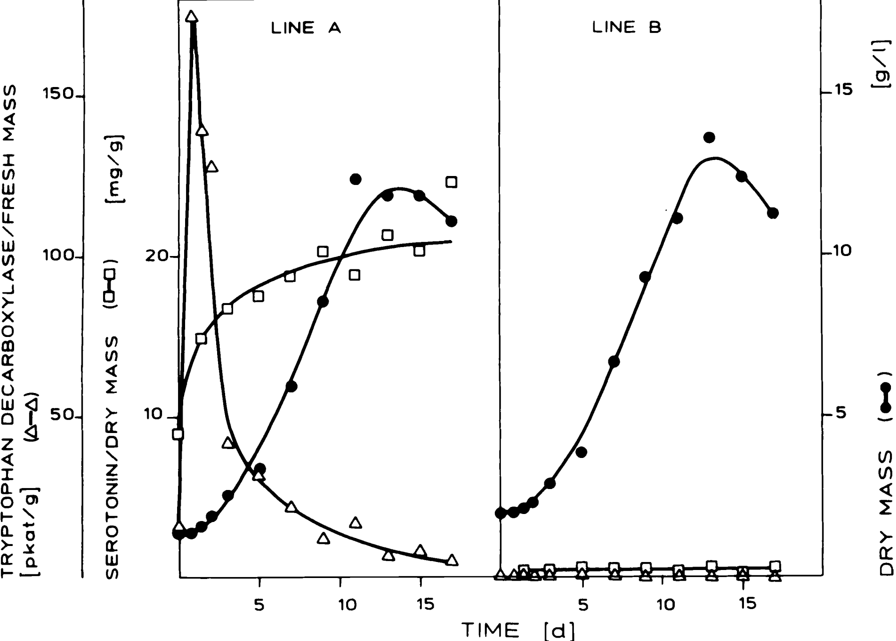

Fig. 7. Growth, TDC activity, and serotonin accumulation of two different 4-fluorotryptophan-resistant cell lines of *P. harmala*. Despite its high resistance, line B shows no difference from the wild-type culture regarding these parameters.

VI. OUTLOOK

The overall impression of β-carboline alkaloid and indole alkylamine formation by *Peganum* cells is that this culture reflects the common problem of secondary metabolite production in cultured cells. Nevertheless, we feel that this culture system deserves further attention. The absence of one biosynthetic enzyme activity appears sometimes to be the reason why certain secondary products are not found in cell culture. One could imagine that the lack of such enzymes may be overcome by the techniques of genetic engineering (Berlin, 1984). Although we have achieved an easier and quicker way of establishing cell lines with high TDC activity, we believe that serotonin biosynthesis in *Peganum* cells represents an ideal system for studying the requirements of integrating foreign gene products into a biosynthetic pathway (Berlin *et al.*, 1985). Such simple pathways may provide the clues of how to turn low-producing cell lines into better-producing ones. The simplicity, not the complexity, make *Peganum* cells an attractive culture for such investigations.

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CHAPTER 21

Monoterpene Indole Alkaloids from Apocynaceae other than *Catharanthus roseus*

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I. INTRODUCTION

The monoterpene indole alkaloids represent a large and diverse group of plant products, the majority of which have been isolated from species belonging to three families (Table I): the Loganiaceae, Apocynaceae, and Rubiaceae (Leeuwenberg, 1980). Reports on their production by cultured cells derived from various species (belonging to different genera)

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Table I

Botanical Classification of the Plant Families Apocynaceae, Loganiaceae, and Rubiaceae[a,b]

Family	Apocynaceae
Subfamily	Plumerioideae
Tribe	Carisseae *Carpodinus, Hunteria, Landolphia, Melodinus, Picralima, Pleiocarpa, Polyadoa*
Tribe	Tabernaemontaneae *Anacampta, Bonafousia, Callichilia, Capuronetta, Conopharyngia, Crioceras, Ervatamia, Gabunia, Hazunta, Hedranthera, Muntafara, Pagiantha, Pandaca, Peschiera, Phrissocarpus, Rejoua, Schizozygia, Stemmadenia, Stenosolen, Tabernaemontana, Tabernanthe, Voacanga*
Tribe	Plumerieae *Alstonia, Ammocallis, Amsonia, Aspidosperma, Catharanthus, Craspidospermum, Diplorhynchus, Geissospermum, Gonioma, Haplophyton, Lochnera, Plumeria, Rhazya, Tonduzia, Vinca*
Tribe	Rauvolfieae *Bleekeria, Cabucala, Excavatia, Kopsia, Neiosperma, Ochrosia, Rauvolfia, Vallesia*
Family	Loganiaceae
Tribe	Strychneae *Gardneria, Strychnos*
Tribe	Gelsemieae *Gelsemium, Mostuea*
Family	Rubiacea
Subfamily	Rubioideae
Tribe	Psychotrieae *Palicourea*
Tribe	Urophylleae *Pauridiantha*
Subfamily	Cinchonoideae
Tribe	Naucleeae *Adina, Anthocephalus, Cephalanthus, Haldina, Mitragyna, Nauclea, Neonauclea, Ourouparia, Pertusadina, Sarcocephalus, Uncaria*
Tribe	Cinchoneae *Cinchona, Corynanthe, Pausinystalia, Pseudocinchona, Remijia*
Tribe	Mussaendeae *Isertia*

(*continued*)

Table I (*Continued*)

Subfamily	Guettardoideae
Tribe	Guettardeae *Antirhea, Guettarda*
Subfamily	Hillioideae

[a] Only those genera that contain indole alkaloids, together with their synonyms, are given.
[b] From Leeuwenberg, 1980.

have been proliferating, although by and large, researchers have focused most of their attention in this area on relatively few of the species, in particular, *Catharanthus roseus* (Apocynaceae) and *Cinchona* species (Rubiaceae) (see Chapters 19 and 22, this volume). Accordingly, in this chapter the status of work from about 1980 through 1986 involving indole alkaloid production by cultured plant cells derived from species of genera other than *Catharanthus* and *Cinchona* is reviewed. Where possible, comments on how alkaloid profiles of the cell cultures have compared with those of the parent plant or closely related species have been included.

II. STRUCTURAL TYPES OF ALKALOIDS

The monoterpene indole alkaloids are formally derived from a unit of tryptamine and a C_9/C_{10} unit of terpenoid origin (secologanin). The basis of their classification has rested on the geometric arrangement of the C_9/C_{10} carbon skeleton with the three main configurations (Fig. 1) being categorized as the *Corynanthe, Aspidosperma,* and *Iboga* types. The

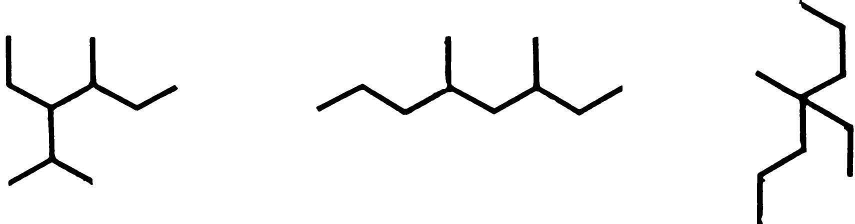


Fig. 1. Three main configurations of the nontryptamine portion of monoterpene indole alkaloids: *Corynanthe* type (left), *Ilboga* type (center), and *Aspidosperma* type (right).

I Corynanthe group

Ia Yohimbine type

Ib Heteroyohimbine type

Ib

Ic

Id Sarpagine type

Ie Ajmaline type

If Akuammiline type

Ig Pleiocarpamine type

Ih

Ih Strychnine type

Ii

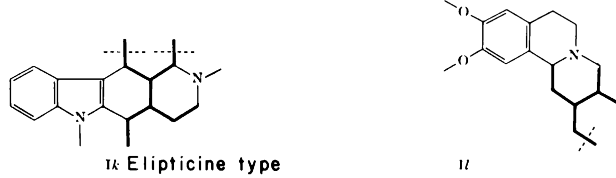
Ij Cinchonamine type

Ij Quinine type

Ik Uleine

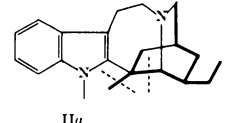
Ik Apparicine type

Fig. 2. Various alkaloid structures found in the *Corynanthe, Iboga,* and *Aspidosperma* types. (Adapted from Snieckus, 1968.)



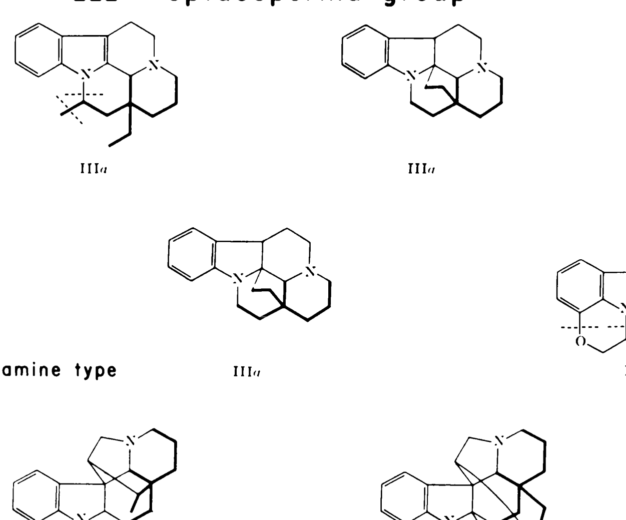
I*k* Elipticine type I*l*

II Iboga group



II*a*

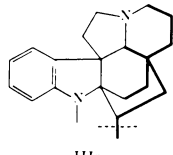
III Aspidosperma group



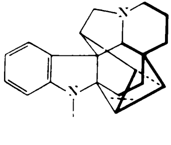
III*a* III*a*

III*a* Eburnamine type III*a* III*b*

III*d* III*c*



III*c*



III*c* III*d*

manner in which these basic units are joined to the tryptamine portion leads to the variety of subtypes (Fig. 2) that have been listed here according to the classification suggested by Snieckus (1968).

Some comment is necessary regarding structural types that do not appear to contain a tryptamine unit (e.g., quinine and ellipticine types). These alkaloids have indeed arisen from the ubiquitous monoterpene indole alkaloid precursor strictosidine, and at some later stage in their biosynthesis the tryptamine portion has undergone modification.

Several types that have not been listed here (although certainly important) are the bisindole group, a large and diverse array of structures arising from the union of two "monomeric" indole alkaloids. The complexity of these types of alkaloids is due not only to the monomers of which they are composed but also to the manner in which the "monomers" are linked. For our purposes, however, consideration of bisindole alkaloids from the perspective of the monomers of which they are composed will suffice. Cordell and Saxton (1981) have reviewed this area.

III. ALKALOIDS FROM CELL CULTURES

A. *Amsonia tabernaemontana*

Amsonia tabernaemontana Walter, a North American perennial, was used by a Polish group (Furmanowa and Rapczewka, 1981) to study growth of, organogenesis from, and alkaloid production by the corresponding cell suspension cultures. The authors were also able to obtain excised root cultures and to regenerate roots from cell aggregates. Detailed alkaloid analyses were not performed although it was noted that the root cultures produced an alkaloid pattern (as observed via thin-layer chromatography) similar to that of roots of the parent plant. Propitious production of alkaloids from the cell suspension cultures was not observed, although in retrospect the use of alkaloid production medium (Zenk *et al.*, 1977) in a two-step protocol might have remedied the situation. The authors employed two media for their study, WB (Wood and Braun, 1961) and MS (Murashige and Skoog, 1962).

B. *Ochrosia elliptica*

Two groups have thus far studied the production of indole alkaloids from cultured cells of *Ochrosia elliptica* Labill. Interestingly, the results

reported were quite different. Kouadio *et al.* (1984, 1985) reported the production of elliptinine (tentative assignment), ellipticine, 9-methoxy-ellipticine, reserpiline, and isoreserpiline from their cell suspension cultures, suggesting a profile very similar to that of the aerial parts of the plant. From their cell suspensions, Pawelka and Stöckigt (1986a) isolated eight alkaloids, none of which was the same as that of the other group's. These alkaloids consisted of the following types: Ib, Ig, Ih, and Ik (Fig. 2). Six of the compounds had not previously been isolated from *O. elliptica*, and two (norfluorocurarine and pleiocarpamine) had not previously been obtained from any species of *Ochrosia*. Typically, plants of the genus *Ochrosia* produce Corynanthe alkaloids of types Ia, Ib, Id, and Ik (Snieckus, 1968).

C. *Picralima nitida*

There is one report of alkaloid production by a cell suspension culture derived from *Picralima* (Arens *et al.*, 1982). Plantlets of *P. nitida* (Stapf) Th. & Hel. Dur. (synonym, *P. klaineana* Pierre) were used as the explant source, and callus formation was achieved on modified VM medium (Veliky and Martin, 1970). Suspension cultures were subsequently established on the same medium, and cells were transferred to alkaloid-production medium (Zenk *et al.*, 1977) to induce formation of alkaloids. An opiate-receptor binding assay (Pert and Snyder, 1974) was then employed to screen various fractions obtained from the methanolic extract of the cells. Two "active" alkaloids were isolated and identified as pericalline and a new closely related derivative that was named pericine (type Ik). "Inactive" alkaloids produced by the cell suspension were not mentioned. Pericalline (and of course, pericine) had not previously been detected in *Picralima*, although most phytochemical reports appear to have dealt with the contents of seeds, which in turn have been used by West African natives as a specific for malaria as well as an antipyretic (Henry and Sharp, 1927; Robinson and Thomas, 1954). The major alkaloid of *P. nitida* seeds is akuammine (Saxton, 1965). Alkaloids isolated from *P. nitida* have been of the *Corynanthe* group and included the types Ib, Id, If, and Ih.

D. *Rauwolfia serpentina*

The genus *Rauwolfia* and in particular the species *R. serpentina* Benth. have been well examined phytochemically (Court, 1983; Schittler, 1965),

due largely to the discovery of the antihypertensive properties of reserpine and its subsequent utilization as a tranquilizer. Although originally isolated from the Indian species, *R. serpentina,* the African species, *R. vomitoria,* has proven to be a superior source. To date, only culture work with *R. serpentina* has been carried out. Both cell suspensions and multiple shoot cultures have been reported. From the cell suspensions were isolated not only several alkaloids, but also three enzymes involved in some steps of the biosynthetic pathways. The enzymes isolated were polyneuridine aldehyde esterase (Pfitzner and Stöckigt, 1983), vinorine synthase (Pfitzner *et al.*, 1986), and vellosimine reductase (Pfitzner *et al.*, 1984). The first mentioned enzyme is involved in the pathway leading to the sarpagine–ajmaline group of alkaloids, the second in the formation of the ajmaline skeleton from the sarpagine skeleton, and the third in the reduction of a 16-aldehyde group (to the corresponding alcohol) in the sarpagine pathway.

Regarding alkaloids, Roja *et al.* (1985) were able to obtain multiple shoot cultures that afforded a profile differing from either those of the roots or leaves. In particular the shoot culture profile was less complex. Alkaloids identified were yohimbine, ajmaline, and ajmalidine (relative amounts not given). The yield of alkaloids from the shoots was 0.71% as compared to 0.54 and 2.64% in leaves and roots, respectively.

There are two reports dealing with alkaloids from cell suspensions. In the earlier one (Stöckigt *et al.*, 1981), 12 alkaloids representing the ajmaline (Ie), yohimbine (Ia), heteroyohimbine (Ib), and sarpagine types (Id) were isolated from two cell lines. The profiles of the two cell lines differed only slightly from each other but significantly from the plant. The major alkaloid obtained from the cell suspension was vomilinine (0.22% dry weight), an ajmaline type. In contrast, the plant produces a preponderance of yohimbine and heteroyohimbine types. Interestingly, vomilenine had not previously been observed in *R. serpentina* plants, but had been reported from *R. vomitoria* (Taylor *et al.*, 1962). It had also been detected in a callus culture of *R. serpentina* (Shimolina and Minina, 1981).

In the second report (Schübel and Stöckigt, 1984), a glycoalkaloid, raucaffricine (vomilenine galactoside), was obtained as the major product from cell suspensions in alkaloid production medium (Zenk *et al.*, 1977) and could be produced in concentrations of 0.5 gm/liter of medium. The authors were able to isolate large quantities (40 g) by using rotation locular countercurrent chromatography. Raucaffricine had previously been observed as a constituent of *R. caffra* (Khan and Ahan, 1972; Habib and Court, 1974).

E. *Rhazya stricta*

The genus *Rhazya* consists of only two species: *R. orientalis* A. DC. and *R. stricta* Decaisne. The latter is a small erect shrub located in the northwest of the Indian subcontinent and has been used in the indigenous system of medicine in Pakistan and India (Chopra *et al.*, 1956). There is one report on the production of alkaloids from cell suspensions of *R. stricta* (Pawelka and Stöckigt, 1986b). Seedlings were used to generate callus material on modified 4× medium (Gamborg *et al.*, 1968; Ulbrich and Zenk, 1979), and cell suspensions were subsequently obtained in the same medium. Analysis of the cell suspensions indicated the presence of at least 26 alkaloids, of which 11 were isolated, characterized, and compared to those of the parent plant. All but one of the alkaloids were found to be typical *Rhazya* alkaloids, and that one (akuammicine) was suggested as a likely precursor to the *Rhazya* alkaloid sewarine (11-hydroxyakuammicine). The authors also noted that improved alkaloid production could be achieved by using alkaloid production medium (Zenk *et al.*, 1977).

F. *Stemmadenia tomentosa*

There is one report dealing with alkaloids from a suspension culture of *Stemmadenia tomentosa* var. *palmeris*. Stöckigt *et al.* (1982) initiated callus tissue from seed and were subsequently able to establish cell suspensions in modified B5 medium (Rüffer *et al.*, 1981). Eight alkaloids were isolated and found to include representatives of the *Corynanthe, Iboga,* and *Aspidosperma* groups. Although the alkaloid types were analogous to those produced by the plant (i.e., Ih, Ii, IIa, IIIb), the profiles of the cell suspension culture and the plant were completely different. Thus, stemmadenine (which occurs in most *Stemmadenia* species) was specifically looked for and not found, whereas vinervine and norfluorocurarine were present but had not previously been detected in *Stemmadenia*.

G. *Tabernaemontana divaricata* and *T. elegans*

At least fifty species belonging to the genus *Tabernaemontana* have been chemically investigated (Danielli and Palmisano, 1986) and found to contain a wide array of alkaloid types, including bisindoles. Two

studies on the production of alkaloids by cultured cells of *Tabernaemontana* have been reported. Working with *T. divaricata*, Pawelka and Stöckigt (1983) were able to obtain cell suspensions in modified B5 medium (Zenk *et al.*, 1977) and were able to isolate the following alkaloids: apparicine (type Ik, 2.5 μg/liter), tubotaiwine (type Ii, 10 μg/liter), vinernine (type Ih, 4.7 μg/liter), conoflorine (type IIIb, 3.9 μg/liter), coronaridine (type IIa, 10.4 μg/liter), and catharanthine (type IIa, amount not given). Tubotaiwine, although found in several species of *Tabernaemontana*, had not previously been observed in *T. divaricata*, whereas vinervine and catharanthine had not previously been observed in any species of *Tabernaemontana*. In the other study, Van der Heijden *et al.* (1986a,b) obtained callus material from *T. elegans* and compared the alkaloid profile with that of the plant. Although the major components of the callus (tabernaemontanine, apparicine, and vobasine) were also major components of the plant, there were some significant differences. Thus, whereas seven bisindole alkaloids could be detected in the plant, only two were found in the callus, of those two only one was in common with the plant. Furthermore, several of the minor callus alkaloids were not detected in the plant although they had been observed in other *Tabernaemontana* species: 3-hydroxyisovoacangine in *T. eglandulosa* Stapf. (Agwada *et al.*, 1975) and 3-hydroxycoronaridine in *T. sananho* Ruiz et Pav. (Delle Monache *et al.*, 1977).

H. *Tabernanthe iboga*

Tabernanthe iboga Baill. is a shrub indigenous to western Africa. The root bark, of which the main alkaloid is ibogaine, has been used by natives to increase resistance to fatigue. There is to date only one report concerning alkaloid production by the corresponding cultured cells. Pawelka and Stöckigt (1983) established suspension cultures in 4× medium (Rüffer *et al.*, 1981) and were able to isolate and identify the two major alkaloids as conoflorine (voaphylline, type IIIb) and tubotiwine (type Ii). In contrast to the cultured cells, the plant produces only iboga (type IIa) alkaloids.

I. *Voacanga africana* and *V. thouarsii*

There have been three reports concerning alkaloids from cell cultures of *Voacanga*. Ferchel *et al.* (1983), in a short communication, noted the production of tabersonine by calli of *V. thouarsii*. Two other papers dealt

with cell suspensions of *V. africana*. Stöckigt *et al.* (1982) were able to isolate and identify three *Aspidosperma* alkaloids: tabersonine, lochnericine, and minovincinine. Later, Stöckigt *et al.* (1983) were further able to isolate and determine the structures of two new bisindole alkaloids, which they named voafrine A and B. The bisindoles were determined to be dimers of tabersonine. Taken as a whole, these results were quite interesting, as in stark contrast to the cultured cells, *Voacanga* plants do not contain "monomeric" alkaloids of the *Aspidosperma* type. A bisindole consisting of two *Aspidosperma* units had, however, been isolated from *V. africana* (Gorman *et al.*, 1966).

IV. CONCLUSIONS

In spite of some fine efforts to date, the examination of indole alkaloid production by cultured cells derived from species of various genera remains a relatively unexplored territory. Results that have been obtained, however, have supported the contention that *a priori* prediction of products is not possible (Pawelka and Stöckigt, 1986a). Indeed, cell cultures that yielded alkaloid profiles similar to those of the parent plant were exceptions; in fact, the isolation of alkaloids atypical of the genus was not an uncommon event. These results were perhaps not surprising when one considers that alkaloid production and/or storage is normally tissue specific, that is, roots, stems, leaves, and flowers generally afford different alkaloid profiles (e.g., Court, 1983), and cultured cells represent a rather unique state of differentiation. Add to this the fact that environmental conditions can profoundly affect the quantity and type of secondary metabolites produced (Waller and Nowacki, 1978; Trease and Evans, 1983), and one has a reasonable rationale for the observed differences (i.e., variation as a result of epigenetic effects). There remains, however, the possibility that profile differences are largely a consequence of mutations that in turn might have arisen as a result of the cell culture process itself. Whatever the reasons, that cultured cells can produce complex alkaloids that *a priori* are not predictable makes this area an attractive one for further study.

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CHAPTER 22

Monoterpene Indole Alkaloids (*Catharanthus* Alkaloids)

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I. INTRODUCTION

A great number of indole alkaloids produced by *Catharanthus roseus* (L.) G. Don (synonym, *Vinca rosea* L.) have been identified. Several of these have been found to be valuable agents in the treatment of hypertension and a number of neoplastic ailments (Farnsworth, 1975). In particular, vinblastine and vincristine, two dimeric indole alkaloids, have been used for many years as chemotherapeutic agents in the treatment of leukemia and Hodgkin's disease. The use of such dimeric indole alkaloids has prompted intensive studies of their chemical and bio-

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chemical synthesis since the 1960s (Gröger, 1985, and references therein). The interest in the production of dimers by plant tissue culture methods has been pursued for almost as many years with mixed results. In this chapter an attempt to give an overview of the work related to the production of indole alkaloids by *Catharanthus* plant cell cultures is made, giving special attention to the enzymatic regulation of synthesis.

II. PRODUCTION OF INDOLE ALKALOIDS

A. Two-Phase Culture System

The first successful screening program investigating the influence of medium composition on indole alkaloid formation in *Catharanthus roseus* cell suspension cultures was performed by both Zenk *et al.* (1977) and Carew and Krueger (1977) (Table I). The studies of Zenk's group resulted in a two-phase culture system that was designed, first, to produce biomass by culturing cells in a growth medium rich in nutrients and containing 2,4-dichlorophenoxyacetic acid (2,4-D), followed by cultivation in an alkaloid-production medium of different nutritional composition. Generally, alkaloid-production media developed by other groups bear resemblance to that of Zenk *et al.* (Table I). The best basal media for biomass production were those of Murashige and Skoog (1962), Linsmaier and Skoog (1965), and Gamborg *et al.* (1968). The alkaloid production media usually lacked the hormone 2,4-D, which was replaced by naphthaleneacetic acid (NAA) or indoleacetic acid (IAA) and contained kinetin and 3–8% sucrose. Whereas previous workers (Carew and Krueger, 1977) could not observe any effect of phosphate on indole alkaloid formation, later work demonstrated that the presence of phosphate above 10 m*M* was inhibitory to secondary metabolite formation (Knobloch and Berlin, 1980). The role of other nutrients on alkaloid accumulation was reviewed by Kurz and Constabel (1985).

The indole alkaloids isolated from cultures of *Catharanthus* cells in alkaloid-production medium are shown in Table II, and their chemical structures are shown in Fig. 1. Earlier reports identified mainly ajmalicine or serpentine as the main products in cell cultures, but by 1980 (Table II), researchers in several laboratories identified the presence of the more complex *Aspidosperma* alkaloids, such as tabersonine and lochnericine, as well as the *Iboga* alkaloid catharanthine. Kurz *et al.* (1981) reported that a cell line produced catharanthine levels at least 3

Table I

Alkaloid-Production Media Developed for *Catharanthus roseus*

Case	Reference	Basal medium[a]	Growth regulator[b] (mg/liter)		Sucrose concentration (g/liter)
1	Carew and Krueger (1977)	B5	2,4-D or IAA 2,4-D	(1.0) (0.5) (0.1)	20, 40, or 60
2	Döller (1978)	MS	IAA Kinetin	(0.2) (2.0)	30
3	Knobloch and Berlin (1980)	Water	None		80
4	Neumann *et al.* (1983)	MS	NAA Kinetin	(2.0) (0.2)	30
5	Petiard (1980)	MS	NAA Kinetin	(1) (1)	30
6	Roller (1978)	NN	IAA Kinetin	(2.0) (0.2)	20
7	Zenk *et al.* (1977)	LS	IAA 6BA	(0.175) (1.125)	50

[a] The basal media used were those of Gamborg *et al.* (1968) (BS), Murashige and Skoog (1962) (MS), Nitsch and Nitsch (1969) (NH), and Lin and Staba (1961) (LS). The screening protocol in cases 2 to 7 identifies media that permitted the accumulation of either ajmalicine, serpentine, or both alkaloids. Carew identified Dragendorff-reagent-positive spots on chromatograms.

[b] 6BA, 6-benzylaminopurine acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indoleacetic acid.

times higher than that of the intact plant. Smith *et al.* (1986) reported that one catharanthine-producing line could be successfully scaled up to 30 liters while still producing catharanthine at 1.28 mg per gram dry weight (75 times higher than in the source plant). In the case of the *Aspidosperma* alkaloids, neither vindoline nor most of the intermediates beyond tabersonine have been reported to occur in cell suspension cultures, and apart from the early work by Richter *et al.* (1965) who reported the presence of vindoline in callus from *Catharanthus*, other studies never could corroborate this finding (Zenk *et al.*, 1977).

Studies by Morris (1986a,b), concerning the loss of the biosynthetic capabilities for vindoline and catharanthine in leaf tissue cultured in various growth media, including Zenk's alkaloid-production medium, showed that during the first 40 days of culture the two main leaf alkaloids, catharanthine and vindoline, were rapidly metabolized, while the alkaloids serpentine and ajmalicine were accumulated. The shift in production from the major leaf alkaloids to the major root alkaloids. when primary callus is initiated from leaf tissue, has thus been estab-

AJMALICINE SERPENTINE AKUAMMICINE LOCHNERIDINE TABERSONINE LOCHNERICINE HÖRHAMMERICINE HÖRHAMMERININE VINDOLININE 19-EPIVINDOLININE CATHARANTHINE VINBLASTINE

Fig. 1. Chemical structures of indole alkaloids produced by *Catharanthus roseus.*

lished (Morris, 1986b). Further evidence as to biosynthetic shifts was shown by Constabel *et al.* (1982), when in callus cultures of *Catharanthus roseus* on redifferentiation to shoots and plants, biosynthesis of catharanthine and vindoline was derepressed. The preservation of genetic information on callus formation as demonstrated is an important prerequisite for the possible production of these alkaloids in tissue culture. These studies also indicate that biosynthesis of the alkaloids takes place in the leaf and that studies related to the biosynthetic regulation of alkaloid formation should be performed on organized, rather than callus, tissue.

B. Single-Phase Culture Systems

Three novel culture systems have been described that may eliminate the need to cultivate cells in two successive media for the production of indole alkaloids (Eilert *et al.*, 1986a,b,c; Morris, 1986a). That a single cell line had never been cultured under conditions employing different alkaloid-production media (Table I) prompted Morris (1986a) to compare

Table II

Indole Alkaloids Produced by Cell Suspension Cultures of *Catharanthus roseus*

Indole alkaloid	Reference	Production media used
Ajmalicine	Patterson and Carew (1969)	
Serpentine	Carew (1975)	Carew and Krueger (1977)
	Scott *et al.* (1980)	Schenk and Hildebrandt (1972)
	Roller (1978)	Roller (1978)
	Döller (1978)	Döller (1978)
	Zenk *et al.* (1977)	Zenk *et al.* (1977)
Akuammicine	Patterson (1968)	
	Scott *et al.* (1980)	Zenk *et al.* (1977)
Lochneridine	Carew (1975)	Carew and Krueger (1977)
12-OH-Akuammicine	Stöckigt and Soll (1980)	Zenk *et al.* (1977)
Tabersonine, lochnericine	Kurz *et al.* (1981)	Zenk *et al.* (1977)
Hörhammericine, hörhammerinine, vindolinine, epi-vindolinine	Stöckigt and Soll (1980)	Zenk *et al.* (1977)
Catharanthine	Kurz *et al.* (1981)	Zenk *et al.* (1977)
	Scott *et al.* (1980)	Schenk and Hildebrandt (1972)
	Stöckigt and Soll (1980)	Zenk *et al.* (1977)
Ajmalicine, epi-3-ajmalicine, desacetyakuammiline, dihydrocondylocarpine, 7-OH-indolenine–ajmalicine, Pseudoindoxyl-ajmalicine, hydroxydesacetyl-akuammiline, mitraphylline, tetrahydroalstonine, isovallesiachotamine, tabersonine, iso(?)sitisirikine	Pétiard *et al.* (1982)	Pétiard (1980)

the quality and quantity of indole alkaloids produced under these conditions. He showed that serpentine and ajmalicine were the major alkaloids produced under all conditions tested and that the accumulation kinetics was similar to that found when Zenk's alkaloid-production medium was used. Morris (1986a) also confirmed the inhibitory effect of 2,4-D on

alkaloid biosynthesis. He found that cells adapted to grow in the presence of NAA instead of 2,4-D showed high growth rates over repeated subcultures as well as stable high production rates for serpentine and ajmalicine. In order to eliminate the need for 2,4-D, hormone-habituated cell cultures and those transformed with *Agrobacterium tumefaciens* were analyzed (Eilert *et al.*, 1986c). Both types of cultures produced indole alkaloid profiles similar to those accumulated in the roots of source plants. serpentine, ajmalicine, and vindolinine being the main alkaloids. On transfer of these hormone-habituated or tumorous cultures to B5 growth medium (Gamborg *et al.*, 1968) containing 1 mg/liter 2,4-D (1-B5), the level of alkaloid production declined rapidly. A marked stimulation of accumulation of serpentine in hormone-habituated *Catharanthus roseus* cultures was also reported by Ramawat *et al.* (1985).

A further single-phase culture system also developed by Eilert *et al.* (1986a), who proposed a novel approach to achieve rapid accumulation of secondary metabolites by using fungal elicitors in cell suspension cultures cultivated on 1-B5 growth medium. Under these conditions the same pattern and level of indole alkaloids were produced as in cultures grown on alkaloid-production medium after Zenk *et al.* (1977). The major advantage in the use of fungal elicitors are the rapid induction of alkaloid accumulation and achievement of optimum yields (hours as compared to weeks in production media), the excretion of product into the medium thus avoiding the sacrifice of cells for product recovery and the need for a single medium for alkaloid production. Further research indicated that elicitor-stimulated alkaloid accumulation occurred as a result of the specific induction of pathway enzymes such as tryptophan decarboxylase and strictosidine synthase (Eilert *et al.*, 1987a).

Stimulation of indole alkaloid accumulation was also observed when cells were submitted to increases in osmotic pressure (Frischknecht *et al.*, 1986), with serpentine being the main product (up to 0.5% dry weight). For more detailed information on elicitor- and other stress-mediated phenomena on secondary metabolite formation, the review by Eilert (see Chapter 9, Volume 4, this treatise) should be consulted.

III. INSTABILITY OF CELL LINES

An important factor for the commercial production of secondary metabolites by tissue culture methods is the retention of high levels of product formation capability in selected cell lines (Sato and Yamada,

1984). Various strategies have been proposed for the maintenance of high yields, such as repeated selection for high-yielding clones, preservation of high-yielding clones by cultivation as slower-growing callus, or cryopreservation of productive cells. Deus and Zenk (1982) used clonal selection to obtain high-producing lines of *Catharanthus;* however, these lines were highly unstable with respect to indole alkaloid synthesis (Deus-Neumann and Zenk, 1984a). Repeated selection during an 8-year period was required in the maintenance of six separate cell lines for production of serpentine in excess of 300 mg/liter. Invariably, they reported rapid loss of productivity over the first few months of cultivation, and noted the unlikelihood of maintaining high productivity when scaling up cell lines to commercial levels. It must be added, however, that whereas this instability holds true for all cell lines selected for production of high levels of serpentine, it does not hold true for cell lines selected for high production of other indole alkaloids. Several cell lines were isolated that produced *Aspidosperma* and *Iboga* alkaloids at high levels (Kurz *et al.*, 1980). These cell lines showed qualitative variability in the pattern of alkaloids produced without, however, losing their quantitative production capability, as reported for the serpentine selected lines (Kurz, 1984). This has been further illustrated with high catharanthine producing lines, which produce remarkable levels of catharanthine even after 5 years in culture, without clonal selection (Smith *et al.*, 1986).

The possible use of hormone autotrophic cultures to maintain stable lines should be investigated. It was reported that a hormone-autotrophic culture that accumulates ajmalicine, serpentine, tabersonine, and vindolinine throughout the growth cycle of the culture, did so in a stable way for more than 1 year (Eilert *et al.*, 1986c). This may be an indication that exogenous hormones affect biosynthetic stability. As indicated previously, hormone-autotrophic cell lines may themselves be screened for accumulation of specific indole alkaloids.

IV. ENZYMOLOGY OF INDOLE ALKALOID BIOSYNTHESIS

Several reviews of the enzymology of indole alkaloid biosynthesis in *Catharanthus* have been published (Gröger, 1985; Scott *et al.*, 1981; Stöckigt, 1981; Zenk, 1980; Madyastha and Coscia, 1979). In recent years enzymology connected with the biosynthesis of indole alkaloids has

progressed rapidly, mainly due to the availability of *C. roseus* cell lines from which enzymes could be extracted and isolated (Table III). It is from such cell lines that Hemscheidt and Zenk (1985) isolated two separate reductases that catalyze the NADPH-dependent formation of stereoisomeric indole alkaloids. Both enzymes used exclusively NADPH as reductant, had pH optima of 6.6, and a molecular weight of 81,000. Whereas one reductase reduced the iminium form of cathenamine to tetrahydroalstonine, the other reduced cathenamine to ajmalicine. Deus-Neumann and Zenk (1984b) used *Catharanthus* cell lines that although unable to synthesize vindoline, showed an active vacuolar uptake system for this alkaloid. This vacuolar uptake system was specific to vindoline and other alkaloids indigenous to the plant, but not to other alkaloids, such as morphine and codeine. The possible localization of indole alkaloids, such as vindoline and catharanthine, in the plant vacuole was in contrast to the cytoplasmic compartmentation of enzymes such as loganic acid *O*-methyltransferase (Madyastha and Coscia, 1979), strictosidine synthase (Deus-Neumann and Zenk, 1984b), and β-glucosidases specific for strictosidine (Deus-Neumann and Zenk, 1984b), the microsomal compartmentation of geraniol hydroxylase (Madyastha and Coscia, 1979), and the chloroplastic membrane associated N-methyltransferase (DeLuca *et al.*, in press) (Table III). Such a variety of sites for biosynthesis of indole alkaloids denotes the complexity of the transport mechanisms that might be required in order to connect the different sites of synthesis of intermediates and final products.

Cell suspension cultures of *Catharanthus roseus* have however, not proven to be useful sources for the enzymes that catalyze the conversion of tabersonine to vindoline. This might be due to the fact that no vindoline-producing cultures have ever been isolated. Two groups (DeLuca *et al.*, 1985 and Fahn *et al.*, 1985a) succeeded in isolating late enzymes of vindoline biosynthesis from the intact plant. Based on substrate specificities, Fahn *et al.* (1985b) suggested that tabersonine was first hydroxylated at three different positions on the molecule followed by *N*-methylation, *O*-methylation, and finally, *O*-acetylation resulting in vindoline biosynthesis. An alternative pathway was proposed by DeLuca *et al.* (1986) and Balsevich *et al.* (1986) based on the isolation of biosynthetic pathway intermediates of vindoline synthesis from dark-grown *Catharanthus* seedlings and their quantitative transformation to vindoline upon greening of etiolated tissue (Fig. 2). It was postulated that tabersonine was 16-hydroxylated, then *O*-methylated followed by hydration of the double bond at position 2,3, followed by *N*-methylation and hydroxylation at position 4 and, finally, the *O*-acetylation. The two proposed pathways have only the final acetylation step in common, and the

Table III

Enzymes Involved in Indole Alkaloid Biosynthesis

Enzyme	Substrate	Product	Source	Reference
Geraniol hydroxylase	Geraniol or nerol + O_2, NADPH	10-OH-Geraniol or 10-OH-nerol	Young seedlings, tissue culture	Madyastha *et al.* (1973)
Loganic acid *O*-methyltransferase	Loganic acid or secologanic acid	Loganin or secologanin	Young seedlings	Madyastha *et al.* (1973)
Tryptophan decarboxylase	Tryptophan	Tryptamine	Young seedlings; tissue culture purified to homogeneity	Scott and Lee (1975) Noé *et al.* (1984)
Strictosidine synthase	Tryptamine + secologanin	Strictosidine	Tissue culture purified to homogeneity	Mizukami *et al.* (1979); Teimer and Zenk (1979)
Strictosidine-specific glucosidase	Strictosidine	Aglycone of strictosidine	Tissue culture	Hemscheidt and Zenk (1980)
Cathenamine reductase	Cathenamine + NADPH	Ajmalicine	Tissue culture	Hemscheidt and Zenk (1985)
Iminium cathenamine reductase	Iminium cathenamine + NADPH	Tetrahydroalstonine	Tissue culture	Hemscheidt and Zenk (1985)
Vindoline vacuolar uptake system			Intact plant, tissue culture	Deus-Neumann and Zenk (1984b)
16-*O*-Demethyl-4-*O*-deacetylvindoline-16-*O*-methyltransferase	16-*O*-Demethyl-4-*O*-deacetylvindoline + SAM[a]	4-*O*-Deacetylvindoline	Intact plant	Fahn *et al.* (1985b)
N(1)Demethyl-16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase	*N*(1)Demethyl-16-methoxy-2,3-dihydro-3-hydroxytabersonine + SAM	*N*(1)-Methyl-16-methoxy-2,3-dihydro-3-hydroxytabersonine	Intact plant	V. DeLuca *et al.* (unpublished)
Deacetylvindoline acetyltransferase	Deacetylvindoline + Acetyl-CoA	Vindoline	Intact plant	DeLuca *et al.* (1985); Fahn *et al.* (1985a)

[a] SAM, *S*-adenosyl-L-methionine.

TABERSONINE → HYDROXYLATION → 16-HYDROXYTABERSONINE → METHYLATION → 16-METHOXYTABERSONINE

HYDROXYLATION → 16-METHOXY-2,3-DIHYDRO-3-HYDROXYTABERSONINE → METHYLATION → DESACETOXYVINDOLINE → HYDROXYLATION

DEACETYLVINDOLINE → ACETYLATION → VINDOLINE

Fig. 2. Proposed biosynthetic pathway for the transformation of tabersonine to vindoline. The numbering system used is as outlined for aspidospermidine derivatives in *Chemical Abstracts*.

correctness of either of the proposed pathways remains to be determined.

The *O*-methylation of 16-*O*-demethyl-4-*O*-deacetylvindoline by crude desalted leaf extracts was demonstrated (Fahn *et al.*, 1985b). Sufficiently detailed substrate specificity studies have not, however, yet been undertaken in order to determine at which stage *O*-methylation occurs. Crude extracts have also been shown to catalyze *O*-methylation of 16-*O*-demethyltabersonine (DeLuca *et al.*, 1986).

Another novel enzyme which has recently been characterized in our laboratory is the *N*-methyltransferase that catalyzes an *S*-adenosyl-L-methionine-dependent transmethylation to form *N*(1)-methyl-2,3-dihydro-3-hydroxytabersonine or 16-methoxy-*N*(1)-methyl-2,3-dihydro-3-hydroxytabersonine from their respective substrates (DeLuca *et al.*, in press). This enzyme has been shown to be localized in chloroplast membranes but its appearance in etiolated seedlings does not coincide with greening of the seedlings when submitted to light (DeLuca *et al.*, in press).

The enzyme which catalyzes the last reaction in vindoline biosynthesis is an acetyl-CoA-dependent acetyltransferase. Its presence in *Ca*-

tharanthus leaf extracts was simultaneously reported by DeLuca *et al.* (1985) and Fahn *et al.* (1985a). This enzyme was further purified more than 300-fold; its molecular weight as determined by gel filtration chromatography is 44,000, its apparent isoelectric point as determined by chromatofocusing is 4.6, and it has a pH optimum between 8 and 9 (DeLuca *et al.*, 1985). The forward reaction demonstrated an absolute requirement for acetyl-CoA and deacetylvindoline derivatives containing a double bond at position 6,7, whereas the reverse reaction occurred only in the presence of free CoA and vindoline containing the same double bond (DeLuca *et al.*, 1985). Similar results were obtained by Fahn *et al.* (1985a); deacetyl vindoline was shown to be the best substrate for this acetylation reaction, whereas in the reverse reaction acetyl-CoA would be formed. However, no evidence was presented to support this conclusion. Substrate saturation studies resulted in Michaelis–Menton kinetics, giving K_m values of 5.4 and 0.7 *M*, respectively, for acetyl-CoA and deacetylvindoline. The forward reaction was subject to product inhibition by CoA, with an apparent K_i of 8 *M*, but was not inhibited by up to 2 m*M* vindoline. The enzyme appears, therefore, to be regulated by the level of free CoA rather than the alternate product of the reaction.

The role of light in the induction of late vindoline biosynthetic enzymes was demonstrated (DeLuca *et al.*, 1986). In germinating seedlings the induction of tryptophan decarboxylase activity was unaffected by light, whereas tabersonine was the major *Aspidosperma* alkaloid in dark-grown seedlings (Balsevich *et al.*, 1986; DeLuca *et al.*, 1986). When 5-day-old etiolated seedlings were transferred to the light, there was a quantitative transformation of tabersonine and other vindoline precursors to vindoline. This may have occurred as a result of the light-induced increase of acetyl-CoA-dependent acetyltransferase (DeLuca *et al.*, 1986). Whether the other four intermediary steps in the vindoline biosynthesis are induced by light remains to be demonstrated.

The developmentally regulated induction of enzymes involved in tabersonine synthesis could be separated temporally and spatially from that of the last six steps in vindoline synthesis. Tabersonine accumulation reached a maximum rate by day 5, whereas vindoline biosynthesis only commenced after day 5 whether seedlings were grown in the dark or light. Also, tabersonine synthesis could be shown to occur in all plant parts studied, whereas vindoline synthesis could only be shown to occur in the aerial parts of the plant (DeLuca *et al.*, 1986).

The development of enzyme assays for the late stages of vindoline biosynthesis has permitted the establishment of screening programs in order to determine whether these activities are expressed in tissue cultures. The *N*-methyltransferase and O-acetyl transferase that catalyze

the third and last step in vindoline biosynthesis, respectively, could not be detected in any cell line studied (Fahn *et al.*, 1985a; DeLuca *et al.*, 1985; DeLuca *et al.*, 1987). It remains to be seen whether other enzymes involved in the late stages of vindoline biosynthesis are also repressed in cell cultures.

Because dimeric alkaloids, vincristine and vinblastine, as well as the monomeric precursor, vindoline, could be extracted from leaves of the intact plant but were consistently absent from heterotrophic cell suspension cultures, it became apparent that culture of photoautotrophic cells might result in production of these secondary metabolites. A report has described for the first time the development of a photoautotrophic cell line from a green photoheterotrophic cell suspension culture (Tyler *et al.*, 1986). Conditions for photoautotrophy included elimination of sucrose and replacement of 2,4-D with NAA and cytokinin in a CO_2-enriched environment. The photoautotrophic cell suspension cultures thus generated were heavily laden with starch as a result of growth under continuous light. Photoautotrophic periwinkle cells accumulated neither vindoline nor dimeric alkaloids. Trace amounts of vindolinine and 19-epivindolinine as well as other *Aspidosperma*-type alkaloids were tentatively identified in photoautotrophic cultures, indicating that the pattern of alkaloids was similar to that found in the much higher producing photomixotrophic cultures. These results support the conclusions of the seedling work (DeLuca *et al.*, 1986), that differentiation other than, or in addition to, the presence of active chloroplasts may be required to confer upon cells the ability to produce certain secondary compounds. Stability of the photoautotrophic trait was demonstrated as this cell line was maintained for more than 1 year in this state.

V. ENZYMATIC SYNTHESIS OF VINDOLINE AND DIMERIC INDOLE ALKALOIDS

Reports by Stuart *et al.* (1978) and Kutney *et al.* (1982) have claimed that cell-free extracts of young *Catharanthus* shoots were able to form [^{14}C]vindoline using [^{14}C]tryptamine and secologanin as substrates. Contrary to these findings, Stöckigt *et al.* (1985) observed that radiolabeled products actually contained impurities accounting for such radioactivity. In our hands, the 30,000 *g*, 20-min centrifugation step (Kutney *et al.*, 1982) most definitely precipitated the membrane-bound preparation containing the specific *N*-methyltransferase (Table III), making supernatants devoid of this enzyme activity.

Many efforts were made to determine the biosynthetic pathway for formation of dimeric indole alkaloids from their precursors, catharanthine and vindoline. Thus far, several attempts to demonstrate significant incorporation of labeled catharanthine and vindoline into dimers have been unsuccessful or have achieved very low incorporation rates (Stuart *et al.*, 1978; Kutney *et al.*, 1982). Reports by Kutney (1986) and Scott (1986) have claimed improved incorporation of these intermediates into dimers. Whereas Kutney (1986) proposed dimer biosynthesis's taking place cytoplasmically, Scott (1986) indicated the biosynthesis's occurring with membrane-bound enzymes. Due to such conflicting reports, much confusion remains concerning the biochemical basis for dimer formation and the presence of coupling enzyme(s) in both the intact plant (Kutney, 1986; Scott, 1986) and in cell suspension cultures (Kutney, 1986).

VI. CONCLUSIONS

There have been marked advances in the development of novel culture systems. In particular, the area of single-phase culture using elicitors or hormone-habituated cultures for the production of indole alkaloids has still to be tested for possible commercial exploitation.

It has been realized that it is necessary to understand the mechanisms of biosynthesis in order to be able to regulate the production of targeted compounds. In order to do this it is important to make use of tissue cultures as well as intact plants in the isolation and characterization of enzymes involved in specific biosynthetic pathways. By studying the regulation of these pathways we will gain insight into the developmental controls of product synthesis and accumulation and will eventually be able to use this information to trigger specific reactions for targeted product synthesis *in vitro*.

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CHAPTER 23

Purines

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I. INTRODUCTION

Purine alkaloids are widely distributed within the plant kingdom and have been detected in at least 90 species belonging to about 30 genera. Their occurrence, however, is limited to dicotyledonous species, preferentially in tropical and subtropical climates. Caffeine (Fig. 1, **1**) and theobromine (**2**), methylated derivatives of xanthine, are generally the main purine alkaloids and are regularly accompanied in low concentrations by the two methylxanthines theophylline (**3**) and paraxanthine (**4**) as well as by methylated uric acids such as theacrine (**5**), methylliberine (**6**), and liberine (**7**) (Kappeler and Baumann, 1985). Although the meth-

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Fig. 1. Structure formula of purine alkaloids found in the plant kingdom:

1	$R_1 = R_2 = R_3 = Me$	Caffeine
2	$R_1 = H, R_2 = R_3 = Me$	Theobromine
3	$R_1 = R_2 = Me, R_3 = H$	Theophylline
4	$R_2 = H, R_1 = R_3 = Me$	Paraxanthine
5		Theacrine
6	$R = Me$	Methylliberine
7	$R = H$	Liberine

yluric acids are the main purine alkaloids (Wanner *et al.*, 1975; Petermann *et al.*, 1977) in West African coffee species, collectively termed liberioexcelsoids (Charrier, 1978), theacrine was first isolated from tea by Johnson (1937), being present in very young leaves in the parts-per-million range (Citroreksoko *et al.*, 1977). As regards the metabolic relation between methylxanthines and methyluric acids, we refer the reader to the articles by Baumann *et al.* (1976) and Petermann and Baumann (1983).

Purine alkaloids are, botanically and geographically, a widespread component of human diet. Plant species of different families have been refined from East to West into pleasant stimulants, coffee [*Coffea arabica* L. and *C. canephora* Pierre ex Froehner (synonym, *C. robusta* used in this text)], tea (*Camellia sinensis* (L.) O. Kuntze), cocoa (*Theobroma cacao* L.), maté (*Ilex paraguariensis* St. Hil.), guarana (*Paullinia cupana* H.B.K.), and cola (*Cola nitida* Schott et Endl.).

The biosynthetic link of purine alkaloids to primary metabolism is

only partially known. A critical discussion of the extensive literature on this topic would be beyond the limits of this chapter. The last two steps of caffeine biosynthesis, however, have been well investigated. Experiments with leaf disks of coffee (Looser *et al.*, 1974) and with cell-free extracts of tea (Suzuki and Takahashi, 1975) have shown that two methylation steps catalyzed by two discrete methyltransferases (Baumann *et al.*. 1983) lead from 7-methylxanthine via theobromine to caffeine. Tissue cultures of purine-alkaloid-containing plants were established with the predominant aim of *in vitro* mass propagation, either by regeneration of meristems or by embryoid formation (reviewed for the coffee species by Dublin, 1984; Sondahl *et al.*, 1984). Formation of purine alkaloids in cell cultures was first described by Ogutuga and Northcote (1970) for primary callus cultures of the tea plant. Keller *et al.* (1972) first reported on the unexpected potency of *Coffea arabica* cultures to produce caffeine in large amounts, that is, up to 2% of dry weight. As a result a whole series of studies on *in vitro* purine alkaloid formation was initiated in our laboratory. The biotechnological application of coffee cell cultures has been reviewed by Prenosil *et al.* (1987).

II. EXPERIMENTAL

A. Cell Cultures

Plant material is derived from young (6- to 24-month-old) plants that are grown either in the greenhouse or in a controlled environmental chamber under the conditions described by Frischknecht *et al.* (1982). Segments, 5–10 mm long, of apical orthotropic internodes are most suitable in starting a primary callus culture (Frischknecht *et al.*, 1977). Chemosterilized explants are transferred onto a commercially available Murashige and Skoog medium (Flow Laboratories, Irvine, Scotland) supplemented with (mg/liter) sucrose, 30,000; cysteine, 10; thiamine HCl, 1.0; 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0; and kinetin, 0.2, and solidified with agar (8 g/liter). Before autoclaving, the pH of the medium is adjusted to 5.7 to 5.8. The cultures are kept in darkness at 27 ± 1°C. These environmental conditions, together with the medium composition mentioned before, are designated as "standard conditions." They allow good growth and alkaloid production rates in both callus and suspension cultures. When primary callus cultures are transferred to a liquid medium, the growing cell mass becomes heterogeneous as re-

gards the size of the cell aggregates. By decantation large aggregates (5–10 mm in diameter) can be separated from small ones (0.1–1.0 mm). This simple selection step yields two cell lines with a relatively stable type of tissue organisation. Routinely, cell lines are maintained in 100-ml Erlenmeyer flasks by transferring every 12 to 15 days an aliquot of 10 to 15 ml of the suspension to 50 ml of fresh medium. Cultures used for experiments are started with a ratio of cells to nutrient medium of 1 to 5.5.

B. Alkaloid Analysis

Dried cell material is extracted with 0.006 *M* H_2SO_4 and cleaned on a diatomaceous earth column (Extrelut, Merck, Darmstadt, Federal Republic of Germany), according to Frischknecht and Baumann (1980). Samples taken from the liquid medium are filtered (0.2 μm) and directly chromatographed. As a consequence of the methodical progress since the late 1970s, two different kinds of separation and quantification were used, namely, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), as described by Frischknecht and Baumann, in 1980 and 1985, respectively.

III. GROWTH AND PRODUCTIVITY

A. *Coffea arabica*

The purine alkaloid content of about 1 to 2% (dry weight) found in callus cultures corresponds to that of young leaves (1.5%; Frischknecht *et al.*, 1982) and ripe coffee beans (1.3%; Kappeler and Baumann, 1985). For more-detailed information as regards alkaloid production in callus cultures of *Coffea arabica* we refer the reader to Keller *et al.* (1972) and Frischknecht *et al.* (1977). Alkaloid formation in callus cultures was reexamined by Waller *et al.* (1983).

Productivity of suspension cultures varies from cell line to cell line in a wide range, from 0.03 to 0.7%, that is, from 5 to 130 mg/liter. High- and low-producing cell lines may easily be obtained by selecting cells of a culture according to their aggregate size (Section II,B). This leads to cell lines of the high-producing/large-aggregate type and of the low-producing/small-aggregate type. This correlation between cell aggregate size and alkaloid production, may, however, disappear after repeated sub-

culture. Coffee cells growing in different fermenter types (J. E. Prenosil, personal communication) show production characteristics similar to batch cultures. A low alkaloid content (0.038%) was found in suspension cultures by Buckland and Townsley (1975) and may be attributed to the applesauce-like cell morphology.

Alkaloid and dry-weight increase for the high-producing/large-aggregate culture type is shown in Fig. 2. After a lag phase of 4 or 5 days, dry-weight increases rapidly to 16 to 18 g/liter. At the end of the cultivation period of 19 days it decreases slightly. Purine alkaloid formation accelerates during the entire cultivation time, which means that most of the final amount of purine alkaloids is synthesized after the exponential period of growth. Prenosil *et al.* (1986) found that intensive secondary metabolite production begins shortly after sugar depletion in the medium. As in the intact plant, caffeine is the main alkaloid *in vitro*, but about 25 to 50% of the total alkaloid content is theobromine, a value considerably higher than in leaves (Frischknecht *et al.*, 1982) and beans (Kappeler and Baumann, 1985) of *Coffea arabica*. The absolute amount of theobromine is relatively stable, between 6.5 and 9 mg/liter within a long period, and increases markedly at the end of the cultivation period.

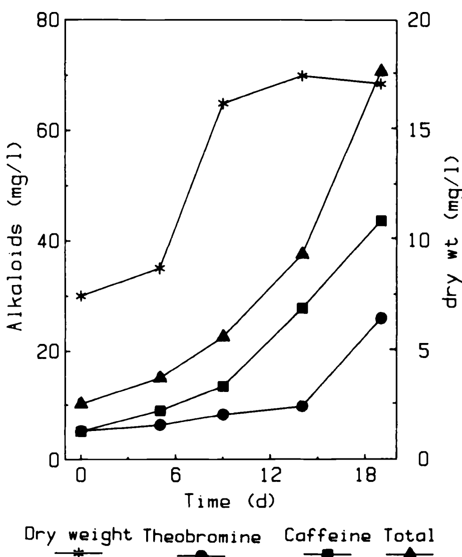

Fig. 2. Growth (*, dry weight) and purine alkaloid (●, theobromine; ■, caffeine; ▲, total) production in a high-producing/large-aggregate cell line of *Coffea arabica* during a cultivation period of 19 days.

Theobromine and caffeine are always dispersed in amounts equal to the ratio of tissue volume to nutrient medium volume. This free exchange was found to occur under all experimental conditions, with the exception of theobromine accumulation in dividing cells, when the sucrose level is increased to 5%. In leaves, 40–60% of caffeine is stored as a molecular complex with chlorogenic acid (A. W. Kappeler and T. W. Baumann, unpublished result). In suspension cultures the formation of an analogous complex was not found, probably due to the low equilibrium constant (44 liter/mol, Sondheimer *et al.*, 1961; Kappeler *et al.*, 1987). Moreover, Buckland and Townsley (1975) reported considerably lower chlorogenic acid levels in suspension cultures than those found in the coffee plant. It was of interest that they observed that in batch cultures chlorogenic acid decreases during active cell multiplication and increases when cell division stops. The release of caffeine into the liquid medium may be associated with the biochemical ecology of purine alkaloids: germinating seeds excrete caffeine into the surrounding substrate as soon as the radicle starts to grow (Baumann and Gabriel, 1984). Because in laboratory tests caffeine has been shown to have "herbicidal" activities, inhibiting seed germination of species relevant to the natural habitat of caffeine-producing plants (Rizvi *et al.*, 1981), Baumann and Gabriel (1984) postulated that caffeine acts as a chemical defence compound against competitors. The ecological biochemistry of purine alkaloids has been reviewed recently by Baumann and Frischknecht (1987).

Coffea arabica suspension cultures need the presence of 2,4-D for satisfactory growth (van de Voort and Townsley, 1974). We found that optimal dry-weight increase is achieved by an initial concentration of 1 mg/liter. Omission or substitution of 2,4-D affects cell growth, which ceases after a few subcultures. With regard to purine alkaloid production, naphthaleneacetic acid (NAA) (1 mg/liter) instead of 2,4-D leads to a two- to threefold stimulation. Omission of auxins or replacement of 2,4-D by indoleacetic acid (IAA) (1 mg/liter) considerably lowers productivity.

B. Other Species

Table I gives a survey of the productivity of a number of purine-alkaloid-containing species and of closely related species. Growth of callus cultures was satisfactory overall, but establishment of suspension

Table I

Survey of Purine Alkaloid Production in Cell Cultures

Species	Total purine alkaloid[a] content (% dry weight)		
	Young leaves[b]	Callus culture	Suspension culture
Coffea arabica	1.5 ca	1.0 ca	0.03–0.7 ca
Coffea robusta	1.9 ca	1.6 ca[c]	0.04–1.5 ca
Coffea congensis	2.7 ca	0.3 ca	0.4 ca
Coffea humilis	0.6 tb	Trace tb	Trace tb
Coffea eugenioides	Trace ca	——	Trace tb
Coffea stenophylla	1.4 ta	0.2 tb[c]	No growth
Coffea arabusta	0.5 ca	Trace tb	No growth
Coffea liberica	1.5 ta	——	Trace tb
Coffea abeokutae	1.5 ta	Trace tb	No growth
Coffea racemosa	0.1 tb	——	No growth
Paracoffea bengalensis	——	Trace tb[c]	No growth
Psilantus mannii	——	——	Trace tb
Theobroma cacao	0.08 tb	——	Trace tb
Paullinia cupana	1.8 ca	0.6 tb	No growth
Camellia sinensis	3 ca	Trace tb	0.01 tb

[a] ca, main alkaloid caffeine; tb, main alkaloid theobromine; ta, main alkaloid theacrine; ——, alkaloid content beyond detection limit.
[b] Values are dependent on leaf age and environmental condition.
[c] Values from primary cultures.

cultures failed in some cases. To ensure comparability of alkaloid production, cultures of all species were grown under standard conditions. Therefore, it is quite possible that in certain cases initiation of cell growth could be reached by optimizing the cultural conditions.

Alkaloid formation is generally very low and is significant only for *Coffea robusta*, *C. congensis*, and in callus cultures, *Paullinia cupana*. Accumulated compounds in all cultures are caffeine and theobromine. Methyluric acids were detected neither in callus nor in suspension cultures. The results of this screening are in accordance with the observations made by other authors. In primary callus cultures of *Camellia sinensis*, Ogutuga and Northcote (1970) found a caffeine content of 0.1%, a substantial part of which may originate from the content of the inoculum. Jallal and Collin (1979) reported a complete absence of purine alkaloids in cocoa (*Theobroma cacao*) callus and suspension cultures.

IV. BIOSYNTHETIC POTENTIAL

A. Biotransformation of Theobromine

With [2-^{14}C]theobromine prepared in our laboratory (Frischknecht and Baumann, 1979) and added to the nutrient medium, the methylation rate of theobromine to caffeine may be measured. Information on the experimental background is given by Frischknecht and Baumann (1980) and Frischknecht *et al.* (1982). In cultures of the high-producing/large-aggregate type the methylation rate (Table II) is two to four times lower than in leaves of the coffee plant (0.5–1.5 mg theobromine per day per gram dry weight (Frischknecht *et al.*, 1982). The methylation rate can be enhanced enormously by the addition (45 mg/liter) of "cold" theobromine. By this means a biotransformation potential is measured during the "exponential" phase, which is in the range of coffee leaves. Within a culture period the rate of the theobromine–caffeine biotransformation is highest during the exponential phase of growth and, surprisingly, does not coincide with the maximum alkaloid formation rate, which is highest in the stationary phase, as shown in Fig. 2. Moreover, the corresponding *N*-methyltransferase activity decreases sharply (Fig. 3) toward the end of the cultivation period. Therefore, the kinetics of

Table II

Biotransformation Rate of Theobromine to Caffeine in Suspension Cultures of *Coffea arabica*[a]

Growth phase	Addition of theobromine (mg/liter)	Methylation rate (μg/g/day)
Lag	——	40
Lag	45	260
Exponential	——	330
Exponential	45	1120
Stationary	——	190
Stationary	45	330

[a] Cultures of the high-producing/large-aggregate type were either fed with [2-^{14}C]theobromine (——) in order to measure the methylation rate of theobromine to caffeine, or additionally, with 'cold' theobromine (45 mg/liter) for assessment of the biotransformation potential.

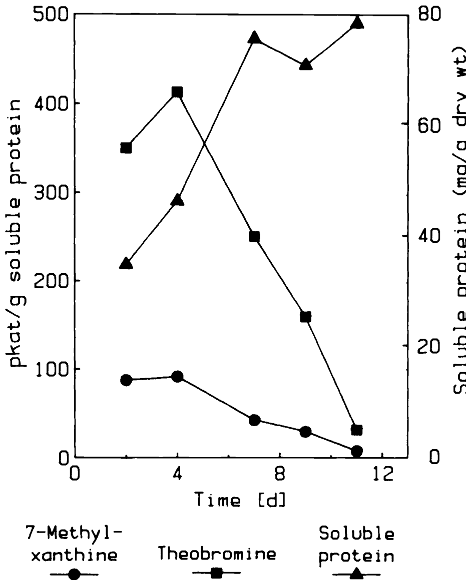

Fig. 3. Time course of soluble protein and of methyltransferase activities as to 7-methylxanthine (●) and theobromine (■) in suspension cultures of *Coffea arabica* during a cultivation period (▲, soluble protein). Adapted from Baumann *et al.* (1983) by permission of Springer-Verlag, Heidelberg.

purine alkaloid formation in cell suspension cultures of *Coffea arabica* may be summarized as follows. Cells in the growth phase have high methyltransferase activities but a deficiency of precursors. When primary processes are reduced, purine metabolites are channeled toward secondary metabolism, coinciding with low methyltransferase activities. This may also explain the increase of theobromine at the end of a cultivation period.

B. Biotransformation of Caffeine

Although caffeine can be regarded as an end product of a biosynthetic chain, the living coffee plant slowly metabolizes this purine alkaloid, preferentially in old leaves (Kalberer, 1965). To test suspension cultures for their metabolizing abilities, 100 mg/liter unlabeled caffeine together with [2-^{14}C]caffeine prepared according to Heftmann (1971) was added at the beginning of the cultivation period (Frischknecht and Baumann, 1980; Baumann and Frischknecht, 1982). Low degradation activity was

Table III

Biodegradation of Caffeine by Suspension Cultures[a]

Species	Degradation rate (μg/g/day)	Degradation products isolated
Coffea arabica	75[b]	Theobromine
Coffea congensis	90[c]	—
Coffea eugenioides	250[b]	Theobromine, paraxanthine
Coffea humilis	800[c]	Theobromine
Coffea liberica	250[b]	Theobromine, paraxanthine
Coffea robusta	40[b]	—
Psilanthus mannii	40[b]	Theobromine, theophylline, paraxanthine
Theobroma cacao	150[c]	Theophylline

[a] Cultures consisting of 6 g of cell material (starting point) and 40 ml of medium were fed with 0.5 μCi of [^{14}C]caffeine with a specific activity of 0.1 μCi/mg.
[b] Maximum degradation rate after the exponential growth phase.
[c] Constant degradation rate during culture period.

characteristic of both culture types of *Coffea arabica*, and of *C. congensis* and *C. robusta*; high degradation activity was observed in *C. eugenioides*, *C. humilis*, *C. liberica*, and *Theobroma cacao* (Table III). *Psilanthus mannii*, which is free of purine alkaloids and closely related to the genus *Coffea*, may serve as a control. In principle, high catabolic activity as to caffeine goes along with a low purine alkaloid production (see also Table I). The metabolizing activity was observed to be either more or less constant during the whole culture period or to reach a maximum after the exponential phase of growth. The *in vitro* pattern of caffeine metabolism is in some respects similar to that in the intact plant of *C. arabica*, especially as seen during leaf development (Frischknecht *et al.*, 1982, 1986a), where a short period of substantial alkaloid biosynthesis in very young expanding leaves (alkaloid content up to 4% dry weight) is followed in fully developed leaves by a phase of breakdown, leading to alkaloid-free leaves at the time of shedding (Weevers, 1930).

V. STRESS-INDUCED PRODUCT FORMATION

Apart from a few exceptions, a profitable exploitation of plant cell cultures in the production of natural compounds has not been achieved.

To overcome these barriers, emphasis was laid on selection or on biochemical research on regulation of secondary metabolism. In an alternative approach we postulate that in cell culture a high complexity of secondary metabolism is reached with a high complexity of the environment (Frischknecht and Baumann, 1985). It is generally accepted that secondary metabolites may protect the plant against the physical and biotic environment (Swain, 1977; Harborne, 1982). It is, therefore, plausible that external factors have a highly modulating effect on secondary metabolism. Indeed, several reports exist on increased levels of secondary compounds under environmental stress conditions (Frischknecht *et al.*, 1986b, and literature cited therein). In ecological terms this is interpreted by a shift from effective but costly defense systems (e.g., tannins) to less costly but less effective defense systems, such as alkaloids, cardenolides, and other substances (Rhoades, 1983). We assume that the *in vitro* creation of physical stress situations comparable to those in nature may influence positively the productivity of cell cultures. This view is supported by the observation that secondary product formation often starts only when the cultured cells are in a nutrient stress, that is, during the stationary phase of growth or if exposed to a deficient medium (Knobloch and Berlin,1980). *In vitro* stress situations in *Papaver somniferum* resulted in a release of thebaine into the culture medium (Lockwood, 1984), and in *Catharanthus roseus* in an increase of indole alkaloid formation (Giger *et al.*, 1985). Imitation of biotic stress by addition of fungal elicitors led to a sanguinarine accumulation of 2.9% (26 times that of the control) in suspension cultures of *P. somniferum* (Eilert *et al.*, 1985).

Screening of a number of physical stress factors showed that high and alternating temperatures had no effect, whereas low temperatures and polyethylene glycol had a negative effect on purine alkaloid formation in *Coffea arabica* suspension cultures. High light intensity (400 μmol/ sec/ m^2) affected in all combinations tested a considerable alkaloid increase (Fig. 4). Highest absolute contents (almost 0.5 g/liter) were found in cultures of the small-aggregate type (Frischknecht and Baumann, 1985). The effect of salt stress (7.5 g per liter of medium, i.e., together with the nutrient solution a water potential of about −10 bar) is directed by the aggregate size: in cultures with small cell aggregates, purine alkaloid production was inhibited, whereas in cultures with large cell aggregates it was increased. The combination of both stress forms, light and sodium chloride, has a cumulative effect: large cell aggregates exhibited an additional increase in alkaloid formation, whereas small cell aggregates had a production intermediate to that with each kind of stress alone. Under stimulating stress conditions the relative alkaloid content shifts from 30–60% theobromine and 40–70% caffeine to 5–20% theobromine and 80–

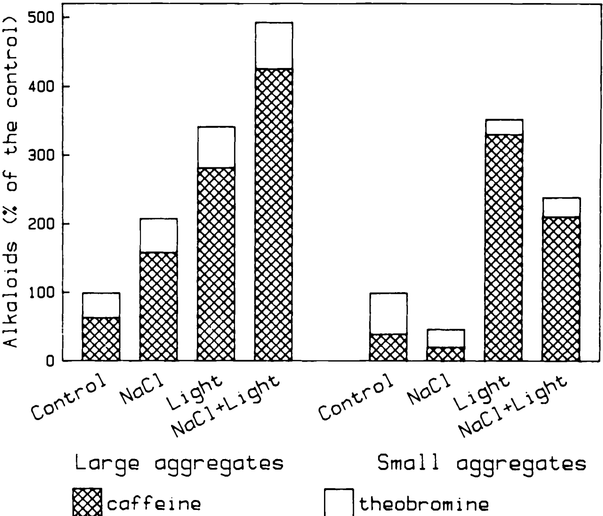

Fig. 4. Effect of stresses such as light and sodium chloride on purine alkaloid (shaded, caffeine; unshaded, theobromine) production in suspension cultures of *Coffea arabica*.

95% caffeine, independent of the culture type. The latter distribution corresponds to that in young leaves (Frischknecht *et al.*, 1982).

VI. CONCLUSIONS

Commercial production of secondary metabolites by plant tissue culture will probably be limited to a few compounds of high value. Caffeine obtained in large quantities by the decaffeination process does not belong to this category. Our motivation in studying purine alkaloid formation in cell culture is based on the fact that the purine alkaloid system is a very suitable model for investigating *in vitro* production of secondary compounds. The key advantages are (a) the presence of only two alkaloids that may easily be analyzed quantitatively by HPLC, simply taking an aliquot of the culture medium, (b) selection of cell lines with relatively stable productivity on the basis of cell aggregation characteristics, and (c) a large body of background information concerning physiological and ecological properties of purine alkaloids that is essential in developing new strategies for synthesis of natural products by means of tissue culture biotechnology.

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CHAPTER 24

Acridones (*Ruta* Alkaloids)

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I. INTRODUCTION

Acridines, known to chemists since the turn of the century, were first detected in plants in 1948 (Hughes *et al.*, 1948). A multitude of different structures has since been elucidated (Mester, 1983). Their occurrence, however, is restricted to members of the Rutaceae, a plant family with an extraordinarily broad spectrum of secondary metabolites.

When pharmacognosists became interested in the potential of tissue culture for production of medicinal compounds, *Ruta graveolens* L., a traditional medicinal plant, was successfully subjected to cell culture (Reinhard *et al.*, 1968). In contrast to many others, *R. graveolens* cell cultures displayed an astonishing diversity of secondary metabolites. Corduan and Reinhard (1972) reported on volatile oil production, von Brocke (1972) on coumarins, and Boulanger *et al.* (1973) on furoquinolines. Scharlemann (1972) discovered three acridone alkaloids while studying chloroplast pigments in *R. graveolens* cultures. Two of these alkaloids, rutacridone and 1-hydroxy-*N*-methylacridone, had previously been isolated from roots. Their occurrence in light-grown, stem-derived

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callus led to the conclusion that cells would express omnipotency when cultured *in vitro* (Czygan, 1975). For a while this generalization nourished hopes of finding high-yielding cell lines by simply screening for them. Since the first report by Reinhard *et al.* (1968), various groups have investigated various aspects of acridone alkaloid formation *in vitro* and have given further impulse to the use of plant cell cultures.

II. ACRIDONES IN TISSUE CULTURES

The acridone alkaloids isolated from tissue cultures are listed in Table I, structures **1–14** (Fig. 1). All 14 acridones isolated occur in *Ruta graveolens* cultures. Four additional *Ruta* species and *Boenninghausenia albiflora* also gave rise to acridone-producing cultures. *Evodia hupehenis* and *Zanthoxylum simulans* cultures proved negative when screened for acridone epoxide production (Engel, 1985); investigations to determine whether other acridones had been accummulated were not performed.

Most alkaloids found in tissue cultures had previously been isolated from plant extracts, whereas rutacridone epoxide (RE), hydroxyrutacridone epoxide (HRE), 1-hydroxyrutacridone epoxide, and rutagravin were first isolated from tissue cultures. Occurrence of the latter two acridones has not been demonstrated in plants. Pyranoacridone-type alkaloids, of interest because of potential cytostatic activity (e.g., acronycine; Gerzon and Svoboda, 1983), have not been found in tissue cultures.

The detection of RE and HRE did not occur by using classical methods. They turned out to be active compounds in extracts of *Ruta graveolens* when screened for antibiotic activity (Wolters and Eilert, 1981). Screening extracts of plant cell cultures for pharmacological activity may prove successful in other systems as well and could help to detect cultures with valuable and perhaps novel compounds.

III. ACRIDONE PATTERNS

The aerial parts of the intact plant contain mostly simple acridones, whereas the roots, the main storage site of acridone alkaloids, harbor compounds of the furano type. Following up on the results of

Table I

Acridone Alkaloids Isolated from Cell Cultures

	Alkaloid	Plant species	Type of tissue culture	Light condition	Medium[a]	Reference
1	1-Hydroxy-*N*-methylacridone	*Ruta graveolens*	Callus	Light	EM	Scharlemann (1972)
			Callus (stem derived)	Light/dark	—	Szendrei *et al.* (1976)
			Callus (root derived)	Dark	—	Szendrei *et al.* (1976)
			Suspension	Dark	—	Kuzovkina *et al.* (1984)
2	1-Hydroxy-3-methoxy-*N*-methylacridone	*Ruta graveolens*	Callus	Light	EM	Scharlemann (1972)
			Suspension	Dark	—	Kuzovkina *et al.* (1984)
		Boenninghausenia albiflora	Callus	—	—	Kuzovkina *et al.* (1983)
3	1-Hydroxy-2,3-dimethoxy-*N*-methylacridone	*Ruta graveolens*	Callus	Light/dark	—	Szendrei *et al.* (1976)
			Callus	Light	EM	Kuzovkina *et al.* (1984)
4	Rutacridone	*Ruta graveolens*	Callus	Light	EM	Scharlemann (1972)
			Callus (stem derived)	Light/dark	—	Szendrei *et al.* (1976)
			Callus (root derived)	Dark	—	Szendrei *et al.* (1976)

(*continued*)

Table I (*Continued*)

Alkaloid	Plant species	Type of tissue culture	Light condition	Medium[a]	Reference
		Callus	—	—	Kuzovkina *et al.* (1979)
		Suspension	—	—	Kuzovkina *et al.* (1984)
		Suspension	—	—	Kuzovkina *et al.* (1980)
		Suspension	Light/dark	MS	Eilert *et al.* (1984)
	Boenninghausenia albiflora	Callus	—	—	Kuzovkina *et al.* (1983)
5 Rutacridone epoxide	*Ruta graveolens*	Callus	Light/dark	EM	Nahrstedt *et al.* (1981)
		Suspension	Light/dark	MS	Eilert *et al.* (1984)
	Ruta chalepensis	Suspension	Light	Various	Eilert *et al.* (1983)
	Ruta corsica	Suspension	Light	Various	Eilert *et al.* (1983)
	Ruta bracteosa	Suspension	Light	Various	Eilert *et al.* (1983)
	Ruta macrophylla	Suspension	Light	Various	Eilert *et al.* (1983)
	Boenninghausenia albiflora	Callus	—	—	Engel (1985)
6 Gravacridonol	*Ruta graveolens*	Callus	—	—	Nahrstedt *et al.* (1985)
7 Hydroxyrutacridone epoxide	*Ruta graveolens*	Callus	Light/dark	EM	Eilert *et al.* (1982)
		Suspension	Light/dark	MS	Eilert *et al.* (1984)
	Ruta chalepensis	Suspension	Light	Various	Eilert *et al.* (1983)
	Ruta corsia	Suspension	Light	Various	Eilert *et al.* (1983)

		Ruta bracteosa	Suspension	Light	Various	Eilert *et al.* (1983)
		Ruta macrophylla	Suspension	Light	Various	Eilert *et al.* (1983)
		Boenninghausenia albiflora	Callus (root derived)	Light	——	Engel (1985)
8	Gravacridondiol	*Ruta graveolens*	Root organ culture	Dark	——	Rosza *et al.* (1976)
9	Gravacridondiol monomethylether	*Ruta graveolens*	Callus (root derived)	Dark	——	Szendrei *et al.* (1976)
10	Gravacridondiol glucoside	*Ruta graveolens*	Root-organ culture	Dark	——	Rosza *et al.* (1976)
		Boenninghausenia albiflora	Callus	——	——	Kuzovkina *et al.* (1983)
11	Gravacridone chlorine	*Ruta graveolens*	Callus (stem and root derived)	Dark	——	Szendrei *et al.* (1976)
12	Isogravacridone chlorine	*Boenninghausenia albiflora*	Callus	——	——	Kuzovkina *et al.* (1983)
13	1-Hydroxyrutacridone epoxide	*Ruta graveolens*	Callus	——	——	Nahrstedt *et al.* (1985)
14	Rutagravin	*Ruta graveolens*	Callus	——	——	Nahrstedt *et al.* (1985)

[a] EM, medium after Scharlemann (1972); MS, medium after Murashige and Skoog (1962); ——, no information given.

I $R_1=R_2=H$
1-HYDROXY-3-N-METHYLACRIDONE

II $R_1=H$; $R_2=OCH_3$
1-HYDROXY-3-METHOXY-N-METHYLACRIDONE

III $R_1=R_2=OCH_3$
1-HYDROXY-2,3-DIMETHOXY-N-METHYLACRIDONE (arborinine)

IV RUTACRIDONE

V RUTACRIDONE EPOXIDE

VI GRAVACRIDONOL

VII HYDROXYRUTACRIDONE-EPOXIDE

VIII GRAVACRIDONDIOL

IX GRAVACRIDONDIOL MONOMETHYLETHER

X GRAVACRIDONDIOL GLUCOSIDE

XI GRAVACRIDONE CHLORINE

XII ISOGRAVACRIDONE CHLORINE

XIII : 1-HYDROXYRUTACRIDONE-EPOXIDE

XIV : RUTAGRAVIN

Fig. 1. Acridone alkaloids.

Scharlemann (1972), Szendrei *et al.* (1976) investigated the influence of origin of the explant on the acridone pattern. The alkaloid pattern in light-grown material reflected the pattern of aerial plant parts, and dark-grown material, independent of the origin of the explant, exhibited a rootlike acridone pattern. Culturing in liquid medium (Table I) seems to result in a shift to formation of compounds earlier in the biosynthetic pathway. The dimethylated acridone arborinine (**3**) has not been found in suspension cultures, and of the furanoacridones only rutacridone (**4**), its epoxide (**5**), and HRE (**7**) have been reported.

IV. CULTURE CONDITIONS AND ACRIDONE YIELD

The common approach to optimize secondary metabolite production in tissue culture is by variation of culture conditions. Only a few reports on the effect of nutrients and light on alkaloid yield have been published; acridone alkaloids are difficult to quantify. The strong lipophilic nature (virtually insoluble in aqueous phases) makes these compounds unsuitable for reversed-phase high-performance liquid chromatography (HPLC). Strong adsorption on silica gel dictates the use of so polar a solvent that sufficient resolution is not obtained (U. Eilert, unpublished results). Gas liquid chromatography (GLC) of underivatized acridones requires extreme conditions and thus is far from ideal, whereas derivatization will cause a loss of information, as discrimination between the epoxides and their hydrolysis products becomes impossible. For rutacridone, however, a method for spectrophotometric determination after thin-layer chromatography (TLC) separation and extraction was developed (Kuzovkina *et al.*, 1979; Eilert *et al.*, 1984). For specific determination of RE and HRE Wolters and Eilert (1982) exploited the strong bactericidal properties of the epoxides to develop a TLC strip bioassay. Other data on quantitative yield are based on gravimetric determination, a method not suited for routine determination.

Kuzovkina *et al.* (1979) as well as Wolters and Eilert (1983) studied the effect of light and hormones on alkaloid production. An extensive study including most major components of the nutrient medium was conducted by Engel (1985).

In general, culturing in continuous dark results in increased acridone alkaloid yields. This result is consistent with the strongly light-regulated expression of the acridone pattern (Table I) and also follows the ex-

pression pattern in the plant. Engel (1985), however, pointed out that the effect of light is also media dependent. Cultures of some *Ruta* species, which produced maximum epoxide yield in continuous dark when grown in Murashige–Skoog (MS) or B5 medium (Gamborg *et al.*, 1968), produced relatively more epoxides on EM medium (see Scharlemann, 1972) when light grown. Looking carefully at the data (Engel, 1985) the EM medium is far from being optimal for acridone production, with RE levels being low and HRE sometimes totally lacking. Furthermore, the level of rutacridone, main alkaloid of the roots and direct precursor of the epoxides, was not determined. Thus the findings should not be interpreted as a contradiction to the general observation of enhanced expression in the dark. Data on rutacridone content would have been desirable to assess whether increased yields are caused by a total increase of accumulation or by a shift in pattern only.

In contrast to many other cell cultures, the type of auxin applied did not affect acridone formation; the presence of an auxin was not favorable at all. A supplement of 1 to 5 mg/liter of kinetin to suspension culture medium was found to be optimal for epoxide production (Engel, 1985). Wolters and Eilert (1983) found highest accumulation in callus cultures on media with low hormone content. Light-grown hormone-autotrophic cultures matched dark-grown callus cultures in epoxide content; their rutacridone content, however, was low. When transferred to the dark, rutacridone accumulation increased whereas epoxide levels did not change significantly (U. Eilert, unpublished results). In hormone-autotrophic cultures a shift to HRE accumulation was observed under all conditions. The presence of hormones, especially auxins, in media has been found antagonistic to secondary metabolite production in many cell cultures (e.g., Kurz and Constabel, 1985). Thus the effect of hormone autotrophy should be investigated further. It may permit achieving a higher degree of expression of secondary metabolism.

Ruta graveolens and *R. macrophylla* cultures were able to use a variety of sugars as a carbon source (Engel, 1985). The content was strongly affected by sugar type and concentration (inverse relation), whereas the HRE level changed with the sugar concentration only. Amount and type of inorganic nitrogen also proved to have a strong influence. Ammonium was found to be inhibitory when supplied as the only nitrogen source, but it was required at a certain ratio to obtain optimum yield. A supplement of organic nitrogen in form of casein proved to be highly favorable in light-grown cultures. This stimulating effect was not obtained in dark-grown cultures. Reduced phosphate levels, thought to be one of the key factors in the alkaloid production medium developed for indole alkaloid formation with *Catharanthus* (Knobloch and Berlin, 1980),

did not result in any increase of epoxide level. Increased levels (10 m*M*) were found to be inhibitory.

After investigating the effect of changing one parameter of the medium at a time, Engel (1985) combined all information when preparing one medium. The effects, however, did not simply add up. Light-grown cultures yielded maximum production on basal MS medium, when supplemented with 1.5% fructose and 5 mg/liter of kinetin, but without casein. Dark-grown cultures performed best on medium that contained 1.5% fructose, 2 mg/liter of kinetin, and 2% casein. These conditions proved optimum for acridone epoxide production by *Ruta macrophylla* cultures as well. Successful replacement of basal MS medium by EM medium required determination of the optimum combination of sugar concentration, kinetin level, and casein supplement anew.

In summary, it can be said that acridone production can be optimized by variation of the culture conditions, and substantial yields (134 mg RE and 80 mg HRE per liter of culture) obtained. These levels exceed alkaloid accumulation in the source plant. Kuzovkina *et al.* (1984) reported rutacridone concentrations in callus that were 20-fold higher than in the plant.

V. ELICITATION OF ACRIDONE EPOXIDE ACCUMULATION

Approach by function follows the modern view of secondary metabolism, which expresses itself in biochemical ecology (e.g., Hartmann, 1985). The plant grows in a complex environ and will respond to environmental signals by expression of certain metabolic capabilities. Tissue cultures are grown under sterile conditions and in as constant an environment as possible. Thus much of the secondary metabolism will not be expressed, or expression will be low. This formed the background for coculturing experiments with *Ruta graveolens* callus and fungi, which resulted in increased accumulation of the strongly antimicrobial acridone epoxides (Wolters and Eilert, 1983). In experiments with callus and suspension cultures, not only living fungi but also autoclaved fungal culture homogenates and fractions of the homogenates stimulated acridone epoxide accumulation; the rutacridone level stayed unchanged. The same effect was also achieved with alginate or chitosan, which both induce phytoalexin accumulation in various different systems (see Chapter 9, Volume 4, this treatise). Studies of the time course

of epoxide accumulation (maximum reached within 24 to 72 hr of elicitation), level of epoxides (up to 2 mg per gram dry weight), spectrum of antimicrobial activity, and range of eliciting agents suggest those compounds to be phytoalexins. A strong structural similarity to isoflavonoids is apparent (Fig. 2). Acridone epoxides present the first example in which elicitor-stimulated accumulation of a secondary metabolite was observed in tissue culture prior to investigation of whether acridone epoxide accumulation is inducible in the plant.

Fig. 2. Glyceollin III.

VI. BIOSYNTHESIS OF ACRIDONE ALKALOIDS

When Gröger (1969) reviewed the biosynthesis of acridone alkaloids, most of the proposed pathway had to be based on speculation. Incorporation of anthranilic acid into acridone alkaloids by plants (Johne *et al.*, 1970; Hall and Prager, 1969) provided evidence for its role as a precursor. Further elucidation of the pathway, however, could not be achieved by feeding studies in plants. In 1978 Reisch proposed a biosynthetic pathway, still on the basis of the chemical structures that had been elucidated and in analogy to the biosynthesis of the related furoquinoline and furanocoumarins. Since then Gröger and his group have elucidated the biosynthesis of acridones using rutacridone-producing suspension cultures of *Ruta graveolens*. They were able to demonstrate anthranilic acid as precursor (Baumert *et al.*, 1982), acetate in form of a polyketo acid (Zschunke *et al.*, 1982), and *S*-adenosylmethionine (SAM) for *N*- and O-methylation (Baumert *et al.*, 1983a) by feeding experiments with radioactively labeled compounds. High incorporation rates of *N*-methylanthranilic acid into rutacridone by these cultures (Baumert *et al.*, 1983a) led to identification of *S*-adenosyl-L-methionine:anthranilic acid *N*-methyltransferase as the first pathway-specific enzyme channeling

anthranilic acid into acridone biosynthesis (Baumert *et al.*, 1983b) (Fig. 3). The enzyme was detectable in acridone-producing cultures only. It is activated by magnesium and shows a broad pH optimum around pH 8 to 9. An investigation over a culturing cycle showed a fairly constant level of enzyme activity, although product accumulation varied. Thus a regulatory role of this enzyme is not assumed.

The next step, activation of *N*-methylanthranilic acid by ATP, forming *N*-methylanthraniloyladenylate (Fig. 3) was shown by Baumert *et al.* (1985). *N*-Methylanthranilic acid proved to be a substrate superior to anthranilic acid, which led to the assumption that *N*-methylacridones are formed via *N*-methylanthranilic acid. Noracridones may be formed from anthranilic acid rather than evolved from *N*-methylated acridones by demethylation. Whereas the activation reaction was inhibited by CoA and required magnesium as a cofactor, no other data were supplied.

Finally, Baumert and Gröger (1985) could report the cell-free synthesis of an acridone alkaloid in a crude enzyme preparation from *N*-methylanthranilic acid and malonyl-CoA (Fig. 3). Although this work presents an important step, much work remains to be done before the regulation of the pathway is understood.

Fig. 3. Pathway of acridone biosynthesis. SAH, *S*-adenosylhomocysteine.

VII. ACRIDONE ALKALOID LOCALIZATION

Lack of secondary metabolite accumulation is often related to lack of special compartments in cultured cells and tissues, as compared to the plant (Lindsey and Yeoman, 1983). As soon as this specialization is

achieved by way of differentiation, eg. laticifers in *Papaver somniferum* (Rush *et al.*, 1985), accumulation of compounds is observed.

Verzar-Petri *et al.* (1976) studied the distribution and storage of acridone alkaloids in plants of *R. graveolens*. Fluorescence microscopy showed isolated parenchyma cells that harbored clusters of droplets that gave a yellow fluorescence specific for acridones. These cells, acridone idioblasts, were found in different organs of the plant but predominantly in root parenchyma. Storage in tracheids was also observed. Several laboratories reported idioblast formation in high-producing cell cultures (Kusovkina *et al.*, 1979; Eilert, 1983; Engel, 1985). They mostly occur among groups of more than 50 cells and frequently are accompanied by tracheids. Electron microscopic examination (Eilert *et al.*, 1986) showed idioblasts differing from other cells by displaying a multitude of vacuoles. A comparative study showed no differences in the morphology of idioblasts of plant or cell cultures. Elicitor stimulation neither induced changes in the ultrastructure of idioblasts nor increased their number. Storage of secondary metabolites in idioblasts is frequent, and they can be regarded as the most primitive of all specialized storage containers (Esau, 1977). Formation of idioblasts and storage of compounds in idioblasts by the plant are, indeed, frequent occurrences in *in vitro* cultures e.g. tannin idioblasts in *Juniperus communis* callus (Constabel, 1969), anthocyanoblasts in cell suspensions of *Ipomoea batatus* (Nozue and Yasuda, 1985), sanguinarine idioblasts in *Macleaya cordata* (Neumann and Müller, 1979), or indole alkaloids in *Catharanthus roseus* (Neumann *et al.*, 1983; Eilert *et al.*, 1986)

Cell cultures, it would appear, simulate storage functions of the root rather than the shoot; it is the root that frequently harbors idioblasts in the plant. Such idioblasts, it seems, are the limit of cellular differentiation prior to organogenesis.

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CHAPTER **25**

Terpenoid and Steroid Alkaloids

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I. INTRODUCTION

The potential of plant cell culture for the production of secondary metabolites has been extensively explored since the 1960s. Much effort has been devoted to cell culture of alkaloid-bearing plants because this group of natural products comprises a great number of physiologically active compounds. The majority of alkaloids are derived from protein amino acids, for example, aromatic amino acids or related compounds such as anthranilic acid and nicotinic acid. In some cases alkaloids are of mixed origin. This means that fragments besides amino acids are involved also in alkaloid formation, for example, acetate or mevalonic acid. The carbon skeleton of the isoprenoid alkaloids is solely derived from mevalonic acid. These "pseudoalkaloids" (Hegnauer, 1964) often co-occur with structurally related terpenoids and steroids. The source of the nitrogen and the timing of the introduction of nitrogen into the framework of mevalonate-derived alkaloids are little understood.

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The formation and metabolism of nitrogen-containing terpenoids and steroids in plant cell and tissue cultures is discussed in this chapter. For a more detailed presentation of chemical, biochemical, and physiological aspects of isoprenoid alkaloids the reader is referred to earlier reviews (Schreiber, 1968; Roddick, 1980; Cordell, 1981; Ripperger and Schreiber, 1981; Gross *et al.*, 1985).

II. TERPENOID ALKALOIDS

Terpenoid alkaloids can be divided into groups based on the number of mevalonate units involved in their biosynthesis, namely, mono-, sesqui-, di-, and triterpenoid alkaloids. Monoterpenoid alkaloids are present in plants belonging to the families Actinidiaceae, Bignoniaceae, Gentianaceae, and Loganiaceae. Well-known sesquiterpenoid alkaloids have been isolated from the genus *Dendrobium* (i.e., dendrobine) and from rhizomes of the water lily *Nuphar luteum* (e.g., nupharidine). The diterpenoid alkaloids are conveniently subdivided in two groups based on chemical structure and biological activity:

1. Highly toxic C_{19} alkaloids, which are substituted by many hydroxyl or methoxyl groups, where some of the hydroxyl groups are esterified; these toxic alkaloids are mainly found in *Aconitum* and *Delphinium* (Ranunculacene)
2. Typical members of the C_{20} alkaloids, for example, atisines and *Garrya* (Garryaceae) alkaloids, which show little oxygen substitution.

The most prominent alkaloids derived from a triterpenoid nucleus are the steroid alkaloids (see Section III). A number of alkaloids possessing a C_{30} or a C_{22} skeleton that are biogenetically related have been isolated from *Daphniphyllum*. Both groups have in common a 2-azabicyclo[3.3.1]-nonane system.

Enormous efforts have been made to clarify the chemistry of these rather complex alkaloids, but knowledge of the biochemistry and physiology of terpenoid alkaloids, including their production in plant cell cultures, is extremely limited.

In callus cultures derived from the roots of *Skytanthus acutus* Meyen, skytanthine (**1**) has been detected by chromatography (Luchetti, 1965). Dohnal (1976a) has grown callus and cell suspension cultures of *Tecoma stans* Juss. on modified Murashige–Skoog (MS) revised tobacco (RT-k)

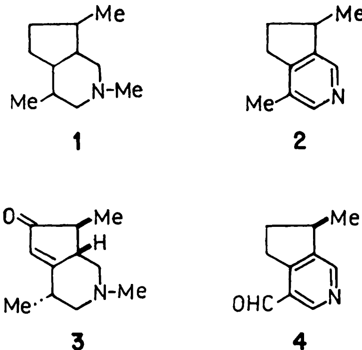

Fig. 1. Various monoterpenoid alkaloids: skytanthine (**1**) actinidine (**2**), tecomanine (**3**), boschniakine (**4**).

medium (Khanna and Staba, 1968) enriched with 0.03 ppm kinetin. Callus cultures propagated on RT-k medium produced in minor quantities actinidine (**2**), tecomanine (**3**), and skytanthine derivatives. Surprisingly, in cultures supplemented with quinolinic acid, boschniakine (**4**) was accumulated also (Dohnal, 1976b) (Fig. 1).

Eight-week-old root-organ cultures of *Delphinium elatum* L. have been found to contain the diterpenoid alkaloid methyllycaconitine (Strzelecka, 1966). Callus tissue cultures of the common larkspur *D. ajacis* L. were incubated under 3000-lux cool-white fluorescent light (15-hr photoperiod) with 28 and 18°C day-and-night cycles and subculture every 4 to 6 weeks (Waller *et al.*, 1981). The authors stated that the calli were not rich in alkaloids but produced copious quantities of sterols, amounting to 8 to 10% of dry weight. Unfortunately, the nature of the diterpenoid alkaloids was not disclosed.

III. STEROID ALKALOIDS

Steroid alkaloids have been found to occur in four families, that is, Solanaceae, Liliaceae, Apocynaceae, and Buxaceae. According to their structure these alkaloids may be divided into major groups

1. Alkaloids with the complete and unaltered C_{27} skeleton of cholestane, showing different heterocyclic ring systems; most promi-

nent are the spirosolanes, solanidanes, and the 3-aminospirostanes, all occurring in Solanaceae and Liliaceae
2. Alkaloids with an altered C_{27} skeleton, e.g., the C-nor-D-homosteroidal alkaloids, mainly found in the genera *Veratrum* and *Fritillaria* (Liliaceae)
3. Alkaloids with a C_{21} skeleton of pregnane and with an amino group at C-3 and/or C-20 (or an imino group between C-18 and C-20), mainly present in Apocynaceae and to a lesser extent in Buxaceae
4. *Buxus* alkaloids, with a C_{21} skeleton, which can be divided into two groups on the basis of whether they have an additional cyclopropane or an expanded ring B

Steroid alkaloids (group 1, *Solanum* alkaloids) and the cerveratrum-type alkaloids (group 2) occur mainly as *O*-glycosides and only rarely as free alkamines (Fig. 2). Cerveratrum-type alkaloids (group 2) are usually esterified with aliphatic or aromatic acids. Alkaloids of the Apocynaceae and Buxaceae are found in plants as free alkamines and occasionally conjugated with acids, forming *N*-acylamides.

The steroidal sapogenin diosgenin is widely used as a valuable starting compound for the production of steroid hormones. Due to the shortage of diosgenin some *Solanum* steroid alkaloids, that is, solasodine (**5**) and tomatidine (**7**), have gained importance as alternative material for

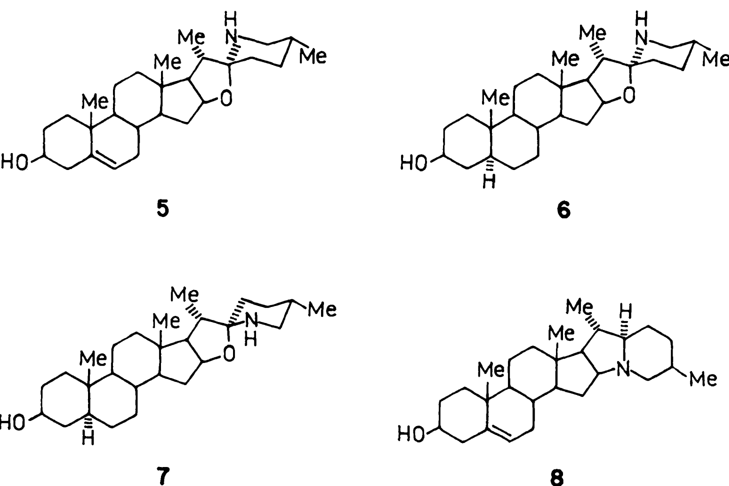

Fig. 2. Structures of some steroidal *Solanum* alkamines: solasodine (**5**), soladulcidine (**6**), tomatidine (**7**), solanidine (**8**).

the manufacture of medicinally useful steroids. Many efforts therefore, have been made to produce steroid alkaloids by plant cell and tissue cultures.

A. *Solanum* Alkaloids

In plant cell cultures of various *Solanum* species, glycoalkaloids and alkamines have been detected that belong to the spirosolane- or solanidane-type alkaloids (Table I). Heble *et al.* (1968) appear to have presented the first evidence for the production of a steroid alkaloid by callus tissues of *S. xanthocarpum*. Four-week-old calli were found to contain solasonine. The frequency distribution of the content of this particular steroid alkaloid in plated colonies of *S. laciniatum* was investigated by Zenk (1978). By using the clonal selection method, strains comparable in solasonine yield (up to 3% of dry weight) to the source plant could be obtained. The screening was performed by means of an efficient radioimmunoassay (RIA), which allows detection of 0.7 ng of solasodine glycosides (Weiler *et al.*, 1980).

Callus cultures of *Solanum acculeatissimum* were cultivated on a modified MS medium (Murashige and Skoog, 1962) supplemented with 1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D), 2 ppm of Δ^5-isopentenyladenine (IP), 10 ppm of adenine sulfate, 10% deproteinized coconut milk and 100 ppm of myoinositol. The cultures were grown for 8 weeks at 25°C with 16 hr of daily illumination (Kadkade and Madrid, 1977). The glycoalkaloids solasonine, solamargine, and the corresponding aglycone solasodine were obtained in crystalline form from callus tissues. The glycoalkaloids are also produced by seeds of the same plant species.

Hosoda and Yatazawa (1979) cultivated callus tissues of *Solanum laciniatum* on a modified MS tobacco medium supplemented with yeast extract (1 g liter^{-1}) and 2,4-D (1 mg liter^{-1}). Besides sterols and a steroidal sapogenin, a new glycoalkaloid was produced by 3-week-old callus cultures. Interestingly, the glycoalkaloid was not identical to solasonine and solamargine, both found in the source plants. The new steroid alkaloid was a glycoalkaloid composed of solasodine as aglycone, rhamnose, and other unidentified sugars.

Tomatine is a glycoside of the steroid alkaloid tomatidine that has been found in a number of species of *Lycopersicon* and *Solanum*. Tomatine was detected in newly initiated *L. esculentum* callus cultures of hypocotyl, radicle, and cotyledon origin that gave rise to large number of roots (Roddick and Butcher, 1972). Established hypocotyl callus tissues

Table I

Production of *Solanum* Steroid Alkaloids by Plant Cell Cultures

Alkaloid[a]	Plant source	Reference
Glycoalkaloids (aglycone plus sugars)		
Solasonine (Solasodine, —Gal—Glu, with Rham branching from Gal)	*Solanum xanthocarpum* Schrad. et Wendl.	Heble *et al.* (1968)
	Solanum acculeatissimum Jacq.	Kadkade and Madrid (1977)
	Solanum aviculare Forst.	Zenk (1978)
	Solanum khasianum C.B. Clarke	Kokate and Radwan (1979)
Solamargine (Solasodine, —Gal—Rham, with Rham branching from Gal)	*Solanum acculeatissium* Jacq.	Kadkade and Madrid (1977)
Unknown alkaloid (Solasodine, Rham and unknown sugars)	*Solanum laciniatum* Ait.	Hosoda and Yatazawa (1979)
Solanine (Solanidine, —Gal, branching to Glu and Rham)	*Solanum tuberosum* L. cv. Wauseon, cv. Merrimack	Zacharias and Osman (1977)
Chakonine (Solanidine, —Glu, branching to Rham and Rham)	*Solanum tuberosum* L. cv. Wauseon, cv. Merrimack	Zacharias and Osman (1977)
Dehydrocommersonine (Solanidine, —Gal—Glu, branching to Glu and Glu)	*Solanum chacoense* Bitt.	Zacharias and Osman (1977)
Tomatine (Tomatidine, —Gal—Glu—Xyl, with Glu branching from Glu)	*Lycopersicon esculentum* Mill, cv. Suttons Best of All	Roddick and Butcher (1972)

(*continued*)

Table I (*Continued*)

Alkaloid[a]	Plant source	Reference
Alkamines		
Solasodine	*Solanum xanthocarpum* Schrad. et Wendl.	Heble *et al.* (1971)
	Solanum aviculare Forst., *nigrum* L.	Khanna *et al.* (1976)
	Solanum xanthocarpum Schrad. et Wendl.	Khanna *et al.* (1976)
	Solanum eleagnifolium Cav., *S. khasianum* C.B. Clarke	Khanna *et al.* (1976)
	Solanum acculeatissimum Jacq.	Kadkade and Madrid (1977)
	S. laciniatum Ait.	Hosoda *et al.* (1979), Chandler and Dodds (1983a)
	Solanum khasianum C.B. Clarke	Uddin and Chaturvedi (1979)
	Solanum verbascifolium L.	Jain and Sahoo (1981)
	Solanum jasminoides Paxt.	Jain *et al.* (1981)
	Solanum nigrum L.	Bhatt *et al.* (1983)
	Solanum aviculare Forst.	Macek *et al.* (1984)
Solasodine	*Solanum dulcamara* L.	Ehmke and Eilert (1986)
Soladulcidine	*Solanum dulcamara* L.	Willuhn and May (1982), Ehmke and Eilert (1986)
Solanidine	*Solanum khasianum* C.B. Clarke	Kokate and Radwan (1979)

[a] Gal, galactose; Glu, glucose; Rham, rhamnose; Xyl, xylose.

that had been cultured for more than 2 years and that had never produced organized structures accumulated only traces of alkaloids (0.013 $\mu g\ mg^{-1}$ dry weight). Suspension cultures failed to produce alkaloids. Apparently, the biosynthesis of tomatine is closely correlated with morphological differentiation, that is, root formation.

In plant cell cultures the most thoroughly investigated steroid alkaloid is solasodine, an aglycone of various spirosolane-type glycoalkaloids. Unfortunately, alkaloidal extracts or dried calli often are subject to hy-

drolysis. Therefore, it is unclear which alkaloids are genuinely present in the plant cells, for example, various glycoalkaloids or a mixture of glycoalkaloids and solasodine. In a number of callus and suspension cultures of various *Solanum* species this particular alkamine, solasodine, has been found in small amounts (Heble *et al.*, 1971; Khanna *et al.*, 1976). When cell suspension cultures of *S. aviculare* were fed with cholesterol (90 mg per 100 ml of medium), a remarkable increase in solasodine content (0.47%) compared to controls (0.026%) could be observed (Khanna *et al.*, 1977).

The effect of culture conditions on the production of solasodine in callus cultures of *Solanum laciniatum* has been investigated by Hosoda *et al.* (1979) and Chandler and Dodds (1983a,b). It was found that solasodine was produced more actively in rapidly proliferating callus tissues. The concentration in the tissue was about 0.05% (dry-weight basis) during the first 5 weeks of culture. The highest yield of the steroid alkaloid was obtained with a 2,4-D concentration of 1 to 2 ppm. Other auxins at the same concentration gave slightly inferior results. Apparently stable cell lines could be maintained by subculturing callus tissues through more than 60 generations. Root- and hypocotyl-derived callus tissues showed no difference in alkaloid production (Hosoda *et al.*, 1979). Chandler and Dodds (1983a) reported on callus and suspension cultures of *S. laciniatum*. The solasodine concentration in both types of culture ranged from 0.5 to 1 mg g^{-1} dry weight. The solasodine concentration was enhanced by the induction of organogenesis (root formation) in callus tissues. Interestingly, abscisic acid (ABA) in a concentration of 0.04 mg $liter^{-1}$ increased solasodine yield in calli. Also, dark-green callus contained more steroid alkaloid than light-grown. In leaf-derived callus cultures, solasodine concentrations increased when medium phosphate or nitrogen concentrations were reduced to one-eigth or when the sucrose concentration was increased from 3 to 4–8% (Chandler and Dodds, 1983b).

Uddin and Chaturvedi (1979) established callus cultures of *Solanum khasianum* from excised radicles, whole seedlings, roots, shoot apices, and leaves in revised MS medium supplemented with 2,4-D (1 mg $liter^{-1}$) and 1% agar. All callus tissues contained solasodine, but maximum yield (0.067%) was observed in callus cultures derived from whole seedlings. Callus cultures of *S. jasminoides* (Jain *et al.*, 1981) and *S. verbascifolium* (Jain and Sahoo, 1981) also were found to produce solasodine, reaching maximum yields after 6 weeks of incubation.

Macek *et al.* (1984) initiated callus tissues from roots, stems, and leaves of *Solanum aviculare* plantlets. The formation of solasodine in calli originating from different parts of the plant was compared. The highest

average production was found in calli derived from roots, the lowest in those from stems, amounting to 0.31 and 0.22 mg g^{-1} dry weight, respectively.

Leaf explants of *Solanum nigrum* were cultured on a basal medium containing inorganic salts (Murashige and Skoog, 1962), vitamins (Gamborg *et al.*, 1968) and 0.8% agar supplemented with combinations of indoleacetic acid (IAA), benzyladenine (BA), and 3% sucrose. The explants and resulting calli were grown at 25°C in either a 16-hr photoperiod or in the dark (Bhatt *et al.*, 1983). The callus tissues formed, under all growth conditions, differentiated structures, for example, shoots and/or roots. Solasodine concentrations were separately measured in the calli and differentiated structures. IAA at 1 μM concentration was generally stimulatory for solasodine formation in callus tissues. Any addition of BA inhibited alkamine biosynthesis. Based on numerous experiments, the authors concluded that light and various combinations of growth regulators in the medium interact in some complex manner to control solasodine biosynthesis in callus and differentiated tissues. Increasing amounts of sucrose in the medium up to 10% favored growth and steroid alkaloid production.

A number of isoprenoid compounds, that is, triterpenes, sterol derivatives, spirostanes, and spirosolanes, as well as fatty acids, were determined in callus cultures of the soladulcidine chemovariety of *Solanum dulcamara* (Willuhn and May, 1982). In callus cultures derived from shoots and leaves, the spirostane tigogenine and, to a lesser extent, the corresponding *N*-analog soladulcidine (**6**) were detected. The alkaloid yield was not given.

A detailed study of steroid alkaloid production in cell cultures of the soladulcídíne chemovariety of *Solanum dulcamara* has been performed by Ehmke and Eilert (1986). Callus and suspension cultures were grown on MS basal medium supplemented with 2,4-D (1 mg $liter^{-1}$, callus; 0.1 mg $liter^{-1}$, cell suspension), kinetin (0.4 mg $liter^{-1}$, callus; 0.25 mg $liter^{-1}$, cell suspension), and 3% sucrose. The cultivation was carried out under heterotrophic and mixotrophic conditions. The heterotrophic cell line contained traces of neutral sapogenins but no alkaloids. The mixotrophic green cultures (calli and cell suspensions) accumulated diosgenin and tigogenin as well as the corresponding spirosolane alkaloids solasodine and soladulcidine. Total alkaloid concentrations amounted to 0.2 and 0.1 mg g^{-1} dry weight in callus tissues and cell suspensions, respectively. A positive correlation was observed between chlorophyll and alkaloid content of the cells. Also, organogenesis promoted steroid alkaloid accumulation.

A number of glycoalkaloids and an alkamine of the solanidane type

were found in some *Solanum* species. Zacharius and Osman (1977) established callus cultures of *S. chacoense* and some varieties of *S. tuberosum*. They were cultured on a modified LS medium (Linsmaier and Skoog, 1965) supplemented with 1 mg liter^{-1} of α-naphthaleneacetic acid (NAA) instead of IAA and 0.2 mg liter^{-1} of kinetin. The calli obtained generated roots after 10 weeks of culture. From 750 mg of cultured roots, 50 mg of dehydrocommersonine was isolated, a hitherto-unknown alkaloid. The callus tissues were devoid of glycoalkaloids. Root formation was also observed in some varieties of *S. tuberosum*. These organs were found to contain solanine and chaconine, both of which are normally present in the tubers. In calli of *S. tuberosum* that failed to initiate roots, alkaloids could not be detected.

Rootlets forming callus tissues of *Solanum khasianum* contained 5.2% (based on dry weight) steroid alkaloids. The mixture is composed of an unknown glycoalkaloid, solasonine, a spirosolane-type alkaloid, and solanidine (Kokate and Radwan, 1979). In undifferentiated callus cultures only traces of solasonine and solanidine (**8**) were present. This is a further striking example that in some cell cultures, morphological differentiation is associated with biochemical "differentiation."

Cell suspension cultures of *Solanum tuberosum* and potato tuber tissues were found to glycosylate labeled solanidine. After 12 hr of incubation the content of *O*(3)-glucosylsolanidine (γ-chaconine) reached a maximum. The onset of the formation of 3-β-*O*-glucosyl(glucosyl)solanidine was observed after 24 hr, reaching maximum yield after 72 hr of incubation. The two major potato glycoalkaloids, that is, α-solanine and α-chaconine, are not formed under these conditions (Osman *et al.*, 1980).

B. Miscellaneous Alkaloids

The first attempt toward a continuous production of glycoalkaloids derived from solasodine was made by Jirku *et al.* (1981). Cells of a suspension culture of *Solanum aviculare* were immobilized by their adsorption and covalent linkage to an activated polymeric adsorbent. Polypropylene oxide activated by 5% glutaraldehyde was used as supporting gel. A packed column was recirculated at 20°C by 100 ml of sterilized sucrose solution (8% v/w). At 24-hr intervals the recirculating medium was replaced with a new one. The released amount of steroid alkaloids was analyzed daily throughout a period of 11 days. An oscillation pattern regarding the amount of eluted alkaloids was recorded. The highest daily yield per 100 mg of cells (dry weight) amounted to 31.9 μg of alkaloid.

Lindsey and Yeoman (1983) described a "flatbed" system in which

cells were cultured in a horizontal mode on the surface of fibrous polypropylene, across which liquid nutrient medium was supplied from a reservoir and recirculated. *Solanum* cells cultured on the flatbed accumulated, in the absence of exogenously supplied precursors, higher yields of steroid glycoalkaloids than did the rapidly dividing suspension cultures. Alternatively, a vertical column packed with cells entrapped in a support matrix was applied. As polymeric matrices, 2% agar or 2% calcium alginate were used. Cells of *S. nigrum* immobilized either in agar or calcium alginate were capable of alkaloid accumulation, with a final content of 11 to 13 mg of alkaloids per gram dry weight of cells after 10 to 12 days of cultivation. The alkaloid yields were reached using illuminated cultures, whereas a significant reduction in the alkaloid content of dark-grown cultures was observed.

Callus cultures of *Holarrhena antidysenterica* (Apocynaceae) accumulated a number of phytosterols but apparently no alkaloids. According to feeding experiments with [4-^{14}C]cholesterol the following biogenetic sequence could be established: cholesterol → 24-methylenecholesterol → 28-isofucosterol → sitosterol → stigmasterol (Heble *et al.*, 1976). Furthermore, radioactive conessine was detected, a C_{21}-skeleton-possessing alkaloid that was not found in untreated callus tissues.

The C_{27} steroid alkaloids are accompanied in plants by analogous steroidal sapogenins (spirostanes). Cholesterol or a biogenetic equivalent of it is the precursor of both the C_{27} steroid sapogenines and alkaloids. Only the late stages of the biosynthetic pathway differ, for example, in the formation of rings E and F. Most work on steroid alkaloid biosynthesis has been performed with intact plants or intact plant organs. Tracer experiments with suitable plant cell cultures seem to be a fruitful area for further research, and high-alkaloid-yielding cell lines may offer the possibility of isolating steroid alkaloid–specific enzymes. Thus it should be feasible to clarify aspects of C_{27} steroid alkaloid biogenesis, for example, the substitution of the 26-hydroxy group of 26-hydroxycholesterol by an amino group of a given amino acid. Aspects of steroid biogenesis and metabolism of steroids in plant tissue cultures have been reviewed (Stohs, 1980; Ripperger and Schreiber, 1981; Heftmann, 1983; Gross *et al.*, 1985).

IV. CONCLUSIONS

The production of terpenoid alkaloids by plant cell cultures is still in its infancy despite the fact that some diterpenoid-derived alkaloids ex-

hibit a remarkably high biological activity. *Solanum* steroid alkaloids have been isolated from nearly 350 plant species. All steroid alkamines of the *Solanum* type possess a C_{27} skeleton of cholestane. The formation of some spirosolanes and solanidanes, which are potential intermediates for the production of steroidal drugs, have been investigated in cell cultures of a few *Solanum* species. Other aforementioned types of steroidal alkaloids have not yet been studied in plant cell cultures.

The *Solanum* alkaloids hitherto investigated certainly do not belong to those groups of natural products that are accumulated by plant cell cultures in high amounts. Therefore, it is necessary to increase the yields if a commercial application is planned. This could be done by using more efficient selection methods and searching for optimal production media. Other conditions also might be favorable for alkaloid accumulation, that is, influence of light (Bhatt *et al.*, 1983; Ehmke and Eilert, 1986) and induction of organogenesis (Roddick and Butcher, 1972; Zacharius and Osman, 1977; Kokate and Radwan, 1979; Bhatt *et al.*, 1983; Chandler and Dodds, 1983a; Ehmke and Eilert, 1986). A modern approach also seems to lead to increases in the alkaloid yield: the application of elicitors to trigger secondary metabolite formation. Rokem *et al.* (1984) could show that in *Dioscorea deltoidea* suspension cultures, diosgenin biosynthesis is stimulated after addition of certain fungal mycelia, especially those of *Rhizopus arrhizus*, to the culture medium.

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NOTE ADDED IN PROOF. Callus autotrophic for both auxin and cytokinin of *Solanum Laciniatum* accumulated solasodine varying from 0.3 to 1.4 mg g^{-1} d.wt. [Chandler, S. F. (1984). *Ann. Botany* **54,** 293–296.] From callus cultures of *Holarrhena floribunda,* a complex mixture of alkaloids was isolated; the main alkaloid was identified as conessine. [Bouillard, L. *et al.* (1987). *Phytochemistry* **26,** 2265–2266.] Callus tissues from different explants of *Solanum eleagnifolium* were found to produce solasodine in a range from 1.0 to 2.15 mg g^{-1} d.wt. [Nigra, H. M. *et al.* (1987). *Plant Cell Rep.* **6,** 135–137.]

CHAPTER 26

Betalains

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I. INTRODUCTION

Betalains represent a group of natural compounds that are particularly fascinating due to their structural and chemotaxonomic properties. Although their structure (Fig. 1) possesses at least one heterocyclic nitrogen atom, betalains do not belong to alkaloids in a strict sense, because they are acidic in nature due to the presence of several carboxyl groups. Interestingly, in microorganisms, as well as in animals, betalains are unknown.

Due to conjugated double bonds betalains are colored. The purple pigments are betacyanins; betaxanthins are yellow to orange colored. Both pigments are characterized by one moiety derived from betalamic acid (Fig. 1a) and can be considered immonium derivatives. Betalain molecules differ from each other by the part bound to the betalamic acid residue. In betacyanins of higher plants this moiety is provided by cyclo-DOPA (Fig. 1b). Its *O*-glycosidation and acylation results in the

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Fig. 1. General betalain formula. (a) Betalamic acid moiety of all molecules. (b) R^1—N—R^2 ≡ residue of cyclo-DOPA (higher plants) or cyclized stizolobic acid (*Amanita muscaria*) in betacyanins. (c) R^1—N—R^2 ≡ Residue of an amino acid or amine in betaxanthins.

formation of a large variety of purple pigments. The condensation of betalamic acid with various amino acids or amines leads to betaxanthins (Fig. 1c), compounds that never show glycosidation. In the mushroom *Amanita muscaria,* several nonprotein amino acids, for example, ibotenic and stizolobic acid, were identified as betaxanthin constituents.

Among higher plants the occurrence of betalains is restricted to the Centrospermae. Most families of this order contain betalains. The finding that they are free of anthocyanins indicates the mutual exclusion of both classes of pigments. Betalains, however, have been detected in association with flavonoid intermediates of the anthocyanin pathway.

Betacyanins and betaxanthins furnish prominent pigmentation of flowers, but also of other plant organs. They are accumulated in the vacuoles of cells. The biosynthetic process can be controlled by several factors and conditions. DOPA is an important precursor (Fig. 2). Light effectively enhances and even induces betalain synthesis.

Betalains have repeatedly been reviewed since the mid-1970s (Piattelli, 1976, 1981; Mabry, 1980; Reznik, 1981; Musso, 1979; Döpp *et al.*, 1982; Schütte and Liebisch, 1985). Still, many open questions exist in the field of betalain biochemistry and stereochemistry. Reasons may be found in the facts that betacyanin and betaxanthin structures have become known only recently, that betalains are of sensitive chemical character, and that betalain analysis is hampered by serious problems despite methodological progress (Döpp and Musso, 1973; Vincent and Scholz, 1978; Schwartz and von Elbe, 1980; Strack *et al.*, 1981).

Cell cultures of higher plants can be advantageous systems for biosynthetic investigations, especially for the isolation of enzymes involved in secondary metabolite formation (Hahlbrock, 1981; Zenk, 1980, 1985). At least from this point of view, betalain-producing cell cultures deserve attention. *In vitro* cultures have been established with several plant species since Constabel (1967) reported successful experiments for the first

Fig. 2. Probable biosynthetic scheme for betalains and stizolobic acid.

time. In this chapter the individual cell cultures are characterized as is their contribution to betalain research and to the knowledge of secondary metabolism of plant cell cultures in general. Finally, cell cultures are considered with respect to a biotechnological production of betalains, that is, as dyes for food.

II. BETALAIN-SYNTHESIZING CELL CULTURES

Betalain formation has been detected in cell cultures of plant species belonging to 5 of the 10 betalain-producing centrospermous families (Table I). Different parts of young and mature plants have given rise to productive *in vitro* systems. Even colorless explants have been useful

Table I

Plant Cell Cultures Showing Betalain Formation

Plant material	Explant	Type of culture	Medium[a]	Pigment	Reference
Amaranthaceae					
Amaranthus caudatus L.	?	Static culture	B5, modified	Betalains	Constabel and Nassif-Makki (1971)
Celosia argentea L.	?	Static culture	B5, modified		
Cactaceae					
Myrtillocactus geometrizans (Mart.) Console	Stem segments	Static culture	LS	Betanin, phyllocactin, indicaxanthin	Colomas *et al.* (1978)
Chenopodiaceae					
Beta vulgaris L.	Seedlings	Suspension culture	B5, modified	Betanin, betaxanthins	Zrÿd *et al.* (1982), Girod and Zrÿd (1985, 1986)
var. *conditiva* Alef.	?	Static culture	B5, modified	Betacyanins, betaxanthins	Constabel and Nassif-Makki (1971)
var. *conditiva* Alef.	Seedlings	Suspension culture	MS, modified	Betanin	McCormick (1972)
var. *crassa* Helm.	?	Static culture	B5, modified	Betacyanins	Constabel and Nassif-Makki (1971)
var. *crassa* Helm.	Root pieces with cambium sections	Static culture	MS, modified	Betacyanins, betaxanthin	Constabel (1967); Constabel and Haala (1968)
var. *rubra* DC. non L. (synonym, var. *conditiva* Alef.)	?	?	Heller, LS	Betacyanins, mainly betanin	Komatsu *et al.* (1975)
Chenopodium album L. var. *centrorubrum*	?	?			

Chenopodium rubrum L.	Meristems of seedlings	Suspension culture	MS, modified	Amaranthin, celosianin, betanin, vulgaxanthin I/II	Berlin *et al.* (1986)
Spinacia oleracea L.	?	?	Heller, LS	Betacyanins, mainly betanin	Komatsu *et al.* (1975)
Phytolaccaceae					
Phytolacca americana L.	Stem segments	Suspension culture	MS, modified	Betacyanins	Sakuta *et al.* (1986)
Phytolacca americana L.	?	Suspension culture	?	Betanin	Misawa *et al.* (1973)
Portulacaceae					
Portulaca grandiflora Hook.	Internode segments	Static culture	White, MS modified	Betanin	Adachi (1970)
Portulaca grandiflora Hook.	Internode segments	Static culture	White, MS modified	Betacyanins	Endress (1976, 1977, 1979), Endress *et al.* (1984)
Portulaca grandiflora Hook.	Seedlings	Static culture	MS, modified	Betacyanins, mainly betanin, betaxanthins	Liebisch and Böhm (1981), Schröder and Böhm (1984, 1987)
Portulaca grandiflora Hook.	Hypocotyls	Static culture	MS, modified		
Portulaca grandiflora Hook.	Seedlings	Static culture	MS, modified	Betaxanthins, betalamic acid, betacyanins	Böhm *et al.* (1987)

[a] B5, Gamborg *et al.* (1968); Heller (1953); LS, Linsmaier and Skoog (1965); MS, Murashige and Skoog (1962); White (1963).

(Constabel, 1967). This confirms the experience that the secondary metabolism of a cell culture is independent of the biochemical status of starting material. Although some experiments have been performed with primary calli transferred a few times at the most (Endress, 1976; Endress *et al.*, 1984; Colomas *et al.*, 1978), other laboratories have maintained cell cultures on solid (Misawa, 1985; H. Böhm, unpublished results) and in liquid media (Berlin *et al.*, 1986) for many years.

The media used for the cultivation of cell material are listed in Table I. In most cases media for long-term culture are identical with the media for callus induction, apart from different auxin concentrations. "Modified" always refers to a change in phytohormone quality and/or quantity; it rarely refers to other components, for example, the carbon source of the medium (Constabel, 1967; Zrÿd *et al.*, 1982). Habituated cell cultures in media free of phytohormones (Zrÿd *et al.*, 1982; Berlin *et al.*, 1986) constitute a special experimental situation.

Some of the cell cultures characterized here did not spontaneously form betalains but showed pigmentation under certain conditions only (see Section III). One prominent example of this group seems to be the callus culture from *Amaranthus caudatus*. This material was reported to contain betalains in trace amounts (Constabel and Nassif-Makki, 1971), but it remained colorless on modified MS media in two other laboratories (H. Böhm, unpublished results; K. H. Köhler, personal communication).

Productive cell cultures have allowed monitoring of betalain composition and quantity during subcultivation. Apparently, the proportion of the individual betacyanins (Berlin *et al.*, 1986) and betaxanthins (Böhm *et al.*, 1987) does not change substantially in the course of a growth cycle. Like other secondary substances in plant cell cultures, betalains generally reach their maximum concentration in the stationary phase of cell growth (Berlin *et al.*, 1986; Böhm *et al.*, 1987). Suspension cell cultures of *Phytolacca americana* showed highest betacyanin levels during the logarithmic growth phase (Sakuta *et al.*, 1986). Such early maxima of secondary metabolite formation in cell cultures have been observed earlier (Böhm, 1977). About 15 days after transfer of *Chenopodium rubrum* cells into fresh medium, 1 liter of suspension culture contained 35 to 45 mg of betacyanins; the yield significantly depended on the ratio of inoculum size to nutrient concentration (Berlin *et al.*, 1986). Neither this nor any other cell culture showed betalain excretion into the medium under normal conditions.

Except for one system the various cell cultures compiled in Table I generally are purple colored. In other words, among betalains the betacyanins dominate; betaxanthins may be absent. As far as individual pigments have been identified, in most cases betanin represents the main component. In cell cultures of *Chenopodium rubrum*, amaranthin

amounts to 80% of betacyanins (Berlin *et al.*, 1986). Minor compounds were rarely analyzed. If betaxanthins are present, they can accumulate in small cell clusters that grow as protuberances (Constabel and Nassif-Makki, 1971). It was possible to isolate and propagate such yellow cell aggregates from a purple *Portulaca grandiflora* callus culture. In this way a brilliant yellow *P. grandiflora* cell culture was established. Its main pigment is probably vulgaxanthin I. Besides minor betaxanthins, free betalamic acid has been identified (Böhm *et al.*, 1987). This finding corresponds with the simultaneous accumulation of betaxanthins and betalamic acid in plants (Reznik, 1978).

III. FACTORS AND CONDITIONS OF BETALAIN FORMATION

As indicated in Section II, cell cultures of betalain-producing plant species have failed to form the characteristic pigments on certain media. For instance, *Portulaca grandiflora* callus remained colorless on media prepared according to Nagata and Takebe (1971) or Schenk and Hildebrandt (1972). Betacyanin formation was observed when cultures were grown on White and MS media (Endress, 1976). On the B5 medium, containing 1 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D), neither cell cultures of *Beta vulgaris* varieties nor *Amaranthus caudatus* and *Celosia argentea* were able to synthesize betalains (Constabel and Nassif-Makki, 1971). The replacement of 2,4-D by α-naphthaleneacetic acid led to betacyanin formation in the cell cultures of some *B. vulgaris* varieties, and on auxin-free medium the cell cultures of all betacyanin-producing *B. vulgaris* varieties showed pigmentation. These processes were paralleled by decreased growth rates. After the addition of gibberellic acid (GA_3), certain *B. vulgaris* cell cultures showed an increased betacyanin accumulation but also the formation of roots (Constabel and Nassif-Makki, 1971).

Root primordia differentiation and betanin synthesis directly followed the transfer of colorless *Beta vulgaris* suspension cultures from an auxin-containing MS medium to one in which auxin was omitted (McCormick, 1972). In both experiments, organogenesis appeared to indicate a level of organization necessary for high betacyanin formation in plant cell cultures. The roots, indeed, may function as sites of betanin biosynthesis, as observed with beet roots (Hamill *et al.*, 1986). The problem is heightened by the fact that the differentiation of roots and cotyledon-like primordia obviously did not lead to betacyanin formation in *Phy-*

tolacca americana callus cultures (McCormick, 1972). Furthermore, GA_3 has been found to affect negatively the secondary metabolism in plant cell cultures (e.g., Yoshikawa *et al.*, 1986), and, therefore, it is doubtful that this phytohormone favors betacyanin accumulation in cultured cell aggregates of *B. vulgaris*. The significant inhibition of betacyanin formation in *Amaranthus caudatus* seedlings by GA_3 is well known (Kinsman *et al.*, 1975).

The betacyanin concentrations of productive cell cultures could only slightly be changed when employing media with various auxin concentrations (Adachi, 1970; Endress, 1976). A kinetin level of about 0.5 mg/liter of medium enhanced the betacyanin concentration considerably above the control value in *Portulaca grandiflora* cell cultures (Endress, 1976; H. Böhm, unpublished results). Cytokinins were shown to exert a stimulating effect on betalain biosynthesis in whole plants. The response partly resembled light action (Piattelli, 1981).

The effect of nitrogen, phosphorus, copper, and carbon sources on betalain formation was investigated. Nitrate and ammonium levels higher than those of the original B5 and MS media changed the growth rate (Berlin *et al.*, 1986), but not the betacyanin concentration of cell cultures of *Beta vulgaris* (Constabel and Nassif-Makki, 1971) and *Chenopodium rubrum* (Berlin *et al.*, 1986). However, the replacement of ammonium by nitrate in the MS medium of short-term suspension cultures of *Portulaca grandiflora* increased the betacyanin concentration substantially (H. Böhm, unpublished results). The elimination of phosphate from the medium resulted in a more intensive pigmentation of *B. vulgaris* callus cultures (Constabel and Nassif-Makki, 1971), whereas it significantly decreased the betacyanin levels in cell cultures from *C. rubrum* (Berlin *et al.*, 1986) and *Phytolacca americana* (Sakuta *et al.*, 1986). The latter two *in vitro* systems showed increased betacyanin concentrations after the addition of phosphate to the medium, up to a phosphate level of 1.25 m*M*, the normal value of the MS medium. Cupric (Cu^{2+}) ions inhibited betacyanin accumulation in *Portulaca grandiflora* calli and acted differently when added to White and MS medium, respectively. This component is considered to be a cofactor rather than a nutrient (Endress, 1976). Testing the effect of increasing carbohydrate levels (2–10%), Berlin *et al.* (1986) found maximum concentration of betacyanins in *C. rubrum* cell cultures in the presence of media with 2% sucrose.

IV. REGULATION OF BETALAIN BIOSYNTHESIS

Tracer experiments with intact plants and plant parts have suggested a biosynthetic pathway of betalains, as illustrated in Fig. 2 (Schütte and

Liebisch, 1985). It is reasonable to assume that the same routes exist in betacyanin- and betaxanthin-producing cell cultures. Indeed, several experiments with *in vitro* systems demonstrated the incorporation of ^{14}C-labeled tyrosine and DOPA into both types of betalains (Liebisch and Böhm, 1981; Zrÿd *et al.*, 1982; Endress *et al.*, 1984). Furthermore, radioactivity could be detected in cyclo-DOPA and in DOPA. The transformation of tyrosine to the latter intermediate is obviously not performed by a colorless callus culture of *Beta vulgaris,* which also shows a very low level of native DOPA. However, both radioactive tyrosine and DOPA are decarboxylated to a much higher extent than in betacyanin-synthesizing *B. vulgaris* cell cultures (Zrÿd *et al.*, 1982). This finding corresponds with the conclusion that the availability of DOPA for the betalain pathway also depends on the intensity of catecholamine biosynthesis, examined in *Portulaca grandiflora* callus (Endress, 1977; Endress *et al.*, 1984). First of all, the hydroxylation of tyrosine influences the endogenous amount of DOPA. An enzyme responsible for this reaction was indirectly characterized by physiological experiments and is probably tyrosinase (Endress, 1977, 1979). The formation of cyclo-DOPA from DOPA could be catalyzed by a phenol oxidase because this enzyme group showed a higher activity in betacyanin-containing cell cultures of *B. vulgaris* than in colorless ones and had a pronounced substrate specificity (Constabel and Haala, 1968). There is no information on further enzymes involved in more typical reactions of betalain biosynthesis. In contrast, an enzyme system catalyzing the synthesis of stizolobic acid (Fig. 2) could be isolated from *Stizolobium hassjoo* seedlings and was extensively characterized (Saito and Komamine, 1978). The α-pyrone amino acid is also formed by *S. hassjoo* callus cultures (Saito *et al.*, 1982) and should arise from the same extradiol cleavage of DOPA as betalamic acid.

After cell cultures from betalain-producing plants were fed with tyrosine or DOPA in substantial amounts, oxidative and degradation processes took place (Constabel and Nassif-Makki, 1971; Berlin *et al.*, 1986). Both precursors were unable to induce betacyanin formation in unproductive cell cultures of several plant species (Constabel and Nassif-Makki, 1971). The feeding of DOPA had practically no effect on betacyanin formation in *Chenopodium rubrum* cell cultures, but tyrosine could remarkably enhance the pigment level of this cell material if it was administered repeatedly (Berlin *et al.*, 1986).

According to experimental data (e.g., Rast *et al.*, 1972) light is not a general prerequisite for betalain biosynthesis in higher plants. However, product accumulation always seems to occur at higher rates under illumination than in the dark.

Cell cultures of *Portulaca grandiflora* (Liebisch and Böhm, 1981; Böhm *et al.*, 1987) and *Beta vulgaris* (Girod and Zrÿd, 1985) intensively pigmented by betalains in light became colorless during subculture in the dark.

After the cultures were returned to light, betalains reappeared due to *de novo* synthesis within a few days. Pigment accumulation on illumination is generally restricted to scattered cell groups and sometimes reveals a change in pigment composition (Liebisch and Böhm, 1981; Girod and Zrÿd, 1985). Surprisingly, *P. grandiflora* callus was reported to incorporate [^{14}C]tyrosine into betacyanins at a much higher rate in the dark than under illumination (Endress *et al.*, 1984). The existence of light-independent betalain-producing cell cultures, however, is apparently exemplified by *Chenopodium rubrum* systems. Callus and suspension cultures continue their betacyanin formation in the dark, the former cell material at a higher level than the latter one. This does not exclude a stimulating effect of illumination, especially of blue light, on the betacyanin biosynthesis in *C. rubrum* cell cultures (Berlin *et al.*, 1986).

V. RESULTS OF GENERAL INTEREST

Betalains are visible, and after their extraction from plant material one can easily determine levels of concentration. Therefore, betalain-containing cell cultures are very suitable for investigations of the secondary metabolism in cells and tissues. Betalains have, for example, successfully been employed in demonstrating the efficiency of selection systems for the maintenance of desirable cell lines. After several transfers, purple- (Constabel, 1967; Liebisch and Böhm, 1981; Zrÿd *et al.*, 1982) and yellow-colored (Böhm *et al.*, 1987) cell cultures were selected and isolated from unproductive, white cell-culture lines (Constabel and Haala, 1968; Liebisch and Böhm, 1981; Zrÿd *et al.*, 1982). In this way comparative experiments could be performed. Besides red cell groups, green ones were also reported in primary calli of *Portulaca grandiflora* explants (Endress, 1976). Isolation and subsequent propagation over a long period of time showed that green cells never contain betalains (H. Böhm, unpublished results).

Liebisch and Böhm (1981) and Girod and Zrÿd (1986) reported weak betacyanin formation in selected white cell cultures. Under the same conditions, cell cultures derived from betalain-deficient mutants remained colorless. These results support the suggestion that the unproductivity of cell cultures from productive plants is caused not by a mutation but by lack of gene expression.

Primary calli of hypocotyl sections of *Portulaca grandiflora*, synthesizing predominantly betacyanins, were used for experiments directed at

the following question: To what extent does the initiation of cell cultures give rise to variation? After several subcultures the betacyanin concentrations of the young *P. grandiflora* cell cultures were determined. As a result the range between the highest and lowest betacyanin concentrations was very narrow. In other words, cell culture initiation with uniform explants resulted in cell groups of nearly homogeneous betacyanin concentrations (Schröder and Böhm, 1984).

On the basis of these data the correlation between the betacyanin content of inbred lines of *Portulaca grandiflora* and of cell cultures derived therefrom was examined. In contrast to most of the data published, the experiments referred to here have not evidenced a positive correlation (Schröder and Böhm, 1987). One can dispute, therefore, that each plant genotype rich in a certain secondary metabolite generates a cell culture with a correspondingly high concentration of this compound.

Sakuta *et al.* (1986) found that the betacyanin concentration of cell aggregates increased with aggregate size in *Phytolacca americana* suspension cultures. In discussing their results the authors have pointed to carrot cell cultures, where the anthocyanin concentration increases with decreasing size of the cell aggregates.

VI. CELL CULTURES FOR PRODUCTION OF FOOD DYES?

Since the 1970s, questions regarding the safety of some artificial dyes in food have been raised by the general public. These questions stimulated ideas and experiments directed at the substitution of synthetic colorants by natural ones. Among suitable plant pigments, betalains, especially betacyanins, have received much attention (e.g., Adams *et al.*, 1976; Havlíková *et al.*, 1985). The only source of importance considered so far is the red beet root (*Beta vulgaris*). Also, patents offering plant cell cultures for the production of betanin and other betacyanins have been filed (McCormick, 1972; Misawa *et al.*, 1973; Komatsu *et al.*, 1975). These systems, it would appear, can hardly compete with the high-yielding red beet, but they may have advantages when compared with various source plants. At least, if the cell cultures are not derived from *B. vulgaris* plants, they are free of the unpleasant smell and taste typical of red beet. The purification of betalain extracts, as recommended for beet roots in several patents (e.g., Behr *et al.*, 1984), would, therefore, not be necessary. Furthermore, betalain-decolorizing enzymes may be absent in cell

cultures. These occur in red beet (Shih and Wiley, 1981) and in *Amaranthus tricolor* seedlings (Elliott *et al.*, 1983), and they complicate the stabilization of betalains as food dyes. Interest in plant cell cultures may increase with the ability of *in vitro* systems to accumulate betaxanthins in high concentrations (Böhm *et al.*, 1987). With respect to this group of betalains no crop plant exists as a competitor to plant cell cultures.

VII. CONCLUDING REMARKS

So far, only a small number of laboratories have become active in research on betalains, and these are mainly interested in establishing new cell culture systems. During the last few years, activity has increased and has been directed at more fundamental questions of betalain formation. In this way it should be possible to clarify more details of the biosynthetic pathway of betacyanins and to study regulation of synthesis, first of all by the isolation of responsible enzymes from cell cultures. Furthermore, the metabolic relationship of betacyanins and betaxanthins is worthy of becoming an object of *in vitro* experiments. In this connection, as in most investigations on betalains, essential insights will depend on work with defined substances. Standardization of the qualitative analysis of betalains, therefore, seems to be an important task. Finally, besides the more theoretical problems, biotechnological production of betalains by cell cultures remains a practical challenge for experimentalists.

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NOTE ADDED IN PROOF. Girod, P.-A. and Zryd, J. P. (1987, *Plant Cell Rep.* **6,** 27–30) have reported the occurrence of betacyanin-containing cell groups in a green, habituated cell culture of *Beta vulgaris*. They studied this process with respect to clonal variability.

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PART **IV**

Glucosinolates, Polyacetylenes, and Lipids

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CHAPTER **27**

Glucosinolates

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I. INTRODUCTION

A. General

Glucosinolates are widespread throughout the families Capparidaceae, Brassicaceae (synonym, Cruciferae), Resedaceae, and Moringaceae, but other plant families also contain glucosinolate-bearing species. Glucosinolates within these plants are responsible for their pungent taste, as hydrolysis occurs on crushing. This autolysis yields one or more degradation products, as shown in Figure 1.

The biosynthesis of more than 15 individual glucosinolates has been studied in plants, and all have been found to be derived from amino acids. In many cases the amino acid undergoes carbon chain elongation prior to incorporation into the glucosinolate.

B. Biological Effects

Both glucosinolates and isothiocyanates have been shown to have antithyroid activity, and damage to liver and kidney has been attributed

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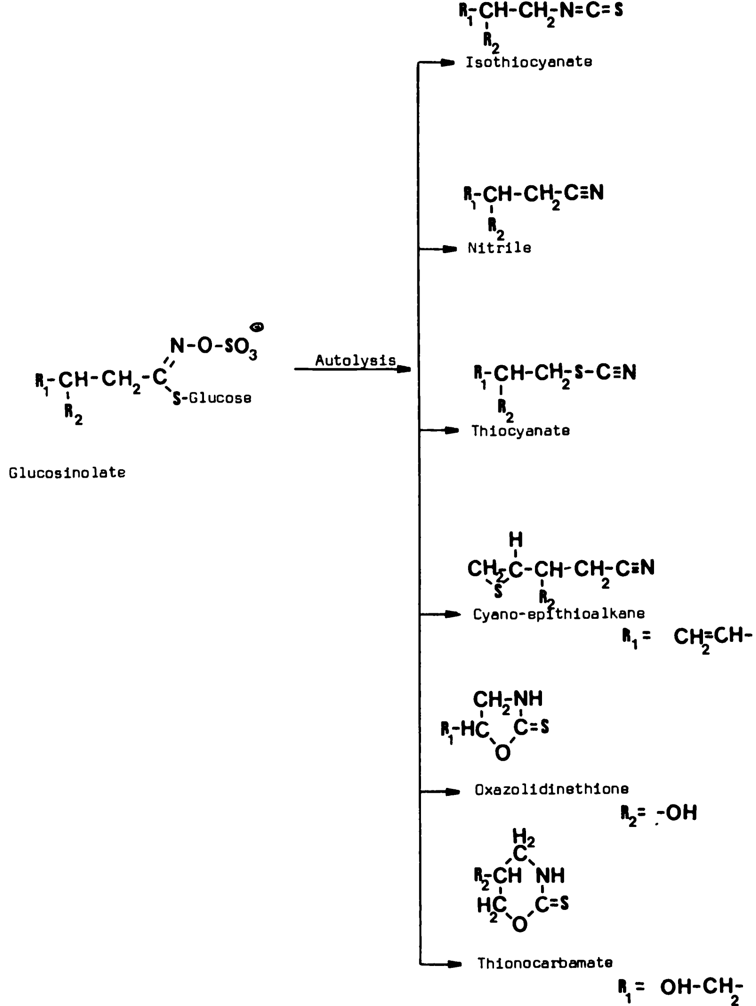

Fig. 1. Degradation products of glucosinolate.

to nitriles (Van Etten and Tookey, 1979). However, possible examples of beneficial glucosinolates have been investigated; the role of benzylisothiocyanate and phenylethylisothiocyanate in inhibiting carcinogen-induced neoplasia in rats and mice through the stimulation of mixed-function oxidases (Wattenberg, 1978) point to the possibility of introduction into our diets of potentially therapeutic chemical constituents.

For some time it has been shown that a number of glucosinolates and isothiocyanates have activity as feeding stimulants in certain insect species, but deterrents in others. This has been shown by both behavioral and electrophysiological data, and allylisothiocyanate has also been shown to attract certain insects to the plant in which it is contained (Schoonhoven, 1972).

II. PLANT CELL CULTURES

Few reports have appeared concerning either the investigation or the identification of glucosinolates in plant cell cultures. Many workers have established cultures of crucifers, however, notably *Sisymbrium irio*, *Sinapis alba*, and numerous species of *Brassica* (Pierik, 1979). Krikorian and Steward (1969) reported the first investigation for glucosinolates, using *Iberis sempervirens* cultures, but no glucosinolates could be detected. Later, Kirkland *et al.* (1971) established suspension cultures from seven plant species and detected myrosinase in all cultures. Only two, those of *Reseda luteola* and *Tropaeolum majus*, were found to contain glucosinolates. Callus cultures from two varieties of rapeseed, *Brassica napus*, have also been established, but the presence of glucosinolates could not be shown by Afzalpurkar (1974). There have been reports concerning the glucosinolates of *Descurainia sophia* (Afsharypuor and Lockwood, 1985; Lockwood and Afsharypuor, 1986a,b) and *Alyssum minimum* (Lockwood and Afsharypuor, 1986a,b) cultures. In both cases the glucosinolates and their degradation products were quantitatively similar to the seed and whole plant, but levels differed, as had been previously found in *R. luteola* and *T. majus* by Kirland *et al.* (1971).

III. ANALYTICAL METHODS

Analysis of glucosinolates is now routinely carried out by identification of their hydrolysis (degradation) products, although previous workers extracted glucosinolates and separated them by partition chromatography prior to identification. The hydrolysis of these glucosides is either carried out by addition of myrosinase or by use of

Table I

Levels of Glucosinolate Degradation Products (μg/g) in Dry Plant Material of *Brassica napus*, Using Dichloromethane Extraction after 17 hr Autolysis

3-Butenylisothiocyanate	13.38
1-Cyano-3,4-epithiobutane	41.04
Isohexylisothiocyanate	9.24
3-Phenylpropionitrile	2.80
Isoheptylisothiocyanate	0.84
2-Phenylethylisothiocyanate	64.84

the endogenous myrosinase enzyme system (autolysis), which is present within plants containing glucosinolates. The liberated volatile hydrolysis products, usually isothiocyanates, nitriles, and/or epithiobutane derivatives, are collected either by distillation or by solvent extraction, and then analysed by gas liquid chromatography. Various workers have studied the factors that influence the products of glucosinolate hydrolysis.

Fresh plant materials, stored or dried at low temperatures, were shown to yield nitriles in favor of isothiocyanates, while hydrolysis of glucosinolates with exogenous thioglucosidase instead of autolysis, and use of high temperatures (up to 75°C), neutral pH, and high dilution with water, favored isothiocyanate production rather than that of nitrile. Using *Lepidium sativum* seed extracts, Gil and Macleod (1980a) had found autolysis temperature and pH to have had little effect on isothiocyanate level, while maximum level occurred after 2 hr, and application of heat drastically reduced isothiocyanate. Later, during extraction of *Nasturtium officinale,* (Gil and Macleod, 1980b) they found that application of heat caused increased nitrile formation in dominance over the isothiocyanate. Both nitriles and isothiocyanates can also be obtained nonenzymatically using heat (Macleod *et al.*, 1981). However, using solvent extraction methods, up to five different hydrolysis products have been detected and quantified in seeds and callus cultures of *Descurainia sophia* (Lockwood and Afsharypuor, 1986a). Table I lists levels of six hydrolysis products detected in a 30-mg sample of *Brassica napus* (Afsharypuor, 1986). The latter solvent extraction method has an added advantage of allowing simultaneous estimation of individual fatty acids and hydrocarbons of cell cultures.

IV. CULTURE CONDITIONS

Kirkland *et al.* (1971) established seven species of cultures, all using Gamborg's B5 medium, containing 1 ppm 2,4-dichlorophenoxyacetic acid (2,4-D), and maintained cell suspensions with the same hormonal composition. These cultures were grown under continuous light at 27°C, and callus was found to have optimum growth rate after about 6 months. Non-glucosinolate-producing callus of *Brassica napus* was grown in Murashige and Skoog medium supplemented with 1 ppm 2,4-D, but culture conditions were not reported by Afzalpurkar (1974). MS medium was also used for callus and suspension cultures of *Descurainia sophia* and *Alyssum minimum*, and a wide range of auxin and kinetin combinations were employed by Lockwood and Afsharypuor (1986a). The latter cultures were generally incubated at 27°C under intermittent lighting (12 hr light, 12 hr darkness). No obvious relationship was found between hormonal supplementation and glucosinolate levels, however; many other hormonal combinations were tested but resulted in either plantlet formation or no growth. Similar media and cultural conditions have been employed with cultures of *B. napus* and *Sinapis alba*, but no glucosinolates could be detected by Afsharypuor (1986). Three-week-old callus of *D. sophia* has been subcultured onto media containing 3 m*M* K_2SO_4 in an attempt to raise available sulfate levels (increasing normal levels by more than 50%), but no improvement of glucosinolate levels was noted (Afsharypuor and Lockwood, 1985). The latter technique had previously been successfully used to produce a dramatic effect on isothiocyanate levels of sand cultured *B. juncea* plants by Freeman and Mossadeghi (1972).

V. CONCLUSIONS

Levels of cell-culture glucosinolates are generally much lower than those of intact plants or seeds. As has been suggested by Kirkland *et al.* (1971), factors or conditions that are conducive either to their biosynthesis or accumulation are often inadequate or totally lacking. It is possible that either some biochemical or morphological differentiation may be necessary for production of reasonable amounts of glucosinolates, as both glucosinolate and myrosinase have been identified in dif-

ferent cytomorphological areas by Matile (1980). There are various studies with callus and suspension cultures in which the accumulation of mixtures of monoterpenes from a range of plants has been reported, but generally these concentrations are much lower and the compositions differ greatly from those in the whole plant, again probably due to association with differences of differentiation.

There may be two additional problems that require solutions before routine accumulation of cell-culture glucosinolates will occur. First, glycosides that yield volatile aglycones are elusive; there is a pronounced lack of reports concerning monoterpene or cyanogenic glucosides. Second, conditions of incubation of callus and suspension cultures are very similar to conditions for autolysis of the glucosinolates. In the future, yields may be improved by solving these problems by use of liquid or gaseous trapping techniques, as have been employed for volatile terpenoids (Bisson *et al.*, 1983).

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CHAPTER 28

Allium Compounds

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I. INTRODUCTION

The application of genetic engineering and plant cell culture techniques to the production of flavorings and seasonings is now part of research programs in the flavor industry (Van Brunt, 1985). The most important commercial flavor sources are plants of *Allium* species, particularly *A. cepa*, the onion, which is used either as a fresh vegetable, as dried flakes, or as concentrated onion oil. It is thought that the application of the new techniques to the production of onion flavor could lead to an increase in the level of flavor by two methods: first, by the develop-

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ment of a new, more highly flavored variety, and second, by the synthesis of onion flavor in large-scale culture of onion cells. A major problem with any attempt to apply these new techniques to increase the yields of secondary products in intact plants, or tissue cultures, is that information on secondary pathways is often very limited. Onion flavor, however, is derived from a small number of compounds, each with a relatively simple biosynthetic pathway. This fact, and the large commercial interest in onion, makes onion a suitable crop for the application of such new techniques to improve flavor production. In this chapter the basic information on the characteristics and biosynthesis of flavors in intact *Allium* is described, followed by an examination of flavor production in tissue cultures of onion.

II. FLAVOR PRODUCTION IN INTACT PLANTS OF *ALLIUM* SPECIES

A. Source of Flavor

Flavor is produced from all parts of the plant, but particularly bulbs, when the tissue is crushed or damaged. On crushing, an enzyme, alliinase, is released that reacts with a number of compounds or flavor precursors, the *S*-alkylcysteine sulfoxides, to produce volatile sulfides, each of which has a characteristic odor. The *S*-alkylcysteine sulfoxides are (+)-*S*-methyl-L-cysteine sulfoxide, $H_3C—SO—CH_2—CH(NH_2)—COOH$ (methylalliin); (+)-*S*-propyl-L-cysteine sulfoxide, $H_3C—CH_2—CH_2—SO—CH_2—CH(NH_2)—COOH$ (dihydroalliin, or propylalliin); *trans*-(+)-SO-(l-propenyl)-L-cysteine sulfoxide, $H_3C—CH{=}CH—SO—CH_2—CH(NH_2)—COOH$; and (+)-*S*-allyl-L-cysteine sulfoxide, $H_2C{=}CH—CH_2—SO—CH_2—CH(NH_2)—COOH$ (alliin). (+)-*S*-Allyl-L-cysteine sulfoxide (All Cys SO) was the first to be isolated (Stoll and Seebeck, 1947, 1948). It is the principal flavor compound in garlic and is present in small amounts in other *Allium* species, including onion. (+)-*S*-Methyl-L-cysteine sulfoxide (Me Cys SO) was isolated from onion in 1959 by Virtanen and Matikkala but is widely distributed in all *Allium* species and in several other members of the Cruciferae, and Liliaceae. (+)-*S*-Propyl-L-cysteine sulfoxide (Pr Cys SO), detected by Virtanen and Matikkala (1959), occurs in smaller amounts and is largely restricted to a number of *Allium* species. *trans*-(+)-*S*-(1-Propenyl)-L-cysteine sulfoxide

(*trans*–Pren Cys SO) was isolated from onion in 1961 by Virtanen and Spare. This compound, at about 4 mg g^{-1} fresh weight in bulb tissue, is the major flavor precursor in onion and contributes to the lachrymatory or tear-producing factor in this species. Of the remaining sulfoxides, (+)-*S*-ethyl-L-cysteine sulfoxide (Et Cys SO) has not been found in *Allium* species, whereas the cyclic sulfoxide cycloalliin is present in large amounts in onion, particularly in dormant tissue, but it makes no contribution to flavor. The other major sulfur-containing compounds are the γ-glutamyl peptides, of which γ-L-glutamyl-*trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide is the most important (Virtanen, 1969). The γ-glutamyl peptides may well represent a storage reserve for nitrogen, because the peptides disappear from the bulb during sprouting (Matikkala and Virtanen, 1965). The significance for the onion flavor is that a considerable proportion of *trans*-Pren Cys SO is locked up as the peptide and cannot contribute to flavor production.

B. Biosynthesis of Flavor Precursors

The most extensive work on the biosynthesis of the flavor precursors was by Granroth (1970), who examined flavor precursor synthesis in intact tissue of onion, garlic, and chives. Using mainly leaf tips, and sometimes bulb scales, Granroth followed the path of carbon-14 labeling after uptake of [^{14}C]cysteine, [^{14}C]serine, and [^{14}C]valine. The onion flavor compounds and amino acids were extracted, then separated by electrophoresis and thin-layer chromatography, according to a method developed by Bieleski and Turner (1966) (Fig. 1). Granroth proposed a biosynthetic route for each of the precursors, *trans*-Pren Cys SO, All Cys SO, Pren Cys SO, and Me Cys SO, in which *trans*–Pren Cys SO arose from valine and cysteine, and the other three sulfoxides from serine (Fig. 2). Thus when [^{14}C]serine was fed to the leaf tip, bulb tissue radioactivity was incorporated into Pr Cys SO, All Cys SO, and Me Cys SO, but not when [^{14}C]cysteine was used, whereas the uptake of [^{14}C]valine or [^{14}C]cysteine led to labeling of the *trans*–Pren Cys SO. The route for biosynthesis of *trans*–Pren Cys SO from valine, α-ketoisovalerate, and methacrylyl-CoA is part of the pathway of valine oxidation (Fig. 3). Methacrylyl-CoA is thought to combine with cysteine to form *S*-(2-carboxypropyl)-L-cysteine (CPC), which represents the beginning of the secondary pathway leading to *trans*–Pren Cys SO (Fig. 3). Thus feeding

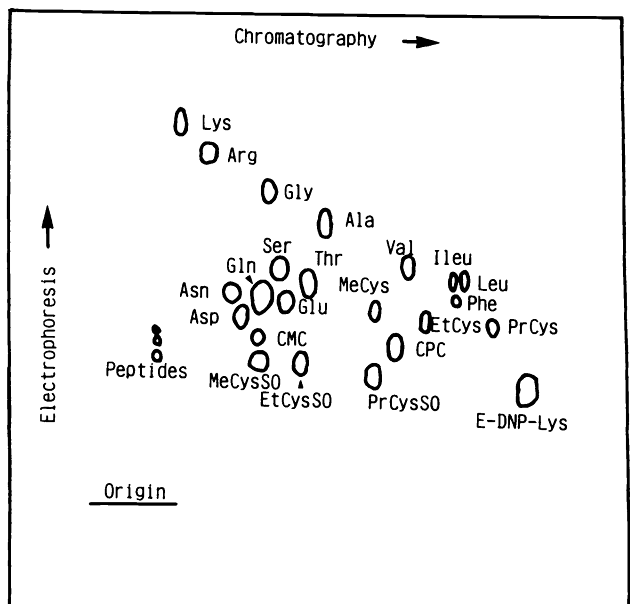

Fig. 1. Trace of electrophoretic thin-layer chromatographic separation of amino acids and flavor-precursor compounds in the onion bulb (Selby *et al.*, 1979).

[^{14}C] CPC, or [^{14}C]*trans*-*S*-(1-propenyl)-L-cysteine, caused radioactivity to appear in *trans*–Pren Cys SO. Although the intermediates on the primary and secondary pathway have been established by Granroth (1970), none of the enzymes involved has been isolated and characterized.

C. Production of Onion Flavor

Undamaged leaf or bulb tissue from *Allium* species has only a slight odor. Immediately on cutting or crushing the tissue, however, there is a rapid development of an odor that is characteristic of each species, and in the onion there is the production of the lachrymatory factor. Thus All Cys SO, Me Cys SO, and Pr Cys SO give rise to the sulfinates diallylsulphinate, methylmethanesulfinate, and propylpropanethiosulfinate, respectively, and aminoacrylic acid, which hydrolyzes spontaneously to ammonia and pyruvic acid. The *trans*–Pren Cys SO leads to

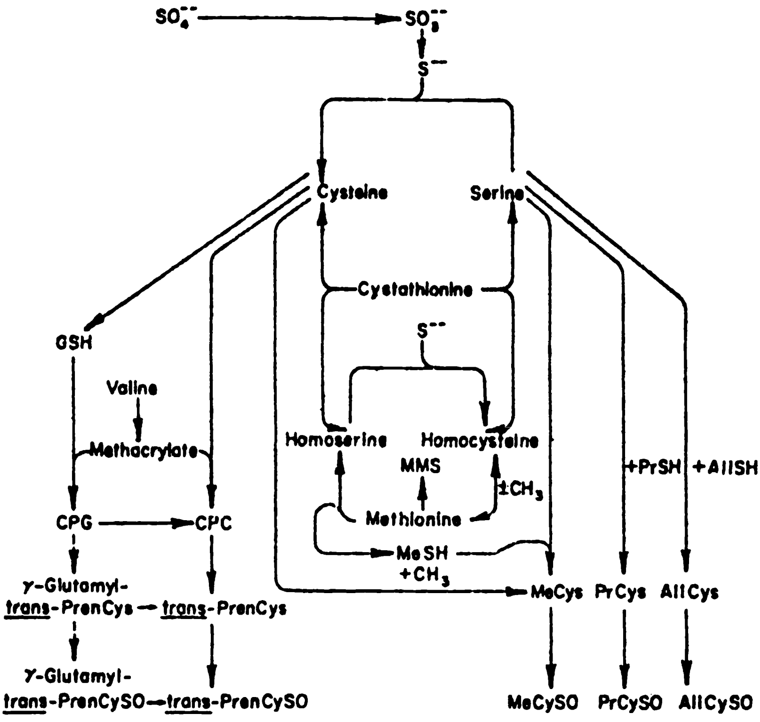

Fig. 2. Biosynthesis of flavor compounds, the *S*-alkylcysteine sulfoxides (*trans*–Pren Cys SO, Pr Cys SO, Et Cys SO, and Me Cys SO) in *Allium* (Granroth, 1970).

the formation of 1-propenylsulfinate (lachrymatory factor), pyruvic acid, and ammonia (Schwimmer, 1968). Although the lachrymatory factor is the most powerful source of flavor in onion, the other sulfoxides are still major contributors to fresh onion flavor. The primary products of the alliinase action, the thiosulfinates, are unstable and undergo spontaneous reactions to form volatile onion odors. The major compounds produced by garlic are diallyldisulfide and allylmethyldisulfide (Brodnitz *et al.*, 1971), and in onion it is dipropyldisulfide followed by methylpropyl disulfide, dimethyldisulfide, and allypropyldisulfide (Boelens *et al.*, 1971).

The liberation of these volatile flavors and odors from the flavor precursors is brought about by the action of an enzyme, alliinase, which is released after cutting or crushing the tissue. The alliinase in garlic (Stoll and Seebeck, 1947; Nock and Mazelis, 1986) and onion (Schwimmer and

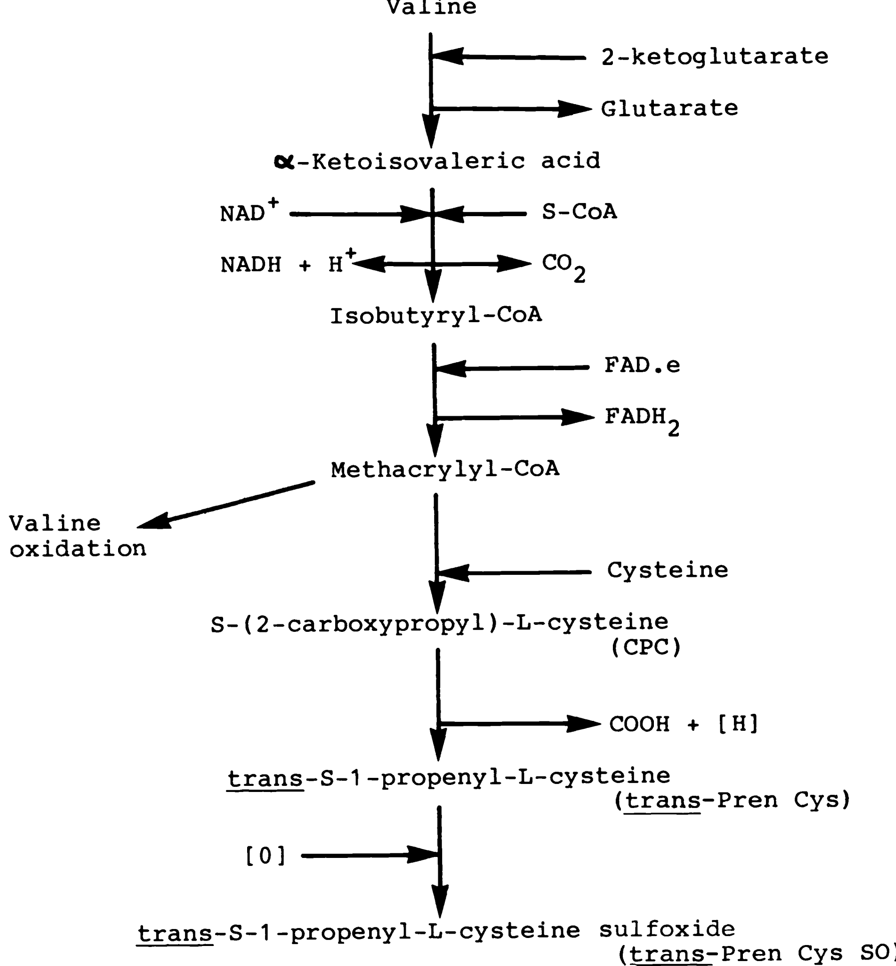

Fig. 3. Biosynthesis of the major flavor-precursor compound in onion, *trans*-(+)-*S*-1-(1-propenyl-L-cysteine sulfoxide (*trans*–Pren Cys SO) (Granroth, 1970).

Mazelis, 1963) has been most extensively studied. The enzyme is a pyridoxal-requiring enzyme, inhibited by hydroxylamine (Karazan *et al.*, 1981) and specific for the cysteine sulfoxides. The alliinase must also be spatially separated from the cysteine sulfoxides in the intact tissue and only released when the tissue is damaged.

D. Intracellular Location of Flavor Precursors and Alliinase

In order to understand the regulation of secondary pathways in plants, it is important that the site of the biosynthetic pathway and site of accumulation of the product be established. In an examination of alliinase, Schwimmer and Mazelis (1963) showed that the particulate fraction from homogenates of etiolated shoots that sedimented at 11,000 *g* contained alliinase activity. This was confirmed in a later paper by Schwimmer (1969), who found that one-half of the activity in the original extract was in the particulate fraction. The activity was heterogeneously distributed among the cellular components, because the activity of the precipitates increased with time and speed of centrifugation.

When the onion bulb scales were fractionated into protoplasts and vacuoles (Fig. 4), however, the activity of alliinase was found in the isolated vacuoles in amounts that accounted for the level of activity in the protoplasts (Lancaster and Collin, 1981). This meant that the alliinase was located in the vacuole and not in the cytoplasm or particulate fraction. The presence of alliinase in the particulate fraction of a total homogenate found by Schwimmer (1969) is likely to be due to the adhesion of the alliinase with this fraction during homogenization. Lancaster and Collin (1981) found the vacuole to contain negligible amounts of *S*-alkylcysteine sulfoxides, whereas there were considerable amounts of these flavor precursors in the protoplasts. Both the synthesis and accumulation of flavor precursors must occur, therefore, in the cytosol. In an electron-microscope examination of the onion bulb cells, J. E. Lancaster and H. A. Collin (personal communication) found large vesicles in the cytoplasm (Fig. 5). These vesicles may be storage sites for food reserves, such as the γ-glutamyl peptides, or for accumulation of flavor compounds. The presence of these vesicles has now been confirmed in 1-month-old germinating seedlings. The vesicles have been isolated by isopycnic density centrifugation on continuous sucrose gradients and found to contain *trans*-Pren Cys SO (Musker, Britton, and Collin; unpublished results, 1987). Becker and Schuphan (1975) suggested that the flavor compounds and alliinase are spatially separated in the cell, with the alliinase possibly in special lysosomes in the cytoplasm, and the flavor precursors in the vacuole. In fact, the situation appears to be the reverse, with the alliinase in the vacuole and the flavor precursor accumulating in the cytoplasm in structures such as the vesicles.

The work by Granroth (1970) did not contain any information on the intracellular site of either the primary or secondary pathways associated

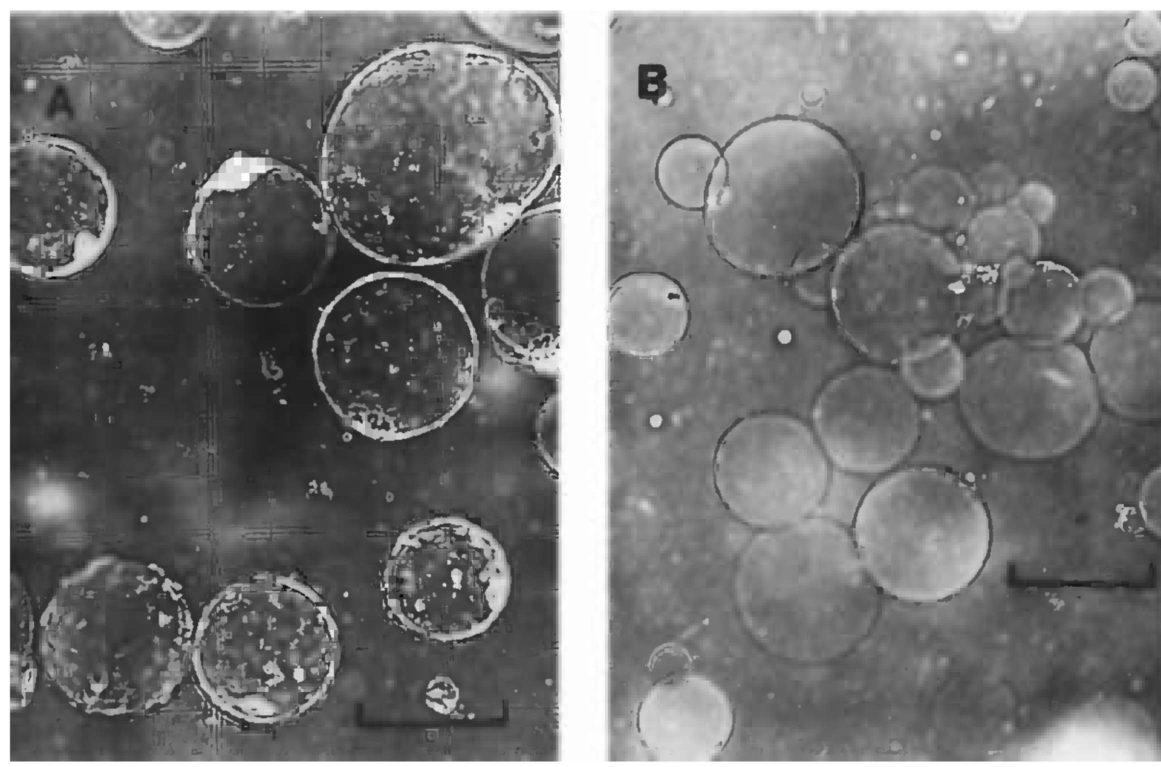

Fig. 4. Protoplasts (A) and vacuoles (B) from inner scale leaf of onion bulb (Lancaster and Collin, 1981).

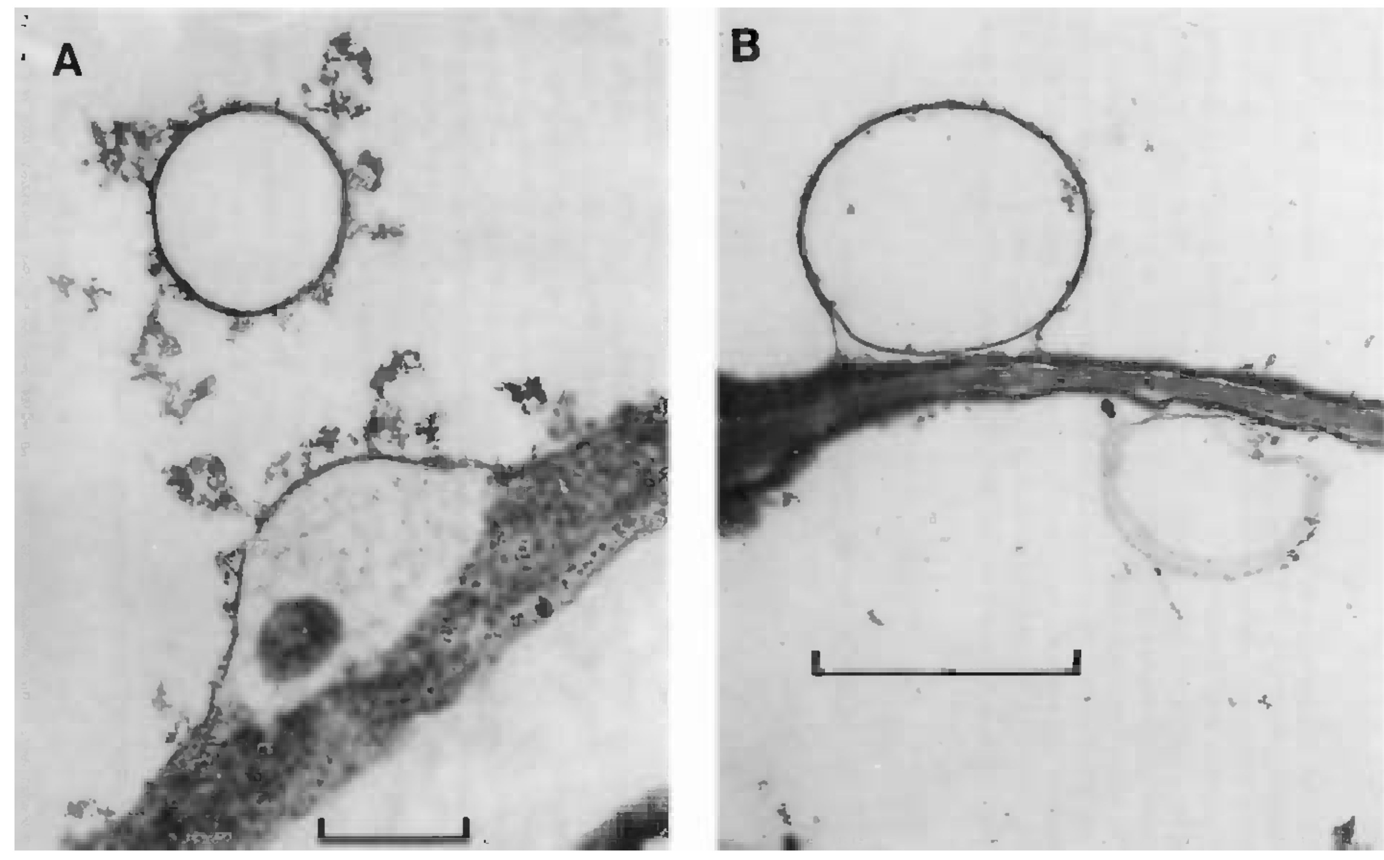

Fig. 5. Cytoplasmic vesicles in onion bulbs (A) and differentiating callus (B).

with flavor precursor synthesis. The evidence for the intracellular location of the pathways is therefore indirect. In mammalian systems, the primary pathway, valine oxidation, is localized within the mitochondrion (Ikeda and Tanaka, 1983). However, the key regulatory enzyme complex, α-ketoisovalerate dehydrogenase, which is bound to the inner mitochondrial membrane, has not been detected in plants. At the start of the secondary pathway, methacrylyl-CoA combines with cysteine to form CPC. The enzyme, cysteine synthase, which is responsible for the formation of cysteine, is found in chloroplasts of green tissue and in protoplastids of nongreen tissue and root tissue (Giovanelli *et al.*, 1980). Further work is required to locate both of these pathways in the onion cell.

III. FLAVOR PRODUCTION IN TISSUE CULTURES OF *ALLIUM* SPECIES

Most of the interest in tissue culture of *Allium* species has been determined by a need for improvements in propagation rather than increases in flavor production. Tissue cultures have been initiated from the major flavor-producing *Allium* species, garlic (Abo El-Nil, 1977), onion (Dunstan and Short, 1978), leek (Dunstan and Short, 1980), and chives (Yamane, 1983). Growth was by callus, with no reports of a fine cell suspension, but regeneration of the callus into plants has been achieved with all of these species. Analysis of flavor production has been restricted to onion tissue cultures, where it was found that flavor-precursor levels were less than 10% of those in the intact plant, but activity of the enzyme alliinase was comparable (Davey *et al.*, 1974; Freeman *et al.*, 1974; Selby and Collin, 1976). It was suggested that the reduced concentration of flavor precursors was due to a specific inhibition of the primary or secondary pathways involved in flavor production. Most of the subsequent work on the onion tissue culture was devoted to establishing the basis for reduced accumulation of flavor precursor in this tissue.

A. Analysis of Flavor Production in Callus

The production of flavor in the onion is determined by a number of components. These are the activity of alliinase enzyme, the total amount of flavor precursors, and the amount of individual flavor precursors.

The methods used to assess the three components of flavor production in onion tissue cultures are described fully by Collin and Watts (1983), but no quantitative method for the estimation of the individual flavor precursors was given. Such a method, based on an electrophoretic and thin-layer chromatographic separation, has been described by Lancaster and Kelly (1983) and Lancaster *et al.* (1986). However, a more rapid and sensitive method for both intact onion and tissue culture has been developed by Musker *et al.* (1987) that makes use of an isocratic, aqueous reverse-phase high-performance liquid chromatography (HPLC) system. In a new extraction procedure incorporating hydroxylamine to inhibit alliinase, approximately 1 g of undamaged tissue was frozen in liquid nitrogen at −20°C and added to 10 ml of prechilled extractant, methanol–chloroform–water (MCW, 12:5:3), at pH 6.8, including 10 m*M* hydroxylamine. The tissue was left for 24 hr, extracted with a further volume of MCW for 4 hr, then the extracts were combined and the phases separated by the addition of a mixture of chloroform (4.5 ml) and water (5.5 ml) per 10 ml of extractant. The upper methanol phase was preserved. The extract was purified by passing through an Amberlite column (IR 120 H^+) and eluted with 2 *M* NH_4OH (aqueous). The eluate was freeze dried, then resuspended in the HPLC mobile phase (50 m*M* NaH_2PO_4; buffer pH, 2.5). The separation, using an injection volume of 10 μl, was on an HPLC column (Lichrosorb RP-18, 5 μm, 250 × 4.6 mm). Monitoring wavelength was 214 nm, with a flow rate of 0.9 ml min^{-1} (pump load, 1500 psi). Although the extraction showed 80–100% recovery, losses were experienced on the ion-exchange column so that recoveries were reduced to 50 to 75%. A known marker compound (carboxymethylcysteine) was included routinely to accommodate for losses during purification (Fig. 6). This method has been used routinely by Musker *et al.* (1987) for the separation and estimation of *trans*–Pren Cys SO in callus.

B. Production of Alliinase

The alliinase enzyme in onion callus culture was found by Davey *et al.* (1974), Freeman *et al.* (1974), and Selby and Collin (1976) to have an activity comparable to that in the intact plant. A more detailed examination of the substrate specificity and *K*m values of the alliinase from callus was made by Selby *et al.* (1979), who compared the substrate specificity for *S*-methyl, *S*-ethyl, *S*-propyl, and *S*-allyl-L-cysteine and the corresponding L-cysteine sulfoxides of the alliinase from onion callus and onion bulb (Table I). None of the unoxidized thioethers was able to act

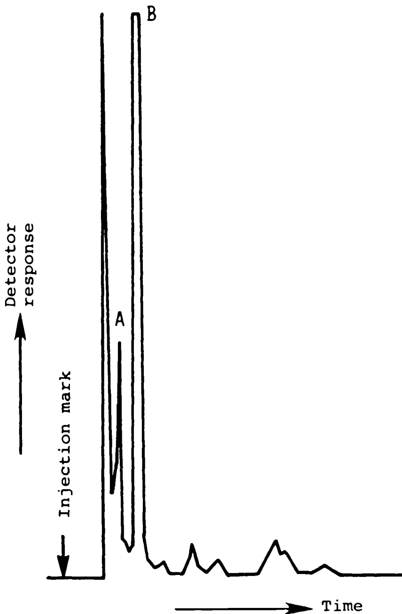

Fig. 6. Trace of HPLC separation of flavor-precursor compounds in the onion bulb. A, carboxypropylcysteine (marker); B, *trans*–Pren Cys SO.

Table I

Substrate Specificities of Allinase Enzymes of Onion Bulb and Callus Origins[a]

Substrate[b]	Pyruvate liberated (μmol ml^{-1} 10 min^{-1}); alliinase source: Onion bulb	Callus
S-All Cys SO	4.13	4.06
S-Pr Cys SO	4.33	4.00
S-Et Cys SO	1.95	1.80
S-Me Cys SO	1.50	1.35
S-All Cys	0.01	0.02
S-Pr Cys	0.01	0.02
S-Et Cys	0.02	0.01
S-Me Cys	0.01	0.01

[a] From Selby *et al.* (1979).
[b] All, allyl; Cys, cysteine; Et, ethyl; Me, methyl; Pr, propyl; SO, sulfoxide.

as a substrate for alliinase from either source, which accords with the characteristics of alliinase from onion bulb tissue established by Schwimmer and Mazelis (1963). The specificity of the alliinase for the different *S*-alkyl-L-cysteine sulfoxides was comparable for the bulb and tissue culture source. A Lineweaver–Burk double reciprocal plot for the hydrolysis of *S*-propyl-L-cysteine sulfoxide showed that each alliinase enzyme obeyed Michaelis–Menten kinetics with little difference between the two sources. The K_m for *S*-propyl-L-cysteine sulfoxide was calculated to be 15.8 and 12.5 m*M* for bulb and callus tissue, respectively, which accords well with a previous value of 11 m*M* for onion provided by Whitaker (1976). The culture of the onion cells obviously had no affect on the activity of alliinase.

C. Biosynthesis of Flavor Precursors

Onion callus contains less than 10% of the total flavor compounds in the intact bulbs (Davey *et al.*, 1974; Freeman *et al.*, 1974; Selby and Collin, 1976). Of the *S*-alkylcysteine sulfoxides, Pr Cys SO, Et Cys SO, and *trans*–Pren Cys SO were absent in the callus, but Me Cys SO was present in small amounts (Selby *et al.*, 1980). The presence of Me Cys SO confirmed earlier results in which it was shown that the volatiles released by crushed onion callus were characteristic of those from Me Cys SO (Freeman *et al.*, 1974). The callus also contained peptides that were similar to those found in onions by Granroth (1970). The amount of valine, one of the amino acid precursors of *trans*–Pren Cys SO, was reduced to 25 to 36% of the concentration in the bulb whereas the other amino acid precursor, cysteine, could not be detected in the callus. In an effort to stimulate flavor-precursor synthesis, the internal concentration of valine and cysteine was increased by adding these two amino acids to the nutrient medium (Selby *et al.*, 1979). Although there was an increase in concentration of valine and cysteine in the callus tissue, there was still no detectable onion odor when the callus was crushed. In a further effort to increase flavor production in callus, later key intermediates of the pathway to *trans*–Pren Cys SO synthesis, namely methacrylic acid and CPC, were added in turn to the nutrient medium. The addition of methacrylic acid generated no odor when the callus was crushed, whereas CPC did. The presence of small amounts of *trans*–Pren Cys SO within the callus was confirmed by electrophoresis and thin-layer chromatography of the callus extract (Selby *et al.*, 1980). Clearly, the secondary pathway leading to synthesis of *trans*–Pren Cys SO was functional in the callus. This was confirmed by adding the intermediate of the second-

ary pathway, *S*-propenylcysteine, to callus when analysis of the callus extract showed the formation of *trans*–Pren Cys SO (Turnbull *et al.*, 1980). These feeding experiments suggested that the secondary pathway was able to function in the callus but that the primary path of valine oxidation from valine to methacrylyl-CoA was either not functional or that the link between the two pathways at CPC formation was rate limiting. The activity of the primary pathway was investigated by feeding [^{14}C]valine and [^{14}C]cysteine to callus and leaf tip. Radiolabeling of the precursors [^{14}C]valine and [^{14}C]cysteine led to a low level of radioactivity in *trans*–Pren Cys SO and Me Cys SO in callus, but with a high level of radioactivity in the *trans*–Pren Cys SO in leaf tips (Turnbull *et al.*, 1980). The radiolabeling data showed that the primary and secondary pathway leading to synthesis of *trans*–Pren Cys SO was operating at a very low level in the callus.

D. Effect of Culture Conditions on Flavor Production

There are a number of culture variables that affect the accumulation of any secondary product. These include the variation between explants, the length of time the tissue has been subcultured, and the degree of differentiation and morphogenesis in the culture. The effect of these variables on flavor production has been examined in onion tissue culture.

1. Explant Source and Age of Culture

The role of somaclonal variation has been discussed extensively for its contribution to crop improvement (Larkin and Scowcroft, 1981). The selection for high-yielding clones of tissue cultures that produce important secondary products has also made use of this variation (Deus and Zenk, 1982). In order to examine the variation in flavor production both within and between tissue cultures from different onion varieties, Selby and Collin (1976) initiated callus from seedlings of three varieties, main crop (Rijnsburgher), spring onion (White Lisbon), and a strongly flavored variety (Red Italian) and subcultured the callus every 6 weeks for 10 subcultures. The original callus lines, each derived from one explant, were grouped according to their growth rate, friability, consistency, and color, and the alliinase and flavor precursor levels were estimated for each line. Both alliinase and flavor-precursor levels showed only limited variation and were not correlated with the wide variation in the physical characteristics of the separate lines. Often, slow-growing, nonfriable

callus generates much higher levels of secondary products (Lindsey and Yeoman, 1983), but this trend was not apparent in the onion. The variation generated within a tissue culture may also contribute to a decline in the ability to synthesize secondary products (Deus and Zenk, 1982). Where recently isolated onion cultures were compared with older cultures, the ability to synthesize flavor precursors did decline with time (Davey *et al.*, 1974).

2. Differentiation of Onion Tissue Culture

Differentiation of tissue cultures into specific tissues, embryos, roots, and shoots, is one factor known to stimulate secondary product formation (Yeoman *et al.*, 1982). The effect of initiating root regeneration in onion callus was to stimulate flavor-precursor synthesis (Freeman *et al.*, 1974). Turnbull *et al.* (1980) also found an increase in flavor levels in differentiated callus and in differentiated roots and shoots.

In a more detailed analysis of the change in flavor-precursor production during cell and tissue differentiation, onion callus was initiated and maintained for 8 months by Musker *et al.* (1987) on the medium of Dunstan and Short (1978), which contained 0.55 mg liter^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) as the auxin source. The callus was then transferred to a medium designed to initiate rapid redifferentiation, or to maintain the callus in an undifferentiated state (Phillips and Luteyn, 1983). The regeneration medium contained 0.03 mg liter^{-1} picloram and 0.5 mg liter^{-1} benzylaminopurine (BAP), whereas the maintenance medium contained 5 mg liter^{-1} picloram and 2 mg liter^{-1} BAP. The accumulation of flavor precursor was assessed weekly by the HPLC method, as outlined. On the regeneration medium the callus developed shoot and root initials at 6 weeks (Fig. 7) but remained non-green. On the maintenance medium no regeneration was observed. Analysis of the callus on both media showed an increase in *trans*–Pren Cys SO up to 7 to 8 weeks (Fig. 8). Both differentiated and undifferentiated tissue showed approximately the same increase in flavor precursor. The flavor was sufficiently strong to be easily detected when either the undifferentiated callus, or particularly the differentiated callus, was crushed. Neither Turnbull *et al.* (1981) nor Musker *et al.* (1987) found flavor accumulation to occur in callus grown on the Dunstan and Short (1978) medium. The change in source of growth regulator to picloram and BAP had obviously initiated the synthesis of *trans*–Pren Cys SO. Turnbull *et al.* (1981) examined the cell structure of intact bulb and undifferentiated tissue to establish whether the reason for the reduction in flavor accumulation in undifferentiated callus was due to a major dif-

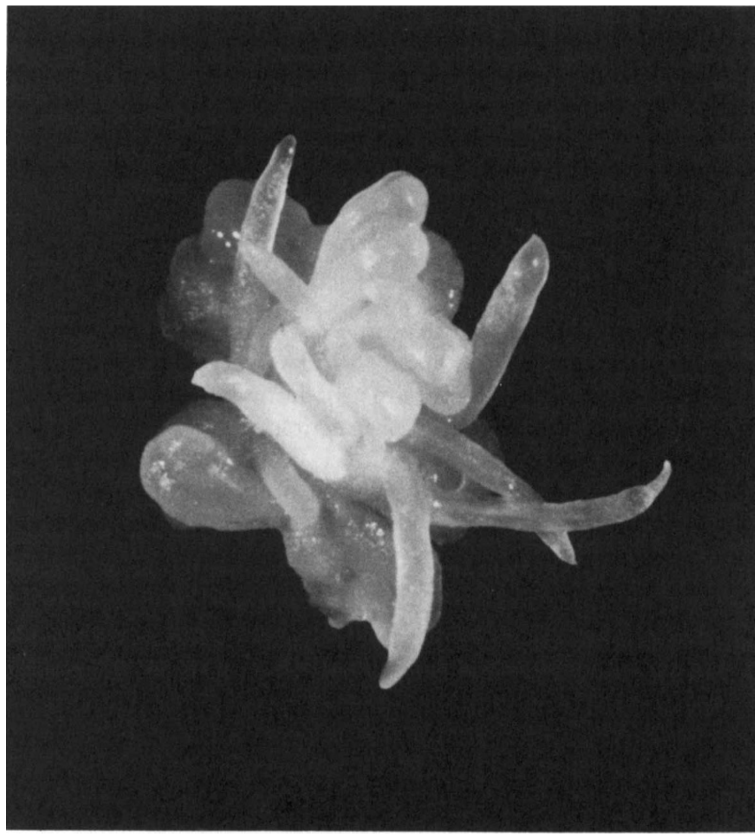

Fig. 7. Early stage in differentiation of onion callus maintained for 6 weeks on a regeneration medium [0.03 mg liter^{-1} of picloram, 0.5 mg liter^{-1} (of benzylaminopurine (BAP)].

ference in structure. They found that the callus cells were more tightly packed, with no intercellular spaces, and had a dense cytoplasm, prominent nuclei, and several small vacuoles instead of a large central vacuole. The cells were much smaller, were connected by plasmodesmata, and possessed a large, lobed nucleus. The vesicles noted in the onion cells were largely absent from the undifferentiated callus cells. The callus tissues grown on both the picloram- and BAP-containing media were examined by Musker *et al.* (1987) 8 weeks after transfer. The structure of the cells was very similar on both media, and comparable to the structure found by Turnbull *et al.* (1981). There were, however, a number of

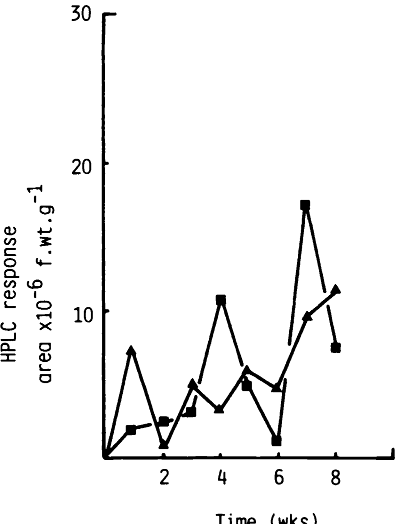

Fig. 8. Increase in accumulation of *trans*–Pr Cys SO in onion callus after transfer to a regeneration medium (0.03 mg liter^{-1} of picloram, 0.5 mg liter^{-1} BAP, ■, and a maintenance medium [5 mg liter $^{-1}$ of picloram and 2 mg liter^{-1} of benzylaminopurine (BAP), ▲].

cytoplasmic vesicles comparable to those found by Turnbull *et al.* (1981) in the intact plant (Fig. 5). The effect of picloram and BAP on differentiation, cell structure, and flavor-precursor biosynthesis needs to be examined in more detail.

IV. CONCLUSIONS

Flavor production in onion is a very interesting system. Besides its intrinsic interest it also provides a useful model system for the analysis of the control of secondary product formation in plant cell cultures. In order to examine the mechanism of control of flavor production, it is important that future work should concentrate on (1) location of both the site of biosynthesis and the site of accumulation of *trans*–Pren Cys SO, and (2) the assay and isolation of the rate-limiting enzymes on the primary and secondary pathways of *trans*–Pren Cys SO biosynthesis.

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CHAPTER 29

Polyacetylenes

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I. INTRODUCTION

The majority of polyacetylenes, or polyines, of higher plants occur in members of the plant families Asteraceae (Compositae), Araliaceae, and Apiaceae (Umbelliferae), and these, so far, have been the most studied

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(for reviews, see Sorensen 1968, 1977; Bohlmann *et al.*, 1973; Hansen and Boll, 1986). Like many other hydrophobic secondary metabolites, they accumulate outside of plant cells, in intercellular spaces. They share this characteristic with volatile oils, resins, and latices, all of which have proven difficult to obtain in plant cell cultures.

Polyacetylene accumulation may occur in all plant organs and appendages, the roots being usually the most active in this respect. The patterns of compounds observed, however, are usually highly organ specific (Sorensen, 1968; Bohlmann *et al.*, 1973; Norton, 1984; Marchant *et al.*, 1984). Their site of accumulation within a plant organ may be related to their function. Constitutive polyacetylenes of higher plants accumulate in specific structures known as "resin canals," which occur in the leaves, stems, and roots of polyacetylene-containing plants of the Asteraceae and Apiaceae (Sorensen, 1968; Van Fleet, 1970). They have also been reported to accumulate as oily droplets in the periderm of roots, possibly originating in the pericyclic oil ducts (Garrod and Lewis, 1979). On the other hand, fungal polyacetylenes are usually excreted directly into the medium. This also occurs with polyines of higher plants, which function as phytoalexins.

As with certain other types of secondary products, for example, isoflavonoids, a "constitutive" polyacetylene of one species may be an "inducible" compound in another. Falcarindiol, a characteristic polyacetylene of the Apiaceae and Araliaceae (Bohlmann *et al.*, 1973), is not normally produced in members of the Solanaceae but may be synthesized in tomatoes in response to fungal infection (de Witt and Kodde, 1981; Elgersma and Overeem, 1981). These cases appear to involve only aliphatic polyines, however, not the photoactive aromatic or thienyl compounds. The apparent need for morphogenesis in some cases, as opposed to simple excretion into the medium in others, has to be kept in mind when considering the production of these compounds in cultured cell lines.

The common origin of most acetylenes as derivatives of oleic acid is considered to be an established fact (Bohlmann *et al.*, 1973). The general outline of their biosynthetic pattern has been elucidated through feeding experiments using radioactively labeled acetate and oleic acid, but there is only one report on an enzymatic step involved in their metabolism (Sütfeld and Towers, 1982). The desaturating system responsible for triple bond formation has not been characterized so far. However, conversion of oleic acid to crepenynic acid has been observed in preparations of chopped *Crepis rubra* seeds (Haigh *et al.*, 1968).

II. POLYACETYLENES IN TISSUE CULTURES

Attempts have been made to obtain cultures active in the synthesis and accumulation of acetylenic compounds. Both organ (root) cultures and unorganized tissues, such as callus or crown gall tumors, have been studied. In both cases, the initial goal has been either to investigate the factors governing their accumulation in plant cells or to obtain a reliable source of compounds, many of which have unusual biological activity. A list of the major polyacetylenes found in long-term plant tissue cultures is given in Fig. 1.

Attempts to obtain the production of a number of leaf polyacetylenes with phototoxic properties, such as 1-phenylhepta-1,3,5-triyne (PHT), have been unsuccessful so far, except for very small amounts produced in kinetin-treated roots (Norton, 1984). The problems encountered in obtaining cultures active in the production of secondary compounds typical of leaves are not unique to this system, as they have been experienced with the complex leaf alkaloids of *Catharanthus roseus* (Krueger *et al.*, 1982). The apparent morphogenetic requirements seem difficult to control in relatively unorganized cultures, such as calli, and the maintenance of actively growing "shooter" lines in liquid culture is difficult and offers no guarantee that the compounds of interest will be present.

A. Root Cultures

The relative ease with which root cultures can be induced and maintained for certain plants has resulted in their being the only organ culture experimented with so far for the production of polyacetylenes. Published reports include the polyacetylenes of *Bidens alba* (Norton and Towers, 1986). *Eriophyllum lanatum* (Norton *et al.*, 1985a), and *Chaenactis douglasii* (Cosio *et al.*, 1986). The compounds so obtained, along with their values in culture, are listed in Table I. The reported values, on a dry-weight basis, vary from about 40 to 200% of those seen in plant roots.

There is also significant variation in the rates of accumulation and total values seen, depending not only on medium composition (Section II,B) but also on the origin of the explant. Various types of inocula have been used: these include rootlets from germinating seeds, from rooting stem segments, and from callus. All liquid media for these cultures contain

I	$CH_2{=}CH{-}(C{\equiv}C)_2{-}CH{=}CH_2OAc$	EDE-OAc
II	$CH_2{=}CH{-}(C{\equiv}C)_4{-}CH{=}CH{-}Me$	ETE
III	$CH_2{=}CH{-}(C{\equiv}C)_4{-}CH{=}CH{-}CH_2OAc$	ETE-OAc
IV	$C_6H_5{-}(C{\equiv}C)_2{-}CH{=}CH{-}CH_2OAc$	PDE-OAc
V	$Me{-}C{\equiv}C{-}[C_4H_2S_2]{-}(C{\equiv}C)_2{-}CH{=}CH_2$	Thiarubrine A
VI	$Me{-}(C{\equiv}C)_2{-}[C_4H_2S_2]{-}C{\equiv}C{-}CH{=}CH_2$	Thiarubrine B
VII	$Me{-}C{\equiv}C{-}[C_4H_2S]{-}(C{\equiv}C)_2{-}CH{=}CH_2$	Thiophene A
VIII	$Me{-}(C{\equiv}C)_2{-}[C_4H_2S]{-}C{\equiv}C{-}CH{=}CH_2$	Thiophene B
IX	$[C_4H_3S]{-}[C_4H_2S]{-}C{\equiv}C{-}CH{=}CH_2$	BBT
X	$[C_4H_3S]{-}[C_4H_2S]{-}C{\equiv}C{-}CH_2{-}CH_2OAc$	BBT-OAc
XI	$[C_4H_3S]{-}[C_4H_2S]{-}C{\equiv}C{-}CH_2{-}CH_2OH$	BBT-OH

Fig. 1. Structures, systematic names, and abbreviated names of major polyacetylenes found in plant tissue cultures.

I —Trideca-5,11-diene-7,9-diyn-13-acetate
II —Trideca-1,11-diene-3,5,7,9-tetrayn
III —Trideca-1,11-diene-3,5,7,9-tetrayn-13-acetate
IV —1-Phenylhepta-1,3-diyn-5-ene-7-acetate
V —1-(Methylethyn)-4-(hex-1,3-diyn-4-ene)-2,3-dithiacyclohexa-4,6-diene
VI —1-(4-Methylbut-1,3-dyin)-4-(but-1-yn-3-ene)-2,3-dithiacyclohexa-4,6-diene
VII —1-(2-Methylethyn)-5-(hex-1,3-diyn-4-ene)-thiophene
VIII—1-(4-Methylbut-1,3-diyn)-5-(but-1-yn-3-ene)-thiophene
IX —5-(3-Buten-1-enyl)-2,2′-bithienyl
X —5-(4-Acetoxy-1-butenyl)-2,2′-bithienyl
XI —5-(4-Hydroxy-1-butenyl)-1-2,2′-bithienyl

small amounts of an auxin, usually naphthaleneacetic acid (NAA), at concentrations between 0.3 and 0.5 mg liter^{-1}. The amount of callus tissue in the cultures varies from none in the case of cultures of *E. lanatum* to about 30% of the fresh weight for *C. douglasii*. Callus growth at the low auxin concentrations employed takes place only on the cut

Table I

Major Polyacetylenes Found in Root cultures[a]

Source	Compound	Average yield (mg g^{-1} dry weight)[b]
Bidens alba	EDE-OAc	0.25
	ETE-OAc	1.68
	ETE	0.07
	PDE-OAc	1.27
Chaenactis douglasii[c]	Thiarubrine A	3.60
	Thiarubrine B	0.90
	Thiophene A	0.16
	Thiophene B	0.03

[a] Cultures were grown in liquid SH medium. *Bidens alba* medium contained 0.5 mg $liter^{-1}$ NAA: *Chaenactis douglasii* medium contained 0.3 mg $liter^{-1}$ NAA.

[b] For more specific values see references indicated in the text.

[c] Root cultures of *Eriophyllum lanatum* produced the same compounds but with one-tenth the yields; see Norton *et al.* (1985a).

surfaces of the rootlets used as inoculum. Usually a balance has to be reached between excessive callus growth at high NAA levels and slow growth of the roots in the absence of auxin. The callus tissue present in the cultures contains no acetylenes.

The many advantages that differentiated root cultures possess over unorganized tissue cultures have made it possible to study a number of factors affecting polyacetylene accumulation in cultured tissues. The acetylene content of roots in culture seems to remain constant even after several years. Additionally, there is no need for constant selection of productive tissue, something that has been found necessary for many cultures of unorganized tissue. The effect of dedifferentiation on polyacetylene accumulation in cultures has been studied using high levels of either auxins or cytokinins (Norton, 1984; Cosio *et al.*, 1986). In all cases, the disappearance of vascular tissue and accompanying resin canals results in elimination of polyacetylene accumulation.

Transformed plant tissues at the level of root cultures have also been a subject of study in our laboratory. Initial studies involved root cultures from regenerated plantlets of *Bidens alba* (Norton, 1984). These plantlets were regenerated from crown gall tumor cultures, an infrequent occurence given the high endogenous hormonal levels often observed in tumor tissues. The transformed roots differed in their increased sensitivity to external hormonal levels and in their propensity to form callus on older root tissue. Polyacetylene levels and other characteristics were

similar to those of normal root cultures. Obtaining tissues exhibiting fast, hormone-independent growth but still retaining a defined organ morphology would be advantageous when trying to establish a permanent source of phytochemicals in culture. Attempts along this line have been reported (Flores and Filner, 1985). These involve alkaloid production in hairy-root cultures induced by infection with *Agrobacterium rhizogenes*. Evaluations of normal and hairy-root cultures of *Chaenactis douglasii* for the production of antibiotic disulfur polyines seen in the roots of this species are being conducted in our laboratory (P. Constabel *et al.*, unpublished results.

B. Unorganized Tissue Cultures

As discussed above, the accumulation of constitutive polyacetylenes in higher-plant tissues takes place in resin canals. It is not known whether there are any minimum morphogenetic requirements for the accumulation of these compounds in "unorganized" tissue cultures. All published reports to date of normal callus cultures involve very low amounts of polyacetylenes in short-term callus cultures (Jente, 1971; Ichihara and Noda, 1977). Reports on the accumulation of biosynthetically related compounds, such as unusual fatty acids or nonacetylenic thiophenes, seem to indicate that instances in which these compounds are produced by callus tissue are limited (Yano *et al.*, 1976; Mangold, 1977; Setia, 1978; Mangold and Spencer, 1980). Our group has attempted, unsuccessfully, to obtain callus cultures that accumulate polyacetylenes in *Bidens alba*, *Eriophyllum lanatum*, and *Chaenactis douglasii*. Small amounts of thiophene polyacetylenes have been observed in long-term *Tagetes patula* callus cultures (Norton *et al.*, 1985b) grown in SH medium with 4 mg liter^{-1} NAA. In this particular case, however, the culture in question was a rooting callus containing thiophene acetylenes characteristic of *T. patula* roots. Similar results have been obtained also in callus cultures of *B. cervicata* and *B. hillebrandiana*, which produce rootlets (unpublished results).

There have been few reports on acetylenic compounds in crown gall tumors (Reichling *et al.*, 1979; Norton *et al.*, 1985b; Norton and Towers, 1985; Cosio *et al.*, 1986). These tissues arise through transformation of plant cells by *Agrobacterium tumefaciens* (see Nester *et al.*, 1984, for review). The insertion of the T-DNA segment of the Ti plasmid into the plant nuclear genome results in hypertrophic growth of the transformed cells and in the synthesis of opines, which when secreted outside the cells can be metabolized by the bacterium.

The tumorlike appearance of the transformed tissues is caused by the altered production of auxins and cytokinins, which is in turn a result of the expression of genes within the T-DNA coding for key enzymes of their biosynthetic pathways (Barry *et al.*, 1984; Thomashow *et al.*, 1984). The endogenous levels of these growth regulators have been shown to exert control over tumor morphology (Amasino and Miller, 1982; Akiyoshi *et al.*, 1983).

Early studies on the presence of secondary metabolites in crown gall tumors were purely descriptive (Klein and Link, 1955; Kovacs *et al.*, 1964; Kado, 1976). Tumors are auxotrophic for growth regulators, and analyses of the secondary metabolites present were performed with the tumors still attached to the plant or after prolonged culture in medium without hormones. It was speculated that the production of secondary metabolites might be a carryover from the tissue where the tumor originated, resembling the case with tumorous endocrine tissues in mammalian cell cultures (Teuscher, 1973). No information was available, in these early studies, on the factors affecting the synthesis of secondary products by the transformed cells, or whether these bore any relationship to those observed in normal cultures.

Table II lists the polyacetylenes that have been obtained from crown gall tumor cultures in our laboratory and the amounts usually observed. These tumor lines are polyclonal in origin and have been maintained over a period of at least 3 years. The tumors were either induced in greenhouse-grown plants or were induced *in vitro* in surface-sterilized stem or petiole segments followed by antibiotic treatment.

The most interesting acetylenes obtained from tumor cultures are two thiarubrines, A and B. These disulfur polyines, present in the roots of *Chaenactis douglasii*, have been a focus of interest because of their antifungal activity (Towers *et al.*, 1985). The intense red color that characterizes them facilitates their detection in cultured tissues. The presence of these compounds in a tumor culture of *C. douglasii* made it possible to study their distribution within the tissue in relation to the degree of differentiation. The coloration also permitted the selective transfer of tissue active in their accumulation, which resulted in establishing a line with yields of thiarubrines consistently equal to those of plant roots (Cosio *et al.*, 1986), the highest yield of polyines yet obtained in tumor or callus tissues.

The thiarubrine-producing culture provided the initial evidence for a correlation between differentiation and polyacetylene production in tumor tissue. The accumulation of product took place in intercellular spaces in close spatial relationship with tracheids. Experiments using exogenously supplied auxins to suppress xylogenensis resulted in dis-

Table II

Major Polyacetylenes Found in Crown Gall Tumor Cultures[a]

Source	Compound	Yield (mg g^{-1} dry weight) A277[b]	A208
Bidens alba	EDE-OAc	0.02	0.03
	ETE-OAc	0.03	0.04
	PDE-OAc	0.17	0.10
Chaenactis douglasii	Thiarubrine A	1.96	——
	Thiarubrine B	0.59	——
	Thiophene A	0.19	——
	Thiarubrine B	0.06	——
		μg g^{-1} fresh weight	
Tagetes patuia	BBT-OH	1.10	52.4
	BBT-OAc	0.80	52.6
	BBT	5.80	91.0

[a] *B. alba* and *C. douglasii* cultures were kept in SH medium without hormones; *T. patula* cultures were grown in MS medium without hormone.
[b] *A. tumefaciens* strains used to induce the tumors.

appearance of thiarubrines from the tissue. Similar results were obtained when selecting for fast, unorganized growth, suggesting an explanation for diminishing yields obtained after successive transfers of cell suspension lines of the tumors. A variable likely to control morphology and polyacetylene production in tumor tissues is the expression of the auxin and cytokinin genes in the T-DNA. This could be achieved either through direct control of transcription of these genes by unknown factors, possibly a result of the insertion site in the genome, or through T-DNA copy number. In the latter case the higher the number of copies the higher the hormone levels in the tissue, and the less likelihood of any degree of tissue organization. We are investigating, using immunochemical techniques,the endogenous auxin and cytokinin levels in various tumor lines of *Chaenactis douglasii* and their relationship to the presence or absence of thiarubrines (E. G. Cosio *et al.*, unpublished results). Research in this area may provide information about minimum morphogenetic or growth regulator levels necessary to obtain polyacetylene production in systems other than differentiated organ cultures.

III. GROWTH MEDIUM AND ENVIRONMENTAL EFFECTS

A. Medium Composition

A number of media of defined composition have been used in cultures that produce polyacetylenes: these include MS, SH, White's, B5, and combinations of these. SH medium (Schenk and Hildebrandt, 1972) has been used most successfully in our laboratory with polyacetylene-producing cultures, however, both for tumor and root cultures. Although the data that follow have been obtained with root cultures, the same effects have been observed in tumor cultures.

Figure 2 shows the effects of SH, MS, and White's medium on the accumulation of acetylenic thiarubrines and thiophenes in root cultures of *Chaenactis douglasii*. Both SH and White's are acceptable media for the production of these compounds in culture. White's medium supports active growth and polyacetylene accumulation for a maximum of 3 weeks, with the accumulation of product taking place during the early phase of culture. SH can support growth for up to 5 weeks.

The fact that both a low- (White's) and high- (SH) salt medium support polyacetylene accumulation suggests that similarities in their mineral nutrient ratios rather than absolute amounts may be the main effectors. Sucrose does not seem to exert major effects, as seen by varying its concentration from 2 to 3% in White's medium. Neither do myo-inositol levels nor vitamin formulations from other media (MS, SH, or B5) (not shown). Evidently, the inefficacy of MS medium to support polyacetylene accumulation has to do with its mineral salt formulation rather than with organic supplements. The results are even more dramatic in tumor cultures. No polyacetylenes can be detected in *Chaenactis douglasii* tumor lines after four subcultures in MS medium, whereas those grown in SH medium produce levels very similar to those of plant roots. A comparison of the mineral salt composition, in millimolar equivalents, reveals a high level of ammonium and nitrate in MS medium. The NH_4^+ levels are 20.6, 2.6, and 0 m*M* for MS, SH, and White's, respectively. For NO_3^- they are 39.4, 24.5, and 2.06 m*M* in the same order. High nitrogen-to-carbon ratios have been shown to be inhibitory to polyacetylene accumulation in *Bidens alba* root and tumor cultures (Table III) (Norton, 1984; Norton and Towers, 1985). The pH of the culture medium does not seem to affect polyacetylene accumulation

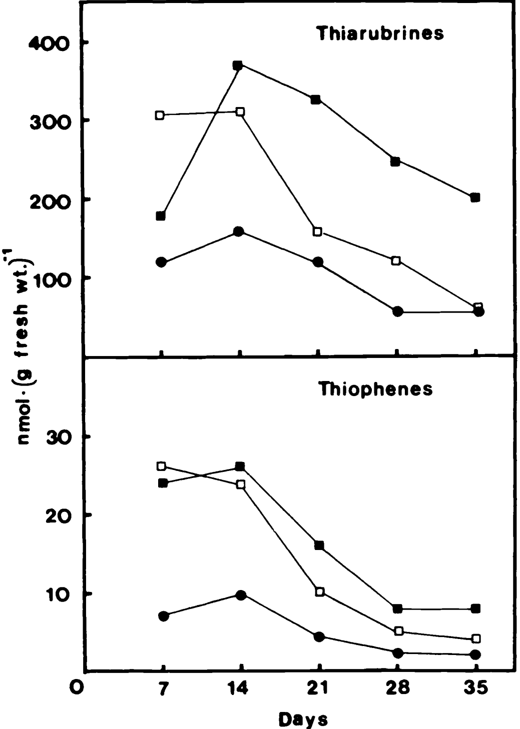

Fig. 2. Polyacetylene accumulation in root cultures of *Chaenactis douglasii*. Cultures were grown in liquid SH (■), MS (●), and White's (□) medium, in the dark at 25°C. All media contained 0.3 mg liter^{-1} NAA. Data points are the average of three flasks.

significantly in the range between 4.5 and 6, nor does the starting pH of the medium (Norton, 1984).

B. Environmental Factors

Among the environmental factors, light has been reported to be important in the synthesis of acetylenes in callus cultures (Jente, 1971), especially the dark–light cycle to which they are exposed. In the case of *Bidens alba* cultures, light seems to affect the type of acetylenic products that will accumulate. The final products are also determined by the type of growth regulator used to support the culture. Most of the acetylenes obtained to date in culture are root acetylenes or biosynthetically close intermediates, and their accumulation is inhibited by light. This light-

Table III

Effect of Changes in Carbohydrate/Nitrate Ratios on Polyacetylene Levels of *B. alba* Root Cultures[a]

mM		Molar ratio	Final root dry weight per flask	Total polyacetylenes[b]
[Sucrose]	[KNO_3]	C/N	(g)	(mg g^{-1} dry weight)
44	25.0	1.8	0.19	2.85b
88[c]	25.0	3.5	0.31	3.12b
176	25.0	7.0	0.33	3.91a
352	25.0	14.0	0.26	0.56c
88	12.5	7.0	0.34	3.89a
88	6.3	14.0	0.30	4.28a

[a] Adapted from Norton and Towers (1986), with permission from Gustav Fischer Verlag. Cultures were grown in SH medium modified as described. Values are averages for three flasks.

[b] Values followed by the same letter are not significantly different at the 5% level by Duncan's multiple range test.

[c] Standard SH medium.

mediated inhibition of polyacetylene accumulation is quite clear in tumor and root cultures of *Chaenactis douglasii* and *Eriophyllum lanatum*, where the main products are the "root" compounds. The inhibition by light has been shown to be reversible, at least within two culture cycles (Cosio *et al.*, 1986), but it has not been established whether it is the result exclusively of photodestruction of unstable acetylenes or if there is also a direct effect on the biosynthetic pathway.

The use and the effects of auxins and cytokinins in cultures that synthesize polyacetylenes have to be considered because all of the cultures obtained in our laboratory exhibit a correlation betweeen the degree of tissue organization and product accumulation (Cosio *et al.*, 1986, and unpublished data). Product levels decrease sharply with reduced tissue organization, and changes in the composition favoring the accumulation of intermediates rather than typical end products have been observed in root cultures of *Bidens alba* treated with high levels of kinetin. An unusual effect was observed in this case: small but significant amounts of phenylheptatriyne, a leaf acetylene, appeared in kinetin-treated root cultures containing a large proportion of callus (Norton, 1984). The use of small amounts of auxins to maintain growth in other root cultures has been discussed above.

The effect of temperature on polyacetylene accumulation in root cultures has been reported (Norton and Towers, 1986). In *Bidens alba*, the

amount of polyacetylenes per unit dry weight decreases as the temperature increases from 15 to 33°C.

IV. CONCLUSIONS

There is considerable paucity in the literature concerning the synthesis of polyacetylenes in plant tissue cultures. The majority of published reports concern the presence of these compounds in root cultures and in crown gall tumor lines. Except for short-term cultures and phytoalexin acetylenes, these compounds have not been observed to accumulate in normal callus, cell suspensions, or other unorganized tissue cultures in amounts that can be considered close to values seen *in planta*. The presence of polyacetylenes in crown gall tumors correlates well with xylogenesis, and it appears that a crude level of tissue organization is sufficient to result in amounts of acetylene accumulation similar to those seen in plant organs. Growth media and environmental factors will also have significant effects on polyacetylene accumulation in plant cell cultures, although the mechanisms involved have not been studied in any detail. Low temperatures and media with low nitrogen-to-carbon ratios seem to favor polyacetylene accumulation. Media with high levels of ammonium or nitrate ions seem to inhibit their accumulation. Our present lack of knowledge of the enzymes and the main regulatory steps involved in polyacetylene biosynthesis makes it extremely difficult to study the minimum morphogenetic aspects required for the expression of this pathway in plant tissues. More information in these areas will be necessary before progress is made on the independent expression of polyacetylene biosynthesis, and accumulation, in unorganized plant cell cultures.

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CHAPTER **30**

Lipids

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I. INTRODUCTION

In the 1970s several laboratories were engaged in the characterization of lipids in plant cell cultures and in studies of the biosynthesis of these compounds. The results of these early investigations on aliphatic lipids were discussed in two reviews (Radwan and Mangold, 1976, 1980). Another review described work on steroids (Stohs, 1980). Interest in the lipids of plant cell cultures decreased drastically when it was realized

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how slowly these cultures grow and how little lipid they contain. Enthusiasm faltered further when it was found that plant cells in culture do not, as a rule, produce all of the substances that occur as constituents of the seeds, leaves, and other parts of intact plants (Staba, 1980).

In the wake of developments in biotechnology, interest rekindled in the potential of plant cell cultures as a source of valuable products (Anderson *et al.*, 1985; Hüsemann, 1985; Kurz and Constabel, 1985; Mangold, 1986; Misawa, 1985; Yamada, 1985). It has become obvious that novel approaches are needed to assess the capabilities of plant cells in culture and their utilization.

As a continuation of two previous reviews (Radwan and Mangold, 1976; 1980), the present chapter summarizes chemical and biochemical aspects of lipids in plant cell cultures and describes in more detail some recent studies that have led to the production of biologically active compounds of great current interest and high value.

II. LIPIDS IN PLANT CELL CULTURES

The lipids of heterotrophic cell cultures are quite similar to those of photosynthetically inactive plant tissues, whereas lipids of photoautotrophic cells in culture closely resemble those of green leaves.

A. Total Lipids

Plant cell cultures usually contain 2–5 mg total lipids per gram fresh weight, i.e. 30–80 mg total lipids/g dry weight. The lipid content of the cultures is dependent upon the photosynthetic capacity of the cultures. Heterotrophic cell cultures contain about half as much total lipids as photoautotrophic cultures (for review see Radwan and Mangold, 1980).

B. Lipid Classes

The lipid classes found in heterotrophic and photoautotrophic cell cultures are similar to those of photosynthetically inactive or photo-

synthetically active plant tissues, respectively—except storage tissues. It is evident from the data in the literature that typical storage lipids, such as triacylglycerols*, occur in rather small proportions, whereas membrane lipids, i.e. ionic and nonionic polar lipids (phospholipids and glycolipids, respectively), are the predominant lipid classes of plant cells in culture.

The proportion of triacylglycerols in cultured plant cells varies greatly. As a rule, they occur at a level of <0.5 mg/g cells fresh weight, i.e. <7 mg triacylglycerols/g dry weight; yet stearic acid that was added in a concentration of 50 μM to the culture medium doubled the triacylglycerol content of callus cultures of cocoa bean (*Theobroma cacao*) (Tsai *et al.*, 1982). Accumulation of triacylglycerols was observed in *Papaver* spp. cell cultures during embryogenesis that had been induced by omitting 2,4-dichlorophenoxyacetic acid from the medium (Schuchmann and Wellmann, 1983). It is of interest that tri*acetyl*glycerols, prominent constituents of spindle tree (*Euonymus europaeus*) seed, do not occur in cell cultures derived therefrom (Gemmrich and Schraudolf, 1980).

The proportions of phospholipids in various cell suspension cultures range from 0.5 to 1.5 mg/g fresh weight, i.e. about 5–25 mg phospholipids/g dry weight (Yamada *et al.*, 1979).

The major glycolipids in dark-grown, heterotrophic soya (*Glycine max*) cell suspension cultures are present—dependent on the age of the culture—at levels between 1.6 mg/g cells (7 d after transfer) and 0.1 mg/g cells (21 d after transfer), on a dry weight basis (Sabinski *et al.*, 1982). Galactolipids as well as diacylglycerophosphoglycerols and sulfoquinovosyldiacylglycerols that are known to be typical constituents of chloroplasts of photosynthetically active plant tissues are not restricted to these organelles. These lipids are also present in proplastids of dark-grown cells of tobacco, soya, and *Datura innoxia* (Manoharan *et al.*, 1987; Martin *et al.*, 1984; Sabinski *et al.*, 1982; Siebertz *et al.*, 1978). As an example, Table I shows the typical composition of lipid classes in heterotrophic and photoautotrophic cell suspension cultures of *Peganum harmala*.

Both the composition of lipid classes and the patterns of their constituent fatty acids in cell cultures of, for example, *Chenopodium rubrum* are reversible in response to alternate shifts in carbon supply (Radwan *et al.*, 1979).

*Lipids are named according to the 1976 recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1977).

Table I

Lipid Classes of Heterotrophic and Photoautotrophic Cell Suspension Cultures of *Peganum harmala*[a]

Lipid class	Cell suspension culture (mg/g dry weight)	
	Heterotrophic	Photoautotrophic
Nonpolar lipids		
Triacylglycerols	1.6	1.9
Sterols	3.0	1.8
Sterylesters	0.1	0.6
Sterylglycosides	0.15	0.1
Esterified sterylglycosides	1.3	3.5
Polar lipids		
Diacylglycerophosphoethanolamines	1.7	4.1
Diacylglycerophosphocholines	3.85	8.65
Diacylglycerophosphoinositols	0.8	1.1
Diacylglycerophosphoglycerols	0.15	1.8
Monogalactosyldiacylglycerols	0.7	4.1
Digalactosyldiacylglycerols	0.5	4.6
Sulfoquinovosyldiacylglycerols	0.25	1.1
Chlorophyll	——	1.6

[a] Modified from Barz *et al.* (1980).

C. Constituent Fatty Acids

The fatty acid pattern of lipids in plant cell cultures is generally similar to that of cells of intact plant organs and tissues but there are some quantitative differences. Palmitic acid (16:0)*, oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) are the predominant fatty acids of lipids in plant cell cultures; other fatty acids are usually detected as minor constituents only. The reason why the composition of acyl moieties in lipids of cultured plant cells is restricted to these few species of fatty acids may be that the lipid classes in rapidly growing plant cells are predominantly phospholipids and glycolipids, i.e. membrane lipids. These lipid classes are known to contain almost exclusively the afore-

*Fatty acids are characterized by number of carbon atoms: number of double bonds. The geometry of double bonds of fatty acids is indicated by the prefixes (*Z*) and (*E*) instead of *cis* and *trans*, respectively, following IUPAC-IUB recommendations.

mentioned fatty acids with variations caused, for instance, by changes in temperature or other environmental conditions. The composition of fatty acids is not affected by subculturing suspension cultures of, e.g. *Nicotiana tabacum* and *Catharanthus roseus* in various synthetic media (MacCarthy and Stumpf, 1980a).

Relatively few plant cell cultures are known to contain lipids having acyl moieties other than those mentioned above. Isomeric octadecenoic acids (18:1) such as petroselinic, oleic, and vaccenic acids can be found in heterotrophic and photosynthetically active parsley cell cultures (Ellenbracht *et al.*, 1980). Hexadecatrienoic acid (16:3) which is known to be a rather prominent constituent fatty acid of lipids of Solanaceae and some other plant species occurs in cell suspension cultures of tobacco in appreciable amounts as well (Matsuzaki *et al.*, 1984; Siebertz *et al.*, 1978). It is striking that cell cultures of the moss *Leptobryum pyriforme* contain relatively large proportions of polyunsaturated fatty acids having 20 carbon atoms, such as arachidonic acid (all *(Z)*-5, 8, 11, 14–20:4) and icosapentaenoic acid (all *(Z)*-5, 8, 11, 14, 17–20:5) which are predominantly esterified in glycerophospholipids (Hartmann *et al.*, 1986).

The rather unusual α-elaeostearic acid ((Z)-9, (*E*,*E*)-11, 13–18:3) that constitutes two thirds of the total fatty acids of cotyledons of *Momordica charantia* (Cucurbitaceae) is not present in callus cells of this plant (Halder and Gadgil, 1983). "Very long-chain" fatty acids (>18 carbon atoms), such as behenic acid (22:0) and erucic acid (22:1) which are found in appreciable amounts in seeds of the cruciferous plant *Descurainia sophia* occur only in traces in cultured cells of this plant (Afsharypuor and Lockwood, 1985); similar observations were made on cell cultures of other cruciferous plants. Reportedly, rather large proportions of very long-chain fatty acids are esterified in the neutral lipids including glycolipids of cultured rape and soya cells (Ezzat and Pearce, 1980). The total fatty acids of callus cultures of *Alnus incana* contain as much as 12% lignoceric acid (24:0) (Simola and Koskimies-Soininen, 1984). Fatty acids having fewer than 16 carbon atoms are, as a rule, detected in small proportions only (Halder and Gadgil, 1983; Pandey *et al.*, 1986).

Cell cultures derived from various Malvaceae contain fair amounts of cyclopropane and cyclopropene fatty acids, whereas small proportions of cyclopentenyl fatty acids are found in callus cultures of Flacourtiaceae (for review see Radwan and Mangold, 1980). Oxygenated fatty acids are found only in minor lipid classes of plant cell cultures (Radwan and Mangold, 1980). Ricinoleic acid (12-hydroxyoleic acid), the major constituent fatty acid of castor oil, is not formed in callus cultures of castor bean (*Ricinus communis*) (Gemmrich, 1982).

D. Molecular Species of Glycerolipids

Studies concerned with the stereospecific distribution of acyl moieties in various glycerolipids show results resembling those found with glycerolipids in various tissues of intact plants. Saturated (16:0, 18:0) and monounsaturated (18:1) acyl moieties are esterified mainly in position 1 of the glycerol backbone (*sn*-1)*, whereas polyunsaturated (18:2; 18:3) acyl moieties are bound predominantly in *sn*-2 position. Molecular species of triacylglycerols were studied in cell cultures of cocoa bean. It was found that 16:0/18:1 (*sn*-1 + *sn*-3/*sn*-2) and 16:0/18:2 are the main molecular species of triacylglycerols of both callus and suspension cultures of this plant; 18:0/18:1 and 18:0/18:2, which are the main molecular species of natural cocoa butter, are also present in appreciable amounts (Tsai *et al.*, 1982).

In heterotrophic cell suspension cultures of *Rauwolfia serpentina* and *N. tabacum* the major fraction, generally more than 50% of each, diacylglycerophosphoethanolamines, diacylglycerophosphocholines, and diacylglycerophosphoinositols are the 16:0/18:2 (*sn*-1/*sn*-2) species. Appreciable proportions of 18:2/18:2 species are also detected in the phospholipids of cell cultures of both plants, whereas fairly high levels of 16:0/18:3 species are found in *N. tabacum* cells (Yamada *et al.*, 1979). Similar results are obtained with heterotrophic cell suspension cultures of soya (Nishihara and Kito, 1978). The diacylglycerophosphocholines of photomixotrophic cell suspension cultures of rape (*Brassica napus*) contain predominantly the 16:0/18:3 molecular species, but significant proportions of 16:0/16:1 + 18:1, 18:1/16:1 + 18:1, and 18:1/18:3 are also detected in diacylglycerophosphocholines of these cells (Weber and Benning, 1985).

Both the monogalactosyldiacylglycerols and digalactosyldiacylglycerols of heterotrophic cell cultures of tobacco contain larger proportions of saturated fatty acids in *sn*-1 position than those of photoautotrophic ones. Linolenoyl moieties (18:3), which are present in lower amounts in galactolipids of heterotrophic tobacco cells than in those of photoautotrophic ones, are almost equally distributed between the *sn*-1 and *sn*-2 positions of monogalactosyldiacylglycerols in the two cultures, whereas linolenoyl moieties are absent in *sn*-1 position of digalactosyldiacylglycerols of heterotrophic tobacco cells (Siebertz *et al.*, 1978).

*The carbon atoms of the glycerol backbone are designated by stereospecific numbering, *sn*, according to IUPAC–IUB recommendations.

III. BIOSYNTHESIS, METABOLISM, AND TURNOVER OF LIPIDS IN PLANT CELL CULTURES

Plant cells in culture are eminently suitable for studying the biosynthesis, metabolism, and turnover of lipids. In contrast to slices of various plant tissues, cells grown in suspension culture take up fatty acids from the medium within a few minutes (Stumpf and Weber, 1977), and other lipids, such as long-chain alcohols and alkylglycerols (Weber and Mangold, 1982; 1983) as well as cholesterol (Weber, 1978), within a few hours. The subsequent incorporation of these exogenous compounds into various lipid classes of plant cells is a function of both internal transport and the activities of various enzymes.

A. Lipid Classes

The biosynthesis and subcellular distribution of neutral glycerolipids was studied in photoautotrophic soybean cells. Diacylglycerols and triacylglycerols were found to be primarily located in chloroplasts, which are known to contain diacylglycerol acyltransferase, the final enzyme of triacylglycerol biosynthesis (Martin *et al.*, 1984).

Glycerophospholipids, e.g., diacylglycerophosphoethanolamines and diacylglycerophosphocholines, are the most prominent lipid classes in heterotrophic and photoautotrophic cell cultures. The existence of phosphorylated diacylglycerophosphoinositols ('polyphosphoinositides') has been demonstrated in various plant cell cultures (Boss and Massel, 1985; Heim and Wagner, 1986; Strasser *et al.*, 1986) (Table II and Fig. 1). In animals, the cleavage product of phosphatidylinositol-4,5-diphosphates (PIP_2), i.e. *myo*-inositol-1,4,5-triphosphate (IP_3), has been found to play an eminently important role in mediating hormonal signals (Scheme 1). Both the cytokinin-induced biosynthesis of diacylglycerophosphoinositols in soybean cells (Connett and Hanke, 1987) and the variation of levels of phosphorylated diacylglycerophosphoinositols during the growth cycle of *C. roseus* cells in culture suggest an involvement of inositolphospholipids in the regulation of cell proliferation in plants (Heim and Wagner, 1986). In addition, breakdown of membrane-bound diacylglycerophosphoinositols in homogenates of soybean callus stimulated by detergent attack or Ca^{2+} suggests an involvement of inositolphospholipids in cell activation (Connett and Hanke, 1986). In this

weight) an that are much lower than levels of alkaloid normally found

Table II

Inositolphospholipids Isolated from Wild Carrot (*Daucus carota*) Cells Labeled Overnight with [2-^{3}H]Myoinositol[a]

Inositolphospholipid	Counts per minute, [^{3}H]inositol[b]	Percentage of recovered [^{3}H]inositol[c]	Percentage of total inositolphospholipids, based on P_i[c]
Phosphatidylinositol	83,873	92.7 ± 1.4	77.2 ± 13
Lysophosphatidylinositol	3,446	3.6 ± 0.7	11.6 ± 7.7
Phosphatidylinositol 4-phosphate	1,807	1.70 ± 0.6	5.7 ± 3.2
Phosphatidylinositol 4,5-biphosphate	738	0.76 ± 0.3	1.4 ± 0.6

[a] Modified from Boss and Massel (1985).
[b] Representative data from one experiment.
[c] The data are means of four to six samples from three separate experiments.

context, it is of interest that IP_3 mobilizes Ca^{2+} from fusogenic carrot protoplasts (Rincon and Boss, 1987) and vacuolar membrane vesicles of oat roots (Schumaker and Sze, 1987). Yet, it seems that "polyphosphoinositides" do not play a role in signal transduction of elicitor-induced synthesis of phytoalexins in cell suspension cultures of soybean

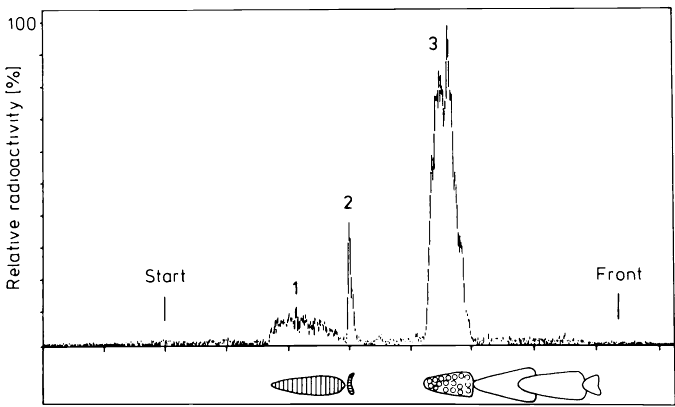
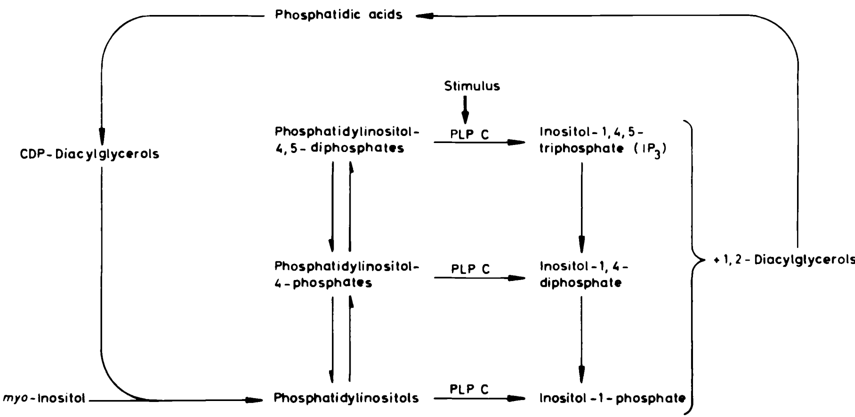


Fig. 1. Separation of [2-^{3}H]inositol-labeled inositolphospholipids from cultured parsley (*Petroselinum crispum*) cells on oxalate-impregnated silica gel layers with chloroform–methanol–4 *N* ammonia (9:7:2, v/v/v). (Upper part) Scan of radioactivity. (Lower part) Detection by iodine vapor (**1** ≡ phosphatidylinositol 4,5-biphosphate; **2** ≡ phosphatidylinositol 4-phosphate; **3** ≡ phosphatidylinositol). (Modified from Strasser *et al.*, 1986.)



Scheme 1. Possible influence of exogenous or endogenous stimuli on the phosphatidylinositol (diacylglycerophosphoinositol) cycle in plant cells (PLP C, phospholipase C).

and parsley (Strasser *et al.*, 1986). Further work along these lines can be anticipated.

In higher plant cells, cardiolipin is localized exclusively in the mitochondrial inner membranes. A decrease of this phospholipid in heterotrophic sycamore (*Acer pseudoplatanus*) cells during sucrose depriviation parallels the progressive diminution of the number of mitochondria (Journet *et al.*, 1986).

The age dependent dynamics of galactolipids in plastids of heterotrophically grown soybean suspension cells were also studied. It was found that a decrease of galactolipids in plastids which correlated with senescence of the cells was reversed by daily irradiation of the cells for a short period of time (Fig. 2); yet, chlorophyll formation was not induced under these conditions (Sabinski *et al.*, 1982).

Exogenous long-chain alcohols are oxidized to fatty acids and esterified to wax esters both by heterotrophically grown soya cells and photomixotrophically grown rape cells. Under nearly anaerobic conditions large amounts of wax esters are produced whereas oxidation of the substrate remains neglegible. The esterification reaction is catalyzed by an acyl-CoA : long-chain alcohol acyltransferase. The substrate specificity of this enzyme is dependent on the chain length of the alcohol supplied as substrate (Weber and Mangold, 1982). It is interesting to note that triacontanol (i.e. a saturated alcohol with 30 carbon atoms) is able to promote the growth of plant cells as has been demonstrated with tobacco, potato, bean, and barley cells in culture (Hangarter *et al.*, 1978).

Higher plants do not contain significant amounts of ether lipids, if any

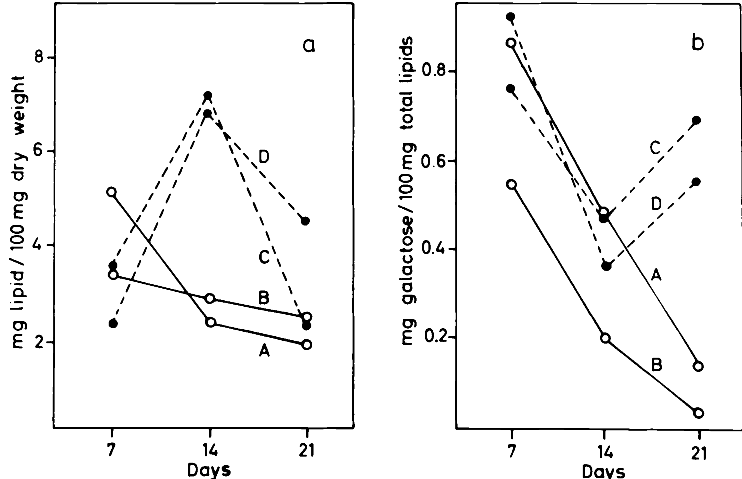


Fig. 2. Influence of light on total lipid (a) and galactolipid (b) contents of soybean (*Glycine max*) suspension cells during extended stationary phase. A and B (——), cultures grown in the dark; C and D (- - -), cultures exposed to white light for 20 min, several times each day. (Modified from Sabinski *et al.*, 1982.)

(Scheme 2). Yet, neutral and ionic ether glycerolipids, predominantly alkylacylglycerols and alkylacylglycerophosphocholines, are synthesized from exogenous 1-*O*-alkyl-*sn*-glycerols [IV] and 2-*O*-alkylglycerols by cell suspension cultures of rape and soya (Table III). The cells specifically incorporate the "natural" 1-*O*-alkyl-*sn*-glycerols from racemic mixtures into 1-*O*-alkyl-2-acyl-*sn*-glycerols, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines [II] and other ether lipids (Fig. 3) (Weber and Benning, 1983; Weber and Mangold, 1985), whereas 2-*O*-alkylglycerols form stereospecifically pure 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines (Weber *et al.*, 1984). In contrast, the "unnatural" 3-*O*-alkyl-*sn*-glycerols are not incorporated at all into ether glycerophosphocholines of rape cells.

The extent of bioconversion of 1-*O*-alkyl-*sn*-glycerols [IV] to ether glycerolipids is dependent on both chain length and degree of unsaturation of their alkyl chains ($C_{14:0} \sim C_{18:1} > C_{16:0} > C_{18:0}$) (Weber *et al.*, 1984). The incorporation of homologous saturated 1-*O*-alkyl-*sn*-glycerols from an equimolar mixed substrate of four racemic alkylglycerols into the various classes of ether glycerolipids broadly reflects the individual preference of enzymes that are involved in the biosynthesis of these lipids (Fig. 4) (Weber, 1983; Weber and Mangold, 1983). The ster-

I II III

IV V

Scheme 2. Ester glycerolipids and ether glycerolipids. R, R_1, R_2, R_3, saturated and unsaturated carbon chains.

- I 1,2-Diacylglycerophosphocholines, naturally occurring in plants and animals
- II 1-*O*-Alkyl-2-acylglycerophosphocholines, naturally occurring in animals, but not in higher plants; main products of the metabolism of exogenous IV in rape *(Brassica napus)* cell suspension cultures
- III 1-*O*-(1′-Alkenyl)-2-acylglycerophosphocholines, naturally occurring in animals, but not in higher plants
- IV 1-*O*-Alkylglycerols, substrates added to rape cell suspension cultures
- V 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholines ("platelet activating factor," PAF)

eospecific distribution of acyl moieties in the above ether glycerolipids reveals that alkylacylglycerophosphocholines [II] can replace the corresponding natural membrane lipids, i.e. diacylglycerophosphocholines [I], to an appreciable extent (Weber, 1985; Weber and Benning, 1985). In this context, it is worth noting that 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (Platelet Activating Factor, PAF) [V] is able to stimulate the activity of 1,3-β-D-glucan synthase of microsomes from cultured soya cells (Kauss and Jeblick, 1986). Moreover, PAF induces fluorescence emission changes in thylakoids by modulating the organization of the photosynthetic units (Argyroudi-Akoyunoglou and Vakirtzi-Lemonias, 1987). It may be speculated that PAF will find use in various areas of work on plant cell cultures, such as the regeneration of plantlets from single cells.

Table III

Incorporation of Radioactivity from *rac*-1-*O*-[1′-^{14}C]Hexadecylglycerol into the Lipids of Photomixotrophic Rape (*Brassica napus*) Cells Grown in Suspension[a]

Lipid class	Distribution of radioactivity (%) in the various lipid classes after *x* hr					
	3	6	12	24	36	48
Choline glycerophospholipids	23	30	43	49	52	50
Ethanolamine glycerophospholipids	2	3	6	6	16	15
Glycerol glycerophospholipids	—	—	Tr[b]	Tr	1	3
Unidentified polar lipids	8	7	9	9	11	11
1-*O*-Hexadecyl-2-acylglycerols[c]	5	3	3	4	4	5
1-*O*-Hexadecyl-3-acylglycerols	2	2	2	1	1	Tr
Triacylglycerols	Tr	1	1	3	3	5
Hexadecyldiacylglycerols	1	1	2	4	4	5
rac-1-*O*-[1′-^{14}C]Hexadecylglycerol	58	55	34	24	8	6
Activity of medium	1	Tr	Tr	Tr	Tr	Tr

[a] From Weber *et al.* (1984).
[b] Tr, trace (<1%).
[c] Contained traces of diacylglycerols.

B. Constituent Fatty Acids

The distribution of fatty acid synthase was studied in subcellular fractions of heterotrophic cells of *Idesia polycarpa* suspension cultures. The biosynthesis of straight-chain fatty acids was found to occur mainly in proplastids, whereas the formation of cyclopentenyl fatty acids, by a different enzyme system appears to take place in the cytosol. These unusual fatty acids are synthesized in cell cultures of *I. polycarpa* from α-ketopimelate via cyclopentenylglycine (Tober and Spener, 1982). The activity of fatty acid synthase in soybean cells is increased by the addition of either soybean acyl carrier protein (ACP) or *E. coli* ACP to incubation mixtures with subcellular fractions (MacCarthy and Stumpf, 1980c; Nothelfer and Spener, 1979). The metabolism of unsaturated acyl-CoAs was studied in proplastid and microsomal fractions of cell suspension cultures of soya. Oleoyl-CoA was metabolized by 3-hydroxylase as well as Δ^{12}-dehydrogenase and α,β-dehydrogenase to 3-hydroxyoleoyl-CoA, octadeca-9,12-dienoyl-CoA, and octadeca-2,9-dienoyl-CoA. The corresponding hydroxylated and dehydrogenated products were also formed from linoleic acid. High acyl-CoA thiolase activities were found in both the proplastid and microsomal fractions (Ferrante and Kates, 1986a,b).

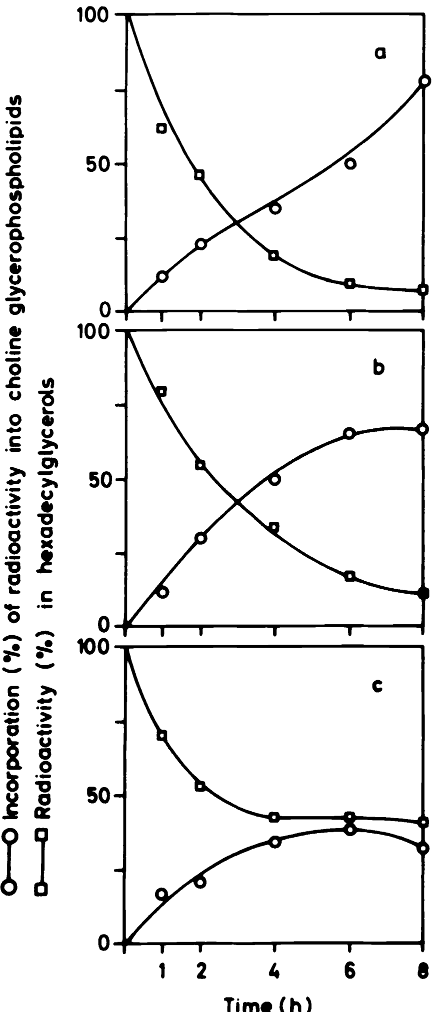


Fig. 3. Incorporation of radioactivity from 1-*O*-[1′-^{14}C]hexadecyl-*sn*-glycerol or *rac*-1-*O*-[1′- ^{14}C]hexadecylglycerol into choline glycerophospholipids of rape (*Brassica napus*) cells and soybean (*Glycine max*) cells in culture, as well as the decrease of radioactive substrate during incubation. (a) Rape cells + 1-*O*-[1′-^{14}C]hexadecyl-*sn*-glycerol. (b) Soybean cells + 1-*O*-[1′-^{14}C]hexadecyl-*sn*-glycerol. (c) Rape cells + *rac*-1-*O*-[1′-^{14}C]hexadecylglycerol. (From Weber and Mangold, 1985.)

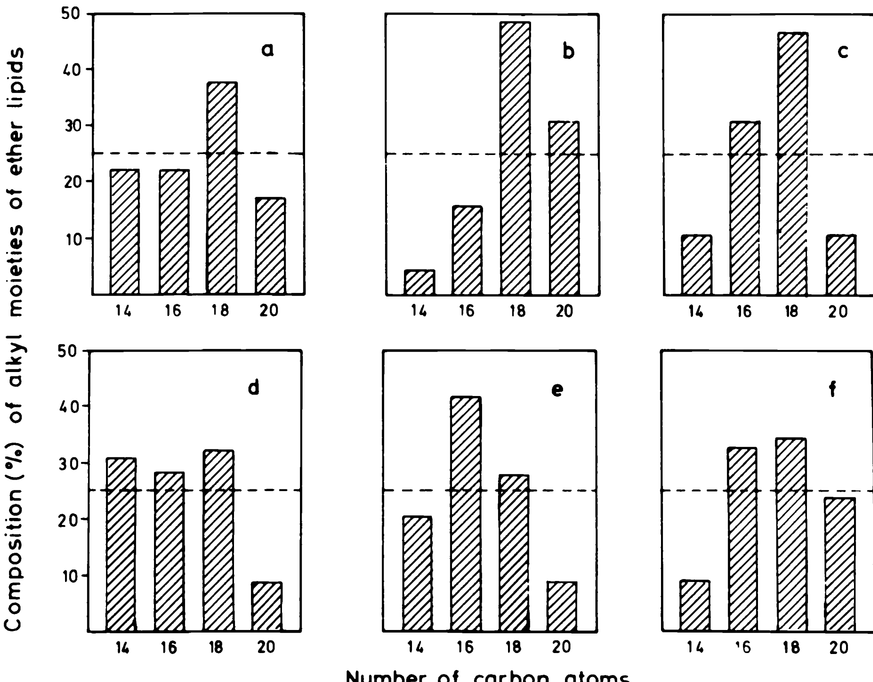


Fig. 4. Incorporation of homologous saturated *rac*-1-*O*-[1′-^{14}C]alkylglycerols from an equimolar mixed substrate into the lipids of the 48-hr incubation of photomixotrophic rape (*Brassica napus*) cells in suspension. (a) 1-*O*-Alkyl-2-acylglycerols. (b) 1-*O*-Alkyl-3-acylglycerols. (c) 1-*O*-Alkyl-2,3-di-acylglycerols. (d) 1-*O*-Alkyl-2-acylglycerophosphocholines. (e) 1-*O*-Alkyl-2-acylglycerophosphoethanolamines. (f) 1-*O*-Alkyl-2-acyl-glycerophosphoglycerols. The dotted line represents the percentage composition of each substrate in the equimolar mixture. (From Weber and Mangold, 1983.)

Radioactive acetate is incorporated about three times more rapidly into fatty acids of cell suspension cultures of soya and tobacco than into those of *C. roseus* (MacCarthy and Stumpf, 1980a). Elongation and desaturation of endogenous fatty acids prelabelled by administration of [^{14}C]acetate or of exogenous radioactive fatty acids was found to be optimal at 20–25°C. The biosynthesis of fatty acids from [2-^{14}C]malonyl-CoA was studied in a stable and highly active cell-free extract from *C. roseus* cells containing the enzymes for *de novo* synthesis, elongation, and desaturation of fatty acids (MacCarthy and Stumpf, 1980b,c). Obviously, fatty acid biosynthesis in this enzyme extract is influenced by several cofactors; desaturation, for instance, was stimulated by NADPH and ferredoxin. Desaturation of stearate to oleate was found to be op-

timal at 25°C. The corresponding desaturase is unstable at 41°C, whereas fatty acid synthase and elongase are unaffected by this temperature (MacCarthy and Stumpf, 1980c).

The incorporation of radioactive acetate and laurate (12:0) into lipids was studied in cell suspension cultures of cocoa bean. The kinetics of fatty acid labeling suggested that formation of linolenic acid (18:3) may occur via desaturation of linoleic acid (18:2) as well as chain elongation of dodecatrienoic acid (12:3) (Tsai and Kinsella, 1982).

The transfer of acyl groups from Tween esters to the various classes of polar lipids in cultured soya cells may be a useful system for manipulating the composition of acyl moieties of membrane phospholipids (Terzaghi, 1986a, b).

Heterotrophic soybean suspension cells esterify isomeric (*Z*)- and (*E*)-octadecenoic acids (18:1) predominantly into triacylglycerols and phospholipids. The incorporation of fatty acids into these lipids was found to be specific with regard to both positional isomers (varying in the position of the C=C double bond) and geometrical isomers (varying in the geometrical arrangement of the C=C double bond, i.e. (*Z*)- and (*E*)-isomers). Apparently, the two naturally occurring Δ^9-isomers, i.e. oleic acid, (*Z*)-18:1, and elaidic acid, (*E*)-18:1, are the preferred substrates (Weber *et al.*, 1979).

The activities of key enzymes of fatty acid degradation by β-oxidation, i.e. isocitrate lyase and malate synthase, are induced in glyoxysomes of anise (*Pimpinella anisum*) cells by adding acetate as a carbon source to the culture medium; homogenates of anise cells grown in the presence of sucrose, however, do not show β-oxidation of palmitoyl-CoA (Lutzenberger and Theimer, 1983).

IV. BIOTECHNOLOGICAL APPLICATIONS OF PLANT CELL CULTURES

Intensive research efforts aimed at using plant cell cultures for the production of valuable lipids have had little success. Recently, a few studies have raised hopes, however, for a more successful application of such cell cultures in the production of lipids by biosynthesis or biotransformation. In view of the low levels of lipids in plant cell cultures it is obvious that only valuable compounds are worth any consideration. These include radioactively labeled compounds, biologically active

phospholipids, as well as arachidonic acid (20:4) and other polyunsaturated fatty acids that can serve as precursors of icosanoides such as prostaglandins, prostacyclins, and leukotrienes. Moreover, it may be found possible to use plant cell cultures as sources of enzyme that are involved in the biosynthesis and metabolism of fatty acids and more complex lipids.

A. Biotransformation of Lipids

Large proportions of radioactively labeled diacylglycerophosphoethanolamines and diacylglycerophosphocholines are produced within 30 min by incubating cell suspension cultures of soya with [1-^{14}C]linoleic acid (Weber *et al.*, 1985). The labeled glycerophospholipids are isolated from the total lipids by thin-layer chromatography on silica gel. Mixtures of soybean glycerophospholipids are known to increase the yield of curd during cheese-making. A radioactively labeled misture was used for studying the distribution and turnover of "lecithin" during this process (Weber *et al.*, 1985; Wiechen *et al.*, 1985). Radioactive lecithin may also be helpful in tests of the activity and alteration of this common emulsifier in food and feed.

It has been reported that radioactively labelled glycerophospholipids can be obtained biosynthetically by incubating, e.g., [methyl- ^{3}H]-choline, [1-^{3}H]ethanolamine, and [2-^{3}H]inositol, with carrot (*Daucus carota*) suspension cells (Kleinig and Kopp, 1978).

Plant cell cultures are certainly useful for the production of randomly or uniformly labeled lipids from exogenous [^{14}C]acetate or, if photosynthetically active cultures are available, from $^{14}CO_2$. It must be realized, however, that any lipid constituent, such as long-chain fatty acids, glycerol, and bases, including ethanolamine, choline, and serine (also inositol) are not only incorporated into complex lipids as intact entities, but that they can also serve (and often serve predominantly) as sources of "organic carbon". Therefore, the incubation of plant cell cultures with such radioactively labeled precursors should not exceed an hour or two. In some cases, even an incubation period of half an hour leads to excessive degradation of the precursor and, consequently, to the appearence of radioactivity in various moieties of the complex lipids produced. Therefore, it is mandatory to establish the pattern of labeling in all radioactively labeled compounds that are produced by biosynthesis in plant cell cultures (Mangold and Radwan, 1980). This is of course not necessary if radioactive or stable heteroatoms, such as N, P, and S, are

supplied for the production of, e.g. ^{15}N-labeled ethanolamineglycerophospholipids or cholineglycerophospholipids.

Research on phosphorylated lipid derivatives of *myo*-inositol in plant cells is of great current interest because of their possible role in membranous signal transduction (Boss and Massel, 1985; Heim and Wagner, 1986; Strasser *et al.*, 1986). Rice (*Oryza sativa*) cells in culture are able to produce a series of labeled *myo*-inositol phosphates including phytic acid (*myo*-inositol hexaphosphate) from exogenous ^{3}H-labelled *myo*-inositol or inorganic ^{32}P (Igaue *et al.*, 1980). Phytic acid has negative nutritional effects because of its ability to bind physiologically important bivalent ions, e.g. Ca^{2+} and Mg^{2+}; radioactively labeled phytic acid may be of interest for studying the metabolism and nutritional effects of this substance as well as technological processes for its removal (Mukherjee, 1986).

The preparation of biologically active ether lipids by means of plant cell cultures constitutes an exciting new development which is being dealt with in some detail in this chapter. It has become apparent that complex ether lipids that do not occur in plant cell cultures can be produced by biotransformation of alkylglycerols, simple compounds that are easily obtained by organic synthesis. This finding opens a field for further exploration.

1-*O*-Alkyl-2-acyl-*sn*-glycero-3-phosphocholines [II] isolated from rape cells in suspension after incubation with 1-*O*-alkylglycerols can serve as starting material for the semisynthesis of physiologically active ether glycerophospholipids, such as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (PAF) [V] and 1-*O*-alkyl-*sn*-glycero-3-phosphocholines (lyso-PAF) (Table IV). The method recommends itself especially for the preparation of radioactive 1-*O*-[1'-^{14}C]alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (Scheme 3) (Weber and Mangold, 1985; 1986) as well as 1-*O*-(1'-alkenyl)-2-acyl-*sn*-glycero-3-phosphocholines ('choline plasmalogens') [III]. PAF is a harassing substance and much effort is being devoted to the production of antagonists that can be used in counteracting the harmful effects of PAF (Benveniste and Arnoux, 1983; Braquet *et al.*, 1987). Several of the synthetic antagonists known, so far, are ether lipids. Results from our laboratory indicate that plant cell cultures may be used for the production not only of PAF, but of positional isomers as well. Some "unnatural" ether glycerophospholipids exhibit antiphytoviral properties (Kluge *et al.*, 1984). 2-*O*-[1'-^{14}C]Alkyl-1-acetyl-*sn*-glycero-3-phosphocholines, for example, may be prepared following a sequence of reactions similar to that shown in Scheme 3 (Weber and Mangold, 1987).

Table IV

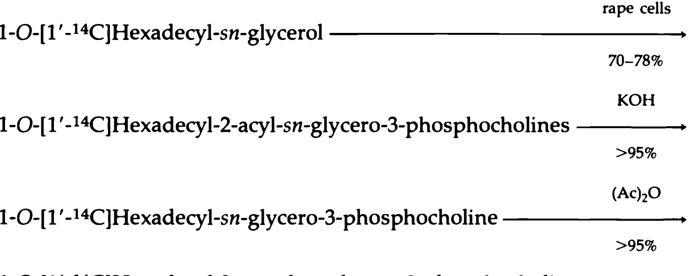
Specific Optical Rotations of Ether Glycerolipids Isolated from Rape (*Brassica napus*) Cell Suspension Cultures and Subsequently derived by Alkaline Hydrolysis and Acetylation[a]

1-*O*-Alkyl-*sn*-glycero-3-phosphocholines (lyso-PAF)[b]		1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (PAF)[b]	
Alkyl chains	$[\alpha]_D^{20}$	Alkyl chains	$[\alpha]_D^{20}$
1-*O*-Tetradecyl	−6.0	1-*O*-Tetradecyl	−1.1
1-*O*-Hexadecyl	−5.8	1-*O*-Hexadecyl	−1.1
1-*O*-Octadecyl	−5.3	1-*O*-Octadecyl	−1.5
1-*O*-[(Z)-9′-Octadecenyl]	−5.3	1-*O*-[(Z)-9′-Octadecenyl]	−1.1
1-*O*-Alkyl (mixture, derived from ratfish liver oil)	−5.5	1-*O*-Alkyl (mixture, derived from ratfish liver oil)	−1.1

[a] Modified from Weber *et al.* (1984).
[b] Optical rotations of alkylglycerophosphocholines were determined in chloroform–methanol (1:1, v/v), and those of alkylacetylglycerophosphocholines in chloroform (c = 1).

The isolation of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines and other glycerophospholipids from plant cells constitutes a rather tedious job, as these compounds occur predominantly in the cell membranes. It may be of advantage, therefore, to combine the preparation of PAF from the latter phospholipids with the production of substances that are excreted in the culture medium.

Lipids containing polyunsaturated, very long-chain fatty acids, e.g. arachidonic acid (20:4) and icosapentaenoic acid (20:5), are synthesized by cell suspension cultures of the moss *L. pyriforme* (Hartmann *et al.*,



Scheme 3. Schematic representation of the various steps involved in semisynthetic preparation of 1-*O*-[1′-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine ("platelet activating factor," PAF), using cell suspension cultures of rape. (From Weber and Mangold, 1985.)

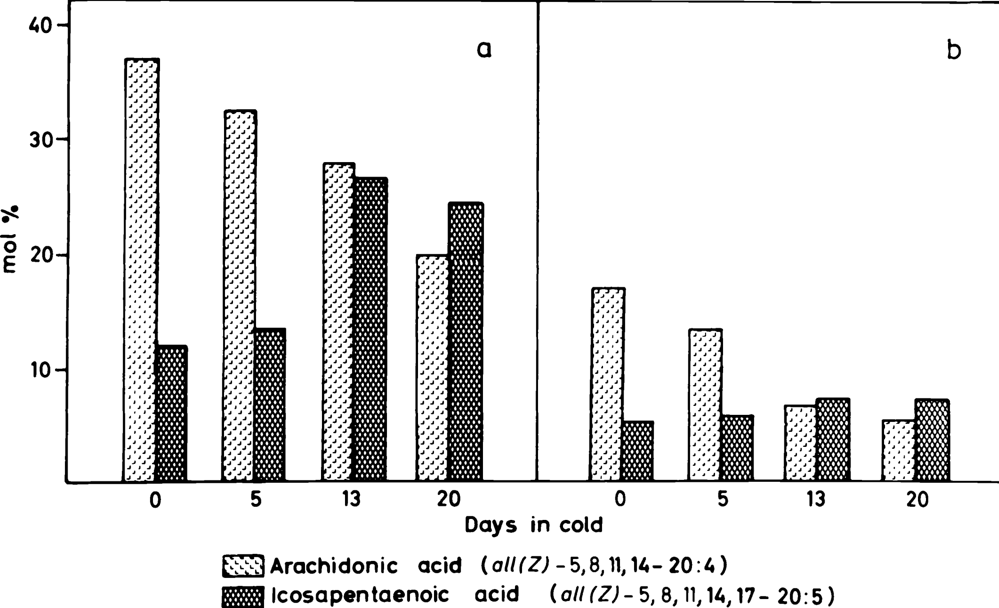


Fig. 5. Modulation of the arachidonic acid (checked bars; 20:4 ω6) and icosapentaenoic acid (cross-hatched bars; 20:5 ω3) contents of diacylglycerophosphoethanolamines (a) and diacylglycerophosphocholines (b) after transferring *Leptobryum pyriforme* cells form normal growth condition to a temperature of 1°C. (Modified from Hartmann *et al.*, 1986.)

1986). The proportions of these two polyunsaturated fatty acids are determined by the temperature during growth of the cultures (Fig. 5). The two polyunsaturated fatty acids are valuable starting materials for the semisynthesis of icosanoids (prostaglandins, prostacyclins, thromboxanes, leukotrienes) (Corey, 1987) that are used in biomedical studies and clinical therapy. A recent survey on the occurrence of these biologically active lipids in the plant kingdom is of interest (Panossian, 1987).

Another promising field of investigation is the production of specific enzymes that are useful for the biochemical preparation and analysis of lipids. For example, Kleinig and Kopp (1978) demonstrated the presence of phospholipase D in various cell suspension cultures; this enzyme may be helpful for the biosynthetic preparation of radioactive glycerophospholipids. Plant cell cultures exhibit activities of various acyltransferases that are able to esterify fatty acids into glycerolipids (Stumpf and Weber, 1977), including ether glycerolipids (Weber *et al.*, 1984). They also catalyze the esterification of long-chain alcohols (Weber and Mangold, 1982), sterols (Weber, 1978), and glycolipids (Heinz *et al.*, 1979). Lipases

and acyltransferases of plant cells (Wink, 1984) may be of interest for the interesterification of triacylglycerols, yielding more valuable products.

B. Production of Lipids

The production of lipids using plant cell cultures is hampered by the fact that it is necessary to extract whole cells, a fairly complex and expensive procedure. The following techniques may be suitable to produce lipophilic substances from suspension culture media without destroying the cells.

The "two-phase culture" uses nontoxic lipophilic solvents or adsorbents to concentrate lipophilic compounds from the medium during the growth of plant cells in culture (Beiderbeck and Knoop, 1987; Knoop and Beiderbeck, 1983; Maisch *et al.*, 1986). This technique can be employed for the isolation of secondary products, such as lipophilic terpenoids, from cell suspension cultures. It is rather doubtful, however, whether this procedure is applicable for the extraction of lipids that are fixed in membranes and not normally excreted by plant cells in culture.

A procedure that may be more successful for the production of certain lipids uses intact plant cells or protoplasts that are contained in a matrix of agar, alginate, protein, or a nontoxic organic polymer (Brodelius, 1983, Brodelius and Mosbach, 1982, Hulst *et al.*, 1985; Mavituna and Park, 1985, Rosevear and Lambe, 1985). These immobilized cells may be continuously rinsed with a nutrient medium containing fairly simple lipids. One may speculate that these precursors can be metabolized to more complex lipids by enzyme systems located in the outer membrane of the cells, and that the products, glycerophospholipids for instance, may then be extracted from the culture medium. In this context it should be mentioned that certain deteriorative processes of lipids catalyzed by lipoxygenase and lipid acylhydrolase are inhibited in *Vicia faba* protoplasts by immobilization in alginate (Fig. 6) (Schnabl and Youngman, 1985).

It can be expected that the biotransformation of lipophilic compounds by immobilized plant cells will be rendered more effective if techniques are developed that permit the cells to react with lipid precursors in aqueous organic solvents. The bioconversion of lipids by immobilized microbial cells in organic solvents has been described (Fukui and Tanaka, 1985). Techniques of downstream processing, e.g. affinity chromatography, high-performance liquid affinity chromatography, and affinity precipitation, may be helpful for the separation of valuable lipids from complex biological mixtures (Lowe, 1984).

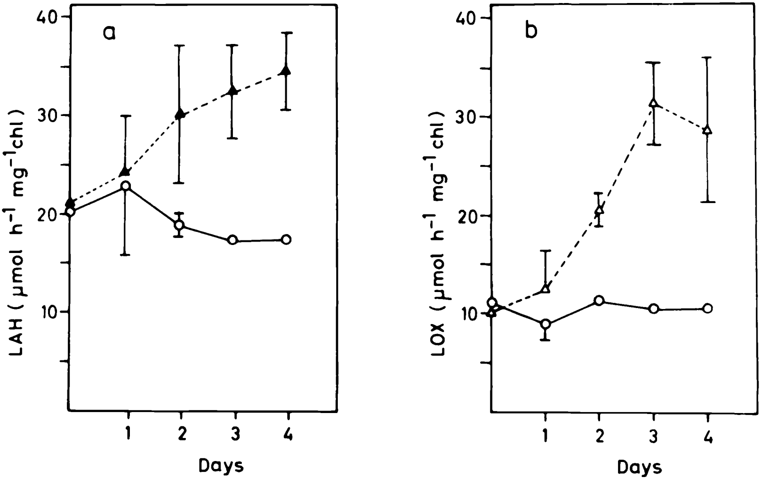


Fig. 6. Lipid acyl hydrolase (LAH) activity (a) and lipoxygenase (LOX) activity (b) in suspended protoplasts (▲) and in alginate-matrix-immobilized protoplasts (○) of *Vicia faba* during storage (chl, chlorophyll). (Modified from Schnabl and Youngman, 1985.)

Accumulation of some lipid classes by elicitor-induced enzymes may also function. Arachidonic acid (20:4) is known to be an elicitor in plant cells (Eilert, 1987).

V. CONCLUSIONS

Plant cell cultures have certainly been of great value in the vegetative propagation of palms and other commercially important oil bearing plants. The breeding of new varieties and hybrids will, no doubt, also profit substantially from cell culture techniques. Work on the biosynthesis of lipids has been much less rewarding, yet the results may be of help in various areas. For example, the incorporation of exogenous polyunsaturated fatty acids into the complex lipids of membranes of plant cells in culture would certainly improve the plasticity of these membranes, and thus, it may promote the viability of the cells during their cryopreservation. Reference is made to a recent review on the low-temperature storage of plant cell cultures (Withers, 1985).

The modification of membrane lipids in plant cells may aid in improving the release of secondary products into the culture medium.

PAF [V], an ether glycerophospholipid typical of human and animal

cells, can be produced by plant cell culture techniques, as described above. The same compound may find application as a phytohormone in improving the growth of plant cell cultures and in regenerating plants, including trees and other woody plants from single cells; work along these lines is in progress in various laboratories.

In the lipid field, major advantages of plant cells in culture may be attributed to the rapid absorption of lipophilic precursors from the culture medium and their incorporation into the cells. This is the prime condition for the use of plant cells in studies concerned with the biosynthesis and metabolism of lipids as well as the biotechnological production of complex compounds by biotransformation of fairly simple precursors. In addition to plant cell cultures, bacteria, yeasts, and algae should be considered for the biosynthetic preparation of valuable lipids.

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PART V

Biologically Active Compounds

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CHAPTER **31**

Insecticidal Phytochemicals

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I. INTRODUCTION

Throughout the world there is a long-established use of local plants for making insecticidal preparations. More than 2000 plant species belonging to 170 families have insecticidal properties (Feinstein, 1952). However, only from a few plants have the insecticides been isolated and their chemistry elucidated. These compounds can be complex esters

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(pyrethrins), alkaloids (nicotine, anabasine), or heterocyclic aromatic compounds (rotenoids). They occur in many plant parts, eg. pyrethrins in the flower heads of pyrethrum, nicotine in the leaves of tobacco, rotenoids in the roots of *Derris*, and ryanodine in the stem of *Ryania*.

Commercial insecticides of plant origin are few. They include the pyrethrins found in some members of Compositae, like *Chrysanthemum*, *Tagetes*, etc. (Compositae), the rotenoids found in *Derris*, *Lonchocarpus*, *Tephrosia*, (Leguminosae), the alkaloid nicotine from *Nicotiana* spp. (Solanaceae), and anabasine from *Anabasis aphylla* (Chenopodiaceae). Others of lesser importance are ryanodine from *Ryana speciosa*, jervin from *Veratrum album*, quassin from *Quassia amara*, veracevin and germine from *Schoenocaulon officinale*, azadirachtin from *Azadirachta indica*, mammein from *Mammea americana*, mundulone from *Mundulea sericea*, and pachyrrhizin from *Pachyrrhizus eroseus*.

Plant insecticides represent only a small fraction of the insecticidal material used each year, but they are effective against many insects that are not successfully controlled by synthetic insecticides. They are often relatively nontoxic to man and to other plants. They exert their insecticidal effect principally by interfering with the physiology of insects, deterring the insects from feeding, or interfering with their normal development.

In this chapter we concentrate only on the plant insecticides, pyrethrins, nicotine, rotenoids, ecdysterones, and limonoids. For a comprehensive list of plant insecticides the reader is advised to consult "Insecticides," by A. J. Fuell (1965) or *Insecticides from Plants: A Review of the Literature from 1954 to 1971*, by M. Jacobson (1971). A comparison of the yields of insecticides from plants and tissue cultures is given in Table I.

II. PYRETHRINS

The most economically important natural plant insecticides are the pyrethrins. These compounds are of great interest because of their lethal activity against insects, low toxicity to mammals, and low persistence after use. They paralyze flying insects very rapidly, and this knockdown effect is especially valued. A further property of value is their repellency, which may be more important than the killing effect when protecting foods (Crombie, 1980).

Pyrethrins have been reported from a number of Compositae: *Chrysanthemum cinerariaefolium C. coccineum*, *Tagetes erecta*, *T. minuta*, *Calen-*

Table I

Insecticides from Plants and Tissue Culture

Insecticide	Species	Source	Content	Reference
Pyrethrins	*Chrysanthemum cinerariaefolium*	Flowers	1–2%	Casida (1973)
		Callus	0.023–0.113%	Zieg *et al.* (1983)
		Shoot cultures	0.050–0.341%	Zieg *et al.* (1983)
	Tagetes erecta	Flowers	0.9%	Khanna *et al.* (1975)
		Suspension culture	1.16%	Khanna *et al.* (1975)
Nicotine	*Nicotiana tabacum*	Leaves	2–5%	Fuell (1965)
		Callus	2.14%	Ohta *et al.* (1978b)
	Nicotiana rustica	Leaves	5–14%	Fuell (1965)
Rotenoids	*Derris elliptica*	Roots	5–9%	Metcalf (1955)
		Callus with rootlets	0.016%	Kodoma *et al.* (1980)
	Lonchocarpus utilis	Roots	8–11%	Metcalf (1955)
	Crotalaria burhia	Callus	1.35%	Uddin and Khanna (1979)
	Tephrosia purpurea	Suspension culture	2.8%	Sharma and Khanna (1975)
	Tephrosia vogelii	Roots	1.2%	Sharma and Khanna (1975)
Phytoecdysones	*Trianthema portulacastrum*	Callus	0.036%	Ravishankar and Mehta (1979)
	Achyranthes sp.	Callus	<0.002%	Hikino *et al. (1971)*

dula officinalis, Zinnia elegans, Z. linnearis, etc. Of these, *C. cinerariaefolium* (pyrethrum) is the principal source of pyrethrins.

A. Pyrethrum

1. Botany

Pyrethrum (*Chrysanthemum cinerariaefolium* Vis.) is a small perennial herb, with deeply lobed leaves of variable shape and length, cultivated mainly in Kenya, Tanzania, and Ecuador at an altitude of at least 1900 m above sea level. At this elevation and low temperature, the flower heads (capitula) are borne on branched leafy stems rising from a compact crown of foliage, whereas at higher temperatures in lower regions, the plants almost exclusively develop vegetatively (Gnadinger, 1945; Glover, 1955).

Pyrethrins are located in all aboveground plant parts, but the ovaries and achenes of the disc and the ray florets of the capitulum contain by far the highest and largest amount (Head, 1966; Brewer, 1973). The flowers are harvested at 2- to 3-week intervals over a period of 2 to 4 years. An estimated 150 million pyrethrum flowers are harvested daily to supply the world demand for pyrethrum, 60% of which is met by Kenya (Balandrin *et al.*, 1985). The fresh flower yield is determined by many factors, such as genotype, climate, soil, plant diseases, and pests. The content of pyrethrins is chiefly influenced by the genotype, picking interval, flowering stage, climate, and drying methods (Zieg *et al.*, 1983).

2. Chemistry

Pyrethrins are a mixture of six structurally related insecticidal esters formed by a combination of two acids (chrysanthemic acid and pyrethric acid) and three alcohols (pyrethrolone, cinerolone, and jasmolone). The esters of chrysanthemic acid are called pyrethrin I, cinerin I, and jasmolin I, respectively, and are together known as pyrethrins I, whereas the esters of pyrethric acid are called pyrethrin II, cinerin II, and jasmolin II, and together, the pyrethrins II. Collectively, these compounds are known as rethrins, and a typical pyrethrum extract may have equal amounts of rethrins I and II and pyrethrins, cinerins, and jasmolins in the proportion 10:3:1 (Crombie, 1980). Pyrethrins I are responsible for killing insects (Elliott, 1971), whereas pyrethrins II provide much of the knockdown action against flying insects (Sawicki and Thani, 1962).

Pyrethrins can be effectively separated by gas liquid chromatography (GLC) and detected selectively by their electron capture response. Mass spectral data are well documented, making gas chromatography–mass spectrometry a useful tool for the study of these compounds. Pyrethrins can also be separated by other chromatographic procedures, such as high-performance liquid chromatography and thin-layer chromatography (Crombie, 1980).

3. Biosynthesis

The pyrethrum plant is efficient in its biosynthesis and/or storage of pyrethrins, producing 2–4 mg of pyrethrins per flower head, or 1–2% of pyrethrins relative to the flower dry weight (Casida, 1973). The chrysanthemic acid portion of pyrethrins I is derived biosynthetically from acetate via mevalonic acid. Incorporation of radiolabeled acetate, mevalonate, and chrysanthemate into pyrethrins I has been accomplished by cut flower stems and isolated achenes. Further, radiolabeled

chrysanthemate has also been incorporated into pyrethric acid and pyrethrins II, thus establishing the relationship between the two types of pyrethrins. The rethrolene portion of the pyrethrins has been shown to be derived from acetate, and because no intermediates were detected, it is presumed that the acetate is incorporated via the polyketide pathway. This work was also done using isolated ovules (achenes) from the flower heads (Casida, 1973). A cell-free system prepared from flower buds or shoot cultures of pyrethrum was able to incorporate radioactivity from [1-^{14}C]isopentenyl pyrophosphate into pyrethrins I and chrysanthemyl alcohol (Staba and Zito, 1985). This study established the intermediacy of pyrethrins I in the biochemical pathway.

4. Tissue Culture

a. Micropropagation. Reports on the *in vitro* micropropagation of *Chrysanthemum cinerariaefolium* have originated from the Netherlands (Roest and Bokelmann, 1973), India (Grewal and Sharma, 1978; Karki and Rajbhandary, 1984; Pal and Dhar, 1985). Ecuador (Levy, 1981), and Kenya (Wambugu and Rangan, 1981). Kenya and Ecuador have field-grown selected pyrethrum clones derived through the tissue culture techniques. The tissue culture micropropagation method was preferred because it is more rapid than multiplication from splits and ensured nematode free plants for a period of time.

i. *Explant Source.* Capitulum explants were used to induce pyrethrum shoot cultures by Roest and Bokelmann (1973), whereas shoot tips were used by Earle and Langhans (1974) to propagate ornamental *Chrysanthemum*. Wambugu and Rangan (1981), Grewal and Sharma (1978), and Karki and Rajbhandary (1984) also used shoot tips, whereas Pal and Dhar (1985) used leaf and petiole explants to induce pyrethrum shoot cultures.

ii. *Media Formulations.* Roest and Bokelmann (1973) used Knop's macronutrients and Heller's micronutrients (half-strength) (George and Sherrington, 1984), with sucrose and benzylaminopurine (BAP) to induce shoot cultures. The optimum sucrose concentration was found to be 0.5% and the optimum BAP concentration was 1 mg/ml. Earle and Langhans (1974) used Murashige and Skoog's (MS) medium (George and Sherrington. 1984) with 2 mg/liter kinetin and 0.02 mg/liter naphthaleneacetic acid (NAA), or 0.5 mg/liter kinetin and 0.8 mg/liter indoleacetic acid (IAA) to induce callus, which was differentiated into plantlets in a liquid MS medium containing 2 mg/liter kinetin and 0.02 mg/liter NAA.

Karki and Rajbhandary (1984) used MS medium with 5 mg/liter BAP

and 0.5 mg/liter IAA to induce multiple shoots from shoot tips. Wambugu and Rangan (1981) were able to induce shoot cultures from shoot tips on an MS medium containing BAP (0.02 mg/liter). Low concentration of BAP enhanced multiple shoots. Similar results were obtained by Grewal and Sharma (1978). Pal and Dhar (1985) induced shoot differentiation from leaf explants on an MS medium containing 0.1 mg/liter indolebutyric acid (IBA) and 0.5 mg/liter BAP, or 8 mg/liter NAA and 1 mg/liter kinetin. They also induced shoots from petiole explants on an MS medium containing 2 mg/liter NAA, 0.25 mg/liter kinetin, and 0.75 mg/liter BAP. Rapid proliferation of plantlets occurred when these shoots were placed on a medium containing 3 mg/liter NAA, 1 mg/liter IAA, and 1 mg/liter BAP.

b. Pyrethrin Production. Cashyap *et al.* (1978) could not detect pyrethrins in pyrethrum callus cultures but were able to detect them in shoots differentiated from the callus. Chumsri and Staba (1975) were able to detect pyrethrins chemically and by bioassay tests from aseptic plants of ornamental varieties of *Chrysanthemum cinerariaefolium* and *C. coccineum;* however, neither callus nor root cultures contained pyrethrins. Similarly, Kueh *et al.* (1985) did not detect any pyrethrin in callus cultures and root differentiated cultures, whereas they were present in shoot differentiated callus. In 1976 it was reported in a Japanese patent that extracts of pyrethrum callus and differentiated plants contained pyrethrins (Aoki *et al.*, 1976). Zieg *et al.* (1983) reported that callus from 54% of cultures established from flower and leaf explants contained 35 mg% or less pyrethrins. The highest concentration observed was 113 mg %. The amount of pyrethrins in shoot cultures varied between 0.05 and 0.34%.

i. *Explant Source.* The influence of the explant source on the biosynthetic capacity of pyrethrum was examined by Zieg *et al.* (1983). Explants of various pyrethrum plant organs were taken from high-yielding and low-yielding plant selections. Those explants that required the most manipulation during the isolation procedure, that is, peeled leaves, leaf epidermis, and achene epidermis, faired poorly in culture. Cultures derived from leaves grew only as callus regardless of attempts to redifferentiate them. The plant part used to initiate the cultures appeared to have little influence on pyrethrin production. However, the plant genotype used as a source of the explants influenced *in vitro* pyrethrin production.

ii. *Tissue Organization.* The type of tissue organization exhibited in culture appears to have a bearing on the amount of pyrethrins pro-

duced. It was shown that callus tissue synthesized less product than did organized shoot cultures (Zieg *et al.*, 1983). Tissue organization may be necessary for the formation of specialized structures such as oil glands and internal secretory canals, the involvement of which in pyrethrin production has been reported by Zieg *et al.* (1983) and Zito *et al.* (1983).

iii. *Environmental Conditions.* The pyrethrin plant is a quantitative short-day plant requiring photoperiods of about 12 hr. In addition, Roest (1976) recommended a low temperature (9°C) for 6 weeks to initiate flowering, and higher temperatures (17–25°C) for flowering and vegetative development. Staba and Zito (1985) tried unsuccessfully to induce flowers on pyrethrum shoot cultures. It was observed that light significantly enhanced the production and/or accumulation of pyrethrins in shoot cultures that received 400 foot-candles for 16 hr at 25°C. The light could be removed for 1 or 2 weeks without significantly affecting the pyrethrins produced (Staba *et al.*, 1984).

B. *Tagetes*

Pyrethrins have been reported in the seeds, floral heads, and tissue cultures of *Tagetes erecta* (Khanna *et al.*, 1975) and *T. minuta* (Jain, 1977). Seeds and floral heads contained 0.55 and 0.9% pyrethrins, respectively.

1. Tissue Culture

a. Explant Source. Seedlings were used as the explant source to initiate callus cultures of *Tagetes erecta* and *T. minuta*.

b. Culture Conditions. Callus of *Tagetes erecta* was initiated on an MS medium supplemented with 1 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) (Khanna *et al.*, 1975). The callus was transferred to MS liquid medium containing 0.1 ppm 2,4-D and was maintained by subculture every 4 to 6 weeks. Callus cultures of *T. minuta* were initiated and subcultured on MS medium containing 1 ppm 2,4-D every 6 to 8 weeks (Jain, 1977).

c. Pyrethrin Production. The pyrethrin content of *Tagetes erecta* suspension culture at 4 to 6 weeks of age was 0.90 and 1.16%, respectively, as determined by GLC. The pyrethrin content was found to increase in these tissues in the presence of exogenous ascorbic acid (Khanna and Khanna, 1976). Pyrethrins from *T. minuta* were not quantitated but were isolated and their biological activity confirmed (Jain, 1977).

III. NICOTINE

A crude tobacco extract was used as an insecticide as early as 1763, but the nicotine alkaloid was not isolated until 1828. By 1910, formulations containing up to 40% free nicotine were used as insecticides. Subsequently, pure nicotine (95–99%) and nicotine sulfate (40% base) became the standard commercial materials. Nicotine preparations were among the most important insecticides available and were extensively used until 1945. The high cost of nicotine production, its disagreeable odor, and extreme toxicity to mammals limit its use today.

A. Source

Nicotine occurs in at least 15 species of *Nicotiana* (Solanaceae) as well as other botanical species. It can be obtained commercially from *N. tabacum* or *N. rustica*. The alkaloid is present in most parts of the plant, but at least 60% of the total content is in the leaves (Fuell, 1965). The percentage of nicotine in the leaves varies with the type of tobacco: about 1–5% in *N. tabacum* and about 8% or more in *N. rustica*.

B. Chemistry

The empirical formula of nicotine is $C_{10}H_{14}N_2$, and it is, chemically, *N*-methyl-(3′-pyridyl)-2-pyrrolidine (Fuell, 1965). Pure nicotine is a colorless, odorless liquid that turns brown and develops a tobacco-like smell on exposure to air. Racemic nicotine prepared synthetically is about half as toxic as the natural nicotine, as the levo isomer is more insecticidal than its dextro counterpart. The nicotine content can be determined spectrophotometrically at its 265-nm absorption.

C. Biosynthesis

The biosynthesis of nicotine has been reviewed by Leete (1983). Biosynthesis occurs mainly in the roots of *Nicotiana* (Jacobson and Crosby, 1971). From there the alkaloid is translocated to other parts of the plant and accumulates mainly in the leaves, where it may undergo further transformations (Alworth and Rapoport, 1965). Cell cultures derived

from root, stem, and leaf of *N. tabacum* have been shown to possess the ability to produce nicotine (Speake *et al.*, 1964).

Nicotinic acid has been shown to be the precursor of the pyridine ring of nicotine (Dawson *et al.*, 1960). Nicotinic acid is derived by condensation of 3-phosphoglyceraladehyde and aspartic acid. Decarboxylation of the condensation product, quinolinic acid, yields nicotinic acid, which on further decarboxylation becomes the pyridine moiety of nicotine (Yang *et al.*, 1965). The pyrrolidine ring of nicotine is derived from ornithine or putrescine (Leete and Siegfried, 1957).

D. Tissue Culture

Nicotine production in tobacco tissue cultures has been reported by several investigators (Tabata and Hiraoka, 1976; Ohta and Yatazawa, 1978; Lockwood and Essa, 1984). It is generally accepted that the nicotine content in callus cultures is lower than that of intact plants; however, Ohta *et al.* (1978b) obtained callus tissues that contained greater than 2% nicotine on a dry-weight basis. The nicotine content of the callus tissue has been found to be directly related to the nicotine content of the cultivar of *Nicotiana tabacum* from which the callus was established (Kinnersley and Dougall, 1980).

Culture Conditions

Nicotine production in both cell cultures and callus is influenced by growth promoters. Ohta *et al.* (1978a) found that lower concentrations of auxins were stimulatory to nicotine production and that the optimum concentration of auxin for nicotine production was 0.15 ppm NAA. Ohta *et al.* (1978b) were able to establish a strain of tobacco callus that maintained a high nicotine content (2.14%) for 2-½ years by successively subculturing it on a medium containing 0.15 ppm NAA. The optimal concentration of sucrose and nitrogen in the culture medium were 3% and 840 mg of nitrogen per liter, respectively. Cultures incubated at 25°C produced the highest yield of nicotine. Ravishankar and Mehta (1982) reported enhancement of nicotine biogenesis in tobacco tissue cultures by the administration of urea, which suppressed urea-cycle enzymes and channeled ornithine into nicotine biosynthesis. Light inhibited nicotine production in tobacco callus (Ohta and Yatazawa, 1978). This inhibitory effect increased as the intensity and length of illumination increased. The inhibitory effect of light was completely reversed when the tissue was transferred to the dark.

IV. ROTENOIDS

Rotenoids were used as insecticides as early as 1848. For many centuries the plants containing these compounds were also used as fish poisons in tropical countries. Rotenoids are valued for the control of plant feeding pests and where toxic residues are not permitted.

A. Source

Rotenoids are found in *Tephrosia, Derris, Lonchocarpus, Millettia,* and *Mundulea* (Leguminosae). The principal economically important species are *D. elliptica, D. malaccensis, L. utilis,* and *L. uruca* (Holman, 1940). The rotenoid content of various commercial plant species varies significantly; for example, roots of *D. elliptica* average 5–9% rotenone, whereas *D. malaccensis* contains 0–4%. *Lonchocarpus utilis* contains 8–11% rotenone (Metcalf, 1955).

B. Chemistry

Six rotenoids are known to occur naturally: (1) rotenone, (2) elliptone, (3) sumatrol, (4) malaccol, (5) α-toxicarol, and (6) deguelin. An oxidative product of deguelin, that is, tephrosin, is sometimes present. Chemically, the rotenoids are classified in three groups of two, each group having the same basic ring structure and its hydroxy derivative, that is, rotenone–sumatrol, elliptone–malaccol, and deguelin–toxicarol (Fuell, 1965). They are optically active and generally occur in the levorotatory form. Rotenoids can be analyzed colorimetrically or by GLC.

C. Tissue Culture

Rotenoids have been reported from tissue cultures of *Derris elliptica* (Kodoma *et al.*, 1980), *Crotalaria burhia* (Uddin and Khanna, 1979), *Tephrosia purpurea*, and *T. vogelii* (Sharma and Khanna, 1975).

1. Explant Source

Seedlings were used to establish callus cultures of *Tephrosia purpurea, T. vogelii* (Sharma and Khanna, 1975), and *Crotalaria burhia,* whereas *Derris elliptica* leaves were used as the explant source by Kodoma *et al.* (1980).

2. Culture Conditions

Sharma and Khanna (1975) used a revised MS medium supplemented with 1 ppm 2,4-D to initiate callus cultures, whereas Uddin and Khanna (1979) did not use any growth regulators. Kodoma *et al.* (1980) used MS supplemented with 1 ppm thiamine, 0.3% yeast extract, 100 ppm myo-inositol, 3% sucrose, 2 ppm 2,4-D, and 0.2 ppm kinetin to induce callus from *Derris* leaves. Cell suspension cultures of *Tephrosia* were initiated from callus cultures by using MS liquid medium containing 0.1 ppm 2,4-D (Sharma and Khanna, 1975), whereas Kodoma *et al.* (1980) used the same medium that was used for callus culture but without the yeast extract.

3. Rotenoid Production

The rotenoid content of *Tephrosia vogelii* reported was maximal (2.8%) in 4-week-old cell suspension cultures. The rotenoid content of static cultures was also maximum at 4 weeks in both *T. purpurea* and *T. vogellii* (~1.25%) and gradually decreased in 6- and 8-week-old tissues. Four rotenoids (elliptone, deguelin, rotenone, and tephrosin) were reported present in the cultures (Sharma and Khanna, 1975).

The rotenoid content of *Crotalaria burhia* gradually increased up to 8 weeks to a maximum of 1.35% and then decreased. Six rotenoids (elliptone, deguelin, toxicarol, rotenone, sumatrol, and tephrosin) were reported in the cultures (Uddin and Khanna, 1979).

Trace amounts of rotenoids (2.9 μg per gram dry weight) were found in the callus tissue of *Derris elliptica* subcultured for 4 weeks. The content of rotenoids decreased gradually (0.6 μg per gram dry weight in 14-month-old subcultured callus) and was finally lost completely. Callus with imperfectly differentiated rootlets induced from leaves or stems and subcultured for 6 to 8 months contained 160 μg per gram dry weight rotenoids (rotenone and deguelin) (Kodoma *et al.*, 1980).

IV. PHYTOECDYSONES AND LIMONOIDS

Phytoecdysones are found in plants and are analogs of ecdysteroids, a group of insect hormones that initiates the cycles of ecdysis in insect development. They can disturb the growth cycles of insects and can result in the formation of abnormal adults. Limonoids are insecticidal compounds, found in members of Meliaceae and Rutaceae. They include azadiractin, limonin, nomilin, obacunone, and gedunin (Balandrin *et al.*, 1985). These compounds primarily deter insects from feeding, and some interfere with their molting cycle.

A. Source

Ecdysterone, a widely occurring phytoecdysone, has been reported in *Polypodium vulgare, P. virginianum, Achyranthes aspera, Trianthema portulacastrum, Sida carpinifolia, Sesuvium portulacastrum,* and *Gomphrene celosiodes.* Azadirachtin is obtained from *Azadirachta indica* and *Melia azedarach* (Nakanishi, 1975; Warthen, 1979).

B. Tissue Culture

Ecdysterone has been reported from tissue culture of *Trianthema portulacastrum* (Ravishankar and Mehta, 1979), and ecdysterone and inokosterone from tissue culture of *Achyranthes* sp. (Hikino *et al.*, 1971).

1. Explant Source

Seedlings were used as the source of explants to initiate callus cultures of *Trianthema* and *Achyranthes.*

2. Medium Formulations

Hikino *et al.* (1971) used White's medium supplemented with coconut milk (10%) and 1 or 4 ppm 2,4-D to induce callus from various species of *Achyranthes.* Maximal callus growth, however, was observed on MS medium supplemented with coconut milk (10%) and 1 or 4 ppm 2,4-D and 1 ppm kinetin. Ravishankar and Mehta (1979) used MS medium containing 2% sucrose supplemented with 2,4-D and kinetin to induce callus of

Trianthema. Of the three auxins used, maximal growth of callus was obtained with 0.2 ppm NAA. Kinetin at a concentration of 0.4 ppm or gibberellic acid (GA) at a concentration of 50 ppm also gave good callus growth.

3. Phytoecdysone Production

Although the phytoecdysone content (ecdysterone and inokosterone) of callus tissues of *Achyranthes* was low (<0.002%), the ecdysterone content of *Trianthema* callus was four times higher than that in differentiated plants. The growth regulator 2,4-D (2 ppm) increased ecdysterone level to 0.0349%, kinetin (0.001 ppm) increased the level to 0.0217%, and GA (100 ppm) increased it to 0.0117%.

Sanyal *et al.* (1981) have reported nimbin production in cultured tissue of *Azadirachta indica*. Nimbin is a tetranortriterpenoid similar to azadirachtin that is obtained from the bark of the plant (0.04%). Callus cultures were initiated from the bark grown on MS medium containing IAA and BAP. Nodular outgrowths were observed in all cultures except 0.5 ppm IAA, where roots appeared after 40 days. The callus was analyzed for nimbin by GLC. Nimbin was detected only in callus cultures containing roots (0.025%).

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CHAPTER 32

Antitumor Compounds

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I. INTRODUCTION

Since the dawn of history, plant materials have been used in the treatment of illnesses referred today as tumors and cancer. In recent years, extensive screening of antitumor agents from plants has been undertaken. One of the most intensive of such projects was carried out by the National Cancer Institute (NCI) in the United States. NCI began the project in 1956, and by 1981 nearly 114,000 plants of about 1550 genera had been screened; 4.3% of these plants showed antineoplastic activity (Suffness and Douros, 1982). From these screening projects, vinblastine, vincristine, and podophyllotoxin were selected and used in

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Table I

Antineoplastic Compounds Produced by Plants

Compound	Plant
Baccharine	*Baccharis megapotamica*
Bruceantine	*Brucea antidysenterica*
Cesaline	*Caesalpinia gillisesii*
3-Deoxycolchicine	*Colchicum speciosum*
Ellipticine, 9-methoxyellipticine	*Ochrosia moorei*
Fagaronine	*Fagara zanthoxyloides*
Harringtonine, homoharringtonine	*Cephalotaxus harringtonia*
Holacanthone	*Holancantha emoryi*
Indicine *N*-oxide	*Heiotropium indicum*
Maytansine	*Maytenus bucchananii, Putterlickia verrucosa*
Podophyllotoxin	*Podophyllum peltatum*
Taxol	*Taxus brevifolia*
Thalicarpine	*Thalictrum dasycarpum*
Tripdiolide, triptolide	*Tripterygium wilfordii*
Vinblastine, vincristine	*Catharanthus roseus*

clinical treatments of cancer. Some others are under investigation. Among the compounds selected, the most important and promising ones are listed in Table I.

One of the major problems in examining antineoplastic agents of plant origin is the difficulty in obtaining sufficient material to accomplish *in vitro, in vivo,* and clinical trials, because the levels of the active components are generally very low. Moreover, the growth rates of some of the plants are slow, and accumulation pattern and content are highly susceptible to geographical and environmental conditions.

Plant tissue culture is one of the approaches available to provide large amounts and a stable supply of these compounds and has been of major interest since the 1970s. In this chapter the research on antitumor compounds using plant tissue culture techniques is described.

II. METHODOLOGY

A. Culture Conditions

In general, conditions of callus or cell suspension culture for the production of antineoplastic compounds are not special. Optimal conditions can be determined by changing the chemical and physical factors

of the culture system, such as chemical components or phytohomones in media, pH, aeration, temperature, and light. Also, the genetic makeup and physiological status of cells are important factors for secondary metabolite production.

1. Chemical Factors

In most of the reports mentioned in this chapter (see Section III), Murashige and Skoog medium (MS; 1962) was employed. The relatively high concentration of nutrients (especially nitrogen) in this medium seems to enhance the production of antineoplastic compounds.

Suitable combinations and concentrations of phytohormones for the *in vitro* growth of several plant species that produce antitumor compounds were examined by Misawa *et al.* (1983; Table II). Although rapid growth of cultured cells is a prerequisite for production of any kind of secondary metabolite, the optimum condition for growth does not necessarily give maximum production of chemicals. Thus, a two-step culture system has been proposed, first culturing the cells in growth-promoting medium, then in medium for the production of the desirable metabolite. Furthermore, casamino acids and coconut water are sometimes added to the medium. Growth and podophyllotoxin production of

Table II

Phytohormones for Callus Induction with Antineoplastic-Agent-Producing Plants

Plant	Antineoplastic agent	Phytohormones[a] (mg/liter)
Baccharis megapotamica	Baccharine	KIN 1, 2,4-D 0.5
		KIN 1, NAA 10
Brucea antidysenterica	Bruceantine	KIN 1, 2,4-D 6
Caesalpinia gilliesii	Cesaline	KIN 1, 2,4-D 0.5
Cephalotaxus harringtonia	Harringtonine, homoharringtonine	NAA 3
Colchicum speciosum	3-Deoxycolchicine	Not induced
Fagara zanthoxyloides	Fagaronine	Not induced
Heliotropium indicum	Indicine *N*-oxide	KIN 1, 2,4-D 0.5
		KIN 1, NAA 1
Ochrosia moorei	Ellipticine, 9-methoxyellipticine	KIN 1, 2,4-D 0.5
Putterlickia verrucosa	Maytansine	KIN 1, 2,4-D 6
Taxus brevifolia	Taxol	Not induced
Thalictrum dasycarpum	Thalicarpine	KIN 0.1, 2,4-D 0.5
Tripterygium wilfordii	Triptolide, tripdiolide	KIN 1, 2,4-D 6
		KIN 1, NAA 1
		KIN 1, NAA 10

[a] 2,4-D, 2,4-dichlorophenoxyacetic acid; KIN, kinetin; NAA, naphltaleneacetic acid.

the callus of *Podophyllum peltatum* were stimulated by addition of 500 mg/liter casamino acids (Kadkade, 1982). Coconut water (10–30 ml/liter) promoted the growth of *Tripterygium wilfordii* cell suspension culture but did not seem to enhance the yield of antitumor diterpenes (Kutney *et al.*, 1983).

2. Physical Factors

Podophyllotoxin levels of the callus of *Podophyllum peltatum* were increased by red light (660 nm) and were inhibited by light at 371, 420, and 460 nm (Kadkade, 1982). The production was also dependent on the intensity of the red light. The most effective light intensity of 750 $\mu W/cm^2$. Light of short wavelength also seemed to inhibit the growth of the callus.

The dry-weight to fresh-weight ratio of *Cephalotaxus harringtonia* cells was affected by temperature (15–35°C) (Delfel and Smith, 1980). Initial pH of the culture medium within the pH range 4.5–8.0 had little or no effect on the growth of *C. harringtonia* callus. In the case of *Camptotheca acuminata* cells, however, relatively low pH (4.3) gave the best growth (Sakato and Misawa, 1974).

3. Biological Factors

It is well known that the level of production of secondary metabolites in cultured plant cells varies from one cell line to another. Even in a single culture vessel, differences in morphology and level of production are found. This heterogeneity makes it possible to select higher-producing lines. Kutney *et al.* (1983) selected a cell line of *Tripterygium wilfordii* that produces large amounts of tripdiolide.

Origin of cultured cells may affect the level of production. Kadkade (1982) induced *Podophyllum peltatum* callus from rhizome, leaf, stem, and root segments of the plant and found that the podophyllotoxin content of callus tissues derived from the rhizome was higher than those from other plant parts.

Another important factor that affects the level of secondary metabolites is age of cells. Therefore, studies of the time course of cell growth and production of metabolites are essential. Podophyllotoxin in *Podophyllum peltatum* callus was reported to parallel the growth of cells (Kadkade, 1982). A time-course study of the production of tripdiolide in *Tripterygium wilfordii* suspension cultures showed that older inoculum (21 days old) was preferable for production than younger inoculum (11 days old) (Kutney *et al.*, 1983).

B. Assay Systems

In most cases the amount of the antitumor compounds produced in cultured plant cells is far below that of the source plants. Therefore, highly sensitive assay methods, such as a bioassay using mammalian tumor cell cultures or a radioimmunoassay (RIA), have advantages.

Cytotoxicity analysis using KB (human epidermoid carcinoma of nasopharynx) cell culture has been widely employed because of its higher sensitivity than that of other tumor cell systems, such as mouse leukemias P388 or L1210. This system has been successfully applied in the screening of crude plant extracts by NCI. Active compounds that do not show significant antitumor activity, however, such as cardenolides, saponins, and aconitic alkaloids, were picked up by KB methods. HeLa S-3 cells were used to detect vinblastine produced by *Catharanthus roseus* cells by Miura and Okazaki (1983). In any case, bioassay systems are not specific to individual compounds, and further analyses with high-performance liquid chromatography (HPLC), gas chromatography, mass spectrometry, (GC), and nuclear magnetic resonance (NMR) are essential for identification of the products.

To determine the amount of cephalotaxine esters in cultured *Cephalotaxus harringtonia* cells, an RIA system was established (Misawa *et al.*, 1983). This system made it possible to quantify up to 1 ng per 0.1 ml of cephalotaxine and its esters.

III. ANTITUMOR COMPOUNDS IN CULTURED PLANT CELLS

A. Camptothecine

A cytotoxic alkaloid, camptothecine (Fig. 1), was isolated from the tree *Camptotheca acuminata* (Nyssaceae), native to north China (Wall *et al.*, 1966). It is highly active against Walker 256 rat carcinosarcoma and mouse leukemias P388 and L1210, and causes gastrointestinal tract toxicity in the mouse.

Effects of chemical and physical factors of culture conditions on growth of *Camptotheca acuminata* callus and suspension cells were reported by Sakato and Misawa (1974). MS medium containing 0.1 mg/liter 2,4-dichlorophenoxyacetic acid (2,4,-D), 3 mg/liter kinetin, and 0.05 mg/liter gibberellic acid (GA_3) produced optimum cell growth in suspen-

Fig. 1. Camptothecine.

sion cultures. Camptothecine was identified from the cells cultured in liquid medium (Sakato *et al.*, 1974); the amount was 2.54 μg per gram dry-cell weight, which was about 5% of the level in the source plant.

B. Vinblastine and Vincristine*

Vinblastine and vincristine (Fig. 2), dimeric indole alkaloids isolated from periwinkle (*Catharanthus roseus*, Apocynaceae), are now well accepted in the treatment of various cancers. Vincristine is one of the most active agents in the treatment of acute leukemia, lymphomas, and solid tumors. Vinblastine shows similar activity and is an active agent against Hodgkin's disease. These compounds are obtained only from periwinkle plants grown in the field, and the yield in the plants is extremely low. Consequently, they are among the most expensive drugs on the pharmaceutical market. Considerable effort, therefore, has been focused on the investigation of their production by plant tissue culture, as well as by chemical processes.

Miura and Okazaki (1983) reported the accumulation of vinblastine by tissue cultures. The cells cultured in MS medium supplemented with 1.0 mg/liter indolencetic acid (IAA) and 1.0 mg/liter benzylpurine (BA) contained 10.2 μg/g (dry weight, as vinblastine) of the cytotoxic compound, though this compound has not yet been identified. Many researchers have been working on the production of various indole alkaloids by cultured cells of *Catharanthus roseus* (Zenk *et al.*, 1977; Kurz *et al.*, 1980; Kutney *et al.*, 1980), but except for Miura's group, all have failed to detect these antitumor alkaloids. Vinblastine-type alkaloids are synthe-

*See also Chapter 22, this volume.

CATHARANTHINE

VINDOLINE

3',4'-ANHYDROVINBLASTINE

VINBLASTINE R=Me
VINCRISTINE R=CHO

LEUROSINE

CATHARINE

Fig. 2. Biosynthetic pathway of dimeric indole alkaloids.

sized through coupling of two indole alkaloids, vindoline and catharanthine (Fig. 2). Although catharanthine was demonstrated in cultured cells of *C. roseus* (Kutney *et al.*, 1980), it is generally found that cultured cells with undifferentiated tissues are incapable of synthesizing vindoline (Fahn *et al.*, 1985). This observation may provide an explanation for the difficulty of finding dimeric alkaloids in cultured cells.

As an alternative, enzymatic synthesis of the dimeric alkaloids was reported at Allelix (Endo *et al.*, 1986; Smith *et al.*, 1986), in collaboration with Kutney, Kurz, and colleagues. According to our results, 86 mg/liter catharanthine was accumulated by *Catharanthus roseus* suspension cultures (450 g fresh weight of cells per liter of cell suspension). Subsequently, this monomeric alkaloid was coupled with commercially available vindoline, using crude or purified enzyme from the suspension cultures to form 3',4'-anhydrovinblastine, an immediate precursor of vinblastine (see Fig. 2), with a conversion yield of 50%. Other dimeric alkaloids, leurosine, catharine, vinamidine, and 3-(*R*)-hydroxyvinamidine, were also found as by-products in this system. All these dimeric alkaloids are expected to have antineoplastic activity, and the activity of some of them has been already established.

C. Homoharringtonine

An evergreen tree, *Cephalotaxus harringtonia* (Cephalotaxaceae), contains several alkaloids that have shown significant activity against leukemias in mice (Powell *et al.*, 1969). These active alkaloids, deoxyharringtonine, harringtonine, isoharringtonine, and homoharringtonine, are all esters of cephalotaxine (Fig. 3).

Delfel and Rothfus (1977) showed that callus cultures of *Cephalotaxus harringtonia* produced the same antitumor alkaloids as the intact plants. Yield of cephalotaxine and its esters found in 6-month-old callus was 0.3 μg/g on a fresh-weight basis. But after the initial success, their studies seem to have focused on the improvement of the growth of cultured cells by manipulating culture conditions and medium components (Delfel, 1980; Delfel and Smith, 1980), but no further reference to the stability and/or improvement of alkaloid production has been made.

Misawa *et al.* (1985) detected 0.55 ng/g (dry weight) of cephalotaxine and its esters with an RIA method in suspension cells grown in MS medium with 1% sucrose and 3 mg/liter NAA.

O N RO H OMe

HARRINGTONINE

R= $-C(=O)-C(OH)(CH_2-CO_2Me)-CH_2-CH_2-C(OH)Me_2$

DEOXYHARRINGTONINE

R= $-C(=O)-C(OH)(CH_2-CO_2Me)-CH_2-CH_2-CHMe_2$

ISOHARRINGTONINE

R= $-C(=O)-C(OH)(CH(OH)-CO_2Me)-CH_2-CH_2-CHMe_2$

HOMOHARRINGTONINE

R= $-C(=O)-C(OH)(CH_2-CO_2Me)-CH_2-CH_2-CH_2-C(OH)Me_2$

CEPHALOTAXINE

R= H

Fig. 3. Structures of harringtonine and its derivatives.

D. Maytansine

Several members of the Celastraceae, such as *Maytenus buchanaii* and *Putterlickia verrucosa*, contain maytansine (Fig. 4), which shows high inhibitory activity against several murine tumors (Kupchan *et al.*, 1972). Kutney *et al.* (1981a) reported that extracts from cultured tissues of *M. buchanaii* showed cytotoxic activity against KB cells, but thin-layer chromatography did not indicate the presence of maytansine. Misawa *et al.* (1985) isolated a compound that showed identical ultraviolet (UV) spectra, R_f's on TLC plates, and retention times in HPLC to authentic maytansine from callus of *P. verrucosa*. The amount of this compound was low (50 μg per kilogram dry weight, based on its cytotoxicity, however, which was about one-two hundred fortieth of that of the mother plant. According to a report by Suffness and Douros (1982), the compound did not show potent activity in clinical trials.

E. Podophyllotoxin

Podophyllum peltatum (Berberidaceae), a rhizomatous herb that grows in deciduous forests of eastern North America, has been known to produce lignanes such as podophyllotoxins (Fig. 5), which are used against virus and skin cancers. In the NCI program, some of the semisynthetic analogs were tested clinically and were found to have produced responses to brain tumor, lymphosarcoma, and Hodgkin's disease (Nissen *et al.*, 1972).

Kadkade (1981, 1982) examined growth and podophyllotoxin production of *Podophyllum peltatum* callus tissues under various culture condi-

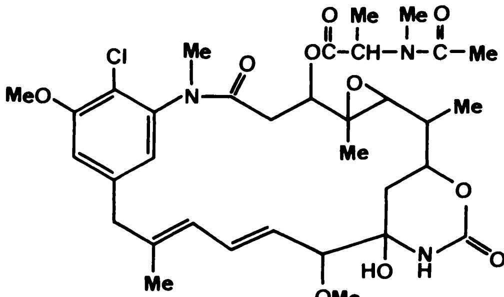

Fig. 4. Chemical structure of maytansine.

Fig. 5. Podophyllotoxin.

tions. Explants were cultured on solidified MS medium with combinations of growth regulators and casamino acids (500 mg/liter). Eight-week-old calluses were harvested, and lignanes were extracted. A combination of 2,4,-D (1 mg/liter) and kinetin (0.2 mg/liter) was optimal for podophyllotoxin production. The maximum production achieved with the optimized condition was 0.74% on a dry-weight basis. If this high yield is stable in long-term culture, this antitumor compound can be one of the most promising targets for commercial production in this field.

F. Tripdiolide and Triptolide*

Tripterygium wilfordii (Celastraceae) is a climbing shrub native to eastern Asia that produces diterpene triepoxides, tripdiolide and triptolide (Fig. 6). These terpenoids showed antineoplastic activity against L1210, P388, and KB cells (Kupchan *et al.*, 1981).

Kutney *et al.* (1981) selected a high-producing cell line that yielded tripdiolide and triptolide, respectively, 16 and 6 times more so than the source plants. They further investigated culture conditions for high production, and the maximum level of tripdiolide (4.0 mg/liter) was achieved when MS medium with 2% sucrose, 0.5 mg/liter naphthaleneacetic acid (NAA), and 0.5 mg/liter kinetin was used as a production medium (Kutney *et al.*, 1983). Dujack and Chen (1980) studied the effects of precursors of the terpenoids on *Tripterygium wilfordii* tissue culture and

*See also Chapter 9, this volume.

R=H TRIPTOLIDE
R=OH TRIPDIOLIDE

Fig. 6. Chemical structures of triptolide and tripdiolide.

demonstrated that addition of pyruvic acid and sodium acetate to the medium increased the cytotoxic activity of the cultured tissue.

Misawa *et al.* (1983, 1985) isolated tripdiolide and triptolide from callus and suspension cultures of *Tripterygium wilfordii.* Culture medium used was MS medium with 1 mg/liter NAA and 1 mg/liter kinetin. *N*-Phenyl-*N*′-(2-chloro-4-pyridyl)urea (4-PU-30), a compound that showed cytokinin-like activity, increased the production of tripdiolide up to 70%. Also, a precursor of the diterpenoids, farnesol, stimulated tripdiolide synthesis (Table III). The level of the diterpenoids in the callus seemed to be higher than that in the suspension culture: 100.6 μg of tripdiolide and 69.8 μg of triptolide were found in 1 g of dried callus, about 10 and 7 times higher than levels in the source plant, respectively.

Table III

Effect of Farnesol on Growth and Production of Tripdiolide by *Tripterygium wilfordii* Cells in Suspension[a]

Farnesol added (μg/ml)	Growth (mg/ml)	Tripdiolide (μg per gram of cells, dry weight)
0	14.3	6.3
30	12.7	13.0
100	10.1	20.4

[a] Cells were cultivated for 3 weeks.

G. Other Antineoplastic Agents

Misawa *et al.* (1983) reported that crude extracts of cultured cells of *Baccharis megapotamica, Holacantha emoryi,* and *Brucea antidysenterica* were found to have cytotoxicity against KB cells. The preliminary experiments showed that products of these cultured cells might be baccharine, holacanthone, and bruceantine, respectively.

IV. PROSPECTS

As seen in Table IV, antineoplastic compounds from plants are classified into wide chemical categories. The prominent compounds vigorously examined now fall into categories of alkaloids (cephalotaxine esters, camptothecine, etc.), maytansine, diterpenes (tripdiolide, triptolide), and lignanes (podophyllotoxin), but it is noteworthy that many new compounds are still being selected from higher plants in extensive

Table IV

Classification of Antitumor Compounds from Higher Plants

Tannins
Sterols (including simple glycosides, excluding saponins)
Quinones (including quinoids and quinols)
Terpenes
Iridoids
Sesquiterpenes
Diterpenes
Tripterpenes (including cucurbitacins, excluding saponins)
Lignans
Flavonoids
Saponins and their aglycones
Steroidal
Triterpenoid
Steroid lactones (including cardenolides, bufadienolides, withanolides, and their aglycones)
Quassinoids (simaroubolides)
Maytansine
Proteins
Alkaloids
Miscellaneous

screening programs. Plant tissue culture may provide a promising method for the production of stable amounts of antitumor compounds.

In general, an antitumor agent shows some cytotoxicity to any living cells, but especially to actively dividing cells. Cultured cells are normally rapidly dividing and proliferating, compared with somatic cells of intact plants. It is, therefore, reasonable to question whether an antineoplastic compound might inhibit the growth of the cells in which it is accumulated. Although *Colchicum autumnale* was known to tolerate colchicine at concentrations toxic to most other species (Blakeslee, 1939; Levan and Steinegger, 1947), Constabel *et al.* (1981) demonstrated that cultured *Catharanthus roseus* cells, as well as other seed plants and mammalian cells, were sensitive to vinblastine. This paradox may be overcome by employing a two-step culture (separating growth and production phases), immobilized cells, or cell-free systems to carry out transformation of metabolites. Alternatively, this cytotoxicity of antitumor compounds to the host cells may be positively utilized to select a cell line tolerant to the compounds, as Constabel *et al.* (1981) proposed.

One promising possibility in exploiting tissue cultures is the discovery of novel antitumor agents. A new cephalotaxine ester, homodeoxyharringtonine (Delfel and Rothfus, 1977), and several terpenoids (Kutney *et al.*, 1983) have been found in cultured cells of *Cephalotaxus harringtonia* and *Tripterygium wilfordii*, respectively, though their antitumor activities have not yet been established.

In most cases the amounts of antineoplastic compounds in callus and cell suspension cultures are much lower than those in the source plants. For example, *Heliotropium indicum* cells that had been expected to produce indicine *N*-oxide failed to synthesize this antineoplastic compound at a detectable amount (Misawa *et al.*, 1983). There may be two explanations for this phenomenon. First, genetic heterogeneity and spontaneous selection for cells that grow best in a given medium may result in the reduction of cells that produce the metabolite but grow poorly in the medium. To counteract this, repeated selection for high production may be required. Deus-Neumann and Zenk (1984) demonstrated the necessity of repeated selection to maintain the production level of ajmalicine and serpentine in cultured *Catharanthus roseus* cells. Employing positive utilization of genetic heterogeneity in cultured cells, cell lines that produce larger amounts of secondary metabolites than do the intact plants have been obtained by cell cloning and selection of small cell aggregates in many plant species (Yamada, 1984). And if an appropriate regeneration system is available, breeding of medicinal plants that produce large amounts of antitumor compounds in another possibility for the utilization of this genetic fluctuation in cultured cells.

The second explanation of low production in cultured cells is the epigenetic supression of expression of genes that encode the key enzymes of the synthesis of the target compounds. Sometimes, synthesis of secondary metabolites is intrinsically associated with organogenesis. Misawa *et al.* (1985) showed that the level of cephalotaxine and its esters in plantlets regenerated from suspension cultures of *Cephalotaxus harringtonia* was more than 60 times higher than that of the original cell suspension culture (Table V). In *Catharanthus roseus,* shoot cultures regenerated from callus produce vindoline, a key intermediate in synthesis of antitumor alkaloids, vinblastine and vincristine, whereas the original callus failed to produce this alkaloid (Constabel *et al.*, 1982). To break through this genetic barrier, mutagenesis using chemical and physical mutagens or random insertion of Ti plasmid into the plant genome to destroy the regulatory gene of the key enzyme could be employed. In such mutagenesis study, a highly sensitive and efficient assay system, such as RIA and tumor cell bioassay, is essential to analyze large numbers of mutagenized cell populations. An alternative possibility to overcome this genetic barrier is the use of organ cultures. Organ cultures such as shoot and root culture have not attracted much attention by researchers seeking industrial application of plant tissue culture because of the difficulty of large-scale culture of these organized systems and their slow growth. However, rapidly growing organ cultures that produce large amounts of secondary metabolites have been reported. Endo and Yamada (1985) described root cultures of *Duboisia leichhardtii* that produce substantial amounts of scopolamine and hyoscyamine. Hagimori *et al.* (1984) have succeeded in jar fermenter culture of *Digitalis purpurea* shoots that grew rapidly and produced a high level of digitoxin. Finally, culture of "hairy roots" of medicinal plants (Shimomura *et al.*, 1986) promises to be a novel method for the exploitation of *in vitro* systems for the production of phytochemicals—among them, antitumor agents.

Table V

Levels of Cephalotaxine and Its Esters in *Cephalotaxus harringtonia*

Source	Concentration (μg per kilogram dry weight)
Suspension-cultured cells	0.55
Plantlet grown *in vitro*	36.0
Plants grown in the field	180.0

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CHAPTER 33

Flavors

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I. INTRODUCTION

The production of flavors is vital to the manufacturing component of the food industry. No packeted, powdered, tinned, or processed food, sweet, biscuit, or drink, is without its added flavor or flavor enhancer. Many flavors are present as a complex mixture of compounds in an essential oil, for example, cardamon, cloves, marjoram, sage, citrus, and peppermint, or an essential oil containing nonvolatile lipids, for example, dill, coriander, caraway, mace, nutmeg, pimento, and celery, or a pungent ingredient, for example, capsicum, ginger, and pepper, or coloring matter, for example, turmeric and paprika, or amino acid derivatives, for example, onion and garlic (Salzer, 1975). The beverage flavors

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of cocoa and coffee are also very complex mixtures but are not found as an essential oil, whereas the bittering agents used to flavor drinks are single compounds.

Most of the natural flavors are obtained from parts of the world where, for a number of reasons, yields and supply are often variable. It has been argued that such secondary products, or flavors, would be better produced under much more controlled conditions so as to ensure a more constant and uniform supply. However, the statement that only a few of the flavor compounds would justify the high initial cost involved in producing the flavors from large-scale tissue culture (Van Brunt, 1985) will be true for sometime. It is likely that the technical problems of large-scale plant cell culture will be overcome so that the feasibility of using tissue cultures as a source of flavor products will be determined by the yield of secondary products in the cells. One of the major limitations with any attempts to increase the yields, which in most cases are well below those of the parent plant, is that the factors controlling the activity of the secondary pathways are largely unknown and in many examples the path of synthesis has not been established. Because flavors form such a diverse group (terpenes, terpenoids, phthalides, amino acid derivatives, alkaloids, and polyphenols), it is impossible to identify a common approach for increasing yields.

In this chapter a number of species producing a flavor have been selected and are used to illustrate the past and current research on flavor production in tissue cultures. In some instances a fuller treatment is given for individual compounds in other chapters of this volume (*see* Alliums, Monoterpenes, Sesquiterpenes, Purine Alkaloids, Quinoline Alkaloids, Immobilization).

II. HERB FLAVORS

The flavors collectively referred to as herbs form a very large group. Normally, the flavor is derived from an essential oil that is synthesized or accumulated in specialized cells. The amount of attention given to the production of these flavors in tissue cultures has been determined by economic considerations, such as the size of the market and the cost of the product (Collin and Watts, 1983). Thus there has been most investigation of the flavors of chamomile, aniseed, peppermint, and celery.

A. Chamomile

Matricaria chamomilla is an herb that is used as dried leaves to make a refreshing beverage as well as providing an extract with known pharmacological properties. The tea is thought to be a carminative, a tonic, and a sedative. The active component is an essential oil that is made up of the following sesquiterpenes: chamazulene, farnesenes, cadinene, cubibene, calamene, murolene, chamavioline, spathulenol, bisabolols, bisabololoxides, caryophyllenes, caryophylleneoxide, and chamomillol, and the polyines *cis/trans*–EN-IN dicycloether and chamomillaesters. In view of its wide usage there has been interest in the possiblity of producing biomass and essential oil from tissue culture. Although Becker (1970) first referred to tissue cultures of *M. chamomilla*, more extensive studies were carried out by Szöke and coworkers (1977). Callus cultures were isolated from all parts of the plant, including the inflorescence, on a Murashige and Skoog medium in the presence of 1 mg liter^{+1} kinetin and 1 mg liter^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Szöke *et al.*, 1977). Szöke *et al.* (1978a,b) attempted to stimulate the essential oil component of undifferentiated callus tissue by modifying the nutrient and cultural conditions. Newly initiated callus was compact and light green. During successive subcultures it became more friable. It showed only occasional tracheid formation and no obvious oil cells. The callus contained large amounts of oil initially (0.12% dry weight compared with 0.3 to 0.6% dry weight in the inflorescence), but this level declined on subculture. Essential oil from the callus contained the same components as in the whole plant, that is, the sesquiterpenes, farnesenes, bisaboloxide, and bisabolols. There were also unidentified sesquiterpenes not found in the plant. The amount and composition of the oil was responsive to the ratio of 2,4-D and kinetin. High kinetin caused a decrease in the concentration of oil in the callus to 0.03% dry weight but an increase in the proportion of sesquiterpenes, whereas high auxin tended to decrease the proportion of sesquiterpenes. Thus auxin favored the synthesis of more simple structures in the oil. These changes were not associated with changes in growth or differentiation, so the phytohormones were having a direct effect on synthesis of the oil. The effect of light was similar to auxin because it stimulated synthesis of farnesenes, bisabolol, spathulenol, and bisabololoxide, whereas dark caused a very large increase in spathulenol and a decline in farnesenes and bisabolol. Cell suspension cultures of *M. chamomilla* were established by Szöke *et al.* (1979) and the amount and composition of the oil

compared with more differentiated callus and excised root cultures. The root cultures contained 0.5% dry weight, comparable to the intact plant, but the callus only contained 0.14% dry weight, and the cell suspension, 0.06% dry weight. Besides producing less oil than the callus, the cell suspension also showed reuced farnesene and bisabolol levels.

The production of essential oil in tissue cultures has generally been correlated with the presence of oil cells in the undifferentiated tissue, or in the differentiated leaves, stems, or roots. However, the early work of Szöke *et al.* (1978b) showed quite clearly that oil accumulation could occur in undifferentiated nongreen tissue. Reichling *et al.* (1983) examined the relationship between differentiation and oil production. Using callus that was intensely green, compact, slow growing, but morphologically undifferentiated, they were able to detect yellow-orange oil cells in the callus comparable to the parent plant. The contents of separated oil cells and callus without oil cells were compared. It was found that the accumulation of essential oil in intact callus was due to that present in the oil cells. The proportion of oil in the callus was very high (0.2% dry weight) and comparable to the inflorescence. The oil only accumulated during the first 5 days of a subculture when little growth occurred, and the accumulation declined with successive subculture. Because the callus was differentiated in the sense that it was slow growing, green, and possessed specialized oil cells, there was still a relationship between oil production and differentiation. Besides accumulating a large proportion of oil, this callus also synthesized a more complex mixture than that found by Szöke *et al.* (1978b). Thus the oil contained farnesenes, chamomillol, caryophyllenoxide, spathulenol, *cis*–EN-IN dicycloether, and chamomillaesters. This composition was closest to the oil derived from the intact root.

Because significant yield of oil from tissue cultures is dependent on the presence of differentiated oil cells, Bisson *et al.* (1983) attempted to replace the natural sites of accumulation within tissue with synthetic sites. The purpose of these sites was to absorb lipophilic or streamvolatile substances and encourage further synthesis within the cell. The sites were created by adding a neutral oil, (Miglycol 812), which consists of C_8C_{10} fatty acids from coconut oil, to the cell suspension. The oil, which has a low viscosity, is stable to autoclaving and is nontoxic. In liquid medium it breaks up into fine droplets that attach themselves to the cells without having any effect on growth. Lipophilic substances are absorbed by the Miglycol, which at the end of the subculture can be separated from the cells and media and extracted by organic solvents. Habituated and crown gall cultures of *Matricaria chamomilla* grown in the

presence of Miglycol accumulated a wide variety of steam volatiles. In comparison, the cell suspension cultures without Miglycol synthesized only trace amounts of these compounds. This is a very valuable technique that may overcome the problems of stimulating essential oil production in tissue cultures.

B. Aniseed

Aniseed is an essential oil extracted from *Pimpinella anisum*. The oil is used as a flavoring agent in confectionery, spirits, and liqueurs. The active components are a mixture of phenylpropenoids, monoterpenes, and sesquiterpenes [anethol, pseudoisoeugenol-(2-methylbutyrate), epoxipseudoisoeugenol 2-methylbutyrate, β-bisabolene, myristicin, and methylchavicol]. Tissue cultures have been isolated from *P. anisum* by Becker (1970) on a Murashige and Skoog medium (1962) containing 0.1 mg liter^{-1} kinetin and 1 mg liter^{-1} auxin [2,4D,indolole-3-acetic acid (IAA), or 1-naphthaleneacetic acid (NAA)]. However, examination of the undifferentiated callus showed none of the essential oil components. Limited differentiation into shoots, roots, or leaves initiated the production of the essential oil with the same compounds as the intact plant but in different relative amounts. The age of the culture had no effect on the composition because tissue that had been in culture for 3 years showed the same composition as newly initiated cultures. An interesting aspect of oil production was that root- and leaf-forming cultures synthesized an oil of similar composition, whereas the oil from the leaves and roots of the plant differed widely. In the intact plant the level of anethol in the essential oil usually increases with age. Only trace levels of anethol were detected in the differentiated leaf-forming tissue culture, and even after the eighth week of a subculture no increase in anethol could be detected. Although differentiation in tissue cultures does act as a stimulus to oil production, not all the secondary pathways appear to be active as in the intact plant. The stimulatory effect of differentiation on oil production in other herb species (*Foeniculum vulgare, Levisticum officinale, Origanum vulgare, Salvia officinalis, Mentha piperita, Mentha pulegium,* and *Rosmarinus officinalis*) was noted.

Using habituated cultures on a Murashige and Skoog medium without added kinetin or auxin, Reichling *et al.* (1985) found that the undifferentiated callus and cell suspension synthesized an essential oil. The major components in both callus and cell suspension cultures were epoxipseudoisoeugenol 2-methylbutyrate, β-bisabolene, and pseudoiso-

eugenol 2-methylbutyrate, and additionally, anethol and myristicin in the callus. The production of the last three compounds was very variable. The synthesis of the oil occurred during the first half of the culture period of both callus and cell suspension, before any significant accumulation of fresh weight had occurred. Examination of the culture showed no differentiation except for the presence of limited tracheid formation and occasional leaf development. No oil-forming cells were present. The accumulation of oil in the callus was 3–4 mg per 100 g fresh weight, and in the suspension culture, 1.0–2.0 mg per 100 g fresh weight. The use of a two-layered system was also investigated in the cell suspension, using the triglyceride Miglycol in an effort to concentrate the essential oil components. The two major compounds, epoxipseudoisoeugenol and β-bisabolene, were accumulated in the triglyceride layer. The use of a lipophilic phase may have many advantages for accumulating other essential oils produced by cell suspensions (Bisson *et al.*, 1983).

C. Peppermint

Peppermint is a widely used flavor in the confectionery industry, in which it is added as an oil. The essential oil is found in special glands, cells, and hairs of the shoot of *Mentha* species, particularly *M. piperita*. The active components of this oil are menthol and menthane, but the oil also contains isomenthol, isomenthane, menthyl acetate, pulegone, and piperitone, as well as terpenes, such as limonene. Tissue cultures were initiated by Lin and Staba (1961) and Becker (1970), but no essential oil could be detected in the undifferentiated cultures. This early work led to the view that synthesis of peppermint oil could only take place in tissue cultures when some form of cell or organ differentiation had occurred. The situation was not clear because Bricourt and Paupardin (1975) found that undifferentiated cultures of *M. piperita* on a Murashige and Skoog medium supplemented with 3% glucose and 0.1 mg liter^{-1} benzyladenine produced an essential oil. In the oil from the intact plant the major components were menthone (0.02% fresh weight) and menthol (0.03% fresh weight), and trace levels of the precursor, pulegone, whereas in the callus there were only trace levels of menthone and menthol, and increased levels of pulegone (0.03% fresh weight). Menthofurane was also found in trace quantities in the plant, but it had accumulated in the callus (0.01% fresh weight). It was suggested that the normal path of synthesis from pulegone to menthone and menthol was blocked and this led to an accumulation of pulegone, part of which was diverted to

synthesis of menthofurane. A more detailed examination of the structure of the undifferentiated callus was made by Kireeva *et al.* (1978). They showed that the callus consisted of normal and giant-size parenchyma-like cells. In newly initiated callus the giant cells constituted 45–50% of the cultures, but this increased to 80% after a number of subcultures. The significance of the giant cells is not clear except that a proportion (6%) of these cells did contain oil droplets. The oil was not synthesized in special oil-producing cells, but the giant cells may represent a more differentiated state. Oil levels were 1.8% dry weight of callus, with an equivalent concentration in the medium. If the oil released into the medium is taken into account, the concentration of oil produced by the callus cultures was comparable to that in the intact plant. However, the composition of the oil from each source differed because the proportions of menthol (49%) and menthone (27%) were higher in the plant than in the callus. The highest levels of essential oil in the callus was at the end of the subculture period when of the total oil content, menthol constituted 8% and menthone 5%, whereas the proportion of pulegone had increased from 6% in the plant oil to 15% in the callus oil. Some synthesis of menthol and menthone had occurred in the undifferentiated callus, but the pattern was essentially the same as that found by Bricourt and Paupardin (1975).

The more recent approach to optimizing the yield of menthol and menthone has been to examine the potential for biotransformation of the precursor, pulegone, to menthone (Aviv and Galun, 1978). Cell lines, or chemotypes, show variation in their ability to biotransform pulegone to menthol and menthone so that it is possible to select lines with specific biotransforming ability. In combination with the technique of cell immobilization (Galun *et al.*, 1983), this approach has great potential for synthesis of these important flavor compounds.

D. Celery

Celery (*Apium graveolens* has a long history of cultivation and use as a medicinal herb and as a vegetable. According to folklore remedies, the celery seed oil, conserve of the blanched stalks and decoction of tea, is able to cure rheumatism, gout, bronchitis, asthma, flatulence, and colic. It is a diuretic, abortifacient, antiseptic, deobstruent, antiinflammatory, cardiac tonic, sedative, and aphrodisiac. The main present-day use of celery, however, is in the food industry, where it is added as a seed oil or oleoresin. Celery oil can also be distilled from the vegetative structures, but the concentration is very low. The major constituents of the oil

are the terpenes, for example, selinene, pinene, myrcene, terpinene, elemene, tumelene, cymene, limonene, caryophyllene, of which limonene is the largest component. Although terpenes constitute more than 80% of the essential oil, only selinene has any definite role in celery flavor. The chief flavor constituents of the oil are C_{12} lactones, or phthalides, and of these, sedanoic anhydride, sedanolide, 3-isobutylidene-3*a*, 4-dihydrophthalide, 3-isovalidene-3*a*, 3-butyl phthalide, 3-isobutylidene phthalide, and 3-isovalidene are the most important (Wilson, 1965). Mitsuhashi and Nomura (1966) tested the possibility that the phthalides are formed from head-to-tail linkages of acetate or malonate units by feeding labeled acetate to *Ligusticum acubilobum*. The resulting alternate-carbon labeling pattern found in 3-butyl phthalide confirmed this mechanism of synthesis.

Although commercial preparations of the oil are mostly derived from the seed, the efficiency of extraction from the herb tissue has been improved, so that now, vegetative tissue can be regarded as a suitable source of oil. If a rapid, large-scale production method for growth of tissue culture were available, prospects for herb oil could be further improved.

Tissue cultures of celery were initiated by Williams and Collin (1976) on a Murashige and Skoog medium (1962) supplemented with 0.5 mg $liter^{-1}$ 2,4-D and 0.6 mg $liter^{-1}$ kinetin. The cultures could be regenerated via embryogenesis by omitting the growth regulators from the medium. Analysis of a long-term undifferentiated cell suspension at the stationary phase showed that all components of the essential oil were absent (Al-Abta *et al.*, 1979). A differentiated suspension, however, containing predominantly torpedo embryos, and differentiated callus possessing shoots and roots contained terpenes and phthalides. It was apparent that even the early stage of differentiation as embryos was sufficient to stimulate secondary product formation. Sections of the torpedo embryos showed tissue differentiation but no highly specialized oil ducts (Al-Abta and Collin, 1978). Thus oil formation in celery tissue cultures was not dependent on the presence of specialized oil cells or channels.

Where the nutrient medium of a cell suspension culture was modified by replacing 2,4-D with 3,5-dichlorophenoxyacetic acid (3,5-D) there was an initial stimulus to phthalide production (3-butyl phthalide and sedanenolide) during the stationary phase of the first subculture (Watts *et al.*, 1985). In fact, the levels of sedan enolide in the medium were comparable to the level in the plant. After this stage in the subculture the phthalides were absent or present only in trace amounts. Conversely, limonene was only found in trace amounts in the first subculture but

increased in the exponential phase of the second and third subcultures to reach levels in the medium comparable with that in the plant. The cultures in 3,5-D were not comparable with those in 2,4-D because the cells had become aggregated and green although there was no indication of tissue differentiation or embryo formation. In the intact plant, greening of the petioles favors limonene accumulation but has no effect on phthalide accumulation, which is comparable with the effect of greening in cell cultures (Watts *et al.*, 1985). The effect of aggregation was examined in the absence of greening (Watts *et al.*, 1984). Aggregates in a 2,4-D medium contained limonene, 3-butyl phthalide, and sedan enolide comparable to the composition of aggregated green cultures in the 3,5-D medium. The effect of 3,5-D on stimulating phthalide synthesis was likely to be through changes in the pattern of cell differentiation in the culture, because greening and aggregation are an early stage in the differentiation process.

In an effort to stimulate flavor formation in an undifferentiated culture, the cell suspension was exposed to a period of stress (Watts *et al.*, 1984). The newly inoculated cell suspension was maintained by 4°C for 5 days then returned to 25°C for 10 days. Limonene accumulation in the medium was significant and as high as in the plant, but there were only traces of 3-butyl phthalide. It was suggested that the effect of a period of low temperature was to stress the cells and cause a diversion of substrate from the primary to the secondary pathways, although only limonene synthesis appeared to be stimulated by this treatment. Attempts to provide additional precursor as acetate to stimulate phthalide accumulation were unsuccessful.

An alternative approach was to attempt to increase the genetic variability of the cultures and then to select for high-yielding clones. Callus initiated from 1500 seeding petiole explants was transferred to liquid medium and screened for celery aroma (M. J. Watts and H. A. Collin, personal communication). A variety of morphologically different clones were established and maintained. As might be expected, the maximum number of clones possessing an aroma were those showing some form of differentiation. Interestingly, recently initiated undifferentiated cell suspensions also showed an aroma, but the proportion of cell suspension with aroma declined on subculture. An analysis of a number of cell suspensions showed a wide variation in terpenes (limonene, selinene, and pinene) and phthalides (methyl phthalide, 3-butyl phthalide, and sedan enolide) in the cells and media. However, after 12 subcultures even a selected high-yielding clone showed a progressive decline in the flavor compounds. Within a subculture, limonene was accumulated during the period of exponential growth, then declined, whereas methyl

phthalide accumulated during the stationary phase. The phthalides appear to be accumulated in the cell cultures when growth is reduced. Methyl phthalide is not found in the intact plant, so it may be either a precursor or a breakdown product of the phthalides. Although flavor precursors were found in undifferentiated cell suspensions, the ability to accumulate these compounds was short lived. After only 12 subcultures, no flavor compounds could be detected in the original high-yielding lines.

It appears that a more stable and biosynthetically active system is provided by slow-growing, aggregated, greening, or partially differentiated cultures. Although the biomass is less, yields appear to be higher than in undifferentiated cell suspensions. Immobilization provides a method for maintaining partially differentiated cultures for long periods. Celery cell cultures have been immobilized in calcium alginate gel beads in order to compare cells in a 2,4-D medium with those in a 3,5-D medium, which stimulates partial differentiation (Watts and Collin, 1985). Immobilized celery cells remained viable in both 2,4-D and 3,5-D, as shown by the increased uptake of nutrients, respiration rate, and dry weight. Although no analyses were performed on the cells or medium, immobilization of cells may provide a valuable system for stimulating and maintaining the production of celery flavor.

III. PUNGENT FLAVORS

The pungent flavors are well-known food additives and flavor enhancers. Examples are the spices, such as ginger, pepper, and nutmeg and the vegetables capsicum, or chillies, and onion. Most work on flavor production in tissue culture has been on capsicums and onion (see Chapter 28, this volume).

Capsaicin

The fruits of *Capsicum annuum* and *C. frutescens* contain an essential oil with a pungent flavor. The fruits, or chillies, are valued mainly as a flavor additive but do have pharmacological properties as a general stimulant and decongestant. The active component of the oil is capsaicin, which is synthesized from the combination of two precursors, vanillylamine (derived from phenylalanine) and isocapric acid (derived from valine). In view of its wide culinary use and the fact that the flavor

source is a single compound, there has been detailed investigation of capsaicin production in tissue culture of *C. frutescens* by Yeoman and coworkers (1980). The callus and cell suspensions were maintained on a medium of Schenk and Hildebrandt (1972) containing 2,4-D (0.5 mg $liter^{-1}$), *para*-chlorophenoxyacetic acid (2.0 mg $liter^{-1}$) and kinetin (0.1 mg $liter^{-1}$). Analysis of the undifferentiated cell suspension cultures showed trace quantities of capsaicin (Yeoman *et al.*, 1980). It was suggested that the yield of capsaicin was determined by the growth rate and degree of cellular organization and differentiation in the tissue. The difference between fast- and slow-growing cultures is that in the fast-growing cell mass, the primary pathways are most active and utilize all common precursors to the almost total exclusion of the secondary pathways so that few secondary products are synthesized. In slow-growing, compact callus cultures, or redifferentiated cultures, the primary pathways are less active, hence there is a supply of common precursors available for secondary product synthesis (Yeoman *et al.*, 1980, 1982). In recognition of this control on capsaicin production, two approaches to stimulate production have been considered. One approach has been to provide precursors to rapidly growing cell and callus cultures. The other approach has been to alter the growth rate of the cultures to reduce the internal demand of the primary pathways on the supply of precursors. Thus addition of the precursors, vanillylamine or isocapric acid, increased the yield of capsaicin (Yeoman *et al.*, 1980). Equally, where the growth of the culture has been restricted by the use of the protein-synthesis inhibitor cyclohexamide, sucrose and nitrate limitation in the media (Yeoman *et al.*, 1982), or immobilization of the cells in polyurethane foam (Lindsey and Yeoman, 1984), the uptake of radioactive phenylalanine or valine into capsaicin, or the accumulation of capsaicin, has been increased. In all these instances growth of the culture has been reduced, so that more precursors are made available for synthesis of capsaicin. One particularly fruitful approach has been cell immobilization. Here the cells are allowed to aggregate and undergo limited growth in a protected environment. Significant increases in yield of capsaicin have been obtained. This approach could well be applied to stimulating the yield of other flavor compounds.

IV. BEVERAGE FLAVORS

The two major beverage flavors are coffee and cocoa. Besides providing a refreshing beverage, both are used to flavor convenience foods,

and additionally, cocoa is used to make chocolate confectionery. Coffee has had considerable investigation as a tissue culture, but the work on secondary product formation has concentrated on the production of caffeine (see Chapter 23, this volume). Cocoa tissue cultures have been investigated for a wide range of flavor precursors and are considered in more detail.

Cocoa

The flavor of cocoa is obtained from the cocoa bean, which is removed from the fresh, ripe pods, allowed to ferment, then roasted. Flavor precursors in the cocoa bean have been divided into three major categories: the carbohydrates (fructose, glucose, sucrose, and two unidentified sugars), the flavonoids (epicatechin, catechin, gallocatechin and epigallocatechin, leucoanthocyanins, quercitin, and quercetrin), the phenolic acids (*para*-coumaric, caffeic, and chlorogenic), and free amino acids (Rohan, 1969). The bitter and mildly addictive component of cocoa is provided by the purine alkaloids, caffeine, theophylline, and theobromine. Although all these flavor precursors are soluble compounds, a major insoluble component of the bean, cocoa butter, is an important component of cocoa flavor as well as being a secondary compound in its own right. Cocoa butter is a storage lipid to distinguish it from other cell lipids, such as the membrane phospholipids. It consists of triglycerides that contain palmitic (16.0), stearic (18.0), and oleic acids (1O:1), making *cis*-palmito-oleostearin the predominant triglyceride.

During fermentation of the cocoa beans there is breakdown of protein, starch, and sucrose. Some loss of amino acids and sugars occurs during roasting, but this stage also causes recombination of amino acids, sugars, and polyphenols to give the volatile cocoa aroma. After roasting, the cocoa bean is ground, and most of the cocoa butter is extracted by pressing. The remaining powder provides the basis for cocoa flavor of commerce. Production of a desirable chocolate flavor at this stage is dependent on the interaction between a large number of compounds rather than one single compound.

Tissue cultures of cocoa were initiated primarily to investigate the potential for micropropagation. Archibald (1954) was the first to establish callus cultures, then Hall and Collin (1975) initiated both callus and suspension cultures from different parts of the seedling. Since then, Jalal and Collin (1979) and Tsai and Kinsella (1981) have described synthetic media for maintaining cocoa callus and cell suspension cultures. The most successful medium consists of Murashige and Skoog medium sup-

plemented with 0.5 mg liter^{-1} 2,4-D and 0.1 mg liter^{-1} kinetin (Tsai and Kinsella, 1981). In an examination for flavor precursor compounds, Jalal and Collin (1977) found that polyphenol composition of callus was much more limited in comparison with explant tissue from the seedling plant. Explants from cotyledons, stems, and roots showed a wide range of polyphenols, whereas the callus isolated from these different sources showed fewer polyphenols, but all had the same composition. In a comparison of the callus tissue and explants, only the polyphenols, leucoanthocyanidins, and epicatechin were common to both callus and parent tissue. The other polyphenols present in the callus could not be detected in the plant. When the compositions of polyphenols from callus and suspension culture were compared, the suspension culture contained fewer polyphenols than the callus. A restricted polyphenol composition was also shown by tissue cultures of tea compared with the intact plant (Forrest, 1969).

A group of compounds that also contribute to cocoa flavor are the purine alkaloids, theobromine, caffeine, and theophylline. Analysis of the cocoa callus and cell suspension cultures showed that no purine alkaloids could be detected (Jalal and Collin, 1979). This is in contrast with the cultures of coffee (Frischknecht *et al.*, 1977) and tea (Ogutuga and Northcote, 1970), in which caffeine and theobromine accumulated in the cells and media. In cocoa cultures, however, when precursors of the purines, 7-methylxanthosine and methionine, were included in the medium, theobromine was synthesized by the callus, indicating that part of the purine biosynthetic pathway was active (Jalal and Collin, 1979).

Another component of cocoa flavor is cocoa butter, which is also added separately to chocolate confectionery. Analysis of cocoa cell suspensions showed that the lipids corresponded to membrane lipids rather than those of cocoa butter (Tsai and Kinsella, 1982). When cocoa tissue cultures were exposed to radiolabeled precursors of cocoa butter, that is, palmitic, oleic, linoleic acid, and stearic acids, there was ready incorporation into cellular lipids (Tsai and Kinsella, 1982). That there was no incorporation of precursors into cocoa butter suggests that the cocoa suspensions are not suitable for synthesis of cocoa butter.

Even though the production of flavor precursors was limited in the cocoa tissue cultures, when the cultures were maintained at a roasting temperature a cocoa aroma was produced. This response only occurred when the cocoa cell suspension was at the lag or stationary phase of growth and not during the rapidly growing exponential phase (Townsley, 1974). The presence of cocoa aroma in the tissue culture does provide an opportunity to select for more highly aromatic clones.

It has not been possible to redifferentiate cocoa tissue cultures in order to investigate the effect of differentiation on flavor production. Somatic embryos of cocoa can be isolated and cultured, however, which provides a differentiating system without an initial callus phase. Somatic embryos are initiated from the embryonic axis or cotyledons of zygotic embryos in the presence of auxin and coconut milk and develop in the same way as the zygotic embryos (Pence *et al.*, 1980). The somatic embryos, whether maintained on solid or in liquid medium, can be used for studying the regulation of morphological and biochemical development. One important aspect of biochemical change in the zygotic embryo is the change in fatty acid composition of the lipids. During development of the embryo, the fatty acid composition becomes more saturated as cocoa butter is synthesized, in addition to the normal membrane lipids. An increase in sucrose concentration in the medium of the somatic embryos causes a change in composition of the lipid synthesis toward synthesis of tryglycerides, which make up cocoa butter (Pence *et al.*, 1981). By modifying the nutrient media in this way, the somatic embryos can be induced to proceed through the same biochemical developmental pattern as the zygotic embryos. It was suggested that cultured somatic embryos may be useful for synthesis of cocoa flavor compounds. One way of overcoming the low yields of secondary products and instability of undifferentiated large-scale tissue cultures may be to use such partially differentiated systems.

V. CONCLUSIONS

Many of the commercial flavors are present as essential oils, which are accumulated in specialized cells, glands, channels, or specific tissues and organs of the plant. In undifferentiated, fast-growing cell suspension cultures no such structures can be found. In contrast, the structure of the callus favors more stable cell–cell associations, larger aggregates, low growth rates, greening, and also differentiation of oil cells. As a result the concentration of oil and other flavor compounds is always higher in callus than in cell suspensions.

Growing callus is not a commercial possibility, however, whereas large-scale culture of cell suspensions is. The problem with using cell suspensions for oil production, or for the production of specific flavor compounds, is that yields are low, variable, and usually decline with

repeated subculture. Alternative approaches are being examined to overcome these problems and to stimulate increased yields. These are

1. Cell immobilization. The culture is provided with an environment that leads to cell aggregation, a reduced growth rate, and even partial differentiation.
2. Two-phase aqueous and lipophilic system. The lipophilic phase in a cell suspension culture provides a site of accumulation for the oil, and indirectly stimulates oil synthesis in the undifferentiated cells.
3. Biotransformation of precursors. Selected cell culture chemotypes that can make specific biotransformations are used to biotransform cheap precursor compounds to give the desired product.

Besides applying these approaches to flavor production in tissue culture, it is also important that studies be made of the basic cell biology and biochemistry of the flavor-producing plant and its tissue culture. It is essential to have more information on the intermediates, enzymes, activity, and intracellular location of the secondary pathways. This information will provide the background for a more direct manipulation of the secondary pathways by the techniques of molecular biology.

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CHAPTER **34**

Phytohormones in Cell and Tissue Cultures

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I. INTRODUCTION

In most plant cell and tissue culture systems the nutrient medium is supplemented with growth regulators, mostly 2,4-dichlorophenoxy-acetic acid (2,4-D) as a synthetic auxin, or a cytokinin such as kinetin [or 6-benzyladenine (6BA)], or both. These substances are necessary to promote vigorous growth, mainly by cell division, or are required to initiate organogenesis. Sometimes, after a prior pulse, the withdrawal of one or the other of these substances is required to promote differentiation processes, for example, to induce embryogenesis in cultured somatic cells. Here, after prior culture in a 2,4-D–supplemented medium, cell material is transferred to an auxin-free medium to produce embryos (Nomura and Komamine, 1985; Ozeki and Komamine, 1985).

A vast literature is available on the influence of growth regulators (mostly synthetic auxins and cytokinins) on cultured cell material of a great variety of plant species, to control growth and differentiation. In these reports the reactions of cells were tested mostly empirically, how-

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ever, and only limited information is available on the metabolic fate of these exogenous growth regulators after uptake by the cells (cf., e.g., Giles *et al.*, 1986; Bender and Neumann, 1979; Laloue *et al.*, 1977; McGraw and Horgan, 1983; Horgan, 1987). Even less is known of endogenous phytohormones and the possible interactions of such an endogenous phytohormone system with the exogenous growth regulators supplied to the nutrient medium. It seems to be safe to hypothesize that the empirically observed responses of cell culture systems following a growth regulator supplement will be related to such interactions.

In many ways, cell culture systems resemble meristematic tissues of intact plants, such as a high cell division activity or a high potential for organogenesis, that is, to produce shoot, root, or flower primordia. In the intact plant such meristematic tissues are recognized as centers of hormonal synthesis and as sources for the endogenous hormonal system. Consequently, cultured cells also should be able to synthesize phytohormones or at least intermediates in metabolic pathways of hormone synthesis. This agrees with the isolation of some cell lines with no response to exogenous auxins or cytokinins, lines that were consequently described as autonomous (habituated) for these phytohormones (e.g., Gautheret, 1946, 1955; Kutacek *et al.*, 1981; Szabo *et al.*, 1981; Meins, 1982; Meins and Foster, 1985; Horgan, 1987). Such cultures as well as the crown gall system with a high capacity to synthesize endogenous hormones as well as genetic tumor cultures were recently reviewed by Horgan (1987) and these will not be considered in this article. Culture systems with an initial requirement for exogenous growth regulators also develop an endogenous hormonal system, however, and the purpose of this chapter is to outline some characteristics of this endogenous system. A more detailed discussion will focus on two systems from our laboratory, used as examples, that is, a carrot callus system derived from root explants and a highly embryogenic culture system derived from carrot petioles.

II. CALLUS CULTURES DERIVED FROM CARROT ROOT EXPLANTS

To induce high cell division activity in freshly isolated explants from carrot roots, with about a 100-fold increase in cell number within a 3-week culture period, the nutrient medium is supplemented with 2 ppm indoleacetic acid (IAA), 50 ppm inositol, and 0.1 ppm kinetin. A first peak in the number of cells passing through the various stages of the cell

cycle, indicating the initiation of cell division activity, is reached about 20 hr after explantation and transfer to the nutrient medium (Fig. 1). The exponential phase of cell division activity commences usually 3 or 4 days later, and after about 3 weeks the cultures pass into a stationary phase (Fig. 2). As can be seen from Fig. 1, the IAA of the medium is readily broken down by photooxidation, and after 3 days the cultures grow in a medium virtually free of auxins (Bender and Neumann, 1978; Bender *et al.*, 1982). In Table I, data are summarized on the duration required in an IAA-supplemented nutrient solution to initiate high cell division activity, as shown in Fig. 2. Here it is quite obvious that only a few days of contact with IAA is sufficient. Obviously, these cultures require either only a short pulse of an auxin to promote high cell division activity, or if a continuous requirement to sustain cell division exists, then the cultured cells should be able to synthesize the required substance or substances. On the basis of experiments with ^{14}C-labeled, though exogenous, IAA and the determination of endogenous IAA concentrations, a balance of contributions from endogenous and exogenous IAA sources was attempted for the initial 24 hr of the cultural cycle, which clearly indicates a considerable contribution of newly synthesized IAA (Table II).

Apparently, within the first few days following the initiation of cell division by the hormones of the nutrient medium, that is, the induction of a "meristematic" state in the explants, a metabolic pathway to synthesize IAA is established. The ability to synthesize IAA persists at least up to the twelfth day in culture (Bender and Neumann, 1978), that is, during the exponential phase of cell division. Data on later stages of the culture cycle are not available.

In Fig. 3, information on the production of ethylene by this callus system is summarized. The very high values of ethylene produced during the first hours of explant culture can be visualized as some kind of wounding effect induced by cutting the explants. Such effects have also been described, following the wounding of intact plants. After a rapid decrease of ethylene production during the following period, however, with a minimum at about the second day, a maximum is reached on the sixth day. Interestingly, here influences of the hormonal regime in the nutrient medium at t_0 seem to exert some influence on the capacity to produce ethylene, indicating a general tendency paralleling the growth intensity induced by the various combinations of the three growth regulators used. A strong influence of IAA on ethylene production can also be derived from the data in Fig. 3, which indicates an acceleration of synthesis following the application of the auxin during later stages of the cultures, with a rather low level of ethylene production before IAA application (Dougal, 1986).

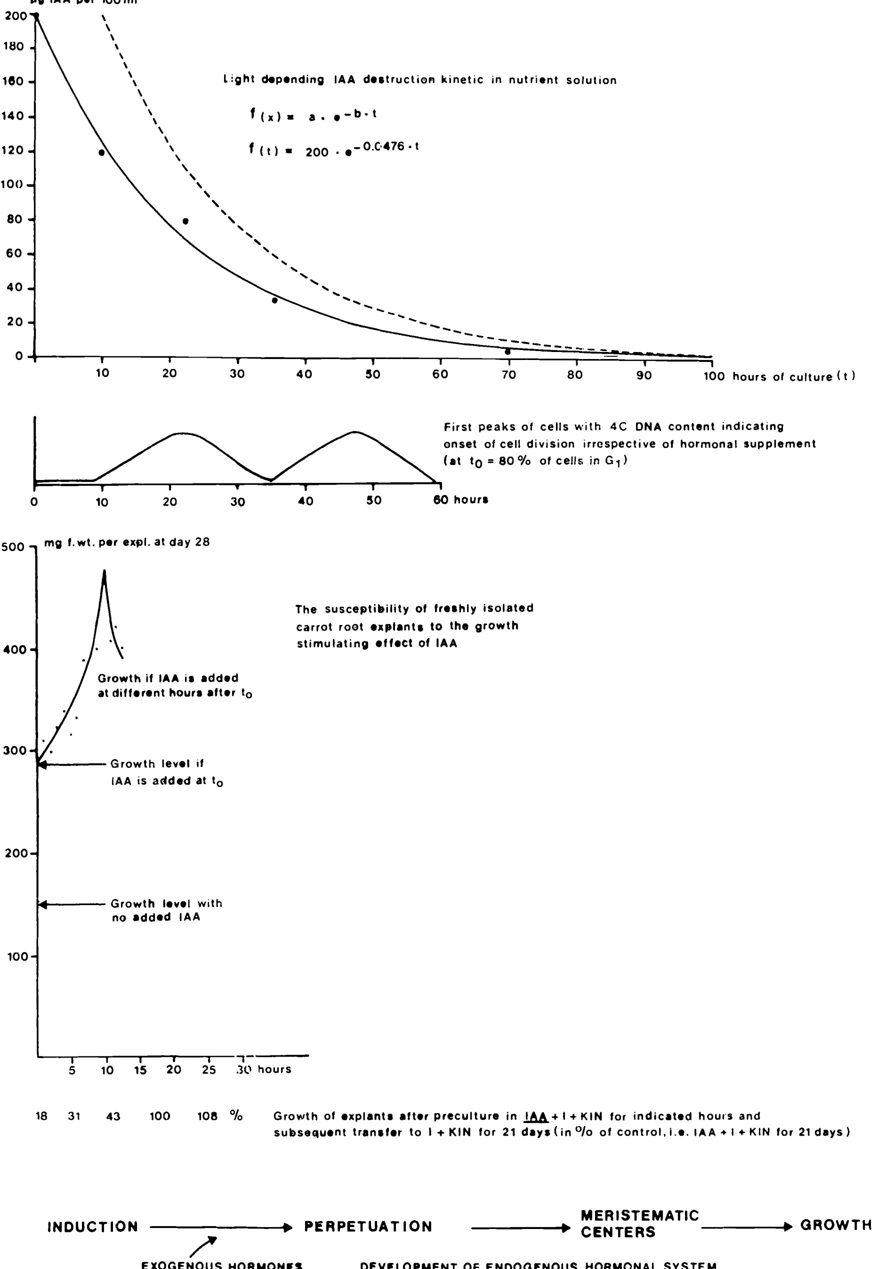


Fig. 1. Interaction of exogenous IAA concentration, onset of cell division and susceptibility to IAA of the cultured carrot tissue. The dashed line (upper graph) indicates the concentration kinetic of exogenous IAA if added 10 hr after t_0 at maximal susceptibility of the explants.

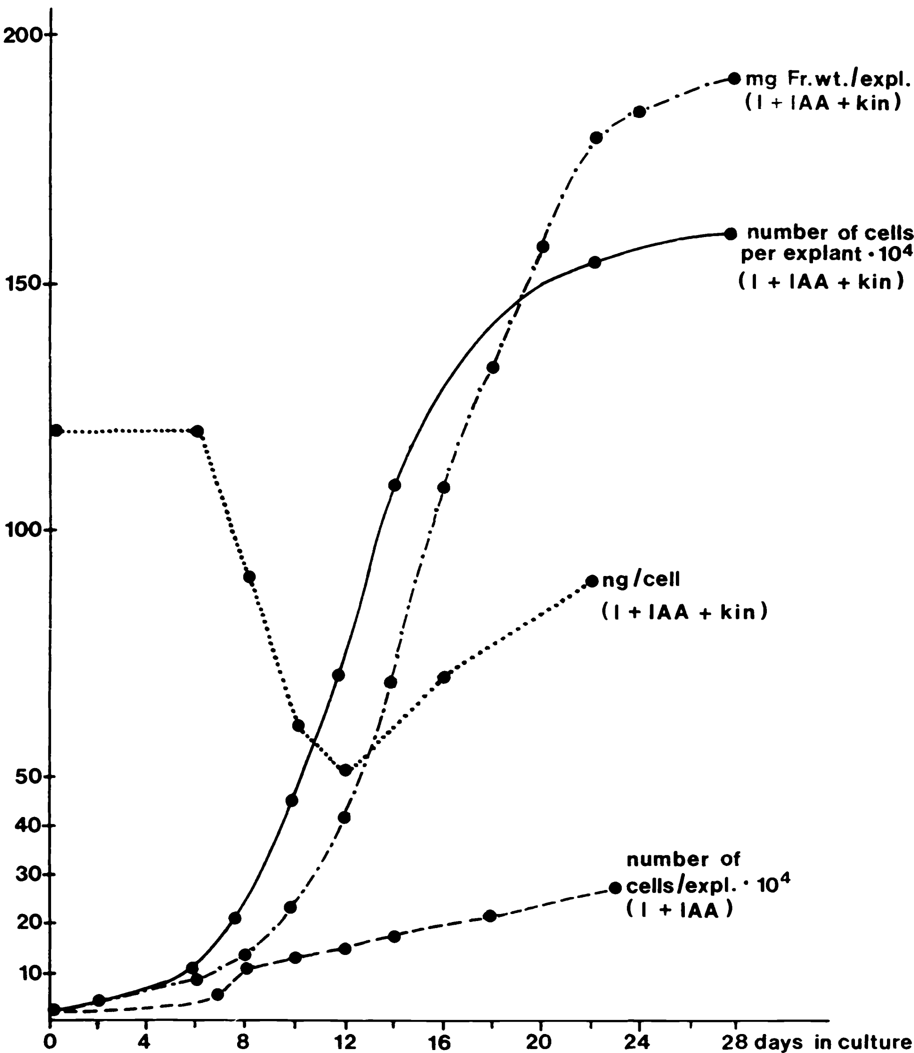


Fig. 2. Fresh weight, number of cells per explant and average weight of cells of cultured carrot root explants (secondary phloem) during a culture cycle of 28 days (kin, 0.1 ppm kinetin; I, 50 ppm *m*-inositol, 2 ppm IAA).

Apparently, carrots belong to the group of plant species that accumulate (2iP) and its riboside, and not zeatin, as the dominating native cytokinin. At least by gas liquid chromatography–mass spectrometry (GLC–MS), zeatin and its derivatives could not be detected (Stiebeling and Neumann, 1986; Einset, 1986a). Interestingly, in the shoot, 2iP and

Table I

Influence of Kinetin and Preculture Period of Various durations (6, 24, 144 hr) in an IAA-Containing Medium on the Fresh Weight and Number of Cells per Explant[a]

	Explants transferred from			
	IAA + I to I;		IAA + I + Kin to I + Kin;	
Time (hr) of preculture	Milligram per explant	Cells per explant ($\times 10^3$)	Milligram per explant	Cells per explant ($\times 10^3$)
6	19.9	133	73.4	1830
24	11.2	84	71.1	1830
144	12.1	105	138.3	3370
Control	12.9	101	118.8	3570

[a] Controls were cultured in the original nutrient solution throughout the experimental period. Harvest was at the fifteenth day of the cultural cycle; original explants, 4 mg fresh weight.
[b] I, 50 ppm *meta*-inositol; IAA, 2 ppm indoleacetic acid; Kin, 0.1 ppm kinetin.

Table II

Indoleacetic Acid Balance in Cultured Carrot Root Explants during the First 24 hr after Inoculation in Nutrient Solution[a]

	Micrograms per gram fresh weight
Content in original explants	0.1
Content in nutrient medium at t_0	700.0
	Micrograms per gram fresh weight per hour
Uptake	1.0
Synthesis total	2.5
Breakdown total	2.0
	Micrograms
Light mediated	1.4
Biogenic	0.6
	Micrograms per gram fresh weight per hour
Net synthesis (including efflux)	0.5
	Micrograms per gram fresh weight[b]
Total IAA passing through the tissue within 24 hr	84
Content in explants after 24 hr	1.4

[a] Plus 50 ppm *meta*-inositol, 2 ppm indoleacetic acid, 0.1 ppm kinetin.
[b] Uptake plus synthesis total.

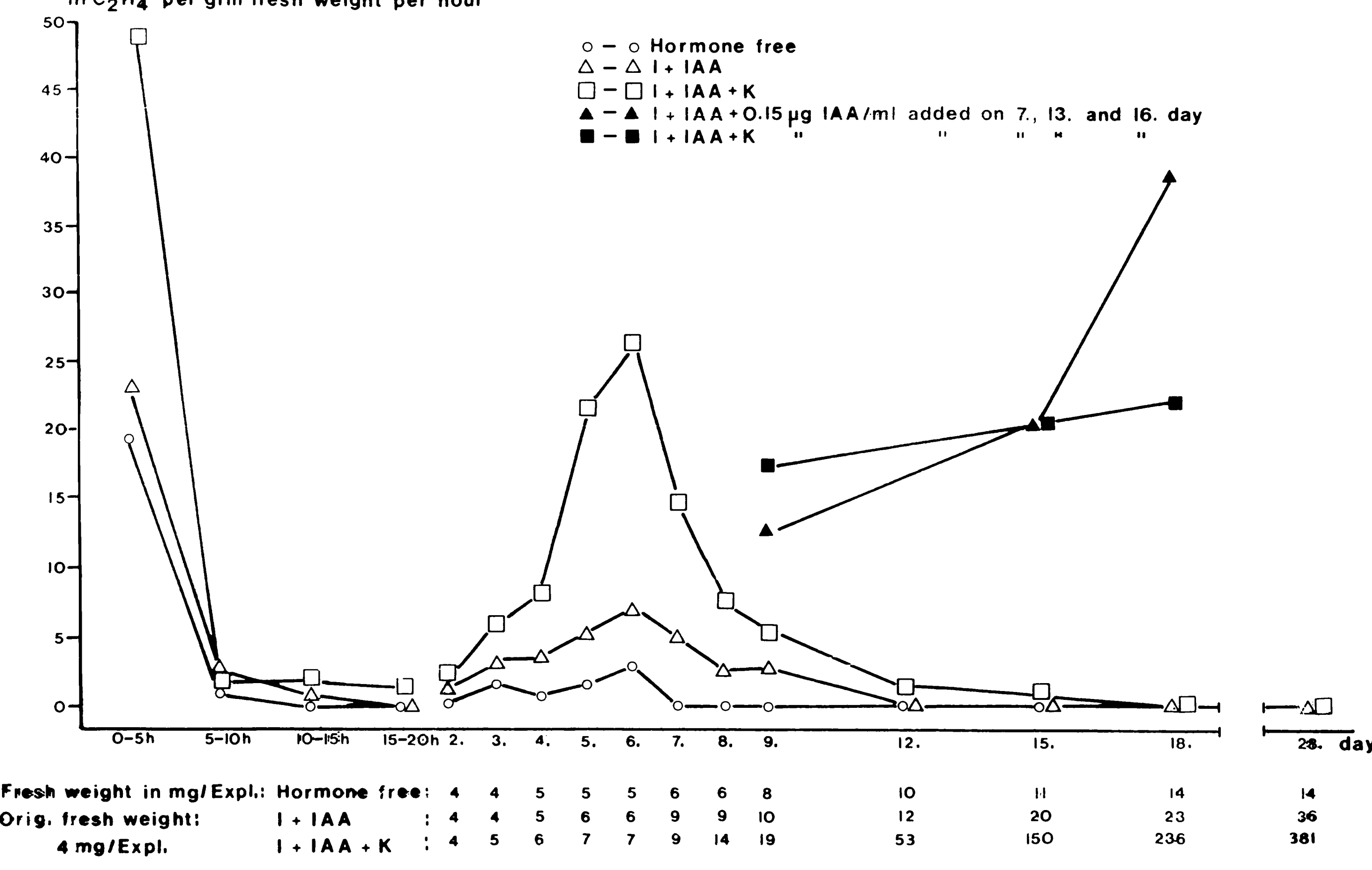


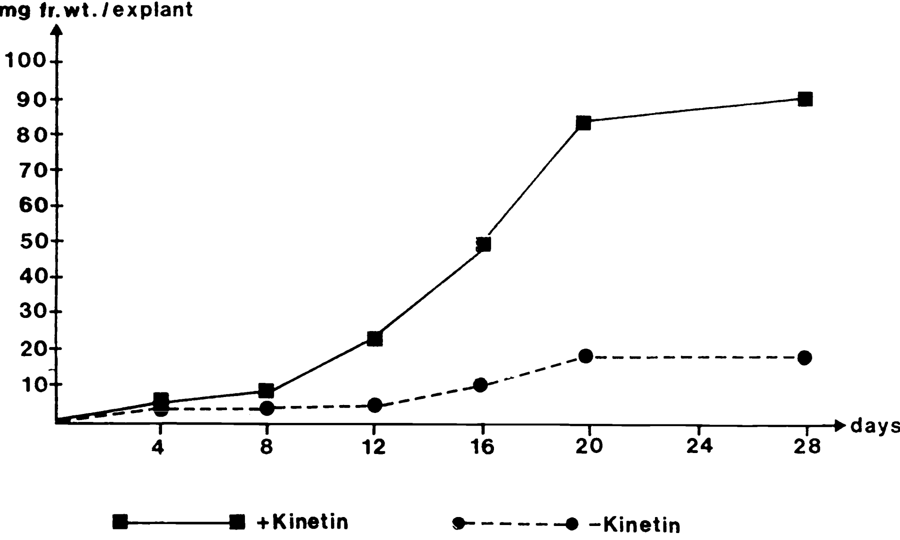
Fig. 3. Ethylene production and growth of cultured carrot root explants (secondary phloem) during a 28-day culture period with various hormonal treatments. In two experiments, 0.15 μg IAA/ml nutrient solution were added on the 7th, 13th, and 16th day, respectively, and ethylene production was determined 2 days later (fr.wt. of original explants: 4mg).

2iPA occur, whereas in the roots only the 2iP base was found. Also, in cultured root explants only 2iP occurs (Stiebeling *et al.*, 1986), indicating that in this aspect cultured explants may somehow preserve some characteristics of the original tissue from which the explants were derived. As reported elsewhere (Kumar *et al.*, 1984), the cells of these cultured explants contain well-developed chloroplasts capable of photosynthesis, and therefore, it seems to be unlikely that the existence of chloroplasts in shoot tissue can be related to the accumulation of the 2iP–riboside. In this callus system the response to 2iPA is even stronger than to the base (Stiebeling and Neumann, 1986), which, however, is in disagreement with other results (Laloue *et al.*, 1977).

The 2iP concentration in the explants indicates a peak during the exponential phase of cell division activity after about 12 days of culture (Fig. 4). This peak coincides with the ability of the cultures to grow in a cytokinin-free medium from this stage onward, up to the same fresh weight and number of cells per explant as those in a cytokinin-supplemented medium, till the end of the culture cycle 28 days after the beginning of the experiment (Bender and Neumann, 1978). If this is taken as an indication for a cytokinin-autonomous status of the cultured cells from this stage of the culture cycle onward, then the establishment of a cytokinin-synthesizing system seems to require a longer preculture than the initiation of the IAA- and ethylene-synthesizing mechanism.

For gibberellins only few data are available. Some rather weak gibberellin activity could be detected by a variety of bioassays for this group of phytohormones, and with a combination of gas liquid chromatography (GLC) and thin-layer chromatography (TLC) combined with bioassays, a component with gibberellin activity could be isolated. However, the latter remains chemically unidentified. By the same method an unidentified inhibitor of gibberellins was also detected in intact carrots in root tissue only, and a second substance with gibberellin activity in shoot tissue. Contrary to cytokinins (see above), in cultured cells all three substances occur. This is in agreement with ideas on the role of chloroplasts abundant in cultured root explants, in gibberellin metabolism (Palussek and Neumann, 1982; Palussek, 1982).

All the data discussed so far indicate the establishement of an endogenous hormonal system in cultured explants, and it should be of interest to follow the concentrations of these various groups of substances at the various stages of the growth cycle. Based on such data, the problem of interaction of the hormonal regime in the nutrient medium with the endogenous hormonal system of cultured cells, to bring about the growth responses and developmental pattern observed, could be inves-



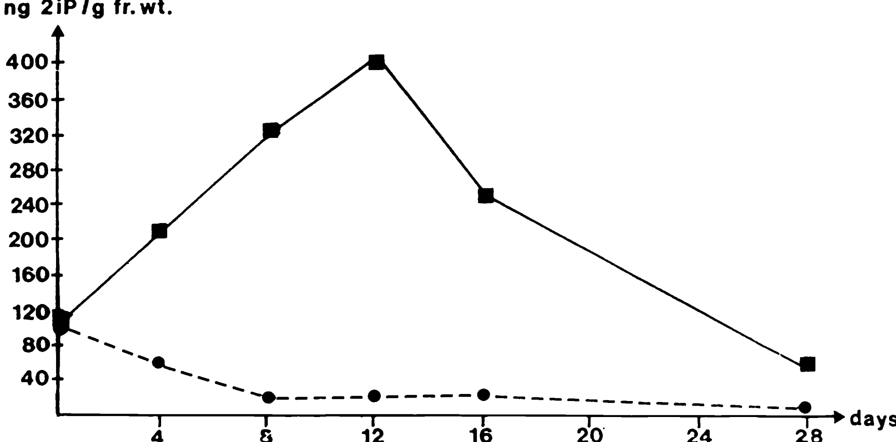


Fig. 4. Growth response and endogenous 2iP-concentrations of carrot tissue cultures under the influence of a kinetin treatment.

tigated in more detail. In intact carrot plants an autonomous circadian rhythm of cytokinin concentration was observed (Stiebeling and Neumann, 1986), and it remains to be seen to what extent such variations in endogenous concentrations of hormones also occur in cultured tissues and cells.

Table III

Developmental Stages of Embryogenesis in Petiole Explants and Concentrations of Endogenous IAA and 2iP[a]

Developmental process	Developmental stage	Nutrient medium	IAA concentration (ng/g fresh weight)	2iP concentration (ng/g fresh weight)
t_0 fresh petiole explants	Parenchymatic cells	High auxin, 2 ppm IAA	—	103.6
Adventitious root formation	Parenchymatic cells with meristematic regions		524	48.0
Induction phase	Embryogenic cells—tetraoidal stage	Medium auxin, 0.1 ppm 2,4-D	76	30.7
Transformation phase	Tetraoidal—globular stage	Low auxin, 0.01 ppm 2,4-D	145	26.4
Embryo development phase	Heart-shaped stage—torpedo-shaped stage	No hormone supplement	—	39.6

[a] IAA, indoleacetic acid; 2iP, N^6 (Δ^2-isopentyl)adenin; 2,4-D, 2,4-dichlorophenoxyacetic acid.

III. CULTURED PETIOLE EXPLANTS OF CARROTS

Cultured petiole explants obtained from carrot seedlings in an IAA- and inositol-supplemented medium at the beginning of the experiment will produce embryos on transfer to a medium containing a low concentration of 2,4-D (0.01 ppm) that is otherwise free of additional phytohormones (Li *et al.*, 1984; Li and Neumann, 1985; Schaefer *et al.*, 1985). The embryoids originate from subepidermal cells, which are originally vacuolated, and the first indications of a transformation into the embryogenic state is vigorous growth of cytoplasm. By microscopic observation, several characteristic stages of embryo development can then be discerned at which the concentration of IAA and 2iP have been determined (Table III). The data were obtained by analysis of entire explants,

and these certainly will not represent the concentration of the embryogenic cells, which may be considerably different. However, the results in Table III clearly show differences in the concentration of IAA and 2iP at various stages of the culture cycle of embryogenic explants. Interestingly, the concentration and the ratio of these two phytohormones, obtained from a highly embryogenic carrot variety ('Rotin'), differ considerably from data derived during the same period after the beginning of the experiment from cultured petiole explants of a variety that hardly produces any embryos (Lobbericher, Li and Neumann, 1985). Apparently, therefore, genetic influences also have to be considered in evaluating the endogenous hormonal system of cultured cells. (See also genetic tumors).

IV. CONCLUSIONS

The tissue culture systems described above demonstrate that cultured cells are able to develop an autonomous endogenous hormonal system. Further, the concentrations of representatives of the various groups of phytohormones change during the culture cycle in a rather characteristic pattern at various stages, associated with characteristic cytological events, for example, cell division activity in root explants or embryo development in petiole explants. Such changes in the endogenous concentrations have also been reported for other systems (e.g., Rajasekaran *et al.*, 1987a,b; Einset, 1986b). This conclusion, however, is in disagreement with results published by Barz and Hüsemann (1982) for established cultures of *Chenopodium rubrum*. In this system no correlations of the phytohormone level with the growth cycle were observed. It remains to be seen whether this discrepancy results from differences in the culture system used for the analysis. In cultures of freshly isolated explants, a considerable number of cells of the original tissue always remain that contribute to the composition of cultured explants and that are absent in established cultures. Still, as clear differences in the concentration and the ratio of various phytohormones of cultured explants during the growth cycle and of fresh explants before culture indicate, however, during culture, cells establish their own distinct hormonal system (e.g., see Tables II and III and Fig. 4). This conclusion is also supported by the production of 2iP in callus cultures in a kinetin-supplemented medium, or in embryogenic petiole explants in a cytokinin-free nutrient medium. It remains to be seen to what extent this

is just an expression of the biochemical and cytological status of the newly produced cells, or whether this hormonal system contributes to and is involved in the development of nonhabituated explants during the culture cycle. At any rate, as compared to metabolites of the "secondary metabolism," such as glycosides or alkaloids, which accumulate mostly during stationary growth, the accumulation of phytohormones seems to be rather characteristic of cells actively growing by cell division.

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