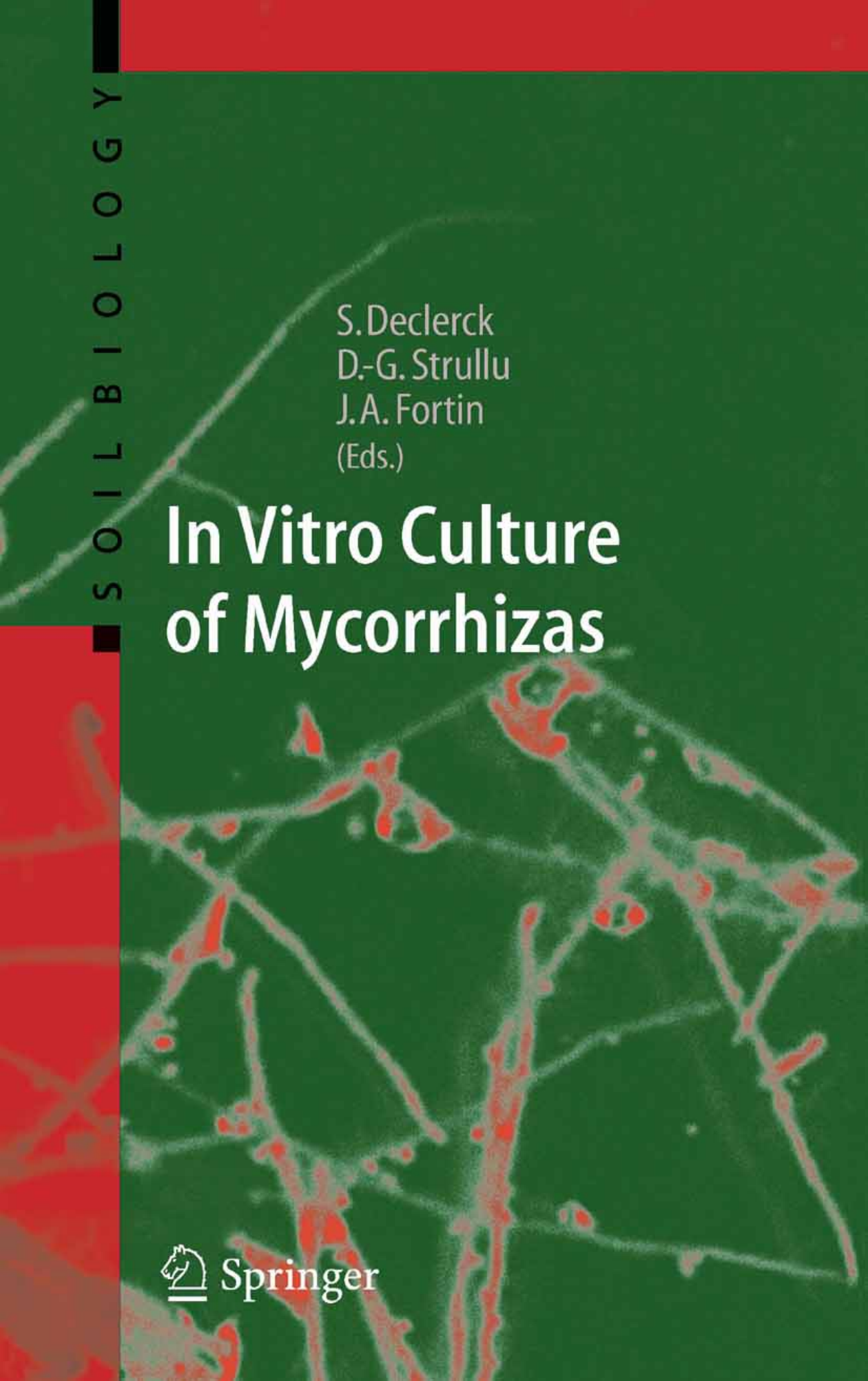
SOIL BIOLOGY

S. Declerck
D.-G. Strullu
J. A. Fortin
(Eds.)

In Vitro Culture of Mycorrhizas



Springer

Soil Biology

Series Editor: Ajit Varma

4

Stéphane Declerck
Désiré-Georges Strullu
J.-André Fortin (Eds.)

In Vitro Culture of Mycorrhizas

With 84 Figures, 13 in Color

Springer

Prof. Dr. Stéphane Declerck
Université catholique de Louvain (UCL)
Mycothèque de l'Université catholique
de Louvain (MUCL)
Unité de microbiologie
3 Place Croix du Sud
1348 Louvain-la-Neuve
Belgium
e-mail: declerck@mbla.ucl.ac.be

Prof. Dr. Désiré-Georges Strullu
Université d'Angers
Laboratoire de Phytonique
2 Bd. Lavoisier
49045 Angers Cedex
France
e-mail: strullu@univ-angers.fr

Prof. J. André Fortin
Université Laval
Département de Sciences du Bois
et de la Forêt
Centre de Recherche en Biologie Forestière
Sainte-Foy, Québec G1K 7P4
Canada
e-mail: j.andre.fortin@videotron.ca

Library of Congress Control Number: 2004116751

ISSN 1613-3382
ISBN-10 3-540-24027-6 Springer Berlin Heidelberg New York
ISBN-13 978-3-540-24027-3 Springer Berlin Heidelberg New York

Springer is a part of Springer Science + Business Media
springeronline.com

Cover design: *design&production*, Heidelberg, Germany
Typesetting and production: LE-TEX Jelonek, Schmidt & Vöckler GbR, Leipzig, Germany
31/3150-YL - 5 4 3 2 1 0 - Printed on acid-free paper

Foreword

The first 30 cm of the earth's surface represents a fragile and valuable ecosystem, thanks to which terrestrial plants, and indirectly animals and humans, can live. The microbial activity occurring in soil is largely responsible for its physical and nutritional quality. Among the micro-organisms living in soil, the arbuscular mycorrhizal (AM) fungi play a major role. They are present in all types of soil, everywhere on the planet, living in symbiotic association with the roots of most plant species. They have co-evolved with plants for 400 million years, improving their nutrition and resistance to various types of stress. Present practices in conventional agriculture, which introduce great amounts of chemicals, have eliminated or underexploited the AM symbiosis. The rational exploitation of AM fungi in sustainable agriculture, to help minimize the use of chemical fertilizers and pesticides, has been hampered by several biological characteristics of these micro-organisms: they cannot be grown in the absence of a plant host and their genetic structure is very complex.

Despite these limitations, biologists have made important progress in understanding better the functioning of AM fungi. An in vitro technique has been developed using mycorrhizal root organ cultures, which made it possible to investigate the genetics, cell biology and physiology of AM fungi. We can now be objective enough to critically evaluate the impacts the in vitro technique has had to improve our knowledge on mycorrhizal symbiosis. Moreover, more experiences in using the technique allows us to appreciate its limits, as well as its yet unexploited scientific potential. A review on the subject has been recently published by Fortin et al (2002).

Along the same lines, but in a much more comprehensive way, this book, through contributions from experienced specialists in the field, offers valuable insights into the most recent uses of the technique. It illustrates how important questions regarding germplasm collection, taxonomy, physiology and metabolism of arbuscular mycorrhizal fungi can be cleverly addressed by taking advantage of the in vitro system. It also reports how the technique has been extended to the culture of other symbiotic fungi. In a unique way, a root/fungus symbiosis normally occurring in soil is made accessible for various investigations: e.g. non-destructive microscope observations, reliable cell physiology studies, clean biochemical and molecular analyses, and highly controlled interaction studies with other micro-organisms. Be-

cause the system provides a way to cultivate in vitro an obligate biotrophic micro-organism, it can even be used to produce aseptically, for the first time, AM fungal inocula on an industrial scale.

Young scientists interested in mycorrhizal symbiosis will find in this book, not only valuable technical information, but also a rich source of inspiration for their research and for the further exploitation of the potential of mycorrhizal in vitro cultures. Like microscopy for cell biology, and the polymerase chain reaction for molecular biology, the mycorrhizal root organ culture system can be considered a critical step in the scientific history of mycorrhiza R&D. This book will certainly provide convincing evidence to support this assertion.

Guillaume Bécard

Acknowledgements

We would like to thank the authors for their contributions and for all their efforts to produce and up-date a literature overview of so many different topics. The success of this book lies in their remarkable contributions. We owe special thanks to Professor Ajit Varma, who invited us to edit this book in the Series, *Soil Biology*. His constant encouragement is highly appreciated. We address special thanks to Dr. Jutta Lindenborn for her patience, dedicated editorial assistance and professionalism during all steps of this book production and to Dr. Dieter Czeschlik, who found our research a valuable topic for this Springer series.

S.D. gratefully acknowledge the financial support from the Belgian Federal Science Policy Office (contract BCCM C3/10/003).

S. Declerck, D.G. Strullu, J.A. Fortin
November 2004

Contents

Part I State of the Art

Part II Systematics

Part V Root Organ Culture of Other Fungal Symbioses

Part VII Methodology

Contributors

Alok Adholeya
Centre for Mycorrhizal Research, Biotechnology and Management of Bioresources Division The Energy and Resources Institute (TERI), DS Block, India Habitat Centre, Lodhi Road, 110003 New Delhi, India

Bert Bago
Estación Experimental del Zaidín (CSIC), Dpto. Microbiología del Suelo y Sistemas Simbióticos, calle Profesor Albareda 1, 18008 Granada, Spain

Céline Bivort
Université catholique de Louvain (UCL), Unité de microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Custodia Cano
Estación Experimental del Zaidín (CSIC), Dpto. Microbiología del Suelo y Sistemas Simbióticos, calle Profesor Albareda 1, 18008 Granada, Spain

Andrew P. Coughlan
Université Laval, Centre de Recherche en Biologie Forestière, Pavillon C.-E.-Marchand, Sainte-Foy, Québec G1K 7P4, Canada

Sylvie Cranenbrouck
Université catholique de Louvain (UCL), Unité de microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Yolande Dalpé
Agriculture and Agri-Food Canada, Environment Health, 960 Carling Avenue, Ottawa K1A 0C6, Canada

Stéphane Declerck
Université catholique de Louvain (UCL), Mycothèque de l'Université catholique de Louvain (MUCL), Unité de microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Ivan Enrique de la Providencia
Université catholique de Louvain (UCL), Unité de microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Bruno Delvaux
Université catholique de Louvain (UCL), Unité des Sciences du Sol, 2/10 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Yves Desjardin
Université Laval, Centre de recherche en horticulture, FSAA, Québec G1K 7P4, Canada

Francisco Adriano de Souza
Empresa Brasileira de Pesquisa Agropecuária, Embrapa Agrobiologia, BR 465-RJ, km 7, Caixa Postal 74505, CEP 23851-000 Seropédica, RJ, Brazil.

David D. Douds Jr.
US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA 19038, USA

Hervé Dupré de Boulois
Université catholique de Louvain (UCL), Unité de microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Roger Durand
Université du Littoral côte d'Opale, Laboratoire Mycologie/Phytopathologie/Environnement, 17 avenue BLERIOT, 62228 Calais, France

Annemie Elsen
Katholieke Universiteit Leuven, Laboratory of Tropical Crop Improvement, Kasteelpark Arenberg 13, 3001 Leuven, Belgium

Joël Fontaine
Université du Littoral côte d'Opale, Laboratoire Mycologie/Phytopathologie/Environnement, 17 avenue BLERIOT, 62228 Calais, France

J.-André Fortin
Université Laval, Département de Sciences du Bois et de la Forêt, Centre de Recherche en Biologie Forestière, Sainte-Foy, Québec G1K 7P4, Canada

Amar Prakash Garg
Ch. Charan Singh University, Department of Microbiology, Meerut, Uttar Pradesh, India

Giovanna Maria Giomaro
Università degli Studi di Urbino "Carlo BO", Istituto di Botanica & Orto Botanico "Pierina Scaramella", Via Bramante 28, 61029 Urbino, Italy

Anne Grandmougin-Ferjani
Université du Littoral côte d'Opale, Laboratoire Mycologie/Phytopathologie/Environnement, 17 avenue BLERIOT, 62228 Calais, France

Solveig Hehl
Carl Zeiss Jena GmbH, Advanced Imaging Microscopy Division, Carl-Zeiss Promenade 10, 07745 Jena, Germany

Cinta Hernandez-Sebastia
University of North Carolina at Chapel Hill, Dept. of Biology, Coker Hall CB# 3280, Chapel Hill, NC-27701, USA

Erik Joner
Agricultural University of Norway, Department of Biotechnological Sciences, N-1432 Aas, Norway

Nathalie Kruyts
Université catholique de Louvain (UCL), Unité des Sciences du Sol, 2/10 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Rina Kumari
Jawaharlal Nehru University, School of Life science, 110067 New Delhi and Ch. Charan Singh University, Department of Microbiology, Meerut, Uttar Pradesh, India

Gerald Nagahashi
US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pennsylvania 19038, USA

Ralf Oelmüller
University of Jena, Institute für Allgemeine Botanik, Dornburger Strasse 159, Jena, Germany

Tatjana Peskan
University of Jena, Institute für Allgemeine Botanik, Dornburger Strasse 159, Jena, Germany

Huong Giang Pham
Jawaharlal Nehru University, School of Life science, 110067 New Delhi, India

Yves Piché
Université Laval, Centre de Recherche en Biologie Forestière, Pavillon C.-E.-Marchand, Sainte-Foy, Québec G1K 7P4, Canada

Ram Prasad
Jawaharlal Nehru University, School of Life science, 110067 New Delhi and Ch. Charan Singh University, Department of Microbiology, Meerut, Uttar Pradesh, India

Laurent Renard
Université catholique de Louvain (UCL), Unité de microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Gervais Rufyikiri
Belgian Nuclear Research Centre (SCK*CEN, Foundation of Public Utility), Radiation Protection Research Department, Radioecology Section, Boeretang 200, 2400 Mol, Belgium.

Minu Sachdev
Jawaharlal Nehru University, School of Life-Sciences, 110067 New Delhi, India

Arthur Schüßler
Darmstadt University of Technology (TUD), Department of Biology, Institute of Botany, Schnittspahnstrasse 10, 64287 Darmstadt, Germany

Sylvie Séguin
Agriculture and Agri-Food Canada, Environment Health, 960 Carling Avenue, Ottawa K1A 0C6, Canada

Nathalie Séjalon-Delmas
UMR 5546, Equipe de Mycologie Végétale, Pole de Biotechnologie Végétales, Chemin de Borde-Rouge, B.P. 42617, 31 326 Castanet-Tolosan, France

Irena Sherameti
Univesity of Jena, Institute für Allgemeine Botanik, Dornburger Strasse 159, Jena, Germany

Anjana Singh
Tribhuvan University, Department of Microbiology, Kathmandu, Nepal

Reena Singh
Centre for Mycorrhizal Research, Biotechnology and Management of Bioresources Division The Energy and Resources Institute (TERI), DS Block, India Habitat Centre, Lodhi Road, 110003 New Delhi, India

Davide Sisti
Università degli Studi di Urbino "Carlo BO", Istituto di Botanica & Orto Botanico "Pierina Scaramella", Via Bramante 28, 61029 Urbino, Italy

Marc St-Arnaud
Institut de Recherche en Biologie Végétale, Jardin Botanique de Montréal, 4101 est, Montréal, Québec HIX 2B2, Canada

Désiré-Georges Strullu
Université d'Angers, Laboratoire de Phytonique, 2 Bd. Lavoisier, 49045 Angers Cedex, France

Yves Thiry
Belgian Nuclear Research Centre (SCK*CEN, Foundation of Public Utility), Radiation Protection Research Department, Radioecology Section, Boeretang 200, 2400 Mol, Belgium.

Pragati Tiwari
Centre for Mycorrhizal Research, Biotechnology and Management of Bioresources Division The Energy and Resources Institute (TERI), DS Block, India Habitat Centre, Lodhi Road, 110003 New Delhi, India

Ajit Varma
Jawaharlal Nehru University, School of Life science, New Delhi 110067, India. *Current Address:* Amity Institute of Herbal and Microbial Research, Noida, India

Horst Vierheilig
Universität für Bodenkultur Wien (BOKU), Institut für Pflanzenschutz, Peter-Jordan-Strasse 82, 1190 Wien, Austria

Liesbeth Voets
Université catholique de Louvain (UCL), Unité de microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Elke Wolf
Darmstadt University of Technology (TUD), Department of Biology, Institute of Botany, Schnittspahnstrasse 10, 64287 Darmstadt, Germany

Vikas Yadav
Jawaharlal Nehru University, School of Life-Sciences, 110067 New Delhi, India

Alessandra Zambonelli
Università degli Studi di Bologna, Dipartimento di Protezione e Valorizzazione Agroalimentare, Via Fanin 46, 40127 Bologna, Italy

Part I
State of the Art

1 In Vitro Culture of Mycorrhizas

J. André Fortin[1], Stéphane Declerck[2], Désiré-Georges Strullu[3]

1 Introduction

Symbiosis with fungi has been determinant for the evolution of vascular plants since their apparition on land. Devonian Rhynia fossils (400×10^6 years old) permit one to observe, in the lower part of their stems, fungal structures closely resembling modern Glomales (Pirozynsky and Malloch 1975). Molecular clocks also permitted one to date the early evolution of Glomales back to about 400×10^6 years (Simon et al. 1993). It seems that associations with some soil fungi were a prerequisite for the evolution of autotrophic land plants, as was also the case with lichens. Plant fossils from several geological periods show the presence of mycorrhizal structures.

During this evolution, arbuscular mycorrhizal (AM) fungi became totally dependent on their host, i.e. obligate symbionts. Today, at our present state of knowledge, it is impossible to grow these fungi independently from a host plant. This also explains why the understanding of the significance of AM fungi in the life of vascular plants and ecosystem dynamics came so late in the second part of the 20th century.

The obligate nature of the AM fungi has always, and still is making it difficult to study most aspects of the biology of these ubiquitous and fundamentally important fungi, including their functioning and roles in terrestrial ecosystems.

Since the mid-1980s, the use of root-organ culture has opened new vistas on several aspects of the AM symbiosis (Fortin et al. 2002). This review gives an idea of the work accomplished but, above all, what remains to be achieved. We feel that this contribution will also encourage more scientists

[1]Université Laval, Faculté de Foresterie et Géomatique, Centre de Recherche en Biologie Forestière, Ste-Foy, QC G2A 1M2, Canada, Tel.: +1-418-6562131, ext. 6119, E-mail: j.andre.fortin@videotron.ca
[2]Université Catholique de Louvain, Mycothèque de l'Université Catholique de Louvain (MUCL), Unité de Microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium
[3]Laboratoire de Phytonique, Université d'Angers, 2 Bd. Lavoisier, 49045 Angers cedex, France

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

to use this approach for increasing innovative research. It also convinced us that there was a need for a more extensive document reporting precise methodologies, disseminating more thoroughly the new knowledge being gleaned, elucidating the potential for diversified use of the method, and also identifying new avenues for further research.

It has become obvious that all areas of AM fungi biology per se, as well as the biology of the symbiotic relationship, have been revisited using monoxenic cultures. Cultivation of AM fungi on root cultures has shed new light on their molecular biology, cytology, genetics, physiology, systematics and phylogeny, which has since received a tremendous innovative momentum. Large-scale industrial production of biologically clean AM inocula produced on root cultures has also become a reality in some countries, including India and Canada.

This first chapter aims to summarize some of the principal findings extensively discussed in the chapters of this book. Several terms related to the so-called in vitro culture of AM fungi have been used in the literature to designate one and the same concept (in vitro, monoxenic, monoaxenic root-organ culture and root culture). For clarity and uniformity throughout this volume, we propose the following standard definitions. A monoxenic culture of an arbuscular mycorrhizal (AM) fungus is a reproducible and contaminant-free, in vitro co-culture between a root organ and a glomalean species. This co-culture should be regarded as continuous if "*the endophyte is maintained* in vitro *indefinitely. It must be subcultured in order to maintain and increase its biomass*" (Bécard and Piché 1992). A root-organ culture is the indefinite culture on a synthetic medium of a transformed or non-transformed, excised root.

The interest of aseptically grown root organs to cultivate AM fungi was communicated to other organisms, namely ectomycorrhizal fungi, where a large number of species can be grown without a host but where several entities see their development improved (e.g. Tuberales). We took the opportunity in this book to underline the interest on basidiomycetes belonging to Sebacinae. Although easily cultivated axenically, these fungi mimic the effects of quite a number of AM fungi on plant growth.

2
A Tool for Germplasm Collection

For the study of micro-organisms, researchers, regardless of their field of interest, must have access to reliable sources of aseptic, properly identified and properly conserved germplasm banks. Such banks must have the recognition of the World Federation of Culture Collection (WFCC). Pot culture-based banks such as BEG and INVAM are most useful and will

remain so, until successful monoxenic cultivation of most existing Glomeromycota is achieved. In the light of Chapter 2, presented by Declerck, Séguin and Dalpé, this is not likely to happen very rapidly, since the number of cultivated species is less than 10% of the estimated 180 species existing in the world. However, increasing numbers of species belonging to most genera are documented in the literature, and are expected to rapidly become available to the scientific community.

3
A Tool for Systematics and Biodiversity

AM fungal taxonomists represent a rare breed, and the support they receive rarely compares to the importance of the issues. Yet, new approaches will have to be developed if successful cultivation of more diversified species of AM fungi is to be achieved. Observations of AM fungi behaviour in bi-compartments suggest that, paradoxically, the mycorrhizal root vicinity is not favourable for the development of extra-matrical mycelium or spore production (Fig. 2 in Fortin et al. 2002). This suggests that bi-compartments should be more widely used in an attempt to cultivate recalcitrant species. Not all soil microbes can grow in completely synthetic medium, thus the presence of soil extracts is often a key to successful cultivation. It should not be assumed that the relationship with the host plant fulfils all the nutriment requirements of AM fungi. Genetic derivation of subcultured AM fungi is often evoked and usually assumed. There is a need for rigorous research regarding this question, along with the development of methods for long-term conservation. In biology, every scientific activity must be based on precise knowledge of the systematic position of the organisms being studied; reliable nomenclature is a prerequisite for organizing knowledge in a useful manner, and assuring continuity and reproducibility of results. AM fungi taxonomists are dealing with fungi living in the soil, a complex environment showing a minimum of morphological characters. Above all, these organisms cannot be cultivated in the absence of a host plant. In Chapter 3, Dalpé, Cranenbrouck, Séguin and Declerck present the problematics of AM fungi systematics, demonstrating the usefulness of monoxenic culture for precise morphological, biochemical and molecular observations of the different steps of their lifecycle. Obviously, monoxenic cultures of AM fungi play a key role in improving our knowledge of their taxonomic classification, their biodiversity and their functionality, in natural as well as managed ecosystems of the world. More graduate students should be encouraged to make a career in AM fungal taxonomy, adding molecular tools to classical approaches.

4
Life Cycle of *Glomus* spp.

The most abundant, at least in managed ecosystems, and the most easily captured, isolated and maintained AM fungi on root cultures belong to the genus *Glomus*. Cultivation of several species and strains has permitted us to trace the life cycle of these species. Dalpé, de Souza and Declerck present (Chap. 4) a detailed step-by-step description of a typical *Glomus*, putting together virtually all the research published up to now on morphological, structural and biochemical aspects of their biology. They also present specific conditions necessary for promoting the development of given stage of the life cycle, i.e. spore germination. Since several *Glomus* spp. can be obtained, observed and maintained on root explants, this should encourage some scientists to cultivate an ever-increasing number of *Glomus* species; the framework recommended in Chapter 2, on the maintenance of AM fungal germplasms, should be strictly followed.

5
Life History of Gigasporaceae

Glomus spp. are rather easy to cultivate monoxenically, but this is not the case with the majority of other AM fungal genera. de Souza, Dalpé, Declerck, de la Providencia and Séjalon-Delmas put together their experience with Gigasporaceae and present an overview of their life cycle (Chap. 5). The fact that most information is based on non-aseptic systems illustrates the challenge that these AM fungi present for their continuous monoxenic cultivation. One of the difficulties is that they often require a longer cultivation period (several months) to produce their first spores on root organs, as compared to only 10 weeks in pot culture. The authors of this chapter mention that spore production comes after senescence of the root. We suggest that selectively weakening or killing (physically or chemically) the root might possibly trigger spore production.

6
Effects of Environmental Factors on Hyphal Growth and Branching

AM fungi must find a compatible host plant to complete their life cycle. In Chapter 6, Nagahashi and Douds present the environmental factors, including light, gaseous or volatile compounds and non-volatile chemical compounds, which affect pre-symbiotic hyphal branching and growth.

Purified chemicals such as some flavonols can stimulate the growth of AM fungi. These authors review the germ tube responses to different interactions between: (1) a gaseous compound and chemicals, (2) different soluble chemical compounds and (3) chemical compounds and light. It appears that AM spores can generally germinate without the presence of root exudates, but the components of the exudates can stimulate fungal growth, hyphal branching and root colonization. It has been demonstrated that multiple genes are expressed when a germinated spore is treated with host root exudates. Recent evidence suggests that we should be aware that there might be different factors for elongation growth and hyphal branching. Not every environmental factor affects AM fungi positively. In addition to chemical components of exudates and volatile compounds, the authors demonstrated that a third physical factor, light, stimulates hyphal branching. In particular, blue light and root exudates appear to trigger the same second messenger involved in the hyphal branching response.

7
Questioning the Value of Monoxenic Cultures

In Chapter 7, Bago and Cano present an interesting discussion concerning seven main questions:

Are AM monoxenic cultures devices too artificial to trust? Does primary colonization by AM fungi occur in young roots? Do hyphae exit the root after symbiosis begins? Are branched absorbing structures (BAS) formed by all glomalean fungi or are artefacts formed under monoxenic conditions? Are there any differences in the development of AM fungi in monoxenic vs. soil cultures? Are AM monoxenic liquid cultures accurate enough to use? What else can monoxenic cultures offer regarding the study of AM fungal biology? In this chapter, the authors present an overview on subjects of high potential interest for those working with AM fungi, either for scientific or commercial purposes.

8
AM Fungi; Host and Non-Host

Arbuscular mycorrhizal fungi can be found in the roots of 80% of all vascular plant species. Generally, Brassicaceae are described as being non-mycorrhizal, but numerous conflicting papers report mycorrhizal associations in many taxa of the Brassicaceae (*Arabidopsis*, *Brassica*, *Cardamina*) and the Chenopodiaceae (sugar beet and spinach). Chemical factors may be involved in reducing the infection. The establishment of mycorrhizal

symbiosis involves a process leading to the recognition and compatibility between the two partners, but the mechanism governing these phenomena is not well understood. In Chapter 8, Vierheilig and Bago discuss the host and non-host impact on the physiology of the AM symbiosis. The authors identify several phases of the root–AM fungal interaction: (1) asymbiotic phase (axenic culture), when the fungus germinates and grows in the absence of plant signals, (2) pre-symbiotic phase, when the fungus germinates and grows in the presence of signal exudates, and (3) symbiotic phase, when the fungus has penetrated the root and formed intraradical arbuscules. The latter phase is difficult to obtain in monoxenic culture, and fewer physiological data are available. The effects of pH, temperature, CO_2 and light on spore germination and hyphal asymbiotic growth of AM fungi are presented first. In a second point concerning pre-symbiotic AM fungus growth, the data discussed show the importance of root exudates favourable to AM fungi for the successful establishment of the symbiosis. At least at the pre-symbiotic phase of the association, some AM non-host plants and myc$^-$ plants seem to share mechanisms affecting their susceptibility to AM fungi. The perception of AM fungi by the plant before root colonization is poorly documented. It has been recently hypothesised that a more favourable environment for root penetration is created by the host in the presence of fungal signals.

9
Carbon and Lipid Metabolism

Great possibilities are offered by monoxenic culture to study different aspects during the formation of the AM association. The knowledge of these interactions progresses at cellular, molecular and biochemical levels. It is generally accepted that up to 20% of the photosynthetically fixed carbon is transferred from the plant to the AM fungi. Intraradical hyphae incorporate plant-derived hexose, which is converted to typical storage forms, trehalose and glycogen, but extraradical mycelium is incapable of taking up sugars. A gene encoding for a transmembrane sugar transporter was cloned from mycorrhizal roots of *Medicago trunculata*. According to Harrison (1996), this transporter (*Mtst1*) was designed as a hexose transporter by activity measured in yeast. The failure of AM fungi to complete their life cycle in the absence of roots could originate from the control by the plant of fungal genes involved in carbon transport and metabolism. On this basis, Grandmougin-Ferjani, Fontaine and Durand (Chap. 9) present the monoxenic culture technique as a tool for the establishment of the lipid composition of AM fungi. Lipid droplets are abundant in spores and vesicles of AM fungi, and biochemical studies indicate that lipids can represent up

to 45% of the fungal dry biomass. The authors give a comparison between lipid analyses of AM fungi (*Glomus intraradices*) obtained by in vitro and in vivo systems. They also propose the use of monoxenic cultures as a tool for the evaluation of AM fungi in host root tissue. AM monoxenic cultures, combined with isotopic labelling techniques, enable a better understanding of lipid metabolism of AM fungi. Moreover, these authors note that the lipid metabolism of AM fungi is still unclear, since results from ^{14}C and ^{13}C labelling seem to be contradictory. RMN studies of lipids suggest that obligate biotrophy of AM fungi could be due to a lack of, or insufficient ability of neutral lipid biosynthesis in both germinating spores and extraradical mycelium. Cloning and expression analysis of genes encoding enzymes involved in lipid biosynthesis are now required. The use of AM monoxenic cultures has clarified some aspects of the symbiotic interactions. Moreover, there are certainly some differences in AM fungi development when grown in vitro (monoxenically) and in vivo, but these could be reduced.

10
Monoxenic Culture and Physiology of in Vitro Grown Plants

Desjardins, Piché and Sebastia (Chap. 10) illustrate how AM fungi produced on root cultures can be useful in the study of the comparative physiology of in vitro cultivated plants, especially in relation to water stress and sink–source relationships. The data demonstrate that the mycorrhizal inoculation of in vitro propagated plants is very promising in acquiring healthy plants, and improves the adaptation of such plants when transferred under natural conditions. In a different context, a review on this subject would be of great practical interest.

11
Nutrient Dynamics in AM Monoxenic Cultures

According to Rufyikiri, Kruyts, Declerck, Thiry, Delvaux, Dupré de Boulois and Joner (Chap. 11), the monoxenic culture system offers three major advantages for element transport studies: (1) bio-sorption and affinity studies at low concentration; (2) modification of the speciation of a defined element due only to its interactions with the AM fungus; and (3) determination of specific uptake and flux rates. Monoxenic culture systems are useful in studies involving essential elements (N and P) and radionuclides (U and Cs). AM fungi take up and translocate these elements. As AM fungi are an important part of the rhizospheric micro-organism biomass, the uptake of radionuclides by the extraradical mycelium has ecological significance –

these fungi intercept radionuclides and influence their migration in the soil and their accumulation by plants. The capacity to exploit certain forms of a nutrient (precipitated P, organic forms of N or P), as explained in this chapter, might be involved in the distribution of the microbial population in the mycorrhizosphere.

12
AM Fungi and Rhizosphere Micro-Organisms

Interactions between AM fungi and other rhizosphere microbes have many effects on the host plant (especially the alleviation of root diseases and the access to nutrients through interactions with N-fixing and P-solubilizing bacteria). AM monoxenic culture represents a new tool to elucidate complex interactions between soil inhabitants. In Chapter 12, St. Arnaud and Elsen elucidate the interaction of AM fungi with soil-borne pathogens and non-pathogenic rhizosphere micro-organisms. More than 40 years ago, Mosse (1962) reported that a *Pseudomonas* isolate was necessary for AM root colonization and in vitro growth of AM fungi. Later, many other soil micro-organisms were shown to stimulate or inhibit AM fungal spore germination and hyphal growth. The authors comment on numerous results relating interactions between AM fungi and soil bacteria, and between AM fungi and other fungi. Concerning interactions between fungi and nematodes, there are few data; the in vitro activity of the cyst nematode *Globodera pallida* was studied, and these first results indicated that the mycorrhizal inoculation of potato plants could stimulate the production of hatching chemicals. Recently, using dixenic cultures, the interactions between *Glomus intraradices* and the nematodes *Radopholus similis* and *Pratylenchus coffeae* have been studied, showing that in the presence of AM fungi the populations of both species have been reduced. An interesting conclusion is made, since it is now suggested that symbiosis regulation might also impact other soil microbes. Actually, it is necessary to document the variability in growth, nutritional kinetics and physiology of the model used to study the interaction between AM symbiosis and its environment.

13
Cistus Incanus Root Organs to Study Ectomycorrhizal Fungi

The increasing use of root cultures for the study of AM fungi prompted a few workers to develop such an approach for the study of ectomycorrhizae (ECM). In Chapter 13, Coughlan and Piché review the attempts made to

date to produce ECM on root organs, and they relate the recent success of their own and other groups in obtaining such mycorrhizae. They were thus able to obtain *Tuber melanosporum* ECM formation within 5 days of inoculation, using *Cistus incanus* roots. Obviously, this approach opens new vistas on the study of ECM. A large number of ECM fungi (e.g. *Russula* spp.) cannot be cultivated under axenic conditions – however, root cultures are likely to make this possible in the near future. This approach will no doubt permit a better understanding of mutual physiological activities as well as biochemical exchanges between partners, and facilitate some studies in molecular biology. The use of bi-compartments will open new possibilities for the study of interactions with soil components, including physico-chemical factors as well as soil-borne micro-organisms, both useful and harmful.

14
Monoxenic Culture of Edible Ectomycorrhizal Fungi

In Chapter 14, Giomaro, Sisti and Zambonelli discuss the problems encountered in the study of edible ECM, with major emphasis on truffle. Although their references to root cultures are brief, reading their chapter, with the idea of using such cultures, suggests a number of promising avenues for research. One of the difficulties encountered in studying species such as *Tuber* or *Cantharellus* spp. is that their biology involves interactions with a large number of soil bacteria. Root cultures should prove useful in determining which species are critical for the development of the mycelia as well as fruit bodies. Understanding signalling, biochemical exchanges, and mutualist physiology should be improved using this approach. The maintenance and production of inoculums should also benefit from monoxenic culture.

15
The Unique *Geosiphon* Symbiosis

Geosiphon pyriformis represents a unique symbiosis between a Glomeromycota and nostocs. Schußler and Wolf (Chap. 15) describe the biology of this fascinating symbiosis. We learn that, in nature, such nostocs are closely associated with *Anthoceros* and *Blasia*, often considered representatives of the ancestors of vascular plants. Even if they have been unsuccessful in obtaining AM structures in vitro between *Geosiphon* and *Anthoceros*, the use of DNA markers suggests that some plant roots as well as *Anthoceros* thalli obtained in nature do contain this organism. For those who want to know more about this strange symbiosis, the authors give a full

account of the methodologies they developed as well as of experiments they carried out on the physiological exchanges between partners. They discuss their phylogeny as well as their possible role in the evolution of AM fungi. Clearly, this is a story to be followed, and which is likely to shed new lights on plant symbiosis in general.

16
Should We Consider Root-Inhabiting Sebacinaceae as Mycorrhizal Fungi?

For several years, a number of papers on *Pirimorphospora indica* have originated from an Indian research team. Since this research remained somehow "captive" within the group, their findings have perhaps not received all the attention they deserve. Therefore, the group was offered the possibility to present this problematic in a scientific light. If the impression was given at the beginning that *P. indica* is a sort of magic fungus, Prasad, Pham, Kumari, Singh, Yadav, Sachdev, Garg, Peskan, Hehl, Sherameti, Oelmuller and Varma (Chap. 16) rather demonstrate its interest, and cover not only *P. indica* but more generally all the Sebacinaceae, where other species share the properties of *P. indica*. We hope that this presentation will permit the readers to evaluate the credibility of the claims often made by the authors and will convince other groups of researchers to evaluate these fungi more seriously, and to objectively establish their role in the life of plants.

17
Industrial-Scale Monoxenic AM Fungus Production

Of course, the perspective of using monoxenic cultures for the large-scale production of AM inocula has been in the mind of most scientists interested in the more applied aspects of mycorrhizas. Such developments have already taken place in Canada as well as in India. Adholeya, Tiwari and Singh describe in Chapter 17 how they achieved such an industrial endeavour. We would have liked that the authors describe their methodology more extensively. However, since this is an industrial process, run by the users on a profit base, we understand that it was not possible for the authors to totally "open their books". Still, this is a story not only of the successful production of AM fungi on root cultures at a large scale, but also of the success obtained in the field by thousands of farmers, who were willing to pay for such inocula. If, at present, the number of species that can be produced by this technology is limited, progress is being made with other species. The use of genetically transformed roots for this production is

sometimes discussed within the framework of governmental regulations, but the fact that hairy roots already exist in nature should make them totally acceptable, at least with respect to legal aspects.

18
Precise Techniques for Successful Development of AM Monoxenic Culture

The closing Chapter 18 will be most useful for all those who want to familiarise themselves with the diverse techniques used to establish, maintain and experiment with AM fungi on root organs. Cranenbrouck, Voets, Bivort, Renard, Strullu and Declerck have combined their expertise to present a precise and thorough description of methodologies for those who seek an introduction to the art. In addition, this chapter should prove useful for all those who have already cultivated AM fungi on root organs, permitting them to identify pitfalls and alternative methodologies.

19
Conclusion

Obviously, the use of monoxenic cultivation of AM fungi on root culture, whether genetically modified or not, has become an essential tool for the study of mycorrhizae and their practical uses. One of the greatest challenges we are facing is to achieve the monoxenic cultivation of most, if not all glomalean fungi. This is the sort of challenge being met in molecular biology, when the world scientific community undertakes the total sequencing of a species' genome, human or other. The maintenance of a reliable, rigorously documented bank of isolates is a prerequisite for establishing the database necessary for the rapid molecular identification of any given species of glomalean fungi. The availability of precise and rapid molecular identification of AM fungal species will open a new era for AM fungal ecology, both in natural and managed ecosystems.

The intraspecific genetic variation of glomalean species is another challenge which needs to be faced. In monoxenic cultures, different isolates of *Glomus intraradices*, under the same set of conditions, produce largely different numbers of spores, a phenotypic variation very easy to identify. What we need to know is the genetic basis and extent of this variation, its source, and the feasibility of using this or other phenotypes experimentally. Although most of us assume that genetic derivation is likely to occur over several generations of a glomalean isolate, this remains to be demonstrated.

Axenic culture of glomalean fungi is often considered as the ultimate challenge, mainly because it would show that we have understood the nature of the critical biochemical exchanges at the very basis of this ubiquitous symbiosis. Monoxenic cultures will undoubtedly be most useful in attaining this goal.

Monoxenic cultures of glomalean species have been used, up to now, to study only a small part of their biology, but several fundamental aspects remain to be elucidated. In soils, spores as well as extra-matrical mycelia are in close contact with physical (e.g. clay), chemical (e.g. humic acids) and biological (e.g. bacteria) components. These factors are all likely to influence the development and functioning of AM fungi. We propose that bacterial biofilm formation is a fundamental and universal phenomenon in the life of AM fungi living under natural conditions. Such biofilms can easily be observed in monoxenic culture using bi-compartment dishes.

Ultimately, all advances in our knowledge of monoxenic AM fungi will be useful for the large-scale production of reliable, microbiologically clean inocula. This is likely to permit the reduction of production costs, and make AM fungal inocula as widely available as rhizobia have been for decades. There is little doubt that a more general use of AM fungal inocula in agriculture could substantially increase financial support for research on this fundamental and universal phenomenon in all natural and managed terrestrial ecosystems.

Among the numerous other subjects which this book could not address is the use of monoxenic culture in the mass production of in vitro propagated plants. A need remains for a review of this subject.

References

Bécard G, Piché Y (1992) Establishment of vesicular-arbuscular mycorrhiza in root organ culture: review and proposed methodology. Methods Microbiol 24:89–108

Fortin JA, Bécard G, Declerck S, Dalpé Y, St Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Harrison MJ (1996) A sugar transporter from *Medicago truncatula*: altered expression pattern in roots during vesicular-arbuscular (VA) mycorrhizal associations. Plant J 9:491–503

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Pirozynski KA, Malloch DW (1975) The origin of land plants through the ages. Can J Bot 59:1824–1827

Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular plants. Nature 363:67–69

Part II
Systematics

2 The Monoxenic Culture of Arbuscular Mycorrhizal Fungi as a Tool for Germplasm Collections

Stéphane Declerck[1], Sylvie Séguin[2], Yolande Dalpé[2]

1 Introduction

It is not yet routine to cultivate all arbuscular mycorrhizal (AM) fungi in vitro on root organs. This is because these fungi are unable to complete their life cycle in the absence of a suitable host plant, and the mechanisms underlying the obligatory biotrophy of the fungal partner are so far not fully understood. Since the mid-1970s, three major breakthroughs have been accomplished in the field of in vitro cultivation of AM fungi, which are of paramount importance for culture collections. Mosse and Hepper (1975) performed the first AM fungi in vitro culture, i.e. a monoxenic culture. Strullu and Romand (1986) achieved the first subcultivation of an AM fungus in a system of successive isolations from mycorrhizal roots and re-associations with various root systems. This step allowed envisioning the indefinite culture-controlled conservation of AM fungi. Subsequently, Bécard and Fortin (1988) adapted the *Agrobacterium rhizogenes* Conn. transformed root tissue technology to the growth and development of AM fungi. Combining these findings has opened the route to the cultivation of various species in nearly all genera, and offers tremendous perspectives for germplasm collections. In this chapter, we will recall some rules essential to establish a culture collection, and clearly demonstrate the usefulness of monoxenic cultures for germplasm collections.

2 Historical Perspective of AM Fungi Culture Collections

A culture collection starts with the acquisition, propagation, characterization and maintenance of a single species. Thus, it is tempting to extrapolate

[1]Université Catholique de Louvain, Mycothèque de l'Université Catholique de Louvain (MUCL), Unité de Microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium, Tel.: +32-10-474644, Fax: +32-10-451501, E-mail: declerck@mbla.ucl.ac.be
[2]Environment Health/Biodiversity, Agriculture and Agri-Food Canada, 960 Carling Avenue, Ottawa K1A 0C6, Canada

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

by saying that there are probably as many collections throughout the world as there are laboratories working with AM fungi. It is obvious that the overwhelming number of strains maintained within laboratories and sampled from various ecosystems help in harnessing the global biodiversity. However, the potential impact of these circum-world, laboratory-independent collections of strains is to date largely outweighed by the generally poor knowledge of the simplest rules to produce and maintain strains. Such maintenance necessitates strict control measures (e.g. VIPS: viability – identity – purity – stability), an appropriate infrastructure to maintain contaminant-free material, and accurate characterization. In reality, the acquisition, propagation, characterization and maintenance of germplasms of AM fungi in living cultures are far more complicated than commonly assumed, and should follow strict rules or codes of conduct. Two collections have faced these criteria, and have made formal commitments for the distribution of quality-controlled, well-identified germplasms for basic and applied research. These are the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM, http://invam.caf.wvu.edu/) in the United States, and the International Bank of Glomeromycota (BEG, http://www.kent.ac.uk/bio/beg/), a collection of glomalean fungi organized by a network of European laboratories. Both collections share an important number of accessions belonging to all the genera, and representative of numerous ecosystems all over the world. However, and without any prejudice to their indubitable importance, these AM fungal collections cannot strictly guarantee the absence of undesirable microbial contaminants or the purity of inoculum, simply because AM fungi are maintained in pot cultures – on a living plant, in a soil-based substrate. This annoying situation, paralleled with the breakthrough of monoxenic cultivation of AM fungal species in the last decade, has led to the development of The Glomeromycota In Vitro Collection (GINCO; http://www.mbla.ucl.ac.be/ginco-bel/, http://res2.agr.gc.ca/ecorc/ginco-can) in 2001. GINCO is the first in vitro culture collection of AM fungi, hosted within two international filamentous fungi and yeast collections [the Mycothèque de l'Université catholique de Louvain in Belgium (MUCL, part of the Belgian Co-ordinated Collections of Micro-organisms, BCCM), and the Canadian Collection of Fungal Cultures in Canada, Canada], registered in the World Federation of Culture Collections (WFCC). It is not hard to believe that in the coming decade, sister collections to GINCO will emerge, providing the international community with increasingly quality-controlled AM fungi. It will be the joint task of taxonomists and culture collection specialists to pave the way for correct management, with high quality standards such as the VIPS, of this new type of biological material provided to the scientific community.

3
Prerequisite to Include AM Fungi in Monoxenic Culture Collections

Almost all AM fungi tested up to now have been brought in monoxenic culture, if one considers the definition of culture as the growth of one organism of a group of organisms for the purpose of experiments (Kirk et al. 2001). This is, for instance, the case of *Glomus mosseae* (Strullu and Romand 1986; Douds 1997), producing mycelia when cultured in association with a suitable excised host root, but usually failing to produce mature spores. Only Raman et al. (2001) reported and illustrated the production of a few

Table 1. Monoxenic cultures with production of viable mature spores and continuous culture by subcultivation of arbuscular mycorrhizal fungi[a]

Species	Production of viable mature spores	Continuous culture by subcultivation	References
Acaulospora rehmii	Yes	No	Dalpé and Declerck (2002)
Gigaspora gigantea	Yes	No	Mosse (1988)
Gigaspora margarita	Yes	No	Miller-Wideman and Watrud (1984)
Gigaspora rosea	Yes	No	Bécard and Piché (1989)
Scutellospora reticulata	Yes	Yes	de Souza and Declerck (2003)
Glomus caledonium	Yes	Yes	Karandashov et al. (2000)
Glomus cerebriforme	Yes	Yes	Samson et al. (2000)
Glomus clarum	Yes	Yes	de Souza and Berbara (1999)
Glomus etunicatum	Yes	Yes	Pawlowska et al. (1999)
Glomus fasciculatum	Yes	Yes	Strullu and Romand (1986)
Glomus intraradices	Yes	Yes	Strullu and Romand (1987)
Glomus macrocarpum	Yes	Yes	Declerck et al. (1998)
Glomus mosseae	Yes	No	Raman et al. (2001)
Glomus proliferum	Yes	Yes	Declerck et al. (2000)
Glomus versiforme	Yes	Yes	Diop et al. (1994)

[a] Data refer to first citation in the literature, with priority to those references in which spore production has been obtained and subcultures have been achieved, followed by references in which only culture has been achieved. The data include only cultures with clear reference to new, mature spore production

spores of this widely used species. Therefore, such material is in practice unusable in a culture collection for distribution purposes. A first prerequisite is therefore the capacity of the fungi to complete its life cycle with the production of sufficient spores and intraradical structures characteristic of the genera considered. Considering this rule, and strictly referring to published papers only, 15 species have so far been cultured with success under monoxenic conditions (Table 1), with the production of mature spores. It should be noted that species such as *G. constrictum* (Mathur and Vyas 1999) and *G. deserticola* (Mathur and Vyas 1995) were reported to produce extraradical mycelia and intraradical vesicles and arbuscules when associated with *Ziziphus mauritiana* in vitro-raised plants, but no text reference was made to spore production, and therefore these species are not considered in Table 1. Production of spores in the first generation does not preclude the capacity of the fungus to be maintained constantly under monoxenic culture. Indeed, numerous species monoxenically cultured failed to be subcultivated under the same growth conditions over several generations, requesting re-sampling of the pot culture inoculum to re-establish a monoxenic culture. Such regular solicitation of in vivo material increases the risk of culturing an AM fungal contaminant species. The second prerequisite is therefore the capacity of the fungal material produced monoxenically to be subcultured, i.e. cultured continuously (see Chap. 1 for definition) under the same monoxenic conditions favouring multiplication of material, necessary for the distribution and durability of the strain. Following this rule, only ten species have been published with reference to subcultivation, and most are *Glomus* species producing rather small spores and an important intraradical phase, i.e. vesicles. In summary, strictly referring to published papers, 8.3% of the approximate 180 described species have been monoxenically cultured, and 5.5% have been maintained over several generations.

4
Culture Properties: Viability – Identity – Purity – Stability (VIPS)

"*The purpose of a collection is to maintain biological material in a viable and stable state, retaining all original properties*" – with these words, Smith and Onions (1994) stressed the necessity for a collection to guarantee the viability of pure, identified strains over long generations, without loss of intrinsic properties.

Viability should be confirmed by growing the AM fungi on the correct medium and host. In the case of monoxenic cultures, two commonly used media are the modified Strullu-Romand (MSR) medium (Declerck

et al. 1998, modified from Strullu and Romand 1986) and the M medium (Bécard and Fortin 1988). Hosts roots are from excised, transformed or non-transformed roots of carrots (*Daucus carota* L.), tomato (*Lycopersicon esculentum* L.), white clover (*Trifolium repens L.*), barrel clover (*Medicago truncatula Gaertn.*) and some other mycotrophic plants (Bago and Cano, Chap. 7). The assessment of strain viability can be performed through spore germination tests or colonized root sub-cultivation, done either before or after a preservation period. Growth dynamics, mycelium organization, level of sporulation, and percent of root colonization are all data used to evaluate viability performance and the maintenance of the strain mycorrhizal potential through generations of sub-cultivation.

The identity of AM fungi has been based for decades almost entirely on spore morphology from material directly sampled from substrate or soils. Incomplete descriptions related to the quality of the material, i.e. punctual harvesting, the absence of spore wall layers or subtending hyphae (Fortin et al. 2002), the absence of living cultures of type specimens, the lack of data on spore ontogeny, together with the restriction to morphological tools, have made species identification a challenge for taxonomists. The accessibility to monoxenic cultures has renewed interest in taxonomy by opening the possibility of efficiently integrating multiple tools such as ultrastructure, molecular biology, biochemistry, mycelium architecture and spore dynamics into the traditional species description (Declerck et al. 2000; de Souza and Declerck 2003). With the current limitation of morphological spore features able to sustain morphological characterization of AM fungi isolates, the combination of cultural with molecular and biochemical characteristics may yield taxonomically valuable tools, once proven to be stable, and heritable through generations and growing conditions. Indeed, the major advantage of monoxenic cultures lies in the standardisation of growing conditions and, as such, helps to distinguish heritable from acquired characters.

The purity of AM fungal strains can be ascertained by establishing the monoxenic culture from a single spore (Declerck et al. 1998, 2004) or a single isolated vesicle (Strullu and Romand 1987; Declerck et al. 1998). Such propagules permit to obtain a fungal colony representing a unique organism, deprived from any other living contaminant. Purity then refers to "contaminant-free monospecies fungal culture". In the case of mycorrhizal root fragments containing multiple vesicles, it is suggested to establish the culture with the mycorrhizal root fragment and to sub-culture with a single propagule. In this case, the purity of the culture will be the first spore daughter generation. Even though monosporal cultures are established, spore wall-encysted bacteria and non-AM fungi propagules, resistant to surface sterilization procedures or intraspore micro-inhabitants, may remain dormant during subcultivation processes. Their presence may then

considerably alter molecular analyses, and be expressed through aberrant mycelial development. However, once bacteria dormancy is raised, antibiotic treatments usually allow complete decontamination and the recovery of contaminant-free monoxenic cultures. With fungal contaminants, such decontamination cannot be achieved as easily because of the usually detrimental effect of fungicides on AM fungi.

The stability of fungi is an important factor, particularly for the agro-environmental important group of AM fungi, which guarantees preservation of all the symbiotic fungal properties. Continuous culture through subcultivation may allow the organism to adapt to laboratory conditions, while danger of variation – loss of some physiological or morphological characteristics – cannot be discarded as reported by Smith and Onions (1994) with several fungi belonging to all taxa. With the current knowledge and experience linked to AM fungi monoxenic cultures, a severe decrease in infectivity through culture generations has been registered with *Acaulospora* and *Gigaspora* species. Indeed, no successful continuous culture over several generations has been reported, neither with *A. rehmii* (Dalpé and Declerck 2002) nor with *Gigaspora* species, mentioned only as difficult to achieve (Fortin et al. 2002). Subcultivation has been shown successfully with *Scutellospora reticulata* (de Souza and Declerck 2003). The situation with *Glomus* strains is more controversial. While *G. intraradices*, strain DAOM 181602, has been maintained under monoxenic culture since 1992 (Chabot et al. 1992), with no discernable loss of infectivity, colonies of a strain of *G. versiforme* showed a decrease in infectivity through generations (Plenchette et al. 1996). For other *Glomus* AM fungal species, especially with large-spore species strains, a decrease in vitality (lower sporulation and root colonization rates, and reduced extraradical mycelia) has been detected, resulting in the gradual decline in strain viability. The revitalization potential of a 7–14 day cold treatment for *G. intraradices*, as proposed by Juge et al. (2002), may be promising but remains to be demonstrated in other species.

Strictly speaking, stability can only be ascertained by long-term preservation processes, where the metabolism of the AM fungi is halted to avoid any subsequent alteration of properties. Preservation methods such as cryopreservation are discussed below.

5
Long-Term Conservation

"*Preservation techniques range from continuous growth through methods that reduce rates of metabolism to the ideal situation where metabolism is halted*" (Smith and Onions 1994). Procedures to preserve and store fungi

have been reviewed by Smith and Onions (1994), and can be split into three groups: (1) the continuous growth method and methods which delay the need for subcultivation, such as storage on the growth medium in the refrigerator, freezer, under oil or water, (2) drying by air or with silica gel and freeze-drying, and (3) suspension of metabolism, which means reduction of available water in the cells by dehydration (freeze-drying) or freezing (cryopreservation) at low temperature or in liquid nitrogen, so that metabolism is halted. It is obvious that subcultivation is the common method to maintain AM fungi monoxenically (Plenchette et al. 1996; Strullu et al. 1997; Declerck et al. 1998). However, this method is laborious, time-consuming and difficult or even unreported for some species. Moreover, the impact on genetic stability through generations is unknown. Therefore, a method for long-term storage appears essential.

The first group of methods to delay subcultivation consists in the storage of the culture on the growth medium in the refrigerator, freezer, under oil or water. At INVAM, most accessions grown since 1990 are stored at 4 °C. According to this collection, the duration of storage varies considerably between genus, and within genera between species (for details, refer to http://invam.caf.wvu.edu/methods/storage/frigeration.htm). Likewise, strains produced in vitro could be stored in the form of plugs of gel bearing numerous spores, and the mycelia incubated in a saline solution or distilled water at 4 °C for several months. With *G. intraradices*, 100% survival was registered with plugs of gel maintained up to 3 years at 4 °C (Dalpé, pers. comm.). This method appears less efficient with species producing few spores, and has never been tested with genera other than *Glomus*.

The second group of methods, consisting of drying and freeze-drying, has been poorly investigated, with material produced either in vivo or in vitro. L-drying (Tommerup 1988) and single-stage lyophilization were shown effective for some AM fungi (Dalpé 1987) from pot culture. N_2 drying was further tested on alginate beads containing spores of *G. intraradices* produced in vitro (Declerck et al. 1997). In the latter case, 80–100% of the treated beads remained infective (as measured by the percent of potentially infective beads, PIB; Declerck et al. 1996), the relative water content being clearly involved in viability maintenance.

The third group of methods consists of freeze-drying and cryopreservation, resulting in the suspension of metabolism. At INVAM, Douds and Schenck (1990) observed that slow drying of pot culture soil followed by freezing the spores in situ was satisfactory for cryoprotection and cryopreservation at −60 to −70 °C for several AM fungi. They further demonstrated that spores of *G. margarita* isolated from pot culture soil had a certain measure of freeze damage protection at −60 to −70 °C when incubated 2 days in trehalose 0.5 M, 0.75 M or 1.0 M. Kuszala et al. (2001) tested the survival of 20 glomalean isolates belonging to 16 species in 4 gen-

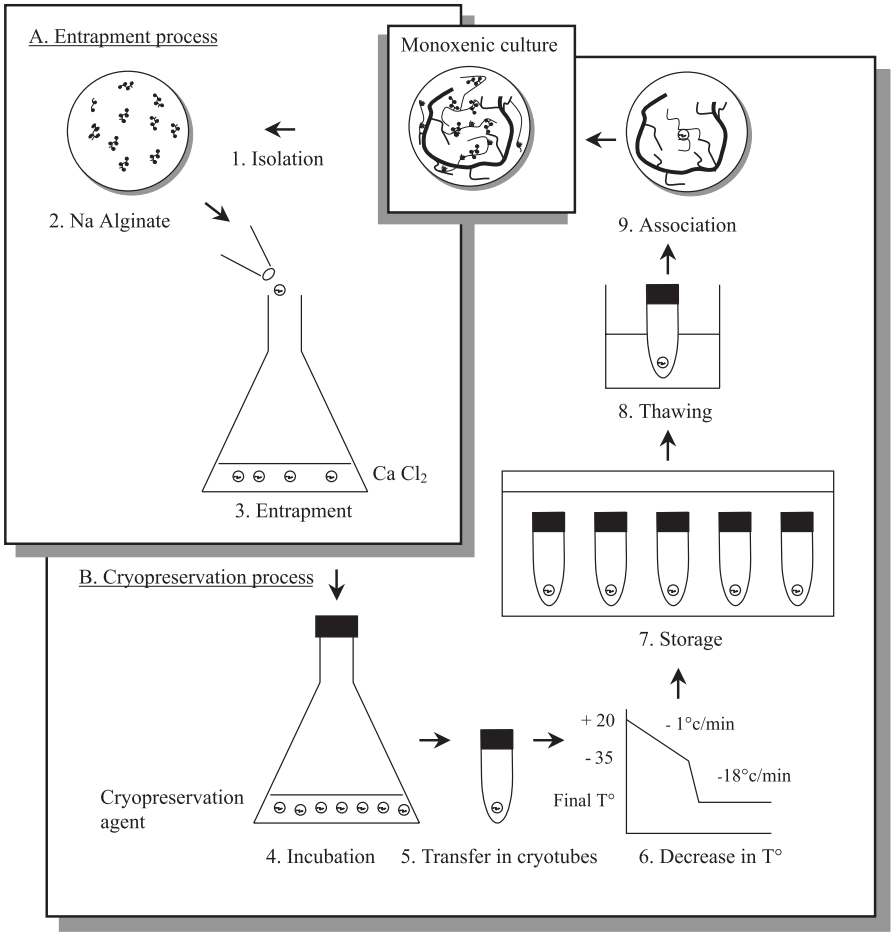


Fig. 1. Schematic view of the entrapment (**A**) and cryopreservation (**B**) processes. **A** Starting from a monoxenic culture, spores are successively isolated with forceps, after solubilization of the growth medium (1), poured in a 20 $g l^{-1}$ sodium alginate solution (2), and dropped in a 0.1 M $CaCl_2$ solution for polymerization (3). After the entrapment of spores, the beads are removed from the $CaCl_2$ solution, stored overnight at 15 °C and cryopreserved. **B** Beads are successively incubated at 4 °C in the cryoprotectant (4), transferred to cryotubes (5), and cold-treated (6) following a two-step decrease from ambient to final preservation temperature. Beads are stored at this temperature for 3 h (7), and thereafter retrieved by fast thawing in warm water (8). The beads are then incubated on the MSR medium, and those showing spore germination are re-associated with a transformed carrot root (9) to start a new monoxenic culture. (Declerck and Angelo-Van Coppenolle 2000, with permission of New Phytologist)

era (*Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora*) under different storage conditions (room temperature, +18–24, +4, −18 and −80 °C, in liquid nitrogen) for the conservation of their germplasm. They observed that sporulation was effective for all isolates stored at all temperatures. Therefore, both proposed methods guaranteed a backup reserve and an alternative supply source to start cultures, which can be applied to a wide range of isolates. These results were also extended to monoxenically cultured strains. In 1998, Addy et al. (1998) demonstrated that the extraradical hyphae of *G. intraradices* produced monoxenically were able to survive at −12 °C when slowly cooled prior to freezing. In 2000, Declerck and Angelo-Van Coppenolle (2000) succeeded in the cryopreservation of monoxenically produced spores of *G. intraradices* (Fig. 1) by adapting the shoot tip, alginate bead entrapment system (Niino and Sakae 1992; Chandler 1994) to *G. intraradices*. The use of cryoprotectant followed by slow (to −4 °C) and fast (to −100 °C) freezing steps allowed 100% germination rate and mycorrhizal establishment. This demonstrates the ability of the entrapped spores to reproduce the fungal life cycle after cold treatment. Although the latter method was shown to be promising, further studies are necessary to include data on long-duration cryopreservation periods, i.e. allowing permanence of living cultures without morphological or physiological changes. In addition, such methods as well as derivatives thereof should be extended to other strains from all genera, with scrupulous attention to the strains which fail to be subcultured over several generations (Table 1), or those with low sporulation levels.

6
Strengths and Weaknesses of Monoxenic Culture Collections

Taking into consideration the two prerequisites, the VIPS control measures and the potential of propagules to be preserved for long periods, monoxenic cultures evidently gain in strength and will become an absolute must for germplasm collections in the near future. Nevertheless, the current development of this technology still faces some major problems, which must first be overcome. Table 2 summarizes the strength and weaknesses of monoxenic versus in vivo culture collections.

Pot cultures present the advantage of being usable, with some exceptions, for the vast majority of species from all genera with production of spores and potential for subcultivation. For instance, the species diversity in INVAM is considerable, with nearly 70 species described and 45 still to be described. Similarly, the BEG has over 200 isolates, representing nearly 50 species. By contrast, monoxenic cultures (if one refers uniquely to published papers and following the rules addressed above) represent

Table 2. Strengths and weaknesses of monoxenic versus in vivo AM fungi germplasm collections

	Pot culture		Monoxenic culture	
	Strength	Weakness	Strength	Weakness
Prerequisite				
Life cycle completion	Most strains	Time and space consuming–destructive sampling	Increasing amount of species and non-destructive observation	Numerous strains with few spores
Sub-cultivation maintenance	Most strains	Time and space consuming–destructive sampling	Time and space saving, and non-destructive observation	Some strains resistant to subculturing
VIPS				
Viability (germination potential)	Numerous strains maintained	Requires substrate sampling	Easy to assess	Low sporulation level for some strains
Identity	Classical tools comparable to literature	Limited array of descriptive tools	Multidisciplinary approach	None
Purity	None	Not guaranteed	High	None
Stability	Relatively easy to maintain	Space and time consuming	Same growth conditions throughout generation	Sub-cultivation may decrease infectivity and effectiveness
Long-term preservation	Demonstrated for various species and genera	High risks of contaminants	Feasible	Investigated for one published species

only 15 species, among which only 5.5% – compared to the 180 species recorded – are actually known to be adapted for continuous culture by subcultivation. This low number, however, is counterbalanced by a considerably higher number of unpublished or even unidentified species, as well as by the improvement of cultivations techniques this last decade, which promises to increase the number of species further. The reduced time and space required for the maintenance of monoxenic cultures, once they are in vitro, offer an indubitable advantage over pot cultures. We calculated that a simple, dark growth chamber with a volume of 2 m^3 would be enough to maintain five replicates, i.e. five Petri plates, of all the 180 known species, while a surface of approx. 100 m^2 would be necessary for the same amount of in vivo cultures. In addition, once in culture, monoxenic species need no

manipulation and should be processed only once a year for subcultivation, while the host plant under in vivo conditions needs regular feeding with water and nutrients, and cleaning and managing against parasites, with a high risk of cross-contamination.

Once the AM fungi have been shown to be cultivable and subcultivable over several generations, thus adapted to enter a culture collection, severe quality measures should be applied. This total quality management of a strain is summarized in four properties, which are (1) viability, (2) identity, (3) purity and (4) stability. In vivo culturing offers strong material for viability assessment and for decades has been the preferred material for the identification of species. However, the below-ground and obligate nature of AM fungi has often restricted identification tools to classical morphology. Techniques such as sequencing of SSU and LSU genes, although promising and powerful, have often resulted in misinterpretation, due to the difficulty of excluding the presence of fungal contaminants. As a result, a number of misleading AM fungal sequences have been obtained, some clustering outside the glomeromycotan taxon (Redecker et al. 1999; Hijri et al. 2002). Other techniques such as fatty acid and sterol profiles, although inconclusive for identification at the species level, are seldom adopted, because of microbial contaminants. In addition, the extraradical mycelium architecture, and sporulation dynamics may represent additional tools for complete description of AM fungi, but these are difficult to use with in vivo cultures. The monoxenic culture offers unique material for multiple descriptive approaches, as demonstrated for *G. proliferum* (Declerck et al. 2000). This material is highly suitable for assessments of morphology, ultrastructure and spore ontogeny. The absence of undesirable micro-organisms makes this material ideal for molecular as well as biochemical analyses. Finally, the possibility of non-destructively following the fungal life cycle helps to add new tools based on mycelium development and architecture and sporulation processes. A major weakness of in vivo cultures lies in the purity of the strains, which is difficult to guarantee. Cultures need to be started with mono-propagules and purity assessed at regular intervals, since AM fungal contaminants may appear transported through the air, or from insects, watering, pot sampling, or simply daily management. In addition, pot cultures are always contaminated with bacteria and frequently with saprophytic fungi, which reduces the level of purity of these cultures. As long as cultures are started with a disinfected, contaminant-free single spore or vesicle, the purity of monoxenic cultures is almost guaranteed. Contaminants may sometimes appear but are easily detectable on the gelled medium, and these cultures can be withdrawn. The stability, i.e. the preservation of all symbiotic fungal properties, is difficult to maintain in vivo as well as with monoxenic cultures, once the organism is transposed to growth conditions which differ from the natural situation where it evolved.

Moreover, different AM fungal strains appear to vary considerably in their stability from generation to generation, as is the case with *G. intraradices* (Chabot et al. 1992) and *G. versiforme* (Plenchette et al. 1996). Such results should be considered with the highest vigilance, however, since no thorough investigation was conducted on other strains cultured under monoxenic conditions and, in both cases, no analysis on genetic stability has ever been done with successive generations. The only reliable method to preserve, almost with certainty, the original properties is the long-term preservation using systems which halt the metabolism. This aspect needs strong improvement for AM fungi cultured monoxenically. Cryopreservation, nowadays the most reliable way to maintain species over long periods, is routinely applied at INVAM, while for monoxenic cultures it has been reported successful for only one species producing high numbers of spores (Declerck and Angelo-Van Coppenolle 2000). Long-term preservation using blocks of gel stored at 4 °C or regular subcultures is nowadays the common method used in GINCO. Thus, research efforts oriented towards simple and reliable methodologies to cryopreserve monoxenic-maintained AM fungal strains are necessary to implement in vitro germplasm collection plans.

7 Conclusion

Germplasm collections, whichever micro-organism is concerned, are an indisputable means to preserve biodiversity, and to improve knowledge on organism properties which may be of direct importance for human beings, as well as for the general welfare of humanity. AM fungi are a particular group of soil micro-organisms which cannot be cultured in the absence of a suitable host. Therefore, germplasm collections are often based on pot cultures, which have shown their importance for decades. The emergence of monoxenic cultures and the increase in cultured strains offer a unique opportunity to enter AM fungi into a new era of quality management, particularly adapted to germplasm collections. The reader should be convinced, as we are, that in vivo and monoxenic culture collections are essential and share, as they function today, complementary properties which should be exploited in the sense of preservation, management and investigation of this important group of soil fungi. Therefore, whichever type of collection is solicited, we plead for the international community to register their strains, and to refer to species located in a collection with a clear number identification, this to facilitate tracing in experimental studies and the compilation of information linked to each species used. Only by working along these lines will the international community benefit from teams dedicated to the preservation of biodiversity of this prominent group of micro-organisms.

References

Addy HD, Boswell EP, Koide RT (1998) Low temperature acclimation and freezing resistance of extraradical VA mycorrhizal hyphae. Mycol Res 102:582–586

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation in Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 112:77–83

Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321

Chandler D (1994) Cryopreservation of fungal spores using porous beads. Mycol Res 98:525–526

Dalpé Y (1987) Spore viability of some Endogonaceae submitted to a single stage lyophilisation. In: Sylvia DM, Hung LL, Graham JH (eds) Proc 7th North Americal Conf Mycorrhiza, Gainsville, Florida, p 279

Dalpé Y, Declerck S (2002) Development of *Acaulospora rehmii* spore and hyphal swellings under root-organ culture. Mycologia 94:850–855

Declerck S, Angelo-Van Coppenolle (2000) Cryopreservation of entrapped monoxenically produced spores of an arbuscular mycorrhizal fungus. New Phytol 148:169–176

Declerck S, Strullu DG, Plenchette C, Guillemette T (1996) Entrapment of in vitro produced spores of *Glomus versiforme* in alginate beads: in vitro and in vivo inoculum potentials. J Biotechnol 48:51–57

Declerck S, Bottu P, Ouwerx C, Mestdagh M, Corbisier-Colson AM, Delvaux B (1997) Short term cold storage and N2 drying of in vitro produced spores of an arbuscular mycorrhizal fungi entrapped in alginate beads. International workshop on bioencapsulation VI: from fundamental to industrial applications. Universitat Autonoma de Barcelona, Spain

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

Declerck S, Bivort C, D'Or D, de Souza FA (2004) Development of extraradical mycelium of *Scutellospora reticulata* under root-organ culture: spore production and function of auxiliary cells. Mycol Res 108:84–92

de Souza FA, Berbara RLL (1999) Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia 91:343–350

de Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012

Diop TA, Plenchette C, Strullu DG (1994) Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Douds DD (1997) A procedure for the establishment of *Glomus mosseae* in dual culture with Ri T-DNA-transformed carrot roots. Mycorrhiza 7:57–61

Douds DD, Schenck NC (1990) Cryopreservation of spores of vesicular-arbuscular mycorrhizal fungi. New Phytol 115:667–674

Fortin JA, Bécard G, Declerck S, Dalpé Y, St Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Hijri M, Redecker D, Petetot JAMC, Voigt K, Wostemeyer J, Sanders (2002) Identification and isolation of two ascomycete fungi from spores of the arbuscular mycorrhizal fungus *Scutellospora castanea*. Appl Environ Microbiol 68:4567–4573

Juge C, Samson J, Bastien C, Vierheilig H, Coughlan A, Piché Y (2002) Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: a critical cold-storage period. Mycorrhiza 12:37–42

Karandashov V, Kuzovkina I, Hawkins HJ, George E (2000) Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots. Mycorrhiza 10:23–28

Kirk PM, Cannon PF, David JC, Stalpers JA (2001) Ainsworth & Bisby's dictionary of the fungi, 9th edn. CABI Bioscience UK Centre, Egham

Kuszala C, Gianinazzi S, Gianinazzi-Pearson V (2001) Storage conditions for the long-term survival of AM fungal propagules in wet sieved soil fractions. Symbiosis 30:287–299

Mathur N, Vyas A (1995) In vitro production of *Glomus deserticola*in association with *Ziziphus nummularia*. Plant Cell Rep 14:735–737

Mathur N, Vyas A (1999) Improved biomass production, nutrient uptake and establishment of in vitro raised *Ziziphus mauritiana* by VA mycorrhiza. J Plant Physiol 155:129–132

Miller-Wideman MA, Waltrud LS (1984) Sporulation of *Gigaspora margarita* on root cultures of tomato. Can J. Microbiol 30:642–646

Mosse B (1988) Some studies relating to "independent" growth of vesicular-arbuscular endophytes. Can J Bot 66:2533–2540

Mosse B, Hepper C (1975) Vesicular arbuscular mycorhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Niino T, Sakai A (1992) Cryopreservation of alginate-coated in vitro-grown shoot tips of apple, pear and mulberry. Plant Sci 87:199–206

Pawlowska TE, Douds DD, Charvat I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Plenchette C, Declerck S, Diop TA, Strullu DG (1996) Infectivity of monoaxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri T-DNA transformed carrot root. Appl Microbiol Biotechnol 46:545–548

Raman N, Sahadevan C, Srinivaan V (2001) Growth of AM fungi on in vitro root organ culture of *Sorghum vulgare* and *Saccharum officinarum*. Indian J Exp Biol 39:1293–1298

Redecker D, Hijri M, Dulieu H, Sanders IR (1999) Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of ascomycete origin. Fungal Genet Biol 28:238–244

Samson J, Dalpé Y, Piché Y (2000) Isolement in vitro de deux nouvelles souches de *Glomus* en co-culture avec des racines de carotte transformées. Coll Mycorhizes 2000, Rivière-du-Loup, Québec

Smith D, Onions AHS (1994) The preservation and maintenance of living fungi. CAB International, Oxon, UK

Strullu DG, Romand C (1986) Méthodes d'obtention d'endomycorrhizes B vésicules et arbuscules en conditions axéniques. C R Acad Sci Paris 303:245–250

Strullu DG, Romand C (1987) Culture axénique de vésicules isolées à partir d'endomycorhizes et ré-association *in vitro* à des racines de tomate. C R Acad Sci Paris 305:15–19

Strullu DG, Diop TA, Plenchette C (1997) Réalisation de collections in vitro de *Glomus intraradices* (Schenck et Smith) et de *Glomus versiforme* (Karsten et Berch) et proposition d'un cycle de développement. C R Acad Sci Paris 320:41–47

Tommerup IC (1988) Long-term preservation by L-drying and storage of vesicular arbuscular mycorrhizal fungi. Trans Br Mycol Soc 90:585–591

3 The Monoxenic Culture of Arbuscular Mycorrhizal Fungi as a Tool for Systematics and Biodiversity

Yolande Dalpé[1], Sylvie Cranenbrouck[2], Sylvie Séguin[1], Stéphane Declerck[3]

1 Introduction

Arbuscular mycorrhizal (AM) fungi systematics and biodiversity studies are amongst areas triggered by the monoxenic culture of AM fungi. The diagram in Fig. 1 schematises the interactions between systematics and biodiversity studies where monoxenic culture can support research advances, from fundamental taxonomy to field application.

During the last decade, an increasing number of strains and species have gradually been made available, and monoxenic cultures of AM fungi have repeatedly demonstrated their suitability for multiple types of investigations. In terms of systematics, monoxenic cultures provide access to abundant and high-quality fungal material suitable for taxonomic and evolutionary studies (Fortin et al. 2002). In terms of biodiversity, monoxenic cultures provide a tool for basic comparative analyses of root populations and strain potential, long-term propagation capabilities, and fungal adaptation to environment. As such, monoxenic culture systems are gradually emerging as a complementary and indispensable tool to investigate AM fungi.

The two major objectives of this chapter are to describe the involvement of AM fungi monoxenic cultures in systematics and biodiversity studies, and to identify working fields where monoxenic cultures may fill some gaps which impede the development of a wide array of characterization tools for classification purposes and functional symbiosis evaluation.

[1]Agriculture and Agri-Food Canada, Environment Health/Biodiversity, 960 Carling Avenue, Ottawa K1A 0C6, Canada, Tel.: +1-613-7591381, Fax: +1-613-7591701, E-mail: dalpey@agr.gc.ca
[2]Université Catholique de Louvain, Unité de Microbiologie, 3 Place Croix du Sud, 1348 Louvain-La-Neuve, Belgium
[3]Université Catholique de Louvain, Mycothèque de l'Université Catholique de Louvain (MUCL), Unité de Microbiologie, 3 Place Croix du Sud, 1348 Louvain-La-Neuve, Belgium

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

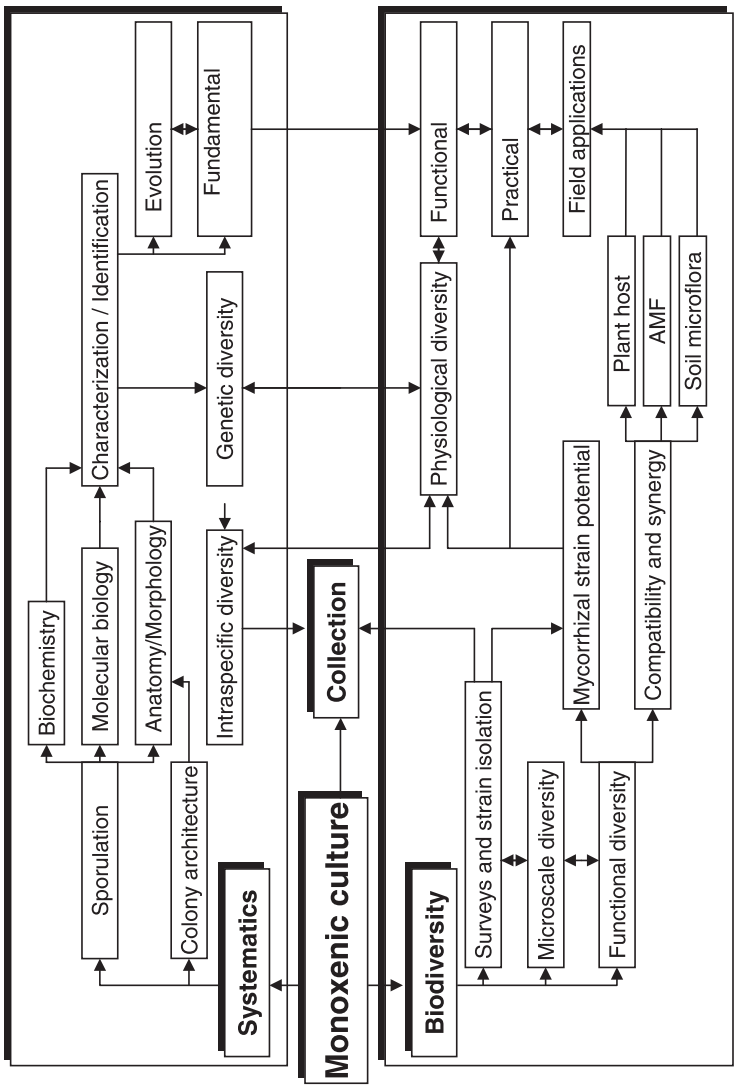


Fig. 1. Diagram of the interrelationships between systematics, biodiversity, and monoxenic cultures

2 Systematics

2.1 Species and Strain Availability

The monoxenic culture methodology has improved tremendously since the pioneering investigations of Mosse (1962) in the early 1960s, followed by several studies in the 1970s and 1980s (Mosse and Hepper 1975; Hepper

and Mosse 1980; Hepper 1981). For the past two decades, several AM fungal species were claimed to have been cultivated monoxenically (Fortin et al. 2002), but some did not support spore production and most were not subcultured successfully over long periods. For the unique *Glomus intraradices* species, several dozen of strains are maintained under monoxenic culture, and for dozens of generations. Species having small-diameter, thin-walled morphotypes, often but not exclusively established in monoxenic culture using the intraradical phase as inoculum, are usually grown easily in monoxenic culture and adapted to sub-cultivation. By contrast, large-spore species such as *Glomus mosseae*, *Glomus caledonium*, and *Acaulospora* and *Scutellospora* representatives, habitually established in monoxenic culture using spores as inoculum, are often shown to gradually lose their colony vitality and decline after two to three subcultures. This is the case with, for example, *A. rehmii*, *S. erythropa*, *G. caledonium*, *G. mosseae* and *Gigaspora spp.* (Dalpé and Declerck 2002; Trépanier and Dalpé, unpublished data). Reasons for the difficulty in obtaining long-term maintenance of most large-spore isolates under monoxenic cultivation remain a matter of speculation. This may be associated, in the case of *A. rehmii*, with a reduced extraradical hypha (EH) network (Dalpé and Declerck 2002), with the inappropriateness of nutritional and/or environmental culture conditions, and with the mis-adaptation of those isolates to the excised root system.

2.2 Fungal Mycelia and Spores

Owing to its inaccessibility, the systematic value of AM fungal colonies has always lagged behind in situ observation using soil-based cultivation methodologies. With monoxenic cultures, the in situ and ongoing micro-scale monitoring of fungal growth can easily be undertaken. Studies can now be performed on (1) the global mycelium architecture of fungal colonies, (2) the micro-morphology of elements constituting the mycelium, (3) the kinetics of development of colonies and of fungal structures, and (4) the intra- and interspecies comparison of the EH architecture.

The comparative morphology grid of Table 1 shows that AM fungal isolates share a common, general mycelium phenotype with runner hypha (RH) skeletons, branched hyphae (BH), branched adsorbing structures (BAS), and vesicle-like structures (VLS), all elements differentiated under a growth continuum with no real, ordered pattern. Major differences between AM fungal cultures deal with the mycelium architecture, such as hyphal network density, pattern of ramification, spore abundance, and

Table 1. Monoxenic culture characteristics of AM fungal colony architecture and spore development

Species	Extraradical mycelium and colonization type							Spore				Sporulation		Reference
	RH	BH	BAS	VLS	AN	AUX	Paris/Arum	INT	API	S	C	AB	As/S	
Small to medium-sized spores (mean size up to 150 µm in diameter)														
Glomeraceae														
Glomus clarum	+	+	–		?	n.a.	A	+	–	+	–	?	As	de Souza and Berbara (1999)
Glomus deserticola	?	+	+	?	?	n.a.	A	?	?	+	–	?	?	Mathur and Vyas (1995)
Glomus fasciculatum	+	+	+	?	+	n.a.	A	+	+	+	+	3	As	Diop et al. (1994), Declerck et al. (1998)
Glomus fistulosum	+	+	?	+	+	n.a.	A	Non-sporulating species						Nuutila et al. (1995)
Glomus intraradices	+	+	+	++	+	n.a.	A	+	+	+	–	3	As	Chabot et al. (1992)
Glomus proliferum	+	+	+	–	+	n.a.	A		–	+		3	As	Declerck et al. (2000)
Glomus versiforme	+	+	+	–	+	n.a.	A	+	+	+		3	As	Declerck et al. (1998)
Glomus sinuosum	+	+	?	+	?	n.a.	?	?	?	–		3	As	Wu (pers. comm.)
Large spores (mean size more than 150 µm in diameter)														
Diversisporeae														
Acaulospora rehmii	+	+	+	+	+	n.a.	n.a.	–	+	+	–	1	As	Dalpé and Declerck (2002)
Gigaspora gigantea	+	+	+	?	+	+	n.a.	–	+	+	–	1	As	Diop et al. (1992)
Gigaspora margarita	+	+	+	?	+	+	n.a.	–	+	+	–	1	As	Karandashov et al. (1999)
Scutellospora erythropa	+(2)	+	+	+	+	+	n.a.	–	+	+	–	1	S	Trépanier (pers. comm.)
Scutellospora reticulata	+	+	+	+	+	+(3)	n.a.	–	+	+	–	1	As	de Souza and Declerck (2003)
Glomus caledonium	+	+	+(1)	+	+	n.a.	P	–	+	+	–	1	S	Hepper (1981), Karandashov et al. (2000)
Glomus constrictum	+	+	?	?	?	n.a.	A	–	+	+	–	?	?	Mathur and Vyas (1999)
Glomus etunicatum	?	?	?	?	?	n.a.	AP	–	+	+	–	1	As	Pawlowska et al. (1999)
Glomus macrocarpum	+	+	–	–	?	n.a.	A	–	+	+	–	2	As	Declerck et al. (1998)
Glomus mosseae	+	+	+	+	+	n.a.	A	–	+	+	–	1	As	Mosse and Hepper (1975), Dalpé (pers. comm.)

RH, runner hyphae; BH, branching hyphae; BAS, branching absorption structures; VLS, vesicle-like structure; AN, anastomose; AUX, auxiliary cells; P/A, Paris/Arum; INT, intercalary position of spores; API, apical position of spore; S, single spore; C, clustered spores; AB, abundance of spores (1, low; 2, medium; 3, high); As/S, asynchronous/synchronous sporulation; +, present; –, absent; n.a., not applicable; (1), stunted type of BAS; (2), hyphae highly pigmented; (3), extra- and intraradical auxiliary cells of different morphology

positioning and clustering of spores. Large-spore species usually exhibit a less dense mycelium and fewer anastomoses. VLS vary, from one isolate to another, by their size, shape and wall pigmentation. Stunted BAS, together with typical Paris-type root colonization, characterize *G. caledonium* isolates (Karandashov et al. 2000).

Spore maturation of monoxenic cultured AM fungi follow similar ontogeny steps as pot-culture ones. Differences reside essentially in the clean, contaminant-free quality of monoxenic cultured spores, with abundant fungal material available at precise age and physiological stages. The monoxenically cultured species comparison of Table 1 shows them readily segregating between large- and small-spore species in terms of their apical mode of development, their single spore differentiation, and their low sporulation levels. By contrast, smaller spore species present a variable growth pattern, mainly with intercalary sympodial spore growth, clustered spores, and high sporulation levels. With the current limitation of morphological spore features able to sustain morphological characterization of AM fungi isolates, such culturing characteristics may become taxonomically valuable tools once proven to be stable, i.e. heritable through generations and growing conditions.

The stability of the ontogenetic intercalary spore character, through successive subculturing, brought de Souza and Berbara (1999) to suggest that this feature may represent a divergent group among *Glomus* species, based on rDNA 18S categorization. This hypothesis has never been demonstrated, due mainly to the restricted number of monoxenically cultured isolates in the *Glomus* subgroups B proposed by Schüßler et al. (2001). None of the dimorphic species described have yet been cultivated in monoxenic cultures; such investigations would help to clarify their taxonomic status. Sporocarp development under monoxenic culture has been detected with a *G. sinuosum* isolate, and initiation of peridial hyphae observed with a *G. mosseae* isolate (Wu and Dalpé, unpubl. data).

Spore wall morphology of monoxenically differentiated spores does not differ fundamentally from field-collected ones, apart from the lower mean spore diameter measured for some AM fungal isolates (Chabot et al. 1992; Pawlowska et al. 1999). With monoxenic cultures, all elements of spore wall architecture remain observable throughout maturation, including the evanescent outer wall, usually absent in soil-propagated AM fungal spores, due to abrasion and/or digestion by soil micro-organisms. The identification performance of AM fungi, using spore morphology and wall anatomy, has resulted in the accumulation of described species, but simultaneously in a reduced capability to segregate between species. Identification, quite difficult to achieve with field-collected spores, is not really simplified with monoxenic cultures. However, descriptions and tentative identifications rely on whole colony parameters, with access to spore ontogeny and spore

maturation data, resulting in a better evaluation of the taxonomic weight of retained characters.

Monoxenic cultures eliminate constraints induced by abiotic growing conditions and, as such, help to distinguish heritable from acquired characters. The comparative analysis of cellular and subcellular changes in colony architecture, when isolates are submitted to different host roots, would help to segregate between plant- and fungal-driven characters. However, care should be taken with the interpretation of such data, because of phenotypic and genotypic differences at fungal isolate level (Cranenbrouck et al. 2000). Morphotyping AM fungal colonies may be taxonomically efficient, but only if host-related behaviour and environmental culture conditions are uniform.

2.3 Biochemical Studies

The usefulness of the integrated analysis of genotypic and phenotypic characters has been emphasized for the study of fungal systematics and evolution within major groups of fungal organisms. The multiplicity of analysis to which monoxenic cultures can be adapted make such multidisciplinary investigations achievable. AM fungi lipid content may reach up to 60% of the fungal biomass and, as such, these are considered oleaginous fungi (Sancholle and Dalpé 1993). Fatty acid profiles were demonstrated to be stable through successive fungal generations and with different host partners (Bentivenga and Morton 1994). The Δ-11 hexadecenoic acid (16:1 Δ-11), never detected in any fungal organism other than *Glomus* where it constitutes 40–80% of total fatty acids, has been considered a potential qualitative and quantitative indicator of *Glomus* root colonization (Gaspar et al. 1997; Jansa et al. 1999) and soil distribution (Olsson 1997). However, the C16:1 ω5 fatty acid spore content was found to be much higher in juvenile than in mature spores of *G. intraradices* (Grandmougin et al. 1996), thus generating mis-interpretation of the spore fatty acid profile through time. A recent study, which used monoxenic cultures, has established that the increment of 24-methyl/methylene sterols was an appropriate indicator of AM-colonized transformed roots (Fontaine et al. 2004).

Fatty acid methyl ester (FAME) profiles were investigated in view of developing tools for species identification (Graham et al. 1995; Bentivenga and Morton 1996). As for the other fungal taxa investigated, AM fungal discrepancies in lipid profiles between juvenile and mature spores, and the variability encountered between isolates of one same species, revealed such chemotaxonomic approaches valuable in discriminating only at the

family level. Fungal sterol profiles have rarely been considered as potential taxonomic tools. AM fungi exhibited a rather stable profile through species (see Sancholle et al. 2001 for a review). Cholesterol, 24-ethylcholesterol, 24-methylcholesterol constitute the primary sterols of AM fungal spores, but variable levels at the isolate level are not supportive of any taxonomic distinction. The 24-ethyl and methylcholesterol AM fungal profile, together with the demonstrated absence of ergosterol, supported the gradually adopted position of AM fungi as a primitive order of Zygomycetes (Grandmougin et al. 1999), and is well served by their separation from Zygomycota and placement in the phylum Glomeromycota (Schüßler et al. 2001).

It has been recently proposed that protein profiles of AM fungi may constitute an accurate tool to characterize species, as this did appear stable through successive fungal generations of a single isolate, and between isolates of different geographic origins and storage conditions (Avio and Giovannetti 1998; Xavier et al. 2000; Giovannetti et al. 2003). To date, this promising protein fingerprinting tool has been investigated only with soil-based propagated material. Although proteins are considered a useful tool in discriminating between isolates and/or species, their profiles vary considerably during life cycle stages (Avio and Giovannetti 1998). Investigations performed on monoxenically propagated AM fungi would allow to follow the chronology of protein profiles through stages of fungal development, and to confirm the potential value of protein profiles as a stable complementary tool to morphological and phylogenetic studies. Similarly, species-specific isozymes have been found to be potentially useful to discriminate between AM fungal species, using root-colonized tissues (Tisserant et al. 1998; Kjoller and Rosendahl 2000). As with lipids, sterols and proteins, isozyme detection was found to vary according to fungal isolates and plant physiological status.

2.4 Molecular Studies

Since the early 1990s, molecular studies of AM fungi showed a rapid expansion which had a profound effect on mycorrhiza studies, particularly in the field of phylogenetic analysis. Molecular techniques based on PCR enable amplification of nucleic acids using minute amounts of fungal material, as little as single spores. Trials to adapt PCR-based techniques to AM fungi detection and identification faced new challenges. The first AM fungi genes to have been sequenced were the small subunit rRNA gene (SSU) and the rDNA comprising ITS1, 5.8S and ITS2 positioned between the 18S and 28S regions.

The SSU and ITS were targeted for most phylogenetic analyses (Simon et al. 1993; Lloyd-MacGilp et al. 1996; Redecker et al. 1997). Specific primers were designed from the sequence data of SSU and ITS genes, enabling detection and identification of AM fungi species from spores and colonized root material (Sanders et al. 1996). Other taxonomic DNA-based approaches were the random amplification of polymorphic DNA (RAPD) analyses with the development of species-specific primer pairs (Lanfranco et al. 1995; Abbas et al. 1996). Detection of AM fungi from soil, including genetic variations, was also performed with amplified fragment length polymorphism (AFLP; Rosendahl and Taylor 1997; Koch et al. 2004), and microsatellite PCR (Douhan and Rizzo 2003). Most of these experiments were conducted with AM fungi DNA originating from pot cultures, with the consequent high risk of contaminant sequences and mis-interpretations of phylogenetic trees (Schüßler et al. 2003).

With the advent of monoxenic cultures of AM fungi, the availability of successive generations of contaminant-free material generated diversified and strengthened molecular studies. For example, Corradi et al. (2004), studying the monophyly of β-tubulin and H+-ATP gene with AM fungi monoxenic cultures, described two β-tubulin and one H+-ATP genes respectively, in contrast to previous investigations, in which three β-tubulin (Rhody et al. 2003) and five H+-ATP genes (Ferrol et al. 2000) were recorded. Corradi and coworkers argued that the additional forms of β-tubulin and H+-ATP genes most likely originated from contaminants.

Recently, the SSU and LSU relationships between four *G. intraradices* isolates and five *G. intraradices*-like isolates cultured monoxenically have been studied by Cranenbrouck et al. (unpubl. data). The results of the SSU sequencing showed a high intraspecific variation in the LSU, as previously described for other *Glomus* species from pot culture (Clapp et al. 2001), but all the strain sequences formed a homogeneous group, including the *G. intraradices* sequence. These data gave the same arrangement as the SSU data in which all studied strains clustered together with a *G. intraradices* and a *Glomus fasciculatum* sequence belonging to the Glomeraceae group A as defined by Schüßler et al. (2001). No sequence was found outside the Glomeraceae group A. This demonstrates the indisputable benefit of using monoxenically cultured species. To date, this is the only system which offers long-term maintenance of the fungi, under strict controlled conditions, without contamination and which permits comparative analysis of morphological, phenotypic, biochemical and molecular studies of one same isolate (Declerck et al. 2000; Koch et al. 2004). However, in phylogenetic studies where it is important to compare a large number of different species, it is important to increase the diversity of AM fungi species cultured monoxenically.

From a taxonomic point of view, it is imperative to crosscheck species characteristics of monoxenic and pot-culture systems, as fungal characteristics may result from environmental adaptation, leading to misinterpretation of fungal identity. With this view, very few specific primers for AM fungi detection and identification have been developed with monoxenic culture. Filion et al. (2003) and Alkan et al. (2004) developed, for example, PCR primers which allow to detect and quantify the fungus *G. intraradices* in soil using the powerful technique of real-time PCR.

3 Biodiversity

The monoxenic cultivation system may, at first glance, not be considered an efficient biodiversity working tool in soil ecosystems. On due reflection, this system may be eminently useful and provide considerable support to biodiversity studies by (1) improving trapping procedures of root-inhabiting AM fungi, (2) providing comparative analysis of AM fungi micro-morphology, and (3) evaluating isolate functional diversity relative to mycorrhizal potential and interactions with the environment. Such a multidisciplinary research tool fits quite well with the claimed usefulness of combining multiple methodologies to help unravel the complexity of diversity studies.

3.1 Trapping of Isolates

Soil AM fungi inventories revealed a surprisingly diverse community, as up to 37 AM fungi morphotypes were trapped from an old, abandoned field by Bever et al. (2001), and 24 AM fungi phylotypes were recovered from roots of two plant species from a grassland ecosystem by Vandenkoornhuyse et al. (2002). Up to three AM fungal isolates of different morphotypes were cultivated monoxenically from 4–5 mm long root segments taken from a single field-collected plant. Roots of the same origin, once pot-cultured, generated quite similar numbers of fungal isolates but not always representing the same morphotypes (Dalpé and Séguin, unpublished data). Such comparisons revealed the complementary role of monoxenic cultures over pot to trap AM fungi, one major advantage residing in the much reduced time lapse required to obtain monoxenic AM fungal cultures, with enough clean fungal material to perform reliable biochemical and molecular analyses. Unfortunately, not all root-inhabiting AM fungi are easily established in monoxenic culture and, as for pot-culture propagated fungi, not all AM fungal isolates survive to successive subculturing.

This is the case with several Acaulosporaceae and Gigasporaceae species isolates (Dalpé and Declerck 2002; de Souza and Declerck 2003) as well as with some large-spore species of the *Glomus* genus. There is in that area a tremendous need to improve cultivation methodologies, notably in developing specific growth media capable of long-term maintenance of cultures.

In terms of AM fungal population studies, there is probably no single case where a full inventory of soil diversity has been achieved. Nonetheless, because of the many advantages attached to AM fungal trapping with monoxenic cultures – amongst others, a constant availability of clean fungal material, an excellent suitability for biochemical and molecular investigations, and a reliable and affordable methodology whose practice requires only basic laboratory equipment – this technology should indeed be seriously considered when biodiversity studies are planned.

3.2 Micro-Morphology

Mycorrhizal associations are recognized as regulators of the structure and functioning of plant communities (Klironomos et al. 2000). Therefore, knowledge of factors influencing AM fungi behaviour gains importance for understanding taxonomic and functional diversities of whole ecosystems. The establishment of AM fungal colonies under monoxenic conditions probably constitutes the simplest working system available for micromorphology in situ studies, where a one-to-one organism interaction can be scrutinized. Of course, the monoxenic system would never allow large-scale investigations with intensive surveys and evaluation of elements which regulate ecosystem processes. Its performance remains restricted to micro-scale studies, to fungal isolate behaviour and simulations of environmental growing conditions.

Colony architecture, intensively described for *G. intraradices* (Friese and Allen 1991; Bago et al. 1998), is shared by all AM fungal isolates cultivated monoxenically (Chabot et al. 1992; Declerck et al. 2000; de Souza and Declerck 2003; Declerck et al. 2004). Elements of interest come from the variability of colony architecture between isolates, in response to biotic and abiotic factors, which allow direct evaluation of the fungal isolate studied. Whatever nutritive or environmental factors are concerned, the system allows in situ observation and direct measurement on AM fungi. With easy accessibility to test fungi, all kinds of measurements can be performed on a variety of AM fungi, providing the basis for isolate evaluation suitable to guide users in the choice of adapted isolates for specific purposes.

3.3 Functional Diversity

AM fungal diversity studies may be achieved by evaluation of either soil fungal biomass, species diversity or species functionality, depending on the objective. Diversity studies can be performed with molecular-based identification tools such as phylogenetic analysis. Such approaches, however, represent an initial step only, as the data obtained refer to species detection and amplitude of diversity. When applied investigations are foreseen, there is an absolute need to have the fungal elements of this diversity at hand. This is where monoxenic cultivation becomes advantageous for the establishment of referenced monoxenic cultures.

The evaluation of fungal diversity is not limited to taxonomy data. The practical measurement of functional diversity can be provided by traits associated with functional symbiosis, such as strain adaptability to media, environment and soil micro-flora. As such, screening among AM fungi monoxenically cultivated isolates for specific functionality can lead to probing for genes which code for a chosen functional symbiotic ability.

With the monoxenic cultivation technology, practitioners have access to a variety of measurable elements suitable for strain performance evaluation. For example, the paradoxical limited independent growing ability of some AM fungi strains may favour their mycorrhizal effectiveness because they can survive longer when the plant host remains non-accessible (Strullu et al. 1997). The rate of appressorium formation by the pre-symbiotic mycelium improves root opportunity to be colonized (Giovannetti and Citernesi 1993). Vegetative compatibility between AM fungal isolates is detectable through anastomoses, which have been detected in all AM fungal genera cultivated monoxenically (Chabot et al. 1992; Diop et al. 1994; Declerck et al. 2000; de Souza and Declerck 2003; Declerck et al. 2004). This self-anastomosing property may be a powerful tool to perform comparison of population diversity at the very fine level of fungal isolates, and to provide data on the spatial distribution of a specific isolate. However, major restrictions come from the fact that such investigations are highly time-consuming, without providing any assurance of the reliability of data collected.

Observations of the developmental dynamics of the EH and root colonization activity are recognized to provide a direct evaluation of the mycorrhizal potential of strains (Dodd et al. 2000). With monoxenic cultures, the lengths of colonized roots have been correlated with EH and spore density (Mugnier and Mosse 1987; Mathur and Vyas 1999; Pawlowska et al. 1999; Glorian 2002). Such results are complemented by a successful dual culture of a high- (*G. intraradices*) and a low-sporulating (*Gigaspora margarita*) isolate, each found to occupy its own niche, without interacting with each

other (Tiwari and Adholeya 2002). EH density, therefore, as observed under monoxenic cultivation, may be retained as a potential AM fungal performance indicator and, with such a scenario at hand, vegetative hyphae of non-sporulating strains would have the same weight as sporulating ones, avoiding any biased evaluation due to sampling procedures.

Technically, EH evaluation can be performed by direct estimation of mycelium density with the help of UV autofluorescence (Sejalon-Delmas et al. 1998), and by histochemistry methods. For example, succinate dehydrogenase activity measures viable intraradical fungal structures (Schaffer and Peterson 1993), fluorescein diacetate is suitable for active cell detection (Dickson and Smith 1998), alkaline phosphatase is used to evaluate the symbiotic efficiency of fungal colonization (Tisserant et al. 1993), and neutral red staining to detect arbuscules (Guttenberger et al. 2000). Carbon, nitrogen, phosphorus and heavy metal absorption and transport can be monitored with monoxenic cultures (Villegas et al. 1996, 2001; Pfeffer et al. 1999; Nielsen et al. 2002; Labour et al. 2003; Rufyikiri et al. 2003), providing access simultaneously to information on substrate acidification and fungal growth and morphology changes (Bago et al. 1996).

AM fungal host-dependent behaviour has been observed under monoxenic culture (Bécard and Piché 1989; Schreiner and Koide 1993). The sporulation intensity, root colonization levels and EH propagation of *G. intraradices* isolates varied according to the transformed tomato lines used (Labour et al. 2003). Host-dependent behaviour can also be attributed to root anatomy. For example, the thick root epidermis of an *Echinacea purpurea* root culture reduced hyphal tip ability to establish symbiotic contact (Dalpé and Séguin, unpubl. data). The differentiation of Paris-type root colonization with carrot root cultures colonized by *G. caledonium* isolates (Karandashov et al. 2000) confirmed the findings of Cavagnero et al. (2001), attributing to both symbiotic partners the control of AM fungal morphology. On the other hand, how mycorrhizal plant dependency would influence AM fungal behaviour remains to be investigated with comparison of AM fungi monoxenic culture response to tomato and carrot root culture having low and high mycorrhizal dependency respectively.

AM fungi share the rhizosphere with a diversity of organisms, all participating in soil formation and soil fertility. The AM fungi monoxenic cultivation system allows the decortication of such a complex system. Species-to-species interactions can be isolated, and measurements performed at both organism and cell levels. Such an approach will never mimic the complexity of natural systems, but may provide species-to-species follow up of the organisms' morphological and nutritional behaviour, leading to a better understanding of biodiversity. Control of rhizosphere interactions by both competition and synergy has been observed with monoxenic cultures. Improved disease resistance (Benhamou et al. 1994; Elsen et al. 2001),

enhanced spore and conidial germination of *Fusarium* and *Trichoderma* isolates (St-Arnaud et al. 1995; Filion et al. 1999), increased stimulation of AM fungal spore germination, and hyphal growth and assimilation of low-soluble P in the presence of soil bacteria (Mayo et al. 1996; Villegas and Fortin 2001; Hildebrandt et al. 2002) have been recorded.

4 Conclusion

As schematized in Fig. 1, systematics and biodiversity are closely interrelated at taxonomic, genetic and physiological levels. Any traits useful to characterize isolates, species or populations provide information at one or the other level of mycorrhizal organization. Exhaustive investigations on systematics and biodiversity using monoxenic cultures remain dependent on the successful cultivation of a maximum number of AM fungal strains, representing a large array of existing spore morphotypes and species genetic categories. The access to such AM fungal monoxenic culture collections would provide a tremendously useful tool for comparison of culture-independent approaches (molecular) with tangible AM fungal reference material, to precisely characterize species and isolate elements, thereby allowing reliable taxonomic studies based on the cross-comparison of data generated from multidisciplinary approaches. Moreover, such banks of inocula would constitute a major key tool for the evaluation of fungal mycorrhizal potential, the development of selected strain performance, and the probing for selected genes.

References

Abbas JD, Gadkar V, Coburn J, Yarden O, Kapulnik Y (1996) Isolate specific detection of mycorrhizal fungi using genome specific primer pairs. Mycologia 88:939–946

Alkan N, Gadkar V, Coburn J, Yarden O, Kapulnik Y (2004) Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. New Phytol 161:877–885

Avio L, Giovannetti M (1998) The protein pattern of spores of arbuscular mycorrhizal fungi: comparison of species, isolates and physiological stages. Mycol Res 102:985–990

Bago B, Vierheilig H, Piché Y, Azcón-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bago B, Azcón-Aguilar C, Piché Y (1998) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bécard G, Piche Y (1989) Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and non-host Ri T-DNA transformed roots. Can J Bot 68:1260–1264

Benhamou N, Fortin JA, Hamel C, St-Arnaud M, Shatilla A (1994) Resistance responses of mycorrhizal Ri T-DNA-transformed carrot roots to infection by *Fusarium oxysporum* f.sp. *chrysanthemi*. Phytopathology 84:958–968

Bentivenga SP, Morton JB (1994) Stability and heritability of fatty acid methyl ester profiles of glomalean endomycorrhizal fungi. Mycol Res 98:1419–1426

Bentivenga SP, Morton JB (1996) Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. Proc Natl Acad Sci USA 93:659–662

Bever JD, Smith SE, Smith FA (2001) Host-specificity of AM fungal population growth rates can generate feedback on plant growth. Plant Soil 244:281–290

Cavagnaro TR, Gao LL, Smith FA, Smith SE (2001) Morphology of arbuscular mycorrhizas is influenced by fungal identity. New Phytol 151:469–475

Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix*in root organ culture. Mycologia 84:315–321

Clapp JP, Rodriguez A, Dodd JC (2001) Inter- and intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. New Phytol 149:539–554

Corradi N, Kuhn G, Sanders IR (2004) Monophyly of [beta]-tubulin and H+-ATPase gene variants in *Glomus intraradices*: consequences for molecular evolutionary studies of AM fungal genes. Fungal Genet Biol 41:262–273

Cranenbrouck S, Corbisier AM, Dalpé Y, Declerck S (2000) Morphological study and R-DNA sequencing herbes three monoxenic cultured herbes *Glomus* species. In: Proc 3rd Int Congr Symbiosis (TICS), August 2000, Marburg University, Germany

Dalpé Y, Declerck S (2002) Ontogeny of *Acaulospora rehmii* spores under root-organ culture. Mycologia 94:850–855

de Souza FA, Berbara RLL (1999) Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia 91:343–350

de Souza F, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

Declerck S, Bivort C, D'Or D, de Souza FA (2004) Development of extraradical mycelium of *Scutellospora reticulata* under root-organ culture: spore production and function of auxiliary cells. Mycol Res 108:84–92

Dickson S, Smith SE (1998) Evaluation of vesicular arbuscular mycorrhizal colonisation by staining. In: Varma A (ed) Mycorrhiza manual. Springer, Berlin Heidelberg New York, pp 77–83

Diop T, Bécard G, Piché Y (1992) Long-term in vitro culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. Symbiosis 12:249–259

Diop TA, Plenchette C, Strullu DG (1994) Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil 226:131–151

Douhan GW, Rizzo DM (2003) Amplified fragment length microsatellites (AFLM) might be used to develop microsatellite markers in organisms with limited amounts of DNA applied to arbuscular mycorrhizal (AM) fungi. Mycologia 95:368–373

Elsen A, Declerck S, De Waele D (2001) Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. Mycorrhiza 11:49–51

Ferrol N, Barea JM, Azcón-Aguilar C (2000) The plasma membrane H+-ATPase gene family in the arbuscular mycorrhizal fungus *Glomus mosseae*. Curr Genet 32:112–118

Filion M, St-Arnaud M, Fortin JA (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytol 141:525–533

Filion M, St-Arnaud M, Jabaji-Hare SH (2003) Direct quantification of fungal DNA from soil substrate using real-time PCR. J Microbiol Meth 55:67–76

Fontaine J, Grandmougin-Ferjani A, Glorian V, Durand R (2004) 24-methyl/methylene sterols increase in monoxenic roots after colonization by arbuscular mycorrhizal fungi. New Phytol 163:159–167

Fortin JA, Bécard B, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. Mycologia 83:409–418

Gaspar L, Pollero R, Cabello M (1997) Variations in the lipid composition of alfalfa roots during colonization with the arbuscular mycorrhizal fungus *Glomus versiforme*. Mycologia 89:37–42

Giovannetti M, Citernesi AS (1993) Time-course of appressorium formation on host plants by arbuscular mycorrhizal fungi. Mycol Res 97:1140–1142

Giovannetti M, Sbrana C, Strani P, Agnolucci M, Rinaudo V, Avio L (2003) Genetic diversity of isolates of *Glomus mosseae* from different geographic areas detected by vegetative compatibility testing and biochemical and molecular analysis. Appl Environ Microbiol 69:616–624

Glorian V (2002) Recherche de polypeptides induits par des champignons endomycorhiziens dans des racines transformées de chicorée (*Cichorium intybus* L.) et de carotte (*Daucus carota* L.). PhD Thesis, Laboratoire de Mycologie/Phytopathologie/Environnement, Université du Littoral Calais, France

Graham J, Hodge N, Morton J (1995) Fatty acid methyl ester profiles for characterization of glomalean fungi and their endomycorrhizae. Appl Environ Microbiol 61:58–64

Grandmougin-Ferjani A, Dalpé Y, Hartmann M, Laruelle R, Couturier D, Sancholle M (1996) Taxonomic aspects of the sterol and Δ11-hexadecenoic acid (C16:1 Δ-11) distribution in arbuscular mycorrhizal spores. In: Williams JP, Khan MU, Lem NW (eds) Physiology, biochemistry and molecular biology of plant lipids. Kluwer, Dordrecht, pp 195–197

Grandmougin-Ferjani A, Dalpé Y, Hartmann M, Laruelle R, Sancholle M (1999) Sterol distribution in arbuscular mycorrhizal fungi. Phytochemistry 50:1027–1031

Guttenberger M (2000) A rapid staining procedure for arbuscules of living arbuscular mycorrhizas using neutral red and acidotropic dye. Plant Soil 226:211–218

Hepper CM (1981) Techniques for studying the infection of plants by vesicular-arbuscular mycorrhizal fungi under axenic conditions. New Phytol 88:641–647

Hepper CM, Mosse B (1980) Vesicular arbuscular mycorrhiza in root organ cultures. In: Ingram DS, Helgeson JP (eds) Tissue culture methods for plant pathologists. Blackwell, New York, pp 167–171

Hildebrandt U, Janetta K, Bothe H (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919–1924

Jansa J, Gryndler M, Matucha M (1999) Comparison of the lipid profiles of arbuscular mycorrhizal (AM) fungi and soil saprophytic fungi. Symbiosis 26:247–264

Karandashov VE, Kuzovkina IN, George E, Marschner H (1999) Monoxenic culture of arbuscular mycorrhizal fungi and plant hairy roots. Russian J Plant Physiol 46:87–92

Karandashov V, Kuzovkina I, Hawkins HJ, George E (2000) Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots. Mycorrhiza 10:23–28

Kjoller R, Rosendahl S (2000) Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). Plant Soil 226:189–196

Klironomos JN, McCune J, Hart M, Neville J (2000) The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecol Lett 3:137–141

Koch AM, Kuhn G, Fontanillas P, Fumagalli L, Goudet J, Sanders IR (2004) High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. Proc Natl Acad Sci USA 101:2369–2374

Labour K, Jolicoeur M, St-Arnaud M (2003) Arbuscular mycorrhizal responsiveness of in vitro tomato root lines is not related to growth and nutrient uptake rates. Can J Bot 81:645–656

Lanfranco L, Wyss P, Marzachi C, Bonfante P (1995) Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. Mol Ecol 4:61–68

Lloyd-MacGilp SA, Chambers SM, Dodd JC, Fitter AH, Walker C, Young JPW (1996) Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol 133:103–111

Mathur N, Vyas A (1995) In vitro production of *Glomus deserticola* in association with *Ziziphus nummularia*. Plant Cell Rep 14:735–737

Mathur N, Vyas A (1999) Improved biomass production, nutrient uptake and establishment of in vitro raised *Ziziphus mauritiana* by VA mycorrhiza. J Plant Physiol 155:129–132

Mayo K, Davis R, Motta J (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78:426–431

Mosse B (1962) The establishment of vesicular arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mosse B, Hepper C (1975) Vesicular arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 7:1045–1050

Nielsen JS, Joner EJ, Declerck S, Olsson S, Jakobsen I (2002) Phospho-imaging as a tool for visualization and noninvasive measurement of P transport dynamics in arbuscular mycorrhizas. New Phytol 154:809–820

Nuutila AM Vestberg M, Kauppinen V (1995) Infection of hairy roots of strawberry (*Fragaria* X *Ananassa* Duch.) with arbuscular mycorrhizal fungus. Plant Cell Rep 14:505–509

Olsson PA, Baath E, Jakobsen I (1997) Phosphorus effects on mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by fatty acid signatures. Appl Environ Microbiol 63:3531–3538

Pawlowska TE, Douds DD, Charvat I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Pfeffer PE, Douds DD Jr, Bécard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol 120:587–598

Redecker D, Thierfelder H, Walker C, Werner D (1997) Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. Appl Environ Microbiol 63:1756–1761

Rhody D, Stommel M, Roeder C, Mann P, Franken P (2003) Differential RNA accumulation of two beta-tubulin genes in arbuscular mycorrhizal fungi. Mycorrhiza 13:137–142

Rosendahl S, Taylor JW (1997) Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. Molec Ecol 6:821–829

Rufyikiri G, Thiry Y, Declerck S (2003) Contribution of hyphae and roots to uranium uptake and translocation by arbuscular mycorrhizal carrot roots under root-organ culture conditions. New Phytol 158:391–399

Sancholle M, Dalpé Y (1993) Taxonomic relevance of fatty acids of vesicular-arbuscular mycorrhizal fungi and related species. Mycotaxon 49:187–193

Sancholle M, Dalpé Y, Grandmougin-Ferjani A (2001) Lipids of mycorrhizae. In: Esser K, Hock B (eds) The Mycota. IX Fungal association. Springer, Berlin Heidelberg New York, pp 63–93

Sanders IR, Clapp JP, Wiemken A (1996) The genetic diversity of arbuscular mycorrhizal fungi in natural ecosystems – a key to understanding the ecology and functioning of the mycorrhizal symbiosis. New Phytol 133:123–134

Schaffer GF, Peterson RL (1993) Modifications to clearing methods used in combination with vital staining of roots colonized with vesicular-arbuscular mycorrhizal fungi. Mycorrhiza 4:29–35

Schreiner RP, Koide RT (1993) Stimulation of vesicular-arbuscular mycorrhizal fungi by mycotrophic and nonmycotrophic plant root systems. Appl Environ Microbiol 59:2750–2752

Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycol Res 105:1413–1421

Schüßler A, Schwarzott D, Walker C (2003) *Glomeromycota* rRNA genes – the diversity of myths? Mycorrhiza 13:233–236

Séjalon-Delmas N, Magnier A, Douds DD Jr, Bécard G (1998) Cytoplasmic autofluorescence of an arbuscular mycorrhizal fungus *Gigaspora gigantea*and nondestructive fungal observations in planta. Mycologia 90:921–926

Simon L, Lévesque RC, Lalonde M (1993) Identification of endomycorrhizal fungi colonizing roots by fluorescent single-strand conformation polymorphism-polymerase chain reaction. Appl Environ Microbiol 59:4211–4215

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995) Altered growth of *Fusarium oxysporum* f.sp. *chrysanthemi* in an in vitro dual culture system with the vesicular arbuscular mycorrhizal fungus *Glomus intraradices* growing on *Daucus carota* transformed roots. Mycorrhiza 5:431–438

Strullu DG, Diop T, Plenchette C (1997) Réalisation de collections in vitro de *Glomus intraradices* (Schenck et Smith) et *Glomus versiforme* (Karsten et Berch) et proposition d'un cycle de développement. C R Acad Sci Paris 320:41–47

Tisserant B, Gianinazzi-Pearson V, Gianninazzi S, Gollotte A (1993) In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res 97:245–250

Tisserant B, Brenac V, Requena N, Jeffries P, Dodd JC (1998) The detection of *Glomus* spp. (arbuscular mycorrhizal fungi) forming mycorrhizas in three plants, at different stages of seedling development, using mycorrhiza-specific isozymes. New Phytol 138:225–239

Tiwari P, Adholeya A (2002) In vitro co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. FEMS Microbiol Lett 206:39–43

Vandenkoornhuyse P, Husband R, Daniell TJ, Watson IJ, Duck JM, Fitter AH, Young JPW (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Mol Ecol 11:1555–1564

Villegas J, Fortin FA (2001) Phosphorus solubilization and pH changes as a result of the interaction between soil bacteria and arbuscular mycorrhizal fungi on a medium containing NH_4 as nitrogen source. Can J Bot 79:865–870

Villegas J, Williams RD, Nantais L, Archambault J, Fortin JA (1996) Effects of N source on pH and nutrient exchange on extramatrical mycelium in a mycorrhizal Ri T-DNA transformed root system. Mycorrhiza 6:247–251

Xavier LJC, Xavier IJ, Germida JJ (2000) Potential of spore protein profiles as identification tools for arbuscular mycorrhizal fungi. Mycologia 92:1210–1213

4 Life Cycle of *Glomus* Species in Monoxenic Culture

Yolande Dalpé[1], Francisco Adriano de Souza[2], Stéphane Declerck[3]

1 Introduction

With respect to the Glomeromycota taxonomy, the genus *Glomus* includes close to 110 described species, making this genus the most important of the order Glomerales (Schüßler et al. 2001). As a consequence, a large number of research investigations on AM fungi are based on *Glomus* species isolates. Even though a limited number of species are cultivated under monoxenic culture, a huge amount of knowledge has already been generated to draw a reliable picture of their life cycle.

The potential of this technology, although still in its infancy, has already influenced and stimulated research investigations notably in colony growth kinetics and sporulation (Fortin et al. 2002). With respect to the *Glomus* species life cycle, this review is subdivided into four major sections: (1) the spore germination, (2) the pre-symbiotic stage, (3) the host root connection, and (4) the symbiotic stage.

2 Life Cycle

Obligate biotrophism triggered the acquisition by arbuscular mycorrhizal (AM) fungi of ingenious morphologies enabling them to survive in the absence of host plants, to adapt to a variety of plant host partners, and to allow synergy with other AM fungal species. For a given monoxenic culture, all phases of the fungal life cycle simultaneously exist and interact with each other. The knowledge we have about the *Glomus* spp. life cycle,

[1]Environment Health/Biodiversity Agriculture and Agri-Food Canada, 960 Carling Avenue, Ottawa K1A 0C6, Canada, Tel.: +1-613-7591381, Fax: +1-613-7591701, E-mail: dalpey@agr.gc.ca
[2]Empresa Brasileira de Pesquisa Agropecuária – Embrapa Agrobiologia, BR 465-RJ, km 7, Caixa Postal 74505, CEP 23851-000 Seropédica, RJ, Brazil
[3]Université Catholique de Louvain, Mycothèque de l'Université Catholique de Louvain (MUCL), Unité de Microbiologie, 3 Place Croix du Sud, 1348 Louvain-La-Neuve, Belgium

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

originating from either in vivo or in vitro systems, remains somehow fragmented between intraradical (root colonization) and extraradical phases (mycelium and sporulation). Methodologically, investigations using in vivo technology are preferentially oriented towards the intraradical phase, with essentially imprecise spore counts as available extraradical data. With the in vitro system, both the intra- and the extraradical phases can be studied, with access to repetitive and non-destructive measurements.

Existing descriptions of AM fungal life cycles are based on morphological observations done either with agar-coated glass slides buried in soil (Powell 1976), with surface-sterilized spores grown in sterile substrate (Garriock et al. 1989) or with monoxenic cultures (Strullu et al. 1997). In the latter case, the proposed life cycles are based on the germination potential of fungal propagules (Strullu and Romand 1986, 1987), linked to species description (Chabot et al. 1992; de Souza and Berbara 1999; Pawlowska et al. 1999; Karandashov et al. 2000; Declerck et al. 2000), nuclei mycelium behaviour (Bago et al. 1999a), and nutritive and environmental conditions (Bago et al. 1996, 1999b; Hildebrandt et al. 2002).

3
AM Fungi Propagule Germination Stage

The AM fungi propagules found capable to germinate, to differentiate mycelium, and to complete the fungal life cycle are the spores, either from inside roots or isolated from the rhizosphere, and the intraradical vesicles, either isolated from the roots or still embedded within tissues of colonized roots. AM fungi hyphae from the peridium and the intraradical mycelium also are suspected to have a regeneration power. As germinating fungal propagules originate, most of the time, from non-sterile substrate, this section is based on data usually obtained from surface sterilized material, and not necessarily from monoxenic cultures.

The long-term survival of AM fungi spores in soil has been associated to their thick wall architecture and their capacity to easily fall into dormancy (Tommerup 1983). Factors involved in AM fungal spore dormancy have been attributed to ageing, physiological status, and harvesting time (Hepper and Smith 1976; Tommerup 1983; Hardie 1984). Breaking dormancy can be achieved by stratification at 4 °C (Camprubi et al. 1990; Juge et al. 2002). A 50% decrease in polyamines content was registered during spore cold treatment, and polyamine treatment did not affect the spore germination potential (El-Ghachtouli et al. 1996).

Glomus spore germination and germ tube elongation are usually not affected by the plant host (Schreiner and Koide 1993; Logi et al. 1998; Giovannetti and Sbrana 1998). *Glomus* spores may take between a few days

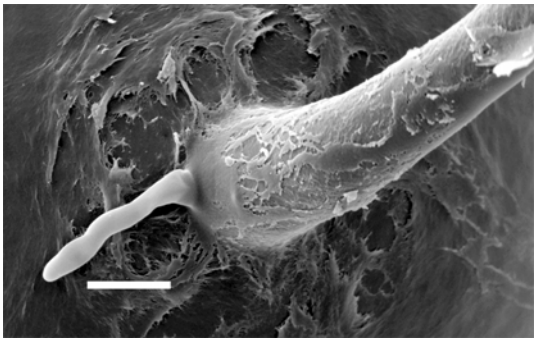


Fig. 1. Scanning electron microscope: spore germination through the subtending hyphal wall, *G. intraradices* spore (*bar* = 7 µm)

up to 6 months to germinate. Germination rates may remain as low as 2–10%.

Germination usually proceeds by the forcing of the inner spore wall through the lumen of the subtending hyphae (Gilmore 1968; Meier and Charvat 1992; de Souza and Berbara 1999), directly through the spore wall (Tommerup and Kidby 1980), or the subtending hyphal wall (Giovannetti et al. 1991; Fig. 1). Spore germination gives rise either to a straight, thick-walled hyphae (de Souza and Berbara 1999) or to stunted hyphae, depending on the spore physiological status (Juge et al. 2002).

Hydration and metabolism activation are prerequisites for AM fungi spore germination (Tommerup 1984). Inhibitors of protein synthesis and of RNA and mtDNA are known to prevent germination (Hepper 1979; Beilby and Kidby 1982; Beilby 1983). Multiple nuclei in quiescent spores (Cooke et al. 1987; Meier and Charvat 1992) and active nuclei replication and DNA synthesis were observed at germination (Bianciotto and Bonfante 1993). Quiescent spore lipid content reaches 40–60% of their biomass (Sancholle et al. 2001). During germination, de novo synthesis of sterols, diacylglycerol, phospholipids and free fatty acids occurs (Beilby and Kidby 1980; Gaspar et al. 1994; Sancholle et al. 2001) whereas triacyl glycerides are consumed (Gaspar et al. 1994).

Extreme dry or wet environmental conditions inhibited germination (Siqueira et al. 1985). Neutral pH usually supported or promoted germination (Green et al. 1976; Tommerup 1983; Pons et al. 1984; Gunasekaran et al. 1987) whereas acidity had an inhibitory effect (Siqueira et al. 1985). Optimum germination temperatures varied in the range 20–30 °C (Daniels and Trappe 1980; Sheik and Sanders 1988). Oxygen tension over 5% (LeTacon et al. 1983) promoted spore germination, whereas 5% CO_2 had no effect. Non-sterile soil filtrates and soil extract agar improved spore germination (Daniels and Trappe 1980; Gunasekaran et al. 1987). Flavonoid compounds (Tsai and Phillips 1991; Leu and Chang 1993; Poulin et al. 1997), low-P

media (Pons et al. 1984), low glucose concentration, D-galacturonic acid (Siqueira and Hubbell 1984), 50% sucrose (Vilarino and Sainz 1997), low xyloglucan concentration (Garcia-Garrida et al. 1999) and thiamin (Hepper and Smith 1976) increased germination rates. The bacteria *Paenibacillus validus*, antagonistic towards soil-borne fungal pathogens (Hildebrandt et al. 2002), *Streptomyces* species (Tylka et al. 1991), bacterial contaminants (Mayo et al. 1986) and nitrogen-fixing bacteria (Tilak et al. 1990) all stimulated germination. Lower mineral content medium (water-agar pH 6.0) favours germination, while high mineral content or rich media inhibit it (Budi et al. 1999).

In addition to spores, several other AM fungi propagules have the potential to germinate. The germination of isolated intraradical vesicles was clearly demonstrated by Strullu et al. (1997), Diop et al. (1994), and Declerck et al. (1998). Such germination occurred through the lumen of their subtending hypha attachment (Declerck et al. 1998), the germ tubes generating runner and ramified hyphae similar to those of AM fungi spore. To date, no systematic investigation has been conducted on factors influencing their germination. Among other fungal structures capable of re-growth are hyphae from the peridium of *G. mosseae* sporocarps which have the capability to elongate and differentiate vesicle-like structures (VLS; Fig. 2; Budi et al.

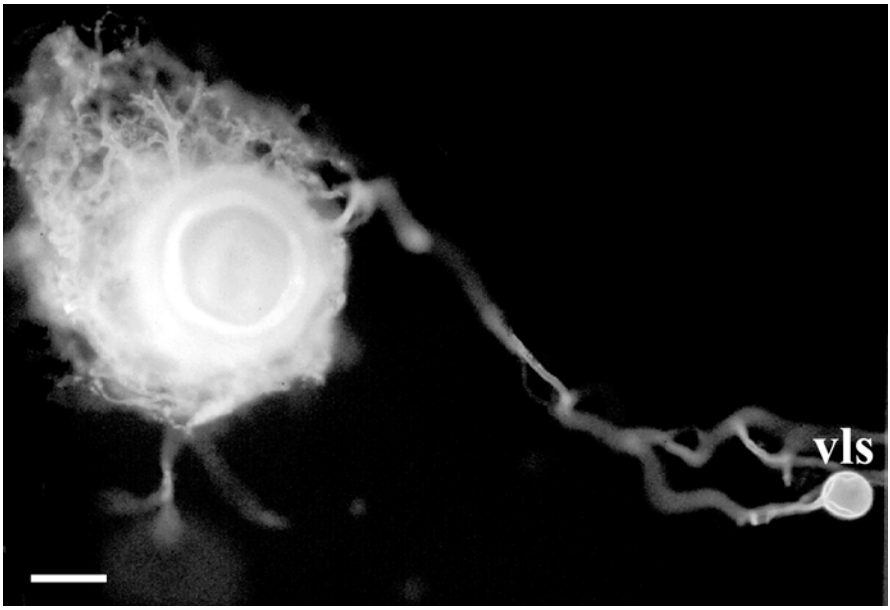


Fig. 2. Dissecting microscope: pre-symbiotic hyphae emerging from *G. mosseae* peridium with vesicle-like structure (*VLS*), UV fluorescence (*bar* = 40 µm)

1999). Moreover, the "germination" of colonized root segments is currently used to replicate AM fungi monoxenic cultures (Strullu and Romand 1986, Strullu et al. 1991). The root vesicles and eventually intraradical spores are certainly the fungal propagules involved in root segment "germination", because colonized root segments deprived of vesicles and spores remained unsuccessful for propagation

4
Pre-Symbiotic Mycelium Stage

Germ tube growth is dependent on the availability of spore reserves (Bécard and Fortin 1988; Sancholle et al. 2001), and the protoplasm contains all the organelles required to ensure development (Meier and Charvat 1992). This consists of a straight growing hypha (runner hyphae, RH) exploring the media by successive branchings into thinner-diameter filaments (Diop et al 1994; de Souza and Berbara 1999; Declerck et al. 2000). In the case of no hyphal root contact or host signal detection, germ tube growth stops within a few days (Bécard and Piché 1989b). The protoplasm shrinks back from the hyphal apex, and is sequestered from the empty hyphae by repeated septation (Logi et al. 1998). Such germinating attempts resemble a well-orchestrated survival scenario, providing repetitive chances for the fungus to establish symbiosis. Low light exposure (Nagahashi et al. 2000), AM fungal spore content (Hepper 1983), plant cell suspension (Hepper 1979; Carr et al. 1985), root exudates and root volatiles (Bécard and Piché 1989b; Giovannetti et al. 1993), phenols and flavonoids (Tsai and Phillips 1991; Vierheilig et al. 1998), amino acids (Hepper and Jakobsen 1983), and thiamin (Siqueira et al. 1982) were all found to enhance hyphal branching. Recently, Tamasloukht et al. (2003) have demonstrated that root factor(s) isolated from root exudates of transformed carrot roots induce higher rates of respiration during the pre-symbiotic stage.

Anastomosis is observed during the AM fungi pre-symbiotic stage (Mosse 1988; Hildebrandt et al. 2002). Demonstrated under in vivo conditions as a fungi segregating factor at the strain level (Giovannetti et al. 2003), vegetative compatibility between the hyphae of a single isolate provides an opportunity for the pre-symbiotic mycelia to connect to the existing soil hyphal network, insuring survival during the time lapse required to establish symbiosis.

Vesicle-like structures (VLS) are small, hyaline thin-walled swellings (Fig. 3) resembling miniature spores (Hepper 1981; Strullu and Romand 1987). Although considered juvenile spores, their germination capability has never been demonstrated. Nevertheless, they differentiate along the pre-symbiotic hyphae of several *Glomus* monoxenic cultures (Mosse

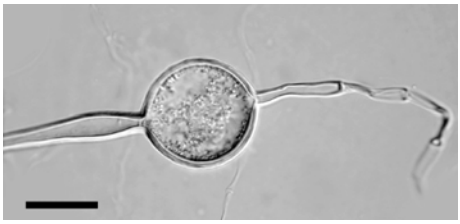


Fig. 3. Differential interference contrast microscope: intercalary vesicle-like structure (*bar* = 15 µm)

1962; de Souza and Berbara 1999; Karandashov et al. 2000). For *G. clarum*, their abundance increased substantially when germinated spores were incubated with *Gluconoacetobacter diazotrophicus* cells (Paula et al. 1994). Hypotheses about the role of VLS range from a survival process during the pre-symbiotic stage to an aborted sporulation tentative.

Thin-walled, densely branched arbuscular-like structures (ALS), renamed branched absorbing structures or BAS by Bago et al. (1998b), are observed along the non-symbiotic mycelium (Mosse and Hepper 1975; Mosse 1988). According to Bago et al. (1998b), BAS are ephemeral (5–7 day lifespan), except for those undergoing spore formation events ("spore-BAS" formation). In contrast to the BAS differentiated on symbiotic hyphae, they rapidly aborted (Bago et al. 1998a), and have been associated with a survival reaction aiming to increase nutrient absorption (Mosse 1988) and have also been attributed a putative saprophytic growth potential (Strullu et al. 1997).

Spore differentiation on pre-symbiotic mycelium has been rarely reported. A mean of 60 *G. versiforme* spores were differentiated from germinated, 5-mm-long root segments (Diop et al. 1994), and newly produced spores were found able to germinate and establish symbiosis. The co-culture of the bacteria *Paenibacillus validus* with *G. intraradices* induced the differentiation of several hundred spores which, however, failed to germinate (Hildebrandt et al. 2002). Such results suggest some ability of AM fungi to develop in the absence of a host partner.

5
Host Root Connecting Stage

The contact between the root and fungal hyphae may take one to several weeks to become established (Declerck et al. 1998). Repetitive investigations under in vivo growth conditions revealed that, once the root–fungi contact is established, the fungal morphology changes drastically, with a reorientation of hyphal apical growth giving rise to either a direct entry point or to an intensive hyphal branching called "fan-like structure" (Garriock et al. 1989; Friese and Allen 1991; Giovannetti et al. 1993). Il-

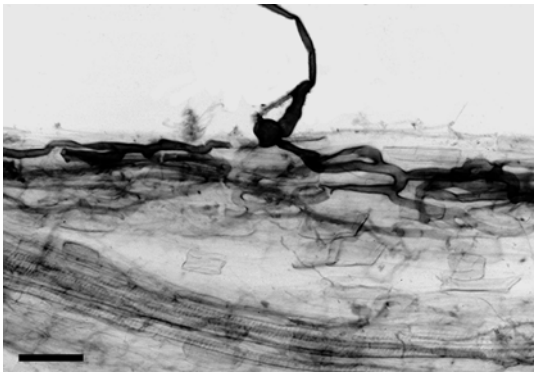


Fig. 4. Differential interference contrast microscope: appressorium with thick-wall extraradical and thin-wall intraradical hyphae (*bar* = 100 µm)

lustrations by Giovannetti et al. (1994) clearly showed the frequent septation of inflated segmental hyphae resembling appressorium structures. Such hyphal architecture never developed with non-host plants (Giovannetti and Sbrana 1998). Germinating hyphae from colonized root segments never differentiated fan-like structures but penetrated roots by single entry points. As suggested by Mosse (1959), germ tubes issued from spores may require other signals for root colonization, different from those required by fungal hyphae and vesicles surrounded by root tissues.

The term "appressorium" refers to swelled or stunted cells differentiated at the contact of host epidermal cells, an easily observable signal of root recognition (Fig. 4). Under in vivo conditions, appressoria may take only 36 h to develop after plant–fungus contact is established (Giovannetti and Sbrana 1998). As for fan-like structures, appressorium induction may be controlled by host signals (Giovannetti and Sbrana 1998). Multiple appressoria can be formed simultaneously, as shown by the capacity of each "fan-like structure" apex to differentiate appressoria (Powell 1976), but not all root penetration tentatives resulted in appressorium differentiation (Harrison 1999). Appressorium differentiation has not yet been studied in AM fungal monoxenic cultures, but the availability to perform non-destructive observations linked to the testing of host root signals or thigmotrophism experiments would merit in-depth investigations.

6
Symbiotic Stage

Ingenious in vivo and in vitro settings have been developed to allow the study of selected symbiotic stages (Hepper and Mosse 1980; Bécard and Piché 1992; St-Arnaud et al. 1996; Bago et al. 1996; Giovannetti et al. 1999;

Fortin et al. 2002). So far, all organizational structures described from in vivo cultures have been observed under monoxenic culture. Specific information related to the nutrition and metabolism of this life-cycle stage is treated in other chapters.

6.1 Intraradical Mycelium

From the appressorium structure, one or more hyphae successfully penetrate the cell wall by enzymatic and mechanical activities, sometimes accompanied by a severe constriction of the hyphae due to the mechanical resistance of the epidermal cells (Fig. 4). Hyphal penetration may occur within a few hours and up to 3 days after initial contact with the root. Based on ultrastructure observations, AM fungi hyphae seem to acquire flexibility once inside root tissues. However, the hypothesis proposed by Bonfante-Fasolo (1987) about the non-fibrillar architecture of the intraradical hyphal wall, and about enhanced chitinase enzyme activity has been partially refuted by Timonen et al. (2001) who demonstrated the similitude of extra- and intraradical hypha microtubule bundle distribution. Once inside the root, the intraradical mycelium pursues its progression, branching and anastomosing within the intercellular root space channel of the root epidermal and cortical parenchyma.

The intraradical vesicles are globose to ellipsoid hyphal swellings differentiated either apically or intercalary along hyphae, in constant connection with the intraradical mycelium, and positioned intercellularly or intracellularly (Pawlowska et al. 1999; Karandashov et al. 1999). Often surrounded by a double wall, 1.0–2.5 µm thick (Fig. 5), the vesicles are filled with lipidic material and organelles required for autonomous growth (Timonen

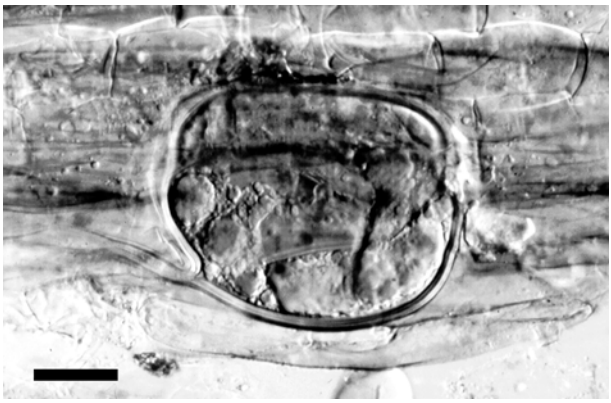


Fig. 5. Differential interference contrast microscope: double-walled vesicle of *G. intraradices* inside a leek root (*bar* = 6 µm)

et al. 2001). As such, vesicles are infective propagules capable of generating a functioning fungal colony (Strullu and Romand 1987; Diop et al. 1994; Declerck et al. 1998). They were observed as early as 5 days and up to 5 months after symbiosis establishment (Chabot et al. 1992). Comparative analysis of enzyme-extracted vesicles obtained in pot cultures with monoxenically grown spores showed comparable mycorrhizal potential; inoculated plants received the same number of propagules, and both spores and vesicles received a 15-day cold treatment before inoculation (Nantais 1997). The role of arbuscules in bi-directional nutrient exchanges with plants makes them an essential structure for the completion of AM fungi life cycles (Bécard and Piché 1989a), a fact which has been confirmed by using mutant plants (Harrison 1999). In monoxenic cultures, arbuscules differentiated inside the deeper layer of cortical cells (Mosse and Hepper 1975; Pawlowska et al. 1999), and exhibited morphological and ultrastructural architecture (Mugnier and Mosse 1987) similar to those observed in vivo. They may take between 4 days and up to 1 month to differentiate (Simoneau et al. 1994). Depending on fungal species, host plant root species, and root age, arbuscules developed either in densely branched structures or remained vestigial with sparse ramifications (Mosse and Hepper 1975). No precise data are available on the life expectancy of arbuscules under monoxenic culture. The monoxenic culture system should considerably facilitate the observation, at cellular level, of the changes occurring in host plant cells during arbuscule implantation. Intensive works on the kinetics of their development, cytoplasmic organization, and metabolism studies such as done under in vivo conditions by Balestrini et al. (1994) and Blancaflor et al. (2001) with in vivo material, would find in monoxenic cultures a powerful and malleable research tool.

In monoxenic cultures, root colonization levels vary according to the root host plant species and fungal isolates (Simoneau et al. 1994; Glorian 2002; Elsen et al. 2003). Acidification of the media directly influences AM fungi development. The pH 5.5 value of standard monoxenic culture systems might limit the growth of some isolates, but a pH increase in the nutritive media may alter the solubility and balance of the media components. Buffered media may counteract such weaknesses.

Most monoxenic culture plants support the Arum-type colonization (Diop et al. 1994; Nuutila et al. 1995; Karandashov et al. 1999; de Souza and Berbara 1999; Glorian 2002). A paradoxical situation occurs with carrot (*Daucus carota*) root culture, an Apiaceae (Umbelliferae) recognized to support both Paris- and Arum-type colonization (Smith and Smith 1997), whereby Arum-type colonization is more differentiated. Only one *G. caledonium* isolate differentiated Paris-type colonization with a carrot root culture (Karandashov et al. 2000), and one *G. etunicatum* isolate had mixed types and differentiated hyphal coils in the first layer of cortical

cells (Pawlowska et al. 1999). Paris and Arum morphotypes were for long considered to be determined by the plant genome (Smith and Smith 1997), but the typical Paris anatomical type observed in carrot root culture colonized by *G. caledonium* emphasizes the impact of the fungal genome on the regulation of fungal morphology (Cavagnero et al. 2001).

6.2 Extraradical Mycelium

The establishment of symbiosis under monoxenic culture triggers, within a few hours, a vigorous extraradical hyphal development and subsequent differentiation of VLS, BAS and spores (Declerck et al. 1998). The structural development of the mycelial phase has been described exhaustively by Bago et al. (1998a), from a single isolate of *G. intraradices*, and has since been used as the reference model to which further descriptions are compared. The basic structure of the mycelium is made of large, straight-growing thick-walled hyphae named runner hyphae (RH), due to their capacity to extend rapidly, to colonize the substrates, and to establish root contact (Figs. 6 and 7). Microscopically, runner hyphae are similar to pre-symbiotic hyphae. Microscopic cellular and subcellular observations allow detection of protoplasmic streaming, nuclei migration and organelle morphology (Mosse 1988; Bago et al. 2001). Hyphae are either single-walled, as with *G. versiforme* (Garriock et al. 1989), or double-walled as found through ultrastructure works on *G. fasciculatum* (Bonfante-Fasolo and Grippiolo 1982). Both walls showed almost equal thicknesses and separated easily at hyphal break (Fig. 6). This morphology recalls the hyphae within in vivo

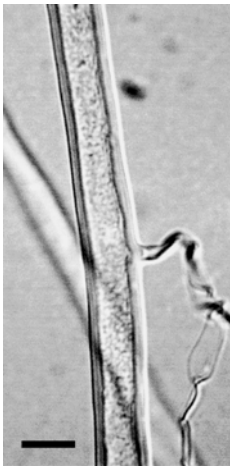


Fig. 6. Differential interference contrast microscope: thick-wall laminated runner hyphae (*bar* = 10 µm)

Fig. 7. Differential interference contrast microscope: collapse thin-wall extraradical hyphae issued from branching of runner hyphae (*bar* = 15 μm)

propagated hyphae of Lim et al. (1983), where the inside hyphal wall was attributed to self-invasion by a hyphal outgrowth following wounding or cutting.

The elongation rates of runner hyphae may attain up to 750 $\mu m\, h^{-1}$ for long periods. With *G. proliferum*, the mycelium network proliferates sometimes so densely that colony extension can almost be seen with the naked eye (Glorian 2002). RH and branched hyphae (BH) abundance determines the mycelium architecture. In spite of a common general organization, finer details of mycelium architecture may vary considerably between *Glomus* species as well as their isolates (Declerck et al. 1998). Experienced microscopists can often differentiate fungal colonies under the binocular by observing branching and sporulation patterns. With some isolates, where the mycelium expansion is restricted to the vicinity of roots, sub-culturing usually failed.

Anastomoses have been reported to occur regularly in the pre-symbiotic and the symbiotic mycelium of monoxenic cultures, and their occurrence has been clearly associated with hyphal growth stimulation (Mosse 1988). This could explain in part the slow growth rate registered with pre-symbiotic hyphae in monosporal tests (Logi et al. 1998; Declerck et al. 2000). From an ecological point of view, anastomoses provide an avenue to understand how AM fungi are building soil hyphal strengthening fungal vitality and perennity under natural constraints or substrate disturbance.

The exchange of nuclei during anastomoses has been observed occurring under in vivo culture conditions only within one and the same isolates (i.e. self-anastomosis), indicating the existence of vegetative compatibility groups in AM fungi (Giovannetti et al. 2003). These findings do not support the proposed heterokaryotic status of AM fungi. Moreover, the homokaryosis of *G. etunicatum* and *G. intraradices* has been recently suggested by typing individual nuclei (Pawlowska and Taylor 2004), Besides, the haploid status of *G. intraradices* was also suggested (Hijri and Sanders

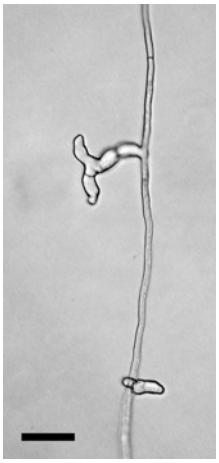


Fig. 8. Differential interference contrast microscope: embryos of branched-absorbing structures (*bar* = 50 µm)

2004). These two papers are good examples of the usefulness of monoxenic cultures for genetic research of AM fungi. However, these results do not rule out the possibility of parasexual recombination, followed by nuclear fusion and the reestablishment of the haploid state, by chromosome losses (see Schardl and Craven 2003 for a review). This implies, however, that the exchange of genetically divergent nuclei is not a common phenomenon in AM fungi, as previously thought (Sanders 2002).

Once a successful symbiosis is established, numerous BAS are differentiated along hyphae (Bago et al. 1998a). Due to their morphological resemblance with arbuscules, they were attributed nutrient absorbing capacities,

Fig. 9. Dissecting microscope: branched-absorbing structures with low level of ramification (*bar* = 50 µm)

reinforced by their association with spores (Bago et al. 1998a; Dodd et al. 2000). Their differentiation occurred within 3–10 days prior to arbuscules, but after hyphal root colonization (Bago et al. 1999b), with a life span of about 7 days (Bago et al. 1998b). BAS may adopt variable morphologies, the most striking being the large, stunted ramified structures of *G. caledonium* (Karandashov et al. 1999). Either not reported (*G. macrocarpum*, Declerck et al. 1998), or sparse and fragile looking (Figs. 8 and 9), the BAS may thus not be a prerequisite for the maintenance of a healthy fungal colony, and their hypothetical support in nutrient absorption not systematically required. When grown with the bacterium *Paenibacillus validus*, "densely packed coils" made of extensively branched hyphae were differentiated by a *G. intraradices* isolate (Hildebrandt et al. 2002), indicating that BAS morphology can be regulated by external factors independent of the plant or AM fungi.

7 Spores

Several of the successfully grown *Glomus* species, including *G. caledonium* (Hepper 1981; Karandashov et al. 1999), *G. clarum* (de Souza and Berbara 1999), *G. fistulosum* (Nuutila et al. 1995) and *G. intraradices* (Chabot et al. 1992), differentiated VLS. In small spore species, these hyaline thin-walled structures may have been confused with juvenile spores. No record of VLS was mentioned for either *G. etunicatum* (Pawlowska et al. 1999), *G. fasciculatum*, *G. macrocarpum* (Declerck et al. 1998) or *G. versiforme* (Declerck et al. 1996). When differentiated, VLS occurred within 2–8 days after root contact. Their size ranged between 20 and 100 µm, depending on the species. Since their detection in *G. mosseae* colonies (Fig. 10; Mosse 1962; Hepper 1981), no precise role has been attributed to these structures, and the early hypothesis expressed by Mosse (1962) as be-

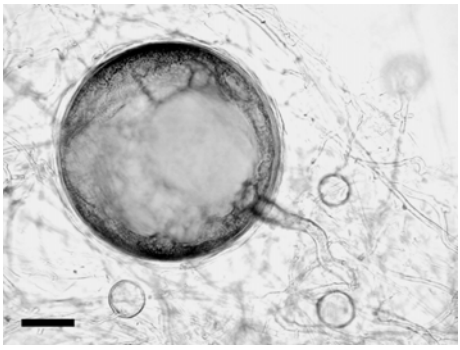


Fig. 10. Differential interference contrast microscope: mature spore and vesicle-like structures of *G. mosseae* (*bar* = 50 µm)

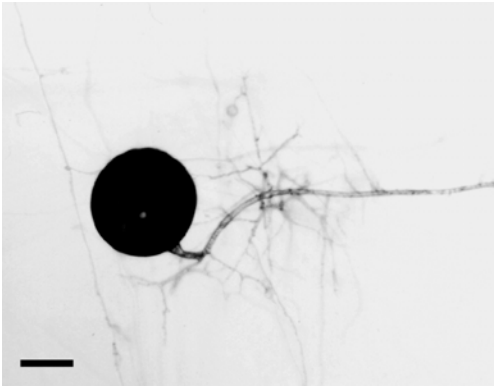


Fig. 11. Dissecting microscope: spore of *G. mosseae* with branched-absorbing structure (*bar* = 60 µm)

ing a remnant structure of a previous saprophytic mode of life remains valuable.

Spore differentiation occurs either apically or intercalary along lateral branches of RH, often in association with BAS (Fig. 11; Mosse and Hepper 1975; Bago et al. 1998b). The outer evanescent spore wall originates then from the hyphal wall. The spore apical hyphae, even though collapsed, remain attached to the spore during most of the maturation process. Intraradical spores have sometimes been observed in monoxenic cultures (Declerck et al. 1996; de Souza and Berbara 1999).

Pot culture and monoxenic culture propagated *Glomus* species share similar ontogeny, but isolates from monoxenic cultures usually differentiated smaller spores with paler pigmentation and thinner laminated spore walls (Chabot et al. 1992; de Souza and Berbara 1999). In monoxenic culture, the possibility of observing all differentiated spores may have biased the mean spore size values, as many of the smaller spores from soil-based culture counting may have been discarded from the measurements. Also, the thicker laminated wall of in vitro differentiated *G. etunicatum* spores, compared to soil-borne spores, may simply be attributable to the immature stage of the spore wall, even though the spores have attained their optimal size (Pawlowska et al. 1999). Moreover, thick-walled spores are recognized to provide long-lasting capabilities and increased protection from adverse environmental conditions. As such, wall thickening may be considered an adaptive feature not fully retained in monoxenic cultures. Based on multiple observation of monoxenic cultures, it appears that in vitro propagated AM fungi usually differentiated thinner walled and sometimes less pigmented spores. Such differences between in vivo and in vitro spore morphology can be attributed to growth environment conditions. Soil rhizospheres, with their multiple micro-organism interactions, their ongoing nutritional and chemical variations, and their variable edaphic conditions induced AM

fungal adaptations directed through long-term survival and competitive behaviour. Such reactions to drastic and severe living competition remain totally absent from the controlled in vitro culture conditions.

Most *Glomus* species exhibit an asynchronous mode of sporulation, i.e. with a lag, log and plateau phase (Declerck et al. 1996, 2001). As a result, spores of various ages occurred simultaneously in a single colony. Ultrastructural study on *G. intraradices* spore maturation revealed spore wall thickening over several months, even though spores could germinate after 30 days (Nantais and Dalpé, unpublished data). For *G. caledonium*, by contrast, Karandashov et al. (2000) demonstrated that all spores were produced within a period of 2–3 days, and sporulation totally stopped thereafter, supporting a synchronous mode of spore production. This apparently contradictory result for *G. caledonium* with the data of Declerck et al. (2001) can be attributed to the consideration of juvenile spores or VLS in the modelling approach of Declerck et al. (2001), while Karandashov et al. (2000) considered only the fully expanded stage, i.e. spore size resembling that of mature spores. Therefore, reliable modelling of growth kinetics should take into account not only the number of spores differentiated but also their maturation process, the proportion of spore-like structures which remain VLS, and those which reach full maturation.

Spore production differs considerably between species and between isolates of a single species, and seems to be related to spore size. With the small- to medium-size spore species *Glomus proliferum* and *G. intraradices*, an average of 7,800 and 8,200 spores were differentiated in mono-compartment (Declerck et al. 2001) and bi-compartment growth systems (St-Arnaud et al. 1996) respectively. Concurrently, a 5-month-old *G. clarum* monoxenic culture (130-µm mean spore size) differentiated 853 spores (de Souza, unpublished data), and a *G. macrocarpum* culture (165 µm) only 250 spores (Declerck et al. 1998). The sporulation rate of *G. caledonium* was reported to increase with successive generations (Karandashov et al. 2000). A case-by-case isolate response to growth conditions should be expected, and the maintenance of a constant sporulation level may be retained as a valuable indicator of strain perennity, once returned to natural environmental conditions.

The presence of aborted or senescent spores has sporadically been observed in healthy monoxenic *Glomus* species cultures, reaching up to 5–10% of spore populations (Pawlowska et al. 1999; Karandashov et al. 2000; Dalpé 2004). Two types of spore senescence were monitored: (1) spore wall disruption discharging granular dextrinoid cytoplasm (Fig. 12) and (2) intrasporal differentiation of coiled hyphae and dispersion in the media at wall break (Figs. 13 and 14). Inadequate in vitro growing conditions and self-strain protection against mutation were proposed as hypothetical explanations of the phenomenon (Marbach and Stahl 1994; Pawlowska et al. 1999).

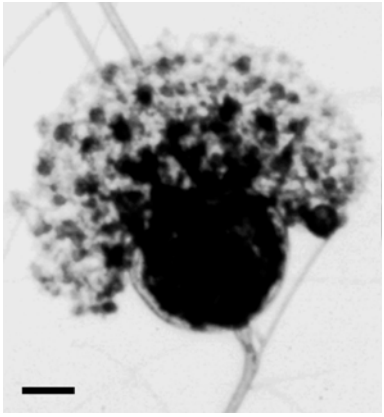


Fig. 12. Differential interference contrast microscope: disrupted spore with extrusion of granular lipidic content reacting to Sudan IV staining (*bar* = 30 µm)

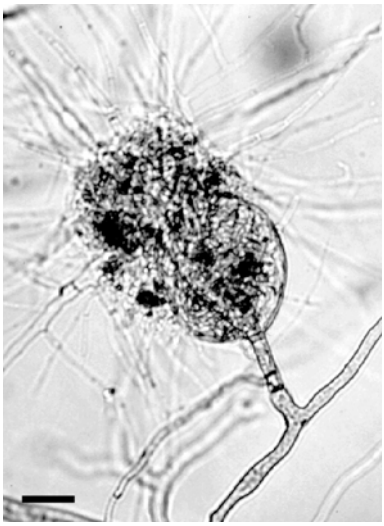


Fig. 13. Differential interference contrast microscope: disrupted spore with differentiation of thin-walled hypha from inside the senescing spore (*bar* = 30 µm)

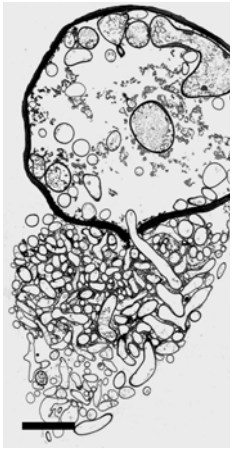


Fig. 14. Ultrastructure view of a newly differentiated hypha extruding from a broken spore (*bar* = 15 µm)

8
Conclusion

Based on the data available from monoxenic culture, both the early presymbiotic and the symbiotic stages of AM establishment seemed to follow similar ontogenic steps as those observed with in vivo growing systems. Intensive cytochemical, ultrastructural and molecular investigations with monoxenic culture systems would be more than welcome in order to allow extrapolation of actual in vivo studies, and confirming hypotheses on a variety of plant–fungi partners. Such investigations would provide strong demonstration as to what extent monoxenic cultures, with their aerial plant part amputated systems, may be successful in mimicking natural symbioses occurring on whole-plant in vitro symbiosis, and may permit to pinpoint and segregate between the metabolic steps directly involved with plant-related metabolism. In any case, monoxenic culture performance in maintaining healthy grown colonies, and the suitability of the system to support developmental and physiological fungal studies should permit many years of investigations.

Acknowledgements. FAdeS thanks the Brazilian Council for Scientific and Technological Development (CNPq) for his financial support (grant #200850/98-9). This represents publication number 3409 NIOO-KNAW Netherlands Institute of Ecology, where FAdeS was formerly associated.

References

Bago B, Vierheilig H, Piché Y, Azcón-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bago B, Azcón-Aguilar C, Piché Y (1998a) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bago B, Azcón-Aguilar C, Goulet A, Piché Y (1998b) Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. New Phytol 139:375–388

Bago B, Zipfel W, Williams RM, Piché Y (1999a) Nuclei of symbiotic arbuscular mycorrhizal fungi as revealed by in vivo two-photon microscopy. Protoplasma 209:77–89

Bago B, Pfeffer PE, Douds DD, Brouillette J, Bécard G, Shachar-Hill Y (1999b) Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. Plant Physiol 121:263–271

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2001) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Balestrini R, Morera C, Puigdomenech P, Bonfante P (1994) Location of a cell wall hydroxyproline-rich glycoprotein, cellulose and β-1,3-glucans in apical and differentiated regions of maize mycorrhizal roots. Planta 195:201–209

Bécard G, Fortin JA (1988) Early events of vesicular arbuscular mycorrhiza formation on Ri-T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989a) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 112:77–83

Bécard G, Piché Y (1989b) Fungal growth stimulation by CO2 and root exudates in vesicular arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Bécard G, Piché Y (1992) Establishment of vesicular arbuscular mycorrhiza in root organ culture: review and proposed methodology. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology, vol 24. Techniques for the study of mycorrhiza. Academic Press, New York, pp 89–108

Beilby JP (1983) Effects of inhibitors of early protein, RNA, and lipid synthesis in germinating vesicular arbuscular mycorrhizal fungal spores of *Glomus caledonium*. Can J Microbiol 29:596–601

Beilby JP, Kidby DK (1980) Biochemistry of ungerminated and germinated spores of the vesicular arbuscular mycorrhizal fungus, *Glomus caledonium*: changes in neutral and polar lipids. J Lip Res 21:739–750

Beilby JP, Kidby DK (1982) The early synthesis of RNA, protein, and some associated metabolic events in germinating vesicular arbuscular mycorrhizal fungal spores of *Glomus caledonius*. Can J Microbiol 28:623–628

Bianciotto V, Bonfante P (1993) Evidence of DNA replication in an arbuscular mycorrhizal fungus in the absence of the host plant. Protoplasma 176:100–105

Blancaflor EB, Zhao LM, Harrison MJ (2001) Microtubule organization in root cells of *Medicago truncatula* during development of an arbuscular mycorrhizal symbiosis with *Glomus versiforme*. Protoplasma 217:154–165

Bonfante-Fasolo P (1987) Vesicular-arbuscular mycorrhizae: fungus-plant interactions at the cellular level. Symbiosis 3:249–268

Bonfante-Fasolo P, Grippiolo R (1982) Ultrastructural and cytochemical changes in the wall of a vesicular arbuscular mycorrhizal fungus during symbiosis. Can J Bot 60:2303–2312

Budi SW, Blal B, Gianinazzi S (1999) Surface-sterilization of *Glomus mosseae* sporocarps for studying endomycorrhization in vitro. Mycorrhiza 9:65–68

Camprubi A, Calvet C, Estaun V (1990) Efecto del almacenamiento en frio de un inoculo de *Glomus mosseae* sobre su infectividad y la germinacion "in vitro" de las esporas de resistencia. Invert Agric Prod Veg 5:337–343

Carr GR, Hinkley MA, LeTacon F, Hepper CM, Jones MGK, Thomas E (1985) Improved hyphal growth of two species of vesicular arbuscular mycorrhizal fungi in the presence of suspension cultured plant cells. New Phytol 101:417–426

Cavagnaro TR, Smith FA, Lorimer MF, Haskard KA, Ayling SM, Smith SE (2001) Quantitative development of Paris-type arbuscular mycorrhizas formed between *Asphodelus fistulosus* and *Glomus coronatum*. New Phytol 149:105–113

Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321

Cooke JC, Gemma JN, Koske RE (1987) Observations of nuclei in vesicular arbuscular mycorrhizal fungi. Mycologia 79:331–333

Dalpé Y (2004) The in vitro monoxenic culture of arbuscular mycorrhizal fungi: a major tool for taxonomical studies. In: Fri'as Hernández JT, Ferrera Cerrato R, Olalde V Portugal (eds) Advances en conocimiento de la biología de las Micorrizas. Universidad de Guanajuato, Mexico

Daniels BA, Trappe JM (1980) Factors affecting spore germination of the VAM fungus *Glomus epigaeus*. Mycologia 72:457–471

Declerck S, Strullu DG, Plenchette C (1996) In vitro mass-production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri-T-DNA transformed carrot roots. Mycol Res 100:1237–1242

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

Declerck A, D'or D, Cranenbrouck S, Le Boulengé E (2001) Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. Mycorrhiza 11:225–230

de Souza FA, Berbara RLL (1999) Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia 91:343–350

Diop TA, Plenchette C, Strullu DG (1994) Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil 226:131–151

El Ghachtouli N, Paynot M, Martin-Tanguy J, Morandi D, Gianinazzi S (1996) Effect of polyamines and polyamine biosynthesis inhibitors on spore germination and hyphal growth of *Glomus mosseae*. Mycol Res 100:597–600

Elsen A, Declerck S, DeWaele D (2003) Use of root organ cultures to investigate the interaction between *Glomus intraradices* and *Pratylenchus coffeae*. Appl Environ Microbiol 69:4308–4311

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. Mycologia 83:409–418

Garcia-Garrida JM, Rejon-Palomares A, Ocampo JA, Garcia-Romera I (1999) Effect of xyloglucan and xyloglucanase activity on the development of the arbuscular mycorrhizal *Glomus mosseae*. Mycol Res 103:882–886

Garriock ML, Peterson RL, Ackerley CA (1989) Early stages in colonization of *Allium porrum* (leek) roots by the vesicular-arbuscular mycorrhizal fungus *Glomus versiforme*. New Phytol 112:85–92

Gaspar L, Pollero RJ, Cabello MN (1994) Triacylglycerol consumption during spore germination of vesicular arbuscular mycorrhizal fungi. J Am Oil Chem Soc 71:449–452

Gilmore AR (1968) Phycomycetous mycorrhizal organisms collected by open-pot culture methods. Hilgardia 39:87–105

Giovannetti M, Sbrana C (1998) Meeting a non-host: the behaviour of AM fungi. Mycorrhiza 8:23–130

Giovannetti M, Avio L, Salutini L (1991) Morphological, cytochemical, and ontogenetic characteristics of a new species of vesicular-arbuscular mycorrhizal fungus. Can J Bot 69:161–167

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infecting stages. New Phytol 125:587–593

Giovannetti M, Sbrana C, Logi C (1994) Early processes involved in host recognition by arbuscular mycorrhizal fungi. New Phytol 127:703–709

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575

Giovannetti M, Sbrana C, Strani P, Agnolucci M, Rinaudo V, Avio L (2003) Genetic diversity of isolates of *Glomus mosseae* from different geographic areas detected by vegetative compatibility testing and biochemical and molecular analysis. Appl Environ Microbiol 69:616–624

Glorian V (2002) Recherche de polypeptides induits par des champignons endomycorhiziens dans des racines transformées de chicorée (*Chicorium intybus* L.) et de carotte (*Daucus carota* L.). PhD Thesis, Laboratoire de Mycologie/Phytopathologie/Environnement, Université du Littoral Calais, France

Green NE, Graham SO, Schenck NC (1976) The influence of pH on the germination of vesicular arbuscular mycorrhizal spores. Mycologia 68:929–934

Gunasekaran P, Sundaresan P, Ubalthoose Raja N, Lakshmanan M (1987) Effect of pH, temperature and nutrients on the germination of a vesicular arbuscular mycorrhizal fungus, *Glomus fasciculatum* in vitro. Proc Indian AS-Plant Sci 97:231–234

Hardie K (1984) Effect of pot-culture age on spore germination in *Glomus mosseae*. In: Proc 6th North American Conf Mycorrhizae, Oregon University, USA

Harrison MJ (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Molec Biol 50:361–89

Hepper CM (1979) Germination and growth of *Glomus caledonium* spores: the effects of inhibitors and nutrients. Soil Biol Biochem 14:269–277

Hepper CM (1981) Techniques for studying the infection of plants by vesicular-arbuscular mycorrhizal fungi under axenic conditions. New Phytol 88:641–647

Hepper CM (1983) Limited independent growth of a vesicular arbuscular mycorrhizal fungus in vitro. New Phytol 93:537–542

Hepper CM, Jakobsen I (1983) Hyphal growth from spores of the mycorrhizal fungus *Glomus caledonius*: effect of amino acids. Soil Biol Biochem 15:55–58

Hepper CM, Mosse B (1980) Vesicular arbuscular mycorrhiza in root organ cultures. In: Ingram DS, Helgeson JP (eds) Tissue culture methods for plant pathologists. Blackwell, New York, pp 167–171

Hepper CM, Smith GA (1976) Observations on the germination of *Endogone* spores. Trans Br Mycol Soc 66:189–193

Hijri M, Sanders IR (2004) The arbuscular mycorrhizal fungus *Glomus intraradices* is haploid and has a small genome size in the lower limit of eukaryotes. Fungal Genet Biol 41:253–261

Hildebrandt U, Janetta K, Bothe H (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919–1924

Juge C, Samson J, Bastien C, Vierheilig H, Coughlan A, Piché Y (2002) Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: a critical cold-storage period. Mycorrhiza 12:37–42

Karandashov VE, Kuzovkina IN, George E, Marschner H (1999) Monoxenic culture of arbuscular mycorrhizal fungi and plant hairy roots. Russian J Plant Physiol 46:87–92

Karandashov V, Kuzovkina I, Hawkins HJ, George E (2000) Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots. Mycorrhiza 10:23–28

LeTacon F, Skinner FA, Mosse B (1983) Spore germination and hyphal growth of a vesicular arbuscular mycorrhizal fungus, *Glomus mosseae* (Gerdemann and Trappe) under decreased oxygen and increased carbon dioxide concentrations. Can J Microbiol 29:1280–1285

Leu SW, Chang DCN (1993) Physiological study of *Glomus* and *Gigaspora* spore germinating on millipore surface. Trans Mycol Soc Rep China 8:1–19

Lim LL, Fineran BA, Cole ALJ (1983) Ultrastructure of intrahyphal hyphae of *Glomus fasciculatum* (Thaxter) Gerdemann and Trappe in roots of white clover (*Trifolium repens* L.). New Phytol 95:231–239

Logi C, Sbrana C, Giovannetti M (1998) Cellular events involved in survival of individual arbuscular mycorrhizal symbionts growing in the absence of the host. Appl Environ Microbiol 64:3473–3479

Marbach K, Stahl U (1994) Senescence of mycelia. In: Wessels JGH, Meinhardt F (eds) Growth, differentiation and sexuality. The Mycota. Springer, Berlin Heidelberg New York, pp 195–210

Mayo K, Davis RE, Motta J (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78:426–431

Meier R, Charvat I (1992) Gemination of *Glomus mosseae* spores: procedure and ultrastructural analysis. Int J Plant Sci 153:541–549

Mosse B (1959) The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular arbuscular mycorrhiza. Trans Br Mycol Soc 42:273–286

Mosse B (1962) The establishment of vesicular arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mosse B (1988) Some studies relating to "independent" growth of vesicular-arbuscular endophytes. Can J Bot 66:2533–2540

Mosse B, Hepper C (1975) Vesicular arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 7:1045–1050

Nagahashi G, Douds D Jr, Buée M (2000) Light-induced hyphal branching of germinated AM fungal spores. Plant Soil 219:71–79

Nantais L (1997) Étude comparative du pouvoir inoculant des spores vs les vésicules du champignon endomycorhizien *Glomus intraradices*. Thèse MSc, Université de Montréal, Canada

Nuutila AM Vestberg M, Kauppinen V (1995) Infection of hairy roots of strawberry (*Fragaria* × *Ananassa* Duch.) with arbuscular mycorrhizal fungus. Plant Cell Rep 14:505–509

Paula MA, dePinto JEB, Siqueira JO, Pasqual M (1994) Benefits of plant cell suspension to vesicular-arbuscular mycorrhizal fungi in vitro. 3. Effects of different culture medium. Pesqui Agropecu Bras 25:1117–1124

Pawlowska TE, Taylor JW (2004) Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature 427:733–737

Pawlowska TE, Douds DD, Charvat I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Pons F, Gianinazzi-Pearson V (1984) Effect of phosphorus, potassium, nitrogen and pH on the in vitro behaviour of vesicular-arbuscular endomycorrhizal fungi. Influence du phosphore, du potassium, de l'azote et du pH sur le comportement in vitro de champignons endomycorhizogenes à vésicules et arbuscules. Cryptogamie Mycol 5:87–100

Poulin MJ, Simard J, Catford JG, Labrie F, Piché Y (1997) Response of symbiotic endomycorrhizal fungi to estrogens and antiestrogens. Mol Plant Microb Interact 10:481–487

Powell CL (1976) Development of mycorrhizal infections from *Endogone* spores and infected root segments Trans Br Mycol Soc 66:439–445

Sancholle M, Dalpé Y, Grandmougin-Ferjani A (2001) Lipids of Mycorrhizae. In: Hock (ed) The Mycota. IX. Fungal associations. Springer, Berlin Heidelberg New York, pp 63–93

Sanders IR (2002) Ecology and evolution of multigenomic arbuscular mycorrhizal fungi. Am Nat 160:S128–S141

Schardl CL, Craven KD (2003) Interspecific hybridization in plant-associated fungi and oomycetes: a review. Mol Ecol 12:2861–2873

Schreiner RP, Koide RT (1993) Stimulation of vesicular-arbuscular mycorrhizal fungi by mycotrophic and non mycotrophic plant root systems. Appl Environ Microbiol 59:2750–2752

Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1403–1421

Sheikh NA, Sanders FE (1988) Effect of temperature on germination mycorrhizal spores and VAM infection in roots. Biologia 34:337–350

Simoneau P, Louisy-Louis N, Plenchette C, Strullu DG (1994) Accumulation of new polypeptides in Ri T-DNA transformed roots of tomato (*Lycopersicon esculentum*) during the development of vesicular arbuscular mycorrhizae. Appl Environ Microbiol 60:1810–1813

Siqueira JO, Hubbell DH (1984) Effect of nutrition on germination and growth of vesicular arbuscular mycorrhizal (VAM) fungi. In: Proc 6th North American Conf Mycorrhizae (NACOM), Oregon, USA, pp 368

Siqueira JO, Hubbell DH, Schenck NC (1982) Spore germination and germ tube growth of a vesicular arbuscular mycorrhizal fungus in vitro. Mycologia 74:952–959

Siqueira JO, Sylvia DM, Gibson J, Hubbell DH (1985) Spores, germination, and germ tubes of vesicular arbuscular mycorrhizal fungi. Can J Microbiol 31:965–971

Smith FA, Smith F (1997) Structural diversity in (vesicular) arbuscular mycorrhizal symbioses. New Phytol 137:373–388

St-Arnaud M, Hamel, C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332

Strullu DG, Romand C (1986) Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. C R Acad Sci Paris 303:245–250

Strullu DG, Romand C (1987) Culture axénique de vésicules isolées partir d'endomycorhizes et ré-association in vitro des racines de tomate. C R Acad Sci Paris 305:15–19

Strullu DG, Romand C, Plenchette C (1991) Axenic culture and encapsulation of the intraradical forms of *Glomus* spp. World J Microb Biot 7:292–297

Strullu DG, Diop T, Plenchette C (1997) Réalisation de collection sin vitro de *Glomus intraradices* (Schenck et Smith) et *Glomus versiforme* (Karsten et Berch) et proposition d'un cycle de développement. C R Acad Sci Paris 320:41–47

Tamasloukht M, Sejalon-Delmas N, Kluever A, Jauneau A, Roux C, Bécard G, Franken P (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from. Plant Physiol 131:1468–1478

Tilak KVBR, Dwivedi BR, Il A, Yindian C (1990) Enhancement of spore germination of *Glomus fasciculatum* by bacterial cell free extracts. J Exp Biol 28:373–375

Timonen S, Smith FA, Smith SE (2001) Microtubules of mycorrhizal fungus *Glomus intraradices* in symbiosis with tomato roots. Can J Bot 79:307–313

Tommerup IC (1983) Spore dormancy in vesicular-arbuscular mycorrhizal fungi. Trans Br Mycol Soc 81:37–45

Tommerup I (1984) Population biology of VA mycorrhizal fungi: propagule behaviour. In: Proc 6th North American Conf Mycorrhizae (NACOM), Oregon, USA, p 331

Tommerup I, Kidby DK (1980) Production of aseptic spores of vesicular arbuscular endophytes and their viability after chemical and physical stress. Appl Environ Microbiol 39:1111–1119

Tsai SM, Phillips DA (1991) Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl Environ Microbiol 57:1485–1488

Tylka GL, Hussey RS, Roncadori RW (1991) Axenic germination of vesicular arbuscular mycorrhizal fungi. Effects of selected *Streptomyces* species. Phytopathology 81:754–759

Vierheilig H, Bago B, Albrecht C, Poulin MJ, Piché Y (1998) Flavonoids and arbuscular mycorrhizal fungi. In: Manthey J, Buslig B (eds) Flavonoids in the living system. Plenum, New York, pp 9–33

Vilarino A, Sainz MJ (1997) Treatment of *Glomus mosseae* propagules with 50% sucrose increases spore germination and inoculum potential. Soil Biol Biochem 29:1571–1573

5 Life History Strategies in Gigasporaceae: Insight from Monoxenic Culture

Francisco Adriano de Souza[1], Yolande Dalpé[2], Stéphane Declerck[3], Ivan Enrique de la Providencia[3,4], Nathalie Séjalon-Delmas[5]

1 Introduction

During the past years, there has been an increased interest in the role of arbuscular mycorrhizal (AM) fungal biodiversity for the functioning of terrestrial ecosystems and in the application of AM fungal technology for agricultural and land rehabilitation schemes. However, one major bottleneck in AM research is the lack of knowledge on ecology, and in particular on life history strategies (LHS) among the different AM fungal families (Hart et al. 2001; Hart and Klironomos 2002).

The LHS of an organism is a product of its evolutionary past, and is expressed in the fungal life cycle, i.e. patterns of growth, differentiation, storage and, especially, reproduction (Begon et al. 1996). In order to understand AM fungal ecology, it is central to appreciate species life history traits and genetic diversity. Monoxenic cultures of AM fungi are exploited here to study these issues, with special emphasis on the family Gigasporaceae.

2 The Family Gigasporaceae and Its Occurrence

The family Gigasporaceae comprises the two genera *Gigaspora* and *Scutellospora*, with approximately 8 and 33 described species respectively. *Scutellospora* is more diverse than *Gigaspora* in terms of described species,

[1]Empresa Brasileira de Pesquisa Agropecuária – Embrapa Agrobiologia, BR 465-RJ, Km 7, Caixa Postal 74505, CEP 23890-000 Seropédica, RJ, Brazil, Tel.: +55-21-26821500, Fax: +55-21-26821230, E-mail: fdesouza@cnpab.embrapa.br
[2]Agriculture and Agri-Food Canada Environment Health/Biodiveristy, 960 Carling Avenue, Ottawa K1A 0C6, Canada
[3]Université Catholique de Louvain, Mycothèque de l'Université Catholique de Louvain (MUCL), Unité de Microbiologie, 3 Place Croix du Sud, 1348 Louvain-La-Neuve, Belgium
[4]Instituto Nacional de Ciencias Agricolas (INCA), Km 3 carretera de Tapaste, Gaveta postal 1, San José de las lajas, La Habana, Cuba
[5]Equipe de Mycologie Végétale, UMR 5546 CNRS Université Paul Sabatier, Pôle de Biotechnologie Végétale, 24 Chemin de Borde-Rouge, B.P. 17, 31326 Castanet-Tolosan, France

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

spore morphological characteristics (Morton 1995), and occurrence in natural ecosystems. Members of this family possess unique intra- and extraradical mycelium morphologies characterized by the absence of intraradical vesicles and the differentiation of extraradical auxiliary cells (AC).

Gigasporaceae occur in terrestrial ecosystems, usually at low spore densities and high species richness in diverse and or stable plant ecosystems (Siqueira et al. 1989; Lovelock et al. 2003; Zhao et al. 2003). In coastal sand-dune ecosystems, Gigasporaceae can be dominant (Stürmer and Bellei 1994; Beena et al. 2000 and references cited therein), while in agricultural soils cultivated with annual crops and in arid ecosystems, they tend to be less abundant or even absent (Sieverding 1991; Helgason et al. 1998; Stutz et al. 2000; Jansa et al. 2002). An adequate explanation for these patterns has yet to be found. Evidence obtained from monoxenic AM fungi cultures was used to clarify these patterns (see Sect. 5).

3 Life Cycle

The AM fungi life cycle can be divided into three main steps: (1) the pre-symbiotic phase and establishment of the symbiosis; this involves propagule activation, host search, appressorium formation, root penetration and arbuscule formation; (2) the vegetative growing phase; and (3) the reproductive phase. Steps 2 and 3 occur almost concomitantly, because in general AM fungi show an iteroparous reproductive phase. Although simple, there is evidence that different AM fungi use different strategies to accomplish each of these steps.

3.1 Pre-Symbiotic Phase

3.1.1 Propagules

In AM fungi, three types of propagules are generally considered: (1) spores, (2) intraradical mycelium within colonized roots, and (3) extraradical mycelium. Spores are the most effective propagules for Gigasporaceae isolate, while Acaulosporaceae and Glomeraceae have been demonstrated to induce new colonization using all three sources of inocula (Biermann and Linderman 1983; Brundrett et al. 1999; Klironomos and Hart 2002). However, it has been suggested that colonized roots of *Scutellospora calospora*

and *S. heterogama* are able to trigger plant root colonization in vivo (Tommerup and Abbott 1981; Braunberger et al. 1996; Klironomos and Hart 2002). Intraradical sporulation has been observed from field and pot-culture *Gigaspora* and *Scutellospora* species (Schenck and Perez 1990; INVAM http://invam.caf.wvu.edu; Dalpé, unpubl. data). One isolate of *Gigaspora margarita* was reported to produce 10–15% of the total number of spores intraradically under monoxenic cultures (Gadkar and Adholeya 2000). Thus, it might be possible that intraradical spores were the cause of the infective capacity of colonized roots of some Gigasporaceae. The infective capacity of the extraradical mycelium of Gigasporaceae has only been demonstrated in vivo with *S. calospora* isolates from Australia (Tommerup and Abbott 1981), while in some other cases, colonization failed (Biermann and Liderman 1983; Klironomos and Hart 2002; Declerck et al. 2004). Declerck et al. (2004) reported, under monoxenic culture conditions, the hyphal re-growth from individual AC of *S. reticulata*, and they suggested that long pieces of intact mycelium harbouring several AC might possibly induce colonization. The apparent discrepancy in these results might be explained by differences in the integrity of the mycelium used to perform these experiments, and the amount of resource available in the mycelial structures. For instance, de Souza and Declerck (2003) observed that, in monoxenic culture, young AC contained lipid drops, while older ones appeared empty.

A comparison of spore diameter of species in the families Gigasporaceae, Acaulosporaceae and Glomeraceae (average diameters 314, 158 and 127 µm respectively) shows that Gigasporaceae species produce, in general, large spores (data from Schenck and Perez 1990; *Glomus* species from the former *Sclerocystis* genus were not included). Common traits related with spore quality are germination rates, survival dormancy, and size. Large spores must contain more resources to support multiple germinations and mycelial growth, and to sustain metabolism while searching for a host.

3.1.2 Spore Germination, Dormancy and Lifespan

The germination process in Gigasporaceae is linked with the spore wall organization (Walker and Sanders 1986; Spain et al. 1989). Multiple germinations were reported for *Gigaspora* species (Koske 1981a; Giovannetti et al. 2000), reaching up to 40 successive germinations for single spores of *Gi. margarita* under in vitro conditions (P. Jargeat, pers. comm.). If the germination tube (GT) does not meet a root, then the cytoplasm may retract (Beilby and Kidby 1980).

Spore germination does not require external factors other than humidity and temperature to germinate. Germination rates reached a 80–100% level for *Gigaspora* isolates (Koske 1981b; Bécard and Piché 1989a; Diop

et al. 1992; Romero and Siqueira 1996; Maia and Yano-Melo 2001), compared to 60% for *Scutellospora* (de Souza, unpubl. data). In the latter case, spores might enter into dormancy, a phenomenon frequently associated with a higher survival capacity. *Gi. gigantea* and *Gi. margarita* spores obtained in monoxenic cultures exhibit a dormancy which does not exist when the spores are produced on whole plants (Séjalon-Delmas, unpubl. data). With *Gigaspora* strains, a 3-week cold treatment may relieve the dormancy (Jargeat and Séjalon-Delmas, unpubl. data). The life span of *Gigaspora* spores has been estimated to be up to 5 months under natural growing conditions (Lee and Koske 1994; Pringle and Bever 2002). A broad comparison of germination and survival capacity of different Gigasporaceae species is still lacking, and it would be interesting to study these traits using a phylogenetic framework, based on morphological and molecular data.

3.1.3
Plant Compounds That Affect the Pre-Symbiotic Phase

Among the studies on the pre-symbiotic stage, increasing numbers are conducted using monoxenic culture and are of key importance to understand the effects of plant compounds on fungal growth (for a review, see Fortin et al. 2002). Buée et al. (2000) showed that mycotrophic plants produced a soluble factor which induced hyphal branching in *Gi. gigantea*. Douds et al. (1996) reported the positive effect of phenolic acids, extracted from carrot hairy roots, on *Gi. gigantea* and *Gi. margarita* germ tube attraction. Some studies revealed the effect of volatiles, from maize root-organ culture or pea Sparkle mutants, on germ tube attraction of *Gi. gigantea* and *Gi. margarita* (Koske 1982 and Boovaraghan et al. 1995 respectively). Synergistic effects of root volatiles, in particular CO_2, with root exudates were also frequently observed (Bécard and Piché 1989b; Suriyapperma and Koske 1995). The effects of root exudates on the AM fungus may be divided into germ tube attraction (Gemma and Koske 1988) and hyphal branching (Nagahashi and Douds 1996; Nagahashi et al. 1999; Buée et al. 2000). The active compounds responsible for the fungal response are still not characterized. Flavonoids have been proposed for several years (Nair et al. 1991). However, the flavonoid family can generate different fungal responses (stimulatory, inhibitory or neutral) and were not detected in carrot hairy roots, suggesting that they are of secondary importance. More recently, a root factor has been found which stimulates the activity of a broad range of AM fungal species (Buée et al. 2000). In the same study, non-mycotrophic plants, like *Brassica*, were devoid of these active root factors. Using the same bioassay, however, Nagahashi and Douds (2000) revealed that the factors derived from the non-host had an inhibitory activity.

Recently, tomato mutants affected in the pre-symbiotic stage have been obtained by fast-neutron mutagenization of seeds. These mutants are resistant to mycorrhization, and spores of *Glomus intraradices* present in their rhizosphere exhibit a percent of germination and appressorium formation lower by 45 and 70% respectively than the rates obtained with the wild-type (David-Schwartz et al. 2001). Monoxenic cultures were developed, and the tomato mutant has been proved to secrete inhibitory compounds. However, the chemical nature of these compounds is still unknown. They delay *G. intraradices* proliferation. Establishment of mycorrhiza with the mutant culture gives abortive spores of *G. intraradices* and *Gi. rosea*, indicating that the mutant lacks a signal essential for the fungus to accomplish its life cycle (Gadkar et al. 2003). Few studies involved molecular fungal response to root exudates. Among the 415 EST deposited today in Genbank, only 47 were obtained at the pre-symbiotic stage. We cannot avoid mentioning the transcriptional studies of Tamasloukht et al. (2003) on *Gi. rosea* and *Gl. intraradices* stimulated by carrot hairy root exudates. Molecular analysis revealed a differential expression of some genes, essentially involved in mitochondrial metabolism, which was correlated to an early increase in respiration activity. This activation may be associated to an increase in the cytoplasmic pool of ATP and lipid catabolism. This would be in accordance with the observations of Bécard and Piché (1989b) on germinating spores of *Gi. rosea* after 3 weeks of stimulation. They noted that spores looked empty, as if the root factor present in root exudates regulated the fungus capacity to use its own reserves.

3.2
Symbiotic Vegetative and Reproductive Growing Phases

In relation to LHS, two interconnected characteristics revealed by studies of Gigasporaceae species under monoxenic culture are highlighted here: the colonization pattern, and the development and maintenance of arbuscules. In addition, the hyphal healing mechanism and anastomosis are also discussed.

3.2.1
Colonization Pattern

Gigasporaceae seem to be slower root colonizers than species of Glomeraceae and Acaulosporaceae (Brundrett et al. 1999; Santos et al. 2000; Tiwari and Adholeya 2002). Hart and Reader (2002) compared the colonization strategy of 21 isolates from the families Acaulosporaceae (4), Gigasporaceae (5) and Glomeraceae (12), using four different host plants under

pot-culture conditions. They reported that Glomeraceae isolates colonized roots before Acaulosporaceae and Gigasporaceae, and the results were independent of the host plant used.

Under monoxenic culture, Gigasporaceae is able to establish contact and colonize a root explant within 3–10 days after coming in the vicinity of an active root. However, the exponential extraradical mycelium growth phase was only observed to begin 3–5 weeks after colonization, with *S. reticulata* (Declerck et al. 2004). An interesting characteristic of Gigasporaceae behaviour is that they increase the overall colonization (number of infection points) and extraradical mycelial growth exponentially when root activity has decreased or ceased (Diop et al. 1992; Declerck et al. 2004).

3.2.2 Maintenance of Arbuscules

Arbuscules are considered short-lived (1–3 weeks) fungal structures found preferentially in young, thin roots during early stages of root colonization (Smith and Read 1997; Harrison 1999). However, arbuscules differentiated by some Gigasporaceae isolates were found, surprisingly, to occur in hairy root cultures that had ceased growth for several months, as observed for *Gi. rosea* and *S. reticulata* (Diop et al. 1992; Declerck et al. 2004). The formation of arbuscules is controlled by the host plant's genetic machinery, and the number of differentiated arbuscules has been found to be dependent on plant identity, the availability of nutrients, and the fungal partner (Smith and Read 1997; Harrison 1999). Are arbuscules controlled in a different way in Gigasporaceae monoxenic cultures and in pot culture? Morton provides information regarding this question via the INVAM website (http://invam.caf.wvu.edu). In Gigasporaceae, arbuscules are "... in pot cultures, still abundant long after plants (and roots) have ceased growth ...", and the persistence of the total arbuscular network in mycorrhizal roots of pot cultures is longer for species of the family Gigasporaceae than for those of Glomeraceae.

3.2.3 Hyphal Healing Mechanism (HHM)

The hyphal healing mechanism (HHM) has been reported in AM fungi since Gerdermann (1955). More recently, Kang-Hyeon et al. (1994) have reported wound healing in *Gi. margarita, S. verrucosa* and *S. heterogama* grown on agar media. Artificial wounding (with a razor blade, Fig. 1) performed on 4–5 day old GT of *Gi. rosea* spores resulted in dead sections of hyphae. The injured section darkened and separated from the living hyphae by a septum

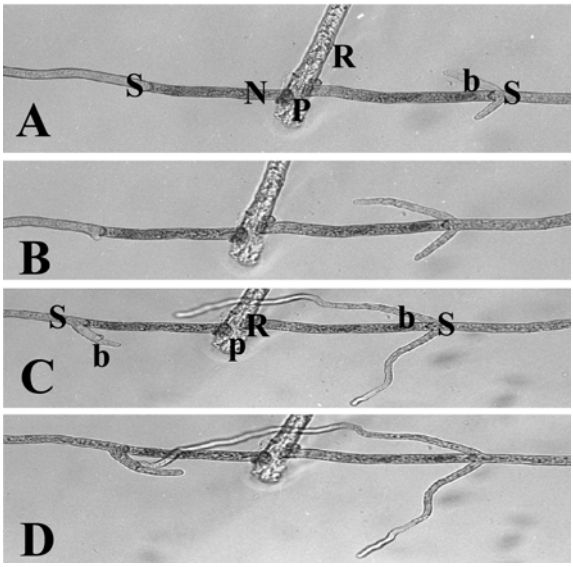


Fig. 1A–D. Hyphal healing mechanisms in germinating hyphae of *Gigaspora gigantea* and *Gi. rosea* (N. Sejalon-Delmas, unpubl. data). **A** Some minutes after wounding, necrosis appears at both hyphal ends. Some cell material forms a plug which obstructed the wounded hypha, preventing cytoplasmic leakage. After 15 min, a septum forms to isolate the hyphal necrotic ends. **B** Emergence, after 4 h, of two lateral branches from one live section. Note the difference of growth of the two branches. **C** Growth of the two lateral branches towards a new single branch emerging at the opposite side of the injured hyphae. **D** Both ends are reconnected (16 h). *B* Lateral branches, *N* necrotic part of the hypha, *P* plug, *R* scrape of the razorblade, *S* septum

within 15 min. After 4 h, two branches grew from both sides of the section, just behind the septum. The branch emerging from the hypha linked to the spore always became dominant. Branches were attracted to each other, and a fusion was observed tip to tip about 7 h after wounding. Average attraction distance may be calculated as distance/number of tip-to-tip contact, established for anastomoses recording. In *Gi. rosea*, this average attraction distance is 396 µm, the value being 512 µm for *Gi. gigantea*. When the distance between the two parts of the hyphae to repair was too long, several branches were formed, while in the case of the wound being too close to the spore, only one new GT was formed and no fusion with the adjacent hyphae was observed. Numerous cellular events are probably involved in HHM: (1) chemical attraction, (2) hydrolysis of the cell wall during cell-to-cell contact, involving autolytic enzymes, and (3) cytoplasmic plasmogamy occurring after membrane fusion, presumably in a manner similar to that of vesicles and organelles fusing with other membranes in eukaryotic cells. Concerning the chemical attraction signal, the proposed hypothesis is that

every growing cell produces a single, specific labile substance, and has a steep concentration gradient. The hyphal tip would be sensitive to a critical concentration of this substance, and responds by growing towards this increased concentration. However, nothing is known about signal transfer, the recognition leading to contact and fusion. The latter event will probably involve cytoskeleton rearrangements and also nuclear exchanges (Giovannetti et al. 1999). The phenomenon described here for germinating spores has also been described on monoxenic cultures of *G. intraradices* (Bago et al. 1999), *Gi. rosea* (Bécard and Piche 1989a) and *S. reticulata* (de Souza and Declerck 2003). The characterization of the mode of action and efficiency of the HHM can give clues about the organisms' LHS, because k-strategists and/or stress-resistant organisms are expected to evolve better defence and repair mechanisms than r-strategists (Pianka 1970).

3.2.4 Anastomosis

Anastomosis is a process of hyphal fusion between compatible fungi, resulting in the formation of mycelial networks and allowing exchange of genetic material. Tommerup (1988) described anastomosis in *G. monosporum* and *A. laevis*. This author demonstrated the absence of anastomosis between different species, and recorded anastomosis events only between isolates of one and the same species. These results were confirmed by Giovannetti et al. (2003) on different *Glomus* strains. As previously reported for *Acaulospora*, no anastomosis could be found on germinating spores of *Gi. rosea* and *S. castanea* (Giovannetti et al. 1999). Recently, anastomosis was observed in *S. reticulata* growing under monoxenic culture, but it was restricted to branches of the same hypha and only observed in thin hyphae linked with branch absorbent structures, never between runner hyphae (de Souza and Decleck 2003), or always correspond to a hyphal bridge (de la Providencia et al. 2005).

4 Genetic Diversity and Phenotypic Variation

The genetic diversity and phenotypic variation in AM fungi need to be understood within a phylogenetic context to be linked with life history traits. In addition, the species concept in AM fungi must be refined to address ecological and evolutionary questions. In this sense, the cultivation of AM fungi in monoxenic culture offers an excellent basis to undertake such studies. Koch et al. (2004) published the first article exploring the potential of monoxenic culture to study quantitative genetic traits in a population

of AM fungi obtained from the field. To relate phenotypic traits with life history, they measured hypha growth rate and spore production between different isolates. The maintenance of isolated individuals from a population in a constant environment (monoxenic culture) for several generations allowed them to directly link phenotypic variation and variation in quantitative genetic traits. In addition, they took advantage of the monoxenic system to produce fungal cultures free of alien DNA, in quantity and quality necessary for applying genomic fingerprint techniques such as amplified fragment length polymorphism (AFLP; Koch et al. 2004).

4.1 Vegetative Compatibility Test (VCT)

In terms of the species concept, VCT can be used to determine species boundaries in fungi. In Gigasporaceae, spontaneous pairing between germinating spores has never been observed due to the absence of anastomoses (Giovannetti et al. 1999). For this reason, Séjalon-Delmas developed a simple and efficient VCT method, based on a wound-healing mechanism. The method consists in germinating two spores side by side. After germination, the apices of the GT of the two spores are cut off. Spore A remains in the plate and its cut GT apex is removed, while spore B is removed and its GT apex remains in the plate. When the two spores are compatible, lateral branches develop from each part, forming a connection bridge. When somatic crossing experiments involved daughter spores from a monosporal monoxenic culture, 80% of fusion was obtained. This result is slightly lower than the percent of repair observed for HHM. The 20% of failure in the crossings may be due to a traumatic cutting. Somatic crossings were never observed between spores from different species, and only seldom between spores originating from different monoxenic cultures (Sejalon-Delmas, unpubl. data). These results strongly suggest that hyphal fusion in Glomeromycota is genetically controlled. It is interesting to note that in *Glomus*, like in *Gigaspora* genera, no tropism occurs between the hyphae of different spores, the incompatibility response being represented by protoplasm retraction and septum formation in the approaching hyphae, prior to any physical contact (Sejalon-Delmas, unpublished data).

The molecular identification of *Gigaspora* at species or even at strain levels has been obtained using PCR-denaturing gradient gel electrophoresis (DGGE). The approach is based on the discrimination of the intragenomic polymorphism of nuclear ribosomal (nrRNA) genes (de Souza et al. 2004). The combination of the VCT and PCR-DGGE methods can be used for the rapid and efficient genetic characterization of Gigasporaceae diversity for field as well as laboratory studies.

5
Life History Strategy (LHS) of Gigasporaceae, as Revealed Using Monoxenic Cultures

The few available data for comparative analyses of Gigasporaceae and Glomeraceae LHS were obtained from experiments focused on growth kinetics and development characteristics, such as the timing of the first daughter spore produced, the rate of sporulation, and the duration of the reproductive phase. These characteristics differed between *S. reticulata* and the following *Glomus* species: *G. caledonium*, *G. intraradices* and *G. proliferum* (Fig. 2A). *G. proliferum* and *G. intraradices* formed their first daughter spores after 1 week in culture, and *G. caledonium* after 2 weeks, while *S. reticulata* produced its first daughter spore only after 12 weeks of continuous culturing. *G. caledonium* and *G. intraradices* reached the stationary phase after 15 weeks, and *G. proliferum* after 17 weeks. In contrast, *S. reticulata* continued to produce spores until week 33, i.e. more than 8 months after starting the culture (Fig. 2A).

Life histories patterns are often related to variation in reproductive activity, or reproductive effort which measures the amount of available resources allocated to reproduction over time (Begon et al. 1996). However, reproductive effort is difficult to measure. One simple way to have an idea about reproductive effort is by calculating the Malthusian fitness (MF). The MF, in this case, compared the instantaneous change of spore production over time in relation to the starting inoculum (Fig. 2B). For a discussion about the application of MF to filamentous fungi, the reader can refer to Pringle and Taylor (2002). The evolution of the MF in *S. reticulata* was clearly different from that of the *Glomus* species (Fig. 2B). The same trend was observed in other species of these two families cultured under monoxenic culture conditions (Fig. 3). Gigasporaceae species studied in monoxenic culture have shown a short overlap of sporulation with the active growing phase of the roots, while *Glomus* species sporulate concomitantly with the root growth (Fig. 3). These observations suggest that, for these species cultured under monoxenic culture, Gigasporaceae and Glomeraceae concentrate their reproductive efforts at different times. Gigasporaceae favour somatic growth, whereas Glomeraceae favour reproduction.

The reproductive phase in Gigasporaceae seems to be linked with a critical extraradical mycelium biomass. For *S. reticulata*, the first daughter spores were produced after 12 weeks, when a biomass of 1360 $\pm$ 625 cm of extraradical mycelium length and 501 $\pm$ 96 AC was reached (Declerck et al. 2004). *Gi. margarita* and *Gi. rosea* produced the first daughter spores after 8–10 weeks of culturing. However, the reproductive phase can be extended

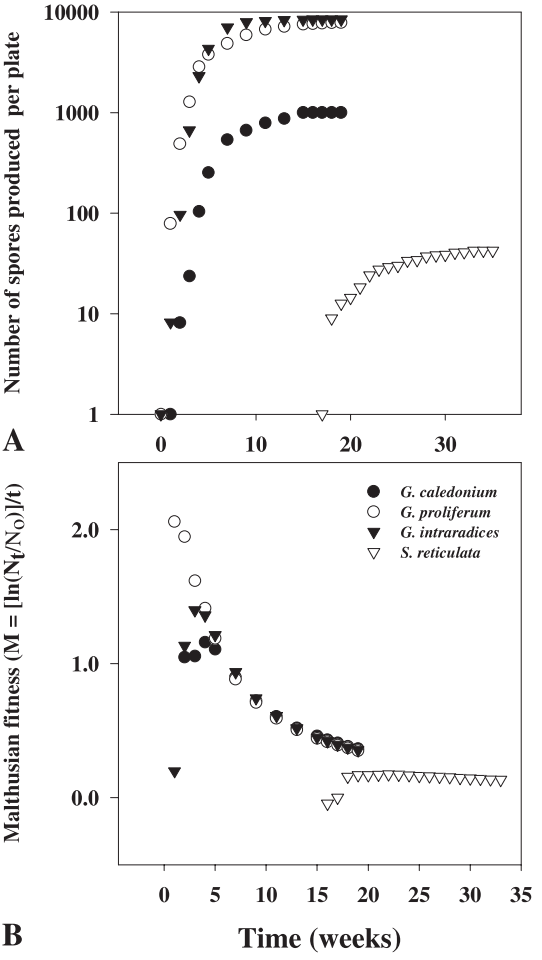


Fig. 2. A Sporulation dynamics of three *Glomus* spp. and *Scutellospora reticulata* under monoxenic culture conditions. **B** Weekly evolution of the Malthusian fitness for the sporulation dynamics showed in **A** (N_o was 1, 10, 10 and 1 for *G. caledonium*, *G. proliferum*, *G. intraradices* and *S. reticulata* respectively). (Modified from Declerck et al. 2001, 2004)

over 1 year, suggesting a long mycelium lifespan (Diop et al. 1992; Gadkar and Adholeya 2000).

An interesting point comes from the fact that most fungal biomass, including spores, obtained from Gigasporaceae isolates was generated after the root had ceased growth. At this time, one part of the resources in the medium was already consumed by the root culture (Diop et al. 1992), indicating a capacity of Gigasporaceae to live and reproduce with a small portion of the resources available (Fig. 3). This scenario is similar to the conditions expected for competitive species (Grime 1979), referring to the k-strategist concept (McArthur and Wilson 1967; Pianka 1970).

The comparative sporulation rates of Gigasporaceae and *Glomus* species revealed a clear negative correlation between the size and the number of

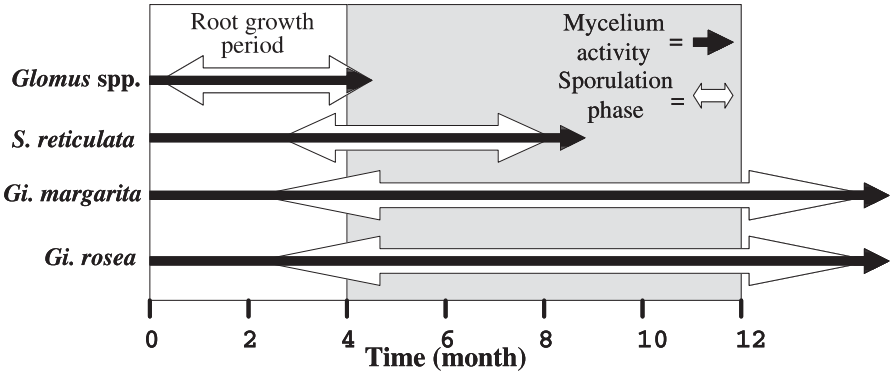


Fig. 3. Schematic representation of mycelium development and activity (*black arrows*) and sporulation (*double-headed arrows*) periods of eight *Glomus* and three Gigasporaceae species under monoxenic culture conditions. *Glomus* and Gigasporaceae species represented were *G. caledonium* (Declerck et al. 2000), *G. clarum* (de Souza, unpubl. data), *G. etunicatum* (Pawlowska et al. 1999), *G. fasciculatum* (Declerck et al. 1998), *G. intraradices* (Declerck et al. 2000), *G. macrocarpum* (Declerck et al. 1998), *G. proliferum* (Declerck et al. 2000), *G. vesiforme* (Declerck et al. 1998), and *Gi. margarita* (Gadkar and Adholeya 2000), *Gi. rosea* (Diop et al. 1992), *S. reticulata* (Declerck et al. 2004) respectively

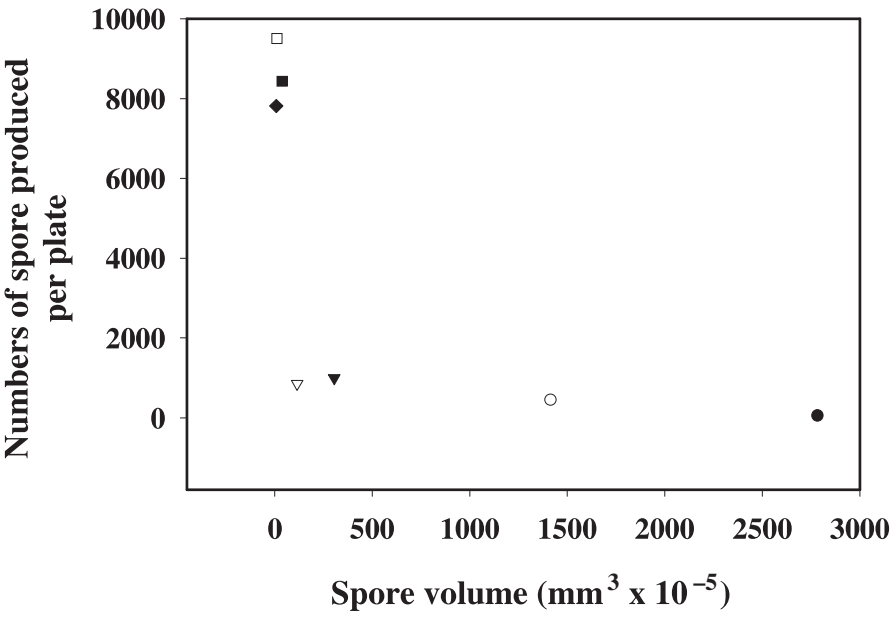


Fig. 4. Spore production in relation to spore volume of five *Glomus* and two Gigasporaceae under monoxenic culture conditions. Data from Declerck et al. 2000, 2004; Diop et al. 1992

differentiated spores (Fig. 4), which is a typical trade-off (Begon et al. 1996). One consequence of a higher allocation of resource for a small progeny is that the offspring will have a higher quality or vigour and, consequently, higher chances of survival than species which produce low energy cost propagules.

5.1 Co-Existence and Competition Experiments Under Dixenic Culture

The direct assessment of coexistence and competition of AM fungi under dixenic culture can be exemplified by the completion of *Gi. margarita* and *G. intraradices* life cycles when co-cultured on an excised root culture (Tiwari and Adholeya 2002). The sporulation patterns observed in this dixenic culture were similar to the patterns reported for monoxenic cultures, i.e. *G. intraradices* started and ceased to form spores earlier than *Gigaspora*. The assessment of fungal competition can be carried out by comparing, for example, the sporulation of two AM fungi growing in monoxenic and dixenic cultures. Such systems can also be adjusted to assess the effects of predators on different AM fungal species, for instance, collembolans. The possibility of studying competition under monoxenic culture might facilitate the implementation, execution and quantification of experiments by allowing a precise control of the resources used, easy maintenance, and direct quantification over time.

5.2 Ecological Implications of the Gigasporaceae Life History Strategy

The Gigasporaceae isolates studied in monoxenic culture exhibited several traits (investment in somatic growth rather than in reproduction, development of large spore size and few offspring) suggesting that they are adapted to live in stable ecosystems where inter- and intraspecific competition is high for resources, and somatic growth is favoured over reproduction. The fast sporulation of *Glomus* isolates differentiating single spores in the soil followed the reversed trend, i.e. they seemed to be adapted for growth in disturbed ecosystems rich in available resources, which favour reproduction over somatic growth. It is important to remember the polyphyletic origin of the genus *Glomus* (Schwarzott et al. 2001), which implies that different subgroups have different evolutionary histories and potentially different LHS. For instance, Brundrett et al. (1999) reported that sporocarp-forming *Glomus* species needed much longer cultivation periods under pot-culture conditions to produce spores than *Glomus* species which formed single

spores in the soil, and this time period was even longer than that observed for species of Gigasporaceae and Acaulosporaceae.

Life history traits reported for Gigasporaceae may imply that these species would be negatively selected in agricultural fields cultivated with annual crops, submitted to frequent plowing which disrupts the mycorrhizal mycelium (Jasper et al. 1989; Fairchild and Miller 1990). On the other hand, fast-sporulating *Glomus* isolates would show the reverse trend, i.e. positive selection. Another important advantage of *Glomus* in adapting to agricultural soils is its ability to survive and propagate well using intraradical vesicles.

Root growth and AM fungi colonization consist in a dynamic process where new and old colonization stages exist in a single root system. Consequently, the coexistence of Gigasporaceae and *Glomus* isolates in one root should be logically facilitated by different LHS which would allow the fungi to explore different phases of their host's life cycle. Coexistence between Gigasporaceae and *Glomus* species can be directly observed under monoxenic culture (Fig. 5). Within the context of this hypothesis regarding the coexistence mechanism, *Glomus* isolates first colonize an active growing root, and differentiate arbuscules which subsequently disappear with root ageing; meanwhile, the colonization evolved forming vesicles. Later, Gigasporaceae colonize the same root fragment, differentiating new arbuscules

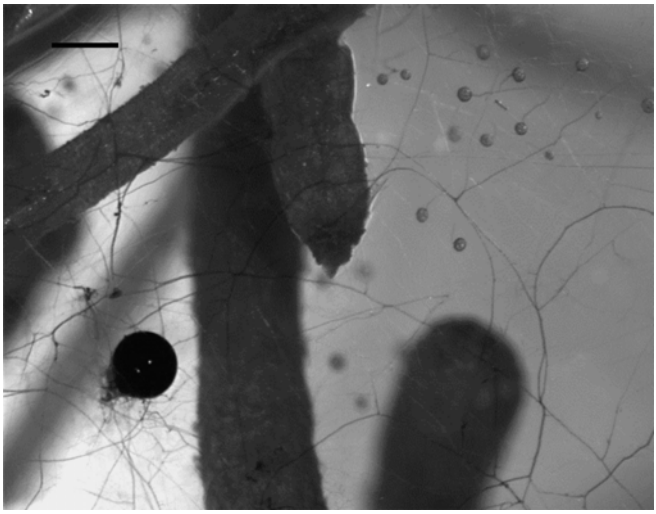


Fig. 5. Co-existence of *Scutellospora reticulata* CNPAB11 and *Glomus intraradices* MUCL 43194 under monoxenic culture conditions. Note the difference in size between the *Scutellospora* (*large dark spore*) and *Glomus* (*smaller, paler spores*). Most of the mycelium shown is from *S. reticulata* (*bar* = 400 µm). De Souza unpublished data

and expanding colonization to other roots and soil. The microcosm experiment of van Tuinen et al. (1998) seems to support this hypothesis. In their experiment, two Gigasporaceae (*Gi. rosea* and *S. castanea*) were usually found only co-colonizing a root fragment together with a *Glomus* isolate. They suggested a mechanism of synergism between the different fungi for colonization. Interestingly, all four species are able to grow and sporulate when cultivated as single species.

6 Conclusions

A tremendous amount of knowledge remains to be acquired through the practice of monoxenic and dixenic cultures of AM fungi. Several of the avenues already explored using monoxenic cultures can be used as steppingstones for future investigations. Whatever discipline is concerned – ecology, genetics, physiology – the cultivation of AM fungi associated with transformed roots appears to be useful for investigating all of these. For example, the advances in genetics of AM fungi will sooner or later be involved in mutant comparisons, where it is easy to foresee the advantages of using monoxenic cultures. This will enable to understand the functional significance of AM fungi genetic variation, as well as the cost and benefits of key phenotypic traits. The evaluation of fungicides, the testing of soil and plant pollutants, the synergy with biocontrol agents, and the behaviour of fungi predators are all practical research avenues. The framework of research constructed using monoxenic culture allows a close follow-up and precise measurement of growth dynamic parameters. However, the relevance of collected data to the complexity of the natural community has to be strictly verified using data from various sources, from a diversity of fungal strains and cultivation methods, in order to confirm the LHS patterns observed using monoxenic cultures. The cultivation of AM fungi in association with transformed roots offers a standard way to compare different AM fungi, in monoxenic or dixenic cultures. In addition, this approach allows detailed observation and long-term experimentations.

Acknowledgements. F.A.deS. thanks the Brazilian Council for Scientific and Technological Development (CNPq) for his financial support (grant #200850/98-9). I.delaP. thanks the Coopération Universitaire au Développement (CUD) from the Université catholique de Louvain for financial support, and the director of INCA for supporting the AM fungal research. This represents publication number 3408 NIOO-KNAW Netherlands Institute of Ecology, where F.A. de Souza was formerly associated.

References

Bago B, Zipfel W, Williams RM, Piché Y (1999) Nuclei of symbiotic arbuscular mycorrhizal fungi as revealed by in vivo two-photon microscopy. Protoplasma 209:77–89

Bécard G, Piché Y (1989a) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 112:77–83

Bécard G, Piché Y (1989b) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Beena KR, Raviraja NS, Arun AB, Sridhar KR (2000) Diversity of arbuscular mycorrhizal fungi on the coastal sand dunes of the west coast of India. Curr Sci 79:1459–1466

Begon M, Harper JL, Townsend CR (1996) Ecology: individual, populations and communities, 3rd edn. Blackwell, London

Beilby JP, Kidby DK (1980) Biochemistry of ungerminated and germinated spores of the vesicular-arbuscular mycorrhizal fungus, *Glomus caledonium*: changes in neutral and polar lipids. J Lipid Res 21:739–750

Biermann BJ, Linderman RG (1983) Use of vesicular-arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. New Phytol 95:97–105

Boovaraghan B, Poulin MJ, Vierheilig H, Piché Y (1995) Responses of an arbuscular mycorrhizal fungus, *Gigaspora margarita*, to exudates and volatiles from the Ri T-DNA-transformed roots of nonmycorrhizal and mycorrhizal mutants of *Pisum sativum* L. Sparkle. Exp Mycol 19:275–283

Braunberger PG, Abbott LK, Robson AD (1996) Infectivity of arbuscular mycorrhizal fungi after wetting and drying. New Phytol 134:673–684

Brundrett MC, Jasper DA, Ashwath N (1999) Glomalean mycorrhizal fungi from tropical Australia. II. The effect of nutrient levels and host species on the isolation of fungi. Mycorrhiza 8:315–321

Buée M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant-Microb Interact 13:693–698

David-Schwartz R, Badani H, Wininger S, Levy AA, Galili G, Kapulnik Y (2001) Identification of a novel genetically controlled step in mycorrhizal colonization: plant resistance to infection by fungal spores but not to extraradical hyphae. Plant J 27:561–569

de la Providencia I, de Souza FA, Fernandez F, Sejalon Delmas N, Declerck S (2005) Arbuscular mycorrhizal fungi reveal distinct pattern of anastomoses formation and hyphal healing mechanism between different phylogenic groups. New Phytologist 165:261–271

de Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012

de Souza FA, Kowalchuk GA, Leeflang P, van Veen JA, Smit E (2004) PCR-denaturing gradient gel electrophoresis profiling of inter- and intraspecies 18S rRNA gene sequence heterogeneity is an accurate and sensitive method to assess species diversity of arbuscular mycorrhizal fungi of the genus *Gigaspora*. Appl Environ Microbiol 70:1413–1424

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

Declerck S, D'Or D, Cranenbrouck S, Le Boulengé E (2001) Modeling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. Mycorrhiza 11:225–230

Declerck S, D'Or D, Bivort C, de Souza FA (2004) Development of extraradical mycelium of *Scutellospora reticulata* under root-organ culture: spore production and function of auxiliary cells. Mycol Res 108:84–92

Diop TA, Bécard G, Piché Y (1992) Long-term in vitro culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. Symbiosis 12:249–259

Douds DD, Nagahashi G, Abney GD (1996). The differential effects of cell-wall associated phenolics, cell walls and cytosolic phenolics of host and non-host roots on the growth of two species of AM fungi. New Phytol 133:289–294

Fairchild MH, Miller GL (1990) Vesicular-arbuscular mycorrhizas and the soil-disturbance-induced reduction of nutrient absorption in maize. III. Influence of P amendments to soil. New Phytol 114:641–650

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Gadkar V, Adholeya A (2000) Intraradical sporulation of AM *Gigaspora margarita* in long-term axenic cultivation in Ri T-DNA carrot root. Mycol Res 104:716–721

Gadkar V, David-Schwartz R, Nagahashi G, Douds DD, Wininger S, Kapulnik Y (2003) Root exudate of *pmi* tomato mutant M161 reduces AM fungal proliferation in vitro. FEMS Microbiol Lett 223:193–198

Gemma JN, Koske RE (1988) Pre-infection interactions between roots and the mycorrhizal fungi *Gigaspora gigantea*: chemotropism of germ tubes and root growth responses. Trans Br Mycol Soc 91:122–132

Gerdermann JW (1955) Wound-healing of hyphae in a phycomycetous mycorrhizal fungus. Mycologia 47:916–918

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575

Giovannetti M, Sbrana C, Logi C (2000) Microchambers and video-enhanced light microscopy for monitoring cellular events in living hyphae of arbuscular-mycorrhizal fungi. Plant Soil 226:153–159

Giovannetti M, Sbrana C, Strani P, Agnolucci M, Rinaudo V, Avio L (2003) Genetic diversity of isolates of *Glomus mosseae* from different geographic areas detected by vegetative compatibility testing and biochemical and molecular analysis. Appl Environ Microbiol 69:616–624

Grime JP (1979) Plant strategies and vegetation process. Wiley, New York

Harrison MJ (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol 50:361–389

Hart MM, Klironomos JN (2002) Diversity of arbuscular mycorrhizal fungi and ecosystem functioning In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology, vol 157. Springer, Berlin Heidelberg New York, pp 225–242

Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344

Hart MM, Reader RJ, Klironomos JN (2001) Life-history strategies of arbuscular mycorrhizal fungi in relation to their successional dynamics. Mycologia 93:1186–1194

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394:431

Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225–234

Jasper DA, Abbott LK, Robson AD (1989) Hyphae of a vesicular-arbuscular mycorrhizal fungus maintain infectivity in dry soil, except when the soil is disturbed. New Phytol 112:101–107

Kang-Hyeon K, Chang-Duck K, Chang-Keun Y (1994) Hyphal growth, auxiliary cell development and hyphal healing process of arbuscular mycorrhizal fungi, *Gigaspora* and *Scutellospora* genera. Korean J Mycol 22:36–45

Klironomos JN, Hart MM (2002) Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza 12:181–184

Koch AM, Kuhn G, Fontanillas P, Fumagalli L, Goudet I, Sanders IR (2004) High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. Proc Natl Acad Sci USA 101:2369–2374

Koske RE (1981a) Multiple germinations by spores of *Gigaspora gigantea*. Trans Br Mycol Soc 76:328–330

Koske RE (1981b) *Gigaspora gigantea*: observations on spore germination of a VA-mycorrhiza fungus. Mycologia 73:288–300

Koske RE (1982) Evidence for a volatile attractant from plant roots affecting germ tubes of a VA mycorrhizal fungus. Trans Br Mycol Soc 79:305–310

Lee PJ, Koske RE (1994) *Gigaspora gigantea*: seasonal abundance and ageing of spores in a sand dune. Mycol Res 98:453–457

Lovelock CE, Andersen K, Morton JB (2003) Arbuscular mycorrhizal communities in tropical forests are affected by host tree species and environment. Oecologia 135:268–279

Maia LC, Yano-Melo AM (2001) Germination and germ tube growth of the arbuscular mycorrhizal fungi *Gigaspora albida* in different substrates. Braz J Microbiol 32:281–285

McArthur RH, Wilson EO (1967) The theory of island biogeography. Princeton University Press, Princeton

Morton JB (1995) Taxonomic and phylogenetic divergence among five *Scutellospora* species based on comparative developmental sequences. Mycologia 87:127–137

Nagahashi G, Douds DD (1999) A rapid and sensitive bioassay with practical application for studies on interactions between root exudates and arbuscular mycorrhizal fungi. Biotechnol Tech 13:893–897

Nagahashi G, Douds DD (2000) Partial separation of the root exudates components and their effects upon the growth of germinated spores of AM fungi. Mycol Res 104:1453–1464

Nagahashi G, Douds DD, Abney GD (1996) Phosphorus amendment inhibits hyphal branching of the VAM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation. Mycorrhiza 6:403–408

Nair MG, Safir GR, Siqueira JO (1991) Isolation and identification of vesicular-arbusculalr mycorrhiza-stimulatory compounds from Clover (*Trifolium repens*) roots. Appl Environ Microbiol 57:434–439

Pawlowska TE, Douds DD, Charvat I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Pianka ER (1970) On r and k selection. Am Nat 104:592–597

Pringle A, Bever JD (2002) Divergent phenologies may facilitate the coexistence of arbuscular mycorrhizal fungi in a North Carolina grassland. Am J Bot 89:1439–1446

Pringle A, Taylor JW (2002) The fitness of filamentous fungi. Trends Microbiol 10:474–481

Romero AGF, Siqueira JO (1996) Activity of flavonoids on spores of the mycorrhizal fungus *Gigaspora gigantea* in vitro. Pesqui Agropecu Bras 31:517–522

Santos AL, de Souza FA, Guerra JGM, Berbara RLL (2000) Estabelecimento e capacidade infectiva de *Gigaspora margarita* e *Glomus clarum* em solo sob erosão. Acta Bot Bras 14:127–139

Schenck NC, Perez Y (1990) Manual for the identification of VA mycorrhizal fungi. Synergistic Publications, Gainesville

Schwarzott D, Walker C, Schußler A (2001) *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is nonmonophyletic. Mol Phylogenet Evol 21:190–197

Sieverding E (1991) Vesicular-arbuscular mycorrhiza management in tropical agrosystems. Germany. Deutsche Gesellschaft für Technische Zusammenarbeit, Eschborn, Germany

Siqueira JO, Colozzi FA, de Oliveira (1989) Occurrence of vesicular-arbuscular mycorrhizae in agro and natural ecosystems in Minas Gerais state. Pesqui Agropecu Bras 24:1499–1506

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, London

Spain JL, Sieverding E, Schenck NC (1989) *Gigaspora ramisporophora*: a new species with novel sporophores from Brazil. Mycotaxon 34:667–677

Stürmer SL, Bellei MM (1994) Composition and seasonal variation of spore populations of arbuscular mycorrhizal fungi in dune soils on the island of Santa Catarina, Brazil. Can J Bot 72:359–363

Stutz JC, Copeman R, Martin CA, Morton JB (2000) Patterns of species composition and distribution of arbuscular mycorrhizal fungi in arid regions of southwestern North America and Namibia, Africa. Can J Bot 78:237–245

Suriyapperuma SP, Koske RE (1995) Attraction of germ tubes and germination of spores of the arbuscular mycorrhizal fungus *Gigaspora gigantea* in the presence of roots of maize exposed to different concentrations of phosphorus. Mycologia 87:772–778

Tamasloukht B, Séjalon-Delmas N, Kluever A, Roux C, Bécard G, Franken P (2003) Root factor induce mitochondrial-related-gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. Plant Physiol 131:1468–1478

Tiwari P, Adholleya A (2002) In vitro co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri-T-DNA transformed roots. FEMS Microbiol Lett 206:39–43

Tommerup IC (1988) The vesicular-arbuscular mycorrhizas. Adv Plant Pathol 6:81–91

Tommerup IC, Abbott LK (1981) Prolonged survival and viability of vesicular-arbuscular mycorrhizae hyphae after root death. Soil Biol Biochem 13:431–434

van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol Ecol 7:879–887

Walker C, Sanders FE (1986) Taxonomic concepts in the Endogonaceae. III. The separation of *Scutellospora* gen. nov. from *Gigaspora* Gerd. & Trappe. Mycotaxon 27:169–182

Zhao ZW, Wang GH, Yang L (2003) Biodiversity of arbuscular mycorrhizal fungi in a tropical rainforest of Xishuangbanna, southwest China. Fungal Divers 13:233–242

Part III
In Vitro Development and Physiology of Glomeromycetes

6 Environmental Factors That Affect Presymbiotic Hyphal Growth and Branching of Arbuscular Mycorrhizal Fungi

Gerald Nagahashi[1], David D. Douds Jr.[1]

Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture

1 Introduction

The presymbiotic growth of arbuscular mycorrhizal (AM) fungi is important because the fungus must find a compatible host to complete its lifecycle. The development and utilization of in vitro culture techniques (see review by Fortin et al. 2002) have provided the way to study how various environmental factors can individually and synergistically affect AM fungal growth during this early developmental stage. Environmental factors which include light, gaseous or volatile compounds, and nonvolatile chemical compounds in the soil can all stimulate either hyphal growth and/or hyphal branching. Although a recent paper has indicated the role of light during the presymbiotic growth of AM fungi (Nagahashi et al. 2000), most of the work on this specific stage of the fungal lifecycle has dealt with the role of exudates and how they affect fungal growth (Elias and Safir 1987; Gianinazzi-Pearson et al. 1989) and hyphal branching (Giovannetti et al. 1993, 1996; Buee et al. 2000; Nagahashi and Douds 2000). Soluble chemicals in the soil are a primary environmental factor which can interact with presymbiotic AM fungal growth in a positive or negative fashion. Although work in this area with host root exudates has shown positive growth promotion of AM fungi, some work has indicated that nonhost roots can produce inhibitors of AM fungus hyphal tip growth (Nagahashi and Douds 2000).

Work with purified chemicals has indicated that some flavonols can stimulate presymbiotic AM fungal growth (Bécard et al. 1992), but little information is available on the interaction among aqueous chemical compounds and their combined effect on fungal growth. This chapter aims

[1]US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA, Tel.: +1-215-2336424, Fax: +1-215-2336581, E-mail: gnagahashi@errc.ars.usda.gov

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

to present some recent results on the presymbiotic hyphal response to interactions between a gaseous compound and chemicals, the interaction between different aqueous chemical compounds, and the interaction between chemical compounds and light.

2
In Vitro Techniques

2.1
Spore Production and Germination

Azygospores of *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe were produced in greenhouse pot cultures on *Paspalum notatum* Flugge (Nagahashi and Douds 1999). Spores were collected, isolated, sterilized, and stored at 4 °C until used (Bécard and Fortin 1988). Upon germination, plugs (each containing one germinated spore) were transferred to a 10-cm square Petri plate of gelled M medium (Bécard and Fortin 1988).

2.2
Root Organ Cultures and Root Exudates

Ri T-DNA transformed carrot roots (*Daucus carota* L.) were cultured on solid support medium as described earlier (Bécard and Piché 1992). Segments of these roots were then transferred aseptically to liquid M medium (minus gellan) in 250-ml Erlenmeyer flasks and grown for 28 days, followed by 7 days in M medium without phosphorus (Nagahashi and Douds 1999). The fresh weight of roots at the end of the growth period was between 10 to 15 g per flask.

Culture filtrates were harvested by filtering through Whatman #4 filter paper and then concentrating in SEPAK C18 cartridges. To semi-purify the active components of the exudate, the C18 cartridge was first eluted with 3 ml of 35% acetonitrile (this fraction was discarded and contained the yellow-brown pigments), followed by 3 ml of 70% acetonitrile. This fraction was dried under N_2, dissolved in 2 ml of 100% acetonitrile, and then passed through a graphitised carbon (GC) cartridge (Supelco ENVI-Carb SPE Tubes) to remove the autofluorescing compounds in the exudate. The material passing through the GC cartridge was then dried under N_2 and dissolved in 0.5 ml of 70% methanol for every 250 ml of original crude exudate. This was called the concentrated exudate fraction and it was diluted 1:10 or 1:100 with 70% methanol for the diluted exudate fractions.

2.3 Incubation Conditions with CO_2 and Exudate Treatments

The plugs containing germinated spores were transferred to new 10-cm square Petri plates containing the same M medium as above, and it took 1 day at 32 °C in 2% CO_2 for the primary germ tubes and main secondary hyphae to grow out of the transferred plugs into the new medium. At this time, the CO_2-exposed Petri plates were kept in the same incubator for an additional 3 days. For the minus CO_2 treatment, the square Petri plates containing the spores were taped to the inside of large, round Petri plates (150 × 15 mm), and two cotton plugs saturated with KOH (10%, w/v) were place inside the round dishes but not touching the square Petri plates. The round Petri plates were placed on edge with the KOH plugs on the bottom edge, and incubated at 32 °C in an ambient air incubator (with an open dish of KOH as the initial CO_2 trap) for 3 days.

Exudate was applied with the microinjection technique (Nagahashi and Douds 1999), except that single holes in the gellan gel were filled with 5 µl of any exudate fraction. For some experiments, the exudate (1:100 dilution) was applied after day 1, and six injections of diluted exudate fraction were made per germinated spore. The injections were made approximately 1 cm from the edge of the plugs, and the spores were allowed to grow an additional 3 days. The minus CO_2 Petri plates were treated with exudate and sealed with KOH traps as above and allowed to grow for three more days. At this time, all hyphal branches of the germinated spores were counted.

For other experiments, a more concentrated exudate fraction (1:10 dilution) was used but in this case, the germinated spores were allowed to grow 2 days at 32 °C in 2% CO_2 before applying the exudate. This allowed the primary germ tubes and main secondary hyphae to grow approximately 2 cm from the original plug, and made it easier to treat individual hyphae. Five µl of exudate was injected into a single hole placed approximately 2 mm from a growing hyphal tip. The Petri plates were either set up for minus CO_2 or placed in the CO_2 incubator. After a further 2 days of growth, the hyphal branches within 1 cm of the injection site were counted. Micrographs were obtained with an Olympus SZH stereomicroscope fitted with a television camera (Dage MT1, Model Series 68, Michigan City, IN 46360). Digital images were achieved with a DT 2853 digitizing board with Image Pro Plus software (Media Cybernetics, Silver Springs, MD 20910).

2.4 Light Experiments

Light source and light meters were described previously (Nagahashi and Douds 2003) but for red light experiments, filtered light was used instead

of monochromatic light. For red light, a 590-nm-long pass filter was used which permitted maximum transmission of red light above 610 nm, and the transmitted light was measured at 640 nm. Monochromatic light at 540 nm was used for green light (Nagahashi and Douds 2003). For hyphal growth studies with blue light, filtered blue light (Nagahashi and Douds 2003) was used to expose the primary germ tube and all major secondary hyphae. To study synergistic effects between light and exudate, the beam of light (monochromatic or filtered) was focused on the first 8 mm of the primary germ tube, as described below. The exudate was applied near the primary germ tube of spores grown in the absence or presence of light (blue, green or red).

2.5 Synergistic Effects Between Chemical Compounds

For these experiments, a filter-sterilized quercetin solution was mixed in M medium while it was still warm after autoclaving, to yield a final concentration of 10 µM. The Petri plates were then filled. Controls for these experiments were without quercetin, plus or minus exudates, while the experimental treatments were with the quercetin, plus or minus exudates.

3 Chemical Components of Exudates or Compounds Found in the Soil Environment That Influence Presymbiotic AM Fungal Growth

It is clear that components of the host root exudates induce the first morphological response in hyphae of germinating AM fungal spores. The fungi respond with the prolific production of hyphal branches before any physical contact with the surface of the host root (Giovannetti et al. 1993). The branching stimulators are constitutive components of the exudate, and the phosphorus stress induced to the host plant drastically increased the level of exuded branching stimulators (Nagahashi and Douds 2000). The concentration of the branching stimulators may directly affect the morphology of the branching pattern, as was demonstrated with the microinjection technique developed earlier (Nagahashi and Douds 1999). These results clearly showed a dose-dependent response in terms of the number of induced branches as well as the type of branching pattern observed. At low concentration, the branches were few but long and at higher concentration, there were many short branches with a very bushy-like morphology. At very

high concentration, the branching pattern was somewhat periodic and the clusters resembled arbuscules (Nagahashi and Douds 2000).

Very little information is available on the interaction between chemicals in the root environment that could influence the growth of germinated AM fungal spores. Natural plant products such as myricetin and especially quercetin (flavonols) have been shown to stimulate spore hyphal growth of *Gigaspora rosea*, *Glomus etunicatum* and *Glomus intraradices* (Bécard et al. 1992) but not *Gi. gigantea* (Douds et al. 1996). Quercetin itself appears to mainly affect the elongation growth of *Gi. rosea* (Bécard et al. 1992). When germinated spores of *Gi. rosea* were treated with quercetin and carrot root exudates separately and compared to a combined treatment, more branching occurred when the treatments were given together compared to the sum of the individual treatments (Table 1). Because of this observation, the dose-dependent morphological branching pattern observed earlier (Nagahashi and Douds 2000) could also be interpreted another way. It is possible that there are two types of hyphal stimulators. One type only induces elongation growth while a second type of compound induces hyphal branches.

There is further evidence which supports the presence of signals which induce different types of morphological responses in hyphal growth. Our recent work with Ri T-DNA transformed roots of tomato mutants indicated that for one type of mutant, the most aqueous fraction (readily soluble in 25% methanol) induced one type of hyphal branching pattern, while a more hydrophobic fraction (soluble in 50% methanol) induced another (Fig. 1). Experiments are under way to examine the number of hyphal branches induced and the type of branching pattern observed when these two fractions are mixed together.

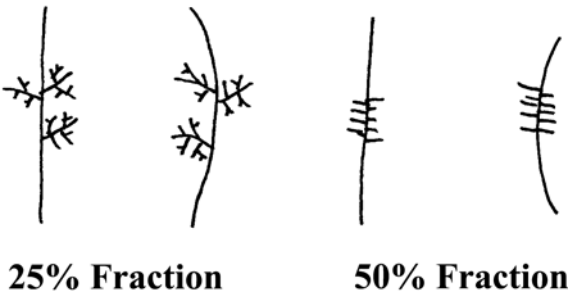


Fig. 1. Tracings of two different types of morphological branching patterns of *Gigaspora gigantea* induced by two different fractions isolated from a transformed tomato root mutant. *Left* The branching pattern induced by a hydrophilic fraction (dissolves readily in 25% methanol). A primary germ tube and a major secondary hypha are shown. *Right* The branching pattern induced by a more hydrophobic fraction (dissolves in 50–70% methanol). A primary germ tube and a major secondary hypha are shown

Table 1. *Gigaspora rosea* spores germinated in plates with or without 10 uM quercetin and then exposed to 70% ethanol (control) or carrot root exudate dissolved in 70% ethanol.[a] (Data from Table 2 of Nagahashi and Douds 2000)

Treatment	Branches off primary hyphae[b]	Branches off secondary hyphae[c]
Control	6.7c	0.1d
Control+exudate	12.2b	1.5c
Quercetin	6.7c	3.6b
Quercetin+exudate	21.6a	15.5a

[a] Numbers in the same column followed by the same letter are not significantly different ($\alpha = 0.05$, Tukey's method of multiple comparisons)
[b] Means of six observations
[c] Means of six spores, four hyphae per spore

4
Effects of Volatile Compounds on Presymbiotic AM Fungal Growth

It has been suggested that volatile compounds also are involved in the establishment of a successful symbiosis (Koske 1982). With the exception of one report, suggesting the beneficial stimulation of hyphal growth with ethylene (Ishii et al. 1996), the only gaseous compound which has been well documented for influencing the symbiosis is CO_2 (Saif 1984). In three host plants grown on soil maintained at 16% O_2 with variable CO_2, the percentage of root length colonized with *Glomus macrocarpum* as well as the number of vesicles in the roots were both greater at 0.5% CO_2 compared to no CO_2 (Saif 1984). The optimum CO_2 concentration ranged from 2 to 4% CO_2, depending on the host plant. Gryndler et al. (1998) showed that the growth of intraradical hyphae of *Glomus fistulosum* was stimulated with 3% CO_2. The growth of the extraradical hyphae also was decreased when CO_2 was removed from the Petri plates containing roots associated with AM fungi in monoxenic culture (Bécard and Piché 1989).

The effect of CO_2 on the free-living or presymbiotic hyphal growth of *Gi. rosea* was demonstrated by Bécard and Piché (1989). A 17.5-fold increase in hyphal length of germinated spores in the presence of 2% CO_2 was observed when compared to controls grown in the absence of CO_2 (Bécard et al. 1992). The synergistic stimulation of AM fungal hyphal length with CO_2 and root exudates was shown for *Gi. rosea* (Bécard and Piché 1989). Indeed, there was a five- to sixfold increase in hyphal growth when exudate and 0.5% CO_2 were given together, compared to the sum of the individual treatments. Autoradiography showed that the $^{14}CO_2$ was taken up and incorporated by the fungus (Bécard and Piché1989). This

was later confirmed by Bago et al. (1999) using ^{13}C NMR spectroscopy. They concluded that $^{13}CO_2$ was metabolized via dark fixation and utilized in gluconeogenesis to synthesize trehalose, or in amino acid synthesis to produce arginine. The exact nature of the role of environmental CO_2 in the stimulation of fungal growth, beyond that of its minor nutritive effect, is unknown.

Other work showed a growth stimulation response with CO_2 and a purified compound (quercetin) for *G. etunicatum* and *G. intraradices* (Bécard et al. 1992; Chabot et al. 1992). One report (Bécard et al. 1992) also showed that *Gi. rosea* had a nice growth response with 2% CO_2 and quercetin or myricetin. More significantly, they showed a 2.5-fold synergistic stimulation of hyphal growth when 2% CO_2 and 10 μM quercetin were given together (206.2 mm), compared to the sum of the individual treatments (38.4 mm for 2% CO_2 and 43.6 mm for 10 µM quercetin).

4.1 Effects of CO_2 on the Hyphal Growth of Germinated *Gigaspora Gigantea* Spores

For the experiments reported here, a germinated spore was transferred in a plug of medium to a fresh Petri plate and allowed to grow 1 day at 32 °C in 2% CO_2. This allowed the primary germ tube to grow out of the plug into the new medium. At this time, CO_2-exposed Petri plates were kept in the same incubator for an additional 3 days, while minus CO_2plates were treated as outlined above. Spores grown for 4 days in the presence of 2% CO_2 exhibited 56% more growth compared to the minus CO_2 control (Table 2). This was not as remarkable as the 17.5-fold increase in growth observed for *Gi. rosea* (Bécard et al. 1992). However, this is yet another AM fungus in which CO_2 stimulation has been verified (Balaji et al. 1995).

Table 2. Growth of *Gigaspora gigantea* for 4 days in the presence and absence of 2% CO_2 and in the presence of a semi-purified root exudate (1:100 dilution) plus and minus CO_2. Branches and auxiliary cells were the total per germinated spore[a]

Treatment	Hyphal length (cm)	Germ tube (cm)	Auxiliary cells	Branches
Minus CO_2	10.9 ± 1.3	5.0 ± 0.1	1.9 ± 0.4	6.3 ± 1.8
Plus CO_2	17.0 ± 0.4	5.2 ± 0.3	1.0 ± 0	8.0 ± 0.6
Exudate–CO_2	19.6 ± 3.4	4.5 ± 0.3	2.5 ± 1.6	23.0 ± 3.1
Exudate+CO_2	35.7 ± 4.8	5.8 ± 0.1	3.8 ± 1.4	60.8 ± 10.2

[a] Each number represents the mean of four observations ± SEM

When exudate was given to *Gi. gigantea* in the presence and absence of CO_2, a synergistic stimulation in hyphal length was observed with CO_2 (Table 2). The basal growth was 10.9 cm, the growth due to CO_2 was 6.1 cm, and the exudate-induced growth was 8.7 cm (total = 25.8 cm), while the average hyphal length induced with exudate and CO_2 given together was 35.7 cm (Table 2). This result showed a 38% increase over the predicted growth of the individually given treatments, but it was not as great as reported for *Gi. rosea* (164%, Becard et al. 1992).

Furthermore, a synergistic stimulation in hyphal branching was observed for *Gi. gigantea* (Table 2). The number of total hyphal branches/spore, in the presence of both CO_2 and exudate, was twice as high as the sum of separate treatments of CO_2 and exudates (Table 2). This observation was verified by treating the individual hypha with a more concentrated exudate fraction (1:10 dilution), and the results showed a 2.7-fold increase in

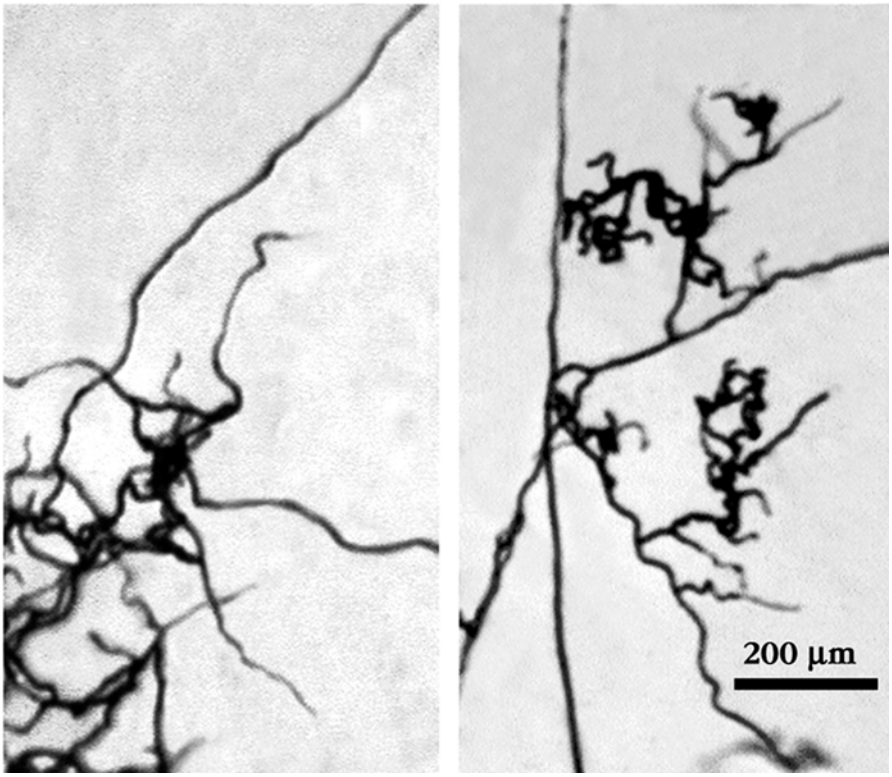


Fig. 2. Micrographs of the hyphal branches of *Gigaspora gigantea* induced by treatment with a concentrated exudate fraction in the absence (*left*) and presence (*right*) of 2% CO_2

branches with CO_2 and exudate together, compared to the sum of individual treatments (data not shown).

A final observation was noted in the morphological change of the hyphae when exposed to a concentrated exudate fraction in the presence of CO_2. Concentrated exudate caused the hyphal branches to take on crinkled and arbuscular-like branched structures (Nagahashi and Douds 2000). These arbuscular-like branches have been observed in extraradical hyphae of colonized roots but, until recently, not in hyphae during the presymbiotic stage. These arbuscular-like branched structures only occur in the presymbiotic stage with concentrated exudate, in the presence of 2% CO_2, and not in the absence of CO_2 (Fig. 2).

5
The Effect of Light on the Presymbiotic Growth of AM Fungi

A recent report (Nagahashi and Douds 2000) has clearly demonstrated that a third physical environmental factor, light, can also stimulate hyphal branching. White light-stimulated hyphal branching in germinating spores of *Gi. gigantea*, *Gi. rosea* and *G. intraradices* (Nagahashi et al. 2000) and subsequent work reported the first action spectrum for light-induced hyphal branching for any fungus (Nagahashi and Douds 2003). These results clearly indicated that near-UV A light (390 nm) and blue light (430 nm) were the most efficient wavelengths for inducing hyphal branching of AM fungi (Fig. 3). There was a small response with green light and a minor response in the red light region (Fig. 3, inset) but these responses were very inefficient when compared to the micromoles of blue or UV A light which induced branching (Nagahashi and Douds 2003). This is mentioned because a recent report has indicated the stimulation of hyphal length of *Gi. margarita* with red light, although an action spectrum was not performed in this case (Yachi et al. 2001).

Recent work with both filtered blue light or filtered red light has indicated that light did not affect the overall hyphal growth of germinated spores. For these experiments, filtered light was used in order to expose the whole Petri plate. The lengths of the primary germ tubes which were exposed to blue (6.8 ± 0.1 cm) or red light (6.6 ± 0.2 cm) were the same as the dark controls (6.2 ± 0.1 cm). The overall growth (including all the major secondary hyphae), measured by the grid-line intersect method, showed the same response (data not shown). However, when blue, green, or red light was used in conjunction with carrot root exudates, a synergistic stimulation between light and host root exudates on the hyphal branching response of AM fungal germ tubes was observed. The results presented here show that the sum of the individual treatments of dilute exudate and long exposure

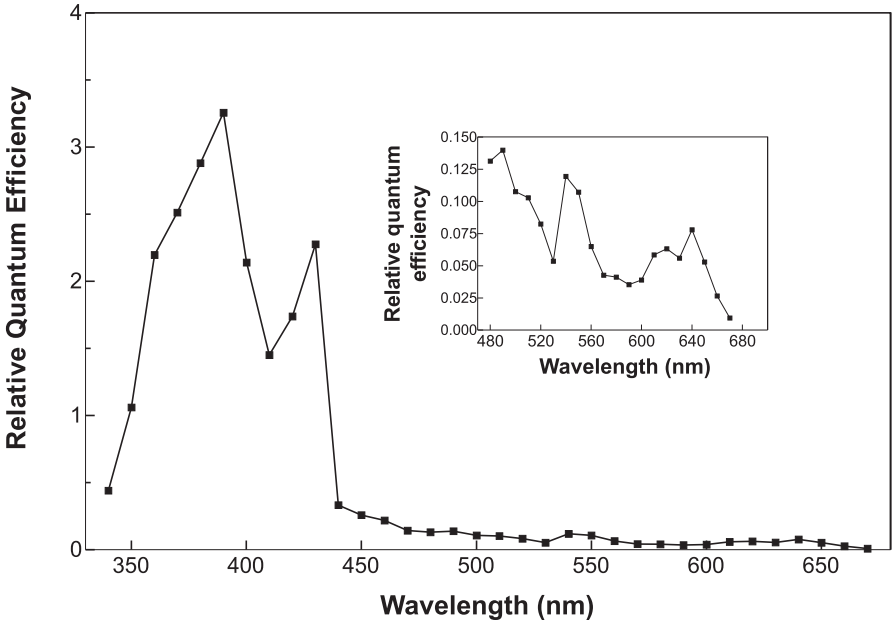


Fig. 3. Action spectrum for the induction of hyphal branches of *Gigaspora gigantea*. Relative efficiency of light-induced hyphal branching between 340 and 700 nm. *Inset* Data taken from the action spectrum reported by Nagahashi and Douds (2003) are plotted here. Although two other peaks are apparent (one at 540 nm and another at approximately 640 nm), these wavelengths were very inefficient for inducing hyphal branches compared to light at 390 and 430 nm

to low-intensity monochromatic blue light was less than the total branches induced when both treatments were given simultaneously (Table 3).

To assess the interaction of green or red light and exudates, considerably higher light intensities were necessary than for studies with blue light, due to their relative efficiencies (Fig. 3). Experimental conditions for green light emitted from a monochromator were 35 µmol s^{-1} m^{-2} with an exposure time of 4 h and, for filtered red light, 1000 µmol s^{-1} m^{-2} for 15–30 min. For green light, the sum of hyphal branches for the individual responses was less than when both treatments were applied together (Table 4). Similarly, the presence of red light and exudate together induced a 2.4-fold increase in hyphal branching over the sum of the individual treatments (Table 4).

There also was a recent report which showed a synergistic branching response between light and a purified chemical compound (Nagahashi et al. 2000). When germinated spores of *Gi. rosea* were exposed to quercetin and white light simultaneously, more branches were induced (2.4-fold increase) when compared to the sum of the individual treatments (Table 5).

Table 3. Synergistic stimulation of hyphal branches of *Gigaspora gigantea* with monochromatic blue light (390 nm, 0.8 μmol s^{-1} m^{-2} for 10 h) and dilute exudate from carrot roots[a]

Treatment	Hyphal branches induced
Exudate	4.25 ± 0.52
Blue light (390 nm)	1.00 ± 0.31
Blue light+exudate	14.00 ± 1.60

[a] The exudate was mixed into the Petri plates and germinated spores were transferred to the plates and grown for 3 days. Only the apical 8 mm of the primary germ tube was exposed. The blue light exposure and exudates treatment were given simultaneously. Branches were counted 16 h after the blue light exposure

Table 4. Synergistic stimulation of hyphal branches of *Gigaspora gigantea* with green light (540 nm, 35 μmol s^{-1} m^{-2} for 4 h) or red light (640 nm, 1,000 μmol s^{-1} m^{-2} for 15 min) and dilute exudates from carrot roots[a]

Treatment	Hyphal branches induced
Exudate	4.00 ± 1.10
Red light	1.00 ± 0.27
Red light + exudate	11.75 ± 1.00
Exudate	3.00 ± 0.42
Green light	1.70 ± 0.37
Green light + exudate	10.90 ± 1.06

[a]The light treatment was given first and immediately followed by a microinjection of exudate. Branches were counted 16 h after injection. Means of ten observations per treatment ± SEM

Table 5. The effect of high light intensity (10,800 μE s^{-1} m^{-2}) on the branching of primary (1) and secondary (2) hyphae of germinated *G. rosea* spores. The germinated spores were grown in the presence and absence of 10 μM quercetin, and either kept in the dark or treated with light.[a] (Data taken from Table 1 of Nagahashi et al. 2000)

Treatment	1	2
Dark control	1.4c	0.0c
Dark + quercetin	1.8c	0.0c
Light (15 min)	5.8b	5.5b
Light + quercetin	15.8a	16.0a

[a] Means of six observations; numbers in the same column followed by the same letter are not significantly different (Tukey's method of multiple comparisons, $\alpha = 0.05$)

Finally, there is some evidence that blue light and compounds in a host root exudate appear to trigger the same second messenger involved in the hyphal branching response (Nagahashi and Douds 2004). This was demonstrated by using the synergistic response between blue light and

exudate. The primary germ tube of *Gi. gigantea* was first exposed to blue light, and then exudate was applied at prolonged intervals after the initial exposure. The loss of the synergistic response was used to determine the stability of the second messenger. These results indicated that the second messenger was stable or active for at least 3 h.

6 Conclusions

In general, AM fungal spores can germinate without the presence of root exudates. However, components of the exudates can stimulate fungal growth, hyphal branching and AM fungal colonization rate (Bécard and Piché 1989, and references therein). The prolific hyphal branching from germinating spores induced by host root exudate components is well documented (Giovanetti et al. 1993; Buee et al. 2000; Nagahashi and Douds 2000), and the types of branching patterns observed with isolated exudate components are the same as when a hyphal tip approaches a host root in monoxenic culture (Nagahashi and Douds 2000). Recent reports have indicated that multiple genes are expressed when a germinated spore is treated with host root exudates (White et al. 2003; Tamasloukht et al. 2003), and the expression of certain fungal genes occurs hours before the intense hyphal branching is observed (Tamasloukht et al. 2003). Both of these reports have used, at best, semi-purified exudate preparations, so purification and identification of a branching stimulator are paramount in determining which genes are specifically related to hyphal branching. Recent evidence also suggests that there are either several different types of compounds or there is a single, major type of compound with variable side groups that can induce hyphal branching (Nagahashi and Douds 2000). We should also be aware that there might be separate factors for elongation growth and hyphal branching.

Not all root exudates and other compounds present in the soil environment affect AM fungi in a positive fashion. A nonhost root can exude hyphal tip inhibitors which delay the rapid growth of AM fungi (Nagahashi and Douds 2000). In addition, some naturally occurring flavonoids can also inhibit AM fungal growth (Bécard et al. 1992). In the presence of a nonhost inhibitor, *Gi. gigantea* exhibits an interesting recovery mechanism. Although the hyphal tip stops growing, a recovery branch behind the inhibited tip forms and starts to grow. This tip inhibition, followed by recovery branch formation, can go through many cycles until the newest tip is far enough from the nonhost root to resume normal growth (Nagahashi and Douds 2000).

The stimulation of AM fungal growth by CO_2 is the only well-documented volatile interaction with germinated spores. The CO_2 synergism is a very

interesting phenomenon. Ecologically, the synergistic interaction is somewhat of a safeguard for the fungus, so that the germinated spores will not exhaust their reserves in the presence of CO_2 generated by soil microflora. On the other hand, the presence of CO_2 from a host root and the simultaneous presence of exudates would stimulate profuse branching and result in much more surface contact with the host root and, hence, successful colonization.

The other interesting observation with CO_2 is its effect on the morphology of a presymbiotic branching cluster. In the presence of CO_2, the branches start to look arbuscular-like, so it is possible that the presymbiotic branching structures (Fig. 3) have a physiological function such as nutrient uptake. The arbuscular-like structure shown is very reminiscent of actual intraradical arbuscules present in the colonized root. The intraradical arbuscules are believed to be the site of nutrient exchange between host and fungus (Bonfante-Fasolo 1984; Smith and Smith 1990). Morphologically similar arbuscular-like branched structures have been observed in the external hyphae of clover root cultures colonized by *Glomus mosseae*(Mosse and Hepper 1975), and this has been verified for another AM monoxenic culture (Bago et al. 1998). These arbuscule-like structures have recently been renamed as branched absorbing structures (BAS). The BAS appear at regular intervals along a runner hypha, and they have a similar morphology, developmental features and cytological features to intraradical arbuscules. Although the BAS and presymbiotic arbuscular branches we observed (Fig. 3; Nagahashi and Douds 2000) are similar in appearance, cytological studies of presymbiotic branching structures have not yet been made. These presymbiotic branching structures also occur with regularity along a major hypha (Nagahashi and Douds 2000), and require CO_2 to form (Fig. 3). Although it has been demonstrated that the removal of CO_2 can inhibit extraradical mycelial growth (Bécard and Piché 1989), a CO_2 requirement for the formation of intraradical arbuscules or BAS has not been demonstrated. The three morphologically similar types of branched structures may all have a similar function in nutrient uptake (or, as in the case of arbuscules, nutrient exchange) but the actual involvement of BAS or presymbiotic arbuscular-like branches in nutrient uptake processes still needs to be determined.

The effect of light on the branching of AM fungi was not totally surprising, since light (especially blue light) can stimulate various processes of other soilborne fungi (Gressel and Rau 1983). The fact that blue light and host root exudates synergistically stimulate hyphal branching can in part help explain why maize seedlings, grown in soil in mini ant farm containers, had a higher percent root length colonization, when roots were directly exposed to light, compared to the dark control (Nagahashi et al. 2000). The fact that hyphal branching from germinating spores induced by

blue light requires only low intensity is consistent with the low penetration of soil by blue light (Bliss and Smith 1985). Much higher-intensity green light or red light is necessary to stimulate hyphal branching, but red light also penetrates deeper and more readily into various soil types (Bliss and Smith 1985). Light penetration into soil in the presence of certain chemical compounds or host root exudates could interact synergistically to increase the chances of a hyphal tip to come in contact with the epidermal surface of a host root, which would lead to the colonization of the root. This phenomenon would be most significant in recently tilled soils or when spores germinate near roots at or very near the soil surface.

A thorough understanding of presymbiotic signalling/recognition events and host root-induced morphogenetic regulation of AM fungi is likely to be essential for the eventual axenic culture of these obligate symbionts. The synergism demonstrated among gaseous compounds, soluble compounds, and physical environmental factors indicates that achieving this understanding will be a challenge. However, synergistic responses to different environmental cues may indicate common steps, such as the activation of particular second messengers during hyphal branching, and this could be unraveled with in vitro culture techniques.

Acknowledgements. We would like to thank Y. Kapulnik for providing roots of tomato mutants used in these studies.

References

Bago B, Azcón-Aguilar C, Piché Y (1998) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bago B, Pfeffer PE, Douds DD, Brouillette J, Bécard G, Shachar-Hill Y (1999) Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. Plant Physiol 121:263–271

Balaji B, Poulin MP, Vierheilig H, Piché Y (1995) Responses of an arbuscular mycorrhizal fungus, *Gigaspora margarita*, to exudate and volatiles from the Ri T-DNA-transformed roots of nonmycorrhizal and mycorrhizal mutants of *Pisum sativum* L. Sparkle. Exp Mycol 19:275–283

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Bécard G, Piché Y (1992) Establishment of vesicular-arbuscular mycorrhiza in root organ culture: review and proposed methodology. In: Norris JR, Read D, Varma AK (eds) Methods in microbiology: techniques for mycorrhizal research. Academic Press, New York, pp 550–568

Bécard G, Douds DD, Pfeffer PE (1992) Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl Environ Microbiol 58:821–825

Bliss D, Smith H (1985) Penetration of light into soil and its role in the control of seed germination. Plant Cell Environ 8:475–483

Bonfante-Fasolo P (1984) Anatomy and morphology of VA mycorrhizae. In: Powell CL, Bagyaraj DJ (eds) VA Mycorrhiza. CRC Press, Boca Raton, pp 5–33

Buee M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The presymbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant Microb Interact 13:693–698

Chabot S, Bel-Rhlid R, Chénevert R, Piché Y (1992) Hyphal growth promotion in vitro of the VA mycorrhizal fungus, *Gigaspora margarita* Becker & Hall, by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. New Phytol 122:461–467

Douds DD Jr, Nagahashi G, Abney G (1996) The differential effects of cell wall-associated phenolics, cell wall, and cytosolic phenolics of host and non-host roots on the growth of two species of AM fungi. New Phytol 133:289–294

Elias KS, Safir GR (1987) Hyphal elongation of *Glomus fasciculatus* in response to root exudates. Appl Environ Microbiol 53:1928–1933

Fortin JA, Bécard G, Declerck S, Dalpé Y, St.-Arnaud, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root organ culture. Can J Bot 80:1–20

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243–255

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during preinfection stages. New Phytol 125:587–593

Giovannetti M, Sbrana C, Citernesi AS, Avio L (1996) Analysis of factors involved in fungal recognition responses to host-derived signals by arbuscular mycorrhizal fungi. New Phytol 133:65–71

Gressel J, Rau W (1983) Photocontrol of fungal development. In: Shropshire W Jr, Mohr H (eds) Encyclopedia of plant physiology, vol 16B. Photomorphogenesis. Springer, Berlin Heidelberg New York, pp 603–639

Gryndler M, HrseLova H, Chvatalova I, Vosatka M (1998) In vitro proliferation of *Glomus fistulosum* intraradical hyphae from mycorrhizal root segments of maize. Mycol Res 102:1067–1073

Ishii T, Shrestha YH, Matsumoto I, Kadoya K (1996) Effect of ethylene on the growth of vesicular-arbuscular mycorrhizal fungi and on the mycorrhizal formation of trifoliate orange roots. J Jpn Soc Hortic Sci 65:525–529

Koske RE (1982) Evidence for a volatile attractant from plant roots affecting germ tubes of a VAM fungus. Trans Br Mycol Soc 79:305–310

Mosse B, Hepper C (1975) Vesicular-arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Nagahashi G, Douds DD Jr (1999) A rapid and sensitive bioassay to study signals between root exudates and arbuscular mycorrhizal fungi. Biotechnol Tech 13:893–897

Nagahashi G, Douds DD Jr (2000) Partial separation of root exudate components and their effects upon the growth of germinated spores of AM fungi. Mycol Res 104:1453–1464

Nagahashi G, Douds DD Jr (2003) Action spectrum for the induction of hyphal branches of an arbuscular mycorrhizal fungus: exposure sites versus branching sites. Mycol Res 107:1075–1082

Nagahashi G, Douds DD Jr (2004) Synergism between blue light and root exudates compounds and evidence for a second messenger in the hyphal branching response of *Gigaspora gigantea*. Mycologia 96(5):948–954

Nagahashi G, Douds DD Jr, Buee M (2000) Light-induced hyphal branching of germinated AM fungal spores. Plant Soil 219:71–79

Saif SR (1984) The influence of soil aeration on the efficiency of vesicular-arbuscular mycorrhizas. III. Soil carbon dioxide and growth and mineral uptake in mycorrhizal and nonmycorrhizal plants of *Eupatorium odoratum* L., *Guizotia abyssinica* (L.F.) Cass. and *Sorghum bicolor* (L.) Moench. New Phytol 96:429–435

Smith SE, Smith FA (1990) Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. New Phytol 114:1–38

Tamasloukht M, Sejalon-Delmas, Kluever A, Jauneau A, Roux C, Bécard G, Franken P (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. Plant Physiol 131:1468–1478

White H, Muratet M, Podila G, Nagahashi G, Douds D (2003) Gene expression profiling of preinfection processes in arbuscular mycorrhizae formation. ICOM IV Abstr, p 146

Yachi Y, Ishii T, Horii S (2001) Red radiation stimulates hyphal growth of vesicular-arbuscular mycorrhizal fungi in vitro. In: Diversity and integration in mycorrhizas. Proc 3rd Int Conf Mycorrhizas, University of Adelaide, pp P1 112

7 Breaking Myths on Arbuscular Mycorrhizas in Vitro Biology

Bert Bago[1], Custodia Cano[1]

1 Introduction

"For a critical study of the effects of vesicular-arbuscular mycorrhiza on plant growth, typical infections must be produced under controlled microbiological conditions". With those initial words, Mosse published in 1962 a research article in which the establishment of "vesicular"-arbuscular mycorrhizas under "aseptic" conditions was first reported. Mosse, together with Hayman and some other early mycorrhizologists, were pioneers in suggesting the importance of this mutualistic symbiosis (Mosse 1953, 1956, 1957; Mosse and Hayman 1971; Hayman and Mosse 1971, 1972). Soil microbiologist and phytopathologist as she was, Mosse was conscious of the necessity of fully understanding the biology of a given micro-organism, in order to obtain some indication for its subsequent manipulation and use in particular situations.

Mosse's first "aseptic" mycorrhizal cultures were, in fact, dixenic (i.e. the AM fungus plus two foreign organisms; Williams 1992) cultures between the AM fungus known at that time as *Endogone* sp., sterile-raised seedlings of different plant species, and *Pseudomonas* sp. The author claimed that the presence of such soil bacteria was necessary for the symbiosis to be established (Mosse 1962; see also Mugnier and Mosse 1987). It was not until 1975 that Mosse and Hepper reported the first in vitro co-culture between a root organ and a contaminant-free inoculum from a glomalean species (*Endogone mosseae*). However, and perhaps due to the difficulty of maintaining such dual cultures, this in vitro technique was almost forgotten for more than a decade.

In 1986 and 1987, Strullu and Romand initiated a series of papers demonstrating the high potential of mycorrhizal root pieces and isolated intraradical vesicles for the establishment of monoxenic cultures. Both papers pioneered the use of this intraradical fungal material for in vitro culturing, nowadays used for some high-number vesicle-forming *Glomus*

[1]Dpto. Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), calle Profesor Albareda 1, 18008 Granada, Spain, Tel.: +34-958-181600, Fax: +34-958-129600, E-mail: abago@eez.csic.es

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

species (Diop et al. 1994; Simoneau et al. 1994; Declerck et al. 1996, 1998, 2000). Great, but unsuccessful, efforts were made in the meanwhile, mainly by Hepper and coworkers, to produce AM fungi in axenic cultures from spores (Hepper and Smith 1976; Hepper 1979, 1981, 1983, 1984, 1987; Hepper and Jakobsen 1983). As a result of these studies, certain physicochemical conditions, such as a 2% CO_2 atmosphere, the addition of some organic acids, amino acids, vitamins, sulphur compounds, root exudates (revised by Hepper 1984; Azcón-Aguilar et al. 1998) or even suspension-cultured plant cells (Carr et al. 1985) to the culture media were found to enhance germ-tube development, but only to a very limited extent. Nowadays, we still have no choice but to consider glomalean fungi as obligate biotrophs (Bago and Bécard 2002).

In 1988, Bécard and Fortin reformulated the experimental conditions for AM monoxenic cultures to be easily maintained, using the idea of Mugnier and Mosse (1982) of using Ri T-DNA transformed carrot roots as the host. Unfortunately, little attention and much disbelieve were given to this major contribution when it was first discussed at the 8th North American Conference on Mycorrhizas (Jackson Hole, WY, USA; Bécard and Piché 1990). As a proof of this, few reports of the use of AM monoxenic cultures were published between 1988 and 1996 (Bécard and Piché 1989a, b, 1992; Chabot et al. 1992; Diop et al. 1992, 1994; Simoneau et al. 1994; Elmeskaoui et al. 1995; Mathur and Vyas 1995; Nuutila et al. 1995), most of them produced by the same research groups and being just modifications on the experimental systems published earlier. In particular, the Diop et al. (1994) paper could be considered as the first report of thousands of *Glomus* spores produced in vitro (with root pieces as inoculum), and the first demonstration of the daughter spores being able to re-establish in vitro and in vivo mycorrhizas.

A crucial date in the widespread use of AM monoxenic cultures was 1996, when an important improvement in this experimental system was reported – by means of bi-compartmented Petri plates, St-Arnaud et al. (1996) achieved the physical separation of the AM fungal extraradical mycelium (ERM) from the host root and its immediate environment. This resulted in the possibility of obtaining large amounts of AM fungal material in one compartment (the "hyphal compartment", HC), especially spores, which nevertheless maintained a symbiotic nature (i.e. the fungus was still connected to the host root, but spatially separated from it). First reports on AM physiology carried out in vitro were published immediately by using that system (Bago et al. 1996; Villegas et al. 1996), and other descriptions on AM fungal cell cycle, ERM morphogenesis and AM fungal colony developmental dynamics promptly followed (Declerck et al. 1996a, b, 1998, 2000; Bago et al. 1998a, b, 1999). Later on, and combining AM monoxenic cultures with other powerful techniques, important advances in our

knowledge of AM fungal cytology, biochemistry and physiology have been achieved (Jolicoeur et al. 1998; Pfeffer et al. 1998, 1999; Bago et al. 1999a, b, 2000, 2002, 2003, 2004a, b; Declerck and Van Coppenolle 2000; Hawkins et al. 2000; Joner et al. 2000; Koide and Kabir 2000; Declerck et al. 2001; Lammers et al. 2001;Nielsen et al. 2002; Tiwari and Adholeya 2002). More recently, molecular biologists have realized that AM monoxenics produced the optimal fungal material to carry out their studies. Indeed, the first successful amplification of ribosomal DNA from an AM fungus was done by using monoxenically produced spores (Simon et al. 1993) and, after bi- and multi-compartmental monoxenics development, an increased number of AM molecular biologists adopted these systems (e.g. Lammers et al. 2001; Maldonado-Mendoza et al. 2001; González-Guerrero et al. 2004). In summary, a change of mood has occurred in mycorrhizologists quite recently with respect to AM monoxenic cultures – from profound skepticism to general acceptance, and from a residual to a widespread use. The danger of all this consists in researchers using AM monoxenics just as a tool to get large amounts of fungal material, paying little or no attention to the type (i.e. developmental stage) and quality of material they are collecting. Indeed, the unadvertised misuse of AM monoxenics could result in poor or inappropriate AM material and, consequently, in inaccurate results and interpretations.

This chapter aims to warn about such misuses, and to address some of the questions, skepticisms and myths raised by AM monoxenic culturing. Final take-home messages should be that (1) AM monoxenics are far more than just a routine technique, (2) easy, but strict protocols should be followed for success and, most importantly, (3) some training/expertise on AM establishment, fungal colony development and hyphal morphogenesis under such conditions is mandatory for researchers aiming to use this technique, to be able to certify the quality of the material obtained and, consequently, the reliability and accuracy of the results obtained.

2
Questioning AM Monoxenic Cultures

2.1
Are AM Monoxenic Cultures Devices Too Artificial to Trust?

One of the most frequent criticisms against AM monoxenic cultures is that these are too artificial to trust any results obtained with them. While recognizing the limitations of the system, which will be addressed below, it is at least curious that most researchers making such statements use whole plants growing in pots in highly controlled environments, which have

little or nothing to do with natural conditions. There are certainly some differences in AM fungal development when growing in Petri plates versus pot microcosms and in nature (see below), but these could be minimized if one is aware of them. Fortunately, the benefits obtained by using AM monoxenic cultures (in terms of increase in our knowledge of AM fungal biology) have by now convinced quite a few of these former skeptics.

The most convincing proof supporting the suitability of monoxenic cultures for AM research is the fact that AM fungi form, both intra- and extraradically, typical symbiotic structures (i.e. appressoria, entry points, intercellular hyphae, arbuscules, intraradical vesicles and spores; Fig. 1a–c), and that they successfully complete their life cycle under these conditions by producing new and infective propagules. It is important to stress here that a given co-culture of a root organ + an AM fungus should not be considered a symbiotic monoxenic culture (SMC) unless the fungal life cycle is completed and new spores, able to establish new AM symbiosis under either monoxenic or soil conditions, are obtained. This is an important rule which should be strictly followed to preserve AM culturing credibility. The second rule is that any report of a new SMC should be presented in a peer-reviewed article and deposited in an appropriate in vitro bank (e.g. the International Bank of Glomeromycota BEG, Colección Iberoamericana de Micorrizas Arbusculares CIMA, Estación Experimental del Zaidín EEZ, Glomeromycota In Vitro Collection GINCO, and Mycothèque de l'Université catholique de Louvain MUCL), and given a deposit code in order to be validated. By following these two simple rules, one could avoid confusion/uncertainty about the AM fungal species/ecotypes actually maintained in SMC, a situation reflected in the following example. When carefully considering the list of glomalean species claimed to be maintained in monoxenics (Fortin et al. 2002), we get some uneasy numbers: only 15 of the 27 cultures listed (55.6%) have been published; to the best of our knowledge, of these 15 at least two (*G. caledonium*, *G. versiforme*) are no longer maintained in monoxenics, and one has serious doubts about the *symbiotic* status of some other such cultures (e.g. *G. etunicatum, G. mosseae, Gi. gigantea, Gi. rosea*) after carefully considering their associated publications. The latter thought is frightening, since tests performed in a culture claimed to be symbiotic, but which is not, will most probably produce misleading results of unpredictable consequences.

Another frequently made comment about AM monoxenic cultures is that while they may be useful to study the extraradical phase of the mycorrhiza formed in vitro (i.e. the fungal extraradical mycelium), this is probably not the case for its intraradical phase, especially at the level of the symbiotic plant–fungal interfaces. We may expect that roots in monoxenic culture have an altered way of acquiring carbohydrates compared to normal roots – their vascular cylinder should be partially or

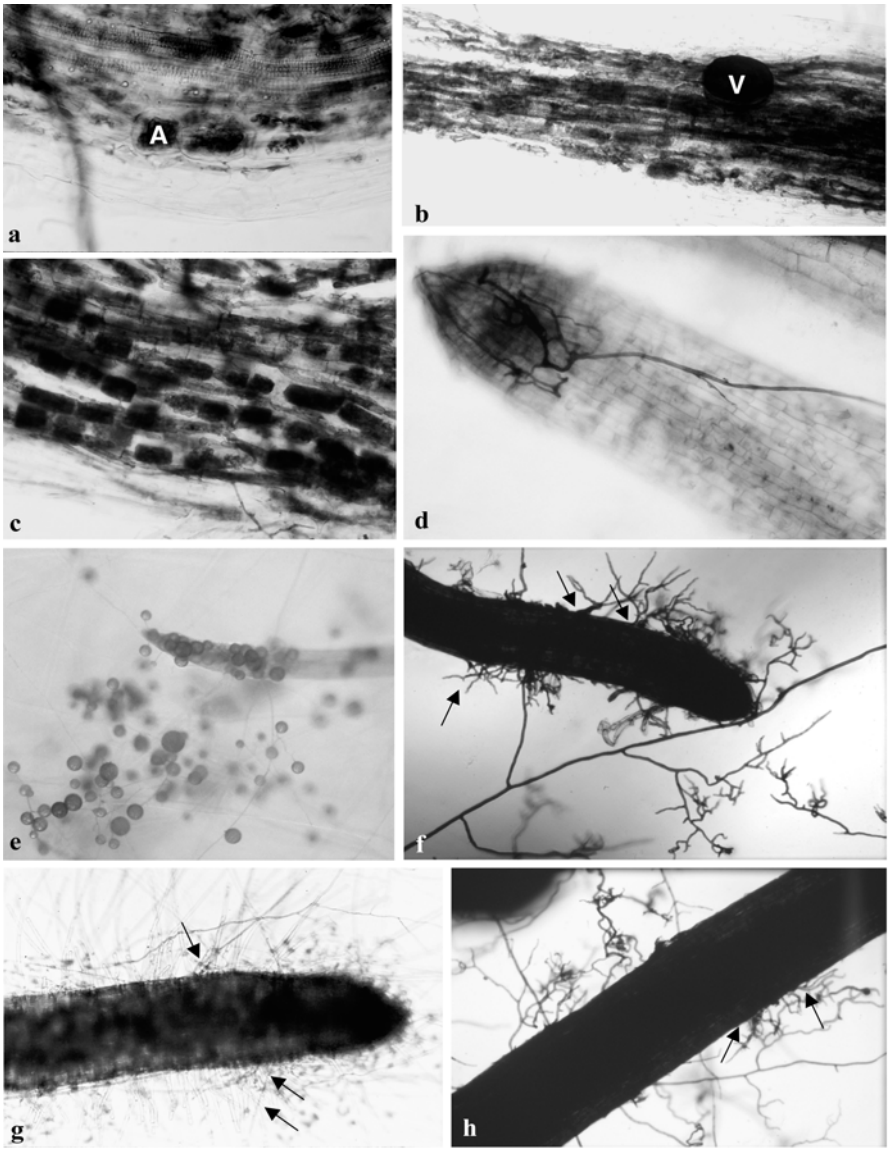


Fig. 1a–c. Intraradical features of three AM fungi grown under monoxenic conditions with a carrot Ri T-DNA transformed root organ culture (ROC, DC-2 clone). **a** *Glomus intraradices* DAOM 181602. **b** *Glomus proliferum* MUCL 41827; **c** *Glomus* sp. DAOM 227023. Colonization develops quite normally in all the species tested, and characteristic fungal structures such as entry points (*EP*), intercellular hyphae, coils, arbuscules (*A*) and intraradical spores/vesicles (*V*) are usually formed. **d–g** Apical colonization of a DC-2 ROC by *Glomus* sp. DAOM 227023 (**d**), *G.* sp. CIMA10 (**e**) or *Gigaspora margarita* CIMA 11 (**f**). **f–h** Pictures showing hyphae exiting different zones of a carrot ROC (DC-2 clone): *Gi. margarita* CIMA 10 (**f**, **h**, *arrows*) and *G. intraradices* DAOM 181602 (**g**, *arrows*)

totally useless, since sucrose is provided to the monoxenic culture homogeneously through the agar medium. Once this has been taken into account, there is no further indication that any physiological, metabolic or genetic process in root organ cells is affected. In a recent work, the cell structure of root organ cultures (ROC) from a tomato variety (c.v. 76R) and its reduced mycorrhizal colonization mutant (rmc, Barker et al. 1998; Gao et al. 2001) have been compared to the corresponding whole-plant roots growing in soil – no remarkable differences were found (Bago et al. 2004a). Also, ROC from roots expressing a Nod^- Myc^- genotype maintained such a phenotype when monoxenically cultured (Balaji et al. 1994). Finally, we should consider the strict requirements of AM fungi to become symbiotic: firstly, a series of pre-symbiotic dialogues between the two partners should occur (Bago and Bécard 2002); secondly, the newly formed interfaces must be fully functional for symbiosis to be established. It seems therefore clear that unless the physiology and functioning of ROC epidermal and cortical cells are preserved, the AM fungus would never acquire its symbiotic status and, consequently, complete its life cycle. In conclusion, while it is crucial to consider case by case the suitability of using AM monoxenic cultures for particular research purposes, there do not seem to be enough reasons to discard by default the use of such experimental systems in the study of AM intraradical/interfacial functioning.

2.2 Are Transformed Root Organs a Good Host Material to Study AM Fungal Biology?

Most of the monoxenic cultures reported to date in mycorrhizal research use Ri T-DNA transformed root organs as hosts (Table 1). These naturally transformed plant roots are obtained by the insertion of the Ri T-DNA plasmid from the ubiquitous soil bacterium *Agrobacterium rhizogenes* into a given plant tissue, which is then induced to morphologically develop as a root (a condition known as "hairy roots"; Giri and Narasu 2000). Transformed root organs usually present a greater growth potential than non-transformed ones, probably due to their modified hormonal balance (Fortin et al. 2002). Ri T-DNA transformed roots show greater AM intraradical colonization and sustain higher extraradical hyphal development than non-transformed ROC, which has led mycorrhizologists to preferentially use these roots rather than the less known non-transformed cultures. However, it has not yet been investigated if transformation could somehow affect AM fungal behaviour, and this is a possibility which should be taken into account (Bago 1998). Due to the increased secondary metabolite produc-

Table 1. Root organ cultures used to date as hosts for AM monoxenic cultures

Plant species	Ri T-DNA transformed	Clone or cv	First citation
Daucus carota L.	Yes	DC1	Bécard and Fortin (1988)
Daucus carota L.	Yes	DC2[a]	Bécard and Fortin (1988)[b]
Medicago truncatula L.	Yes	Jemalong	Boisson-Dernier et al. (2001)
Lycopersicon esculentum Mill.	Yes	–	Simoneau et al. (1994)
Pisum sativum L.	Yes	Lincoln	Balaji et al. (1994)
Pisum sativum L.	Yes	Sparkle E135[c]	Balaji et al. (1994)
Pisum sativum L.	Yes	Sparkle R25[c]	Balaji et al. (1994)
Pisum sativum L.	Yes	Sparkle R72[c]	Balaji et al. (1994)
Fragaria x Ananassa Duch.	Yes	Senga sengana	Nuutila et al. (1995)
Trifolium repens L.	Yes	New Zealand White	De Souza and Berbara (1999)
Linum usitatissimum L.	Yes	Atalante	Karandashov et al. (1999)
Tagetes patula L.	Yes	nana	Karandashov et al. (1999)
Althaea officinalis L.	Yes	–	Karandashov et al. (1999)
Trifolium pratense L.	No	S123	Mosse and Hepper. (1975)
Fragaria ananassa Duch	No	ananassa	Strullu et al. (1986)
Solanum lycopersicon Mill.	No	Saint-Pierre	Strullu and Romand (1987)
Medicago sativa L.	No	Europe	Strullu et al. (1989)
Lycopersicon esculentum Mill.	No	Vendor	Chabot et al. 1992
Lycopersicon esculentum Mill.	No	76R	Bago et al. (2004a)
Lycopersicon esculentum Mill.	No	rmc[c]	Bago et al. (2004a)
Helianthus annus L.	No	HES	Bago et al. (unpublished data)

[a]There might be further Ri T-DNA transformed carrot root clones used for AM monoxenic cultures, but this is not specified in the paper
[b]There is no clear mention in this paper on whether the carrot ROC used corresponds to what it is today known as DC1 or DC2 clones; however, since both clones came from the same authors/laboratory, we use this as first citation
[c]Mutant clones usually impaired for AM symbiosis

tion of transformed root organs (Giri and Narasu 2000), one may expect the final composition of culture media containing such transformed cultures to be different to those containing non-transformed root organs of the same plant species. Moreover, by growing different plant root organs in initially similar culture media, such media will become different in composition,

since each ROC produces specific compounds (see Giri and Narasu 2000, Table 1). Rigorous studies comparing the effects of transformed versus non-transformed root organs on AM fungal development are lacking. Such studies would be important to perform in order to evaluate and better understand the basis of AM symbiosis, and even mandatory in assessments of aspects such as fungal symbiotic gene expression.

2.3 The Downfall of Two Colonization Myths

AM in vitro cultures, in general, and monoxenics, in particular, are exceptional tools for studying the pre-symbiotic and symbiotic steps of the colonization process (Bago and Bécard 2002). Observations difficult to carry out in soil are easily performed in monoxenics, and important information has been acquired as a result of this (Schreiner and Koide 1993; Bago 1998; Bago et al. 1998a, b, 2004b; Jolicoeur et al. 1998; de Souza and Berbara 1999; Declerck et al. 2000, 2001; Nielsen et al. 2002). Taking advantage of this, we want to address very briefly two old "myths" traditionally accepted by mycorrhizologists as true, which become now challenged by direct observation with monoxenic cultures:

1. "*Primary colonization by AM fungi occurs in young roots, but the actual root apices are rarely if ever colonized*" – it has been thought for a long time that preferential colonization of roots by AM fungi occurred in subapical zones (0.5 to 1.5 µm from the root tip; Harley and Smith 1983; Smith and Read 1997), where the root is growing most actively and its cell walls are still loose, and that a sort of "exclusion zone" for AM colonization was formed at the root apical level (see Plate 3, Harley and Smith 1983). Based on colonization modelling, Smith and coworkers (2001) found that such exclusion zones were minimal or zero in most cases. Monoxenic culture observations of different AM fungi confirm these theoretical results, as is clearly shown in Fig. 1d–g. Even more, we could say that apical colonization is quite frequent. Although one may think at first that the homogeneous environmental conditions encountered by both root and fungus in AM monoxenic cultures could make root tips more prone to colonization, the observation of root tips developing in soil also suggests that apical colonization is not a rare event (C. Cano, pers. observ.), and that AM fungi have the potential to colonize root apices with minimal interference on its meristematic growth.
2. *Do hyphae exit the root after symbiosis setup?* – mycorrhizal "lore" establishes that, after symbiosis setup, and synchronously with in-

traradical colonization spreading, the AM fungus acquires a sort of hyphal "invigoration" which makes it possible to develop and explore the soil surrounding the root. In other words, this "lore" states that whereas AM fungal hyphae have the mechanisms to penetrate the root, they are unable to exit it, using instead the already established hyphal penetrating network to develop towards the soil. In contrast, a more intuitive mechanism for AM soil colonization was suggested by Friese and Allen (1991), who referred to "exiting hyphae" when describing hyphal spread in the soil from a host root; such a suggestion has caused some controversy. Monoxenic cultures confirm that AM hyphae are indeed able to exit the root to explore the surrounding media (Fig. 1f–h), and that this is a quite common event. This is in fact no big surprise, since the same mechanisms (both mechanical and enzymatic) described to be used by AM fungi to penetrate host roots (Cox and Sanders 1974; Kinden and Brown 1975; Holley and Peterson 1979; Gianinazzi-Pearson et al. 1981; García-Romera et al. 1990, 1991) could be easily used by the mycobiont to develop in the opposite direction.

2.4 Are Branched Absorbing Structures (BAS) Commonly Formed by all Glomalean Fungi? Are They Artifacts Formed Only Under in Vitro Conditions?

In the first report of the in vitro co-culture of a root organ and a glomalean species (Mosse and Hepper 1975), "... a form of branching strongly reminiscent to arbuscules ..." was already described. Since then, different authors have mentioned such "arbuscule-like structures" (ALS) formed on extraradical runner hyphae of different AM fungi (Mosse 1988; Bécard and Fortin 1988; Chabot et al. 1992; Declerck et al. 1996; Bago et al. 1998a). Strullu et al. (1997) suggested such structures are a thallus emerging from the intraradical mycelium of AM fungi during the saprophytic phase, thus being different from the spore-produced pre-symbiotic hyphae. In 1998, Bago et al. (1998b) studied ALS in depth, finding striking morphological, cytological and developmental similarities between these and intraradical arbuscules. ALS were suggested to be preferential sites for nutrient uptake by the extraradical mycelium of AM fungi (Bago et al. 1998b), and homologous to arbuscules in the context of AM fungi being highly bipolarized organisms (Bago 1998). Following the advice of the two referees revising the paper, Bago et al. (1998b) had to rename arbuscule-like structures to "branching absorbing structures" (BAS): both referees felt that such structures were (1) not similar enough to arbuscules, (2) different in function,

and (3) perhaps artificial structures produced by the AM fungus *Glomus intraradices* under highly controlled in vitro conditions.

Since then, the question of BAS being artificial structures has been raised in different scientific meetings and paper reviewing processes. At the same time, more and more mycorrhizologists using monoxenic cultures realized the omnipresence of BAS on AM extraradical runner hyphae (de Souza and Berbara 1999; Declerck et al. 2000; Nielsen et al. 2002). In this section, we present graphic evidence of (1) the occurrence of BAS in all of the different AM fungal isolates successfully cultivated by us in monoxenics (Fig. 2) and (2) the occurrence of such structures under natural conditions (Fig. 3a–j). Finally, we will show first evidence confirming the hypothesized homology between arbuscules and BAS (Fig. 3k, l).

In Fig. 2, the morphology of extraradical hyphae of 12 different AM fungal isolates is shown. The most striking feature in all of these is the presence of BAS at regular intervals on the runner hyphae. In some of the species/isolates studied, BAS were more difficult to distinguish, due to the frequent anastomoses suffered by extraradical hyphae, which masked BAS occurrence (Fig. 2b, *G. etunicatum*). In all cases, BAS show their typical, slender morphology, with a dichotomous branching pattern and a hyphal tip thickness of approx. 1.5 μm. BAS were slightly different in morphology from one species/isolate to another; this could be of use in the future as a taxonomic character. In agreement with first descriptions (Bago et al. 1998b), BAS were ephemeral (5–7 day life span) in all isolates tested, except for those undergoing spore formation events ("spore-BAS", Bago et al. 1998b; see Fig. 2, insets). BAS are developed not only by *Glomus* species, but also by members of the *Gigaspora* (Fig. 2j–l), *Scutellospora* (de Souza and Declerck 2003) and *Acaulospora* (Dalpé and Declerck 2002) genera tested to date. We can now say that BAS are not just artificial structures formed in vitro by AM fungi – they are also formed under soil ex-vitro conditions, although in such situations they are extremely difficult to observe (Fig. 3a–j). Bago (1998) proposed that, besides the putative physiological role of BAS as preferential soil nutrient scavengers, these structures would also be implicated in maintaining soil structure and aggregate formation, as their thin branches would grow between soil particles, holding them together. This could be the reason why they are usually hidden, and their presence overlooked. Likewise, these structures might also be involved in the excretion of substances possibly involved in the establishment of a microbial mycorhizosphere. Such hypotheses, although not sustained at present, would merit in-depth investigation.

Photomicrographs in Fig. 3a–j illustrate extraradical hyphal structures formed by two AM fungi as they develop in soil containing vermiculite particles. The latter semi-transparent substrate is organized in thin layers, which allows the fungus to develop three-dimensionally between them.

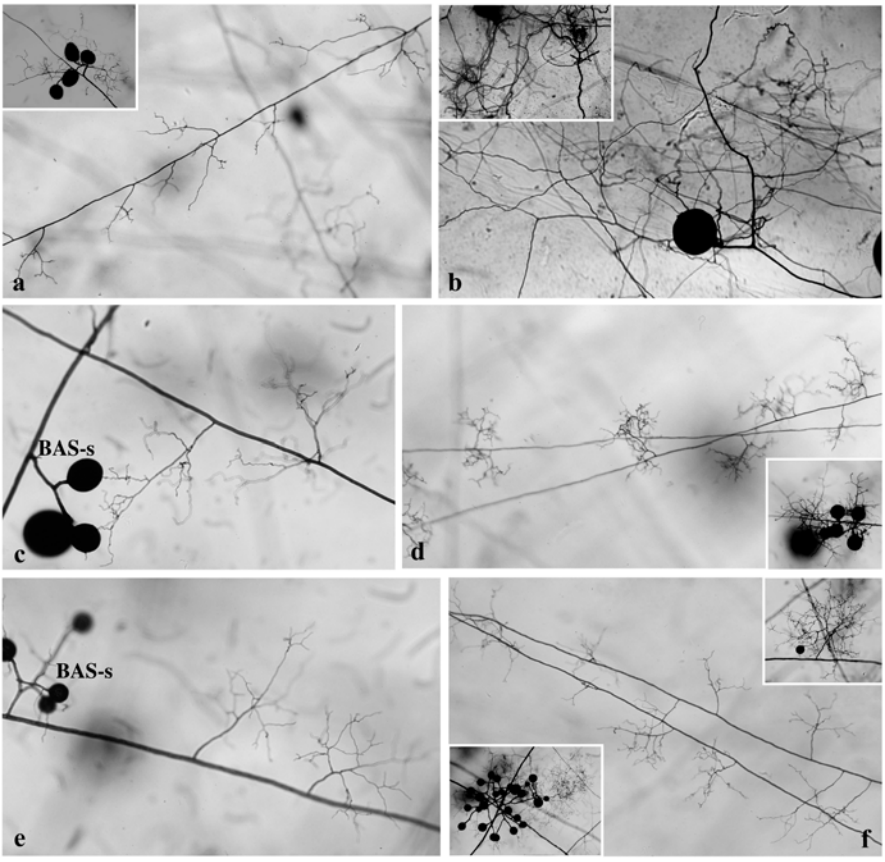


Fig. 2a–l. Branched absorbing structures (BAS, BAS-s) formed by extraradical hyphae of six different AMF grown under monoxenic conditions with a carrot Ri T-DNA ROC (DC-2 clone). **a** *Glomus intraradices* DAOM 181602. **b** *Glomus etunicatum* CIMA 07. **c** *Glomus* sp. MUCL 43195. **d** *Glomus proliferum* MUCL 41827. **e** *Glomus cerebriforme* DAOM 227022. **f** *Glomus* sp. DAOM 227023. **g** *Glomus* sp. CIMA 09. **h** *Glomus* sp. CIMA 10. **i** *Glomus* sp. CIMA 12. **j** *Gigaspora margarita* BEG34. **k** *Gi. margarita* CIMA 05. **l** *Gi. margarita* CIMA 11. While presenting similar general morphogenetic and developmental features (i.e. ephemeral short structures with dichotomous branching pattern formed by runner hyphae at regular intervals), BAS from different fungi differ from each other, which might be of taxonomic interest. In all the cases studied, spore-BAS were observed (**a**, **d**, **f**–**h**, *insets*; **c**, **e**, **i**, *BAS-s*), except for the *Gi. margarita* isolates, where auxiliary cells preferentially developed at the BAS trunk (**j**, *arrow in inset*; **k**, **l**, *arrows*). *G. etunicatum* CIMA 07 presented the most differential developmental pattern (**b**) in which BAS were difficult to observe due to frequent anastomoses of thin branches (**b**, *inset*) (*continued on next page*)

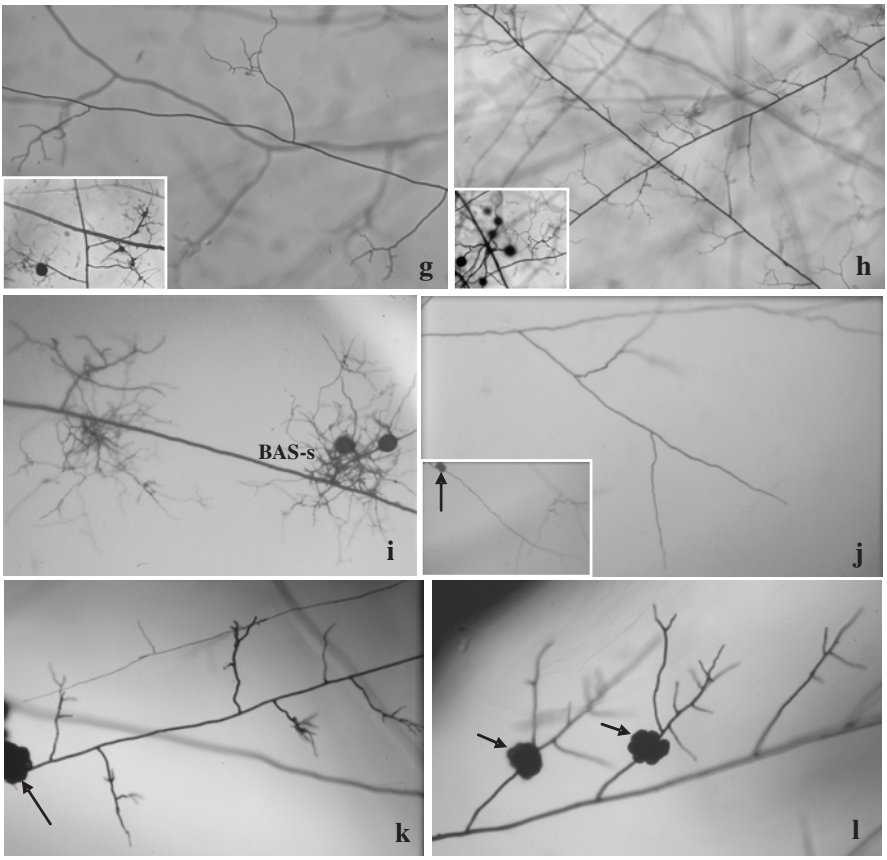


Fig. 2a–l. (*continued*)

Such layers seem to be suitable for BAS to be formed, thus allowing us to show, for the first time, their actual occurrence under ex vitro conditions. Interestingly, preferential sporulation between vermiculite layers has been observed (Vidal et al. 1990; C. Cano, pers. observ.; Fig. 3f, g), suggesting that some or most BAS formed there could, in fact, be spore-BAS.

Arbuscules and BAS do share some features (Bago 1998; Bago et al. 1998b), and this leads us to propose (and this may appear scandalous to some mycorrhizologists) that they are homologous structures, and bipolar extremes of one and the same AM fungal colony; if this were true, then some arbuscules should reflect intraradically the occurrence of spore-BAS extraradically, by supporting vesicle formation on their branches. This is indeed the case, as shown, also for the first time, in Fig. 3k. Further observations should confirm the frequency for such "spore-arbuscules" to occur within the root; nevertheless, their actual presence gives some additional

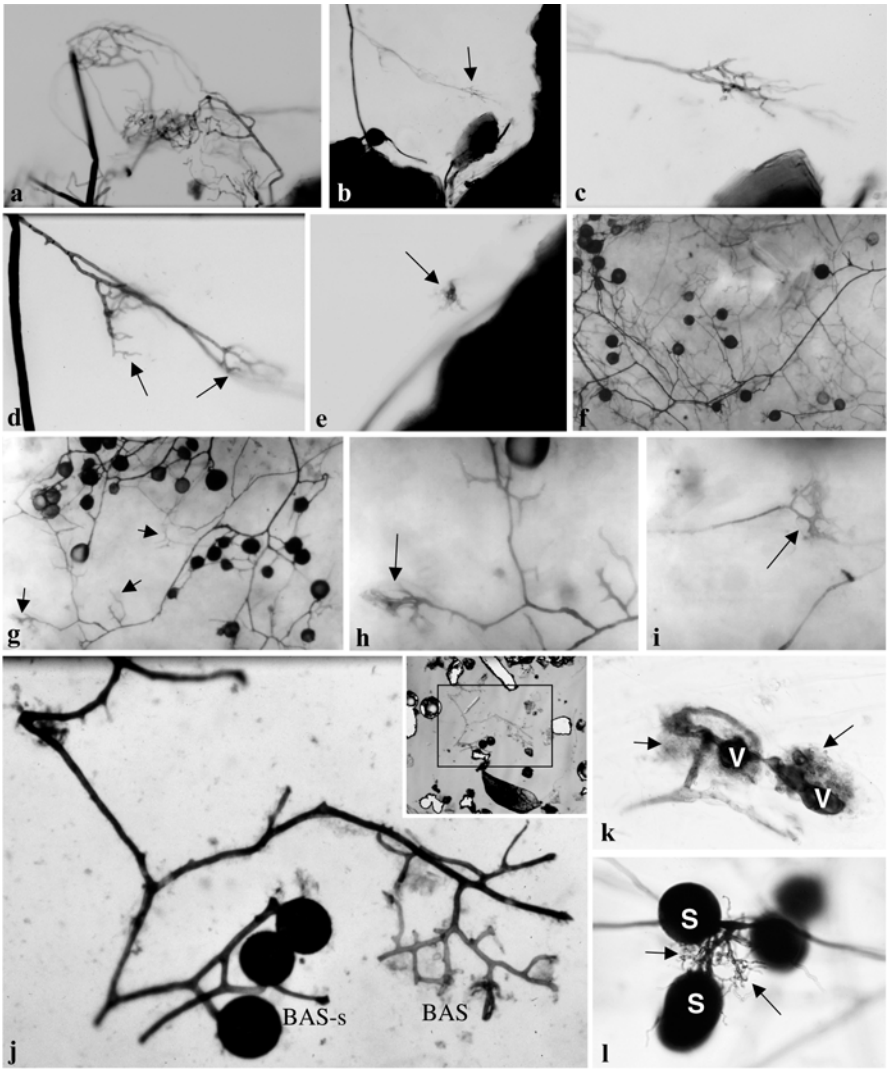


Fig. 3a–l. Occurrence of branched absorbing structures ex vitro, in either soil, or soil-like substrates and under greenhouse or natural conditions (photographs in **f–i** were taken by C. Cano together with C. Azcón-Aguilar). **a–e** *Glomus intraradices* DAOM 181602 grown in soil:vermiculite:sepiolite (1:1:1). **f–i** *G. viscosum* EEZ 34 in sand:vermiculite (1:1). **j** *G.* sp. CIMA 12 extraradical hyphae isolated from natural soil. In all cases, BAS are indicated by *arrows*. **k–l** Comparison between arbuscule-forming vesicles (**k**) and spore-BAS (**l**). Note the striking morphological and developmental similarities of both structures, suggesting their homologous origin and confirming once more the AM fungal colony bipolarity. *Arrows* Arbuscule or BAS thin branches, *V* vesicle, *S* spore

validation to the above-mentioned hypothesis (cf. Fig. 3k, intraradical, with Fig. 3 l, extraradical). Moreover, it is interesting to note here that members of the Gigasporaceae family typically do not form intraradical vesicles; in parallel, BAS of such isolates never support spore formation. However, many of the BAS produced by the *Gigaspora* species formed auxiliary cells at the BAS trunk (Fig. 2j, inset, Fig. 2 l). A recent study has shed some light on the formation and development of auxiliary cells in the Gigasporaceae family (Declerck et al. 2004). More research is needed to reveal their real physiological role, and to understand their possible relationship to BAS.

2.5 Are There Any Differences in AM Fungal Development in Monoxenics Versus Soil?

While insisting that AM monoxenic cultures are valid experimental systems to study AM fungal biology, we cannot deny that highly controlled in vitro conditions could somehow affect fungal development. This is in fact the case for all in vitro-cultured micro-organisms, as they develop in nutrient-supplied, homogeneous agar media under optimal environmental conditions. Concerning AM fungi, observations by Pawlowska et al. (1999) and Dalpé (2001) indicate that monoxenically produced spores may be smaller and less pigmented than soil-borne spores (Fortin et al. 2002). We have also observed that a differential response to Melzer's staining usually occurs in soil versus monoxenically raised spores (Fig. 4a, b, e, f). Perhaps related to this, an important reduction in spore wall thickness is noted under monoxenic conditions (Fig. 4c, d, g, h). The latter could be observed at first as a frightening result, since one may think that monoxenically produced spores are weaker than those obtained from soil. However, this is absolutely not the case – monoxenically produced propagules have been shown to be even more effective in colonizing either seed-raised or micropropagated plants under greenhouse conditions than soil-raised inoculum (Vimard et al. 1999; Filion et al. 2001; Declerck et al. 2002; Jaizme-Vega et al. 2003; C. Cano, pers. observ.), and such material was shown effective to improve growth of plants under greenhouse conditions. Interestingly, tests performed in our laboratory indicate that AM fungi adapt to existing environmental conditions surprisingly quickly. Plants inoculated with monoxenically obtained, thin-walled spores and cultured in soil under greenhouse conditions produced a new generation of thick-walled AM fungal spores (C. Cano and B. Bago, unpubl. data). Such a result indicates once more the incredible plasticity of fungi; AM fungi in particular have been demonstrated to quickly adapt to a changing environment while preserving the integrity of the fungal colony (Bago et al. 2004b).

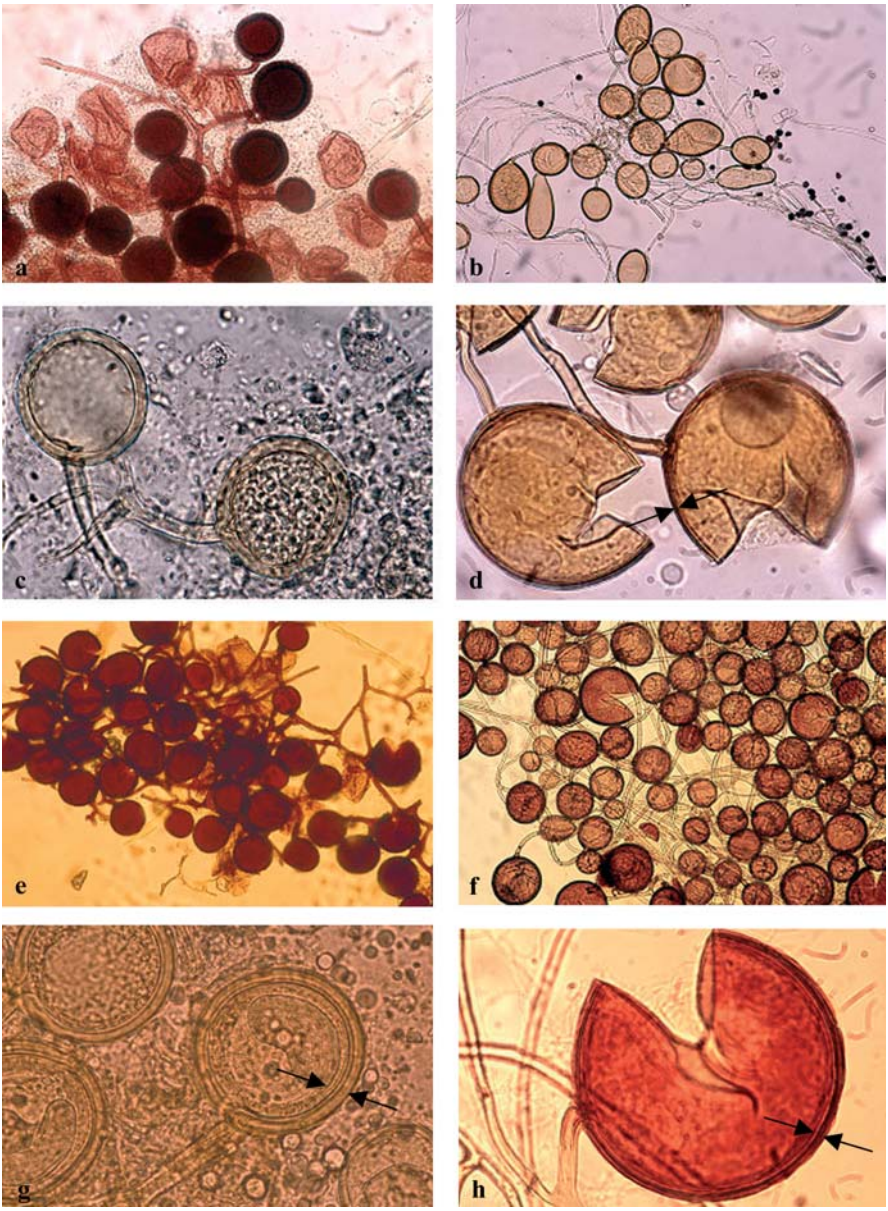


Fig. 4a–h. Comparison between spores of *Glomus intraradices* DAOM 181602 (**a–d**) and *G.* sp. CIMA 12 (**e–h**) produced either in soil (**a, c, e, g**) or under monoxenic cultures (**b, d, f, h**). Spores were extracted from the substrate and treated with either Melzer's reagent (**a, b, e, f, h**) or PVLG (**c, d, g**). Note the intense Melzer's staining of soil-borne spores compared to those obtained from monoxenic cultures (**a** vs. **b**, and **d** vs. **e**). This could be due at least partially to the dramatic reduction in cell wall thickness of soil- compared to monoxenically raised spores (**c** vs. **d**, and **g** vs. **h**, *arrows*)

Thus, careful observations of AM fungal developmental features should be performed in monoxenics in parallel to any physiological or molecular tests we may want to carry out. Fungal morphogenesis is a good indicator of any fungal response, and it is important to have eyes wide open during data interpretation to avoid misleading conclusions. Only on this basis will monoxenic cultures be of value for the advance of our understanding of AM symbioses.

2.6 Are AM Monoxenic Liquid Cultures Accurate?

As indicated above, more and more researchers now have a different perception of AM monoxenic cultures, and consider this experimental system as an extremely useful tool to work with, especially for fine research such as biochemistry and molecular biology of the AM symbiosis. Consequently, modifications of the original monoxenic culture described by Bécard and Fortin have been developed. A clear example of this is the bi-compartmented system (St-Arnaud et al. 1998), allowing the physical separation of extraradical hyphae from the influence of roots, or the multi-compartmented system (Bago et al. 2004b), which allows the testing of the physiological abilities of different parts of a single AM fungal colony growing on spatially heterogeneous media. Another modification of the system, which is now being widely used, is the replacement of the solid medium in the hyphal compartment by a liquid medium. Such a modification was first reported by Maldonado-Mendoza et al. (2001), and is extremely useful for pulse-chase experiments.

Nevertheless, and taking into account the warning notice expressed at the end of the previous section, when observing morphogenesis of extraradical hyphae growing in liquid culture, dramatic morphological changes are noted (Fig. 5). Indeed, the regular pattern of development, with runner hyphae extending from the fungal colony radially, and producing BAS at regular intervals (Fig. 5a, b), is lost in liquid cultures, in which runner hyphae are predominant (Fig. 5c, d) and BAS are scantly and appear much less branched (Fig. 5e). Zones of fine hyphal networks, which could be the consequence of multiple BAS anastomoses, appear instead (Fig. 5f), and on these preferential sporulation seems to take place (Fig. 5g, h). Liquid-growing fungal hyphae are extremely fragile, and it is common to inadvertently stop development of extraradical mycelia simply by slightly moving the Petri plate.

It would be important to make sure that the important morphological changes induced by liquid media do not affect the extraradical fungus either cytologically or functionally. For instance, one may think that under liquid

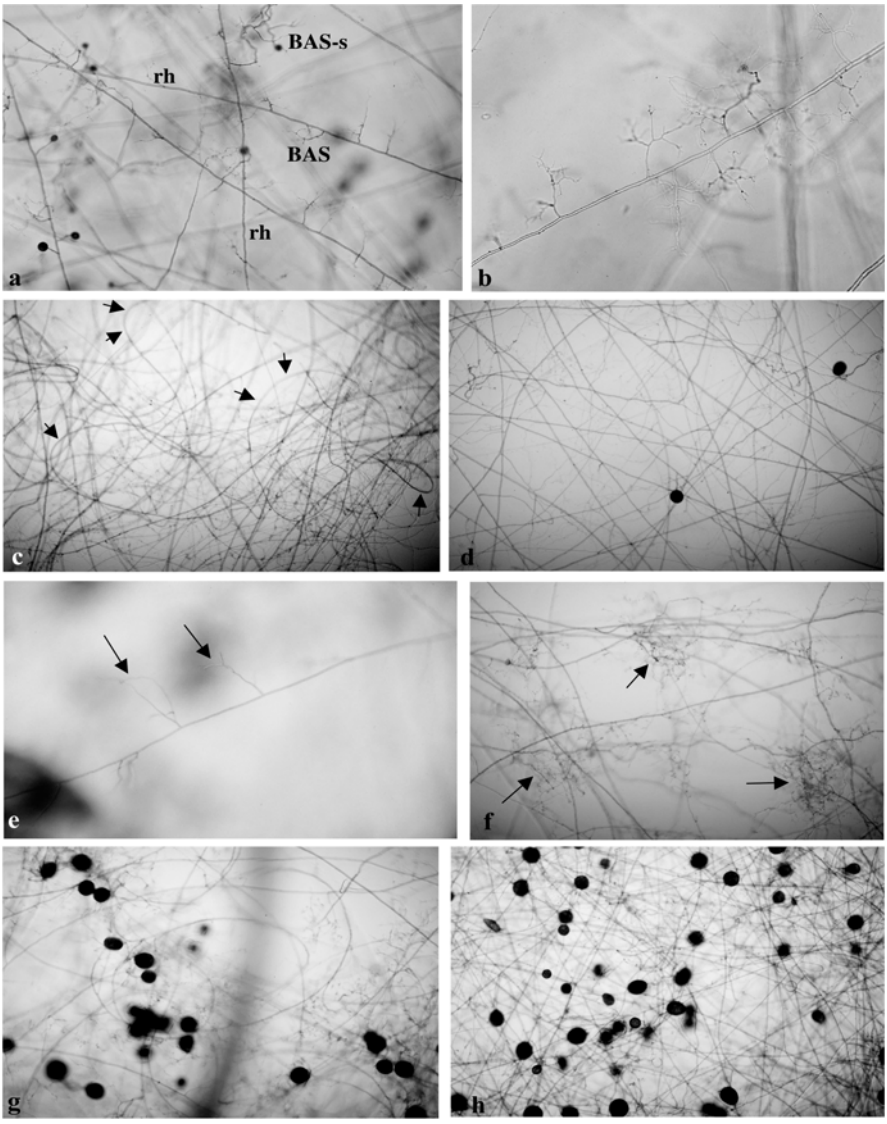


Fig. 5a–h. Developmental features of the extraradical mycelium of *Glomus intraradices* DAOM 181602 growing monoxenically in hyphal compartments containing either solid (**a**–**b**) or liquid (**c**–**h**) M-C culture media. When developing in solid medium, the extraradical AM fungal mycelium consists of straight, leading runner hyphae (*rh*) supporting differentiated *BAS* or *BAS-s* at regular intervals. However, the liquid medium modifies such patterns: runner hyphae become more generalized and prominent (**c**, **d**), and bend easily in the liquid medium (**c**, *arrows*). BAS are formed, but they are less profusely branched (**e**, *arrows*). Disorganized branching events become frequent in the extraradical mycelium (**f**, *arrows*), and seem to be preferential sites for spore formation under these special conditions (**g**). Sporulation is in no case affected by the liquid state of the medium (**h**)

conditions transport proteins would be distributed differently, that cell wall structure would adapt to the new environment, and that gene expression could be consequently affected. We are not challenging the validity of liquid cultures at all; we just warn researchers once more about the importance of considering their working material before just collecting and using it. Much additional information could be obtained, and many misleading results avoided by following this simple practice. On the other hand, the application of cutting edge techniques such as suppression subtractive hybridizations (SSH, Diatchenko et al 1996; Gianinazzi-Pearson et al. 2004), to test whether morphological changes in AM fungi indeed reflect changes in their gene expression, would be of great use.

2.7 The Danger of Contamination in AM Monoxenic Cultures

As is the case for all in vitro cultivation techniques, undesired contamination (either of fungal or bacterial origin) is the most important handicap for AM monoxenic culturing. Adequate manipulation knowledge, exhaustive contamination screening, and immediate removal of the affected plates are sine qua non conditions for any laboratory using AM monoxenic cultures. Even so, the occurrence of some contamination is unavoidable, but this should never exceed 5% as a maximum to ensure reliability of the manipulator. Our experience indicates that, in most cases, just one or two types of contaminating fungi, and two or three different contaminating bacteria appear in AM monoxenic cultures. Preliminary fatty acid profiling analysis of these (Larsen, Cano and Bago, unpubl. data) resulted in the identification of two bacterial isolates, a *Paenibacillus* sp. and a *Bacillus subtilis*. Interestingly, both of these bacteria are well-known PGPRs; moreover, another *Paenibacillus* isolate (*P. validus*, Hildebrandt et al. 2002) has been recently pointed out as an important inducer of morphological differentiation in *G. intraradices*. More research is needed because of the consistent presence of these bacteria in AM monoxenic cultures, which is linked somehow to the Mugnier and Mosse (1987) claim that the presence of some "helper bacteria", may be necessary to succeed in culturing certain AM fungi. In any case, the development of molecular kits for early detection of bacterial contaminations in AM cultures would probably be of great interest, especially for high-quality, certified in vitro AM inoculum production.

2.8 What Else Have Monoxenic Cultures to Offer on the Study of AM Fungal Biology?

Figure 6a shows a general view of AM extraradical mycelium as it develops monoxenically on a hyphal compartment. Here, it is easy to distinguish two phases in the development of extraradical hyphae (separated by the dashed line in the photograph). During the "absorptive phase" (Fig. 6a, ABS), BAS are the predominant fungal structure. This phase is at the forefront of the fungal colony, and it is easy to relate it with the substrate-scavenging abilities shown by AM fungi. Behind this absorptive phase, and probably formed after a given signal which might well be the arrival of storage lipids at important rates (Bago et al. 2002, 2003), we found the sporulative phase (Fig. 6a, SP). On this, the most frequent structures found are spore-BAS and spores which are formed in sporulation waves, according to sporulation dynamics described for AM and most other fungi (Bago et al. 1998; Declerck et al. 2001). It is important to stress here that genes of each of those two developmental phases (i.e. absorptive and sporulative) probably express differentially, according to the stage of hyphae, and therefore according to the younger versus older parts of the colony. As a consequence, caution should be taken when using extraradical hyphae for molecular biology studies – one must be sure of the physiological and developmental situation of the extraradical mycelium in order to avoid a mixture of gene expressions which may lead, once more, to misinterpretation of the obtained results.

Undoubtedly, there are still many surprises reserved for us concerning AM fungal biology. Monoxenic cultures could well be ideal tools for further advances in such knowledge. To conclude this chapter, we would like to show some "strange" growing patterns usually found in monoxenic cultures, but rarely reported due to the difficulty in identification and/or explanation.

2.8.1 Interwoven Hyphae of *Glomus Intraradices* Forming Sporocarp-Like Structures

Such a morphological event (Fig. 6b–f) occurs quite frequently at late developmental stages of monoxenically grown *G. intraradices*. It consists of a sudden re-growth of thin, newly formed hyphae emerging from a given point of a hypha on which, quite frequently, an anastomosis has taken place (Fig. 6b arrow). Such long, thin and tortuous hyphae interweave, giving rise to what we could name "hyphal knots" (Fig. 6c), which grow more compact (Fig. 6d) to support sporulation at latter stages (Fig. 6e), being at the end extremely reminiscent of sporocarps (Fig. 6f). Since the occurrence

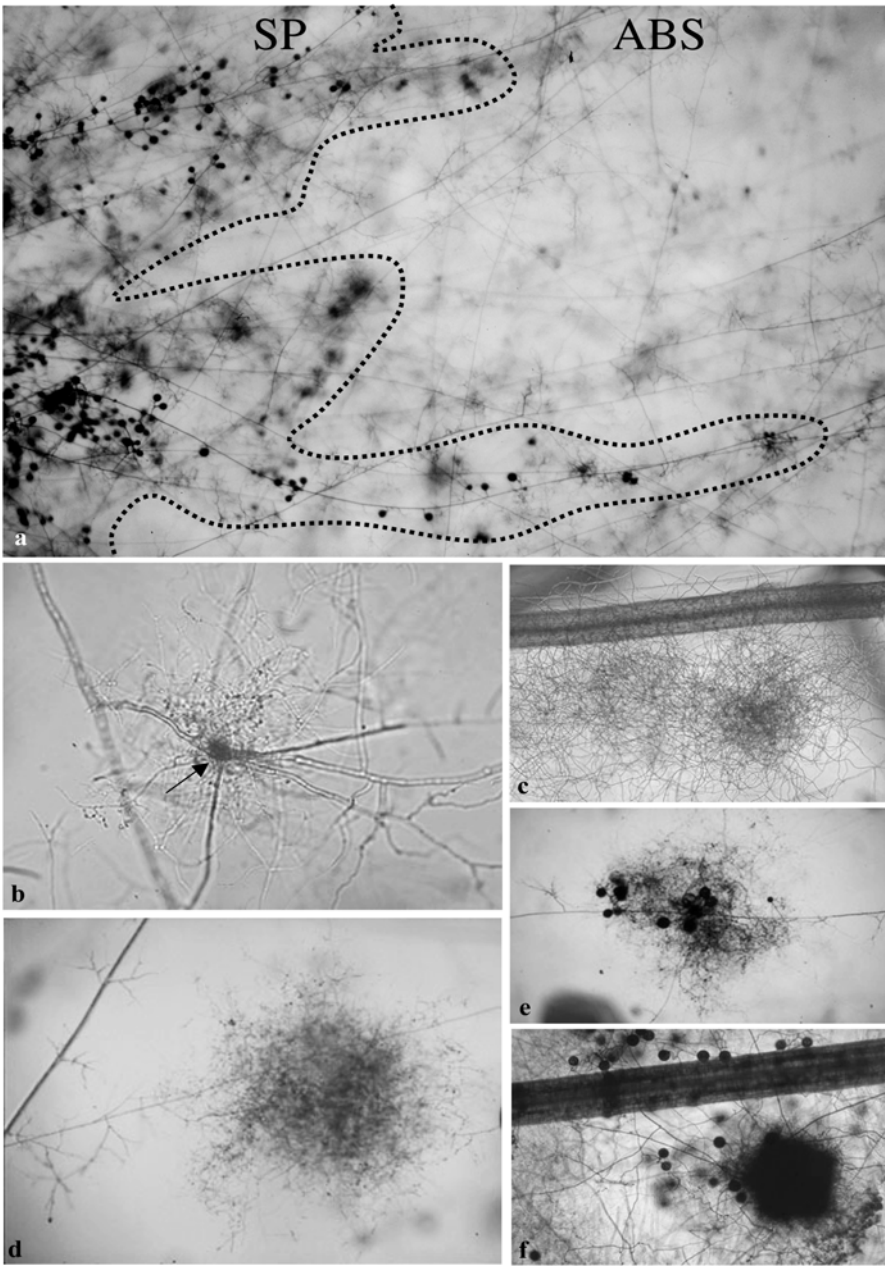
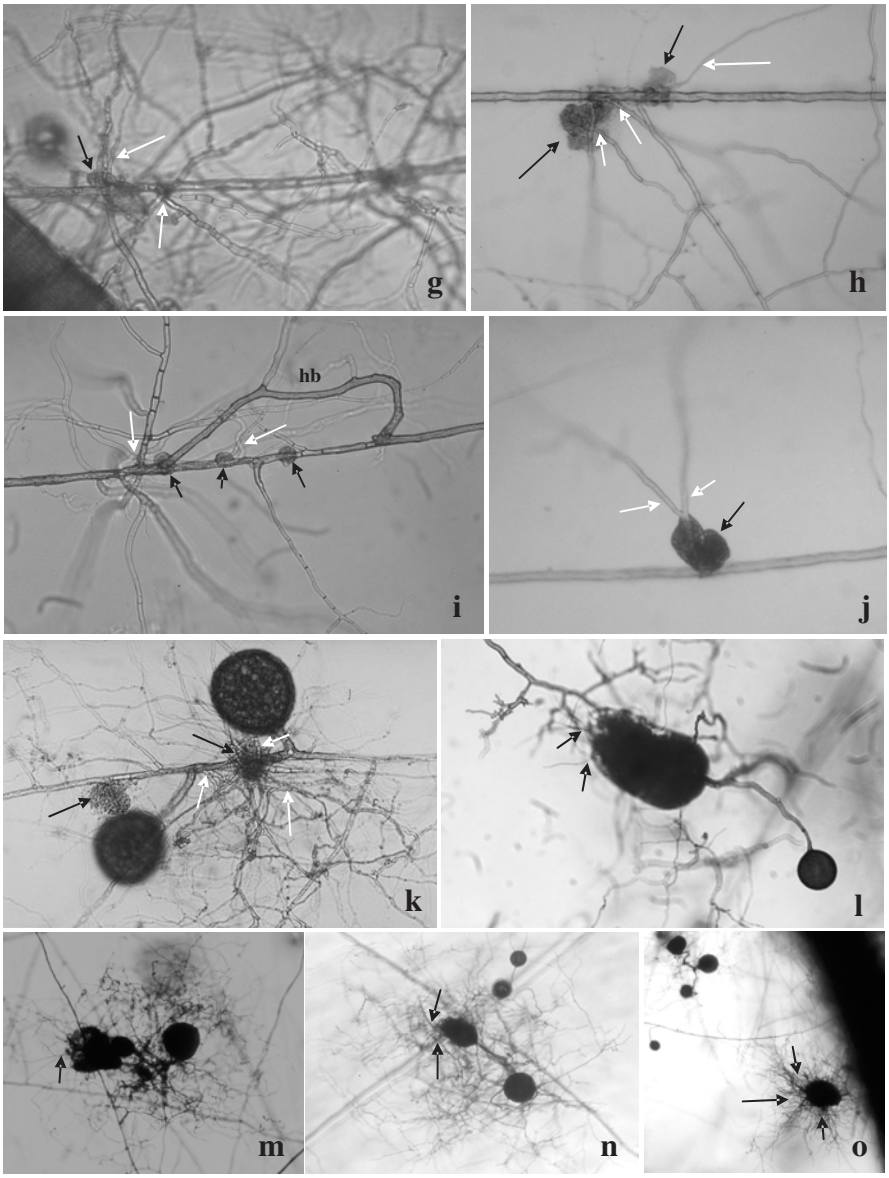


Fig. 6a–o. Morphogenetic events of interest in mature (**a**) and late (**b–o**) stages of AM fungal extraradical mycelium as revealed by monoxenic cultures. **a** Transition from absorptive (*ABS*) to sporulative (*SP*) phases. Note the well-differentiated limit between both developmental stages, which allows one to easily follow the "sporulation wave" undergone by the fungal colony. **b–f** Hyphal knots formed by the extraradical mycelium, usually after an anas-



tomosis event (**b**, *arrow*): hyphae interweave and curl progressively (see transition from **b** to **f**) to finally form a sporocarp-like structure in which preferential sporulation occurs (**f**). **g–k** "Protrusion-and-re-growing" events in old extraradical AM hyphae. Hyphae burst at given points with no apparent cause, and frequent cytoplasmic protrusion occurs (*closed arrows*); from these same points, newly formed, thin hyphae re-grow radially (*open arrows*). Occasionally, hyphal bridges are formed to bypass the affected zone (**i**, *hb*). **l–o** "Protrusion and re-germination" events in mature spores. Spores burst at their distal pole with cytoplasm loss, while newly formed, thin hyphae re-grow from the exploded sites (*arrows*)

of sporocarps in *G. intraradices* has never been described under either greenhouse or natural conditions, we may conclude that (1) the observed structure has a different function than sporocarps, and simply resembles them, or (2) *G. intraradices* has the potential to form sporocarps, but such a potential is rarely used under the experimental/natural conditions studied up to now.

2.8.2 Protrusion and Re-Growing Events

These processes usually occur in older parts of the fungal colony as well. They consist of a burst of a runner hypha in non-apical zones, with protrusion of cytoplasmic material (Fig. 6g–k, closed arrows), which is sometimes followed by "hyphal bridging" (Gerdemann1955; Mosse 1988) events (Fig. 6i). Either from, or at the protruded portion of hyphae, new thin hyphae re-grow (Fig. 6g–k, open arrows), usually in a quite unorganized manner. The most extreme situation of these protrusion and re-growing events is exploded spores (Fig. 6l–o). In this case, it is the distal pole of a given spore which bursts, liberating parts of its content from which new, thin, interwoven hyphae develop (Fig. 6l–o, arrows). This protrusion and re-growth in spores has been noted in all the AM fungal isolates revised, but its real cause/significance remains absolutely obscure to us. It is important to stress here that there is no indication that the bursting and protrusion of either hyphae or spores are a consequence of contamination of the monoxenic cultures by exogenous bacteria – contaminated plates are routinely discarded and never used in our studies.

2.8.3 Are There Sexual Processes Waiting to Be Described in AM Fungi?

Unfortunately, we have no pictures to answer this controversial question; it is, nevertheless, tempting to speculate that, similarly to the fact that there are morphogenetic processes which had never been described to occur in AM fungi before, there might be other significant processes waiting to be described. Could it be that the hyphal knots formed from an anastomosis are in fact the result of a genetic exchange of nuclei within a given hypha? Reports by several authors (Bago et al. 1999a; Giovannetti et al. 1999) seem to support such a possibility since, in very close hyphal tips, nuclei seem to sense and attract each other. Nevertheless, recent reports suggest that no sexual exchange occurs in AM fungi (Sanders 1999; Pawlowska and Taylor 2002), although such observations remain to be fully confirmed. Hopefully, new powerful techniques (molecular/microscopy) will answer

such questions – no doubt monoxenic cultures will be, once again, the selected experimental system to carry out such studies.

3 Conclusion

In this chapter, we tried to give a brief overview on subjects of potential interest for those using, or willing to use, AM monoxenic cultures. This experimental system has already opened, and can further open in the future, new avenues in our knowledge and understanding of AM symbiosis; it has also uncalculated potential as an inoculum source for either scientific or commercial purposes. Nevertheless, only by following some simple common sense rules, monoxenic cultures will retain liability and appropriateness for these purposes. Therefore, caution is needed not to misuse a system which, we could say, has promoted a quiet revolution in the fascinating research field of arbuscular mycorrhizas.

Acknowledgements. We thank the following researchers for providing the different AM monoxenic mother cultures indicated: Julie Samson for *Glomus cerebriforme* DAOM 227022 and *G.* sp. DAOM 227023; Stéphane Declerck for *G. proliferum* MUCL 41827 and *G.* sp. MUCL 43195; and Guillaume Bécard for *Gigaspora margarita* BEG34.

References

Azcón-Aguilar C, Bago B, Barea JM (1999) Saprophytic growth of arbuscular mycorrhizal fungi. In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology and biotechnology. Springer, Berlin Heidelberg New York, pp 391–407

Bago B (1998) AM monoxenic cultures using tomato non-transformed roots. In: Kling M (ed) Development and function of the mycelium of arbuscular mycorrhizal fungi. Method Manual. Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden, pp 41–44

Bago B (2000) Putative sites for nutrient uptake in arbuscular mycorrhizal fungi. Plant Soil 226:263–274

Bago B, Vierheilig H, Piché Y, Azcón-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bago B, Azcón-Aguilar C, Piché Y (1998a) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bago B, Azcón-Aguilar C, Goulet A, Piché Y (1998b) Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. New Phytol 139:375–388

Bago B, Zipfel W, Williams RC, Piché Y (1999a) Nuclei of symbiotic arbuscular mycorrhizal fungi, as revealed by in vivo two-photon microscopy. Protoplasma 209:77–89

Bago B, Pfeffer PE, Douds DD, Brouillette J, Bécard G, Shachar-Hill Y (1999b) Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. Plant Physiol 121:263–271

Bago B, Shachar-Hill Y, Pfeffer PE (2000) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 124:949–957

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2002) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Bago B, Pfeffer PE, Abubaker J, Jun J, Arreola R, Brouillette J, Douds DD Jr, Lammers PJ, Shachar-Hill Y (2003) Carbon export from arbuscular mycorrhizal roots involves the translocation of glycogen as well as lipid. Plant Physiol 131:1496–1507

Bago B, Dickson S, Cano C, Barea JM, Smith SE (2004a) Interactions between wildtype and mycorrhiza-defective mutant tomato non-transformed roots with *Glomus intraradices* in monoxenic cultures. New Phytol (submitted)

Bago B, Cano C, Samson J, Coughlan AP, Piché Y, Azcón-Aguilar C (2004b) Differential morphogenesis of the extraradical mycelium of an arbuscular mycorrhizal fungus grown monoxenically on spatially heterogeneous culture media. Mycologia 96:452–462

Balaji B, Ba AM, La Rue TA, Tepfer D, Piché Y (1994) *Pisum sativum*mutants insensitive to nodulation are also insensitive to colonization in vitro by the mycorrhizal fungus *Gigaspora margarita*. Plant Sci 102:195–203

Barker SJ, Stummer B, Gao L, Dispain I, O'Connor PJ, Smith SE (1998) A mutant in *Lycopersicon esculentum* Mill. with highly reduced VA mycorrhiza colonization: isolation and preliminary characterisation. Plant J 15:791–797

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation in Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989a) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 112:77–83

Bécard G, Piché Y (1989b) Physiological factors determining vesicular-arbuscular formation in host and non-host Ri T-DNA transformed roots. Can J Bot 68:1260–1264

Bécard G, Piché Y (1990) Discussion on VAM systems. In: Proc 8th North American Conf Mycorrhizae, Jackson Hole, Wyoming

Bécard G, Piché Y (1992) Establishment of vesicular-arbuscular mycorrhiza in root organ culture: review and proposed methodology. Methods Microbiol 24:89–108

Boisson-Dernier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker DG (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol Plant Microb Interact 14:695–700

Carr GR, Hinkley MA, Le Tacon F, Hepper CM Jones MGK, Thomas E (1985) Improved hyphal growth of two species of vesicular-arbuscular mycorrhizal fungi in the presence of suspension-cultured plant cells. New Phytol 101:417–426

Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321

Cox G, Sanders FE (1974) Ultrastructure of the host-fungus interface in a vesicular-arbuscular mycorrhiza. New Phytol 73:901–912

Dalpé Y (2001) In vitro monoxenic culture of arbuscular mycorrhizal fungi: a major tool for taxonomical studies. In: Proc 3rd Natl Symp Mycorrhizal Symbioses, Guanajuato, Mexico

Dalpé Y, Declerck S (2002) Development of *Acaulospora rehmii* spore and hyphal swellings under root-organ culture. Mycologia 94:850–855

Declerck S, Van Coppenolle A (2000) Cryopreservation of entrapped monoxenically produced spores of an arbuscular mycorrhizal fungus. New Phytol 148:169–176

Declerck S, Strullu DG, Plenchette C (1996a) In vitro mass-production of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri T-DNA transformed carrot roots. Mycol Res 100:1237–1242

Declerck S, Strullu DG, Plenchette C, Guillemette T (1996b) Entrapment of in vitro produced spores of *Glomus versiforme* in alginate beads: in vitro and in vivo inoculum potentials. J Biotechnol 48:51–57

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

Declerck S, D'Or D, Cranenbrouck E, Le Boulengé E (2001) Modelling sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. Mycorrhiza 11:225–230

Declerck S, Risede JM, Delvaux B (2002) Greenhouse response of micropropagated bananas inoculated with in vitro monoxenically produced arbuscular mycorrhizal fungi. Mycol Res 100:1237–1242

Declerck S, D'Or D, Bivort C, de Souza FA (2004) Development of extraradical mycelium of *Scutellospora reticulata* under root-organ culture: spore production and function of auxiliary cells. Mycol Res 108:84–92

de Souza FA, Berbara RLL (1999) Ontogeny of *Glomus clarum* in Ri T-DNA transformed carrot roots. Mycol Res 100:1237–1242

de Souza FA, Declerck S (2004) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012

Diatchenko L, Chris L, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov K, Gurskaya ND, Siebert (1996) Suppression subtractive hybridization: a method for generating differentially-regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 93:6025–6030

Diop TA, Bécard G, Piché Y (1992) Long term in vitro culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed root of carrot. Symbiosis 12:249–259

Diop TA, Plenchette C, Strullu GD (1994) Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Elmeskaoui A, Damon JP, Poulin MJ, Piché Y (1995) A tripartite culture system for endomycorrhizal inoculation of micropropagated strawberry plantlets. Mycorrhiza 5:313–319

Filion M, St-Arnaud M, Guillon C, Hamel C, Jabaji-Hare SH (2001) Suitability of *Glomus intraradices* in vitro-produced spores and root segment inoculum for the establishment of a mycorrhizosphere in an experimental microcosms. Can J Bot 79:879–885

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. Mycologia 83:409–418

Gao LL, Delp G, Smith SE (2001) Colonization patterns in a mycorrhiza-defective mutant tomato vary with different arbuscular-mycorrhizal fungi. New Phytol 151:477–491

García-Romera I, García-Garrido JM, Martinez-Molina E, Ocampo JA (1990) Possible influence of hydrolytic enzymes on vesicular-arbuscular mycorrhizal infections of alfalfa. Soil Biol Biochem 22:149–152

García-Romera I, García-Garrido JM, Martinez-Molina E, Ocampo JA (1991) Pectolytic enzymes in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. FEMS Microbiol Lett 78:343–346

Gerdemann JW (1955) Wound healing of hyphae in a phycomycetous mycorrhizal fungus. Mycologia 47:916–918
Gianinazzi-Pearson V, Morandi D, Dexheimer J, Gianinazzi S (1981) Ultrastructural and ultracytochemical features of *Glomus tenuis* mycorrhiza. New Phytol 88:633–639
Gianinazzi-Pearson V, Brechenmacher L, Weidmann S, Sanchez L Gianinazzi S, van Tuinen D (2004) Molecular dialogue in arbuscular mycorrhiza: getting to the symbiotic cell programme. Mol Plant Microb Interact (in press)
Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575
Giri A, Narasu L (2000) Transgenic hairy roots: recent trends and applications. Biotech Adv 18:1–22
González-Guerrero M, Azcón-Aguilar C, Moonie M, Valderas A, MacDiarmid CW, Eide DJ, Ferrol N (2004) Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family. Mol Plant Microb Interact (submitted)
Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, London
Hawkins HJ, Johansen A, George E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226:275–285
Hayman DS, Mosse B (1971) Plant growth responses to vesicular-arbuscular mycorrhiza. I. Growth of Endogone inoculated plants in phosphate deficient soil. New Phytol 70:19–27
Hayman DS, Mosse B (1972) Plant growth responses to vesicular-arbuscular mycorrhiza. III. Increased uptake of labile P from soil. New Phytol 71:41–47
Hepper CM (1979) Germination and growth of *Glomus caledonius* spores: the effects of inhibitors and nutrients. Soil Biol Biochem 11:269–277
Hepper CM (1981) Techniques for studying the infection of plants by vesicular-arbuscular mycorrhizal fungi under axenic conditions. New Phytol 88:641–647
Hepper CM (1983) Limited independent growth of a vesicular-arbuscular mycorrhizal fungus in vitro. New Phytol 93:537–542
Hepper CM (1984) Inorganic sulphur nutrition of the vesicular-arbuscular mycorrhizal fungus *Glomus caledonium*. Soil Biol Biochem 16:669–671
Hepper CM (1987) VAM spore germinationa and hyphal growth in vitro: prospects for axenic culture. In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade: practical applications and research priorities. Institute of Food and Agricultural Sciences, Gainesville, pp 172–174
Hepper CM, Jakobsen I (1983) Hyphal growth from spores of the mycorrhizal fungus *Glomus caledonius*: effect of amino acids. Soil Biol Biochem 15:55–58
Hepper CM, Smith GA (1976) Observations on the germination of Endogone spores. Trans Br Mycol Soc 66:189–194
Hildebrandt U, Janetta K, Bothe H (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919–1924
Holley JD, Peterson RL (1979) Development of a vesicular-arbuscular mycorrhiza in bean roots. Can J Bot 57:1960–1978
Jaizme-Vega MC, Rodríguez-Romero AS, Hermoso CM, Declerck S (2003) Growth of micropropagated bananas colonized by root-organ culture produced arbuscular mycorrhizal fungi entrapped in Ca-alginate beads. Plant Soil 254:329–335
Jolicoeur M, Germette S, Gaudette M, Perrier M, Bécard G (1998) Intracellular pH in arbuscular mycorrhizal fungi: a symbiotic physiological marker. Plant Physiol 116:1279–1288

Joner EJ, Ravnskov S, Jakobsen I (2000) Arbuscular mycorrhiza phosphate transport under monoxenic conditions using radio-labelled inorganic and organic phosphate. Biotechnol Lett 22:1705–1708

Karandashov VE, Kuzovkina IN, George E, Marshner H (1999) Monoxenic culture of arbuscular mycorrhizal fungi and plant hairy roots. Russian J Plant Physiol 46:87–92

Kinden DA, Brown MF (1975) Electron microscopy of vesicular-arbuscular mycorrhizae of yellow poplar. I. Characterization of endophytic structures by scanning electron stereoscopy. Can J Microbiol 21:989–993

Koide R, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. New Phytol 148:511–517

Lammers PJ, Jun J, Abubaker J, Arreola R, Gopalan A, Bago B, Hernandez-Sebastia C, Allen JW, Douds DD, Pfeffer PE, Shachar-Hill Y (2001) The glyoxylate cycle in an arbuscular mycorrhizal fungus. Carbon flux and gene expression. Plant Physiol 127:1287–1298

Maldonado-Mendoza IE, Dewbre GR, Harrison MJ (2001) A phosphate transporter gene from the extra-radical mycelium of an arbuscular mycorrhizal fungus *Glomus intraradices* is regulated in response to phosphate in the environment. Mol Plant Microb Interact 14:1140–1148

Mathur N, Vyas A (1995) In vitro production of *Glomus deserticola* in association with *Ziziphus nummularia*. Plant Cell Rep 14:735–737

Mosse B (1953) Fructifications associated with mycorrhizal strawberry roots. Nature 171:974

Mosse B (1956) Fructifications of an *Endogone* species causing endotrophic mycorrhiza in fruit plants. Ann Bot 20:349–362

Mosse B (1957) Growth and chemical composition of mycorrhizal and non-mycorrhizal apples. Nature 179:922–924

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mosse B (1988) Some studies related to the "independent" growth of vesicular-arbuscular endophytes. Can J Bot 66:2533–2540

Mosse B, Hayman DS (1971) Plant growth responses to vesicular-arbuscular mycorrhiza. II. In unsterilised field soils. New Phytol 70:29–34

Mosse B, Hepper CM (1975) Vesicular-arbuscular infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mugnier J, Mosse B (1987) Vesicular-arbuscular infections in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Nielsen JS, Joner EJ, Declerck S Olsson S, Jakobsen I (2002) Phospho-imaging as a tool for visualization and non-invasive measurement of P transport dynamics in arbuscular mycorrhizas. New Phytol 154:809–819

Nuutila AM, Vestberg M, Kauppinen V (1995) Infection of hairy roots of strawberry (*Fragaria x Ananassa* Duch.) with an arbuscular mycorrhizal fungus. Plant Cell Rep 14:505–509

Pawlowska TE, Taylor JW (2002) Organization of genetic variation within Glomalean individuals. In: Proc 7th Int Mycological Congress, Oslo, Norway

Pawlowska TE, Douds DD, Charvart I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Pfeffer PE, Shachar-Hill Y (1998) Plant/microbe symbioses. In: Shachar-Hill Y, Pfeffer PE (eds) Nuclear magnetic resonance in plant biology. ASPP Ser, vol 16, pp 77–107

Pfeffer PE, Douds DD, Bécard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol 120:587–598

Plenchette C, Declerck S, Diop TA, Strullu DG (1996) Infectivity of monoxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri T-DNA transformed carrot roots. Appl Microbiol Biotechnol 46:545–548

Sanders IR (1999) No sex, please, we're fungi. Nature 399:737–739
Schreiner RP, Koide R (1993) Stimulation of vesicular-arbuscular mycorrhizal fungi by mycotrophic and non-mycotrophic plant root systems. Appl Environ Microbiol 59:2750–2752
Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69
Simoneau P, Louisy-Louis N, Plenchette C, Strullu DG (1994) Accumulation of new polypeptides in Ri T-DNA transformed roots of tomato (*lycopersicon esculentum*) during the development of vesicular-arbuscular mycorrhizae. Appl Environ Microbiol 60:1810–1813
Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, London
Smith SE, Dickson S, Walker NA (2001) Distribution of VA mycorrhizal entry points near the root apex: is there an uninfectible zone at the root tip of leek or clover? New Phytol 122:469–477
St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *G. intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332
Strullu DG, Romand C (1986) Méthodes d'obtention d'endomycorrhizes à vésicules et arbuscules en conditions axéniques. C R Acad Sci Paris 303:245–250
Strullu DG, Romand C (1987) Culture axénique de vésicules isolées à partir d'endomycorrhizes et réassociation in vitro à des racines de tomate. C R Acad Sci Paris 305:15–19
Strullu DG, Diop T, Plenchette C (1997) Constitution of in vitro collections: a proposed life cycle of *Glomus*. C R Acad Sci Paris 320:41–47
Tiwari P, Adholeya A (2002) In vitro co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed carrot roots. FEMS Microbiol Lett 206:39–43
Vidal MT, Azcón-Aguilar C, Barea JM (1990) Nuevo método para la producción masiva de los hongos formadores de micorrizas VA *Glomus mosseae* y *Glomus fasciculatum*. III Congr Nacional de Biotecnología, Murcia, Spain
Villegas JR, William RD, Nantais L, Archambault J, Fortin JA (1996) Effect of N source on pH and nutrient exchange of extramatrical mycelium in a Ri T-DNA transformed root system. Mycorrhiza 6:247–251
Vimard B, St-Arnaud M, Furlan V, Fortin JA (1999) Colonization potential of in vitro-produced arbuscular mycorrhizal fungus spores compared with root-segment inoculum from open pot culture. Mycorrhiza 8:335–338

8 Host and Non-Host Impact on the Physiology of the AM Symbiosis

Horst Vierheilig[1], Bert Bago[2]

1 Introduction

Whereas in soil (i.e. under non-sterile conditions) the physiology of arbuscular mycorrhizal (AM) associations has been studied quite in detail, few data are available from in vitro systems. Before focusing on this subject, it is important to define correctly what we understand by "in vitro systems" when referring to AM fungi. In fact, "in vitro" is a general term used to describe a series of different experimental systems which should be strictly differentiated. Thus, in vitro systems should be referred to as "axenic systems" when just one organism (the AM fungus) is growing under aseptic conditions; "monoxenic systems" should be used when two organisms (an AM fungus and a root organ) are growing together; the term "dixenic systems" indicates that an AM fungus, a root organ and another organism are grown on the same Petri plate, usually to study the interaction of the AM symbiosis on the third organism or vice versa (Williams 1992; St.-Arnaud et al. 1995; Bago et al. 1996; Elsen et al. 2001, 2003; Villegas and Fortin 2001, 2002).

In this review, we will discuss axenic and monoxenic experimental systems. Dixenic experimental systems are discussed in another chapter of this book (see Chap. 12).

Two types of monoxenic mycorrhizal systems can be distinguished: (1) monoxenic mycorrhizal systems in which the fungus has penetrated the root organ and established a functional symbiosis; below we will call such cultures "symbiotic monoxenic cultures" (SMC); (2) monoxenic mycorrhizal systems where the fungus and the root organ are not in physical contact but signals can be exchanged; below we will call this experimental setup "pre-symbiotic monoxenic cultures" (pre-SMC). By using and combining these two monoxenic systems, different aspects of the symbiosis can be studied. Whereas in SMC the symbiotic phase of the mycorrhizal association can be studied in more detail, in pre-SMC

[1]Institut für Pflanzenschutz, Universität für Bodenkultur Wien, 1190 Wien, Austria, Tel.: +43-1-476543391, Fax: +43-1-476543359, E-mail: nonhorst@boku.ac.at
[2]Estación Experimental del Zaidín, CSIC; 18008 Granada, Spain

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

signalling events before the establishment of the symbiosis can be investigated.

Pot experiments under greenhouse or growth chamber conditions provide fast and, in general, homogeneously colonized mycorrhizal plant material in relevant quantities; however, in such systems, pure fungal material is difficult to obtain. Moreover, plant and fungal material produced under these conditions are accompanied by other micro-organisms, which might affect physiological measurements and observations. In contrast, SMC and pre-SMC are more time-consuming to establish, but provide excellent systems to study the different phases of the establishment of the AM symbiosis under strictly controlled conditions, excluding the presence of other possibly interacting micro-organisms. Despite the relatively low root colonization in SMC compared to soil systems, there are indications that the AM symbiosis is fully functional under these restricted condition (see Chap. 7), which make SMC and pre-SMC the only options to obtain pure (microbial-free or pathogen-free) AM fungal material, and SMC the only option to produce higher quantities of fungal material to perform consistent analyses.

Several phases of the root–AM fungal interaction can be identified and have been studied, at least partially, by using in vitro systems: (1) the asymbiotic phase of the AM fungus, when the fungus germinates and grows in the absence of plant signals (axenic cultures); (2) the pre-symbiotic phase, when the AM fungus germinates and shows hyphal growth in the presence of signals exuded by plants (pre-SMC); and finally (3) the symbiotic phase, when the fungus has penetrated the root and formed its intraradical structures such as the arbuscules (SMC). Whereas axenic AM fungal cultures and even pre-SMC are relatively easy to handle and thus have been used in many studies, the setup of SMC is more complex and, thus, fewer physiological data are available.

2 Asymbiotic AM Fungal Growth

To study the asymbiotic phase of AM fungi, spores are usually surface-sterilized and thereafter grown under axenic conditions on agar-like substrates. The asymbiotic phase is characterized by the spore germination and a limited hyphal growth in the absence of a host root (Azcón-Aguilar et al. 1999). Therefore, only abiotic chemical and physical factors such as pH, temperature, light and CO_2 levels can affect spore germination and hyphal asymbiotic growth of AM fungi (Smith and Read 1997; Giovannetti 2000).

2.1 pH

Optimal pH conditions for spore germination differ between species and genera. *Acaulospora laevis* germinates best between pH 4 and 5 (Hepper 1984), *Gigaspora* spp. at a pH from 4 to 6, and *Glomus* spp. between pH 6 and 9 (Green et al. 1976). The optimal pH for spore germination seems to be somewhat linked to the pH of the soil where the AM fungus is isolated (Giovannetti 2000).

Since the first step to initiate AM monoxenic cultures is to germinate either spores, isolated vesicles or mycorrhizal surface-sterilized roots under in vitro conditions, the initial pH of the agar medium is an important factor to consider. Nevertheless, our experience indicates that despite testing a wide pH range, there are occasionally AM isolates whose propagules do not germinate, even when spores seem healthy and ready to do so. Two examples are different isolates of *Glomus mosseae* and *Gigaspora margarita*, which during certain periods of the year do not germinate in agar media despite changing pH and other environmental conditions (such as those described below; C. Cano and B. Bago, pers. observ.). This indicates that other factors besides the environment could influence AM fungal asymbiotic growth. It is tempting to suggest that, as is the case for most organisms, AM fungi could have "biological clocks" which enable them to germinate and search for a new host root to colonize, just when environmental conditions are best. More research is needed to confirm this hypothesis.

2.2 Temperature

There are some data on the effect of temperature on spore germination and hyphal elongation under axenic conditions. Optimal temperatures seem to vary between AM fungi. Whereas *G. mosseae* and *Acaulospora leavis* can germinate between 10–18 and 30 °C with an optimum between 20 and 25 °C, *Gigaspora* germinates between 10 and 30 °C with an optimum between 20 and 30 °C (Safir 1986), and germination was best at 10–25 °C for *G. caledonium* (Tommerup 1983) and at 25 °C for *G. epigaeum* (Graham 1982). The optimal germination temperature seems to depend on the environment where the fungus has been isolated (this seems similar to the pH). *G. coralloidea and G. heterogama*, both isolated in Florida (USA), germinated best at 34 °C, whereas a *G. mosseae* isolate from cooler regions showed maximal germination at 20 °C and failed to germinate at 34 °C (Schenck et al. 1975).

Few data are available on the effect of low temperatures on AM fungi. Recent tests, on the in vitro growth of two AM fungi (*G. intraradices* and

G. proliferum) at different temperatures ranging from 4 to 30 °C, clearly showed that AM fungi germinations were impaired at temperatures below 12 °C, thus preventing root colonization and development of SMC (Gavito, Bago and Azcón-Aguilar, unpubl. data). A negative effect of low temperatures on AM fungi has been reported before by Safir in 1986: hyphal growth of *Gigaspora calospora* was 50% reduced at 15 °C, and lower temperatures (5–10 °C) reduced hyphal elongation even more.

Apart from a direct effect of temperature on AM fungal spore germination and hyphal growth, the temperature to which dormant spores are exposed can affect spore germination (Hepper and Smith 1976; Gemma and Koske 1988), spore mortality and the hyphal growth pattern (Juge et al. 2002). Cold storage (4 °C) of spores of *G. intraradices* for longer than 14 days increased spore germination, reduced spore mortality and resulted in the growth (at a temperature of 25 °C) of a clearly distinguishable, several centimetre-long main hypha with few branches, whereas hyphal growth of spores stored at a higher temperature (25 °C) was without a visible dominant hypha, and the hyphae emerging from the spore continuously curled and branched heavily (Juge at al. 2002).

2.3 CO_2

Depending on factors such as soil structure and water content, CO_2 levels in the soil are increased (around 2%) compared to the CO_2 concentration of ambient air (0.03%). Although released by roots, CO_2 cannot be regarded as a plant-specific signal for AM fungi, as CO_2 levels in the soil can also be increased from other sources such as the respiration of soil organisms. When initiating AM monoxenic cultures, an enriched CO_2 atmosphere is most surely developed in the Petri plate due to root organ respiration. This CO_2 increase is probably an activator of spore germination and of asymbiotic hyphal growth under these conditions.

The CO_2 effect on AM fungi seems concentration-dependent. Whereas in axenic systems CO_2 levels ranging from 0.5 to 2.5% stimulate hyphal growth of *Gi. margarita* (Bécard et al. 1989; Bécard and Piché 1989a; Bécard et al. 1992; Poulin et al. 1993), a CO_2 level of 0.1% showed no effect on hyphal growth (Poulin et al. 1993), and high CO_2 levels (5%) irreversibly inhibited in vitro growth of *G. mosseae* (Le Tacon et al. 1983).

Interestingly, AM fungi hyphal growth was highest at CO_2 levels around 2% (Bécard et al. 1992; Poulin et al. 1993), a concentration which usually is found in most soils. Bécard and Piché (1989) suggested that during germination CO_2 may be a net source of C for anabolic processes of the spore. Indeed, Bago et al. (1999; 2000) have demonstrated recently that a sig-

nificant rate of trehalose, a short-term fungal storage carbohydrate, was ^{13}C-labelled when $^{13}CO_2$ was supplied to asymbiotic spores. However, after deciphering the metabolic pathways involved, these authors concluded that such ^{13}C incorporation occurred via dark fixation, a side part of gluconeogenesis which does not lead to net gain of C. Nevertheless, such dark fixation seems to have an important role in anaplerotic reactions, thus explaining the better pre-symbiotic AM fungal development shown in the presence of CO_2.

2.4 Light

In nature, the exposure of soil-borne AM fungi to light is an extremely unlikely event (see Chap. 6), as underground roots are the colonized plant organ. Surprisingly, light treatment affects the growth pattern of axenically growing hyphae. Light induced hyphal branching in developing germ tubes of *Gi. gigantea*, *Gi. rosea* and *G. intraradices* (Nagahashi et al. 2000). In a study with *Gi. gigantea*, wavelengths in the blue to UV range were identified as the responsive ones (Nagahashi and Douds 2003). Blue light has been found to regulate all known differentiation processes in Basidiomycetes (Kues et al. 1998). The biological relevance of these findings in nature still remains unclear, and it has been suggested that a general mechanism of stress response could be implicated (Kues et al. 1998); in any case, in vitro systems are ideal to further study such intriguing questions.

3 Pre-Symbiotic AM Fungal Growth

More than 80% of all plant species are hosts for AM fungi. Depending on the host status, plant signals perceived by AM fungi differ. Whereas there are abundant data on the stimulatory effects of plant signals from host plants on AM fungal spore germination, hyphal growth and branching, there are contradictory reports on the effect of signals from non-host plants on AM fungi (Giovannetti and Sbrana 1998; Vierheilig et al. 1998; Giovannetti 2000). To our knowledge, although AM fungi can grow to some extent in the presence of AM non-host plants, in general, they do not grow on their root surface and do not form appressoria on them. As the pre-symbiotic phase is characterized by the presence of both root and AM fungi, still in the absence of physical contact between them, it can be concluded that the non-host status of plants to AM fungi is determined during the pre-symbiotic stage of the symbiosis.

Apart from plant signals perceived by the fungus, more and more data indicate that signal exchange between plants and AM fungi is not unidirectional, but that fungal signals are perceived by plants before the hyphae are in direct contact with the root (Gadkar et al. 2001; Vierheilig and Piché 2002; Vierheilig 2004).

3.1 Plant-to-Fungus Signals

In this section, we will distinguish between two classes of compounds released by roots which might act as signals for AM fungi: gaseous compounds which below we will name root volatiles, and liquid compounds which below we will name root exudates. Different approaches have been taken to study a possible signalling effect of compounds released by roots on AM fungi. A more generalistic approach is to study the effect of root volatiles and root exudates on spore germination and hyphal growth. This is usually done under in vitro conditions (see reviews by Fortin et al. 2002; Vierheilig et al. 1998). Depending on the experimental system, different fractions of root volatiles/exudates are studied.

Co-culture of roots and AM fungi in the same agar medium of a closed Petri plate system means exposing the AM fungi to a combination of root volatiles and exudates. To study the exclusive effect of volatiles on AM fungi, monoxenic closed experimental systems are used where AM fungi are exposed to volatiles, but are physically separated from root exudates (pre-SMC). To study the exclusive effect of root exudates, exudates are collected and thereafter applied to AM fungi under axenic conditions (Vierheilig et al. 1998).

A more specific approach is to test the effect of compounds isolated and identified from root exudates (e.g. in studies on the rhizobial–legume interaction) on the above-described AM fungal parameters.

3.1.1 Signals from AM Host Plants Towards the AM

More than 15 years ago, first reports on the stimulatory effect of collected root exudates from whole citrus and white clover plants on axenic AM spore germination and hyphal growth were published (Graham 1982; Elias and Safir 1987). Thereafter, this effect of root exudates was confirmed with a wide range of host plants (see review by Vierheilig et al. 1998), such as clover (Gianinazzi-Pearson et al. 1989), alfalfa (El-Atrach et al. 1989), onion (Tawaraya et al. 1996) and carrot (Poulin et al. 1993), and with root

exudates collected from Ri T-DNA-transformed carrot root organ cultures (Nagahashi et al. 1996).

Similar results were reported when AM fungi were grown under in vitro conditions in the vicinity of living roots (pre-SMC) by Hepper (1984), Mosse (1988), Bécard and Piché (1989a, b) and Bécard et al. (1989). These root organ cultures are excellent experimental systems to study the presence of signalling compounds from living roots under sterile conditions. However, effects on AM fungal growth observed in root organ cultures can not exclusively be attributed to root exudates, but also to root volatiles. A combination of exudate and volatile factors showed a synergistic stimulatory effect (Bécard et al. 1989; Bécard and Piché 1989a, 1989b, 1990). Between the volatiles released by roots, CO_2 seems to be a key factor for the stimulation of the growth of AM fungi, although the role of other volatile root factors cannot be discarded (Bécard and Piché 1989a).

Apart from affecting hyphal growth, compounds released by host roots have been demonstrated to induce branching of AM hyphae in in vitro and pot experiments (Mosse and Hepper 1975; Powell 1976; Mosse 1988; Giovannetti et al. 1993a, 1994). The data obtained indicated that fungal branching can occur without direct contact between the AM fungi and the root. Recently, Nagahashi and Douds (1999) developed a rapid bioassay to detect compounds in root exudates active on hyphal branching. Using this bioassay, active signals have been reported from a wide range of host plants such as carrot, tobacco, corn, sorghum, pea and tomato (Buee et al. 2000; Nagahashi and Douds 2000). As the presence of AM non-host roots was not effective in inducing hyphal branching, hyphal branching has been proposed as a prerequisite for a successful root colonization (Giovannetti and Sbrana 1998; Buee et al. 2000). The active branching compound(s) has(have) not been identified yet. Recently, Tamasloukht et al. (2003) determined the time course of action of the root-exuded factor. Gene activation in the AM fungi by the root factor occurs after 0.5–1 h, alterations at the physiological level can be measured after 1.5–3 h, and the morphological response – the branching of the hypha – is first visible after 5 h.

Plant roots release a wide range of compounds. Although some of these compounds have been identified, many still remain unknown. Secondary plant compounds, specifically flavonoids, are found in root exudates and have been reported to play a role in the signalling of various plant–microbe interactions. A chemotactic effect towards flavonoids released by peas has been reported for *Aphanomyces euteiches* (Yokosawa et al. 1986), and towards flavonoids released by soybean growing with *Phytophthora sojae* (Morris and Ward 1992; Tyler et al. 1996). Moreover, flavonoids stimulate spore germination of various fungal pathogens (Straney et al. 2002).

As flavonoids are also key signals in the formation of the rhizobial symbiosis (reviewed by Phillips and Tsai 1992), and activate the bacterial nod

gene expression required for subsequent events in nodulation, they have been suggested as important candidates for signalling during the AM symbiosis (Phillips and Tsai 1992; Morandi 1996).

Many flavonoids have been tested for their effect on spore germination and hyphal growth under axenic conditions (reviews by Morandi 1996, and Vierheilig et al. 1998). In a first report, the effect of apigenin, naringenin and hesperitin on AM fungi was tested by Gianinazzi-Pearson et al. (1989). Whereas all three compounds showed a clear stimulatory effect on hyphal growth of *Gi. margarita*, only apigenin and hesperitin stimulated spore germination.

There are indications that the effect of these compounds on AM fungi is flavonoid-, concentration- and AM fungal-specific (reviewed by Vierheilig et al. 1998). Chrysin has always been found to inhibit hyphal growth (Bécard et al. 1992; Chabot et al. 1992), whereas quercetin was nearly always stimulatory at low and at high concentrations (Tsai and Phillips 1991; Bécard et al. 1992, 1995; Chabot et al. 1992; Kape et al. 1992; Morandi et al. 1992; Poulin et al. 1993). Naringenin has been reported stimulatory at low concentrations (Gianinazzi-Pearson et al. 1989), but inhibited hyphal growth at a high concentration (Poulin et al. 1993).

A certain AM fungal specificity has been observed with quercetin and biochanin A. Quercetin seems to stimulate hyphal growth of different *Glomus* spp. and *Gi. gigantea* (see review by Vierheilig et al. 1998), whereas biochanin A inhibits hyphal growth of *Gi. gigantea* and *Gi. margarita* (Bécard et al. 1992; Chabot et al. 1992; Baptista and Siqueira 1994), and stimulates hyphal growth of *Glomus* spp. (Nair et al. 1991; Vierheilig et al. 1998).

So far, two flavonoids, quercetin (Tsai and Phillips 1991) and 4′,7-dihydroxyflavone, have been reported to induce hyphal branching. However, flavonoids seem not to be the active signals in root exudates of host plants inducing AM hyphal branching (Nagahashi and Douds 1999, 2000; Douds and Nagahashi 2000; Buee et al. 2000).

3.1.2 Signals from AM Non-Host and Myc$^-$ Plants Towards the AM Fungi

Non-Host Plants.

Although most land plants are hosts for AM fungi, some plant families, e.g. the Brassicaceae and the Chenopodiaceae, are reported as non-AM plants (Smith and Read 1997). The mechanism responsible for the non-susceptibility of these plants to AM fungi is still controversial. The hypothesis (see Fig. 1) has been put forward that root exudates of some AM non-host plants lack signals essential for root colonization by AM fungi, whereas root exudates of other AM non-host plants contain compounds

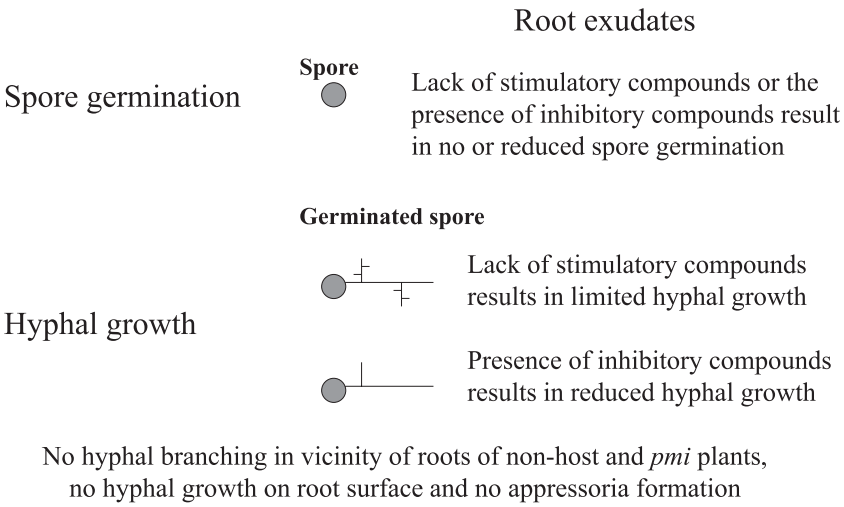


Fig. 1. Effects of root exudates of non-host plants and myc$^-$ (*pmi*) plants on spore germination and hyphal growth

inhibitory to AM fungi (reviewed by Giovannetti 2000; Giovannetti and Sbrana 1998; Vierheilig et al. 1998).

Similarly to the studies with AM host plants, root exudates of non-host plants have been tested under axenic and monoxenic conditions for their effect on AM fungi. Working with root organ cultures of three non-mycothrophic plants, *Brassica kaber*, *B. niger* and sugar beet and the mycotrophic carrot, Schreiner and Koide (1993a) reported that all roots "... stimulated germination and hyphal growth of *Glomus etunicatum* ... however, only roots of the mycothophic species (carrot) supported continued hyphal exploration ...".

Working with sugar beet (a member of the non-mycothrophic Chenopodiaceae) and carrot (mycothrophic) root organ cultures, Bécard and Piché (1990) found that roots of both plants stimulate hyphal growth through root volatiles, and suggested CO_2 as the active factor, whereas the effect of root exudates of the mycothrophic and non-mycothrophic plants seemed to differ. In their experiment, in contrast to carrot root exudates, sugar beet root exudates did not exhibit a stimulatory effect on hyphal growth. In recent studies, not only the lack of stimulatory compounds in root exudates of sugar beet but also the presence of inhibitory compounds have been suggested. In the presence of exudate from Ri T-DNA-transformed sugar beet roots, David-Schwartz et al. (2003) found a drastic inhibition of spore germination of *G. intraradices*, and Nagahashi and Douds (2000) reported an inhibition of hyphal tip growth of *Gi. gigantea* by sugar beet root exudates. The release of inhibitory compounds by members of the non-mycothrophic

Chenopodiaceae has been suggested previously in studies performed under soil conditions. Vierheilig et al. (1995) found that hyphal spreading through the rhizosphere of spinach plants was negatively affected, and recently it has been reported that the application of root exudates of sugar beet to cucumber plants inoculated with *G. mosseae* resulted in reduced root colonization (Vierheilig et al. 2003). The presence of compounds in spinach root inhibiting AM spore germination has been shown before (Vierheilig and Ocampo 1990).

Inhibitory compounds in root exudates also have been suggested for members of the Brassicaceae and for *Urtica dioica*, a member of the Urticaceae family. Observing the retraction of cytoplasm from the hyphal tip of *G. etunicatum* in proximity to transformed roots of *B. nigra*, Schreiner and Koide (1993a) suggested "... the involvement of inhibitory compounds in the rhizoplane interaction of *G. etunicatum* with the nonmycotrophs ...". This hypothesis was confirmed by experiments in soil using the filter "sandwich" method (Tommerup 1984), where spore germination of *G. intraradices* was inhibited in the presence of intact living roots of *B. kaber* and *B. nigra* (Schreiner and Koide 1993b), and under axenic conditions volatiles released by *B. oleraceae* inhibited spore germination of *G. mosseae* (El-Atrach et al. 1989). Moreover, in greenhouse experiments, spreading of *G. mosseae* through the rhizosphere of *B. napus* (Vierheilig et al. 1995) and *Urtica dioica* was reduced (Vierheilig et al. 1996), and the application of root exudates of *B. nigra* to cucumber plants inoculated with *G. mosseae* resulted in reduced root colonization (Vierheilig et al. 2003).

Lupins are exceptions in the mycorrhizal host family of the Leguminosae as they do not form the AM symbiosis (Avio et al. 1990). Several works on lupins have been performed with *Lupinus albus* (Gianinazzi-Pearson et al. 1989; Vierheilig et al. 1995, 2003), showing no signs of inhibitory compounds released by the roots. This was confirmed in a recent work by Oba et al. (2002) on the axenic growth of *Gi. margarita*, showing no negative effect of root exudates of *L. albus*. However, when testing root exudates of a number of other *Lupinus* species, it was found that root exudates of lupins in general do exhibit an inhibitory effect on hyphal growth.

To summarize, although there are still some contradictory reports, recent data from in vitro experiments point towards the presence of inhibitory compounds in root exudates of most AM non-host plants. However, this does not exclude that factors other than root exudates are involved in the determination of the non-host status of these plants.

Myc⁻ Plants

The rhizobia–legume symbiosis has been explored in detail using mutants which fail to form nodules, the so-called nod⁻ mutants. In AM symbioses, due to the obligate status of the fungal symbiont, the control exercised

by each symbiont is poorly understood. When compared to the rhizobia–legume interaction, the use of myc$^-$ mutants in AM research is just at the beginning. Several reports using myc$^-$ mutants (to cite only the earliest papers, Duc et al. 1989; Gianninazzi-Pearson et al. 1991) have shown the potential of plant mutants to give us new insight into the biology of the mycorrhizal symbiosis.

Several types of myc$^-$ mutants have been identified so far. The "early myc$^-$" or myc^{-1} mutants are characterized by an enhanced number of appressoria on the root surface and on aborted infection. The "late myc$^-$" or myc^{-2} show a normal root colonization pattern, but arbuscule formation is blocked (Gianinazzi-Pearson et al. 1991).

Most recently, mycorrhizal mutants from non-legume systems have been identified. So-called *pre*m*y*corrhizal *i*nfection (pmi) tomato mutants are resistant to colonization by single spores and colonized roots (David-Schwartz et al. 2001, 2003). Besides these, the colonization pattern of the "highly reduced mycorrhizal colonization" (rmc) tomato mutants in soil consists of the blockage of AM fungal penetration at root surface or outer root layers for species such as *G. intraradices*, whereas for other AM fungi species such as *G. versiforme*, colonization is established normally after a certain resistance from the root (Barker et al. 1998).

Working with pre-SMC of myc^{-1} peas, Balaji et al. (1995) reported that root volatiles from these mutants significantly stimulated *Gi. margarita* hyphal growth, whereas root exudates produced by both myc$^-$ mutants and wild-type peas inhibited hyphal growth. This is in contrast to a later report by Buee et al. (2000) where in axenic culture purified root exudates from of myc^{-1} and myc^{-2} roots exhibited a similar branching activity as root exudates of wild-type pea roots. These data are in agreement with those obtained in pot experiments. Giovannetti et al. (1993b) reported that root exudates of whole myc^{-1} pea plants had the same stimulatory effect on hyphal growth of *G. mosseae* as exudates from wild-type pea plants.

Interestingly, the myc$^-$ status of the pmi tomato mutants, apparently blocked in a very early event of their symbiotic potential, seems to be regulated differently (Fig. 1). Whereas volatiles of root organ cultures of pmi myc$^-$ tomato mutants and tomato wild-type plants exhibited a similar effect on hyphal growth (Gadkar et al. 2003) as that known from other host plants (Bécard et al. 1989; Bécard and Piché 1989a, b, 1990), root exudates of transformed and non-transformed pmi tomato mutants reduced spore germination of *G. intraradices* (David-Schwartz et al. 2003), and retarded the proliferation of *G. intraradices* in vitro (Gadkar et al. 2003). A preliminary characterization of the exudates with polyvinyl polypyrrolidone suggested that the inhibitory compounds are of phenolic nature.

In a very recent work, Bago et al. (2005) have compared the colonization features of the AM fungus *G. intraradices* growing monoxenically on

wild-type or rmc mutant tomato root organ cultures. When in monoxenic culture, the rmc phenotype showed an increased resistance to fungal penetration, which led the fungus to multiply "attempts" to penetrate. Only few of these attempts were successful, and in such cases the infection unit was rather spatially restricted compared to those formed in the wild type. Since fluorescence microscopy of the rmc root organs showed no signs of increase in cell wall thickness compared to the wild-type root organs, the authors suggested that some type of unknown barrier, other than physical, is involved in the blockage of root colonization by the rmc mutant.

To summarize, some AM non-host plants and the pmi myc$^-$ plants seem to share certain mechanisms affecting their susceptibility to AM fungi, at least at the pre-symbiotic phase of the symbiosis when signals are exchanged between the plant and the AM fungi. The data discussed above clearly show the importance of root exudates favourable to AM fungi for a successful establishment of the AM association.

3.2 AM Fungus-to-Plant Signals

First reports on the perception of AM fungi by the plant before root colonization date about 10 years back. Whereas Simoneau et al. (1994) reported the appearance of new polypeptides in Ri T-DNA-transformed tomato roots challenged with a spore extract of *G. intraradices*, Vierheilig et al. (1994) observed activity changes of two hydrolases, a β-1,3-glucanase and a chitinase, in roots of an AM fungi-inoculated non-host plant. The hypothesis of AM fungus-derived signals perceived by the plant prior to root colonization has been confirmed by recent studies reporting the accumulation of glucosinolates in a range of non-host Brassicaceae plants (Vierheilig et al. 2000), and alterations of the accumulation pattern of several flavonoids in alfalfa roots challenged with spores and hyphal fragments of *G. intraradices* (Larose et al. 2002).

Recently, by using pre-SMC of several AM fungi and *M. truncatula* which were physically separated, Kosuta et al. (2003) have shown that a diffusible factor from different AM fungi induces symbiosis-specific MtENOD11 expression in roots of *M. truncatula*. The diffusible AM fungal factor seems specific to AM fungi, as no MtENOD11 induction was observed with several fungal pathogens.

It is tempting to speculate on the function of changes promoted in roots of AM host plants by AM fungus-derived signals before root penetration. Larose et al. (2002) hypothesized that a more favourable environment for root penetration is created by the host through changes in the flavonoid

pattern in the presence of fungal signals. Further studies are needed to understand the early signalling events during the formation of the AM association. Possibly, the signal exchange cascade between AM fungi and their host plants is as complex as that between rhizobial bacteria and legumes (Vierheilig and Piche 2002), with every step being essential for the outcome of a functional symbiosis.

4 Symbiotic AM Fungal Growth

After reaching the host plant root, AM fungi grow on its surface, form appressoria, penetrate, and finally form intraradical structures such as intercellular hyphae, arbuscules and, in some cases, vesicles. The establishment of the symbiosis in AM non-host plants is stopped at the stage just before a direct contact between the plant and the fungus occurs. Neither hyphal growth on the root surface nor the formation of appressoria can be observed in transformed (Bécard and Piché 1990; Schreiner and Koide 1993a) or non-transformed roots of AM non-host plants (Giovannetti et al. 1993b; Vierheilig et al. 1994, 2000). However, both gene regulation (Yap and Schultze 2003) and the physiology (Vierheilig et al. 1994, 2000a) of the non-host plant are altered in the presence of AM fungi.

Using SMC, the development of the fungus after root penetration can be followed. After root colonization, runner hyphae spread through the in vitro agar media, forming at regular intervals so-called branched absorbing structures (BAS) and spores (Diop et al. 1992; Bécard et al. 1995; Bago et al. 1998a, b).

Further information on sporulation can be acquired from recent experiments in which the intra- and extraradical colonization features of rmc root organ growing monoxenically with *G. intraradices* were examined. As stated above, the AM fungus was able to penetrate and establish symbiosis with the rmc root organ, although the extent of such colonization was greatly reduced (Bago et al. 2005). Intraradical colonization structures in rmc (i.e. coils, arbuscules) appeared normal, as were extraradical structures such as runner hyphae and BAS. However, reflecting the decreased intraradical colonization extent, a reduction in extraradical mycelial development was evident. The authors suggested that a limitation in C uptake and/or translocation by the AM fungus could be at the basis of this, since one of the most significant effects observed was a dramatic reduction in sporulation by extraradical hyphae in rmc compared to wild-type cultures. Nevertheless, this possibility is still to be confirmed.

Interestingly, the hyphal and spore density of the extraradical mycelium is also affected by the presence of the root. When in SMC the extraradical

mycelium of *G. intraradices* is physically separated from the Ri T-DNA-transformed carrot root, hyphal density and spore density are more than doubled (St.-Arnaud et al. 1996), in the absence of sugar and root exudates. St.-Arnaud et al. (1996) suggested that root exudates from colonized roots inhibit extraradical hyphal growth and sporulation in the proximal compartment.

A modification of the bi-compartmental monoxenic system of St.-Arnaud et al. (1996), replacing the physical separation by a nylon screen (60-µm mesh size), thus restricting root growth to one compartment but allowing hyphal growth and the movement of root exudates in both compartments, resulted in a reduction of sporulation on the root-free side compared to sporulation in root-free compartments where root exudates from the Ri T-DNA carrot root were physically excluded (García-Garrido and Ocampo, pers. comm.). These data suggest a negative effect of root exudates of mycorrhizal roots on the sporulation of AM fungi, although the production of self-regulatory compounds by the extraradical mycelium could not be excluded. Filion et al. (1999) reported the presence of biologically active substances in the extraradical mycelium of *G. intraradices*.

In soil systems, alterations of the microbial population around mycorrhizal compared to non-mycorrhizal roots point towards a changed exudation pattern of mycorrhizal roots (Linderman and Paulitz 1990; Bansal and Mukerji 1994; Andrade et al. 1997; Vazquez et al. 2000; Marschner et al. 2001). Data from in vitro systems seem to confirm an altered root exudation of mycorrhizal plants. Root exudates collected from mycorrhizal strawberry plants have been shown to exhibit a reduced stimulatory effect on the sporulation of the fungal pathogen *Phytophthora fragariae*, compared to root exudates of non-mycorrhizal plants (Norman and Hooker 2000). Recently, it has been shown that root exudates collected from non-mycorrhizal tomato root organ cultures exhibit a higher attracting effect on zoospores of *Phytophthora parasitica* than root exudates from mycorrhizal tomato root organ cultures (Lioussanne et al. 2003). An inverse effect was observed with the chemotactic response of plant growth-promoting bacteria. Root exudates collected from non-mycorrhizal tomato plants exhibited a lower attracting effect on *Azobacter chroococum* and *Pseudomonas fluorescens* than exudates from mycorrhizal plants (Sood 2003).

Root exudates of mycorrhizal plants also show an altered effect on AM fungi. Root exudates collected from mycorrhizal cucumber plants exhibited a reduced stimulatory effect on the axenic growth of AM hyphae (Pinior et al. 1999), whereas in a soil system root exudates of mycorrhizal cucumber plants exhibited a suppressive effect on root colonization by AM fungi (Pinior et al. 1999; Vierheilig et al. 2003).

The importance of the altered effect of root exudates of non-mycorrhizal and mycorrhizal plants still remains unclear. It has been suggested recently

that an altered root exudation is possibly involved in the autoregulation of mycorrhization and the enhanced resistance of mycorrhizal plants towards soil-borne pathogens (Vierheilig and Piché 2002; Vierheilig 2004).

5 Conclusion

In this review, data obtained from soil, asymbiotic, pre-symbiotic and symbiotic in vitro AM cultures have been discussed. Such data show the great possibilities offered by the in vitro culturing approach to study different aspects during the formation of the symbiosis. In many cases, data obtained with in vitro systems have been confirmed or do confirm results obtained in non-axenic experimental systems. However, besides a huge potential to answer questions on asymbiotic and pre-symbiotic events, few data on symbiotic events from in vitro systems are available as yet. Although somehow artificial, it is obvious that in vitro cultures offer new avenues in our understanding of the plant–AM fungus dialogue leading to AM establishment and functioning.

Acknowledgements. Special thanks go to Maria Jesus Delgado and Paco Lara for hosting H.V. during his working stay in Granada, Spain.

References

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ (1997) Bacteria from rhizosphere and hyphosphere soils of different arbuscular mycorrhizal fungi. Plant Soil 192:71–79

Avio L, Sbrana C, Giovannetti M (1990) The response of different species of *Lupinus* to VAM endophytes. Symbiosis 9:321–323

Azcón-Aguilar C, Bago B, Barea JM (1999) Saprophytic growth of arbuscular mycorrhizal fungi In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology and biotechnology. Springer, Berlin Heidelberg New York, pp 391–407

Bago B, Vierheilig H, Piché Y Azcón-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bago B, Azcón-Aguilar C, Piché Y (1998a) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bago B, Azcón-Aguilar C, Goulet A, Piché Y (1998b) Branched absorbing structures (BAS): a feature of the extraradical mycelium of the symbiotic arbuscular mycorrhizal fungi. New Phytol 139:375–388

Bago B, Pfeffer PE, Douds DD Jr, Brouillette J, Bécard G, Shachar-Hill Y (1999) Carbon metabolism in arbuscular mycorrhizal spores as revealed by NMR spectroscopy. Plant Physiol 121:263–271

Bago B, Shachar-Hill Y, Pfeffer PE (2000) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 124:949–957

Bago B, Dickson S, Cano C, Barea JM, Smith SE (2005) Interactions between wildtype and mycorrhiza-defective mutant tomato non-transformed roots with *Glomus intraradices* in monoxenic cultures. New Phytol (in press)

Balaji B, Poulin MJ, Vierheilig H, Piché Y (1995) Responses of an arbuscular mycorrhizal fungus, *Gigaspora margarita*, to exudates and volatiles from the Ri T-DNA-transformed roots of nonmycorrhizal and mycorrhizal mutants of *Pisum sativum* L. sparkle. Exp Mycol 19:275–283

Bansal M, Mukerji KG (1994) Positive correlation between VAM-induced chances in root exudation and mycorrhizosphere mycoflora. Mycorrhiza 5:39–44

Baptista MJ, Siqueira JO (1994) Efeito de flavonóides na germinacão de esporos e crescimento assimbiotico do fungo micorrízico arbuscular *Gigaspora gigantea*. Rev Bras Fisiol Veg 6:127–134

Barker SJ, Stummer B, Gao L, Dispain I, O'Connor TJ, Smith SE (1998) A mutant in *Lycopersicon esculentum* Mill. with highly reduced VA mycorrhizal colonization: isolation and preliminary characterisation. Plant J 15:791–797

Bécard G, Piché Y (1989a) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Bécard G, Piché Y (1989b) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 112:77–83

Bécard G, Piché Y (1990) Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and nonhost Ri T-DNA transformed roots. Can J Bot 68:1260–1264

Bécard G, Piché Y, Fortin AJ (1989) Some aspects on the biotrophy of VAM fungi. Agric Ecosyst Environ 29:29–33

Bécard G, Douds DD, Pfeffer PE (1992) Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of $CO_2$and flavonols. Appl Environ Microbiol 58:821–825

Bécard G, Taylor LP, Douds DD, Pfeffer PE, Doner LW (1995) Flavonoids are not necessary plant signal compounds in arbuscular mycorrhizal symbiosis. Mol Plant Microb Interact 8:252–258

Buee M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant Microb Interact 13:693–698

Chabot S, Bel-Rhlid R, Chênevert R, Piché Y (1992) Hyphal growth promotion in vitro of the VA mycorrhizal fungus, *Gigaspora margarita* Becker & Hall, by the activity of structurally specific flavonoids compounds under CO_2-enriched conditions. New Phytol 122:461–467

David-Schwartz R, Badani H, Wininger S, Levy AA, Galili G, Kapulnik Y (2001) Identification of a novel genetically controlled step in mycorrhizal colonization: plant resistance to infection by fungal spores but not extraradical hyphae. Plant J 27:561–569

David-Schwartz R, Gadkar V, Wininger S, Bendov R, Galili G, Levy AA, Kapulnik Y (2003) Isolation of a premycorrhizal infection (pmi2) mutant of tomato, resistant to arbuscular mycorrhizal fungal colonization. Mol Plant Microb Interact 16:382–388

Diop TA, Bécard G, Piché Y (1992) Long-term in vitro culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. Symbiosis 12:249–259

Douds DD, Nagahashi G (2000) Signaling and recognition events prior to colonization of roots by arbuscular mycorrhizal fungi. In: Podila GK, Douds DD (eds) Current advances in mycorrhizae research. APS Press, St. Paul, Minnesota, pp 11–18

Duc G, Trouvelot A, Gianinazzi-Pearson V, Gianinazzi S (1989) First report of non-mycorrhizal plant mutants (Myc-) obtained in pea (*Pisum sativum* L. and fababean (*Vicia faba* L.) Plant Sci 60:215–222

El-Atrach F, Vierheilig H, Ocampo JA (1989) Influence of non-host plants on vesicular-arbuscular mycorrhizal infection of host plants and on spore germination. Soil Biol Biochem 21:161–163

Elias KS, Safir GR (1987) Hyphal elongation of *Glomus fasciculatus* in response to root exudates. Appl Environ Microbiol 53:1928–1933

Elsen A, Declerck S, De Waele D (2001) Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. Mycorrhiza 11:49–51

Elsen A, Declerck S, De Waele D (2003) Use of root organ cultures to investigate the interaction between *Glomus intraradices* and *Pratylenchus coffeae*. Appl Environ Microbiol 69:4308–4311

Filion M, St.-Arnaud M, Fortin JA (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytol 141:525–533

Fortin JA, Bécard G, Declerck S, Dalpé Y, St.-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Gadkar V, David-Schwartz R, Nagahashi G, Douds DD, Wininger S, Kapulnik Y (2003a) Root exudate of *pmi* tomato mutant M161 reduces AM fungal proliferation in vitro. FEMS Microbiol Lett 223:193–198

Gadkar V, David-Schwartz R, Kunik T, Kapulnik Y (2003b) Arbuscular mycorrhizal fungal colonization. Factors involved in host recognition. Plant Physiol 127:1493–1499

Gemma J, Koske R (1988) Seasonal variation in spore abundance and dormancy of *Gigaspora gigantea* and in mycorrhizal inoculum potential of a dune soil. Mycologia 80:211–216

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243–255

Gianinazzi-Pearson V, Gianinazzi S, Guillemin JP, Trouvelot A, Duc G (1991) Genetic and cellular analysis of resistance to vesicular arbuscular (VA) mycorrhizal fungi in pea mutants. In: Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions. Kluwer, Dordrecht, pp 336–342

Giovannetti M (2000) Spore germination and pre-symbiotic mycelial growth. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, pp 47–68

Giovannetti M, Sbrana C (1998) Meeting a non-host: the behaviour of AM fungi. Mycorrhiza 8:123–130

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993a) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytol 125:587–594

Giovannetti M, Avio L, Sbrana C, Citernesi AS (1993b) Factors affecting appressorium development in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. New Phytol 123:115–122

Giovannetti M, Sbrana C, Logi C (1994) Early processes involved in host recognition by arbuscular mycorrhizal fungi. New Phytol 127:703–709

Graham JH (1982) Effect of citrus exudates on germination of chlamydospores of the vesicular-arbuscular mycorrhizal fungus, *Glomus epigaeum*. Mycologia 74:831–835

Green NE, Graham SO, Schenck NC (1976) The influence of pH on the germination of vesicular-arbuscular mycorrhizal spores. Mycologia 68:929–934

Hepper CM (1984) Regulation of spore germination of the vesicular-arbuscular mycorrhizal fungus *Acaulospora laevis* by soil pH. Trans Br Mycol Soc 83:154–156

Hepper C, Smith G (1976) Observations on the germination of *Endogone* spores. Trans Br Mycol Soc 66:189–219

Juge C, Samson J, Bastien C, Vierheilig H, Piché Y (2002) Breaking spore dormancy of the AM fungus *Glomus intraradices*. Mycorrhiza 12:37–42

Kape R, Wex K, Parniske M, Goerge E, Wetzel A, Werner D (1992) Legume root metabolites and VA-mycorrhiza development. J Plant Physiol 141:54–60

Kosuta S, Chabaud M, Lougnon G, Gough C, Dénarié J, Barker DG, Bécard G (2003) A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots of *Medicago truncatula*. Plant Physiol 131:952–962

Kues U, Granado JD, Hermann R, Boulianne RP, Kertesz-Chaloupkova K, Aebi M (1998) The A mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. Mol Gen Genet 260:81–91

Larose G, Chenevert R, Moutoglis P, Gagne S, Piché Y, Vierheilig H (2002) Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. J Plant Physiol 159:1329–1339

Le Tacon F, Skinner FA, Mosse B (1983) Spore germination and hyphal growth of a vesicular-arbuscular mycorrhizal fungus, *Glomus mosseae* (Gerdeman and Trappe), under decreased oxygen and increased carbon dioxide concentrations. Can J Microbiol 29:1280–1285

Linderman RG, Paulitz TC (1990) Mycorrhizal-rhizobacterial interactions. In: Hornby D, Cook RJ, Henis Y, Ko WH, Rovira AD, Schippers B, Scott PR (eds) Biological control of soil-borne plant pathogens. CAB International, Wallingford, pp 261–283

Lioussanne L, Jolicoeur M, St. Arnaud M (2003) Effects of the alteration of tomato root exudation by *Glomus intraradices* colonization on *Phytophthora parasitica* var. *Nicotianae* zoospores. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Marschner P, Crowley DE, Lieberei R (2001) Arbuscular mycorrhizal infection changes the bacterial 16S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11:297–302

Morandi D (1996) Occurrence of phytoalexins and phenolic compounds on endomycorrhizal interactions, and their potential role in biological control. Plant Soil 185:241–251

Morandi D, Branzanti B, Gianinazzi-Pearson V (1992) Effect of some plant flavonoids on in vitro behaviour of an arbuscular mycorrhizal fungus. Agronomie 12:811–816

Morris PF, Ward EWB (1992) Chemoattraction of zoospores of the soybean pathogen, *Phytophthora sojae* by isoflavones. Physiol Mol Plant Pathol 40:17–22

Mosse B (1988) Some studies relating to "independent" growth of vesicular-arbuscular endophytes. Can J Bot 66:2533–2540

Mosse B, Hepper CM (1975) Vesicular-arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Nagahashi G, Douds DD (1999) Rapid and sensitive bioassay to study signals between root exudates and arbuscular mycorrhizal fungi. Biotechnol Tech 13:893–897

Nagahashi G, Douds DD (2000) Partial separation of root exudate components and their effects upon the growth of germinated spores of AM fungi. Mycol Res 104:1453–1464

Nagahashi G, Douds DD (2003) Action spectrum for the induction of hyphal branches of an arbuscular mycorrhizal fungus: exposure sites versus branching sites. Mycol Res 107:1075–1082

Nagahashi G, Douds DD, Abney GD (1996) Phosphorus amendment inhibits hyphal branching of the VAM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation. Mycorrhiza 6:403–408

Nagahashi G, Douds DD, Buee M (2000) Light-induced hyphal branching of germinated AM fungal spores. Plant Soil 219:71–79

Nair MG, Safir GR, Siqueira JO (1991) Isolation and identification of vesicular-arbuscular mycorrhiza stimulatory compounds from clover (*Trifolium repens*) roots. Appl Environ Microbiol 57:434–439

Norman JR, Hooker JE (2000) Sporulation of *Phytophthora fragariae* shows greater stimulation by exudates of non-mycorrhizal than by mycorrhizal strawberry roots. Mycol Res 104:1069–1073

Oba H, Tawaraya K, Wagatsuma T (2002) Inhibition of pre-symbiotic hyphal growth of arbuscular mycorrhizal fungus *Gigaspora margarita* by root exudates of *Lupinus* spp. Soil Sci Plant Nutr 48:117–120

Phillips DA, Tsai SM (1992) Flavonoids as plant signals to rhizosphere microbes. Mycorrhiza 1:55–58

Pinior A, Wyss U, Piché Y, Vierheilig H (1999) Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. Can J Bot 77:891–897

Poulin MJ, Bel-Rhlid R, Piche Y, Chenevert R (1993) Flavonoids released by carrot (*Daucus carota*) seedlings stimulate hyphal development of vesicular-arbuscular mycorrhizal fungi in the presence of optimal CO_2 enrichment. J Chem Ecol 10:2317–2327

Powell CL (1976) Development of mycorrhizal infection from *Endogone* spores and infected root fragments. Trans Br Mycol Soc 66:439–445

Safir GR (1986) VA Mycorrhizae: an ecophysiological approach. In: Safir GR (ed) Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton, pp 1–3

Schenck NC, Graham SO, Green NE (1975) Temperature and light effect on contamination and spore germination of vesicular-arbuscular mycorrhizal fungi. Mycologia 67:1189–1192

Schreiner RP, Koide RT (1993a) Stimulation of vesicular-arbuscular mycorrhizal fungi by mycothophic and nonmycotrophic plant root systems. Appl Environ Microbiol 59:2750–2752

Schreiner RP, Koide RT (1993b) Mustards, mustard oils and mycorrhizas. New Phytol 123:107–113

Simoneau P, Louisy-Louis N, Plenchette C, Strullu DG (1994) Accumulation of new polypeptides in Ri-T-DNA-transformed roots of tomato (*Lycopersicon esculentum*) during the development of vesicular-arbuscular mycorrhizae. Appl Environ Microbiol 60:1810–1813

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, London

Sood SG (2003) Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiol Ecol 45:219–227

St.-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995) Altered growth of *Fusarium oxysporum* f.sp. *chrysanthemi* in an in vitro dual culture system with the vesicular arbuscular mycorrhizal fungus *Glomus intraradices* growing on *Daucus carota* transformed roots. Mycorrhiza 5:431–438

St.-Arnaud M, Hamel C, Vimard B, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332

Straney D, Khan R, Tan R, Bagga S (2002) Host recognition by pathogenic fungi through plant flavonoids. In: Buslig B, Manthey JA, Tyler BM (eds) Flavonoids in cell functions. Kluwer, New York, pp 9–22

Tamasloukht MB, Séjalon-Delmas N, Kluever A, Jauneau A, Roux C, Bécard G, Franken P (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. Plant Physiol 131:1468–1478

Tawaraya K, Watanabe S, Yoshida E, Wagatsuma T (1996) Effect of onion (*Allium cepa*) root exudates on the hyphal growth of *Gigaspora margarita*. Mycorrhiza 6:57–59

Tommerup IC (1983) Temperature relations of spore germination and hyphal growth of vesicular-arbuscular mycorrhizal fungi. Trans Br Mycol Soc 81:381–384

Tommerup IC (1984) Development of infection by a vesicular-arbuscular mycorrhizal fungus in *Brassica napus* L. and *Trifolium subterraneum* L. New Phytol 98:487–495

Tsai SM, Phillips DA (1991) Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl Environ Microbiol 57:1485–1488

Tyler BM, Wu MH, Wang JM, Cheung W, Morris PF (1996) Chemotactic preferences and strain variation in the response of *Phytophthora sojae* zoospores to host isoflavones. Appl Environ Microbiol 62:2811–2817

Vazquez MM, Cesar S, Azcon R, Barea JM (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 15:261–272

Vierheilig H (2004) Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. Can J Bot 82(8):1166–1176

Vierheilig H, Piché Y (2002) Signalling in arbuscular mycorrhiza: facts and hypotheses. In: Buslig B, Manthey J (eds) Flavonoids in cell functions. Kluwer, New York, pp 23–39

Vierheilig H, Alt M, Mohr U, Boller T, Wiemken A (1994) Ethylene biosynthesis and activities of chitinase and β-1,3-glucanase in the roots of host and non-host plants of vesicular-arbuscular mycorrhizal fungi after inoculation with *Glomus mosseae*. J Plant Physiol 143:337–343

Vierheilig H, Alt M, Mäder P, Boller T, Wiemken A (1995) Spreading of *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus, across the rhizosphere of host and non-host plants. Soil Biol Biochem 27:1113–1115

Vierheilig H, Iseli B, Alt M, Raikhel N, Wiemken A, Boller T (1996) Resistance of *Urtica dioica* to mycorrhizal colonization: a possible involvement of *Urtica dioica* agglutinin. Plant Soil 183:131–136

Vierheilig H, Bago B, Albrecht C, Poulin MP, Piché Y (1998) Flavonoids and arbuscular-mycorrhizal fungi. In: Manthey JA, Buslig BS (eds) Flavonoids in the living system. Plenum, New York, pp 9–33

Vierheilig H, Bennett R, Kiddle G, Kaldorf M, Ludwig-Müller J (2000) Differences in glucosinolate patterns and arbuscular mycorrhizal status of glucosinolate-containing plant species. New Phytol 146:343–352

Vierheilig H, Lerat S, Piché Y (2003) Systemic inhibition of arbuscular mycorrhiza development by root exudates of cucumber plants colonized by *G. mosseae*. Mycorrhiza 13:167–170

Villegas J, Fortin JA (2001) Phosphorus solubilization and pH changes as a results of the interactions between soil bacteria and arbuscular mycorrhizal fungi on a medium containing NH_4^+ as nitrogen source. Can J Bot 79:865–870

Villegas J, Fortin JA (2002) Phosphorus solubilization and pH changes as a results of the interactions between soil bacteria and arbuscular mycorrhizal fungi on a medium containing NO_3^- as nitrogen source. Can J Bot 80:571–576

Williams PG (1992) Axenic culture of arbuscular mycorrhizal fungi. Methods Microbiol 24:203–220

Yap HL, Schultze M (2003) Responses of the non-host plant, *A. thaliana*, to arbuscular mycorrhizal fungi. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Yokosawa R, Kunninaga S, Sekizaki H (1986) *Aphanomyces euteiches* zoospore attractant isolated from pea root prunetin. Ann Phytopathol Soc Jpn 52:809–816

9 Carbon Metabolism, Lipid Composition and Metabolism in Arbuscular Mycorrhizal Fungi

Anne Grandmougin-Ferjani[1], Joël Fontaine[1], Roger Durand[1]

1 Introduction

Arbuscular mycorrhiza (AM) is a compatible interaction between plant roots and fungi (Glomeromycota), and the most widespread symbiosis of land plants in all terrestrial ecosystems. This mutualistic interaction has important implications for plant nutrition, plant health and vegetation dynamics (Smith and Read 1997). The interaction between both symbionts at the cellular, biochemical and molecular levels has received increased interest, with the development of a range of molecular and physiological microtechniques allowing investigations of symbiotic processes at a more and more refined scale (Franken 1999; van Buuren et al. 2000; Bago et al. 2002a; Gollotte et al. 2002). However, obtaining information on the physiology of the two partners of the symbiosis is difficult for technical reasons. The fungus is obligatory biotrophic, and therefore necessitates the presence of the plant partner to fulfil its life cycle, and only small amounts of fungal material is available for analysis [spores, extraradical mycelium (ERM), intraradical mycelium (IRM)]. In addition, the conventional pot culture production of ERM and IRM cannot ascertain the absence of undesirable microbial contaminants, neither in the rhizosphere nor in the host root. In recent decades, the development of monoxenic culture systems (see review by Fortin et al. 2002) and the successful maintenance of tens of strains belonging to most genera have greatly influenced our understanding of the AM symbiosis. In this chapter, we will review the major findings made with monoxenic culture systems in fungal physiology and metabolism, and the differential expression of genes in IRM and ERM.

[1]Laboratoire de Mycologie/Phytopathologie/Environnement, Université du Littoral Côte d'Opale, 17 Avenue Blériot, B.P. 699, 62228 Calais, France, Tel.: +33-3-21964807, Fax: +33-3-21347113, E-mail: grand@univ-littoral.fr

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

2
Sugar Metabolism in AMF

It has been estimated that up to 20% of the photosynthetically fixed carbon would be transferred from the plant to the AM fungi (Jakobsen and Rosendahl 1990). The biochemical nature of the transferred form was determined by ^{13}C nuclear magnetic resonance (NMR) spectroscopy. AM fungus-colonized leek roots were perfused in a solution containing $^{13}C_1$ glucose (Bago et al. 2000). NMR spectra indicated the transformation of glucose by the fungus into glycogen and trehalose. Isolated arbuscules were also shown to use glucose for respiration (Solaiman and Saito 1997). NMR spectroscopy of AM monoxenic cultures revealed information on the sugar fluxes between the AM partners when using cultures grown in divided Petri plates, following the model of St Arnaud et al. (1996). With such systems, the isotopically labelled substrates could be added either to the colonized roots or to the extramatrical hyphae in physically separated compartments. The labelling patterns of each of these samples revealed the carbon metabolic pathways in IRM and ERM (Bago et al. 2000). Intraradical hyphae rapidly assimilated hexose but extraradical hyphae appeared unable to take up sugars (Douds et al. 2000; Bago et al. 2000). Such a strong duality between IRM and ERM implies an efficient uptake of plant-derived hexose, the conversion to typical fungal storage forms, i.e. trehalose and glycogen, and active translocation processes to the ERM (Bago et al. 2000). The labelling experiments were consistent with the enzymatic determinations and molecular techniques, all indicating the functioning of glycolysis, tricarboxylic acid cycle and the pentose phosphate pathway in intraradical structures, whereas a substantial gluconeogenic flux fuelled by the glyoxylate cycle is present in ERM (reviewed by Bago et al. 2000, 2002b). Using the lipid-specific fluorochrome Nile Red, Bago et al. (2002a, c) analysed the flux of triacylglycerides from IRM to ERM, postulating that these neutral lipids were the main products fuelling the metabolism of extraradical hyphae.

The understanding of how the AM symbiosis functions is a key issue in plant development and physiology. However, molecular analyses of the fungus in the symbiotic stages of development were hampered by technical limitations to obtain fungal material, particularly when located in roots. Nevertheless, the combination of advanced molecular techniques and monoxenic cultures of AM fungi with transformed roots has resulted in the recent identification of a number of genes involved in AM functioning (van Buuren et al. 2000; Burleigh 2001; Franken and Requena 2001; Golotte et al. 2002; Delp et al. 2003). Cloning of the symbiosis genes was obtained by targeted approaches investigating molecules with known gene sequences and protein functions. The fungal genes

isolated to date range from nutritionally to morphologically important genes (review in Harrier 2001; Golotte et al. 2002). More recently, non-targeted approaches have been used to isolate AM fungal genes. They include differential display, differential screening, and large-scale EST sequencing (Gollotte et al. 2002). The use of mycorrhizal defective mutants and the application of high-density array technology are promising tools to isolate genes involved in the different steps of plant–AM interactions.

Concerning genes involved in carbon metabolism and transport, the nature of the plant transporters or fungal transporters involved in the movement of glucose from the cortical cells to the interface compartment and to arbuscules and hyphae are still unknown. A gene encoding a transmembrane sugar transporter (*Mtst1*) was cloned from mycorrhizal roots of *Medicago truncatula*. This transporter was designed as a hexose transporter by activity measured in yeast (Harrison 1996). In situ hybridization showed that the sugar transporter transcripts were induced in cells containing arbuscules and in adjacent cells, suggesting that the gene may be active in the process of hexose transport towards colonized plant cells. A phosphoglycerate kinase (PGK) cDNA was isolated from tomato mycorrhizas by differential display (Harrier et al. 1998). Quantitative immunoblotting using a polyclonal antibody specific for the *G. mossae* PGK protein revealed a significantly higher accumulation of the protein during symbiosis compared with presymbiotic development (Harrier and Sawczak 2000). These results suggested that there is a differential regulation of fungal genes during symbiosis. Analysis of the *Gmpgk* fungal promoter showed several regulatory elements homologous to carbon source-controlled upstream activating elements from *Saccharomyces cerevisiae* (Harrier 2001).

AM fungi could cause changes in the regulation of plant genes in roots. Ravnsknov et al. (2003) demonstrated a higher gene expression of both *Sus1* and *Sh1*, the two isoforms of sucrose synthase cleaving sucrose into hexoses, in maize roots colonized by different AM fungal isolates. Higher sucrose synthase gene expression was not related to the concentrations of sucrose, reducing sugars or starch in the root tissue whereas increasing soil phosphorus concentrations decreased this gene expression. The higher gene expression of gene coding for sucrose synthase in AM roots was measured during the earliest phase of root colonization by fungal isolates.

3 Monoxenic Cultivation Techniques as a Tool for the Establishment of the Lipid Composition of AM Fungi

It is obvious that the knowledge of AM fungi lipid composition was a prerequisite for studies on lipid metabolism in AM fungi. Likewise, the biosynthetic lipid pathways could not be analysed as long as the composition of the final products was unknown. The monoxenic culture system offers several advantages for the investigation of the lipid profile of AM fungi. First, the production of these fungi is contaminant-free. Mycoparasites or saprophytic fungal organisms were described in the cell wall of AM fungi (Jeffries and Young 1994; Rousseau et al. 1996) and inside surface sterilized healthy spores isolated from pot cultures (Hijri et al. 2002). Secondly, monoxenic cultures allow the substantial production of fungal biomass that is quick and easy to harvest and permit different biochemical analyses. Finally, the use of divided Petri plates allows the production of extraradical mycelium physically separated from the mycorrhizal roots (St Arnaud et al. 1996), therefore, without interference with the latter.

AM fungi are characterized by thick-walled spores where lipid droplets are predominant in the cytoplasm (Cooper and Lösel 1974). Ultrastructural microscopy (Sward 1981) and biochemical studies confirmed that lipids constitute up to 45% of the dry mass (Beilby 1980; Beilby and Kidby 1980; Jabaji-Hare 1984). Many lipid analyses were conducted on AM fungi isolated from plant roots grown in pot cultures. Relative proportions of lipid classes were determined (Beilby and Kidby 1980; Jabaji-Hare 1984, 1988; Gaspar and Pollero 1994; Gaspar et al. 1994). Total fatty acid (FA) composition of AM fungal spores, auxiliary cells and vesicles were measured (Beilby 1980; Beilby and Kidby 1980; Jabaji-Hare 1984; Sancholle and Dalpé 1993; Gaspar and Pollero 1994; Gaspar et al. 1994; Bentivenga and Morton 1994; Graham et al. 1995; Bentivenga and Morton 1996; Grandmougin-Ferjani et al. 1997; Jansa et al. 1999; Madan et al. 2002). FA profiles from different polar and neutral lipid fractions, such as phospholipids (PL), glycolipid and sphingolipid, mono-, di-, triacylglycerols (TAG), free FA (FFA) and sterol ester FA were also determined (Beilby and Kidby 1980; Jabaji-Hare 1984, 1988; Olsson et al. 1995; Olsson 1999). Few sterol analyses of different species of Glomales were realized (Beilby 1980; Beilby and Kidby 1980; Grandmougin-Ferjani et al. 1999). A recent review has assembled information available on the lipids of mycorrhizal associations and on the possible implication of these substances in mycorrhizal associations (Sancholle et al. 2001).

To date, very few lipid analyses have been performed with AM fungi propagated in monoxenic cultures. Here, we present the first comparative

lipid analyses of AM fungi produced under monoxenic culture and in vivo conditions, with *Glomus intraradices*, the unique AM fungus which is available for lipid data, both from mycelium obtained from pot and monoxenic culture.

3.1 Lipid Classes of *Glomus Intraradices*

Relative proportions of individual lipid classes were reported in intraradical spores isolated from pot cultures by Jabaji-Hare et al. (1988). The most abundant lipid class was TAG (78%), followed by FFA (11%). Major lipid classes of *G. intraradices* extraradical hyphae and spores produced under monoxenic culture conditions were also reported as TAG (57%) and FFA (26%; Fontaine et al. 2001a). PL represented a minor fraction of lipids, about 2% of the total present in spores isolated from roots grown in pot culture or in monoxenic culture.

Radiolabelling experiments using (1-^{14}C) sodium acetate as a lipid precursor were performed with *G. intraradices* grown in monoxenic culture under different experimental conditions, i.e. fungus attached to the host plants, fungus separated from the roots, and at germinating stage (Fontaine et al. 2001a). It was concluded that the fungus in all three stages was able to synthesize de novo its own lipid classes: 1,2- and 1,3-DAG, TAG, PL, sterols and FFA.

3.2 Total FA Profiles of *G. Intraradices*

Table 1 shows the comparison between the FA compositions obtained from pot and monoxenic cultures of *G. intraradices*. The major FA in the 12 strains analysed, except for DAOM 184739 (Sancholle and Dalpé 1993), was the C16:1ω5 (Graham et al. 1995; Fontaine 2001). This FA occurs widely in other species of AM fungi (Jabaji-Hare 1988; Graham et al. 1995; Olsson et al. 1995; Grandmougin-Ferjani et al. 1997; Jansa et al. 1999; Madan et al. 1999). C16:0 is the second most-abundant FA, and a significant percentage of C18:1 was present in the different isolates. A highly similar FA composition for different isolates of *G. intraradices* grown in pot culture and under monoxenic culture conditions was shown. The FA profiles of fungal isolates grown on plant species transformed by *Agrobacterium rhizogenes* or not were similar, suggesting that plant host is not a significant factor in modifying the FA profile of AM fungi.

Bentivenga and Morton (1994) demonstrated the stability of the FA profile of one species subjected to different conditions of growth, except with

Table 1. Fatty acid profiles of total lipid extract of *Glomus intraradices* spores isolated from pot or monoxenic cultures

Isolates of *G. intraradices*	Type of cultures	Root host	14:0	14:1	16:0	16:1	16:1 ω5	18:0	18:1	18:2	18:3	20:3	20:4	20:5	22:0	References
DAOM 184739[a]	Pot culture	Leek	3.13	–	32.96	16.33	–	18.44	16.47	3.4	–	–	–	9.25	–	Sancholle and Dalpé (1993)
INVAM AU109-1[b]	Pot culture	Sudan grass	–	–	22.5	–	68.6	–	2.2	–	–	–	–	–	–	Graham et al. (1995)
INVAM FL208-2	Pot culture	Sudan grass	–	–	12.5	–	80	–	1.1	–	–	–	1	–	–	Graham et al. (1995)
INVAM SW101-1	Pot culture	Sudan grass	–	–	10	–	77	–	4.4	–	–	–	–	–	–	Graham et al. (1995)
INVAM SW103-1	Pot culture	Sudan grass	–	–	12.5	–	77	–	4.4	–	–	–	–	–	–	Graham et al. (1995)
INVAM UT118-2	Pot culture	Sudan grass	–	–	15	–	62.5	–	4.9	–	–	–	–	–	–	Graham et al. (1995)
INVAM UT126-1	Pot culture	Sudan grass	–	–	12.5	–	74.3	–	5.4	–	–	–	–	–	–	Graham et al. (1995)
INVAM UT143	Pot culture	Sudan grass	–	–	18.8	–	68.6	–	4.4	–	–	–	–	–	–	Graham et al. (1995)
INVAM UT176	Pot culture	Sudan grass	–	–	20	–	74.3	–	2.2	–	–	–	–	–	–	Graham et al. (1995)
INVAMWV994-1	Pot culture	Sudan grass	–	–	11.2	–	80	–	1.8	–	–	–	1	–	–	Graham et al. (1995)
DAOM 197198	Pot culture, young spores	Leek	–	–	32	–	47	14	7	–	–	–	–	–	–	Grandmougin-Ferjani et al. (1997)
DAOM 197198	Pot culture, mature spores	Leek	1	1	30	–	11	13	38	4	1	–	–	–	–	Grandmougin-Ferjani et al. (1997)
DAOM 197198	In vitro	Carrot	Tr[c]	–	9	–	76	1	5	4	1	1	1	1	1	Fontaine (2001)

[a] DAOM, National Mycological Herbarium, Agriculture Canada, Ottawa, Ontario
[b] INVAM, International Culture Collection of Arbuscular and Vesicular-Arbuscular Fungi
[c] Tr, traces

spores with a long period of storage. Apparently healthy spores may harbour different parasites, which cause differences in FA analysis. In this context, monoxenic cultures appear of particular interest. Each fungal sample must not necessarily be examined closely for signs of hyperparasitism before the analysis. Different authors (Jabaji-Hare et al. 1988; Grandmougin-Ferjani et al. 1997) detected changes due to spore age in the FA profile, whereas Olsson and Johansen (2000) detected no large differences between 1- and 3-month-old mycelium. Studies performed with monoxenic cultures could highlight the correlation between lipid analysis and the stage of growth of the cultures.

Very few analyses were performed with other species of AM fungi produced under monoxenic culture. The total FA profile of a recent species described, *Glomus proliferum* (Declerck et al. 2000), was published. The same major FA were found – C16:0 and C16:1ω5 represented respectively 34 and 41% of the total content.

3.3 FA Profiles of *G. Intraradices* After Separation of Different Lipid Fractions

Total lipid extracts could be fractionated by chromatography (thin layer chromatography, silica column). Different lipid fractions were obtained depending of the method employed.

3.3.1 Phospholipid FA

Table 2 shows major FA in the PL fraction: C16:0, C16:1ω5, C18:0 and C18:1. FA PL presented significant differences between *G. intraradices* grown in monoxenic cultures and in pot cultures. Higher proportions of 18:0 were detected in monoxenic cultures. The presence of C18:2, the dominant FA of saprophytic fungi (Larsen et al. 1998), was detected for *G. intraradices* grown in pot cultures. Surprisingly, this FA was also present in mycelia obtained from monoxenic cultures. So, this excluded a saprophytic fungal origin. Nevertheless, C16:1ω7 detected in pot culture was missing in monoxenic cultures, implying that this FA could have originated from some contaminant micro-organisms of AM fungi in pot cultures.

Table 2. Data on phospholipid fatty acid profiles of *Glomus intraradices* grown in pot culture and in monoxenic culture

Isolates of *G. intraradices*	Type of cultures	Structure of fungus analysed	15:0	16:0	16:1 ω5	16:1 ω7	18:0	18:1	18:2	18:3	20:0	20:2	20:4	20:5	References
Gift of Dr. G. Safir	Pot culture	External hyphae	–	28.4	24.6	1.6	–	32.8	–	–	–	–	0.6	6.6	Johansen et al. (1996)
Isolate 28a from Dr. S. Rosendahl	Pot culture	Mycelium	1	34	15.6	6.2	1	9.9	2	2	–	–	2	5.2	Larsen et al. (1998)
BEG 87[a]	Pot culture	Hyphae	–	42.8	18.2	1.1	3.5	21.1	1.3	–	–	8.2	–	–	Olsson and Johansen (2000)
BEG 87	Pot culture	Spores	–	50.7	12.7	2.3	6.3	20.4	5	–	–	0.9	–	–	Olsson and Johansen (2000)
DAOM 197198[b]	In vitro culture	Mycelium	–	14	13	–	21	16	10	1	–	–	3	–	Fontaine (2001)
Not provided	In vitro culture	Mycelium in free-P medium	–	31	23.2	–	11.4	21.3	1.8	–	11.2	–	–	–	Olsson et al. (2002)
Not provided	In vitro culture	Mycelium in high-P medium	–	40	14.7	–	18.1	20	–	–	7.1	–	–	–	Olsson et al. (2002)

[a] BEG, The International Bank for the Glomeromycota
[b] DAOM, National Mycological Herbarium, Agriculture Canada, Ottawa, Ontario

3.3.2 Neutral Lipid FA

This fraction contains lipids of storage as main lipids. TAG are the major type of neutral lipids found in large amounts in spores and vesicles. These lipids are enriched in 16-carbon FA as compared to PL (Tables 2 and 3). C16 FA represent 75–95% of the FA profile. Homogeneity of FA composition was observed between the lipid analyses obtained from mycelia grown in pot cultures and in monoxenic cultures.

There are few reports detailing the FA content of neutral lipid fractions: mono-, di-, TAG, FFA (Beilby and Kidby 1980). The results obtained by Fontaine (2001) on *G. intraradices* isolated from monoxenic cultures were in agreement with data reported by Beilby and Kidby (1980) on *G. caledonius* spores isolated from non-monoxenic cultures. The predominant FA remains C16:1 in all the fractions, except for the FFA. The proportions of C16:1 in DAG and sterol ester FA decrease in comparison with the TAG fraction.

3.4 Phospholipid Composition

Very little information is available on the PL of AM fungi. Phosphatidylethanolamine was the major PL described in AM fungal spores of *G. mosseae* (Cooper and Lösel 1978), *G. caledonius* (Beilby and Kidby 1980) and *G. versiforme*(Gaspar et al. 1994) obtained from plant cultures, and *G. intraradices* (Fontaine et al. 2001a) grown in monoxenic culture. Other PL detected were phosphatidylcholine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylserine and phosphatidic acid (Cooper and Lösel 1978; Beilby and Kidby 1980).

3.5 Sterol Composition

Little attention has been paid to the sterol composition of AM fungi. The first analyses on sterol content of AM fungi were performed by Beilby (1980), Beilby and Kidby (1980) and Nordby et al. (1981) on three different species – *G. caledonius*, *G. mosseae* and *Acaulospora laevis* – and revealed 24-ethylcholesterol as major sterol. Grandmougin et al. (1999) studied the sterol distribution in spores of 16 species of AM fungi, including representatives of Glomaceae, Gigasporaceae and Acaulosporaceae propagated in pot cultures. The main sterol identified in all the species was 24-ethylcholesterol.

Table 3. Fatty acid profiles of neutral lipids of *Glomus intraradices*

Isolates of *G. intraradices*	Type of culture	Structure of fungus analysed	Type of lipid fraction	14:0	15:0	16:0	16:1	16:1 ω5	16:1 ω7	18:0	18:1	18:2	18:3	19:0	20:0	20:1	20:2	20:3	20:4	20:5	References
Gift of Dr. G. Safir	Pot culture	External hyphae	Neutral fatty acids	–	–	28.4	–	63.2	3	–	4.2	–	–	–	–	–	–	–	0.6	0.7	Johansen et al. (1996)
Isolate 28 a from Dr. S. Rosendahl	Pot culture	Mycelium	Neutral fatty acids	–	0.5	33	–	54	0.7	1	9	0.8	–	–	–	–	–	–	0.1	0.7	Larsen et al. (1998)
BEG 87[a]	Pot culture	Hyphae	Neutral fatty acids	–	–	35.8	–	38.8	0.2	0.2	6.2	1.6	–	–	–	0.5	15.9	0.2	–	0.5	Olsson and Johansen (2000)
BEG 87	Pot culture	Spores	Neutral fatty acids	–	–	41.6	–	33.8	2.5	2.7	9.6	2.9	–	–	–	0.1	5.4	–	–	0.1	Olsson and Johansen (2000)
BEG 87	Pot culture	Mycelium	Neutral fatty acids	–	–	47	–	37	–	3	9	3	–	–	–	–	–	–	–	–	Aarle and Olsson (2003)
Not provided	In vitro culture	Mycelium in free-P medium	Neutral fatty acids	–	–	17.7	–	77.6	–	0.7	3.2	0.4	–	–	0.5	–	–	–	–	–	Olsson et al. (2002)
Not provided	In vitro culture	Mycelium in high-P medium	Neutral fatty acids	–	–	19.8	–	74.6	–	1.4	2.6	0.3	–	–	1.1	–	–	–	–	–	Olsson et al. (2002)
DAOM 186602[b]	Pot culture	Spores	Triacylglycerol fatty acids	0.2	–	11.8	82.1		–	0.5	2.4		0.7	2	–	–	–	0.3	–	–	Jabaji-Hare (1988)
DAOM 197198	In vitro culture	Mycelium	Triacylglycerol fatty acids	–	–	7	–	86	–	1	3	1	1	–	–	–	–	1	Tr[c]	1	Fontaine (2001)
DAOM 197198	In vitro culture	Mycelium	1,2 Diacylglycerol fatty acids	–	–	11	–	24	–	21	17	6		–	–	–	–	9	8	5	Fontaine (2001)
DAOM 197198	In vitro culture	Mycelium	1,3 Diacylglycerol fatty acids	–	–	15	–	46	–	11	14	6	1	–	Tr	–	–	3	3	1	Fontaine (2001)
DAOM 197198	In vitro culture	Mycelium	Fatty acids from esterified sterols	–	–	18	–	28	–	9	20	8	3	–	1	–	–	5	5	1	Fontaine (2001)
DAOM 197198	In vitro culture	Mycelium	Free fatty acids	–	–	10	–	9	–	10	25	4	1	–	3	–	–	18	6	4	Fontaine (2001)

[a] BEG, The International Bank for the Glomeromycota [b] DAOM, National Mycological Herbarium, Agriculture Canada, Ottawa, Ontario [c] Tr, traces

Other sterols identified were cholesterol, 24-methylcholesterol, 24-methylcholesta-5,22-dienol and Δ5-avenasterol. Ergosterol was not detected in any of the analysed species, except for traces in *G. mosseae* (Nordby et al. 1981), despite the fact that this sterol was recognized to be the most common sterol found in fungi (Weete 1989). Generally, the presence of ergosterol was restricted to the more advanced fungal taxa (Weete and Gandhy 1997). Nevertheless, the presence of ergosterol in roots colonized by AM fungi and in extraradical mycelia was published by Frey et al. (1992, 1994), Antibus and Sinsabaugh (1993) and Fujiyoshi et al. (2000). Methods which used ergosterol analysis to estimate AM fungal biomass in the root and soil were proposed (Hart and Reader 2002a, 2002b). These different experiments were conducted with material obtained from pot cultures susceptible to have been contaminated with some saprophytic or parasitic fungal organisms.

The use of monoxenic cultures allowed the investigation of sterol content without contaminating fungi. The sterol composition of *G. intraradices* and *G. proliferum* spores was established (Declerck et al. 2000; Fontaine et al. 2001b, 2004). Predominant sterols were found to be 24-alkylsterols. No detectable ergosterol was found. Recently, Olsson et al. (2003) estimated the ergosterol content of *G. intraradices* and *Gigaspora margarita* mycelia as well as colonized and non-colonized roots developing in monoxenic cultures. They used two different methods, but neither revealed the presence of ergosterol, consistent with the earlier findings of Fontaine et al. (2001b, 2004). The monoxenic culture of AM fungi also allowed the exploration of the particular sterol metabolism of these fungi as *G. intraradices* under different experimental conditions (Fontaine et al. 2001b). Feeding experiments with a labelled precursor of lipids were performed. A de novo synthesis of two main AM fungal sterols, 24-methylcholesterol and 24-ethylcholesterol, and different metabolic intermediates, lanosterol and 24-methylenelanosterol, was detected for the first time. Absence of synthesis of ergosterol was pointed out.

Δ5-sterols – terminal products of the sterol pathway – found in *G. intraradices* developing in pot cultures and in monoxenic cultures were cholesterol, 24-methylcholesterol, 24-ethylcholesterol-5,22-dienol, 24-ethylcholesterol and 24-ethylidenecholesterol (Grandmougin-Ferjani et al. 1999; Fontaine et al. 2001b, 2004). However, changes in the relative proportions of main sterol were observed between the sterol profiles obtained in spores of *G. intraradices* grown in pot cultures and in monoxenic cultivation systems (Grandmougin-Ferjani et al. 1997; Fontaine et al. 2001b, 2004). This change is still poorly understood. It could be hypothesized that the sterol metabolism could be affected by culture conditions or during the ontogeny of spores, as described in a biotrophic pathogen *Blumeria graminis* (Muchembled et al. 2000).

4
Monoxenic Culture Techniques as a Tool for the Establishment of Lipid Indicators of the Presence of AM Fungi in Roots

The abundance of lipids in AM fungi is a potential tool to evaluate AM fungi in host root tissue. Root staining methods, followed by microscopic examinations, are traditionally used to evaluate the presence of AM fungi and root colonization levels. However, these methods, based on differential staining of roots and estimation of the percentage or total length of the root, are all tedious, time-consuming (Sylvia et al. 1994) and dependent on the visualization technique employed (Gange et al. 1999). For these fungi, the signature FA C16:1ω5 was proved to be a tool for the estimation of AM fungal biomass in soil and in roots. This point was the subject of a review by Olsson (1999).

Schmitz et al. (1991) have observed a significant increase of 24-methyl/methylenecholesterol upon mycorrhizal colonization of roots obtained from pot cultures. A recent study, which used monoxenic cultures, established that the increment of 24-methyl/methylene sterols was an appropriate indicator of AM colonized transformed roots (Fontaine et al. 2004).

5
Lipid Metabolism

Lipids are the main sources of carbon in AM fungi, and therefore they are in the centre of the C metabolism. Isotopic labelling has long provided a powerful approach to study metabolism in living systems. The application of these methods to study AM systems was made difficult because of the obligate biotrophic nature of these fungi, and the necessity to culture them in the presence of their plant partner. The first studies using labelled precursors were performed during the 1970s with whole plants. Ho and Trappe (1973) demonstrated the transfer of photosynthates labelled by $^{14}CO_2$ to mycelium of *Glomus mosseae*. In 1975, Cox et al., using autoradiography of mycorrhizal roots and external mycelium, showed that hyphae, vesicules and arbuscules contained a greater proportion of ^{14}C, after translocation of ^{14}C-labelled photosynthate. These authors suggested that photosynthates were used by the mycorrhizal fungus for lipid synthesis. Bevege et al. (1975) examined the assimilation of ^{14}C-labelled photosynthates and its distribution among carbohydrates and other fractions in AM fungi and in non-mycorrhizal roots. They concluded that much of the ^{14}C-lipid fraction was concentrated in extraradical hyphae. Lösel and Cooper (1979) were

the first to demonstrate that lipids of mycorrhizal onion became labelled when plants photosynthesised in presence of $^{14}CO_2$ or were supplied with labelled lipid precursors (acetate, glycerol, sucrose).

These experiments provided information on the classes of compounds labelled in colonized roots by $^{14}CO_2$ or by different substrates, but they did not elucidate the lipid metabolism of AM fungi and did not perceive where the synthesis of lipids took place.

In the last years, the development of the monoxenic cultivation systems stimulated interest in the lipid metabolism of AM fungi. The use of AM monoxenic cultures has allowed longer-term labelling experiments without interference with other micro-organisms, and has also facilitated the selective application of labelled substrates to either the extraradical mycelium or to the mycorrhizal roots in using bi-compartmented Petri plates. Two types of isotope labelling were used to study AM fungi lipid metabolism, i.e. ^{14}C and the ^{13}C.

5.1 ^{14}C Labelling

Acetate is the universal precursor of lipid synthesis. Labelled acetate is frequently used to study the lipid metabolism of plants or fungi. The lipid labelling in these organisms is obtained after short period (hours or days) of radiolabelled precursor application. The first study on AM fungi using [1-^{14}C] acetate was realized in 1983 by Beilby on germinating spores. Previous biochemical works have shown that AM fungi contained between 45 and 72% of lipids, with TAG as major lipid class (Beilby and Kidby 1980). During germination, the concentration of these neutral lipids was decreased while the polar lipids increased (Beilby and Kidby 1980; Gaspar et al. 1994). In the same way, free sterol fraction was obtained in greater quantity than sterol ester in germinating spores (Beilby and Kidby 1980), these esters being considered to be a storage form for sterols in fungi (Parks and Weete 1991). The lipid synthesis during germination of *G. caledonium* spores isolated from soil was shown by Beilby (1983). The incorporation of [1-^{14}C] acetate into the lipids was detected 2 h after spore imbibition. By 5 h, all lipid class contained low levels of isotope. The rate of incorporation into TAG and DAG increased significantly between 19 and 35 h, while the label of other lipid fractions showed little variation (FFA, PL, sterols). No label was detected in either the sterol ester or the monoacylglyceride fractions. Therefore, these results supported biochemical works described above. Under monoxenic culture conditions, Fontaine et al. (2001a, 2001b) have shown that in germinating spores of *G. intraradices*, [1-^{14}C] acetate was incorporated predominantly into the PL and free sterol fractions. This

asymbiotic phase is characterized by a high synthesis of membranes for the development of the germ tube. This strong synthesis requires the use of lipid reserves, probably under the action of hydrolytic enzymes of the li-

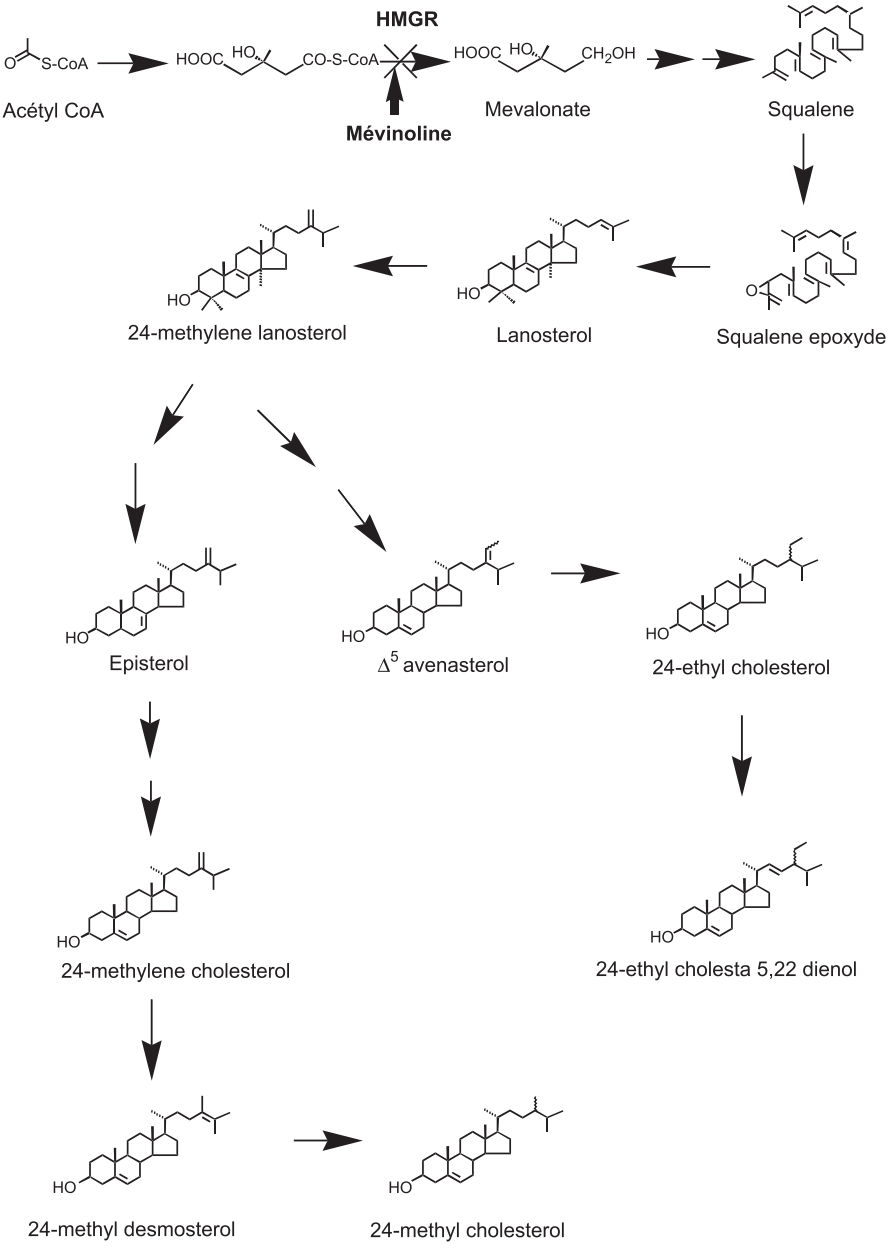


Fig. 1. Possible biosynthetic pathway of sterols in *Glomus intraradices*

pase type as described by Gaspar et al. (1997b), but also a de novo synthesis of polar lipids and free sterols from exogenous precursors. Labelling experiments using [1-^{14}C] acetate were realized also on ERM (Fontaine et al. 2001a), and have shown an incorporation into various lipid classes (FFA, DAG, TAG, PL and sterols). The AM fungus was therefore able to synthesize its own lipids in this symbiotic phase, with a major rate of incorporation into PL, DAG, TAG and sterols. These results were in agreement with those of Gaspar et al. (1994a) who demonstrated that when the fungus was in symbiotic stage, the development of ERM (hyphae and spore maturation) needs a high amount of TAG and PL. When acetate was added to the fungal compartment, a part of radioactivity was rapidly found in lipids of mycorrhizal roots. This result demonstrated that a slight part of [1-^{14}C] acetate uptake by ERM or of labelled lipids could be transferred to IRM. The study of the distribution of radioactivity among the sterol classes (free sterols and sterol ester) showed that radioactivity was associated with the two main AM fungal sterols, 24-methyl cholesterol and 24-ethyl cholesterol, but also with biosynthetic intermediates, lanosterol and 24-methylene lanosterol (Fontaine et al. 2001b). By using mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a high inhibition of the radioactivity incorporation in AM fungal sterols was shown. These results indicated that acetate was metabolised into sterols by the classical mevalonate pathway, as in filamentous fungi (Dish and Rohmer 1998). Labelled biosynthetic intermediates identified in the extracts of *G. intraradices*ERM allowed to propose a hypothetical sterol pathway (Fig. 1). So, AM fungi synthesize sterols though the mevalonic acid pathway via HMG-CoA from an acetyl-CoA carbon source.

5.2 ^{13}C Labelling

In order to investigate the ability of AM fungi to synthesize lipids, labelling experiments using ^{13}C labelling and NMR spectroscopy were performed by several workers. NMR spectroscopy is among the most informative methods allowing the study of several metabolisms at the same time. In order to work for long periods of time and without possible contamination, these experiments have been realized under monoxenic culture conditions. In 1999, Bago et al. demonstrated that the synthesis of storage lipids in the asymbiotic stage of *G. intraradices* was not significant. None of the substrates (glucose or acetate) provided during spore germination resulted in detectable labelling of storage fungal lipids, in spite of the label of acetyl-CoA or trehalose. These authors concluded that the synthesis of storage lipid was blocked or greatly reduced in the asymbiotic fungus, and that lipid synthesis

was largely or entirely confined to lipid constituents of the membranes. In 1999, Pfeffer et al. showed that when ^{13}C-Glc was added to the root compartment of the bi-compartmental monoxenic culture system (St Arnaud et al. 1996), neutral lipids from both the root and fungal compartment were labelled. Glucose taken up by the IRM was metabolized via glycolysis and was labelled from acetyl-CoA, from labelled pyruvate. When ^{13}C-glucose was added to the fungal compartment, neither fungal nor root compartment contained labelled lipids, nor other labelled compounds. They concluded that glucose was taken up by the fungus only within the root, and was metabolized to storage lipids. On the other hand, the extraradical mycelium was unable to take up exogenous sugars. Curiously, in using ^{13}C-acetate, these researchers obtained low levels of labelling in both host and fungi extracted from the root compartment when acetate was added to the root compartment. No labelling was observed in fungal or host storage lipids when acetate was added to the fungal compartment in spite of the uptake of acetate by the ERM demonstrated by the labelling of trehalose in IRM. Using $^{2}H_2O$ labelling, a more permeable precursor than acetate, they demonstrated that little or no storage lipid synthesis occurred in AM extraradical mycelium. AM lipids were synthesized in IRM and then transferred from IRM to ERM. Bago et al. (2002c) showed a significant bidirectional translocation of lipid bodies containing storage lipids. They concluded that large amounts of lipids were translocated between IRM and ERM, with a possible recirculation of these lipid bodies throughout the fungus.

5.3 Lipid Synthesis in Arbuscular Mycorrhizae: the Controversy

Actually, lipid metabolism of AM fungi appears still unclear. Results from ^{14}C and ^{13}C labelling seem to be contradictory. In NMR studies, only the most abundant FA, i.e. C16:1ω5, was used to follow the lipid metabolism in AM fungi. This FA, predominantly associated with storage lipid, cannot be used in order to study lipid metabolism as a whole. The studies with the ^{14}C labelling used a global label on lipid compounds, and not only on one FA. Using [1-^{14}C] acetate, recent studies have shown that germinating spores and extraradical hyphae were completely unable to synthesize 16-carbon FA, but were able to elongate and desaturate FA already present (Trépanier et al. 2003). These results could explain the controversy and showed that, in using [1-^{14}C] acetate or [1-^{14}C] sucrose, AM fungi were able to synthesize their own FA only inside the root. FA synthase genes of AM fungi could hypothetically be exclusively expressed in the intraradical mycelium. Trépanier et al. (2003) suggested that root signal or massive hexose influx could activate the fungal FA synthase.

6 Conclusions

The use of AM monoxenic cultures in combination with isotopic labelling techniques has been primordial to clarify the lipid metabolism of AM fungi, and may be at the cutting edge of technologies used to understand the obligate biotrophic nature of these fungi. Bago et al. (2000) have suggested that AM fungi are characterized by morphologic and metabolic differentiations between IRM and ERM. The differentiation program could be induced by specific physical or chemical factors in the particular environment where the IRM developed, the root apoplast (Bago and Bécard 2002). In order to understand why the IRM is biosynthetically competent, genes encoding the key enzymes governing lipid metabolism, such as FA synthase, should be examined and host signal(s) should be identified. To date, studies on lipid metabolism under monoxenic culture conditions have been realized on *G. intraradices* Schenck & Smith (DAOM 197198). Futures studies should be extended to other AM fungal genera.

AM monoxenic cultures have also offered unique advantages for investigating carbon metabolism (^{13}C–^{14}C labelling) and lipid translocation in hyphae. In the future, this technique will continue to provide contaminant-free plant and fungal materials.

The most recent results obtained in the understanding of the AM symbiosis by using different molecular biological techniques (Franken and Requena 2001; Harrier 2001; Burleigh 2001; Delp et al. 2003) have demonstrated a spatial and temporal regulation of the expression of plant and fungal genes. There is a mutualistic control of gene expression when symbiosis is established. The presence of IRM and arbuscules in plant cortical root cells modulates the expression patterns of plant genes involved in carbon metabolism (sucrose synthase: Ravnskov et al. 2003), and genes involved in nutrient transport (Burleigh 2001). Quantification of fungal genes in mycorrhizal roots is possible when using a combination of molecular techniques (quantitative RT-PCR and immunoblotting). Unfortunately, antibodies corresponding to fungal proteins have been utilized in an attempt to identify specific fungal species or detect AM fungi in plant roots (Harrier 2001). There is also a lack of data on proteins corresponding to functional metabolic genes. It is of crucial importance to develop new tools to understand nutrient transport and carbon and lipid metabolism in AM symbiosis. AM monoxenic cultures offer unique advantages for investigating the differential expression of genes in IRM and ERM. The genome sequencing of *G. intraradices* will allow, from identified genes, the design of specific primers for quantitative expression and recombinant protein and antibody production for immunodetection.

From RMN studies of lipid metabolism, Bago et al. (1999) and Bago and Bécard (2002) hypothesized that the failure of AM fungi to complete their life cycle in the absence of plants (obligate biotrophy) could be due to a lack (or insufficient ability) of neutral lipid biosynthesis of both ERM and germinating spores. Cloning and expression analyses, at both mRNA and protein levels, of genes encoding enzymes involved in lipid biosynthesis are required to support or invalidate this hypothesis. RMN studies after labelling of ERM or IRM in transformed monoxenic cultures demonstrated that the hyphae in both phases of development behave differently with respect to carbon metabolism and glucose transport (Bago et al. 2000, 2002b; Bago and Bécard 2002). Germ tubes produced during germination are able to take up limited amounts of hexose (Bago et al. 1999), and hexose transporters should be present in the fungal membrane. Intraradical AM fungal hyphae acquire hexose very efficiently from the plant, and the expression of hexose transport proteins should be correspondingly high. Extraradical hyphae which are produced after the colonization of roots behave differently, ERM being unable to acquire exogenously provided hexose. Are the glucose transporter proteins absent in ERM? Are the corresponding genes down-regulated in ERM? It could be postulated that when associated to plant cells, the fungal nuclei undergo a plant imprinting leading to a modified expression of fungal genes. This plant imprinting could be maintained in nuclei of ERM produced by successive division of nuclei originating from IRM, but should disappear during spore formation. Thus, the base of the obligate biotrophy of AM fungi could originate from the control by the plant of fungal genes involved in carbon transport and metabolism, rather than in intrinsic limitations of AM fungi in these processes.

Acknowledgements. We are grateful to Corinne Aït-Saïd for typing the manuscript. The Region Nord Pas de Calais and FEDER provided financial support for which we are most grateful.

References

Antibus R, Sinsabaugh RL (1993) The extraction and quantification of ergosterol from ectomycorrhizal fungi and roots. Mycorrhiza 3:137–144

Bago B, Bécard G (2002) Bases of the obligate biotrophy of arbuscular mycorrhizal fungi. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel, pp 33–48

Bago B, Pfeffer PE, Douds DD, Brouilette J, Bécard G, Shachar-Hill Y (1999) Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. Plant Physiol 121:263–271

Bago B, Shachar-Hill Y, Pfeffer PE (2000) Dissecting carbon pathways in arbuscular mycorrhizas with NMR spectroscopy. In: Podila GK, Douds DD Jr (eds) Current advances in mycorrhizae research. APS Press, St Paul, pp 111–126

Bago B, Pfeffer PE, Zipfel W, Lammers P, Shachar-Hill Y (2002a) Tracking metabolism and imaging transport in arbuscular mycorrhizal fungi. Plant Soil 244:189–197

Bago B, Pfeffer PE, Shachar-Hill Y (2002b) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 124:949–958

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2002c) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Beilby JP (1980) Fatty acid and sterol composition of ungerminated spores of the vesicular-arbuscular mycorrhizal fungus, *Acaulospora laevis*. Lipids 15:949–952

Beilby JP (1983) Effects of inhibitors on early protein, RNA, and lipid synthesis in germinating vesicular-arbuscular mycorrhizal fungal spores of *Glomus caledonium*. Can J Microbiol 29:596–601

Beilby JP, Kidby DK (1980) Biochemistry of ungerminated and germinated spores of the vesicular arbuscular mycorrhizal fungus, *Glomus caledonius*: changes in neutral and polar lipids. J Lipid Res 21:739–750

Bentivenga SP, Morton JB (1994) Stability and heritability of fatty acid methyl ester profiles of glomalean endomycorrhizal fungi. Mycol Res 98:1419–1426

Bentivenga SP, Morton JB (1996) Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. Proc Natl Acad Sci USA 93:659–5662

Bevege DI, Bowen GD, Skinner MF (1975) Comparative carbohydrate physiology of ecto- and endomycorrhizas. In : Sanders FE, Mosse B, Tinker PB (eds) Endomycorrhizas. Academic Press, London, pp 175–195

Burleigh SH (2001) Relative quantitative RT-PCR to study the expression of plant nutrient transporters in arbuscular mycorrhizas. Plant Sci 160:899–904

Cooper KM, Lösel DM (1978) Lipid physiology of vesicular-arbuscular mycorrhiza I. Composition of lipids of onion, clover and ryegrass infected with *Glomus mosseae*. New Phytol 80:143–151

Cox G, Sanders FE, Tinker PB, Wild JA (1975) Ultrastructural evidence relating to host-endophyte transfer in a vesicular-arbusular mycorrhiza. In: Sanders FE, Mosse B, Tinker PB (eds) Endomycorrhizas. Academic Press, London, pp 297–311

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

Delp G, Timonen S, Rosewarne GM, Barker SJ, Smith S (2003) Differential expression of *Glomus intraradices* genes in external mycelium and mycorrhizal roots of tomato and barley. Mycol Res 107:1083–1093

Disch A, Rohmer M (1998) On the absence of the glyceraldehydes 3-phosphate/pyruvate pathway for isoprenoid biosynthesis in fungi and yeasts. FEMS Microbiol Lett 168:201–208

Douds DD Jr, Pfeffer PE, Shachar-Hill Y (2000) Application of in vitro methods to study carbon uptake and transport by AM fungi. Plant Soil 226:255–261

Fontaine J (2001) Etude du métabolisme lipidique d'un champignon endomycorhizien à vésicules et arbuscules: *Glomus intraradices*. PhD Thesis, Université du Littoral Côte d'Opale, Calais

Fontaine J, Grandmougin-Ferjani A, Sancholle M (2001a) Métabolisme lipidique du champignon endomycorhizien: *Glomus intraradices*. C R Acad Sci Paris 324:847–853

Fontaine J, Grandmougin-Ferjani A, Hartmann MA, Sancholle M (2001b) Sterol biosynthesis by the arbuscular mycorrhizal fungus *Glomus intraradices*. Lipids 36:1357–1363

Fontaine J, Grandmougin-Ferjani A,Glorian V, Durand R (2004) 24-methyl/methylene sterols increase in monoxenic roots after colonization by arbuscular mycorrhizal fungi. New Phytol 163:159–167

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Franken P (1999) Trends in molecular studies of AM fungi. In: Varma A, Hock B (eds) Mycorrhiza : structure, function, molecular biology and biotechnology. Springer, Berlin Heidelberg New York, pp 37–47

Franken P, Requena N (2001) Analysis of gene expression in arbuscular mycorrhizas: new approaches and challenges. New Phytol 150:517–523

Frey B, Buse HR, Schüepp H (1992) Identification of ergosterol in vesicular-arbuscular mycorrhizae. Biol Fertil Soil 13:229–234

Frey B, Vilarino A, Schüepp H, Arines J (1994) Chitin and ergosterol content of extraradical and intraradical mycelium of the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices*. Soil Biol Biochem 26:711–717

Fujiyoshi M, Nakatsubo T, Ogura S, Horikoshi T (2000) Estimation of mycelial biomass of arbuscular mycorrhizal fungi associated with the annual legume *Kummerowia striata* by ergosterol analysis. Ecol Res 15:121–131

Gange AC, Bower E, Stagg PG, Aplin DM, Gillam AE, Bracken M (1999) A comparison of visualization techniques for recording arbuscular mycorrhizal colonization. New Phytol 142:123–132

Gaspar ML, Cabello MN (1994) *Glomus antarcticum*: the lipids and fatty acid composition. Mycotaxon 51:129–136

Gaspar ML, Pollero RJ, Cabello MN (1994) Triacylglycerol consumption during spore germination of vesicular-arbuscular mycorrhizal fungi. J Am Oil Chem Soc 71:449–452

Gaspar L, Pollero R, Cabello M (1997a) Variations in the lipid composition of alfalfa roots during colonization with the arbuscular mycorrhizal fungus *Glomus versiforme*. Mycologia 89:37–82

Gaspar ML, Pollero R, Cabello M (1997b) Partial purification and characterization of a lipolytic enzyme from spores of the arbuscular mycorrhizal fungus *Glomus versiforme*. Mycologia 89:610–614

Golotte A, Brechenmacher L, Weidmann S, Franken P, Gianinazzi-Pearson V (2003) Plant genes involved in arbuscular mycorrhiza formation and functioning. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkäuser, Basel, pp 87–102

Graham JH, Hodge NC, Morton JB (1995) Fatty acid methyl ester profiles for characterization of glomalean fungi and their endomycorrhizae. Appl Environ Microbiol 61:58–64

Grandmougin-Ferjani A, Dalpé D, Hartmann MA, Laruelle F, Couturier D, Sancholle M (1997) Taxonomic aspects of the sterol and Δ11 hexadecenoic acid (C16:1, Δ11) distribution in arbuscular mycorrhizal spores. In: Williams JP, Khan MU, Lem NW (eds) Physiology, biochemistry and molecular biology of plant lipids. Kluwer, Dordrecht, pp 195–197

Grandmougin-Ferjani A, Dalpé Y, Hartmann MA, Laruelle F, Sancholle M (1999) Sterol distribution in arbuscular mycorrhizal fungi. Phytochemistry 50:1027–1031

Harrier LA (2001) The arbuscular mycorrhizal symbiosis: a molecular review on the fungal dimension. J Exp Bot 52:469–478

Harrier L, Sawczak J (2000) Detection of the 3-phosphoglycerate kinase protein of *Glomus mosseae*. Mycorrhiza 10:81–86

Harrier LA, Wright F, Hooker JE (1998) Isolation of the 3-phosphoglycerate kinase gene of the arbuscular mycorrhizal fungus *Glomus mosseae*(Nicol. and Gerd.) Gerdemann and Trappe. Curr Genet 34:386–392

Harrison MJ (1996) A sugar transporter from *Medicago truncatula* altered expression pattern in roots during vesicular arbuscular (VA) mycorrhizal associations. Plant J 9:491–503

Hart MM, Reader RJ (2002a) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344

Hart MM, Reader RJ (2002b) Is percent root colonization a good measure of colonization for all arbuscular mycorrhizal fungi? Mycorrhiza 12:297–301

Hijri M, Redecker D, MacDonald-Comber Petetot JA, Voigt K, Wöstemeyer J, Sanders IR (2002) Identification and isolation of two ascomycete fungi from spores of the arbuscular mycorrhizal fungus *Scutellospora castanea*. Appl Environ Microbiol 68:4567–4573

Ho I, Trappe JM (1973) Translocation of ^{14}C from *Festuca*plants to their endomycorrhizal fungi. Nature 244:30–31

Jabaji-Hare S (1988) Lipid and fatty acid profiles of some vesicular-arbuscular mycorrhizal fungi contribution to taxonomy. Mycologia 80:622–629

Jabaji-Hare S, Deschene A, Kendrick B (1984) Lipid content and composition of vesicles of a vesicular-arbuscular mycorrhizal fungus. Mycologia 76:1024–1030

Jakobsen I, Rosendahl L (1990) Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. New Phytol 115:77–83

Jansa J, Gryndler M, Matucha M (1999) Comparison of the lipid profiles of arbuscular mycorrhizal (AM) fungi and soil saprophytic fungi. Symbiosis 26:247–264

Jeffries P, Young TWK (1994) Ecological aspects of mycoparasitism. In: Jeffries P, Young TWK (eds) International parasitic relationships. CAB International, Wallingford, pp 147–180

Larsen J, Olsson PA, Jakobsen I (1998) The use of fatty acid signatures to study mycelial interactions between the arbuscular mycorrhizal fungus *Glomus intraradices* and the saprotrophic fungus *Fusarium culmorum* in root-free soil. Mycol Res 102:1491–1496

Lösel DM, Cooper KM (1979) Incorporation of ^{14}C-labelled substrates by uninfected and VA mycorrhizal roots of onion. New Phytol 83:415–426

Madan R, Pankhurst C, Hawke B, Smith S (2002) Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. Soil Biol Biochem 34:125–128

Muchembled J, Lounès-Hadj Sahraoui A, Grandmougin-Ferjani A, Sancholle M (2000) Changes in sterol composition with ontogeny of *Blumeria graminis* conidia. Can J Bot 78:1–6

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Nordby HE, Nemec S, Nagy S (1981) Fatty acids and sterols associated with *Citrus* root mycorrhizae. J Agric Food Chem 29:396–401

Olsson PA (1999) Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiol Ecol 29:303–310

Olsson PA, Johansen A (2000) Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycol Res 104:429–434

Olsson PA, Baath E, Jacobsen I, Söderström B (1995) The use of PL and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycol Res 99:623–629

Olsson PA, Larsson L, Bago B, Wallander H, van Aarle IM (2003) Ergosterol and fatty acids for biomass estimation of mycorrhizal fungi. New Phytol 159:1–10

Parks LW, Weete JD (1991) Fungal sterols. In: Patterson GW, Nes DW (eds) Physiology and biochemistry of sterols. Am Oil Chem Soc, Champaign, Illinois, pp 158–171

Pfeffer PE, Douds DD, Becard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol 120:587–598

Ravnskov S, Wu Y, Graham JH (2003) Arbuscular mycorrhizal fungi differentially effect expression of genes coding for sucrose synthases in maize roots. New Phytol 157:539–545

Rousseau A, Benhamou N, Chet I, Piché Y (1996) Mycoparasitism of the extramatrical phase of *Glomus intraradices* by *Trichoderma harzianum*. Phytopathology 86:434–443

Sancholle M, Dalpe Y (1993) Taxonomic relevance of fatty acids of arbuscular mycorrhizal fungi and related species. Mycotaxon 49:187–193

Sancholle M, Dalpé Y, Grandmougin-Ferjani A (2001) Lipids of mycorrhizae. In: Hock B (ed) The Mycota IX. Springer, Berlin Heidelberg New York, pp 63–93

Schmitz O, Danneberg G, Hundeshagen B, Klingner A, Bothe H (1991) Quantification of vesicular-arbuscular mycorrhiza by biochemical parameters. J Plant Physiol 139:106–114

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, San Diego

Solaiman MDL, Saito M (1997) Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. New Phytol 136:533–538

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332

Sward RJ (1981) The structure of the spores of *Gigaspora margarita*. I. The dormant spore. New Phytol 87:761–768

Sylvia DM, Wilson DO, Graham JH, Maddo JJ, Millner P, Morton JB, Skipper HD, Wright SF, Jarstfer AG (1993) Evaluation of vesicular-arbuscular mycorrhizal fungi in diverse plants and soils. Soil Biol Biochem 25:705–713

Trépanier M, Bécard G, Moutoglis P, Gagné S, Willemeot C, Rioux JA (2003) Fatty acid synthesis on the Glomerales: key role of the intraradical phase. In: Proc 4th Int Conf Mycorrhizae (ICOM 4), Montréal

van Buuren ML, Trieu AT, Baylock LA, Harrison MJ (2000) Isolation of genes induced during arbuscular mycorrhizal associations using a combination of subtractive hybridization and differential screening. In: Podila GK, Douds DD Jr (eds) Current advances in mycorrhizae research. APS Press, St Paul, Minnesota, pp 91–99

Weete JD (1989) Structure and function of sterols in fungi. Adv Lip Res 23:115–167

Weete JD, Gandhi SR (1997) Sterols of the phylum Zygomycota: phylogenic implications. Lipids 32:1309–1316

10 Monoxenic Culture as a Tool to Study the Effect of the Arbuscular Mycorrhizal Symbiosis on the Physiology of Micropropagated Plantlets in Vitro and ex Vitro

Yves Desjardins[1], Cinta Hernández-Sebastià[2], Yves Piché[3]

1 Introduction

The development of in vitro culture systems for arbuscular mycorrhizal (AM) fungi has revolutionized the way we approach the complexity of mycorrhizal interaction, and has provided a powerful model for the study of the relationship between the mycorrhizal partners. In their recent review, Fortin et al. (2002) provide an in-depth overview of the impact that these systems have had on research in the field of AM symbiosis. Indeed, as explained in other chapters of this book, the development of such systems has been used extensively in AM fungal systematics, in mycelium development and sporulation studies, in signalling studies between symbiotic partners, in physiological studies of the fungal partner, in microbial-free inoculum production, and in the study of interactions between soil micro-organisms, including soil-borne pathogens, and mycorrhizal roots. Another area where this technique has been used with some success is the study of the impact of the mycorrhizal symbiosis on the physiology of host plants. For instance, Elmeskaoui et al. (1995) described an in vitro culture system, named "tripartite", consisting of the culture of a strawberry plantlet, an AM fungus (*G. intraradices*) and a carrot root organ under in vitro conditions. With this system, it has been possible to study the impact of mycorrhizal inoculation on water relations, mineral nutrition and carbon metabolism of plantlets produced in vitro. We will thus describe in this chapter (1) how this system has been adapted to carry out physiological research on mycorrhiza, and (2) the effect of mycorrhizal colonization on in vitro whole-plantlet physiology. The main scope of this chapter is to demonstrate how this tripartite

[1]Centre de Recherche en Horticulture, FSAA, Université Laval, Québec G1K 7P4, Canada, Tel.: +1-418-6562131, Fax: +1-418-6563515, E-mail: yves.desjardins@plg.ulaval.ca
[2]Robarts Research Institute, P.O. Box 5015, 100 Perth Drive, London, Ontario N6A 5K8, Canada
[3]Centre de Recherche en Biologie Forestière, Département des Sciences du Bois et de la Forêt, Université Laval, Québec G1K 7P4, Canada

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

culture system can constitute a powerful tool for the study of many complex physiological whole-plant responses under aseptic conditions.

2 The Tripartite Culture System

Despite a few successful pioneer reports of AM fungal inoculation on clover seedlings germinated in vitro or on tomato roots (MacDonald 1981; St-John et al. 1981; Strullu and Romand 1986), attempts to inoculate in vitro micropropagated plants using surface-sterilized germinated spores of AM fungi placed close to in vitro-formed roots have generally been less successful (Pons et al. 1983; Ravolanirina et al. 1989; Chavez and Ferrara-Cerrato 1990; Cassells et al. 1996). This approach may have failed for a number of reasons which include (1) the different nutrient requirements of both organisms, i.e. the plant for root initiation, and the fungus for AM establishment (Rapparini et al. 1994; Vestberg and Estaun 1994), (2) the complex and time-consuming manipulation of the AM propagules (MacDonald 1981), and (3) the very low rate of colonization due to the limited number of propagules normally used to inoculate plantlets (St-John et al. 1981).

The presence of high sucrose and nutrient (N and P) contents in conventional tissue culture media – for example, 60 mmol NO_3^- in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) vs 3.8 mmol in MSR (Declerck et al. 1998) – generally inhibits the germination of spores or the establishment of mycorrhizal roots in vitro (Schubert et al. 1987; Lubraco et al. 2000; Bressan 2002). The presence of 3% sucrose in the medium reduced hyphal growth compared to media with lower concentrations of sucrose (Bécard and Fortin 1988). Bressan (2002) observed that high salt concentrations in the medium prevented mycorrhizal colonization of sweet potato grown on different substrates (vermiculite, agar, hydroxyethyl cellulose). Media with a higher salt content and, in particular, with a higher N and P content, like MS medium and Hoagland solution (Hoagland and Arnon 1950), completely inhibited root colonization (Bressan 2002). Pons et al. (1983) found that the presence of sterilized soil was necessary to successfully inoculate propagated plants of *Prunus avium* L. grown on agar-solidified media.

Cassells et al. (1996) developed a successful system to establish AM fungi in vitro. This system took into consideration some of the pitfalls described above, which have hindered the formation of mycorrhiza in vitro. The originality of their approach was in the use of an autotrophic culture system alleviating the necessity to supplement sugar in the medium, concurrently decreasing the risks of bacterial contamination and competition in the cultures. However, apart from showing that the in vitro inoculation improved

the growth of plantlets during acclimatization, and that the mycorrhizal symbiosis had no effect on in vitro photosynthesis, the system was not used further in physiological studies.

Using the same premises and in order to induce active symbiosis before transfer to the acclimatization stage, Elmeskaoui et al. (1995) developed a system by which it was possible to rapidly and consistently achieve a very high level of in vitro mycorrhization. The basis of this system is to place actively growing roots from micropropagated plantlets directly in contact with actively growing mycorrhizal hyphae originating from monoxenic cultures. AM fungi associated with a root organ provide a vigorous and uniform fungal mycelium capable of faster root colonization than isolated germinated spores or surface-sterilized AM colonized root fragments (Bécard and Piché 1992). This tripartite culture system was demonstrated to be useful for physiological studies, while only having limited usefulness in a practical micropropagation setup since it is technically demanding, requiring the simultaneous culture of three actively growing partners. Innovations are still sought to improve its efficiency and profitability for large-scale operations.

The tripartite culture system has been described in detail by Elmeskaoui et al. (1995), and was used by Hernández-Sebastià (1998) and Hernández-Sebastià et al. (1999; 2000). Briefly, spores of an AM fungus are obtained from a monoxenic carrot (*Daucus carota* L.) root culture. This monoxenic culture was routinely produced on minimal (M) medium (Bécard and Fortin 1988), according to the procedure described by Chabot et al. (1992). In vitro micropropagated explants are established according to a normal tissue culture protocol and, following subculture, these explants are transferred to Sorbarod cellulose plugs (Baumgartner Papiers SA, Lausanne, Switzerland) in test tubes containing a rooting liquid medium. The cellulose plug acts as a support for the plant in vitro cultures, and also permits the rapid medium exchange necessary for the following steps of the tripartite culture. These liquid cultures are incubated for 2 weeks in a growth room ($23 \pm 1\,^{\circ}C$), under a photosynthetic photon flux density (PPFD) of $60\,\mu mol\,m^{-2}\,s^{-1}$ for a photoperiod of 16 h, provided by cool-white fluorescent lamps until inoculation, or until the onset of roots on the explant. At the same time, in separate culture containers (Magenta boxes, Chicago), 7-cm-long Ri T-DNA transformed roots of carrot (*Daucus carota* L.) are inoculated with 40 monoxenically produced spores of *Glomus intraradices* inoculated in the middle of a Magenta vessel on M medium (Figs. 1A, 2A). The vessels are sealed and incubated at $27\,^{\circ}C$ in the dark for 5 weeks. After the induction of roots on micropropagated plantlets on the paper plugs, and mycorrhizal establishment on the carrot root organs, the colonization of micropropagated plantlets is achieved as follows. The rooting medium is removed from the cellulose plugs by suction and by rinsing three times

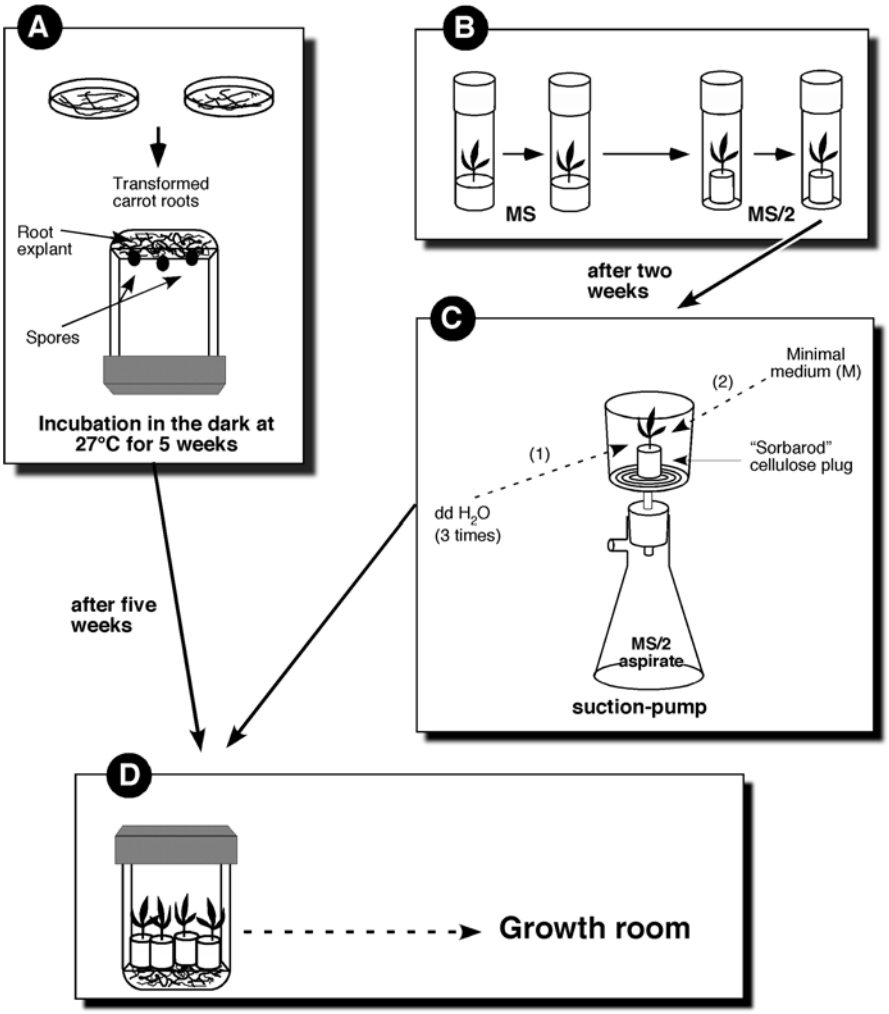


Fig. 1A–D. In vitro tripartite culture system used for establishment of mycorrhizae on micropropagated plantlets. **A** Inoculation of Ri T-transformed carrot roots (primary inoculation). **B** Micropropagation of plantlets. **C** Transfer of plantlets to rooting medium on Sorbarod plugs. **D** Inoculation of plantlets on Sorbarod plugs onto the primary inoculated roots to establish the tripartite culture (secondary inoculation)

with sterile distilled water under a laminar flow hood (Fig. 1C). After washing, liquid M medium is added aseptically to the Sorbarods supporting micropropagated plantlets. The cellulose plugs are then placed in contact with the mycorrhizal carrot root organs in culture vessels under aseptic conditions (Figs. 1D and 2B, E). Tripartite cultures are normally grown at

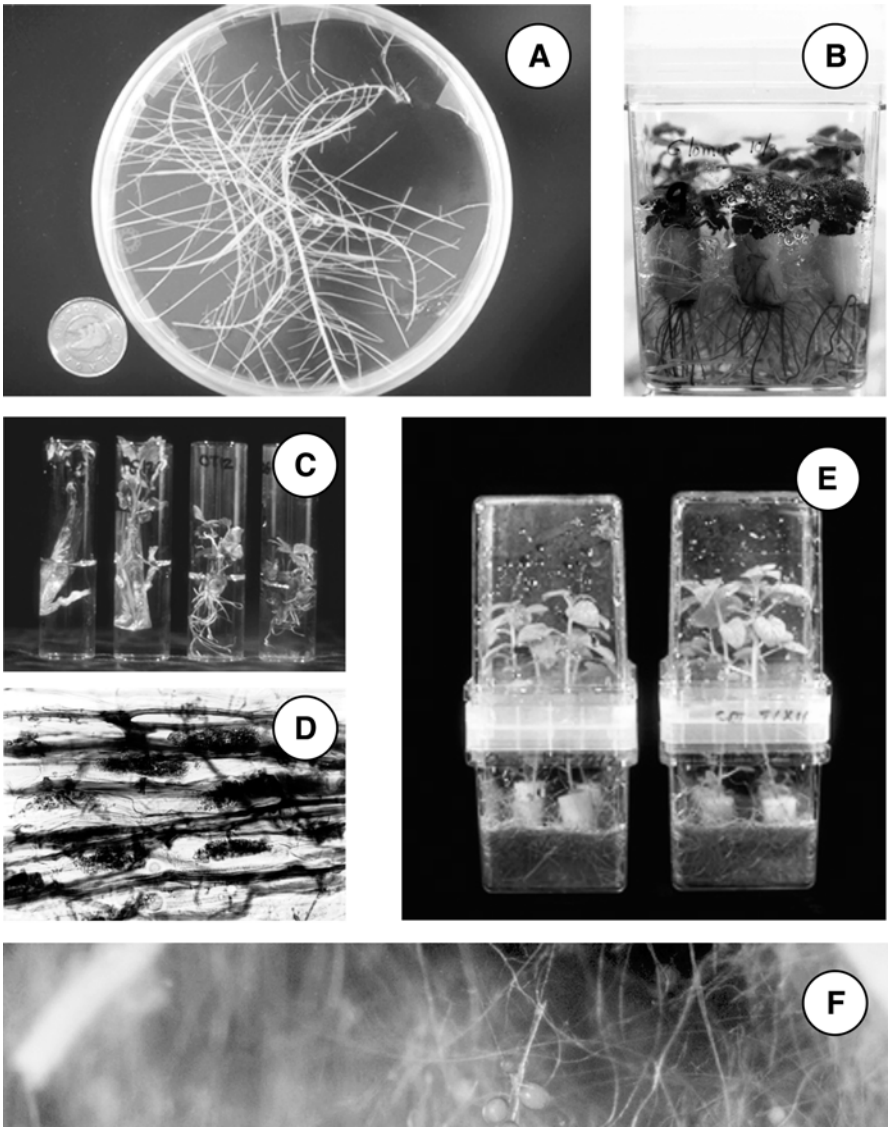


Fig. 2. **A** Monoxenic culture on a Petri plate. **B** Strawberry plantlets on Sorbarod plugs in a tripartite culture system. **C** Strawberry plantlets transferred from tripartite culture system to PEG solutions to induce water stress. **D** Arbuscules in a mycorrhizal strawberry root. **E** Potato plantlets on Sorbarod plugs in the tripartite culture system. **F** *Glomus intraradices* hyphae actively growing in the tripartite culture system

25 °C in small growth chambers with a PPFD of 60 µmol s^{-1} m^{-2} provided by cool-white fluorescent lights, for a 16-h photoperiod (Laforge et al. 1990)

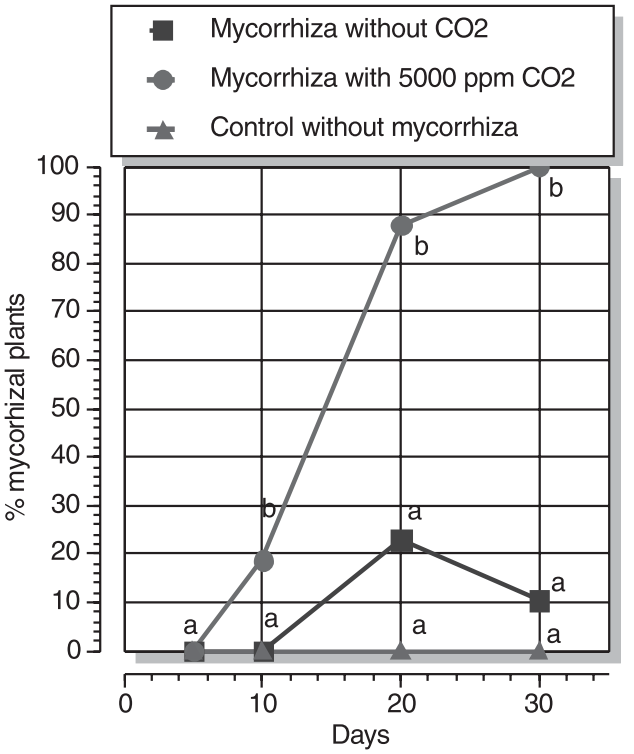


Fig. 3. Percentage of strawberry plants colonized with AM mycorrhizal fungi after 5, 10, 20, 30 days following in vitro inoculation in the presence or absence of 5000 ppm CO_2. (Adapted from Elmeskaoui et al. 1995)

with 5000 ppm CO_2. This high CO_2 concentration has been shown to enhance colonization of roots by the AM fungi (Poulin et al. 1993; Elmeskaoui et al. 1995). In the case of strawberry plants, root colonization was observed after 10 days, and a high level of colonization was obtained after 20 days (Fig. 3). We anticipate that any AM-susceptible host plant, if micropropagated without difficulties, especially in the rooting phase, should provide high rates of AM colonization in the tripartite culture.

Elmeskaoui et al. (1995) demonstrated that the age of the mycorrhizal carrot root organs is an important factor to optimize the colonization of plantlets in vitro. For instance, 30-day-old mycorrhizal carrot roots offer a better source of inoculum than younger ones. These authors attributed the higher colonization potential to a more infective mycelium possessing the secondary infective structure described by Friese and Allen (1991). Moreover, older roots may also support more hyphae which, in turn, provide higher inoculation potential.

One of the most interesting features of the tripartite culture system is the possibility to obtain mycorrhizal plantlets in vitro, allowing the study of the physiology and behaviour of these plants in controlled environments. It provides, for example, an interesting model system to study the hypothesis that colonization of roots in vitro could provide a certain degree of stress tolerance during acclimatization ex vitro.

3
The Tripartite Culture System to Study the Adaptation of Plants to Water Stress

Despite the potential usefulness of measuring the water status of tissue-cultured plants for understanding physiological changes occurring during acclimatization, such data are seldom provided (Diaz-Pérez et al. 1995). One reason for this may be the inherent difficulty of measuring the water status of small, fragile, in vitro plantlets with classical analytical instruments. Furthermore, very few studies have investigated the potential use of beneficial rhizospheric micro-organisms, such as AM fungi, to reduce drought stress during acclimatization. This may be due to the absence of an in vitro model to study the effect of AM fungal colonization on the water status of in vitro-produced plantlets under controlled conditions.

AM fungi, especially species belonging to *Glomus*, are able to modify water relations in native plants (Augé 2001). For instance, the stomatal conductance (Gs), transpiration rate and leaf water potential (ψ_w) are often higher in mycorrhizal plants under drought conditions, due to a higher water uptake (Augé et al. 1987) which allows mycorrhizal plants to maintain higher rates of photosynthesis and higher water contents than non-mycorrhizal controls. However, the mechanisms involved in the modification of water relations induced by AM fungi remain uncertain. The effects of AM fungi on plant water parameters are frequently subtle, transient and subject to highly variable conditions, including the degree of colonization, the inherent resistance of the plant species to water stress, the efficacy and competence of the fungus (Ruíz-Lozano and Azcón 1995), soil types and pH (Al-Agely and Reeves 1995). A number of different hypotheses have been tested: (1) an indirect effect of improved P nutrition in mycorrhizal plants (Koide 1993; Wright et al. 1998), (2) an improvement of water uptake by mycorrhizal root systems, either via the extraradical hyphal phase (Ruíz-Lozano and Azcón 1995), by increasing effective root hydraulic conductivity (Sands et al. 1982), or by modifying root architecture (Kothari et al. 1990), (3) a biochemical modification of water control in the host plant following changes in hormonal signalling (Duan et al. 1996; Goicoechea et al. 1997), and (4) an induction of osmoregulatory responses in mycorrhizal plants

compared to non-inoculated controls (Augé et al. 1986; Goicoechea et al. 1997). However, the results of these studies are inconclusive.

Augé et al. (1986) showed that leaves of mycorrhizal rose plants were able to enhance their osmotic adjustment under water deficits. Moreover, in a preliminary study using the tripartite culture system, Elmeskaoui et al. (1995) showed that colonization with *G. intraradices* induced changes in the water status of strawberry leaves and root tissues, under low vapour pressure deficits (Fig. 4). These authors were able to show that AM fungi could induce an osmotic adjustment in vitro in the absence of stress, as indicated by Sánchez-Diaz (1994). It was suggested that this adjustment could have contributed to the improved adaptation to ex vitro transfer observed in mycorrhizal plants. However, the mechanism of this adaptation is still unknown.

Hernández-Sebastià et al. (1999) took this investigation one step further and evaluated the osmotic potential ($\psi_{\pi L}$) of leaves and root tissues, as well as the relative water content (RWC) of whole plants, leaf discs and roots,

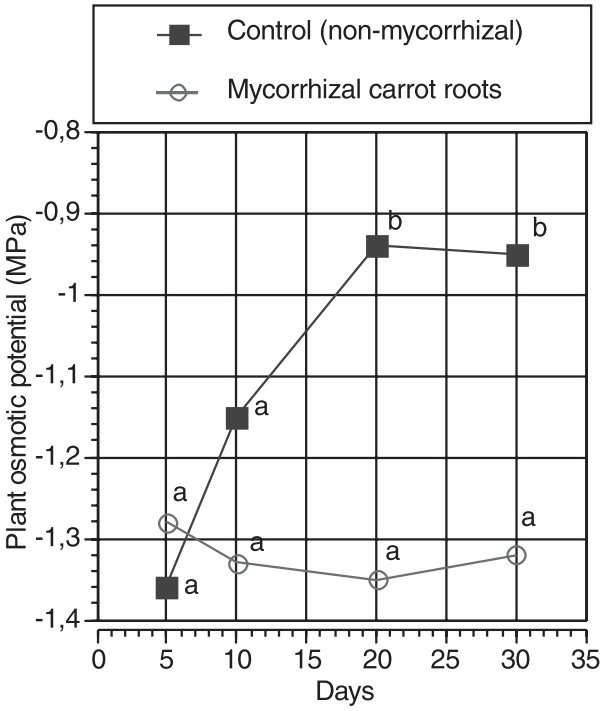


Fig. 4. Effect of inoculum source on the osmotic potential of strawberry plants after in vitro inoculation in the presence of 5000 ppm CO_2. *Squares* Control non-mycorrhizal plants, *circles* mycorrhizal carrot roots. Mean separation is by Duncan multiple range test (Pv < 0.05). (Redrawn from Elmeskaoui et al. 1995)

and the leaf stomatal conductance (Gs) of mycorrhizal plants cultured in the tripartite culture system under high humidity levels. The authors also evaluated whether modification of the plant water status of tissue-cultured mycorrhizal plants was associated with differences in plant mineral nutrition. Their results showed that *G. intraradices* increased the RWC of whole plantlets. Since neither turgid weight nor dry weight were affected by the fungal inoculation, it was concluded that mycorrhizal colonization did not alter the plantlet's maximal water retention capacity, but rather allowed it to hold more water during normal growth (Fig. 5). Since foliar RWC (leaf blade and petiole of plantlets) was not affected by the inoculation treatment, enhanced RWC of the whole plantlet was attributed to an increase in water retention in the roots. Several possible mechanisms have been advanced to explain the higher water volume recorded in mycorrhizal roots. Duan et al. (1996) suggested that mycorrhizal root systems have an improved ability to scavenge water in drier soil. Such an ability could be the result of an increase in turgor in AM roots, or a decrease in xylem-sap abscisic acid (ABA) concentrations of mycorrhizal plants during drought. Higher root water content in AM colonized plants could be due to changes in biochemical or metabolic pathways which alter the root osmoregulation process. Mycorrhizal fungi have previously been shown to alter root cytokinins (Drüge and Schönbeck 1992; Goicoechea et al. 1995) and calcium concentrations (Augé et al. 1992). It seems conceivable that AM could also modify water partitioning between the apoplast and symplast in roots (Augé and Stodola 1990; Bonfante and Perotto 1995). Diaz-Perez

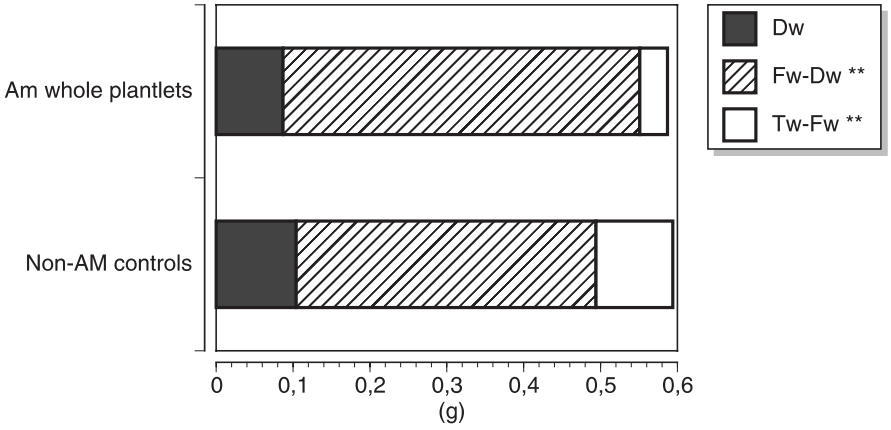


Fig. 5. Compartments of dry matter (*Dw*), absolute freshwater content (*Fw–Dw*) and water absorbed during full hydration in deionized water (*Tw–Fw*) for inoculated (*AM*) whole plants and non-inoculated (*non-AM*) controls. ** Highly significant between inoculated and control plants ($P < 0.001$). (Redrawn from Hernández-Sebastià et al. 1999)

et al. (1995) reported that higher RWC in leaves of micropropagated apple plants were correlated with improved resistance to transplantation stress, although the mechanism involved remains unknown.

3.1 Intraradical Phase of AM Fungi

In a further investigation using the tripartite system, Hernández-Sebastià et al. (2000) investigated whether the increase in RWC caused by the mycorrhizal colonization in vitro could confer an advantage to plants subjected to the sudden water stress encountered during ex vitro acclimatization. To do so, mycorrhizal plantlets produced in the tripartite culture system were transferred to a polyethylene glycol (PEG) solution to simulate and induce rapid water stress. Transfer of the plantlets from the Sorbarod plugs to the PEG solution resulted in the loss of the fragile extraradical phase of *G. intraradices*, which remained within the plugs. Consequently, colonization comprised only intraradical hyphae, arbuscules and vesicules (see Fig. 2D). Under the PEG-induced water stress, the authors observed no significant differences in water stress adaptations between mycorrhizal and non-mycorrhizal plantlets under in vitro conditions. It was concluded that the extraradical phase of the fungus played an important role in improving water stress. Despite the difficulty of showing reduced water potential in roots and leaves, the authors were able to show important changes in amino acid metabolism as a result of the concomitant presence of the mycorrhiza and the application of an osmotic stress. The intraradical phase of *G. intraradices* caused a large increase in the total amino acid pool under water stress, while there was no increase in non-mycorrhizal controls. More precisely, mycorrhizal plants showed a marked increase in asparagine in the roots, and a corresponding decrease in the leaves (Table 1). The inverse response was observed in non-mycorrhizal plants. These results suggest the presence of an important mobile pool of asparagine which moves from leaves to roots, and vice versa, in response to drought stress, depending on the plant's mycorrhizal status. Simultaneously with the increase in asparagine concentration in mycorrhizal roots, the authors recorded an increase in starch accumulation within roots. The results thus strongly suggest that the mycorrhizal association induces a coordinated adjustment of the nitrogen and carbon metabolism, representing an adaptation to water stress. However, the adaptive response of plantlets to water stress revealed by the authors is of limited efficiency, and has been largely overwhelmed by the severity of the PEG-induced osmotic stress of this study. The authors restricted the scope of their findings to the induction of an adaptation to water stress induced by the presence of the intraradical phase of the fungi,

Table 1. Effect of the intraradical phase of *G. intraradices* on concentrations of asparagine (*Asn*), aspartic acid (*Asp*) and starch in leaves and roots (mmol kg^{-1}) of in vitro strawberry plantlets cultured in a tripartite culture system, and submitted or not to a strong water stress caused by a 15% PEG solution for 4 h[a]. (Adapted from Hernández-Sebastià et al. 2000)

		Leaves		Roots		
In vitro mycorrhizal inoculation	PEG (%)	Asn	Asp	Asn	Asp	Starch
Control	0	12.8 ± 0.6	3.2 ± 0.1	29.5 ± 0.2	3.4 ± 1.1	9.7 ± 0.2
	15	32.7 ± 0.6	2.0 ± 0.1	17.4 ± 0.2	2.0 ± 1.1	7.8 ± 0.2
Glomus intraradices	0	69.5 ± 0.7	3.4 ± 0.1	6.0 ± 0.2	3.4 ± 1.1	6.6 ± 0.2
	15	11.9 ± 0.7	5.4 ± 0.1	52.5 ± 0.2	5.9 ± 1.1	33.6 ± 0.2

[a] Means ± SE

and suggested that a different response might have been observed in the presence of the extraradical mycelium.

3.2 Extraradical Phase of AM Fungi

The extraradical phase of mycorrhizal fungi appears to play an important role in determining the adaptation of the plants to stress conditions. However, it is still not clear as to the mechanism by which the AM symbiosis contributes to water uptake. Some AM fungi have been shown to improve water uptake and conductivity through the soil–plant–atmosphere continuum via the hyphae in the soil (Ruíz-Lozano and Azcón 1995). By contrast, Georges et al. (1992) showed that given the small diameter of the hyphae and the considerable transpiration flux of the plant, the hyphae cannot supply enough water to improve plant performance significantly. With this in mind, Hernández-Sebastià (1998) investigated the potential role of a preexisting extraradical mycelium of *G. intraradices* in the acclimatization substrate in improving water relations, osmotic adjustment, photosynthetic rate and mineral uptake of plantlets during their transfer to soil and adaptation to drought cycles. To test this, AM and non-AM strawberry plantlets under the tripartite culture system were transferred to substrates containing, or not containing, the same strain of an extraradical mycelium of *G. intraradices*. The plants were exposed weekly to drought-re-watering cycles.

In short, in order to compare the effect of the presence of the extraradical mycelium on stress adaptation during acclimatization, 1 liter recycled

Table 2. Mineral content of non-mycorrhizal (Nmmix) and mycorrhizal (Mycorimix) peat substrates used to compare the effect of the presence of active mycorrhizal hyphae on growth and stress adaptation of non-mycorrhizal and mycorrhizal strawberry plantlets grown in vitro, under a tripartite culture system. (Adapted from Hernández-Sebastià 1998)

	N	P	K	Ca	Mg	Fe	Zn
NMmix	103	6	59	138	26	1.32	0.16
	(5)[b]	(2)	(7)	(8)	(2)	(0.06)	(0.02)
Mycorimix	127	5	61	151	35	1.55	0.20
	(30)	(1)	(6)	(34)	(5)	(34)	(0.04)

	Cu	Mn	B	Na	pH	EC[a]
NMmix	< 0.1	0.47	0.33	21	5.31	1.25
		(0.04)	(0.03)	(4)	(0.14)	(0.07)
Mycorimix	< 0.1	0.56	0.33	28	5.56	1.30
		(0.09)	(0.04)	(3)	(0.08)	(0.17)

[a]Electrical conductivity (mmho cm^{-1})

[b]Mineral contents are in ppb (SE); note that 1%=10,000,000 ppb

cellulose culture pots were filled with a peat substrate (Mycorimix, Premier Tech Inc., Rivières-du-Loup, Québec, Canada) containing at least four propagules of *G. intraradices* DAOM 181602 (as dry pieces of colonized leek roots) per gram of peat. Control pots were filled with an identical but non-mycorrhizal peat mix (NMmix). Substrate nutrient concentrations, pH and conductivity are shown in Table 2.

To obtain peat soil substrate with AM mycelia well developed at the beginning of the experiment, rooted non-mycorrhizal strawberry plantlets were transplanted into the Mycorimix and NMmix pots (three plants per pot). The strawberry plantlets were fertilized weekly with 250 ml pot^{-1} of Long Ashton solution modified by Hewitt (1966). After 1 month in a growth chamber, the strawberry plantlets were cut at the base of the stems, leaving their roots in the soil. The presence of mycorrhiza was verified in each pot by sampling one whole root system. Mycorrhizal colonization exceeded 25% in plantlets grown on Mycorimix. All NMmix plants were non-mycorrhizal. In vitro plantlets, grown in the presence or absence of mycorrhiza under the tripartite culture system, were transplanted to the two acclimatization substrates. The authors assumed that formation of anastomoses between the hyphae already in the soil and those present in or on the roots of the in vitro plantlets could occur, as AM mycorrhizae are known to undergo anastomosis between compatible hyphae (Tommerup and Sivasithamparam 1990; Giovannetti et al. 2001). The rational was that anastomoses could provide the in vitro-produced mycorrhizal plantlets with an important extrarad-

Table 3. Effect of the presence of the extraradical phase of *G. intraradices* in the acclimatization substrate, and of in vitro inoculation of in vitro strawberry plantlets cultured in a tripartite culture system, on leaf water potential (ψ_w), stomatal conductance to H_2O, water use efficiency (*WUE*) and leaf sucrose concentration under different irrigation regimes (control and 20% water saturation). (Adapted from Hernández-Sebastià 1998)

Ex vitro mycorrhizal inoculation	Water stress	ψ_w (MPa)	Gs H_2O ($mm\,s^{-1}$)	WUE (mol CO_2 $mmol^{-1}$ H_2O)
NM[a]	–	-0.73 ± 0.03	36.03 ± 2.37	1.58 ± 0.29
	+	-1.30 ± 0.03	17.96 ± 2.37	2.63 ± 0.29
Glomus intraradices	–	-0.77 ± 0.03	36.91 ± 2.37	1.55 ± 0.29
	+	-1.61 ± 0.03	7.04 ± 2.37	3.90 ± 0.29

Ex vitro mycorrhizal inoculation	In vitro mycorrhizal inoculation	Sucrose concentration in leaves ($mg\,g^{-1}$ dry weight)
NM substrate	–	22.3 ± 6.4
	+	22.5 ± 6.4
Mycorimix	–	16.8 ± 6.4
	+	34.3 ± 6.4

[a]Non-mycorrhizal. Means ± SE

ical structure, thus replacing that lost during transfer to acclimatization, thereby conferring an added advantage under drought. The results of this study confirmed the crucial role of the extraradical phase in water uptake, mineral absorption and osmotic adjustment of strawberry plants adapted to drought. We suggest, even if we did not observe it as such, that a well-developed AM fungus in the acclimatization substrate may have formed anastomosis with in vitro mycorrhizal hyphae of *G. intraradices*, which may have contributed to the modified water relations of acclimated strawberry plantlets with a water deficit. Under dry cycles, where pots were left to dry for 3–4 days until the substrate had reached 20% water saturation, leaf water potential (Ψ_w), stomatal conductance and transpiration rates were lower in plants grown in a substrate containing the extraradical mycelium of an AM fungus than in controls (Table 3). Under drought, both stomatal conductance and intercellular CO_2 were reduced by the Mycorimix treatment. However, the rates of carbon dioxide assimilation (A) were maintained at levels similar to those of the plants growing on NM-mix. This resulted in higher instantaneous water use efficiency (WUE) in the Mycorimix-grown plants under drought stress conditions, compared to those growing on NMmix. Interestingly, simultaneous use of in vitro mycorrhizal planlets and ex vitro acclimatization mycorrhized substrate caused an increase in compatible osmolytes, most noticeably of sucrose,

in the leaves (Hernández-Sebastià 1998). The results thus demonstrate the value of the tripartite culture system with mycorrhizal monoxenic cultures to test complex hypotheses under controlled conditions.

4 The Tripartite Culture System to Study Sink–Source Relationships

The tripartite culture system has also proved useful in the study of the benefits of the mycorrhizal association for carbon metabolism, and the stimulation of photosynthetic C uptake by plants. It is generally assumed that the increased photosynthetic rates exhibited by mycorrhizal plants is the result of increased P uptake (Fitter 1991). However, such potential increases of photosynthesis are modulated by a number of other environmental factors, including atmospheric CO_2 and light levels. For the host plant, these factors can modify the cost-benefit balance of a mycorrhizal relationship. For example, long-term CO_2 enrichment – which otherwise would stimulate photosynthesis through the suppression of photorespiration (Gerbaud and André 1980) – can, in a situation of insufficient sink strength, cause sugar accumulation in source leaves and trigger down-regulation of photosynthesis (Bowes 1991). Some authors have suggested that mycorrhizal fungi constitute a significant sink for excess assimilates (Hodges 1996; Wright et al. 1998), which is sufficient to decrease the plant's susceptibility to the down-regulation of photosynthesis under prolonged exposure to elevated CO_2. Other authors have suggested that mycorrhiza can further delay down-regulation of photosynthesis by increasing the uptake of nutrients required to sustain the stimulated plant growth and the formation of new sink organs (Lewis and Strain 1996).

Louche-Tessandier et al. (1999) have used the tripartite culture system to investigate the interactive effects of different CO_2 concentrations (350 and 10,000 ppm) and PPFD (60 and 300 $\mu mol\, m^{-2}\, s^{-1}$) on the relationship between the AM fungus *G. intraradices* and potato plantlets (*Solanum tuberosum*). Using this system, these authors were able to show how changes in the mycorrhizal colonization rate and in plant source–sink relationships alter cost-benefit in mycorrhizal potato plantlets. For example, the effect of AM on weight accumulation was highly dependent on the CO_2 enrichment and light levels during tri-culture. Under normal CO_2 concentrations, the AM fungus had no effect on dry matter production, stomatal conductance or pigment content. Under a CO_2-enriched atmosphere and high light levels, however, the presence of the AM fungus increased dry weight accumulation of the plantlets by 25 % (Table 4). At the same time, the increased availability of photosynthates under high light and CO_2 increased

Table 4. Effect of in vitro inoculation with *Glomus intraradices* and of different levels of CO_2 and photosynthetic photon flux (*PPF*) on percent root colonization, production of dry matter (*DM*), chlorophyll a+b content, and maximum quantum efficiency of PSII photochemistry (Fv/Fm) of potato plantlets cultured in a tripartite culture system. (Adapted from Louche-Tessandier et al. 1999)

CO_2 enrichment (ppm)	PPF mycorrhiza (µmol m^{-2} s^{-1})	In vitro colonization	Percent (28 days)	Dry matter (g)	Chl a+b (mg g^{-1} FW)	Fv/Fm
350	60	NM[a]	0	42.9 ± 6.1	3.2 ± 0.8	0.760 ± 0.005
		M	4.4 ± 0.6	43.3 ± 9.0	3.3 ± 0.9	0.770 ± 0.003
	300	NM	0	91.8 ± 36	1.9 ± 0.6	0.725 ± 0.005
		M	4.8 ± 0.6	90.6 ± 18	1.9 ± 0.3	0.750 ± 0.014
10,000	60	NM	0	61.4 ± 9.4	3.2 ± 0.9	0.763 ± 0.008
		M	5.7 ± 0.7	43.9 ± 14	3.7 ± 0.9	0.761 ± 0.002
	300	NM	0	122 ± 19	0.5 ± 0.2	0.514 ± 0.054
		M	7.8 ± 1.0	136 ± 36	0.8 ± 0.6	0.580 ± 0.111

[a] NM, Non-mycorrhizal plantlets; M, mycorrhizal plantlets. Means ± SE

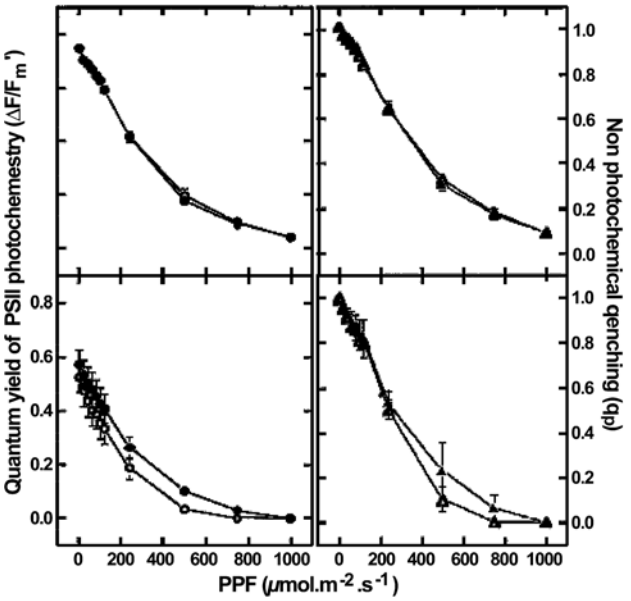


Fig. 6. Effect of the presence (*closed symbols*) or absence (*open symbols*) of *Glomus intraradices* and of different levels of CO_2 and photosynthetic photon flux (*PPF*) on the quantum yield of photosystem II electron transport (ΔF/Fm′; *circles*) and photochemical quenching (q_n; *triangles*) of in vitro potato plantlets. (Adapted from Louche-Tessandier et al. 1999)

AM colonization rates. In general, plantlets submitted to high light and CO_2 showed symptoms of photoinhibition (estimated by the chlorophyll fluorescence ratio Fv/Fm) caused by insufficient sink activity. The negative effects of high light and CO_2 were lower in the leaves of mycorrhizal plantlets than in those from non-mycorrhizal plantlets grown under the same conditions. The presence of mycorrhiza also protected the photosynthetic system from over-reduction and photoinhibition problems, showed by a smaller decrease in the effective photochemical yield of photosystem II electron transport (estimated by the parameter Fv/Fm′; Genty et al. 1989; Fig. 6). These results demonstrated not only that the CO_2 concentration during the tripartite culture affected the degree of mycorrhizal colonization in in vitro potato roots, but also that the mycorrhiza affected the physiology of the potato plantlets. On a practical side, the authors concluded that high CO_2 should be maintained at least in the first week of the tri-culture in order to stimulate mycorrhizal colonization, and that light levels should be adjusted so that the source capacity matches the sink strength of the plantlets.

5 Conclusions

In this chapter, we have highlighted some examples of the useful application of the monoxenic culture system and its extension, the tripartite culture system, to study the effects of mycorrhization on micropropagated plantlet physiology under controlled in vitro conditions. This system alleviates some of the problems encountered in sampling roots for in vivo studies and, particularly, under conditions of water stress. This simplified in vitro model constitutes a versatile approach allowing the study of metabolites involved in the symbiosis or induced by mycorrhizal plants under different types of stress. The tripartite culture system could also be interesting for the study of molecular and genetic interactions involved in the mycorrhizal symbiosis, since it allows the analysis of the response of both partners under sterile conditions.

Acknowledgements. The authors wish to thank Andrew Coughlan for critically reviewing the text. Y.D. and Y.P. thank the Natural Science and Engineering Research Council of Canada for its financial support.

References

Al-Agely AK, Reeves FB (1995) Inland sand dune mycorrhizae: Effects of soil depth, moisture and pH on colonization of *Oryzopsis hymenoides*. Mycologia 87:54–60

Augé R (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Augé RM, Stodola JW (1990) An apparent increase in symplastic water contributes to greater turgor in mycorrhizal roots of droughted *Rosa* plants. New Phytol 115:285–295

Augé RM, Schekel KA, Wample RL (1986) Osmotic adjustment in leaves of VA mycorrhizal and nonmycorrhizal rose plants in response to drought stress. Plant Physiol 82:765–770

Augé RM, Schekel KA, Wample RL (1987) Rose leaf elasticity changes in response to mycorrhizal colonization and drought acclimatization. Physiol Plant 82:765–770

Augé R, Stodola JW, Brown MS, Bethlenfalvay GJ (1992) Stomatal response of mycorrhizal cowpea and soybean to short-term osmotic stress. New Phytol 120:117–125

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1992) Establishment of VA mycorrhizae in root organ culture: review and proposed methodology. In: Varma A, Norris JR, Read DJ (eds) Methods in microbiology: experiments with mycorrhizae. Academic Press, New York, pp 89–108

Bonfante P, Perotto S (1995) Tansley review no. 82 – strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol 130:2–21

Bowes G (1991) Growth at elevated CO_2 – photosynthetic response mediated through RuBisCO. Plant Cell Environ 14:795–806

Bressan W (2002) Factors affecting "in vitro" plant development and root colonization of sweet potato by *Glomus etunicatum* Becker & Gerd. Braz J Microbiol 33:33–34

Cassells AC, Mark GL, Periappuaram C (1996) Establishment of arbuscular mycorrhizal fungi in autotrophic strawberry cultures in vitro. Comparison with inoculation of microplants in vivo. Agronomie 16:625–632

Chabot S, Bel-rhild R, Chénevert R, Piché Y (1992) Hyphal growth promotion in vitro of the VA mycorrhizal fungus, *Gigaspora margarita* Becker & Hall, by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. New Phytol 122:461–467

Chavez MCG, Ferrara-Cerrato R (1990) Effect of vesicular-arbuscular mycorrhizae on tissue culture-derived plantlets of strawberry. Hortic Sci 25:903–905

Declerck S, Strullu DC, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Diaz-Pérez JC, Shackel KA, Sutter E (1995) Relative water content and water potential of tissue-cultured apple shoots under water deficits. J Exp Bot 46:111–118

Drüge U, Schönbeck F (1992) Effect of vesicular-arbuscular mycorrhizal infection on transpiration, photosynthesis and growth of flax (*Linum usitatissiumum* L.) in relation to cytokinin levels. J Plant Physiol 14:40–48

Duan XG, Neuman DS, Reiber JM, Green CD, Saxton AM, Augé RRM (1996) Mycorrhizal influence on hydraulic and hormonal factors involved in the control of stomatal conductance during drought. J Exp Bot 47:1541–1550

Elmeskaoui A, Damont JP, Poulin MJ, Piché Y, Desjardins Y (1995) A tripartite culture system for endomycorrhizal inoculation of micropropagated strawberry plantlets in vitro. Mycorrhiza 5:313–319

Fitter AH (1991) Cost and benefits of mycorrhizas – implications for the functioning under natural conditions. Experimentia 47:350–355

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. Mycologia 83:409–418

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of fluorescence. Biochem Biophys Acta 990:87–92

Georges E, Haussler KU, Vetterlein D, Gorgus E, Marschner H (1992) Water and nutrient translocation by hyphae of *Glomus mosseae*. Can J Bot 70:2130–2137

Gerbaud A, André M (1980) Effect of CO_2, O_2 and light on photosynthesis and photorespiration in wheat. Plant Physiol 66:1032–1036

Giovannetti M, Fortuna P, Citernesi AS, Morini S, Nuti MP (2001) The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytol 151:717–724

Goicoechea N, Dolezal K, Antolin MC, Strnad M, Sánchez-Díaz M (1995) Influence of mycorrhizae and rhizobium on cytokinin content in drought-stressed alfalfa. J Exp Bot 46:1543–1549

Goicoechea N, Antolin MC, Sanchez-Diaz M (1997) Gas exchange is related to the hormone balance in mycorrhizal or nitrogen-fixing alfalfa subjected to drought. Physiol Plant 100:989–997

Hernández-Sebastià C (1998) Adaptation au stress hydrique des vitroplants de fraisier inoculés avec *Glomus intraradices* Schenk & Smith dans un système de culture tripartite. PhD Thesis, Université Laval, Québec

Hernández-Sebastià C, Piché Y, Desjardins Y (1999) Water relations of whole strawberry plantlets in vitro inoculated with *Glomus intraradices* in a tripartite culture system. Plant Sci 143:81–91

Hernández-Sebastià C, Samson G, Bernier PY, Piché Y, Desjardins Y (2000) *Glomus intraradices* causes differential changes in amino acid and starch concentrations of in vitro strawberry subjected to water stress. New Phytol 148:177–186

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Farnham Royal (Bucks) Commonwealth Agricultural Bureau, London

Hoagland DR, Arnon HI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Circ 347:1–32

Hodges A (1996) Impact of elevated CO_2 on mycorrhizal associations and implications for plant growth. Biol Fertil Soil 23:388–398

Koide R (1993) Physiology of mycorrhizal plant. Adv Plant Pathol 9:33–54

Kothari SK, Marschner H, George E (1990) Effect of VA mycorrhizal fungi and rhizosphere microorganisms on root and shoot morphology, growth and water relations in maize. New Phytol 116:303–311

Laforge F, Desjardins Y, Graham MED, Gosselin A (1990) Miniature growth chambers for the study of environmental conditions in vitro. Can J Plant Sci 70:825–836

Lewis JD, Strain BR (1996) The role of mycorrhizas in the response of *Pinus taeda* seedlings to elevated CO_2. New Phytol 133:431–443

Louche-Tessandier D, Samson G, Hernandez-Sebastia C, Chagvardieff P, Desjardins Y (1999) Importance of light and CO_2 on the effects of endomycorrhizal colonization on growth and photosynthesis of potato plantlets (*Solanum tuberosum*) in an in vitro tripartite system. New Phytol 142:539–550

Lubraco G, Schubert A, Previati A (2000) Micropropagation and mycorrhization of *Allium sativum*. Acta Hortic 530:339–343

MacDonald RM (1981) Routine production of axenic vesicular-arbuscular mycorrhizas. New Phytol 89:87–93

Murashige T, Skoog F (1962) A revised medium, for rapid growth and bio-assay with tobacco tissue cultures. Physiol Plant 15:473–497

Pons F, Gianinazzi-Pearson V, Navatel JC (1983) Studies of VA mycorrhizae in vitro: mycorrhizal synthesis of axenically propagated wild cherry (*Prunus avium L.*) plants. Plant Soil 71:217–221

Poulin MJ, Bel-Rhlid R, Piché Y, Chênevert R (1993) Flavonoids released by carrot (*Daucus carota*) seedlings stimulate hyphal development of vesicular-arbuscular mycorrhizal fungi in the presence of optimal CO_2 enrichment. J Chem Ecol 19:2317–2327

Rapparini F, Baraldi R, Bertazza G, Branzanti B, Predieri S (1994) Vesicular-arbuscular mycorrhizal inoculation of micropropagated fruit trees. J Hortic Sci 69:1101–1109

Ravolanirina F, Gianinazzi S, Trouvelot A, Carre M (1989) Production of endomycorrhizal explants of micropropagated grapevine rootstocks. Agric Ecosyst Environ 29:323–327

Ruíz-Lozano JM, Azcón R (1995) Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. Physiol Plant 95:472–478

Sánchez-Diaz M, Honrubia M (1994) Water relations and alleviation of drought stress in mycorrhizal plants. In: Gianinazzi S, Schuepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhäuser, Basel, pp 167–178

Sands R, Fiscus EL, Reid CPP (1982) Hydraulic properties of pine and bean roots with varying degrees of suberization, vascular differentiation and mycorrhizal infection. Aust J Plant Physiol 9:559–569

Schubert A, Mazzitelli A, Gribaudo I (1987) Effect of inoculation with vesicular-arbuscular mycorrhizal fungi on micropropagated *Vitis berlandieri* X V. *rifaria* "Kober 5 BB". In: Ducate G, Jacob M, Simeon A (eds) Plant micropropagation in horticultural industries: preparation, hardening and acclimatization processes. Belgian Plant Tissue Culture Group, Florizel 87, Arlon, Belgium, pp 144–153

St-John TV, Hays RI, Reid CPP (1981) A new method for producing pure vesicular-arbuscular mycorrhiza-host cultures without specialized media. New Phytol 89:81–86

Strullu DC, Romand C (1986) Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. C R Acad Sci Paris 305:15–19

Tommerup I, Sivasithamparam K (1990) Zygospores and asexual spores of *Gigaspora decipiens*, an arbuscular mycorrhizal fungus. Mycol Res 94:897–900

Vestberg M, Estaun V (1994) Micropropagated plants, an opportunity to positively manage mycorrhizal activities. In: Gianinazzi S, Schuepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhäuser, Basel, pp 217–225

Wright DP, Read DJ, Scholes JD (1998) Mycorrhizal sink strength influences whole plant carbon balance of *Trifolium repens* L. Plant Cell Environ 21:881–891

11 Uptake, Assimilation and Translocation of Mineral Elements in Monoxenic Cultivation Systems

Gervais Rufyikiri[1], Nathalie Kruyts[2], Stéphane Declerck[3], Yves Thiry[1], Bruno Delvaux[2], Hervé Dupré de Boulois[4], Erik Joner[5]

1 Introduction

While searching for optimal means to study the transport processes of nutrients and non-essential elements, diverse in vivo systems were developed using bi-compartmental containers where extraradical mycelium (ERM) of mycorrhizal fungi was separated from the plant roots. These systems generated some major results (reviewed in Smith and Read 1997), but suffered several limitations such as (1) the presence of undesirable micro-organisms which could influence element bioavailability or the transport processes; (2) the difficulty to visualize the ERM dynamic development and the bi-directional translocation processes in hyphae; (3) the difficulty to collect ERM and to distinguish thin hyphae of arbuscular mycorrhizal (AM) fungi from other fungi. As a consequence, some innovative approaches were tried out, such as the system of Pearson and Tinker (1975), used by Cooper and Tinker (1978) to study the transport of P, Zn and S by AM fungi. This system was based on a bi-compartment Petri plate in which a mycorrhizal plant (*Trifolium repens* L.) was grown on sterilized soil, while the ERM was allowed to cross the partition wall to develop in an agar medium without roots. This ingenious system kept the plant and the AM fungus (*Glomus mosseae*) under sterile conditions but, as the seedling developed, a hole was made in the lid and the plant grew out from it, making the system difficult to maintain free from other micro-organisms. This system was improved by St Arnaud et al. (1996), by growing the AM fungus in bi-compartmental Petri plates under monoxenic culture conditions on

[1]Belgian Nuclear Research Centre (SCK/CEN), Radiation Protection Research Department, Radioecology Section, Boeretang 200, 2400 Mol, Belgium
[2]Université Catholique de Louvain, Unité des Sciences du Sol, Place Croix du Sud 2/10, 1348 Louvain-la-Neuve, Belgium
[3]Université Catholique de Louvain, Mycothèque de l'Université Catholique de Louvain (MUCL), Unité de Microbiologie, Place Croix du Sud 3, 1348 Louvain-la-Neuve, Belgium, Tel.: +32-10-474644, Fax: +32-10-451501, E-mail: declerck@mbla.ucl.ac.be
[4]Université Catholique de Louvain, Unité de Microbiologie, Place Croix du Sud 3, 1348 Louvain-la-Neuve, Belgium
[5]Norwegian Forest Research Institute, Hogskoleveien 8, 1432 Aas, Norway

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

a synthetic medium. This major technical progress, which allowed the spatial separation of mycorrhizal roots growing in a root compartment (RC) and the ERM ramifying into a neighbour, root-free hyphal compartment (HC), rapidly became a powerful system for studies of physiological and element transport processes in AM symbioses.

In transport studies, the medium added in the HC containing, for example, a labelled element, may be either liquid or solid, depending on the experimental objective. On the one hand, a solid medium facilitates the manipulation of the cultures and repeated measurements at specific sites to monitor hyphal growth and observations of branching patterns are feasible (Bago et al. 1998). Indicator dyes, which can reveal active sites of enrichment/depletion of specific ions or molecules, may also be included (e.g. for pH, see Bago et al. 1996). On the other hand, a liquid medium allows the modification of the concentration of specific ions at any given time, for example, when studying the effect of nutrient starvation (Joner et al. 2000). In addition, a liquid medium allows precise control of the elements added, avoiding disturbance by ions commonly present in the gelling agent of solid media. These elements may be problematic while working under carrier-free conditions or may cause confounding effects with analogous ions (e.g. K for Cs, see below). Finally, the recovery of AM fungal biomass is easier in liquid medium, and prevents possible leakage of elements into the medium during extraction from a gel.

In summary, the monoxenic culture system offers three major advantages for element transport studies:

1. The element in question may be provided at a highly precise concentration and will not interact with any matrix which reduces its bioavailability, as it is the case in soil-based systems. This allows bio-sorption and affinity studies at low concentrations.

2. The element may be supplied as defined species, so that any modification of its speciation would be due only to its interactions with the AM fungus. The measurements of uptake from organic sources or chemically precipitated forms are highly relevant in this context.

3. Specific uptake rates and flux rates may be determined with a high precision, as labelled or unlabelled elements may be provided over a short period of time, and hyphal length and cross-sections area of living runner hyphae can be determined simultaneously.

Before continuing, a precise definition of element transport by AM fungi is necessary to clearly identify the different processes behind this term. Here, we adopt the definition proposed by Cooper and Tinker (1978) and by Smith and Smith (1990), which comprises three distinct steps: (1) the uptake of an element by the extraradical hyphae; (2) the translocation of the element

from the site of uptake to the intraradical hyphae; (3) the transfer of the element from the intraradical hyphae to the adjacent plant cell apoplasm.

In this chapter, we will present and discuss available data on monoxenic studies involving the uptake and translocation of elements by AM fungi. These elements include the nutrients P and N, and the non-essential elements U and Cs.

2
Nutrient Uptake and Translocation by AM Fungi

2.1
Phosphorus

Phosphorus is a major plant nutrient with limited availability in soil, due to its slow diffusion and its propensity to fix more or less irreversibly to various components of the soil matrix. The function of roots in the uptake of this element, and the mechanisms involved have been the subject of hundreds of studies. Similarly, the role of mycorrhizal fungi in the acquisition of this element by plants has been widely investigated and remains today a major area of research on AM fungi.

Phoshorus transport in soil-filled compartmental systems began in 1973 (Hattingh et al. 1973), and saw an expansion in the 1990s. Transport studies in monoxenic cultures first started in the late 1990s. For instance, two studies on the transport of P originating from organic sources brought an end to the controversy of AM fungal capacity for exploiting organic P sources (Joner et al. 2000; Koide and Kabir 2000). Thereafter, the hyphal efficiency in P translocation has been determined in some studies. Nielsen et al. (2002), using compartmented Petri plates and gelled medium containing 50 kBq ^{32}P and a concentration of 50 µM non-radioactive P, found a maximum P flux rate corresponding to 2.9×10^{-3} mol m^{-2} s^{-1} based on the cross-section area of active (confirmed by cytoplasmic streaming) runner hyphae of *G. intraradices*. Translocation by *G. intraradices* during this 9-day transport study accounted for 96% of the P added to HC, whereas a less efficient fungus, *G. proliferum*, had depleted the HC by only 58% of the added ^{32}P. In a similar system, but using a liquid medium containing 0.4 kBq ^{33}P at 50 µM P in the HC and the fungus *G. intraradices*, Rufyikiri et al. (2004c) observed a translocation to the root compartment (RC) corresponding to 70% of the initially supplied ^{33}P. Here, activity in hyphae located in the HC was also measured, and accounted for an additional 16% of the label. Resulting P flux rates in hyphae were estimated on the basis of the hyphae cross-section area at the partition between the two compartments (0.013 mm^2, calculated for the average 137 crossing hy-

phae). For the 14-day duration of the experiment, the average P flux rate was 3.8×10^{-5} mol m^{-2} s^{-1}. This value represents about 13% of the P flux rate reported by Nielsen et al. (2002). It should be noted that in this later experiment, ^{32}P was observed in the RC already after 14 h for both fungi, with an almost complete depletion of ^{32}P in the HC after 60 h in the case of *G. intraradices*. The maximum flux rate noted by Nielsen et al. (2002) was based on flux during this most active period, and should thus not be compared as such to the data of Rufyikiri et al. (2004c). Nielsen et al. (2002) further elegantly demonstrated P transfer from fungal cells to root cells by using two imaging techniques carried out on actively growing monoxenic cultures at 24-h intervals. For a start, the cultures were photographed with a digital camera and thereafter, they were placed in a digital scintillation imager which registered the location and intensity of ^{32}P irradiation over 6 h. When the two images were superimposed, the ^{32}P activity could be quantified at precise locations, i.e. in < 1-mm-long root segments. By comparing images recorded on consecutive days, growing root apexes were observed to contain significant amounts of ^{32}P. Since actively growing root apexes were not mycorrhizal, the activity emanating from them could only be contained within plant cells, thus following P transfer from the fungus to the host.

2.2 Nitrogen

Nitrogen is perhaps the element which limits plant growth to the largest extent in most terrestrial ecosystems, but N transport has still received far less attention than P transport in the mycorrhizal literature (Hawkins et al. 2000). One reason for this may be the high cost and cumbersome analysis of the stable isotope ^{15}N which is used as a tracer in such studies. Nitrogen transport by AM fungi was first demonstrated in compartmented pots by Ames et al. (1983), using ^{15}N-labelled NH_4. The mean N flux rate through hyphae of *G. mosseae* over a 30-day labelling period was calculated to be 7.4×10^{-8} mol cm^{-2} s^{-1}, and accounted for 25% of the added ^{15}N. This value was quite similar to the P flux value of 3.8×10^{-8} mol cm^{-2} s^{-1} obtained with soil-based systems some years earlier (Sanders and Tinker 1973). The amount of N transported to the host plant was highly correlated to the number of hyphae crossing into the labelling compartment, and to the total hyphal length in the labelling compartment at harvest. In later experiments, transport of NH_4, NO_3 and amino acids (Hawkins et al. 2000) has been demonstrated in different compartmented systems featuring whole plants on solid substrates. In monoxenic cultures, uptake and translocation have been demonstrated for the amino acids glycine and glutamate, with

the highest uptake/translocation efficiency observed for glycine (Hawkins et al. 2000). Further, it was shown that NO_3 was a competing N source which reduced N uptake from amino acids when present in similar concentrations. The use of a proton gradient uncoupler (carbonyl cyanide m-chlorophenyl hydrazone) to distinguish between active and passive uptake of amino acids into hyphae showed that uptake was only partially an active process, and that as much as 50% of the absorbed ^{15}N from glycine and glutamate was entering the hyphae, in spite of the lack of a proton gradient. This was also the case for the uptake of ammonium, whereas the uptake of nitrate was mainly a passive process (Hawkins et al. 2000). In their experiments on N uptake in monoxenic cultures, the comparison of root uptake and hyphal uptake showed that roots were apparently far more efficient in the absorption of both amino acids, but in these comparisons no account was made for the fact that hyphal biomass, and particularly the hyphal cross-section area available for translocation, was far lower than for roots (Hawkins et al. 2000). Contrary to amino acids, for which both hyphal uptake and translocation have been shown, only uptake has been demonstrated for NH_4 (Villegas et al. 1996) and NO_3 (Bago et al. 1996). These experiments were carried out by analysing remaining N in a hyphal compartment, without any regard for whether N was adsorbed or absorbed by hyphae, nor to which extent absorbed N was translocated towards roots. The capacity of hyphae to extrude OH^- to maintain electrochemical charge when N was taken up as NO_3^- was clearly demonstrated (Bago et al. 1996), and complements the previous finding of Li et al. (1991) that extraradical hyphae can extrude H^+ when NH_4^+ is the dominant form of N taken up from soil. It is known that uptake of NH_4 and NO_3 is to some extent followed by incorporation of N into amino acids, as demonstrated in excised hyphae (Johansen et al. 1996). But overall very little precise information is available on the N transport processes (see Hawkins et al. 2000). There is thus a great potential in describing N transport based on monoxenic cultures.

3 Non-Essential Element Uptake and Translocation by AM Fungi

3.1 Uranium

Uranium is the most abundant of the naturally occurring actinides, the others being actinium, thorium and protactinium. Its concentration in the earth's crust ranges from 1–4 $mg\,kg^{-1}$ in sedimentary rocks, to tens or even

hundreds $mg\,kg^{-1}$ in phosphate-rich deposits (Langmuir 1997; Qureshi et al. 2001) and in U ore deposits (Plant et al. 1999). Although there is no known biological function of U, the accumulation of this element has been reported in living organisms including plants (Ebbs et al. 1998; Huang et al. 1998; Shahandeh et al. 2001; Rufyikiri et al. 2004a), bacteria, algae, lichens and fungi (Abdelouas et al. 1999; Suzuki and Banfield 1999). Among fungi, recent studies, using monoxenic culture systems, demonstrated the ability of AM fungi to take up U (Fig. 1; Rufyikiri et al. 2002, 2003, 2004c). This uptake was influenced by the pH of the growth medium in the HC. Indeed, Rufyikiri et al. (2002) demonstrated that U uptake by the ERM of the AM fungus *G. intraradices* was two times higher at pH 5.5 than at pH 4 or pH 8.

The pH of the bathing solution contained in the HC is a determinant factor on the process of U uptake because of its simultaneous effects on surface charges of the mycelium (Gadd 1990; Zhou 1999) and on U speciation (Grenthe et al. 1992; Suzuki and Banfield 1999). Dominant U species in the HC were predicted within the pH range 4.0–8.0 (Bethke 2001), and the thermodynamic data of U (Grenthe et al. 1992). These dominant species were identified as uranyl cation and uranyl-sulphate at pH 4.0, uranyl-phosphate at pH 5.5, and anionic uranyl-carbonate at pH 8.0 (Rufyikiri et al. 2002). Furthermore, living hyphae can induce pH changes due to NH_4^+ and NO_3^- uptake, thereby affecting U speciation. An increase in pH of the growth medium was reported when the extraradical hyphae of *G. intraradices* developed in the presence of NO_3 as source of N, either in the absence (Bago et al. 1996) or in the presence of U (Rufyikiri et al. 2003), while a decrease is to be expected in the presence of NH_4 as source of N.

The relative extent of U uptake by ERM was compared to that of mycorrhizal and non-mycorrhizal roots (Rufyikiri et al. 2004c), and by comparing the hyphal U uptake to that of P (Rufyikiri et al. 2003). It was observed that the U concentrations for hyphae were 5.5 and 9.7 times higher than for mycorrhizal roots and non-mycorrhizal roots respectively. The higher U concentration in fungal mycelium than in roots could partially be explained by differences in their respective cation exchange capacity (CEC), which were reported to be four times higher for AM mycelium ($187\ cmol_c\,kg^{-1}$ dry weight) than for the carrot host roots ($47\ cmol_c\,kg^{-1}$ dry weight; Rufyikiri et al. 2003). At the same time, Cu-extractable U was 15 times higher for AM fungal mycelia than for carrot roots. However, the contribution of the CEC to the U accumulation appeared to be low, as the Cu-extractable U represented only 6 and 15% of the total U contents in roots and mycelia respectively. A large proportion, representing 47 and 67% of the U contents of carrot roots and AM fungal mycelia respectively, could not be desorbed after successive treatments with 0.01 M $CuSO_4$, 0.01 M HCl and 0.1 M HCl. This suggested that other mechanisms of accumulation in hyphae and

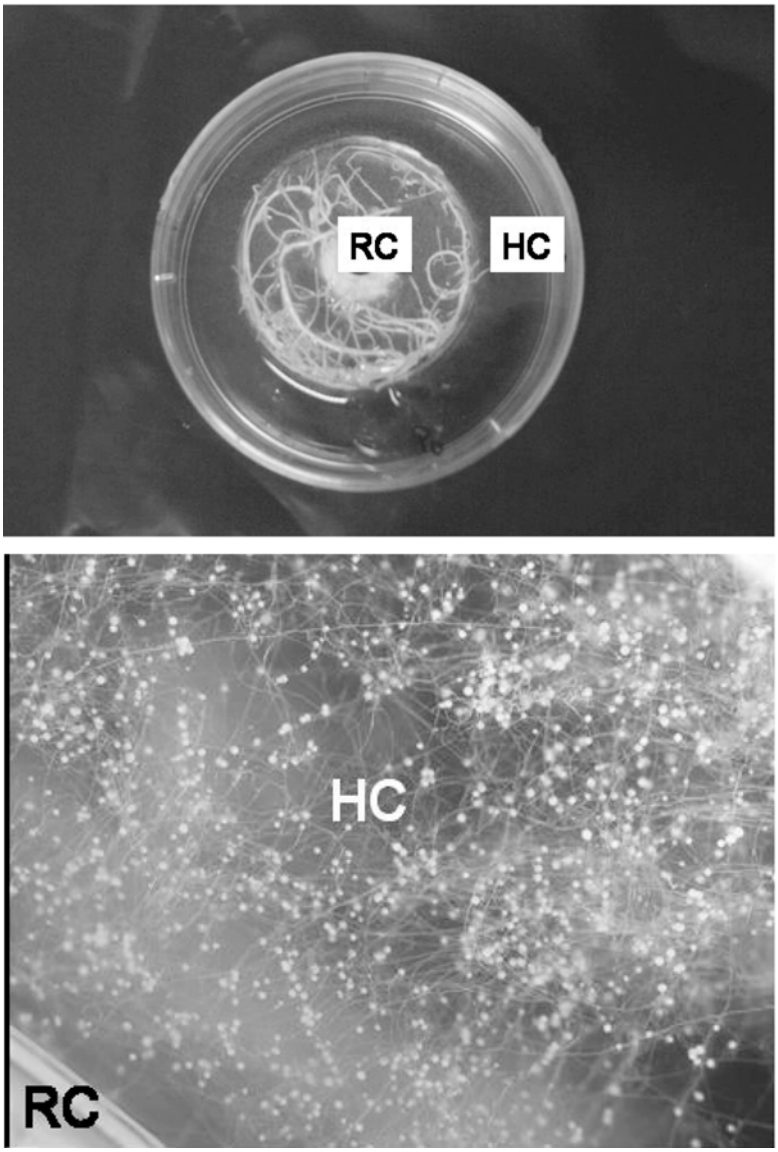


Fig. 1a, b. A bi-compartment monoxenic culture system allowing the spatial separation of a central root compartment (*RC*) for the growth of mycorrhizal roots from a neighbouring external hyphal compartment (*HC*) where extraradical mycelia and/or roots were allowed to grow (**a**). The synthetic growth medium was solid in the RC, while the HC contained a liquid growth medium lacking sucrose and vitamins. Numerous hyphae crossed the partition between the RC and HC. Once in contact with the liquid growth medium, an abundant branched mycelium developed, and thousands of spores were produced. Magnified view of hyphae and spores in the HC **b**

roots were involved, and the formation of stable complexes or precipitates seemed to be the main mechanism of U accumulation in fungal hyphae and roots.

Despite the relatively high capacity of hyphae to take up U, the total fraction of U removed from the labelled medium by the hyphae was very low compared to the capacity of the same hyphae to take up P (Rufyikiri et al. 2004c).

The monoxenic culture system in bi-compartment Petri plates also allowed to demonstrate that the ERM of AM fungi can translocate U towards the host roots (Rufyikiri et al. 2002). These authors observed that the amount of translocated U was higher at pH 4 than at pH 5.5 or pH 8. It was suggested that soluble uranyl cations or uranyl-sulphate species which are stable under acidic conditions were translocated to a higher extent by the fungal hyphae, while phosphate and hydroxyl species, dominating under acidic to near-neutral conditions or carbonate species dominating under alkaline conditions, were rather immobilized by hyphal structures. The efficiency of AM fungal hyphae to translocate U appeared higher than that of carrot roots grown under the same experimental conditions (Rufyikiri et al. 2003). Indeed, on the basis of the average diameter of hyphae (11 µm; Nielsen et al. 2002) and roots (1000 µm), and the number of hyphae (147) and roots (5) connecting the HC to the RC, the total section area of roots at the crossing point was 281-fold higher than that calculated for hyphae, while the amounts of U translocated were 9 times higher for hyphae than for roots (Rufyikiri et al. 2004b).

In another experiment, the efficiencies of hyphae to translocate U and P were compared (Rufyikiri et al. 2004c). To do so, the liquid medium in the HC was labelled with both ^{233}U (total concentration of 0.1 µM) and ^{33}P (in a concentration 50 µM of non-radioactive P). After 2 weeks of contact, 9.8 and 79% of U and ^{33}P initially supplied were translocated to the RC via hyphae respectively. High hyphal efficiency for P translocation was reported in other studies. Cooper and Tinker (1978) compared the uptake and translocation of ^{32}P, ^{65}Zn and ^{35}S by the AM fungus *G. mosseae* with *Trifolium repens* L. as host growing in bi-compartment Petri plates. They found that the molar amounts of P, S and Zn translocated were in the ratio 35:5:1.

The addition of formaldehyde (2% v/v) to the solutions in the HC, 24 h before U was supplied, killed the mycelia. These formaldehyde-killed AM hyphae accumulated U but did not translocate it to roots developing in the root compartment (Rufyikiri et al. 2002, 2003, 2004c). Thus, the U translocation by the extraradical fungal hyphae was not a passive process.

3.2 Caesium

Because of its long half-life (30.2 years), high mobility and chemical behaviour similar to that of potassium, ^{137}Cs is one of the most threatening radio-pollutants released in the environment by nuclear weapon testing (1950s and 1960s) and nuclear accidents (e.g. Chernobyl, 1986; Avery 1996). The chemical similarities between radiocaesium and potassium, the latter being a major plant nutrient, imply an important risk of contamination of the aboveground vegetation (Korobova et al. 1998), as root uptake mechanisms appear to be closely related for these elements (White and Broadley 2000; Zhu and Smolders 2000). Furthermore, as rhizospheric processes involving soil micro-organisms influence root uptake of radiocaesium (Gadd 1996), the obligate AM fungal symbionts could play a key role in plant uptake of radiocaesium (Entry et al. 1996, 1999), even if their participation in plant K nutrition appears to be controversial (Smith and Read 1997). Indeed, if recent results show that K is taken up by extraradical hyphae and could be translocated concurrently with P (Ezawa et al. 2002), transfer of K is not yet demonstrated. Furthermore, Ryan et al. (2003) suggested that the high concentrations of K in intraradical hyphae and arbuscule trunk hyphae could play the role of balancing cation for the predominant P forms, and could be involved in maintaining high hyphal turgor to help hyphae invaginate the root cell plasma-membrane. Therefore, following uptake and translocation, radiocaesium could be accumulated in AM intraradical structures while implicated in these processes. The hypothesis of Berreck and Haselwandler (2001) that AM fungi could sequester radiocaesium radiocaesium could consequently find some support.

In order to decipher the role of AM fungi in the acquisition of radiocaesium by plants, studies have been performed using various in vivo cultivation systems with contaminated soils or nutrient solutions. However, the results obtained have given rise to different conclusions. For instance, Dighton and Terry (1996) observed an increased concentration of radiocaesium in AM *Festuca ovina*, but a decrease in AM *Trifolium repens*. Since plain pot experiments like these assess not only Cs transport but also a whole range of mycorrhizal effects including confounding growth effects, studies on hyphal transport in compartmented pots with soil were undertaken by Joner et al. (2004). Here, different fungus/host plant combinations were used together with double labelling with radiocaesium and either $^{32/33}P$ or ^{65}Zn to verify symbiotic transport activity. The results from three independent experiments unanimously showed that Cs was not transported to the host plants by the AM fungi *G. mosseae* and two different isolates of *G. intraradices*, while all three fungi were highly active in nutrient transport. However, the pot experimental conditions might not have been met

to demonstrate whether AM fungi can actually transport Cs or sequester it. Therefore a clear identification of the transport and sequestration of Cs by AM fungi was required. Monoxenic cultures of AM fungi were considered as the optimal mean to achieve this as K concentrations in the media can be precisely determined and the bioavailability of Cs does not depend on the presence of other micro-organisms or soil characteristics. The monoxenic culture systems were similar to those described for P, N ans U transport studies, but the MSR medium was slightly adapted to decrease the concentration of K. Indeed, increasing the concentration of K in the soil solution results in the rapid decrease in radiocaesium uptake by plants (Smolders et al. 1996, 1997; Delvaux et al. 2001), and analogy of the effect of K concentration was thus assumed for AM radiocaesium uptake. The concentration of K in the RC was thus reduced to 0.18 mM, while the liquid MSR medium in the HC was K-free. Declerck et al. (2003) demonstrated that the ERM of *G. lamellosum* could take up and translocate radiocaesium towards their host root. The uptake represented 1.5% of the initial radiocaesium supply, and 55% of the radiocaesium taken up was translocated towards the roots contained in the RC. However, it was suggested by the authors that the translocation of radiocaesium might have been partially impaired by the low number of hyphae crossing the partition wall between the RC and HC, as the radiocaesium concentration of the ERM (Bq/cm of hyphae) in the HC was much higher than that of the ERM in the RC. Consequently, a slightly modified system was employed in a second study (Dupré de Boulois et al., 2005, in press) in order to assess Cs uptake and translocation under conditions of increased number of hyphae crossing the partition wall, and higher fungal biomass in HC. These authors used pre-colonised, actively growing mycorrhizal roots instead of roots which had to be inoculated with AM fungal spores, and raised the level of the solid medium in the RC to 2 mm above the partition wall, as described by Rufyikiri et al. (2003). The length of hyphae in the RC and HC was increased four and six times respectively compared to those of Declerck et al. (2003), and the number of hyphae crossing the partition wall reached 39, which was comparable to the study by Nielsen et al. (2002) on P transport. The uptake of radiocaesium thus reached 5.2% of the initial radiocaesium supply. From this uptake, 81% of the radiocaesium was translocated to the roots contained in the RC. Accordingly, this study confirmed that AM fungi can translocate radiocaesium towards their host root. Furthermore, statistical analysis showed that the uptake of radiocaesium was positively correlated with the length of hyphae contained in the HC, and that radiocaesium translocation by AM fungal hyphae was correlated to the number of hyphae crossing the partition wall. The rather low translocation of radiocaesium in the study of Declerck et al. (2003), and the apparent high accumulation of radiocaesium in the ERM of the HC could thus be explained by a bottleneck

effect due to the low number of hyphae crossing the partition wall. Still, a substantial sequestration of radiocaesium in the ERM may be possible, as the radiocaesium content of the ERM of the HC was positively correlated with the length of hyphae present in this compartment (Declerck et al. 2003).

In the study of Dupré de Boulois et al. (2005, in press), the relative contribution of AM hyphae, mycorrhizal and non-mycorrhizal roots to the uptake and translocation of radiocaesium was compared. The results obtained showed that if mycorrhizal or non-mycorrhizal roots could take up 33% of the initial radiocaesium supply, its translocation was similar to that of the ERM alone. While 81% of the radiocaesium taken up by the hyphae alone was translocated to the roots in the RC, only 19 and 16% of the radiocaesium taken up by mycorrhizal and non-mycorrhizal roots respectively were translocated towards the roots contained in the RC. The authors thus concluded that AM fungal hyphae have a high radiocaesium translocation capacity compared to roots. However, the low translocation of radiocaesium within the roots could be linked to the lack of shoots in the monoxenic system, which would act as a sink for radiocaesium. Another aspect which might have reduced radiocaesium translocation in roots is the proximity of actively growing root apexes. It has been observed that K taken up by the first centimetres behind the root apexes is poorly translocated, and rather allocated to these apexes (Richter and Marschner 1973). Furthermore, the low K supply might have inhibited the KOR (potassium outwards-rectifying) channels which are responsible for the efflux of radiocaesium from the root cells. This inhibition was suggested to prevent leakage of K from the root cells (Maathuis and Sanders 1997), and could have consequently decreased radiocaesium loading into the xylem.

Finally, Dupré de Boulois et al. (2005, in press) observed a positive correlation between the radiocaesium content of the mycorrhizal roots in contact with radiocaesium and the frequency of root length colonization in the labelled compartment, and a negative correlation for the roots in the RC. On the basis of these results, they suggested that AM intraradical fungal structures might reduce translocation of radiocaesium within the roots.

4 Conclusion

This chapter has synthesized available information on monoxenic studies of element uptake and translocation by AM fungi. Compartmented monoxenic culture systems using root organs were shown to be useful in studies involving essential elements such as P and N, and non-essential

ones such as the radionuclides U and Cs. Results published showed that AM fungi are able to take up and translocate these elements. However, the relative extent of these processes depends on the nature of the element, and on other factors such as the pH of the growth media. The transport and sequestration of radionuclides by the extraradical mycelium can have an ecological significance, as AM fungi are part of the rhizospheric biomass. Mycorrhizal fungi can thus play a role in the interception of radionuclides which may temporarily influence their migration in the soil profile and their accumulation by growing plants.

The monoxenic culture system could also be used to characterize different AM fungi with respect to transport efficiency, to study the capacity of fungi to exploit certain forms of a nutrient (precipitated P, organic forms of N or P), and to verify transport of elements for which the role of mycorrhiza is less well or not yet documented.

References

Abdelouas A, Lutze W, Nuttall HE (1999) Uranium contamination in the subsurface: characterization and remediation. In: Burns PC, Finch R (eds) Uranium: mineralogy, geochemistry and the environment. Mineralogy Society of America, Washington, DC, Reviews in Mineralogy, pp 433–447

Ames RN, Reid CPP, Porter LK, Cambardella C (1983) Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus. New Phytol 95:381–396

Avery SV (1996) Fate of caesium in the environment: distribution between the abiotic and biotic components of aquatic and terrestrial ecosystems. J Environ Radioact 30:139–171

Bago B, Vierheilig H, Piché Y, Azcon-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bago B, Azcon-Aguilar C, Goulet A, Piché Y (1998) Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. New Phytol 139:375–388

Berreck M, Haselwandter K (2001) Effect of the arbuscular mycorrhizal symbiosis upon uptake of cesium and other cations by plants. Mycorrhiza 10:275–280

Bethke C (2001) The Geochemist's Workbench, release 3.2. A user's guide to Rxn, Act2, Tact, React, and Gtplot. University of Illinois Hydrogeology Program, University of Illinois

Cooper KM, Tinker PB (1978) Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. II. Uptake and translocation of phosphorus, zinc and sulphur. New Phytol 81:43–52

Declerck S, Dupré de Boulois H, Bivort C, Delvaux B (2003) Extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus lamellosum* can take up, accumulate and translocate radiocaesium under root-organ culture conditions. Environ Microbiol 5:510–516

Delvaux B, Kruyts N, Maes E, Smolders E (2001) Fate of radiocesium in soil and rhizosphere In: Gobran GR, Wenzel WW, Lombi E (eds) Trace elements in the rhizosphere. CRC Press, Bocan Raton, pp 61–91

Dighton J, Terry GM (1996) Uptake and immobilization of caesium in UK grassland and forest soils by fungi following the Chernobyl accident. In: Frankland JC, Magan N,

Gadd GM (eds) Fungi and environmental change. Cambridge University Press, Cambridge, pp 184–200
Dupré de Boulois H, Delvaux B, Declerck S (2005) Contributions of extraradical mycelium and roots to radiocaesium uptake and translocation by arbuscular mycorrhizal carrot roots under root organ culture conditions. Environmental Pollution (in press)
Ebbs SD, Brady DJ, Kochian LV (1998) Role of uranium speciation in the uptake and translocation of uranium by plants. J Exp Bot 49:1183–1190
Entry JA, Vance NC, Hamilton MA, Zabowski D, Watrud LS, Adriano DC (1996) Phytoremediation of soil contaminated with low concentrations of radionuclides. Water Air Soil Pollut 88:167–176
Entry JA, Astrud LS, Reeves M (1999) Accumulation of ^{137}Cs and ^{90}Sr from contaminated soil by three grass species inoculated with mycorrhizal fungi. Environ Pollut 104:449–457
Ezawa T, Smith SE, Smith FA (2002) P metabolism and transport in AM fungi. Plant Soil 244:221–230
Gadd GM (1990) Heavy metal accumulation by bacteria and other microorganisms. Experientia 46:834–840
Gadd GM (1996) Role of microorganisms in the environmental fate of radionuclids. Endeavour 20:150–156
Grenthe I, Fuger J, Konings RJM, Lemire RJ, Miller AB, Nguyen-Trung C, Wanner H (1992) Chemical thermodynamics of uranium. OECD-NEA, Elsevier, North-Holland, Amsterdam
Hattingh MJ, Gray LE, Gerdemann JW (1973) Uptake and translocation of ^{32}P-labelled phosphate to onion roots by endomycorrhizal fungi. Soil Sci 116:383–387
Hawkins H, Johansen A, George E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226:275–285
Huang JW, Blaylock MJ, Kapulnik Y, Ensley BD (1998) Phytoremediation of uranium-contaminated soils: role of organic acids in triggering uranium hyperaccumulation in plants. Environ Sci Technol 32:2004-2008
Johansen A, Finlay RD, Olsson PA (1996) Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol 133:705–712
Joner EJ, Ravnskov S, Jakobsen I (2000) Arbuscular mycorrhizal phosphate transport under monoxenic conditions using radio-labelled inorganic and organic phosphate. Biotechnol Lett 22:1705–1708
Joner EJ, Roos P, Jansa J, Frossard E, Leyval C, Jakobsen I (2004) No significant contribution of arbuscular mycorrhizal fungi to transfer of radiocesium from soil to plants. Appl Environ Microbiol 70:6512–6517
Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. New Phytol 148:511–517
Korobova E, Ermakov A, Linnik V (1998) Cs-137 and Sr-90 mobility in soils and transfer in soil-plant systems in the Novozybkov district affected by the Chernobyl accident. Appl Geochem 13:803–814
Langmuir D (1997) Aqueous environmental geochemistry. Prentice Hall, Upper Saddle River, NJ
Li XL, George E, Marschner H (1991) Phosphorus depletion and pH decrease at the root-soil and hyphae-soil interfaces of VA mycorrhizal white clover fertilized with ammonium. New Phytol 119:397–404
Maathuis FJM, Sanders D (1997) Regulation of K+ absorption in plant root cells by external K+: interplay of different plasma membrane K+ transporters. J Exp Bot 48:451–458
Nielsen JS, Joner EJ, Declerck S, Olsson S, Jakobsen I (2002) Phospho-imaging as a tool for visualization and noninvasive measurement of P transport dynamics in arbuscular mycorrhizas. New Phytol 154:809–820

Pearson V, Tinker PB (1975) Measurement of phosphorus fluxes in the external hypophae of endomycorrhizas. In: Sanders FE, Mosse B, Tinker PB (eds.) Endomycorrhizas. Acedemic Press, London, pp. 277–287

Plant JA, Simpson PR, Smith B, Windley BF (1999) Uranium ore deposits-products of the radioactive earth. In: Burns PC, Finch R (eds) Uranium: mineralogy, geochemistry and the environment. Mineralogy Society of America, Washington, DC, Reviews in Mineralogy, pp 255–319

Qureshi AA, Khattak NU, Sardar M, Tufail M, Akram M, Iqbal T, Khan HA (2001) Determination of uranium contents in rock samples from Kakul phosphate deposit, Abbotabad (Pakistan), using fission-track technique. Radiat Meas 34:355–359

Richter C, Marschner H (1973) Turnover of potassium in different root zones of corn seedlings.Z Pflanzenernähr Bodenk 70:211–221

Rufyikiri G, Thiry Y, Wang L, Delvaux D, Declerck S (2002) Uranium uptake and translocation by the arbuscular mycorrhizal fungus, *Glomus intraradices*, under root-organ culture conditions. New Phytol 156:275–281

Rufyikiri G, Thiry Y, Declerck S (2003) Contribution of hyphae and roots to uranium uptake and translocation by arbuscular mycorrhizal carrot roots under root-organ culture conditions. New Phytol 158:391–399

Rufyikiri G, Huysmans L, Wanijn J, Van Hees M, Leyval C, Jakobsen I (2004a) Arbuscular mycorrhizal fungi can decrease the uptake of uranium by subterranean clover grown at high levels of uranium in soil. Environ Pollut 130(3):427–436

Rufyikiri G, Thiry Y, Declerck S (2004b) Uptake and translocation of uranium by arbuscular mycorrhizal fungi under root-organ culture conditions. In: Huang PM (ed) Biogeochemisty of trace elements in the rhizosphere. Elsevier, Amsterdam (in press)

Rufyikiri G, Declerck S, Thiry Y (2004c) Comparison of ^{233}U and ^{33}P uptake and translocation by the arbuscular mycorrhizal fungus *Glomus intraradices* in root organ culture conditions. Mycorrhiza 14:203–207

Ryan MH, McCully ME, Huang CX (2003) Location and quantification of phosphorus and other elements in fully hydrated, soil-grown arbuscular mycorrhizas: a cryo-analytical scanning electron microscopy study. New Phytol 160:429–441

Sanders FE, Tinker PB (1973) Phosphate flow in mycorrhizal roots. Pest Sci 4:385–395

Shahandeh H, Lee JH, Hossner LR, Loeppert RH (2001) Bioavailability of uranium and plutonium to plants in soil-water systems and the potential of phytoremediation. In: Gobran GR, Wenzel WW, Lombi E (eds) Trace elements in the rhizosphere. CRC Press LLC, London, pp 93–124

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, San Diego

Smith SE, Smith FA (1990) Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. New Phytol 114:1–38

Smolders E, Kiebooms L, Buysse J, Merckx R (1996) Cs-137 uptake in spring wheat (*Triticum aestivum* L. cv. Tonic) at varying K supply. I. The effect in soil solution culture. Plant Soil 181:205–209

Smolders E, VandenBrande K, Merckx R (1997) Concentrations of Cs-137 and K in soil solution predict the plant availability of Cs-137 in soils. Environ Sci Technol 31:3432–3438

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332

Suzuki Y, Banfield JF (1999) Geomicrobiology of uranium. In: Burns PC, Finch R (eds) Uranium: mineralogy, geochemistry and the environment. Mineralogy Society of America, Washington, DC, Reviews in Mineralogy, pp 393–432

Villegas J, Williams RD, Nantais L, Archambault J, Fortin JA (1996) Effects of N source on pH and nutrient exchange of extramatrical mycelium in a mycorrhizal Ri T-DNA transformed root system. Mycorrhiza 6:247–251

White PJ, Broadley MR (2000) Mechanisms of caesium uptake by plants. New Phytol 147:241–256

Zhou JL (1999) Zn biosorption by *Rhizopus arrhizus* and other fungi. Appl Microbiol Biotechnol 51:686–693

Zhu YG, Smolders E (2000) Plant uptake of radiocaesium: a review of mechanisms regulation and application. J Exp Bot 51:1635–1645

12 Interaction of Arbuscular Mycorrhizal Fungi with Soil-Borne Pathogens and Non-Pathogenic Rhizosphere Micro-Organisms

Marc St Arnaud[1], Annemie Elsen[2]

1 Introduction

The rhizosphere is a site of complex interactions between plants and microorganisms, where environmental factors such as soil physico-chemical parameters as well as fertilization or cultivation practices may have large effects on microbial and microfauna communities. Interactions between arbuscular mycorrhizal (AM) fungi and other rhizosphere microbes have numerous impacts on the host plant. Among these, the alleviation of root diseases and the facilitated access to nutrients through synergistic interactions with N-fixing and P-solubilizing soil microbes are currently of high interest in sustainable agriculture and for the development of better management practice strategies. It is likely that complex species associations play key roles in the stability of natural ecosystems, and contribute to both plant (van der Heijden et al. 1998) and AM fungal diversity (Johnson et al. 2004). Different soluble molecules, mucilage and gas are lost by roots as exudates, lysates or dead cells. Rhizosphere depositions in particular are determinant in structuring and maintaining microbial populations (Grayston et al. 1997; Knee et al. 2001), yet the concentration of active molecules could be extremely low, if taken back by roots or by rhizosphere microbes. While studies have generally focused on bacteria or fungi, many animals also live in the soil and interact with these organisms (Klironomos et al. 1999; Maraun et al. 2003; Schreiner and Bethlenfalvay 2003; Wamberg et al. 2003a, b), but have received far less attention. Multitrophic studies involving more than two microbial organisms are also scarce in the mycorrhiza literature, especially as related to interaction mechanisms.

[1]Institut de Recherche en Biologie Végétale, Jardin Botanique de Montréal, 4101 East Sherbrooke Street, Montréal, QC H1X 2B2, Canada, Tel.: +1-514-8721439, Fax: +1-514-8729406, E-mail: marc.st-arnaud@umontreal.ca

[2]Katholieke Universiteit Leuven, Laboratory of Tropical Crop Improvement, Kasteelpark Arenberg 13, 3001 Leuven, Belgium

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

The AM in vitro systems, referred to as axenic, monoxenic or dixenic, are powerful tools to scrutinize and highlight the fundamental effects of this ubiquitous symbiosis on rhizosphere microbes, and to characterize the mechanisms regulating the plant–microbe as well as microbe–microbe interactions. This rather simplified microcosm has important advantages over soil systems. It is by far easier to precisely place the interacting organisms, monitor changes and sample accordingly, also permitting non-destructive in situ observations of the interactions (St-Arnaud et al. 1995, 1996; Elsen et al. 2001; Pfeffer et al. 2001; Bago et al. 2002; Elsen et al. 2003). Each factor can be adjusted and closely controlled, an attractive way to test hypotheses. Even more important, the use of AM in vitro systems is the most effective way to achieve monoxenic or dixenic experimental conditions with AM fungi, as well as to resolve the problem of appropriate AM fungal controls, since it is now accepted that non-axenically produced AM inocula frequently harbour an associated and often unnoticed microflora (Ruiz-Lozano and Bonfante 2000; Xaviera and Germida 2003). Close associations of specific bacteria with the AM mycelium were often described (Bianciotto et al. 1996b; Bianciotto et al. 2000; Gryndler et al. 2000; Minerdi et al. 2002; Artursson and Jansson 2003; Levy et al. 2003). From this perspective, AM in vitro systems allow the isolation of the organisms under study from other biotic and abiotic effects present in most, if not all, other experimental systems. Of course, the observations gained from such simplified microcosms should always be confirmed in soil mycorrhizosphere systems in order to avoid studying in vitro artefacts. While the understanding of the complex relations between AM fungi, other components of the soil microbial biomass and plants is a prerequisite for sustainable development, the AM in vitro systems are key tools to unravel complex and multitrophic interactions between soil inhabitants.

2
Interaction Between AM Fungi and Soil Bacteria

When the first in vitro growth of AM fungi was achieved more than 40 years ago (Mosse 1962), it was reported that a *Pseudomonas* isolate was essential for AM root colonization and growth of the AM fungus under aseptic conditions. Various types of growing medium filtrates showed a similar effect. Later, many other soil bacteria were shown to promote AM fungi spore germination and hyphal growth, either with or without direct contact between the organisms (Table 1). For example, unidentified bacteria isolated from soil, as well as spore-associated bacteria including *Corynebacterium* sp. and *Pseudomonas* sp. enhanced *Glomus mosseae* and *G. versiforme* spore germination, hyphal growth and sporulation in vitro (Mayo et al. 1986; Azcón

Table 1. Interaction between AM fungi and bacteria in vitro

Bacteria species[a]	AMF species[a]	Interaction type	Reference
Azospirillum brasilense	*Glomus intraradices*	Neutral	Hildebrandt et al. (2002)
Bacillus chitinosporus; *B. pabuli* and other spore-associated bacteria	*G. clarum*	Positive, neutral or negative	Xavier and Germida (2003)
Clavibacter michiganensis ssp. *michiganensis*	*G. intraradices*	Neutral	Filion et al. (1999)
Corynebacterium sp.	*G. versiforme*	Positive	Mayo et al. (1986)
Escherichia coli	*G. intraradices*	Neutral	Hildebrandt et al. (2002)
Paenibacillus validus	*G. intraradices*	Positive	Hildebrandt et al. (2002)
Pseudomonas sp.	*Endogone* sp.	Positive	Mosse (1962)
Pseudomonas sp.	*G. versiforme*	Positive	Mayo et al. (1986)
P. aeruginosa	*G. intraradices*	Positive	Villegas and Fortin (2001, 2002)
P. chlororaphis	*G. intraradices*	Positive	Filion et al. (1999)
P. fluorescens	*Gigaspora margarita*	Positive	Bianciotto et al. (1996b)
P. putida	*G. intraradices*	Positive or neutral	Villegas and Fortin (2001, 2002)
Rhizobium leguminosarum	*Gi. margarita*	Positive	Bianciotto et al. (1996b)
Serratia plymutica	*G. intraradices*	Neutral	Villegas and Fortin (2001, 2002)
Streptomyces avermitilis	*G. mosseae*	Positive	Tylka et al. (1991)
S. avermitilis	*Scutellospora heterogama*	Negative	Tylka et al. (1991)
S. griseus	*G. mosseae*	Positive	Tylka et al. (1991)
S. orientalis	*Gi. margarita*	Positive	Mugnier and Mosse (1987); Tylka et al. (1991)
S. orientalis	*G. mosseae*	Positive	Tylka et al. (1991)
S. orientalis	*S. heterogama*	Negative or positive	Tylka et al. (1991)
Spore-associated bacteria	*G. versiforme*	Positive	Mayo et al. (1986)
Unidentified soil bacteria	*G. mosseae*	Positive	Azcón (1987, 1989)

[a] Species names are those used in the cited references

1987, 1989). *Streptomyces avermitilis*, *S. griseus* and *S. orientalis* were also shown to increase spore germination of *Gigaspora margarita* or *G. mosseae* (Mugnier and Mosse 1987; Tylka et al. 1991). Azcón (1987) further reported that cell-free fractions from rhizosphere bacteria cultures have the same stimulatory effect as living bacteria. These results were used to support the involvement of volatile or diffusible factors excreted by bacteria (Azcón-Aguilar et al. 1986; Azcón 1989). On the other hand, *S. avermitilis* and *S. orientalis* were shown to suppress *Scutellospora heterogama* spore germination when grown in the same growth compartment, but to increase germination in different compartments (Tylka et al. 1991). In this case, the inhibitory effect was attributed to a pH increase in the growth medium, caused by bacterial growth, while the stimulatory effect in a different compartment was postulated to result from a volatile, but without further direct evidences.

Some bacteria were shown to live in close association with AM fungi, and even as obligatory intracellular endophytes of *Gigaspora margarita* spores (Bianciotto et al. 1996a). Other species, such as *Pseudomonas fluorescens* and *Rhizobium leguminosarum*, adhere to and colonize the surface of germinating spores and growing hyphae (Bianciotto et al. 1996b). While these results support the observation that interactions between AM fungi and rhizosphere bacteria may be mediated by either soluble factors or physical contact, these authors hypothesized that AM fungi may be a vehicle for the colonization of plant roots by soil rhizobacteria. Recently, Hildebrandt et al. (2002) have reported that *Paenibacillus validus* frequently develops on surface-sterilized spores of *Glomus intraradices*. The bacteria supported the growth of the fungus on the agar Petri plates, and induced hyphal branching, development of coiled structures and production of new spores. Under the same conditions, *Escherichia coli* and *Azospirillum brasilense* did not display any similar effect. Among various bacteria isolated from spores of *Glomus clarum*, Xavier and Germida (2003) found that most bacteria did not alter AM fungus spore function, while some bacteria inhibited or stimulated spore germination. Moreover, they reported that stimulation of spore germination occurred only when bacteria were in contact with spores, and inhibition of spore germination was the result of volatile bacterial metabolites.

Using the split-plate approach (following St-Arnaud et al. 1995, 1996) to grow *G. intraradices* extraradical mycelia separated from the colonized roots, Filion et al. (1999) concentrated crude extracts from the growing medium of the extraradical mycelial compartment, presumably containing soluble biologically active substances, and tested these against various soil bacteria and fungi. Growth of *Pseudomonas chlororaphis* was stimulated while *Clavibacter michiganensis* was unaffected. Two fungal species were also differentially affected (see below). The measured effects were generally

in direct correlation with extract concentrations. In this case, volatiles were not involved, since the extracts were previously lyophilized before dilution to the desired concentration. Differences in pH were noted between the extracts from the AM fungus mycelium colonized media and the non-AM control. However, no significant pH influence was noted on bacterial growth, which strongly suggested that non-volatile substances, released by the AM fungus in the growth medium, were the main factor explaining differential growth of the micro-organisms tested.

The AM in vitro systems were also helpful to study the capacity of the extraradical mycelium of AM fungi to interact with soil bacteria to take up insoluble forms of phosphate. Using the two-compartment Petri plate approach with NH_4^+ or NO_3^- as N sources, both *G. intraradices* colonized transformed carrot roots or AM fungus extraradical mycelium alone significantly altered the pH of the growth medium, but the magnitude and direction of this change were dependent on the N source available. While in the presence of NH_4^+ the pH was reduced from 5.5 to around 4.5, when NO_3^- was used, pH was increased from 5.5 to 8.0 after 13 weeks of growth. However, these pH changes were not sufficient to solubilize Ca-P in the presence of the AM fungus alone. On the other hand, species-specific interactions were obtained when *G. intraradices* was grown along with *Pseudomonas aeruginosa, P. putida* or *Serratia plymutica*. While the inherent ability of the fungus and the bacteria to solubilize a recalcitrant form of Ca-P was low, *P. aeruginosa* and *P. putida* interacting with the extraradical mycelium markedly increased P availability in the growth medium, and this increase was dependent on the N source (Villegas and Fortin 2001, 2002).

3
Interaction Between AM Fungi and Other Fungi

To the best of our knowledge, no data reported interactions between AM fungi and other fungal species in monoxenic cultures before the 1990s, when different research groups undertook to study the effect of various soil fungi on AM fungi spore germination in vitro, and vice versa (Table 2). Calvet et al. (1992) examined the effect of six fungal species isolated from various organic substrates on *G. mosseae* spore germination and hyphal growth. They observed that *G. mosseae* development was stimulated by two isolates of *Trichoderma aureoviride* and one *T. harzianum*, while isolates of *Aspergillus fumigatus* and *Penicillium decumbens* inhibited AM fungus germination. McAllister et al. (1994, 1995, 1996) reported similar species-specific interactions. They analysed the effect of several soil fungi on *G. mosseae* spore germination and growth, as well as the effect of the AM fungus germinating spores on saprobe development. *Aspergillus niger*

Table 2. Interaction between AM fungi and other fungi species in vitro

Fungus species[a]	AMF species[a]	Interaction type	Reference
Alternaria alternata	*G. mosseae*	Negative, neutral or positive	McAllister et al. (1996)
Aspergillus fumigatus	*G. mosseae*	Negative	Calvet et al. (1992)
A. niger	*G. mosseae*	Negative	McAllister et al. (1995)
Bipolaris sorokiniana	*Gi. margarita*[b]	Neutral	Chabot (1991)
Fusarium equiseti	*G. mosseae*	Negative, neutral or positive	McAllister et al. (1996)
F. oxysporum f. sp. *chrysanthemi*	*G. intraradices*	Negative	Benhamou et al. (1994); Filion et al. (1999)
F. o. chrysanthemi	*G. intraradices*	Positive or negative	St-Arnaud et al. (1995)
F. solani	*Gi. margarita*[b]	Neutral	Chabot (1991)
F. solani	*G. mosseae*	Neutral or positive	McAllister et al. (1994)
Gaeumannomyces graminis	*Gi. margarita*[b]	Neutral	Chabot (1991)
Gliocladium roseum	*G. mosseae*	Neutral	Fracchia et al. (1998)
Ophiostoma ulmi	*Gi. margarita*[b]	Neutral	Chabot (1991)
Paedilomyces farinosus	*G. mosseae*	Positive or neutral	Fracchia et al. (1998)
Penicillium decumbens	*G. mosseae*	Negative	Calvet et al. (1992)
Phytophthora sp.	*Gi. margarita*[b]	Neutral	Chabot (1991)
P. nicotianae	*G. intraradices*	Negative	Lioussanne et al. (2003)
Pythium ultimum	*Gi. margarita*[b]	Neutral	Chabot (1991)
Pyrenochaeta terrestris			
Rhizoctonia solani			
Rhodotorula mucilaginosa	*G. mosseae* *Gi. rosea*	Positive	Fracchia et al. (2003)
Sclerotinia sclerotiorum	*Gi. margarita*[b]	Neutral	Chabot (1991)
Thielaviopsis basicola			
Trichoderma aureoviride	*G. mosseae*	Positive	Calvet et al. (1992)
T. harzianum	*G. intraradices*	Positive	Filion et al. (1999)
T. harzianum	*G. mosseae*	Positive	Calvet et al. (1992)
T. harzianum	*G. mosseae*	Neutral	Fracchia et al. (1998)
T. harzianum	*G. intraradices*	Negative	Rousseau et al. (1996)
T. koningii	*G. mosseae*	Negative or neutral	McAllister et al. (1994, 1996)
T. pseudokoningii	*G. mosseae*	Neutral	Fracchia et al. (1998)
Verticillium albo-atrum	*Gi. margarita*[b]	Neutral	Chabot (1991)
V. dahliae			
Wardomyces inflatus	*G. mosseae*	Negative	Fracchia et al. (1998)
Unidentified soil fungi	*G. mosseae*	Positive	Azcón-Aguilar et al. (1986)

[a] Species names are those used in the cited references

[b] This isolate is now recognized as *G. rosea* (Bago et al. 1998)

induced a significant decrease in spore germination and length of germinating hyphae of *G. mosseae*, while the AM fungus germinated spores did not affect the growth of the saprophytic fungus on water–agar. Similarly, *Trichoderma koningii* inhibited the germination of *G. mosseae* but did not reduce its mycelial development. *Alternaria alternata* and *Fusarium equiseti* also inhibited AM fungal spore germination but had no effect on, or even induced a marked stimulation of the AM fungus hyphal growth, while *G. mosseae* had no effect on *A. alternata*, *F. equiseti* and *T. koningii* hy-

phal growth. Fracchia et al. (1998) isolated various fungi from sporocarps of *G. mosseae*. The recovered fungal species were *Gliocladium roseum*, *Paecilomyces farinosus*, *Trichoderma pseudokoningii*, *T. harzianum*, *Wardomyces inflatus* and an unidentified, dark sterile mycelium. After inoculation with *G. mosseae* spores on water–agar, *W. inflatus* decreased the percent germination of the AM fungus spores, while *G. roseum*, *T. pseudokoningii* and *T. harzianum* had no effect. *W. inflatus* also significantly decreased germinating hyphal length, but not the other species tested. By contrast, *P. farinosus* and the dark sterile mycelium isolate were shown to increase *G. mosseae* spore germination. More recently, Fracchia et al. (2003) have examined the effect of the soil yeast *Rhodotorula mucilaginosa* on *G. mosseae* and *Gi. Rosea* mycelium growth. Hyphal length of *G. mosseae* and *Gi. rosea* spores increased significantly in the presence of the yeast culture. As for bacteria, the role of soluble and volatile compounds on these interactions was often suggested (Calvet et al. 1992; McAllister et al. 1994, 1996). Here again, Fracchia et al. (2003) clearly showed that the exudates of *R. mucilaginosa* alone, as well as yeast cells, stimulated the hyphal growth of *G. mosseae* and *Gi. rosea* spores. Moreover, an increase in hyphal length of *G. mosseae* coincided with an increase in *R. mucilaginosa* exudates. It appears, therefore, that there is a differential effect of rhizosphere micro-organisms on AM fungi, which might be induced by microbial metabolite production. In these studies, however, the AM fungi were always represented by germinating spores (i.e. at a pre-symbiotic stage and with a minimal biomass), which may underestimate the potential impact of a functional AM fungal mycelial network on other fungi growth dynamics in soil.

To the best of our knowledge, Chabot (1991) was the first to use a well-developed AM fungal mycelium grown on transformed roots in vitro following Bécard and Fortin (1988), to test the effect of a mycorrhizal root system on fungal isolates under dixenic conditions. She used gel plugs containing exudates from a well-colonized root-organ culture of *D. carota* inoculated with *Gi. rosea* (formerly identified as *Gi. margarita* in her work) or from equivalent uninoculated control roots, to test for antibiosis-like effects on 25 isolates from 12 different fungal plant pathogenic species (the species list is detailed in Table 2). No growth inhibition or abnormal hyphal development was observed with any of the tested fungi. However, while the gel plugs contained exudates of a functional extraradical mycelium and of the AM root system, they also contained sucrose, which may have masked any inhibitory effect. Benhamou et al. (1994) also successfully used this in vitro system to study the effect of mycorrhizal colonization on plant disease processes induced by a fungal pathogen. A strong stimulation of disease resistance was shown in mycorrhizal carrot roots when challenged with an isolate of *Fusarium oxysporum* f. sp. *chrysanthemi*. Growth of this pathogen was restricted to the root epidermis and outer cortex. By contrast, exten-

sive growth within the root and degradation of plant cells were observed in non-AM control roots. However, in this study, the carrot roots and AM fungus were bathing in the sugar-containing medium required to sustain root growth. As the fungal pathogen used was normally not pathogenic on carrot under soil conditions, it remains possible that the high level of sugar had enhanced its aggressiveness under these conditions. While the results have clearly shown the potential capacity of AM colonization to enhance host disease responses, they also illustrate the requirement to design an experimental system free of sugar.

With this objective, St-Arnaud et al. (1995) modified the Bécard and Fortin (1988) system to study the direct interaction between *G. intraradices* and soil micro-organisms under monoxenic conditions. The aim was to separate the functional extraradical mycelium network from the root compartment containing sugars and root exudates. This approach was used to study the impact of the functional AM extraradical network on the conidial germination, hyphal growth and sporulation of the root pathogen *F. oxysporum* f. sp. *chrysanthemi*. In the absence of root exudates, spore germination of the pathogenic fungus was strongly enhanced after 5 h of incubation with the active and symbiotic *G. intraradices* mycelium. Hyphal growth of the pathogen was also significantly, albeit slightly enhanced, whereas significant negative correlations were found between new conidia formation and the densities of both AM fungal hyphae or AM fungus spore in the gel. While the results suggested that AM fungal exudates might be involved, the experimental design did not permit to rule out the possibility of a pH change in the medium, or of a CO_2 buildup in the agar Petri plates induced by fungal growth.

To test these hypotheses, using the same experimental approach, Filion et al. (1999) concentrated crude extracts from the extraradical mycelial compartment, presumably containing soluble biologically active substances, and tested these on various soil microbes. As the exudates were first lyophilized before dilution to the expected concentrations, volatiles were excluded and only soluble substances released by the extraradical mycelium of *G. intraradices* were considered. Under these conditions, as for bacteria (see above), species-specific responses occurred, with conidial germination of *F. oxysporum* f. sp. *chrysanthemi* being decreased and *T. harzianum* germination being enhanced. Differences in pH were noted between extracts from AM and non-AM samples, but no significant influence of pH on growth or conidial germination was measured within the experimental pH range. These results therefore supported the hypothesis that substances released by the AM fungus in the growth medium induced the differential growth responses of the organisms tested. Recently, the tomato–AM fungus–*Phytophthora nicotianae* association was chosen as a model for the purification of bioactive fractions of the root and mycelium exudates.

The study aimed at characterization of the molecules secreted by the mycorrhiza which potentially provoke shifts in microbe populations. It was found that the attraction of zoospores by exudates from mycorrhizal roots is significantly different from that of non-colonized roots (Lioussanne et al. 2003).

Rousseau et al. (1996) investigated the effect of a *T. harzianum* isolate on a functional mycelium of *G. intraradices* growing on pea roots in vitro. In this study, however, both the root and root-free compartments used to separate the fungi from the roots contained sugar. Under these conditions, TEM observations and gold labelling of cell wall constituents showed a marked antagonism of *T. harzianum* on *G. intraradices* spores and hyphae. The mycoparasite proliferated on the spore surface, penetrated the cell wall, and massively colonized the AM fungal hyphae, inducing disorganization, loss of protoplasm, hyphal bursting, and finally death of the *G. intraradices* hyphae. This study showed that the extraradical phase of AM fungi may also be adversely affected by certain biocontrol micro-organisms, and highlights the necessity to consider this phenomenon when developing biocontrol strategies. However, positive (Datnoff et al. 1995) and neutral (Fracchia et al. 1998) as well as negative (McGovern et al. 1992) interactions between *Trichoderma* species and AM fungal root colonization or biocontrol potential have been reported in soil systems. Therefore, the strong antagonistic interaction described in vitro might not entirely reflect the in vivo situation, and could have been emphasized by the aggressiveness of the biocontrol strain selected or by the in vitro growth conditions. This again highlights the complexity of microbial interactions within the rhizosphere community, and emphasizes the need for additional research.

4
Interaction Between AM Fungi and Nematodes

Until the work by Elsen et al. (2001), no study had reported the interaction between AM fungi and nematodes in AM in vitro systems (cf. Table 3). However, the in vitro hatching activity of the cyst nematode *Globodera*

Table 3. Interaction between AM fungi and nematodes in vitro

Nematode species[a]	AMF species[a]	Interaction type	Reference
Globodera pallida	*Glomus* sp.	Positive	Ryan et al. (2000)
Radopholus similis	*G. intraradices*	Negative	Elsen et al. (2001)
Pratylenchus coffeae	*G. intraradices*	Negative	Elsen et al. (2003)

[a]Species names are those used in the cited references

pallida has been studied using filter-sterilized root leachates from in vivo potato plants (Ryan et al. 2000). In the presence of root leachates from mycorrhizal potato plants, hatching activity was significantly higher compared to hatching in the presence of root leachates from non-mycorrhizal plants. These first results indicated that mycorrhizal inoculation of potato plants could stimulate hatching chemicals.

Recently, non-compartmentalized AM dixenic cultures have been used to study the interaction between AM fungi and nematodes (Elsen et al. 2001, 2003). Ri T-DNA transformed carrot roots colonized with *G. intraradices* were infected with the burrowing nematode *Radopholus similis* (Elsen et al. 2001). Although the two organisms were able to complete their life cycle in dixenic culture, the nematode population density was reduced by 50% relative to that in non-mycorrhizal controls. However, the results were not significant for all developmental stages of the nematode, and the reduced population density was not correlated with AM fungal root colonization, or with mycelial or spore densities. The same non-compartmentalized AM dixenic cultures were used to study the interaction between *G. intraradices* and the lesion nematode *Pratylenchus coffeae* (Elsen et al. 2003). In the presence of the AM fungus, the *P. coffeae* population was reduced to the same extent as in the experiment with *R. similis*. The impact of the AM fungus on the nematode population density was more pronounced in the roots than in the medium. The nematodes, on the other hand, had no impact (at least in terms of visual observations) on the intra-root and extraradical development of the AM fungus under dixenic culture conditions.

While the mechanisms involved in the nematode population reduction were not elucidated, these studies support the potential of the AM dixenic culture system for isolating the factors involved in the interaction between nematodes and AM fungi.

5 Conclusion

The increased ratio of microbes in bulk soil as compared to rhizosphere in AM plants (Posta et al. 1994; Ravnskov et al. 1999) suggests that carbon of plant origin is brought into soil by AM hypha (Johnson et al. 2002). The difference in physiological activity and exudation of AM host roots might also be involved in mycorrhizosphere microbial population shifts (Olsson et al. 1996; Andrade et al. 1997; Ronn et al. 2002). Recently, AM colonized plants were shown to regulate further AM root colonization through altered root exudation (Pinior et al. 1999; Vierheilig et al. 2000) and systemic signalling (Ludwig-Muller et al. 2002; Herrera-Medina et al. 2003), suggesting that symbiosis regulation might also impact other soil microbes.

To efficiently use the AM in vitro culture systems to model the interaction between the AM symbiosis and its environment, it is therefore urgent to thoroughly characterize and assess the variability in growth, nutritional kinetics and physiology of the model organisms. Until recently, most in vitro experiments characterizing mycorrhizas were done with only one transformed root line and very few plant species, including the carrot hairy root line established by Bécard and Fortin (1988), the tomato (Simoneau et al. 1994; Khaliq and Bagyaraj 2000), strawberry (Nuutila et al. 1995) and, more recently, *Medicago truncatula* (Boisson-Dernier et al. 2001). The nitrogen metabolism was recently analysed in mycorrhizal transformed roots. This study showed a significant alteration in N key enzyme activities, N transfer and assimilation between the symbiotic partners, and different GS isoforms in roots and AM mycelium (Toussaint et al. 2004). Labour et al. (2003) also compared several hairy root lines and showed that growth, nutrient uptake rates and mycorrhizal receptiveness of the lines were highly variable. Such results point to the role of intracellular storage and use efficiency in regulating symbiosis, as well as to the importance of controlling these parameters in a model system.

References

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ (1997) Bacteria from rhizosphere and hyphosphere soils of different arbuscular-mycorrhizal fungi. Plant Soil 192:71–79

Artursson V, Jansson JK (2003) Use of bromodeoxyuridine immunocapture to identify active bacteria associated with arbuscular mycorrhizal hyphae. Appl Environ Microbiol 69:6208–6215

Azcón R (1987) Germination and hyphal growth of *Glomus mosseae* in vitro: effects of rhizosphere bacteria and cell-free culture media. Soil Biol Biochem 19:417–419

Azcón R (1989) Selective interaction between free-living rhizosphere bacteria and vesicular-arbuscular mycorrhizal fungi. Soil Biol Biochem 21:639–644

Azcón-Aguilar C, Diaz-Rodriguez R, Barea JM (1986) Effects of soil microorganisms on spore germination and growth of the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. Trans Br Mycol Soc 86:337–340

Bago B, Azcón-Aguilar C, Goulet A, Piché Y (1998) Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. New Phytol 139:375–388

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2002) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Benhamou N, Fortin JA, Hamel C, St-Arnaud M, Shatilla A (1994) Resistance responses of mycorrhizal Ri T-DNA-transformed carrot roots to infection by *Fusarium oxysporum* f. sp. *chrysanthemi*. Phytopathology 84:958–968

Bianciotto V, Bandi C, Minerdi D, Sironi M, Tichy HV, Bonfante P (1996a) An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Bianciotto V, Minerdi D, Perotto S, Bonfante P (1996b) Cellular interactions between arbuscular mycorrhizal fungi and rhizosphere bacteria. Protoplasma 193:123–131

Bianciotto V, Lumini E, Lanfranco L, Minerdi D, Bonfante P, Perotto S (2000) Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family *Gigasporaceae*. Appl Environ Microbiol 66:4503–4509

Boisson-Dernier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker DG (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol Plant Microb Interact 14:695–700

Calvet C, Barea JM, Pera J (1992) In vitro interactions between the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* and some saprophytic fungi isolated from organic substrates. Soil Biol Biochem 24:775–780

Chabot S (1991) Utilisation d'un système de culture monoxénique, comme modèle pour l'étude des endomycorhizes à vésicules et arbuscules. Thèse MSc, Université Laval, Québec

Datnoff LE, Nemec S, Pernezny K (1995) Biological control of Fusarium crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. Biol Control 5:427–431

Elsen A, Declerck S, De Waele D (2001) Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. Mycorrhiza 11:49–51

Elsen A, Declerck S, De Waele D (2003) Use of root organ cultures to investigate the interaction between *Glomus intraradices* and *Pratylenchus coffeae*. Appl Environ Microbiol 69:4308–4311

Filion M, St-Arnaud M, Fortin JA (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytol 141:525–533

Fracchia S, Mujica MT, Garcia-Romera I, Garcia-Garrido JM, Martin J, Ocampo JA, Godeas A (1998) Interactions between *Glomus mosseae* and arbuscular mycorrhizal sporocarp-associated saprophytic fungi. Plant Soil 200:131–137

Fracchia S, Godeas A, Scervino JM, Sampedro I, Ocampo JA, Garcia-Romera I (2003) Interaction between the soil yeast *Rhodotorula mucilaginosa* and the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea*. Soil Biol Biochem 35:701–707

Grayston SJ, Vaughan D, Jones D (1997) Rhizosphere carbon flow in trees, in comparison with annual plants – the importance of root exudation and its impact on microbial activity and nutrient availability. Appl Soil Ecol 5:29–56

Gryndler M, Hrselova H, Striteska D (2000) Effect of soil bacteria on hyphal growth of the arbuscular mycorrhizal fungus *Glomus claroideum*. Folia Microbiol 45:545–551

Herrera-Medina MJ, Gagnon H, Piche Y, Ocampo JA, Garcia-Garrido JM, Vierheilig H (2003) Root colonization by arbuscular mycorrhizal fungi is affected by the salicylic acid content of the plant. Plant Sci 164:993–998

Hildebrandt U, Janetta K, Bothe H (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919–1924

Johnson D, Leake JR, Read DJ (2002) Transfer of recent photosynthate into mycorrhizal mycelium of an upland grassland: short-term respiratory losses and accumulation of 14C. Soil Biol Biochem 34:1521–1524

Johnson, D, Vandenkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young JPW, Read DJ (2004) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. New Phytol 161:503–515

Khaliq A, Bagyaraj DJ (2000) Colonization of arbuscular mycorrhizal fungi on Ri T-DNA transformed roots in synthetic medium. Indian J Exp Biol 38:1147–1151

Klironomos JN, Bednarczuk EM, Neville J (1999) Reproductive significance of feeding on saprobic and arbuscular mycorrhizal fungi by the collembolan, *Folsomia candida*. Funct Ecol 13:756–761

Knee EM, Gong FC, Gao MS, Teplitski M, Jones AR, Foxworthy A, Mort AJ, Bauer WD (2001) Root mucilage from pea and its utilization by rhizosphere bacteria as a sole carbon source. Mol Plant Microb Interact 14:775–784

Labour K, Jolicoeur M, St-Arnaud M (2003) Arbuscular mycorrhizal responsiveness of in vitro tomato root lines is not related to growth and nutrient uptake rates. Can J Bot 81:645–656

Levy A, Chang BJ, Abbott LK, Kuo J, Harnett G, Inglis TJJ (2003) Invasion of spores of the arbuscular mycorrhizal fungus *Gigaspora decipiens* by *Burkholderia* spp. Appl Environ Microbiol 69:6250–6256

Lioussanne L, Jolicoeur M, St-Arnaud M (2003) Effects of the alteration of tomato root exudation by *Glomus intraradices* colonization on *Phytophthora parasitica* var. *nicotianae* zoospores. In: Proc 4th Int Conf Mycorrhizae, Montréal

Ludwig-Muller J, Bennett RN, Garcia-Garrido JM, Piché Y, Vierheilig H (2002) Reduced arbuscular mycorrhizal root colonization in *Tropaeolum majus* and *Carica papaya* after jasmonic acid application can not be attributed to increased glucosinolate levels. J Plant Physiol 159:517–523

Maraun M, Martens H, Migge S, Theenhaus A, Scheu S (2003) Adding to 'the enigma of soil animal diversity': fungal feeders and saprophagous soil invertebrates prefer similar food substrates. Eur J Soil Biol 39:85–95

Mayo K, Davis R, Motta J (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78:426–431

McAllister CB, Garcia Romera I, Godeas A, Ocampo JA (1994) In vitro interactions between *Trichoderma koningii*, *Fusarium solani* and *Glomus mosseae*. Soil Biol Biochem 26:1369–1374

McAllister CB, Garcia-Romera I, Martin J, Godeas A, Ocampo JA (1995) Interaction between *Aspergillus niger* van Tiegh and *Glomus mosseae* (Nicol and Gerd) Gerd and Trappe. New Phytol 129:309–316

McAllister CB, Garcia-Garrido JM, Garcia-Romera I, Godeas A, Ocampo JA (1996) In vitro interaction between *Alternaria alternata*, *Fusarium equiseti* and *Glomus mosseae*. Symbiosis 20:163–174

McGovern RJ, Datnoff LE, Tripp L (1992) Effect of mixed infection and irrigation method on colonization of tomato roots by *Trichoderma harzianum* and *Glomus intraradices*. Proc Fla State Hortic Soc 105:361–363

Minerdi D, Bianciotto V, Bonfante P (2002) Endosymbiotic bacteria in mycorrhizal fungi: from their morphology to genomic sequences. Plant Soil 244:211–219

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infections in transformed Ri T-DNA roots grown axenically. Phytopathology 77:1045–1050

Nuutila AM, Vestberg M, Kauppinen V (1995) Infection of hairy roots of strawberry (*Fragaria* × Ananassa Duch) with arbuscular mycorrhizal fungus. Plant Cell Rep 14:505–509

Olsson PA, Bååth E, Jakobsen I, Soderstrom B (1996) Soil bacteria respond to presence of roots but not to mycelium of arbuscular mycorrhizal fungi. Soil Biol Biochem 28:463–470

Pfeffer PE, Bago B, Shachar-Hill Y (2001) Exploring mycorrhizal function with NMR spectroscopy. New Phytol 150:543–553

Pinior A, Wyss U, Piché Y, Vierheilig H (1999) Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. Can J Bot 77:891–897

Posta K, Marschner H, Römheld V (1994) Manganese reduction in the rhizosphere of mycorrhizal and nonmycorrhizal maize. Mycorrhiza 5:119–124

Ravnskov S, Nybroe O, Jakobsen I (1999) Influence of an arbuscular mycorrhizal fungus on *Pseudomonas fluorescens* DF57 in rhizosphere and hyphosphere soil. New Phytol 142:13–122

Ronn R, Gavito M, Larsen J, Jakobsen I, Frederiksen H, Christensen S (2002) Response of free-living soil protozoa and microorganisms to elevated atmospheric CO^2 and presence of mycorrhiza. Soil Biol Biochem 34:923–932

Rousseau A, Benhamou N, Chet I, Piché Y (1996) Mycoparasitism of the extramatrical phase of *Glomus intraradices* by *Trichoderma harzianum*. Phytopathology 86:434–443

Ruiz-Lozano JM, Bonfante P (2000) A *Burkholderia* strain living inside the arbuscular mycorrhizal fungus *Gigaspora margarita* possesses the vacB gene, which is involved in host cell colonization by bacteria. Microb Ecol 39:137–144

Ryan NA, Duffy EM, Cassells AC, Jones PW (2000) The effect of mycorrhizal fungi on the hatch of potato cyst nematodes. Appl Soil Ecol 15:233–240

Schreiner RP, Bethlenfalvay GJ (2003) Crop residue and Collembola interact to determine the growth of mycorrhizal pea plants. Biol Fertil Soils 39:1–8

Simoneau P, Louisy-louis N, Plenchette C, Strullu DG (1994) Accumulation of new polypeptides in Ri T-DNA-transformed roots of tomato (*Lycopersicon esculentum*) during the development of vesicular-arbuscular mycorrhizae. Appl Environ Microbiol 60:810–1813

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995) Altered growth of *Fusarium oxysporum* f. sp. *chrysanthemi* in an in vitro dual culture system with the vesicular arbuscular mycorrhizal fungus *Glomus intraradices* growing on *Daucus carota* transformed roots. Mycorrhiza 5:431–438

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an *in vitro* system in the absence of host roots. Mycol Res 100:328–332

Toussaint JP, St-Arnaud M, Charest C (2004) Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith and Ri T-DNA roots of *Daucus carota* L. in an in vitro compartmented system. Can J Microbiol 50:251–260

Tylka GL, Hussey RS, Roncadori RW (1991) Axenic germination of vesicular-arbuscular mycorrhizal fungi – effects of selected *Streptomyces* species. Phytopathology 81:54–759

van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Vierheilig H, Garcia-Garrido JM, Wyss U, Piché Y (2000) Systemic suppression of mycorrhizal colonization of barley roots already colonized by AM fungi. Soil Biol Biochem 32:589–595

Villegas J, Fortin JA (2001) Phosphorus solubilization and pH changes as a result of the interactions between soil bacteria and arbuscular mycorrhizal fungi on a medium containing NH_4^+ as nitrogen source. Can J Bot 79:865–870

Villegas J, Fortin JA (2002) Phosphorus solubilization and pH changes as a result of the interactions between soil bacteria and arbuscular mycorrhizal fungi on a medium containing NO_3^- as nitrogen source. Can J Bot 80:571–576

Wamberg C, Christensen S, Jakobsen I (2003a) Interaction between foliar-feeding insects, mycorrhizal fungi, and rhizosphere protozoa on pea plants. Pedobiologia 47:281–287

Wamberg C, Christensen S, Jakobsen I, Muller AK, Sorensen SJ (2003b) The mycorrhizal fungus (*Glomus intraradices*) affects microbial activity in the rhizosphere of pea plants (*Pisum sativum*). Soil Biol Biochem 35:1349–1357

Xavier LJC, Germida JJ (2003) Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biol Biochem 35:471–478

Part IV
Root Organ Culture of Ectomycorrhizal Fungi

13 *Cistus incanus* Root Organ Cultures: a Valuable Tool for Studying Mycorrhizal Associations

Andrew P. Coughlan[1], Yves Piché[1]

1 Introduction

The origin of the ectomycorrhizal (ECM) symbiosis, so important in temperate and boreal biomes (Erland and Taylor 2002), dates back approximately 130×10^6 years (Axelrod 1986; Berbee and Taylor 1993). This symbiotic association is, therefore, much more recent than that formed by the arbuscular mycorrhizal (AM) fungi considered in most of the other chapters of this volume.

While only approximately 190 species from the Glomeromycota are recognized as forming AM associations (http://www.tu-darmstadt.de/fb/bio/bot/schuessler/amphylo/amphylogeny.html), at least 6000 species from the Basidiomycotina and Ascomycotina are considered to form ectomycorrhiza (Molina et al. 1992). In contrast to AM fungi, which are obligate biotrophs, many species of ECM fungi exhibit a certain capacity for saprophytic growth under axenic conditions. This, it has been suggested, makes them useful experimental organisms (Satyanarayana et al. 1996) and has allowed the nutrition, and growth and development of known fungal species to be studied under different physically and/or chemically controlled conditions. The results obtained from certain studies have been extrapolated and used to make inferences about the likely nutrient uptake capabilities and behavioural responses of ECM plants growing in natural ecosystems (e.g. Kernaghan et al. 2002). However, like AM fungi, ECM fungi are unable to complete their life cycle in the absence of a host plant. It therefore seems probable that the enzymes, phytohormones and other secondary compounds produced while growing saprophytically, differ from those produced when growing symbiotically. This theory is supported by differences in nutrient utilization (Rygiewicz et al. 1984; Scheromm et al. 1990; Smith and Read 1997) and fungal gene expression between these two states (Nehls and Martin 1995; Söderström et al. 2003). Therefore, it is not clear to what extent the conclusions drawn from axenic studies with a given ECM

[1]Centre de Recherche en Biologie Forestière, Faculté de Foresterie et de Géomatique, Pavillon C.-E.-Marchand, Université Laval, Sainte-Foy, QC, G1K 7P4, Canada, Tel.: +1-418-6562131, Fax: +1-418-6567493, E-mail: andrew.coughlan@rsvs.ulaval.ca

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

fungus can be accurately applied to plants colonized by the same fungus growing in the field.

Even though there is a current effort to replace traditional "reductionist" techniques used to investigate the ECM association with experimental approaches that have greater ecological relevance (Read 2002), certain fundamental aspects of the symbiosis (e.g. molecular signalling, gene expression, and morphological changes to fungal hyphae prior to, or following, root contact) can only realistically be investigated under simplified aseptic conditions. Furthermore, in vitro techniques can also be used to overcome the problems associated with trying to single out the effects of an individual biotic or abiotic factor on mycorrhizal formation.

Over the last 50 years, a number of aseptic, or semi-aseptic, techniques have been developed which go some way towards palliating the potential bias introduced by the use of axenic ECM fungal cultures for investigating the ECM association. The evolution of these techniques towards the use of isolated roots is briefly reviewed, before highlighting a new method for ECM research based on Ri T-DNA transformed root organ cultures of *Cistus incanus* (Fig. 1).

Fig. 1. Typical club-shaped ectomycorrhiza (*star*) of *Tuber maculatum* on a *Cistus incanus* root organ. Note the non-mycorrhizal short root to the *right*

2
Evolution of Monoxenic Techniques for Investigating ECM Associations

2.1
Whole-Plant Techniques

Many early experimental systems were based on axenically grown seedlings inoculated with a given ECM fungus and grown under aseptic conditions in Erlenmeyer flasks (Marx and Zak 1965; Abuzinadah et al. 1986) or glass tubes (Molina 1979). However, the seeds of certain host species are difficult to sterilize due to endogenous contaminants, and germination rates vary considerably. Furthermore, enclosed systems may be CO_2 limiting (but see Richard and Fortin 1975) and prone to the accumulation of compounds such as ethylene (Peterson and Chakravarty 1991), both of which can significantly affect plant growth rates, root morphology (Reid 1987; Orcutt and Nilson 1996) and, potentially, mycorrhizal formation. These systems also allow the excessive accumulations of root exudates, which may affect fungal activity. Moreover, these systems require growth cabinet conditions and, as with field-grown plants, observation of mycorrhizal roots involves destructive harvesting of the experimental unit.

The use of vented Mason storage jars (Trappe 1967) or modified Petri plates (e.g. Duddridge 1986; Wong and Fortin 1989) allow mycorrhizal roots of whole plants to develop in an aseptic environment, while allowing the shoot to grow under ambient conditions, thereby overcoming the problem of CO_2 acquisition and ethylene accumulation. Nevertheless, the experimental system still requires growth cabinet conditions, and the methods (e.g. sterile lanolin) used to keep the rooting zone contaminant free are not always effective, as certain fungi and bacteria can pass intercellularly via the stem tissue into the rooting zone. Furthermore, these systems are also affected by genetic differences between individual host plants within a given seed lot.

Many of the problems outlined above can be overcome using elaborate experimental systems comprising individual growth units ventilated with filtered forced air and coupled with clonal plant material issued from somatic embryos (e.g. Díez et al. 2000) or microcuttings (e.g. Tonkin et al. 1989). Nevertheless, these can be space-consuming and expensive to establish.

Recently, many investigations into the mechanisms underlying the functioning of the AM symbiosis have used an inexpensive and more easily manipulated culture system based on the in vitro co-culture of a clonal root organ culture associated with contaminant-free inoculum from a glo-

malean species. This system, used either in standard or compartmentalized Petri dishes, and maintained under identical conditions to those used for growing axenic fungal cultures, has provided answers to important questions regarding host/fungus signalling, gene expression, growth and development, and nutrient uptake (for recent review, see Fortin et al. 2002). Similar questions could be usefully addressed using root organ cultures of ECM plants associated to ECM fungi in vitro. Such an approach would improve our understanding of the ECM symbiosis and, in certain cases, allow greater accuracy when drawing conclusions about the likely response of a plant colonized by a given ECM fungus growing in the field.

2.2 Non-Transformed Root Organs and Root Hypocotyl Organs

Ectomycorrhizal host plants are, with few exceptions, trees (Smith and Read 1997). The first root organs of ECM plant species were obtained in the 1940s (Bonner 1942; Slankis 1948a). Unfortunately, one of the main problems limiting the use of these non-transformed root organs for ECM fungal research was that the roots of ECM host plants, which grow well under certain liquid culture techniques, grow relatively poorly on solidified media. This phenomenon is principally linked to the need for pre-treatments with exogenous hormones. For example, the method used by Ulrich (1962) required that roots of *Pinus ponderosa* be incubated in a nutrient solution amended with IAA to initiate the formation of root initials; once achieved, the roots were washed and returned to an IAA-free nutrient medium to allow elongation. Manipulations of this type are practical neither for roots grown on agar-solidified media, nor for ECM studies. Furthermore, certain isolated non-transformed tree roots are highly sensitive to light, and even short exposure can induce senescence (Ulrich 1962).

Slankis (1948b), investigating the effect of ECM fungi on plant roots, grew root organs of *Pinus sylvestris* in liquid culture with mycelium of the ECM fungus *Boletus variegatus*. However, although dichotomous branching of short roots was observed, it is unclear as to whether or not true ectomycorrhiza were formed (Fortin 1966), or whether the branching was due to a build up of ethylene in the root or in response to fungal exudates.

In a further attempt to develop a simple monoxenic system for ECM research, Fortin (1966) adapted the method developed by Raggio and Raggio (1956) for bean (*Phaseolus vulgaris*) roots, and applied it to *P. sylvestris* roots. Briefly, this technique involved applying different media to the two ends of the excised root – the part closest to the hypocotyl was inserted into a medium containing an organic carbon source (i.e. mimicking shoot-derived nutrients), while the distal part was allowed to develop in a carbon-

free medium amended with mineral nutrients. This system recreated the polarity occurring naturally in plants, and allowed roots to develop under conditions closer to those found in nature. While this polarity is not essential for the growth of pine roots in liquid culture, it appears to be a prerequisite for their successful growth on solidified media. However, acceptable levels of root growth were obtained only when part of the hypocotyl was left in place, or replaced by IAA. The system was successfully used to produce mycorrhizas between *Pinus strobus* and four species of ECM fungi, i.e. *Pisolithus tinctorius*, *Suillus granulatus*, *Suillus tormentosus* and *Leccinum chromapes* (Fortin and Piché 1979), and between *Eucalyptus pilularis* and *P. tinctorius* (Bailey and Peterson 1988). Although this method allowed ECM formation under aseptic conditions and in the absence of the aerial part of the host plant, the need for hypocotyl tissue meant that clonal root organs could not be developed.

The first attempt to use truly clonal root organs on solid medium for ECM studies was by Louis and Scott (1987). The authors used hormonal regulators (naphthalene acetic acid and 6-benzylamino purine) to produce root organs from a tissue culture of the tree species *Shorea roxburghii* (Dipterocarpaceae). These root organs were subsequently inoculated with mycelium of an ECM fungus from the genus *Rhodophyllus*. The results obtained were widely reported as the first formation of ectomycorrhiza on a root organ clone. However, at harvest, the 5-month-old roots were approximately the same length as those placed in the Petri plates at the beginning of the experiment and, while the fungal mycelium had grown, perhaps preferentially, over the root surface, there was no clear indication of ECM formation. The lack of root growth and the presence of intracellular hyphae, but no Hartig net, suggest that the ECM fungus might have been using the cell contents for saprophytic growth, as is observed in individual excised ectomycorrhiza. Nevertheless, the fact that these studies were attempted highlighted the need for a model system based on root organs, which would allow the study of certain aspects of the ECM symbiosis which cannot be investigated in situ.

2.3 Ri T-DNA Transformed Root Organs

Although Mosse and Hepper (1975), working with AM fungi, were the first to obtain mycorrhiza using host-plant root organs, it was Mugnier and Mosse (1987) who realized the potential value of Ri T-DNA transformed root organs for the study of mycorrhiza. Transformed root organs result from the natural incorporation into the plant's genome of the Ri T-DNA plasmid from the ubiquitous soil-borne bacterium *Agrobacterium rhizo-*

genes. This plasmid induces the production of growth hormones in the root, hence removing the need to incorporate plant hormones into the culture medium. The resulting transformed roots exhibit increased growth rates and increased branching (Tepfer 1989). While this bacterium can induce the formation of so-called hairy roots in a number of forb, shrub and tree species, its use is limited to work on angiosperms, for it does not naturally infect gymnosperm species. Of the 115 plants listed by Tepfer (1989) as having been transformed by *A. rhizogenes*, only two represent plants capable of forming ectomycorrhiza: *Eucalyptus gunnii* and *Populus tremula x Populus alba*. Work in our laboratory indicates that root organs of tree species have a much higher carbon (i.e. sucrose) requirement for normal growth than those of forbs, which could influence studies into the ECM association. While excised roots of woody species are difficult to grow in vitro, not all ECM plants are trees – some are woody shrubs and others, forbs. This provides the possibility of establishing root organs which might behave in a similar way to the root organs used so successfully for research into the AM association.

2.4
Ri T-DNA Transformed Root Organs of *Cistus incanus*

A team working with the ECM fungus *Tuber melanosporum* at the Ben-Gurion University of the Negev (Israel) was the first to realize the potential use of root organs of *Cistus incanus*, a woody shrub, for studying ECM plant–fungal interactions (Nurit et al. 1999; Wenkart et al. 2001). This team produced nine clones which could be grown on Minimal (M) medium (Bécard and Fortin 1988), a medium typically used for AM-based research under monoxenic conditions. At about the same time, a team at the Institute of Microbiology ASCR (Czech Republic) developed a *Cistus albidus* root organ clone (Milan Gryndler, pers. comm.), and our group at Laval University (Québec) produced four *C. incanus* root organ clones.

3
Production and Maintenance of Transformed *Cistus incanus* Root Organs

In areas around the Mediterranean Basin, *Cistus* species are an important component of the soil seed bank, their seedlings rapidly colonizing recently burned sites (Dansereau 1939). The dormancy of *Cistus* seeds can therefore be easily broken by subjecting them to high temperatures, either by immersion in concentrated sulphuric acid (20 min; Nurit et al. 1999; Wenkart

et al. 2001), or by exposing dry seeds to temperatures of 100 °C (30 min). Surface sterilized seeds are then germinated under aseptic conditions.

Root organs are produced from the resulting axenic seedlings by wounding the stem or leaf surface with a sterile needle, and inoculating the wound with *A. rhizogenes* cells from a 48-h-old liquid (Nurit et al. 1999; Wenkart et al. 2001) or Petri plate culture. The seedlings are then incubated under ambient conditions. A callus develops after 3–5 days, from which transformed roots appear after 8–14 days.

Agrobacterium rhizogenes cells are eliminated from the transformed roots, by transferring root tips (2–3 cm) to M medium amended with ampicillin (200–500 mg/L (Nurit et al. 1999; Wenkart et al. 2001), or modified White's (WM) medium (Bécard and Fortin 1988) amended with rifampicin (50 mg/l), or a mix of cefotaxime (200 mg/L) and carbenicillin (500 mg/L). We have found that bacteria-free root organ cultures are best maintained on WM (pH 6.5) in 150-mm Petri dishes and incubated in the dark at 25 °C. To maintain vigorous growth, *C. incanus* root organ cultures should be subcultured onto fresh media every 14 days.

Different *C. incanus* root organ clones exhibit different growth rates and branching patterns (Wenkart et al. 2001; Roth-Bejerano et al. 2003). Fast-growing clones can accumulate five times as much biomass as slow-growing ones; this is possibly due to differences in the site of insertion of the plasmid into the plants genome, or to the overall number of insertions (Wenkart et al. 2001). However, even slow-growing clones can be distinguished from non-transformed *C. incanus* roots, as the latter fail to grow on WM medium.

4
Use of *Cistus incanus* Root Organs for the Study of Mycorrhizal Associations

4.1
Effect of *Cistus incanus* Root Organs on ECM Fungal Growth

While Coughlan et al. (2001a) found that *C. incanus* root organs showed preferential growth rates at pH 6.5, they also grow well at lower pH values (Nurit et al. 1999; Wenkart et al. 2001). As a given ECM fungus has an optimum pH for mycorrhizal formation (Marx and Zak 1965; Cordell and Marx 1994), the plasticity exhibited by these roots allows their use with a wide range of ectomycobionts. Coughlan et al. (2001b) investigated the effect of *C. incanus* root organs on the development of three species of ECM: two relatively slow-growing species (*Cenococcum geophilum* and *T. melanosporum*) and one fast-growing species (*Laccaria bicolor*). The maximal growth

rate of each species on its optimal axenic culture medium was compared to growth in the presence of a root organ on the nutritionally poorer M medium. In all cases, while the optical density of the colonies remained similar to that on the optimal culture medium, the growth rate was higher: *C. geophilum* and *T. melanosporum* (Fig. 2) exhibited a fourfold increase and *L. bicolor*, a twofold increase. In the same study, the growth rate of *T. melanosporum* was shown to double when a *C. incanus* root organ was allowed to develop on the medium for 1 week and then removed before inoculating the Petri plates with the fungus (Fig. 2). This suggests that either root-induced modifications to the original medium occur and/or that certain non-volatile root exudates remain in the substrate. In a similar study by Ventura et al. (2003) using two different *C. incanus* root organ clones, roots were pre-grown in Petri plates, removed, and the plates inoculated with *T. melanosporum*. The authors observed that the rate of carbohydrate utilization by one of the clones, and in particular the levels of reducing sugars remaining in the media, had the potential to mask any stimulatory effect of the root on fungal growth.

Although the presence of both young (10–25 days) and old (217 days) *C. incanus* roots can increase hyphal growth of *T. melanosporum*, the great-

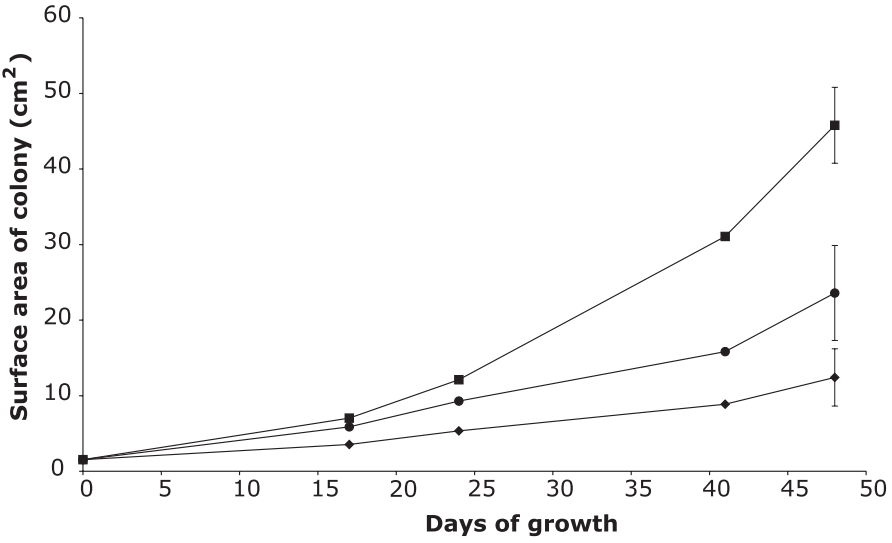


Fig. 2. Growth of mycelium of *Tuber melanosporum* on a potato dextrose malt medium (optimal medium for axenic growth; *filled diamonds*) compared with growth on M medium on which a *Cistus incanus* root organ was grown for 2 weeks and removed prior to inoculation of the Petri plate (*filled circles*), or on M medium together with a *C. incanus* root organ (*filled squares*). Values are means for ten replicate 150-mm Petri plates, and *vertical bars* (day 48) are standard errors of the mean

est stimulation has been observed to occur in the presence of young roots (Wenkart et al. 2001). This possibility to increase the growth rate of relatively recalcitrant species of mycorrhizal fungi has important implications for the production of inocula (see below).

4.2 Differentiation of ECM Fungal Mycelium in the Presence of *Cistus incanus* Root Organs

Initial studies with *C. incanus* root organs have shown that certain ECM fungi may modify hyphal morphology in the presence of a host plant. When grown monoxenically with a *C. incanus* root organ, an isolate of *Tuber maculatum* developed hyphal ramifications similar to the branched absorbing structures (BAS) found on the extraradical hyphae of AM fungi (Bago et al. 1998). These structures may either have been modifications to the hyphae prior to root colonization, or structures serving for nutrient absorption. Whatever their role, it suggests that the morphology of the mycelium in the presence of a plant host may show subtle differences from that observed under axenic conditions.

Fungal differentiation also includes alterations in gene expression, and the monoxenic system can provide sufficient quantities of clean material for investigating such changes. For example, Kagan-Zur et al. (2003) observed that two strains of *Terfezia boudieri* in the presence of a certain *C. incanus* clone (M2) produced ectendomycorrhizal rather than ECM associations. In an elegant study, Zaretsky et al. (2003) identified genes expressed by different *C. incanus* root organ clones and different isolates of *T. boudieri* when growing in the same Petri plate but separated by a sheet of cellophane. By so doing, the authors were able to show that the root–fungus pairings which formed ectendomycorrhiza had different gene expression profiles to those root–fungus pairings which formed ectomycorrhiza.

4.3 Formation of ECM on *Cistus incanus* Root Organs

Wenkart et al. (2001) gave the first published account of the formation of ECM on a *C. incanus* root organ. The authors tested nine clones, and found that all formed ECM associations when grown monoxenically with *T. melanosporum*. A later study confirmed that the clones also formed mycorrhiza with *T. boudieri* (Kagan-Kur et al. 2003). In studies by Coughlan et al. (2001b), the four clones tested all formed ectomycorrhiza with a range of known ECM fungal species. The latter root organs have subsequently

proved valuable in testing the ability of unidentified fungi, isolated from field-collected roots, to form ectomycorrhiza.

The technique used by Wenkart et al. (2001) to form ECM consisted of cultivating a slow-growing *C. incanus* root organ (L2A) on M medium for 3 months in a glass test tube. The root organ was then inoculated with *T. melanosporum* by placing a cube of medium containing fungal hyphae directly onto the root surface. Hartig net formation was observed in long roots after 3 months, and club-shaped ECM short roots were observed after 5 months.

By contrast, Coughlan and Piché (2003a) obtained typical club-shaped *T. melanosporum* ectomycorrhiza as little as 5 days after contact between the *C. incanus* root organ and the fungal hyphae. These authors used a similar technique to that described by Wenkart et al. (2001), but with important modifications. Firstly, a clone exhibiting rapid growth (clone #2) was used. Secondly, root tip segments (2 cm) from an actively growing root organ were initially grown on WM medium (pH 6.5) for 7 days, before being transferred to a 150-mm Petri dish containing minimal (M) medium (pH 6.5). Finally, two 15-mm plugs were cut from the gel, just in front of the developing lateral roots, and replaced with identically sized plugs cut from an actively growing colony of *T. melanosporum*. The reason for the difference in the time taken for mycorrhizal formation between the two techniques may lie in the initial pH of the substrate used (pH 5.5; Nurit et al. 1999; Wenkart et al. 2001) vs. pH 6.5 (Coughlan and Piché 2003a), root age and, perhaps, important genetic differences at the phyto- and mycobiont levels. However, it is also likely that, after 3 months of growth, the *C. incanus* roots in the experimental system employed by Nurit et al. (1999) and Wenkart et al. (2001) had significantly modified the small volume of medium in the test tubes used. Furthermore, root organs of certain plant species which have exhausted the supply of a particular mineral nutrient (e.g. phosphorus) are able, just as roots of whole plants, to liberate organic acids in an attempt to mobilize the deficient ion from less soluble compounds. This is likely to have a negative effect on the growth of *T. melanosporum*, which is a calcicolous species. In the system employed by Coughlan and Piché (2003a), young actively growing roots with abundant emerging laterals were used. Under field conditions, it is these which are rapidly colonized by ectomycorrhizal fungi. Moreover, the pre-treatment on the relatively nutrient-rich WM medium, before being transferred to the relatively nutrient-poor M medium, may also have played an important role in stimulating ECM formation. After 4 weeks, the growth of the *C. incanus* root organ clones developed at Laval University slows, the roots become progressively browner, probably due to a build up of phenolic substances in the tissue, and they are not considered to provide optimal conditions for ECM formation.

4.4
Use of *Cistus incanus* Root Organs for Work with AM Fungi

There are increasing reports of certain AM fungal structures occurring in the roots of species that have traditionally been considered ECM (e.g. Smith et al. 1998; Moyersoen and Fitter 1999; Dickie et al. 2001). Coughlan and Piché (2003c) used *C. incanus* root organs to investigate the nature of the interaction between a typical ECM host and an AM fungus. The roots were grown monoxenically with spores of the AM fungus *Glomus intraradices*. The spores germinated, and the fungus formed appressoria and penetrated the root. However, the colonization was extremely sparse (less that 1%), and no arbuscules and very few vesicles were formed. Nevertheless, because of the transparent nature of the medium (M) used, the authors were able to determine that the extraradical mycelium developed BAS, which are features associated with fully functional AM associations (Bago et al. 1998). Numerous lipid-filled spores, similar in size and general appearance to those obtained in monoxenic cultures with carrot root organs, were also produced. Neither BAS nor spores were observed in control treatments. Although the spores were not tested for their ability to germinate, it seems that the roots of certain typical ECM hosts can trigger all the necessary metabolic pathways allowing carbon and mineral nutrient uptake by AM fungi, and completion of the fungal life cycle. Because of the low levels of colonization, and the problems associated with investigation of the extraradical mycelium in the field, it is unlikely that such information could have been gleaned without the use of this monoxenic system.

5
Practical Applications of *Cistus incanus* Root Organs

5.1
Maintaining Fungal Isolates

The *C. incanus* root organs developed at Laval University formed ECM with all isolates of known ECM fungi tested (Coughlan et al. 2001a, b). Furthermore, the root organs are used to stimulate slow-growing ECM fungal species, and for routine maintenance of all *Tuber* species. However, *C. incanus* root organs not only stimulate the growth of ECM fungi, they can also serve to reduce the number of times that isolates, in particular *Tuber* species (which are relatively intolerant to storage at low temperatures), have to be subcultured onto fresh culture medium. Transfers after 6, 7 and 14 months of monoxenic culture resulted in new fungal growth, whereas mycelium transferred from axenic fungal colonies of the same

age failed to grow (Wenkart et al. 2001). Therefore, *C. incanus* root organs appear to offer a suitable means for the long-term maintenance of ECM fungi.

Several authors have highlighted the fact that the characteristics of a given ECM fungus maintained under axenic conditions, including its ability to form ectomycorrrhiza, may alter with time since isolation (Marx 1981; Thomson et al. 1993; Brundrett et al. 1996). The reduced ability to form mycorrhiza may be overcome by inoculating the fungus onto a host plant and subsequently re-isolating it from the ectomycorrhiza (Marx 1981) or sporocarps (Thomson et al. 1993) formed. Thomson et al. (1993) even proposed that an "alternative method" for the storage of ECM fungal isolates be sought. *Cistus incanus* root organs could provide just such a method, allowing the ECM-forming capacity of fungal isolates which are to be used for inoculation experiments, or for inoculum production, to be maintained (Nurit et al. 1999). Furthermore, the use of *C. incanus* root organs to reinvigorate ECM isolates, under aseptic conditions, is rapid, and it alleviates the need to use antibiotic-amended media to remove the microbial contaminants usually associated with mycorrhiza or sporocarps obtained from pot cultures.

5.2 Inoculum Production

Following an initial study by Nurit et al. (1999), Wenkart et al. (2001) advanced the possible use of *C. incanus* root organs colonized by *T. melanosporum* as a suitable source of inoculum for large-scale in vitro or in vivo inoculation programmes. Coughlan et al. (2001b) successfully used such roots to colonize vitroplants of *Betula populifolia* under semi-aseptic condition. Briefly, the authors trimmed the roots of acclimatized vitroplants growing in minirhizotrons (Peterson and Chakravarty 1991), in a sterilized vermiculite-based substrate, to 10 cm. An inoculum of *C. incanus* root organs colonized by *T. melanosporum* was inserted just below the cut roots; mycorrhiza developed within 8 weeks on the new roots which developed. By contrast, in a similar study by Nurit et al. (1999) using *Cistus* seedlings, ECM formation took 20 weeks, but this may have been due to the small quantity of inoculum used.

In a subsequent study, Coughlan and Piché (2003b) developed a system for the in vitro inoculation of individual vitroplants with ECM fungi. Briefly, working aseptically, ECM *C. incanus* root organs were used to pre-colonize vermiculite-filled 20-mm KIM-KAPs with *T. melanosporum* mycelium prior to the introducing of a vitroplant. This technique allows the developing root system of acclimatizing vitroplants to be colonized by a given ECM

fungus. Furthermore, as the unit is small and remains aseptic, the system is potentially suitable for the large-scale development and export of ECM plants.

6 Conclusion

As outlined in the introduction, there is a move towards experimental approaches that allow the maximum amount of ecologically pertinent information about the ECM association to be gathered. However, it is clear that certain aspects of this association cannot be investigated on whole plants growing in natural substrates under natural or semi-natural conditions. As we have outlined, *C. incanus* root organs provide a valuable tool for the investigation of some of these, and they have already allowed important advances to be made. Continued use of this system should improve our knowledge concerning many molecular, nutritional and morphological aspects of the symbiosis which will, in turn, improve our understanding of plant–fungal interactions at the ecological level.

Although the *C. incanus* root organ culture system described above was developed as a means of stimulating growth of certain ECM fungal species and for the production of inocula (Nurit et al. 1999; Wenkart et al. 2001; Coughlan and Piché 2003a, b), it will, nevertheless, prove valuable for investigating many aspects of the ECM association. Spore germination, which is often difficult to achieve for ECM fungi, may be improved in the presence of host roots (Fries et al. 1987). *Cistus incanus* root organs, just as the transformed root organs of AM host plants (Buée et al. 2000), probably produce stimulatory molecules that will not only enhance hyphal growth but also stimulate spore germination. The nature and effect of these molecules can only be investigated under monoxenic culture conditions. Presently, our knowledge concerning the mechanisms of host–fungus recognition, and the subsequent modifications leading to the development and functioning of the symbiosis is limited (Lammers 2004). The monoxenic system outlined above will allow continued investigation into the differential gene expression of both host and fungus in the pre-symbiotic and symbiotic state that was instigated by Zaretsky et al. (2003). Such investigations should help elucidate important aspects of the communication between ECM fungi and their hosts. The ability of *L. bicolor* to form mycorrhiza with *C. incanus* root organs, coupled with the project to sequence the entire *L. bicolor* genome (Martin et al. 2004) and the ability to detect up-regulated and down-regulated genes, will provide a solid base for investigating inherent molecular and cellular mechanisms underlying the ECM association. This work could be extended to investigate differences between gene expression

of monokaryotic and dikaryotic cultures, and to investigate the ability of a monokaryotic mycelium to form ectomycorrhiza.

Attempts to isolate ECM fungi from field-collected roots often result in the production of large banks of isolates. *Cistus incanus* root organs could be used to rapidly screen isolates for non-host-specific ECM fungi, and to separate out certain saprophytic or pathogenic isolates. Using techniques developed for work with AM fungi (Diop et al. 1994), *C. incanus* root organs could also allow the isolation of fungi which are difficult to grow under axenic conditions, such as species of the genera *Amanita* and *Russula*. Furthermore, they will allow the production of sufficient quantities of aseptic mycelium for use in molecular identification work.

Little in the way of investigation into the differentiation and functioning of the extraradical mycelium under monoxenic conditions has been done. While studies by Wenkart et al. (2001) showed poor growth of extraradical mycelium of *T. melanosporum* on M medium, studies by Coughlan et al. (2001a) showed dense hyphal growth. This disparity was probably due to differences in pH and/or the chemical composition of the media after 3 months, compared to that after a few days. The capacity of many ECM fungi to grow saprophytically will necessitate the use of compartmentalized Petri plates to allow the development of an extraradical mycelium in a carbon-free substrate. Studies of the morphology of extraradical hyphae will be an interesting field for future research because the hyphae of axenically grown ECM fungi are normally simple structures with relatively few ramifications. Preliminary work in our laboratory with members of the genus *Suillus* has shown that, following ECM formation on *C. incanus* root organs, the extraradical mycelium differentiates to form typical mycelial strands.

Using compartmental Petri plates, the monoxenic system will allow investigation not only into nutrient uptake by the extraradical mycelium but also into interactions between different fungal species or isolates of the same species. The system may also prove valuable for ECM fungal fructification studies. Furthermore, the compartmentalized system would allow a pre-screening of large numbers of isolates for their ability to withstand certain edaphic conditions or pollutants, and to investigate interactions with other components of the soil biota.

Acknowledgements. We thank the Universidade Coimbra (Portugal) and the Orto Botanico dell'Universita, Siena (Italy) for providing seeds of *C. incanus*, and Dr. David Tepfer (INRA, Versailles) for providing the *A. rhizogenes* isolate LBA 9402. We also thank NSERC for funding to Y.P.

References

Abuzinadah RA, Finlay RD, Read DJ (1986) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. II. Utilization of protein by mycorrhizal plants of *Pinus contorta*. New Phytol 103:495–506

Axelrod DI (1986) Cenozoic history of some western American pines. Ann Mo Bot Gard 73:565–641

Bago B, Azcón-Aguilar C, Goulet A, Piché Y (1998) Branched absorbing structures: a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. New Phytol 139:375–388

Bailey SR, Peterson RL (1988) Ectomycorrhiza synthesis between isolated roots of *Eucalyptus pilularis* and *Pisolithus tinctorius*. Can J Bot 66:1237–1239

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Berbee ML, Taylor JW (1993) Dating the evolutionary radiation of the true fungi. Can J Bot 71:1114–1127

Bonner J (1942) Culture of isolated roots of *Acacia melanoxylon*. Bull Torr Bot Club 69:130–133

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. Monograph 32, Australian Centre for International Agricultural Research, Canberra

Buée M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant Microb Interact 13:693–698

Cordell CE, Marx DH (1994) Effects of nursery cultural practices on management of specific ectomycorrhizae on bareroot tree seedlings. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. APS Press, The American Phytopathological Society, St Paul, Minnesota, pp 133–151

Coughlan A, Piché Y (2003a) Method for synthesizing ectomycorrhiza in vitro. USA Patent Application no 60/459,995

Coughlan A, Piché Y (2003b) Method for colonizing a plant with an ectomycorrhizal fungus. USA Patent Application no 60/459,993

Coughlan A, Piché Y (2003c) Are arbuscules always characteristic structures of arbuscular mycorrhiza? In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Coughlan AP, Lavaud J, Piché Y (2001a) Racines transformées de *Cistus incanus* colonisées par divers champignons ectomycorhiziens: cas particulièrement favorable au développement de *Tuber melanosporum*. Réunion du Réseau de Mycologie de la Société Française de Microbiologie, Université Paul Sabatier, Toulouse, France

Coughlan AP, Dubé S, Marzitelli F, Piché Y (2001b) Développement in vitro des genres *Tuber*, *Laccaria* et *Cenococcum*. In: Colloq Mycorrhizes 2001, Jardin Botanique de Montréal

Dansereau P (1939) Monographie du genre *Cistus*. PhD Thesis, University of Geneva, Switzerland

Dickie IE, Koide RT, Fayish AC (2001) Vesicular-arbuscular mycorrhizal infection of *Quercus rubra* seedlings. New Phytol 151:257–264

Díez J, Manjón JL, Korvács GM, Celestino C, Toribio M (2000) Mycorrhization of vitroplants raised from somatic embryos of cork oak (*Quercus suber* L.). Appl Soil Ecol 15:119–123

Diop TA, Plenchette C, Strullu DG (1994) Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Duddridge JA (1986) The development and ultrastructure of ectomycorrhizas. III. Compatible and incompatible interactions between *Suillus grevillei* (Klotzsch) Sing. and 11 species of ectomycorrhizal hosts in vitro in the absence of exogenous carbohydrate. New Phytol 103:457–464

Erland S, Taylor AFS (2002) Diversity of ecto-mycorrhizal fungal communities in relation to the abiotic environment. In: van der Heijen MGA, Sanders I (eds) Mycorrhizal ecology. Springer, Berlin Heidelberg New York, pp 163–200

Fortin JA (1966) Synthesis of mycorrhizae on explants of the root hypocotyls of *Pinus sylvestris* L. Can J Bot 44:1087–1092

Fortin JA, Piché Y (1979) Cultivation of *Pinus strobus* root-hypocotyl explants for synthesis of ectomycorrhizae. New Phytol 83:109–119

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Fries N, Serck-Hanssen K, Dimberg LH, Theander O (1987) Abietic acid, an activator of basidiospore germination in ectomycorrhizal species of the genus *Suillus* (Boletaceae). Exp Mycol 11:360–363

Kagan-Zur V, Bustan A, Mills D, Roth-Bejerano N, Wenkart S, Ventura Y, Zaretzky M (2003) Differences between hairy root clones and fungal isolates in forming mycorrhizas. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Kernaghan G, Hambling B, Fung M, Khasa D (2002) In vitro selection of boreal ectomycorrhizal fungi for use in reclamation of saline-alkaline habitats. Restor Ecol 10:43–52

Lammers PJ (2004) Symbiotic signalling: new functions for familiar proteins. New Phytol 161:324–326

Louis I, Scott E (1987) In vitro synthesis of mycorrhiza in root organ cultures of a tropical dipterocarp species. Trans Br Mycol Soc 88:565–568

Martin F, Tuskan GA, DiFazio SP, Lammers P, Newcombe G, Podila GK (2004) Symbiotic sequencing for the *Populus* mesocosm. New Phytol 161:330–335

Marx DH (1981) Variability in ectomycorrhizal development and growth among isolates of *Pisolithus tinctorius* as affected by source, age, and reisolation. Can J For Res 11:168–174

Marx DH, Zak B (1965) Effect of pH on mycorrhizal formation of slash pine in aseptic culture. For Sci 11: 66–75

Molina R (1979) Pure culture synthesis and host specificity of red alder mycorrhizae. Can J Bot 57:1223–1228

Molina R, Massicotte H, Trappe JM (1992) Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: Allen MF (ed) Mycorrhizal functioning. Chapman and Hall, London, pp 357–423

Mosse B, Hepper CM (1975) Vesicular-arbuscular infections in root organ cultures. Physiol Plant Pathol 5:215–223

Moyersoen B, Fitter AH (1999) Presence of arbuscular mycorrhizas in typical ectomycorrhizal host species from Cameroon and New Zealand. Mycorrhiza 8:247–253

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Nehls U, Martin F (1995) Changes in root gene expression in ectomycorrhiza. In: Stocchi V, Bonfante P, Nuti M (eds) Biotechnology of ectomycorrhizae: molecular approaches. Plenum Press, New York, pp 125–137

Nurit R-B, Wenkart S, Mills D, Kagan-Zur V (1999) Mycorrhizal associations between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. In: Actes du Vème Congr Int Science et Culture de la Truffe et des Autres Champignons Hypoges Comestibles, Aix-en-Provence, pp 173–175

Orcutt DM, Nilsen ET (1996) The physiology of plants under stress: soil and biotic factors. Wiley, New York

Peterson RL, Chakravarty P (1991) Techniques in synthesizing ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology. Academic Press, London, pp 75–106

Raggio M, Raggio N (1956) A new method for the cultivation of isolated roots. Physiol Plant 9:466–469

Reid MS (1987) Ethylene in plant growth, development, and senescence. In: Davis PJ (ed) Plant hormones and their role in plant growth and development. Martinus Nijhoff, Dordrecht, pp 257–279

Read DJ (2002) Towards ecological relevance – progress and pitfalls in the path towards an understanding of mycorrhizal functions in nature. In: van der Heijen MGA, Sanders I (eds) Mycorrhizal ecology. Springer, Berlin Heidelberg New York, pp 3–29

Richard C, Fortin, JA (1975) Rôle protecteur du *Suillus granulatus* contre le *Mycelium radicis atrovirens* sur des semis de *Pinus resinosa*. Can J For Res 5: 452–456

Roth-Bejerano N, Kagan-Zur V, Mills D, Zaretzky M (2003) Mycorrhiza formation by hairy root clones with *Terfezia boudieri* isolates. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Rygiewicz PT, Bledsoe CS, Zasoski RJ (1984) Effects of ectomycorrhizae and solution pH on [^{15}N]ammonium uptake by coniferous seedlings. Can J For Res 14:885–892

Satyanarayana T, Gupta V, Garg S (1996) Ectomycorrhizal fungi as experimental organisms. In: Mukerji KG (ed) Concepts in mycorrhizal research. Kluwer, Dordrecht, pp 333–346

Scheromm P, Plassard C, Salsac L (1990) Nitrate nutrition of maritime pine (*Pinus pinaster* Soland in Ait.) ectomycorrhizal with *Hebeloma cylindrosporum* Romagn. New Phytol 114:93–98

Slankis V (1948a) Verschiedene Zuckerarten als Kohlehydratquelle für isolierte Wurzeln von *Pinus silvestris*. Physiol Plant 1:278–289

Slankis V (1948b) Einfluß von Exudaten von *Boletus variegatus* auf die dichotomische Verzweigung isolierter Kiefernwurzeln. Physiol Plant 1:390–400

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, San Diego

Smith JE, Johnson KA, Cázares (1998) Vesicular mycorrhizal colonization of seedlings of Pinaceae and Betulaceae after inoculation with *Glomus intraradices*. Mycorrhiza 7:279–285

Söderström B, Johansson T, Le Quéré A, Wright D, Ahrén D, Tunlid A (2003) The *Paxillus involutus*/*Betula pendula* symbiosis: gene expression in ectomycorrhizal root tissue. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Tepfer D (1989) Ri T-DNA from *Agrobacterium rhizogenes*: a source of genes having applications in rhizoshpere biology and plant development, ecology, and evolution. In: Kosuge T, Nestor EW (eds) Plant-microbe interactions. McGraw-Hill, New York, pp 294–342

Thomson BD, Malajczuk N, Grove TS, Hardy GE St J (1993) Improving the colonization of ectomycorrhizal fungal cultures by association with a host plant and re-isolation. Mycol Res 97:839–844

Tonkin CM, Malajczuk N, McComb JA (1989) Ectomycorrhizal formation by micropropagated clones of *Eucalyptus marginata* inoculated with isolates of *Pisolithus tinctorius*. New Phytol 111:209–214

Trappe JM (1967) Pure culture synthesis of Douglas-fir mycorrhizae with species of *Hebeloma*, *Suillus*, *Rhizopogon* and *Astraeus*. For Sci 13:121–130

Ulrich JM (1960) Auxin production by mycorrhizal fungi. Physiol Plant 13:429–443

Ulrich JM (1962) Cultural requirements for growth of excised ponderosa pine roots. Physiol Plant 15:59–71

Ventura Y, Mills D, Bustan A, Kagan-Zur V, Roth-Bejerano N (2003) Effects of sugars on in vitro growth of *Cistus incanus* hairy roots and of *Tuber melanosporum* mycelium. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Wenkart S, Roth-Bejerano N, Mills D, Kagan-Zur V (2001) Mycorrhizal associations between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. Plant Cell Rep 20:369–373

Wong KKY, Fortin JA (1989) A Petri dish technique for the aseptic synthesis of ectomycorrhizae. Can J Bot 67:1713–1716

Zaretzky M, Sitrit Y, Mills D, Kagan-Zur V, Roth-Bejerano N (2003) Gene expression during establishment of mycorrhizal associations between *Cistus* hairy roots and *Terfezia boudieri* isolates. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

14 Cultivation of Edible Ectomycorrhizal Fungi by in Vitro Mycorrhizal Synthesis

Giovanna Maria Giomaro[1], Davide Sisti[1], Alessandra Zambonelli[2]

1 Introduction

A significant proportion of ectomycorrhizal fungal species form edible fruiting bodies, and several of these species are important commercially. The most highly regarded ectomycorrhizal fungi include truffles, in particular, the Italian white truffle (*Tuber magnatum* Pico) and Périgord black truffle (*Tuber melanosporum* Vittad.), chanterelle (*Cantharellus cibarius* Fr.), porcini (*Boletus edulis* Bull.: Fr. sensu latu), and matsutake [*Tricholoma matsutake* (S. Ito et Imai) Sing.]. Though generally not as highly regarded, species such as the saffron milk cap [*Lactarius deliciosus* (L.: Fr.) S.F. Gray], Caesar's mushroom [*Amanita caesarea* (Scop.:Fr.) Pers.], burgundy truffle (*Tuber uncinatum* Chatin), bianchetto truffle (*Tuber borchii* Vittad.) and shoro [*Rhizopogon rubescens* (Tul.) Tul.] are also popular in some countries.

Very high prices are paid for the truffles. For example, in 2003, a year in which harvest was poor, the prized Italian white truffle (*T. magnatum*) sold for US$ 2,200–4,600/kg (http://www.tuber.it/pagine/comuni/borsa.php), while the Périgord black truffle retailed for US$ 900–2,000/kg and the bianchetto truffle, generally modestly priced, for US$ 300/kg (http://www.Acqualagna.info/). Matsutake is also very highly regarded, and grade 1 can retail for US$ 1,250/kg or more (Martinez-Carrera et al. 2002). Despite these high prices, over the past few decades, there has been a steady upward trend in the price paid for these fungi, due to a general decline in production (Hall et al. 2003a).

Numerous factors have led to this steady decline in production, including deforestation, the introduction of exotic forest species that are not symbiotic with edible mycorrhizal mushrooms, poor forest management and indiscriminate harvesting of fruiting bodies. Other factors, such as

[1]Istituto di Botanica ed Orto Botanico, Università degli Studi di Urbino, Via Bramante 28, 61029 Urbino (PU), Italy, Tel.: +39-722-2428, Fax: +39-722-4092, E-mail: g.giomaro@uniurb.it
[2]Dipartimento di Valorizzazione e Protezione Agroalimentare, Università degli Studi di Bologna, Via Fanin 46, 40127 Bologna, Italy

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

global climate change, pollution and acid rain, may also contribute to this progressive decrease in production.

The dramatic decline in the production of edible mycorrhizal mushrooms, together with an increase in demand for high-priced foods in industrialized countries, has resulted in increased interest in their cultivation – a difficult task because ectomycorrhizal fungi depend on their host plants for much of their nutrition. In an ectomycorrhizal relationship, the fungus fosters the growth of the tree by helping with the uptake of essential elements and the plant, in turn, provides the fungus with carbohydrates. In order to cultivate an ectomycorrhizal fungus, it is therefore not enough to simply grow the mycelium on a suitable culture substrate, as is the case with cultivated saprophytic fungi, but instead the mycelium of ectomycorrhizal edible mushrooms must be grown together with a suitable plant host, under optimal conditions, to first establish the symbiosis and then encourage the production of the mushrooms. This whole process is so difficult that the cultivation of most edible ectomycorrhizal mushrooms, such as *Tuber magnatum* (Hall et al. 1998b; Tibiletti and Zambonelli 1999), *Tricholoma matsutake* (Wang and Hall 2004) and *Boletus edulis* (Hall et al. 1998a), has never been achieved.

The aim of this chapter is to illustrate the current state of the art of edible mushroom cultivation, with particular focus on in vitro ectomycorrhizal synthesis techniques used with the *Tuber* species, a field in which Italian and French research teams have been working for roughly the past 30 years.

2
Methods for Synthesizing Ectomycorrhizas

Modern methods to culture ectomycorrhizal mushrooms first require the production of mycorrhizal plants under semi-sterile or totally sterile conditions. The colonized plants are then transplanted into areas where the soil and climate suit the growth of both the plant and the fungus. Seedlings, cuttings and micropropagated plantlets can be inoculated by spores, infected roots (mother-plant technique), or mycelial pure cultures to produce mycorrhizal plants.

Seedlings of *Quercus* spp., *Pinus* spp., *Corylus avellana* L. and *Corylus colurna* L., *Ostrya carpinifolia* Scop. and other ectomycorrhizal plants have been used extensively for the commercial production of infected plants. Cuttings have been used for species whose seeds are difficult to germinate, such as *Populus* spp., *Salix* spp. and *Tilia* spp. (Zambonelli and Di Munno 1992; Chevalier and Frochot 1997) and, recently, the use of micropropagated plants has been introduced, mainly for research purposes (Sisti et al. 1998; Chevalier 2001).

2.1 Sporal Inoculum

Spores are the preferred inoculum for the colonization of forest tree transplants with *Pisolithus tinctorius*, *Rhizopogon luteolus* and *Rhizopogon vinicolor* (Garbaye 1991). This method has also been the mainstay for producing *Tuber* spp. colonized plants for the past 30 years in Italy, France and New Zealand (Fontana 1967; Chevalier et al. 1973; Mannozzi Torini 1976; Bencivenga 1982; Tibiletti and Zambonelli 1999; Chevalier 2001; Hall et al. 2003b). However, those details of this method ensuring success, such as the amount and treatment of sporal inoculum, potting mix and greenhouse conditions remain trade secrets (Hall et al. 2003b). This inoculation method can be applied to most of the prized *Tuber* species, such as *T. melanosporum*, *T. aestivum* and *T. borchii*, but not to *T. magnatum* because of difficulties with the germination of its spores (Gregori 2002). Although sporal inocula have also been used for *T. matsutake* and some other edible ectomycorrhizal mushrooms, they have not proved successful. Sporal inoculation techniques are unlikely to be used for mycorrhizal synthesis in vitro because the inoculum invariably contains other fungi and bacteria which may inhibit the development of the ectomycorrhizal fungal mycelia in vitro (Bedini et al. 1999; Barbieri et al. 2004, unpubl. data).

2.2 Mother-Plant Technique

This inoculation technique exploits the mycelium's capacity to propagate from an already colonized root to another root, thus spreading the mycorrhizal colonization. A mother plant, already colonized, is planted in the middle of a large container and then surrounded by other plantlets, obtained by seed germination, scion or in vitro micropropagation (Zuccherelli 1990). Alternatively, parts of the mycorrhized roots of the mother plant are collected and placed in contact with the roots of sterile plants (Chevalier and Grente 1973). This method is widely used for the production of plants colonized with *Tuber* spp.

This technique is cheaper than the sporal inoculation method since fruiting bodies do not need to be purchased. Moreover, it has been used extensively in the production of *T. magnatum* colonized plants, which are difficult to obtain using the sporal inoculation method. However, this method carries a high risk of spreading contaminating and possibly very competitive ectomycorrhizal fungi. Even though growers usually control the mother plants or the root sections to be used as inocula, some contaminated roots may escape detection. Indeed, some contaminating fungi,

such as *Sphaerosporella brunnea* (A. & S. ex Fr.) Svrcek & Kubicka (Amicucci et al. 2000), which are often found on plantlets grown in greenhouses, are difficult to distinguish from the desired fungus using morphological methods. It is even more difficult to distinguish among the mycorrhizas produced by different species of truffles using morphological methods, in particular the white and whitish truffles – *T. magnatum*, *Tuber maculatum* Vittad., *Tuber dryophilum* Tul. & Tul. and *Tuber puberulum* Berk. and Broome (Zambonelli et al. 2000). While there are now highly developed molecular identification methods that allow unequivocal identification of the symbiotic fungus (Amicucci et al. 2002), these methods are still very expensive and thus impractical for large-scale quality control of mother plants.

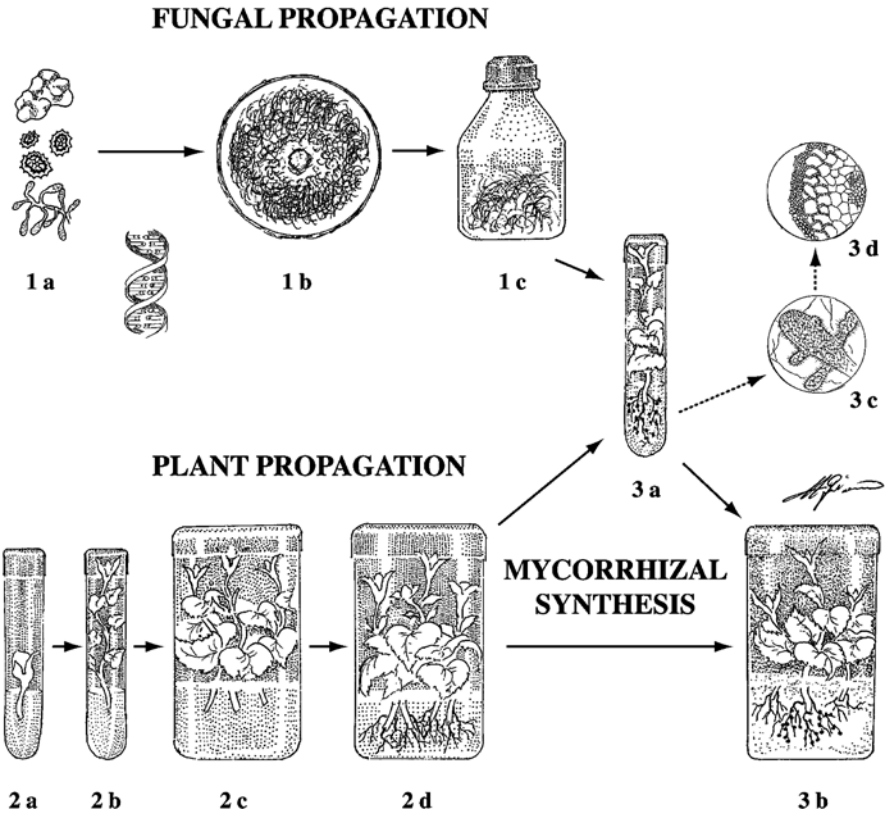


Fig. 1. 1*a* Fungal propagation: sources of mycelial culture: fruit body, spores, mycorrhizas, 1*b* Petri plate mycelial culture after biomolecular identification, 1*c* liquid mycelial culture. 2*a* Plant propagation: breaking dormancy bud phase, 2*b* in vitro plant regeneration, 2*c* micropropagation phase, 2*d* rooting phase. 3*a* Mycorrhizal synthesis: culture tube with inoculated plant, 3*b* mycorrhization using mother-plant method (∗), 3*c* in vitro ectomycorrhiza, 3*d* ectomycorrhiza cross section

In order to avoid contamination, the mother-plant technique could be applied on a larger scale by using colonized mother plants obtained in vitro by mycelial inoculation. In our laboratories, we produce in vitro colonized plantlets of *T. borchii* and *T. brumale* using this method (Fig. 1). It guarantees excellent results, thanks to the high infectivity of the mycelium, already in the symbiotic stage.

2.3 Mycelial Inoculation

For more than 30 years, numerous research teams have tried to isolate the mycelia of edible ectomycorrhizal species in an attempt to avoid the problems and limitations associated with the production of colonized plantlets using the above methods. Currently, the mycelia of many prized species can be cultivated in vitro and, thanks to molecular methods, their identity can be verified, thus avoiding the misidentification problems experienced in the past (Danell 1994; Mello et al. 2001; Amicucci et al. 2002; Guerin-Laguette et al. 2002). The mycelia of ectomycorrhizal fungi can be obtained from spores, sections of tissue from fruiting bodies, or mycorrhizas (Chevalier 1973).

2.3.1 Spores

Obtaining mycelia from spores is extremely complicated because of difficulties related to the germination of dormant spores. The percentage of spore germination in *C. cibarius* modified Fries medium (MFM) with the addition of activated charcoal was only 0.04%, and the spores of *C. tubaeformis* germinated only after 10 months (Danell 1994). Difficulties with the germination of *T. matsutake* spores have also been reported (Wang and Hall 2004). Although the germination of *Tuber* spores has been reported in the past by several authors (Sappa 1940; Guiochon 1959; Grente et al. 1972), unfortunately such reports seem to constitute isolated cases. In fact, these studies did not rigorously establish the conditions for germination and, at that time, researchers did not have the technical means to unambiguously identify the mycelia that were obtained.

2.3.2 Glebal Tissue

Numerous problems are also associated with obtaining mycelia starting from glebal tissue because the fruiting bodies generally contain a considerable microbial population, including several species of bacteria. Whereas

B. edulis sporocarps often appeared to be free of bacteria, the aerobic bacterial population in *C. cibarius* varied from 0.3 to 7.10^6 CFU/g (colony forming units per gram; Danell 1994), with 78% of the total weight consisting of fluorescent *Pseudomonas* bacteria. Likewise, in *T. borchii*, about 300 different *Pseudomonas*, mainly *P. fluorescens*, *P. corrugata* and *P. tolaasii*, were isolated (Bedini et al. 1999). These bacteria have a significant role, because they can alter the growth and morphological characteristics of the mycelium in pure culture (Sbrana et al. 2000), and stimulate mycorrhizal colonization (Founoune et al. 2002). Gazzanelli et al. (1999) found 10^6CFU/g bacteria inside the fruiting bodies, which was considerably higher than bacterial levels in the surrounding soil. Barbieri et al. (2001) characterized the bacteria associated with the fruiting bodies of *T. borchii* using molecular methods and, in addition to *Pseudomonas* spp., found bacteria belonging to the *Bacillus subtilis* group, *B. cereus* group and *Paenibacillus* spp. Furthermore, an uncultivable bacterium, belonging to the *Cytophaga–Flexibacter–Bacteroides* complex, with 6–10 bacterial cells for every fungal hyphal compartment, was associated with the mycelium in the pure culture of five *T. borchii* isolates (Barbieri et al. 2002). This bacterium was also found in *Tuber aestivum* Vittad. (Mello et al. 2002; Minerdi et al. 2002).

Another problem that needs to be overcome during the isolation of mycelia is a growth lag that ranges from a minimum of 8 days to about 1 month for *Tuber* spp., and from 17 to 53 days for *C. cibarius* (Chevalier 1972; Danell 1994). Unfortunately, some isolates, particularly those of *Tuber* spp., fail to grow after the first subculture (Iotti et al. 2002). This is probably because during subculturing and before the regenerating mycelium is adapted to a saprophytic existence, it is detached from the original inoculum providing essential nutrients. This is most pronounced with cultures of *T. magnatum* derived from sections of glebal tissue, which fail to grow after the first subculturing (Zambonelli and Sisti, unpubl. data).

2.3.3 Mycorrhizas

The main problems associated with isolating mycelia from mycorrhizas are ensuring that the mycorrhizas are free of contaminants, and then identifying the isolated fungal species using morphological methods. In the past, the latter problem led to the isolation of contaminants instead of the desired species – a problem discovered only after the application of molecular identification techniques (Mello et al. 2001).

2.4 Culture Media

A wide range of media have been used for the cultivation of ectomycorrhizal fungi. The most frequently used media for *T. matsutake* have been the modified Melin-Norkrans (MMN, Marx 1969; Molina and Palmer 1982; Heinonem-Tanski and Holopainen 1991), potato-dextrose-agar (PDA; Gong et al. 1999), Hamada (Ogawa 1978) and Oyama (Yamada et al. 1999). Isolated matsutake mycelia exhibit features common to many ectomycorrhizal fungi, in that once isolated, it is possible to grow the mycelium on a range of media, albeit at variable rates. Interestingly, Ogawa and Kawai (1976) obtained a better growth of *T. matsutake* after adding a filtrate of needles and roots of *Pinus densiflora*, the typical host plant. *T. matsutake* mycelium culture submerged in a bio-fermenter grew at a faster rate than cultures in a dish or flask (Kawagoe et al. 1999).

Modess agar medium (Modess 1941), used by Chevalier (1972) for the propagation of *T. brumale*, *T. melanosporum*, *Tuber mesentericum* Vittad., *Tuber rufum* Pico and *T. uncinatum*, was subsequently employed by Bonfante and Fontana (1973) in their study of the nutrition of the mycelium of *T. melanosporum*. Other media used for *Tuber* include Melin's for *Tuber brumale* (Melin 1922; Chevalier 1973), Moser's (Palenzona et al. 1972) for *T. brumale*, *T. melanosporum* and *T. rufum*, MS modified medium (Murashige and Skoog 1962) and MMN medium for *Tuber borchii* (Sisti et al. 1998; Giomaro et al. 2000), woody plant modified medium (WPMm; Lloyd and McCown 1980) for *T. maculatum*, *T. rufum*, *T. melanosporum*, *T. aestivum*, *T. brumale* and *Tuber macrosporum* Vittad. (Iotti et al. 2002), and PDA for *T. brumale* and *T. borchii* (Giomaro et al. 2002). PDA, MMN and malt agar have been used for *C. cibarius*, although Danell's (1994) use of MS medium with the addition of 0.05% activated charcoal achieved the best results for isolating mycelia from fruiting bodies, and MFM for stock cultures. Iotti et al. (unpubl. data) were also successful isolating *B. edulis* mycelium with several different media. The optimum pH for the growth of most ectomycorrhizal fungi is between 3.0 and 7.0 (Marx 1973; Ogawa 1978), although species of *Tuber* grow better at higher pH, which is consistent with their ecological requirements (Zambonelli and Di Munno 1992; Hall et al. 2003a). However, the rate of growth of isolated edible mycorrhizal fungi varies according to the species, isolates and medium employed (Giomaro et al. 2000).

2.5 Mycorrhizal Synthesis In Vitro

Numerous in vitro mycorrhizal synthesis methods have been developed and are described in detail by Peterson and Chakravarty (1991), a few of these having been used for edible ectomycorrhizal mushrooms. A system similar to Molina's (1979) test tube technique has been developed for the ectomycorrhizal synthesis of *T. borchii* with *Tilia platyphyllos* Scop. (Sisti et al. 1998), *Populus* and *Cistus* (Zambonelli et al. 2002), and *Quercus* (Giomaro et al. 2002; Fig. 1). The method involves raising plantlets in a medium with a low concentration of auxin (0.2 mg/l of NAA) to allow the differentiation of roots with multiple secondary roots of limited growth. The mycelium of *T. borchii* is propagated in a Petri plate on PDA and, after about 30 days, mycelial plugs are taken from the edge of the actively growing fungal colony and cultivated in MMN liquid medium. The mycorrhizal synthesis is performed in vermiculite moistened with MS/2 liquid medium at pH 6.3. The volume of MS/2 medium is critical – the medium must be sufficiently wet, but without any residual liquid at the bottom of the culture tube, so that the substrate is adequately aerated. In the early stages, the mycelium colonizes the substrate, the first mycorrhizas not differentiating until after about 3 months. Some *T. borchii* isolates grow uniformly throughout the substrate, while others are thicker around the root tips, with full mycorrhizal formation taking about 4 months (Giomaro et al. 2000). The mycorrhizas have a mantle, Hartig net and well-differentiated cystidia, and are morphologically similar to mycorrhizas found in the field (Figs. 2 and 3). A slightly modified version of this system was successfully used for the in vitro mycorrhization of three isolates of *B. edulis* with *Betula pendula* Roth and *Pinus radiata* (Wang et al. 1998), and *L. deliciosus* with *P. radiata* (Wang et al. 2002). Using faster systems of mycorrhizal synthesis, such as

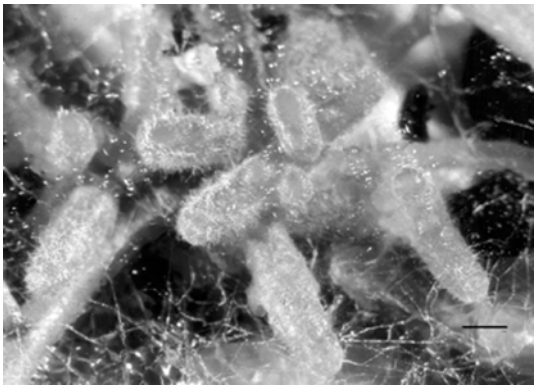


Fig. 2. In vitro mycorrhizas of *Tuber borchii* on *Tilia platyphyllos* under the stereomicroscope. A compact mantle covers the colonized roots, and abundant cystidia are present on the mantle surface. *Bar* 200 µm

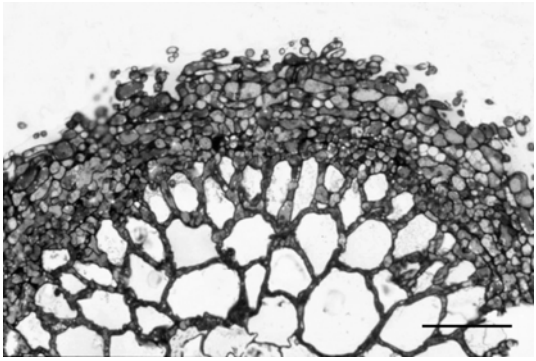


Fig. 3. Cross section of a mycorrhiza of *T. borchii* on *T. platyphyllos* under the light microscope. *Bar* 10 µm. (Photograph courtesy of Paola Bonfante)

paper sandwich techniques (Chilvers et al. 1986), only the beginning of intercellular penetration is achieved and a well-differentiated mantle is not formed (Menotta et al., unpubl. data).

The in vitro formation of mycorrhizas of *C. cibarius* was obtained by Danell (1994) using a culture unit system (CUS) where 10 ml of the 5-l nutrient solution (Ingestad mineral solution) supplemented with glucose was replaced every 90 min. The $C0_2$ concentration in the culture vessel was also raised to 0.2%. This system led to the formation of mycorrhizas after 8 weeks, and they were fully developed after 10–12 weeks. Moore et al. (1989) obtained mycorrhizas in 4–5 months in Erlenmeyer flasks, but these were not fully developed.

Recently, a root organ culture system using transformed roots of *Cistus incanus* L. has been used to obtain mycorrhizal association with *Tuber melanosporum* mycelia (Roth-Bejerano et al. 2001; Wenkart et al. 2001; Coughlan and Piché 2004, see Chap. 13) and with *T. borchii* (Bonfante, pers. comm.).

3 In Vitro Results to Date

Most of the in vitro mycorrhization techniques applied to edible mushrooms have been developed to obtain mycorrhizal plantlets, which are then planted to produce fruiting bodies. Plantlets colonized with *Cantharellus* have produced fruiting bodies in the greenhouse (Danell and Camacho 1997). In New Zealand, the first *Pinus radiata* D. Don plantlets colonized with *L. deliciosus* mycelia in pure culture produced commercially viable fruiting bodies less than 2 years after planting (Wang et al. 2002). Although plants colonized with porcini and *T. matsutake* have been obtained, these have yet to lead to commercial production (Hall et al. 2003b).

Tuber colonized plants are still produced commercially using sporal inocula, even though mycelia of several *Tuber* spp. (Iotti et al. 2002) have been obtained in pure culture and in vitro ectomycorrhizal synthesis was extensively used only for research purposes, specifically, to gain insights into early colonization events and elicitation of biochemical responses by ectomycorrhizal fungi. The technique has shown, for example, that different isolates of *T. borchii* produce varying degrees of colonization and have varying morphological characteristics, whereas the colour, shape and type of ramification of the mycorrhiza and cystidia are species-specific (Giomaro et al. 2002). Furthermore, some cytological characteristics of the cells of the fungal mantle (degree of vacuolization, the greater axis and the chrome affinity) are also isolate-specific and correlated with the development of the plantlets in vitro, which in turn depends on the efficiency of the fungal isolate (Sisti et al. 2003). The host plant can also lead to variations in the morphological characteristics of the mycorrhizas. Indeed, the mycorrhizas of *T. brumale* on *Quercus pubescens* Willd. are darker and shorter with a less lobed micoclena than mycorrhizas on *Tilia americana* L. Ultimately, it is hoped that this method will be employed to elucidate the genetic control of mycorrhizal formation.

It is well known that for ectomycorrhizal symbiosis to take place, the plant and the fungus must exchange signals in order to recognize one another (Tagu et al. 2002). Menotta et al. (2004), using the in vitro *Tuber borchii–Tilia platyphyllos* mycorrhizal system, detected 29 molecules formed specifically when the two organisms interact with each other (without actually coming into contact). In particular, a brassinosteroid was detected. This kind of phytohormone causes cell elongation, xylem differentiation, and enhances resistance to stress. Other molecules, such as benzothiazole, *p*-isopropylbenzaldehyde, beta-pinene and germacrene D, may play a role in the biochemical mechanisms of signal transduction in these systems. The *Tilia platyphyllos–Tuber borchii* model has also been used in order to identify genes induced or up-regulated during symbiosis, since their isolation is a prerequisite for understanding the molecular bases of mycorrhizal development and regulation. The results show that many genes – more plant genes than fungal genes – are expressed at higher levels during the symbiotic phase (Polidori et al. 2002). Moreover, fungal biomass and transcript levels in *T. platyphyllos–T. borchii* ectomycorrhizas were estimated and a decrease in fungal biomass, transcript and protein levels during ectomycorrhizal development was found (Zeppa et al. 2000). Based on this mycorrhizal model, the role of *T. borchii* in nitrate assimilation by ectomycorrhizas was investigated by Guescini et al. (2003). Amino acid determination by HPLC showed higher levels of glutamate, glutamine and asparagine in symbiotic tissues compared with mycelial controls, suggesting that these amino acids may be the

compounds serving to transfer nitrogen to the host plant (Guescini et al. 2003).

4 Conclusion

In vitro mycorrhization provides a study model in which all the biotic and abiotic variables influencing the colonization process are controlled, thus allowing us to broaden our genetic and physiological understanding of mycorrhizal symbiosis with no interference from extraneous factors. For example, we can compare different genotypes in order to obtain the best combination of host plants and symbiont fungi. Furthermore, if we modify the system and make it more complex, additional variables can be introduced, and the effects of single biotic and abiotic variables or of a combination of these variables on the formation of the mycorrhizas can be assessed. It will therefore be possible to study and clarify the role of several nutrients, the reaction of the substrate, temperature, humidity, light, etc., in the formation of symbiosis. It will also be possible to clarify the role of bacteria and other micro-organisms usually found in the mycorrhizosphere in the field. These new insights will provide researchers with useful data to overcome the problems associated with edible ectomycorrhizal mushrooms (e.g. *T. magnatum*), which are difficult to cultivate but also the most interesting mushrooms from a commercial standpoint.

In vitro inoculation might also be used for the commercial production of contaminant-free edible fungi colonized plants. Clonal plantlets could be utilized for the selection of "plus plantlets" (Chevalier 2001; Olivier et al. 2001), i.e. plants that are more susceptible to mycorrhizal infection, able to maintain mycorrhizas in the field, and characterized by an early and abundant production of fruiting bodies.

The use of mycelial pure cultures as inocula might allow us to select fungal strains that are better adapted to a particular combination of soil, climate and host plants.

Acknowledgements. We would like to thank to Dr. Ian Hall for the helpful suggestions and for the English revision.

References

Amicucci A, Guidi C, Zambonelli A, Potenza L, Stocchi V (2000) Multiplex PCR for the identification of white *Tuber* species. FEMS Microbiol Lett 189:265–269

Amicucci A, Potenza L, Guidi C, Rossi I, Bertini L, Zambonelli A, Stocchi V (2002) Molecular techniques for the identification of edible mycorrhizal mushrooms. In: Hall I, Wang Yun,

Danell E, Zambonelli A (eds) Edible mycorrhizal mushrooms and their cultivation. Proc 2nd Int Conf Edible Mycorrhizal Mushrooms, New Zealand Institute for Crop & Food Research, CD-ROM

Barbieri E, Potenza L, Stocchi V (2001) Molecular characterization of cellulosolytic-chitinolytic bacteria associated with fruitbodies of the ectomycorrhizal fungus *Tuber borchii* Vittad. Symbiosis 30:123–139

Barbieri E, Riccioni G, Pisano A, Sisti D, Zeppa S, Agostini D, Stocchi V (2002) Competitive PCR for quantitation of a *Cytophaga-Flexibacter*-Bacteroides phylum bacterium associated with the *Tuber borchii* Vittad. mycelium. Appl Environ Microbiol 68:6421–6424

Bedini S, Bagnoli G, Sbrana C, Leporini C, Tola E, Dunne C, D'Andrea F, O'Gara F, Nuti MP (1999) *Pseudomonas* isolated from within fruit bodies of *Tuber borchii* are capable of producing biological control or phythostimulatory compounds in pure culture. Symbiosis 26:223–236

Bencivenga M (1982) Alcune metodiche di micorrizazione di piante forestali con il tartufo nero pregiato di Norcia o di Spoleto (*Tuber melanosporum* Vitt.). Inform Agrario 38:21155–21163

Bonfante FP, Fontana A (1973) Sulla nutrizione del micelio di *Tuber melanosporum* Vitt. in coltura. Atti Accad Sci Torino 107:731–741

Chevalier G (1972) Obtention de cultures de mycélium de truffe à partir du carpophore et des mycorhizes. Académie d'Agriculture de France, extrait procès-verbal séance 28 juin 1972, pp 981–989

Chevalier G (1973) Synthèse axénique des mycorhizes de *Tuber brumale* Vitt. à partir de cultures pures du champignon. Ann Phytopathol 5:163–182

Chevalier G (2001) La plante mycorrhize I.N.R.A.: passé, présent, avenir. In: Actes Vème Congr Int Science et Culture de la Truffe, FFT, Aix en Provence, pp 300–303

Chevalier G, Frochot H (1997) La Truffe de Bourgogne. Pétrarque, Levallois-Perret

Chevalier G, Grente J (1973) Propagation de la mycorhization par la truffe à partir de racines excisées et de plantes inséminateurs. Ann Phytopathol 5:317–318

Chevalier G, Grente J, Pollaksek A (1973) Obtention de mycorhizes de différents *Tuber* par synthèse à partir de spores en conditions gnotoxéniques et à partir de cultures pures du mycélium en conditions axéniques et gnotoxéniques. Ann Phytopathol 5:107–108

Chilvers GA, Douglass PA, Lapeyrie FF (1986) A paper-sandwich technique for rapid synthesis of ectomycorrhizas. New Phytol 103:397–402

Danell E (1994) *Cantharellus cibarius*: mycorrhiza formation and ecology. Acta Univ Ups, Comprehensive Summaries Uppsala Dissertation Faculty of Science and Technology, Uppsala, pp 35–75

Danell E, Camacho FJ (1997) Successful cultivation of the golden chanterelle. Nature 385:303

Fontana A (1967) Sintesi micorrizica tra *Pinus strobus* e *Tuber maculatum*. Giorn Bot Ital 101:298–299

Founoune H, Ba AM, Sall S, Branget I, Lorquin J, Neyra M, Chotte JL (2002) Mycorrhiza helper bacteria stimulated ectomycorrhizal symbiosis of *Acacia holosericea* with *Pisolithus alba*. New Phytol 153:81–89

Garbaye J (1991) Utilisation des mycorhizes en sylviculture. In: Strullu DG, Garbaye J, Perrin R, Plenchette C (eds) Les mycorhizes des arbres et plantes cultivées. Tech Docum Lavoisier Paris, France, pp 197–248

Gazzanelli G, Malatesta M, Pianetti A, Baffone W, Stocchi V, Citterio B (1999) Bacteria associated to fruit bodies of the ecto-mycorrhizal fungus *Tuber borchii* Vittad. Symbiosis 26:211–222

Giomaro G, Zambonelli A, Sisti D, Cecchini M, Evangelista V, Stocchi V (2000) Anatomical and morphological characterization of mycorrhizas of five strain of *Tuber borchii* Vittad. Mycorrhiza 10:107–114

Giomaro G, Sisti D, Zambonelli A, Amicucci A, Cecchini M, Comandini O, Stocchi V (2002) Comparative and molecular characterization in *Tilia americana* and *Quercus pubescens* with *Tuber brumale*. FEMS Microbiol Lett 216:9–14

Gong MQ, Chen Y, Wang FZ, Chen YL (1999) *Song Rong*. Yunna Science and Technology Press, Kunming

Gregori GL (2002) Problems and expectations with the cultivation of *Tuber magnatum* In: Hall I, Wang Y, Danell E, Zambonelli A (eds) Edible mycorrhizal mushrooms and their cultivation. Proc 2nd Int Conf Edible Mycorrhizal Mushrooms, New Zealand Institute for Crop & Food Research, CD-ROM

Grente J, Chevalier G, Pollacsek A (1972) La germination de l'ascospore de *Tuber melanosporum* et la synthèse sporale des mycorrhizes. C R Acad Sci Paris 275:743–746

Guerin-Laguette A, Vaario L, Gill WM, Matsushima N, Suzuki A, Lapeyrie F (2002) Growth stimulation of a shiro-like mycelium of *Tricholoma matsutake* on artificial substrate following addition of non-ionic surfactants or vegetable oils. In: Hall IR, Wang Y, Danell E, Zambonelli A (eds) Edible mycorrhizal mushrooms and their cultivation. Proc 2nd Int Conf Edible Mycorrhizal Mushrooms, New Zealand Institute for Crop & Food Research, CD-ROM

Guescini M, Pierleoni R, Palma F, Zeppa S, Vallorani L, Potenza L, Sacconi C, Giomaro G, Stocchi V (2003) Characterization of the *Tuber borchii* nitrate reductase gene and its role in ectomycorrhizae. Mol Genet Genom 269:807–816

Guiochon P (1959) Germinations monospermes de la truffe *Tuber melanosporum* Vitt. Mushroom Sci 4:294–297

Hall IR, Lyon JE, Wang Y, Sinclair L (1998a) Ectomycorrhizal fungi with edible fruiting bodies 2. *Boletus edulis*. Econ Bot 52:44–56

Hall IR, Zambonelli A, Primavera F (1998b) Ectomycorrhizal fungi with edible fruiting bodies 3. *Tuber magnatum*. Econ Bot 52:192–200

Hall IR, Stephenson SL, Buchanan PK, Yun W, Cole ALJ (2003a) Edible and poisonous mushrooms of the world. Timber Press, Portland, Oregon

Hall IR, Yun W, Amicucci A (2003b) Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol 21:433–438

Heinonem-Tanski H, Holopainen T (1991) Maintenance of ectomycorrhizal fungi. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology. Academic Press, London, pp 413–422

Iotti M, Amicucci A, Stocchi V, Zambonelli A (2002) Morphological and molecular characterization of mycelia of some *Tuber* species in pure culture. New Phytol 155:499–505

Kawagoe M, Kawakami K, Nakamura Y, Naoe K, Miki K, Noda H (1999) Submerged culture of *Tricholoma matsutake* micelium in bubble column fermentors. J Biosci Bioeng 87:116–118

Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb Proc Int Plant Propagator Soc 30:421–427

Mannozzi-Torini L (1976) Manuale di tartuficoltura. Tartufi e tartuficoltura in Italia. Edagricole, Bologna

Martínez-Carrera D, Morales P, Pellicer-González E, León H, Aguilar A, Ramínez P, Ortega P, Largo A, Bonilla M, Gómez M (2002) Studies on the traditional management, and processing of matsutake mushrooms in Oaxaca, Mexico. Micol Apl Int 14:25–43

Marx DH (1969) The influence of ectotrophic ectomycorrhizal fungi on the resistance of pine roots to pathogenic colonizations. I. Antagonism of mycorrhizal fungi to pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH (1973) Mycorrhizae and feeder root diseases. In: Marks GC, Kozowski TT (eds) Ectomycorrhizae: their ecology and physiology. Academic Press, New York, pp 351–382

Melin E (1922) Untersuchungen über die Larix-Mykorrhiza. I. Synthese der Mykorrhiza in Reinkulture. Sven Bot Tidskr 16:161–196

Mello A, Fontana A, Meotto F, Comandini O, Bonfante P (2001) Molecular and morphological characterization of *Tuber magnatum* mycorrhizas in a long-term survey. Microbiol Res 155:279–284

Mello A, Cantisani A, Vizzini A, Bonfante P (2002) Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environ Microb 4:584–594

Menotta M, Gioacchini AM, Amicucci A, Buffalini M, Sisti D, Stocchi V (2004) Headspace solid-phase microextraction with gas chromatography and mass spectrometry in the investigation of volatile organic compounds in an ectomycorrhizae synthesis system. Rapid Commun Mass Spectrom 18:1–5

Minerdi D, Bianciotto V, Bonfante P (2002) Endosymbiotic bacteria in mycorrhizal fungi: from their morphology to genomic sequences. Plant Soil 244:211–219

Modess O (1941) Zur Kenntnis der Mycorrizabildner von Kiefer und Fichte. Symb Bot Uppsala 5:1–146

Molina R (1979) Pure culture synthesis and host specificity of red alder mycorrhizae. Can J Bot 57:1223–1228

Molina R, Palmer JG (1982) Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, Minnesota, pp 115–129

Moore LM, Jansen AE, Van Griensven LJLD (1989) Pure culture of ectomycorrhizas with *Cantharellus cibarius*. Acta Bot Neerl 38:273–278

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Ogawa M (1978) Biology of matsutake mushroom. Tsukiji Shokan, Tokyo

Ogawa M, Kawai M (1976) Studies on the artificial reproduction of *Tricholoma Matsutake* (Ito et Imai) Sing. III. Effect of growth promoting natural products on the vegetative growth of *T. matsutake*. J Trans Mycol Soc Jpn 17:492–498

Olivier JM, Chevalier G, Guinberteau J, Mamoun M, Lemaire JP, Lemoine MC, Oudin A, Bardet MC, Verlhac A, Giraud M, Navatel JC (2001) Multiplication végétative et clonage des arbres hôtes de *Tuber melanosporum* Vitt. et *Tuber uncinatum* Chatin. In: Actes Vème Congr Int Science et Culture de la Truffe, FFT, Aix en Provence, pp 307–310

Palenzona M, Chevalier G, Fontana A (1972) Sintesi micorrizica tra i miceli in coltura di "*Tuber brumale*", "*T. melanosporum*", "*T. rufum*" e semenzali di conifere e latifoglie. Alliona 18:41–52

Peterson RL, Chakravarty P (1991) Techniques in synthesizing ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology. Academic Press, San Diego, pp 75–106

Polidori E, Agostini D, Zeppa S, Potenza L, Palma F, Sisti D, Stocchi V (2002) Identification of differentially expressed cDNA clones in *Tilia platyphyllos-Tuber borchii* ectomycorrhizae using a differential screening approach. Mol Genet Genom 266:858–864

Roth-Bejerano N, Wenkart S, Mills D, Kagan-Zur V (2001) Mycorrhizal association between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. In: Actes Vème Congr Int Science et Culture de la Truffe, FFT, Aix en Provence, pp 173–176

Sappa F (1940) Ricerche biolgiche sul *Tuber magnatum* Pico. La germinazione delle spore e caratteri della micorriza. Nuovo Giorn Bot Ital 47:155–198

Sbrana C, Bagnoli G, Bedini S, Filippi C, Giovannetti M, Nuti MP (2000) Adhesion to hyphal matrix and antifungal activity of *Pseudomonas* strains isolated from *Tuber borchii* ascocarps. Can J Microbiol 46:256–268

Sisti D, Giomaro G, Zambonelli A, Rossi I, Ceccaroli P, Citterio B, Benedetti PA (1998) In vitro mycorrhizal synthesis of micropropagated *Tilia platyphyllos* Scop. plantlets with *Tuber borchii* Vittad. mycelium in pure culture. Acta Hortic 457:379–387

Sisti D, Giomaro G, Cecchini M, Faccio A, Novero M, Bonfante P (2003) Two genetically related strains of *Tuber borchii* produce *Tilia* mycorrhizas with different morphological traits. Mycorrhiza 13:107–115

Tagu D, Lapeyrie F, Martin F (2002) The ectomycorrhizal symbiosis: genetics and development. Plant Soil 244:97–105

Tibiletti E, Zambonelli A (1999) I tartufi della provincia di Forlì-Cesena, Patron, Bologna

Wang Y, Hall IR (2004) Matsutake – a natural biofertiliser? In: Rai M (ed) Handbook of microbial fertilizer. Haworth Press, Binghamton, NY (in press)

Wang Y, Zambonelli A, Hall IR (1998) Researches on *Boletus edulis* in New Zealand. In: Abstr Vol 2nd Int Conf Mycorrhizae, Uppsala, p 182

Wang Y, Hall IR, Dixon C, Hance-Halloy M, Strong G, Brass P (2002) The cultivation of *Lactarius deliciosus* (saffron milk cap) and *Rhizopogon rubescens* (shoro). In: Hall I, Wang Yun, Danell E, Zambonelli A (eds) Edible mycorrhizal mushrooms and their cultivation. Proc 2nd Int Conf Edible Mycorrhizal Mushrooms, New Zealand Institute for Crop & Food Research, CD-ROM

Wenkart S, Roth-Bejerano N, Mills D, Kagan-Zur V (2001) Mycorrhizal association between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. Plant Cell Rep 20:369–373

Yamada A, Meada K, Ohmasa M (1999) Ectomycorrhizal formation of *Tricholoma matsutake* on *Pinus densiflora* in vitro. Mycoscience 40:455–463

Zambonelli A, Di Munno R (1992) Indagine sulla possibilità di diffusione dei rimboschimenti con specie tartufigene: aspetti tecnico-colturali ed economici. A cura del Ministero dell'Agricoltura e delle Foreste, Ecoplanning s.r.l., Città di Castello

Zambonelli A, Rivetti C, Percurdani R, Ottonello S (2000) TuberKey: a DELTA-based tool for the description and interactive identification of truffles. Mycotaxon 74:57–76

Zambonelli A, Iotti M, Giomaro G, Hall I, Stocchi V (2002) *T. borchii* cultivation: an interesting perspective. In: Hall I, Wang Y, Danell E, Zambonelli A (eds) Edible mycorrhizal mushrooms and their cultivation. Proc 2nd Int Conf Edible Mycorrhizal Mushrooms, New Zealand Institute for Crop & Food Research, CD-ROM

Zeppa S, Vallorani L, Potenza L, Bernardini F, Pieretti B, Guescini M, Giomaro G, Stocchi V (2000) Estimation of fungal biomass and transcript levels in *Tilia platyphyllos-Tuber borchii* ectomycorrhizae. FEMS Microbiol Lett 188:119–124

Zuccherelli G (1990) Moltiplicazione in vitro di cinque varietà di nocciolo e loro micorrizazione con *Tuber melanosporum*. Inform Agrario 66:51–55

Part V
Root Organ Culture of Other Fungal Symbioses

15 *Geosiphon pyriformis* – a Glomeromycotan Soil Fungus Forming Endosymbiosis with Cyanobacteria

Arthur Schüßler[1], Elke Wolf[1]

1 Introduction

The *Geosiphon* symbiosis is the only known fungal endosymbiosis with cyanobacteria and has recently attracted attention because the fungal symbiosis partner phylogenetically belongs to the arbuscular mycorrhizal (AM) fungi. This discovery also led to a project resulting in the new taxonomy for these fungi (the Glomeromycota).

The *Geosiphon* symbiosis with cyanobacteria broadens the spectrum of glomeromycotan symbiosis partners beyond that of vascular plants and bryophytes, and raises interesting questions about the origin of the AM. Moreover, the *Geosiphon* symbiosis also can serve as a model system for AM. This chapter will describe some of these aspects, e.g. regarding partner recognition, nutrient exchange and evolution of the AM symbiosis, and the culture systems we use.

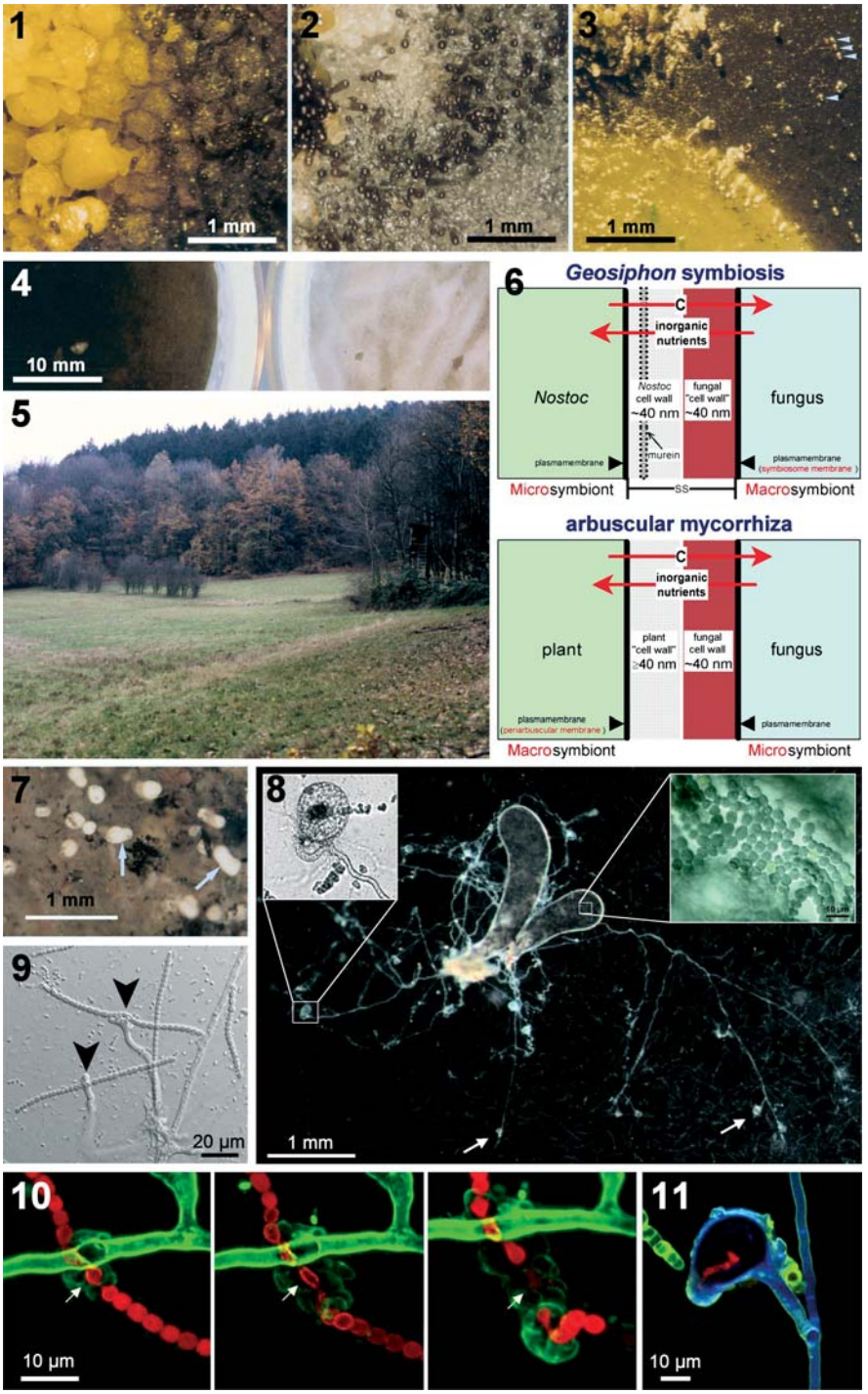
2 Development and Structure of the Symbiotic Consortium

2.1 Structure of the Mature Symbiosis

The *Geosiphon* symbiosis is formed at the surface of damp, nutrient-poor soils. The symbiotic 'organ', the '*Geosiphon* bladder', is a large, multinucleated fungal vesicle which is coenocytic with the mycelium and which can be more than 2 mm long (Figs. 1–11). The bladders are under turgor pressure of about 0.6 MPa (Schüßler et al. 1995) and can live for more than 6 months. Inside the bladder, the cyanobacteria (*Nostoc punctiforme*) cells are located peripherally within a single, cup-shaped compartment, the symbiosome. Its membrane is derived from the plasma membrane by

[1]Institute of Botany, Schnittspahnstrasse 10, 64287 Darmstadt, Germany, Tel.: +49-6151-164568, Fax: +49-6151-164630, E-mail: schuessler@bio.tu-darmstadt.de

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)


1
1 mm
2
1 mm
3
1 mm
4
10 mm
5
6
Geosiphon symbiosis
C
inorganic nutrients
Nostoc
Nostoc cell wall ~40 nm
fungal "cell wall" ~40 nm
fungus
murein
plasmamembrane
plasmamembrane (symbiosome membrane)
Microsymbiont
SS
Macrosymbiont
arbuscular mycorrhiza
C
inorganic nutrients
plant
plant "cell wall" ≥40 nm
fungal cell wall ~40 nm
fungus
plasmamembrane (periarbuscular membrane)
plasmamembrane
Macrosymbiont
Microsymbiont
7
1 mm
8
1 mm
9
20 µm
10
10 µm
11
10 µm

◀ **Fig. 1. 1–11** Positive influence of charcoal on *Geosiphon* symbiosis formation. Bladders are nearly exclusively formed where charcoal is scattered onto the substrate. **1** Culture on coarse, quartz sand. **2** Culture on fine, white quartz sand. **3** Culture on 2% agar (*arrowheads* indicate young bladders). **4** View from the top onto two cultures on fine, white quartz sand (without charcoal layer), only a part of the culture vessels is shown. *Nostoc* growth is much lower (after 12 weeks) in medium with 5 µM phosphate (*right*) compared to 1 mM phosphate (*left*). **5** A typical *Geosiphon* habitat in the Spessart Mountains. **6** Schemes comparing the symbiotic interfaces of the *Geosiphon* and AM symbioses. **7** Spores formed in coarse quartz sand culture. In contrast to culture on fine substrates where spores are regularly round, spores formed in the coarse substrate can be irregularly shaped (*arrows*), showing the potential influence of the substrate on spore morphology. **8** Two *Geosiphon* bladders with newly formed hyphae. At the tips new young bladders develop (*arrows*). *Left inset* One-week-old bladder. *Right inset* Endosymbiotic *Nostoc* arranged at the periphery of the bladders; the *light green cells* are heterocysts. **9** Early stages of recognition (*arrowheads*). Upon contact with a *Nostoc* primordium, the fungus forms several 'projections', eventually engulfing the *Nostoc* cells. **10** Incorporation of a *Nostoc* primorium. Confocal laser scanning microscopy (CLSM); excitation 488 nm, maximum projection (23 optical sections) through 9.5 µm. *Green* indicates ConA-Alexa488 fluorescence (detection: 505–550 nm; stains mannose), *red* indicates *Nostoc* pigment autofluorescence (detection: 620–730 nm). Pictures were taken 27 (*left*), 41 (*centre*) and 60 h (*right*) after start of green light illumination (see text). The *Nostoc* cells deform and bleach during incorporation (*arrow*). **11** CLSM of a very young *Geosiphon* bladder. Maximum projection (11 optical sections) through 5.5 µm in the middle part of the bladder. Simultaneous 2-photon (780 nm) and 1-photon (488 + 568 nm) excitation. Detection: *blue* (405–480 nm) = Calcofluor White (stains chitin), *green* (515–560 nm) = ConA-Alexa488 (stains mannose), *red* (600–775 nm) = *Nostoc* pigment autofluorescence. The endosymbionts begin to recover. The EPS of free-living *Nostoc* as well as the surface of the hyphae contain mannose. From a part of the hypha, cytoplasm was retracted and septae were formed

invagination (see 2.2, Figs. 9–11, and retains the capability to synthesize chitin. This is interesting in the context that the fungus belongs to the Glomeromycota (see Sect. 6.1), and the symbiotic interface is similar in structure and function to that formed in AM fungi (Fig. 6: Schüßler et al. 1996). The endosymbionts are located mainly in the apical three-fourths of the bladder, while the basal part acts as a storage region, containing many lipid droplets. The *Geosiphon* bladders, hyphae and spores contain another endosymbiont, the 'bacteria-like organisms' (BLOs), which are bacteria of unknown phylogenetic affiliation with an ultrastructure identical to those frequently found in AM fungi (for a review, see Schüßler and Kluge 2001).

2.2 Specificity of Partner Recognition and Development of the Symbiosis

The *Geosiphon* symbiosis establishment was first studied by Knapp (1933), who already documented the incorporation of cyanobacteria into hyphal tips (see also Mollenhauer et al. 1996; Mollenhauer and Mollenhauer 1997). The signals leading to the interaction between the symbionts are unknown, although there is progress towards their elucidation (see Sect. 7.1). Free-living *Nostoc* undergoes a characteristic life cycle (for a review, see Rai et al. 2000). Only a particular stage (the early primordium, following the hormogonium stage) is recognized by the fungus, and the heterocysts are 'cut off' when the *Nostoc* filament is ingested, indicating that specific signal perceptions must exist. Since *Nostoc* changes its surface carbohydrate composition during its life cycle, extracellular glycoconjugates could be involved (Schüßler et al. 1997).

After contacting *Nostoc*, the hyphal tip forms an irregular structure (Fig. 9), enclosing the cyanobacteria (Figs. 10, 11). The engulfed cells lose pigmentation and are deformed during the incorporation. Some of the *Nostoc* cells do not survive this process. After a week, the young bladders expand to $\sim$ 100 µm (Fig. 8) and, at least under phosphate limitation (see Sect. 4.1), the recovered endosymbiotic *Nostoc* cells divide faster and are larger than their free-living relatives.

3 Ecology and Distribution of the *Geosiphon* Symbiosis

3.1 Ecology of the *Geosiphon* Symbiosis

Experience with the *Geosiphon* symbiosis in nature and our culture attempts indicate that it is established only on soils poor in phosphate. Eutrophication of the ecosystem results in the disappearance of the symbiosis (Mollenhauer, pers. comm.). At present, this symbiosis is known only from one locality in the Spessart Mountains (Figs. 5, 12; see Sect. 3.2), characterized as follows (Mollenhauer 1992): altitude $\sim$ 300 m, low mountain range; Triassic 'coloured sandstone', mainly silicate and iron compounds, brown earth; soft water; natural vegetation beach wood on acid soil, mostly replaced by grassland and winter grain; annual mean temperature $\sim$ 7 °C; annual mean precipitation $\sim$ 1000 mm. The soil is loamy or silty, pH 4–6, and nutrient-deficient. The *Geosiphon* symbiosis needs damp habitats. This

may be the reason for its appearance mainly in spring and autumn. It also can be found under fresh snow cover and, from data on growth chamber breakdown, we know that it survives frost (about −3 °C) for at least 2–3 days.

The stands are usually small, occupying only some square centimetres at the edges of clods to a few square metres, and can most reliably be found by looking for some plants with which the fungus is always associated, namely the hornwort *Anthoceros*, the liverwort *Blasia*, the moss *Dicranella* and some small vascular plants. In nutrient solution, the germination of the spores (Schüßler et al. 1994) and hyphal outgrowth from the bladders (Mollenhauer 1992) are stimulated by exudates from mosses (*Funaria*, *Dicranella*).

3.2 Reports and Distribution of the *Geosiphon* Symbiosis

Including the presently known stands, only six natural habitats of the *Geosiphon* symbiosis have been reported, which are (for references, see Schüßler 2002):

1. Nordhausen (Thüringen, Germany): in October and November between 1841 and 1849, Kützing found an 'alga' in loamy fields and along the banks of the River Salza which he described as *Botrydium pyriforme*. From herbarium material (Rijksherbarium Leiden), it is clear that this 'alga' represents the *Geosiphon* symbiosis (Mollenhauer 1992).
2. Kremsmünster (Oberösterreich, Austria): in November 1913 and autumn 1914, von Wettstein found the symbiosis, mainly on the edges of clods in cabbage fields, until the first snowfalls. He was able to culture it for ∼ 6 months in glass beakers on humid field substrate.
3. Göda (Sachsen, Germany): in autumn 1916, the symbiosis was found by Drude and Schorler in a loamy stubble field ('Lausitzer *Geosiphon*').
4. Göttingen (Niedersachsen, Germany): Knapp (1933) found the *Geosiphon* symbiosis from September to December in loamy fields and reported that von Wettstein also found it near Göttingen in 1926.
5. Wetter (Hessen, Germany): v. Stosch and Henssen found the symbiosis on 16 November 1963 and cultured it for some time on natural substrate in a north-facing window.
6. Bieber (Hessen, Germany): from 1968 on, *Geosiphon* has been frequently reported from fields around this small village in the Spessart Mountains (see Sect. 3.1), presently the only known natural habitat.

Fig. 12. The six locations in Germany and Austria where the *Geosiphon* symbiosis was reported (*filled dots*)

As shown in Fig. 12, these habitats span a region of 400 × 400 km. Therefore, the symbiosis seems to be geographically widespread but rarely reported.

3.3 An Ecological Network Between Fungi, Cyanobacteria and Plants?

The bryophytes *Anthoceros* and *Blasia* accommodate *Nostoc* in cavities of their thalli (Rai et al. 2000), and this *Nostoc* is also capable of forming *Geosiphon* symbiosis (Mollenhauer 1992). Since *Geosiphon* belongs to the Glomeromycota, it is conceivable that *Geosiphon* forms an AM and delivers nitrogen (fixed by the endosymbionts) to the plants. It is known that *Anthoceros* associated with the *Geosiphon* symbiosis forms an AM with *Glomus claroideum* under laboratory conditions (Schüßler 2000), but such culture attempts failed with *Geosiphon*. Nevertheless, the hypothesis was raised that *Nostoc*, *Geosiphon*, *Anthoceros*, *Blasia* and plant roots might be linked together in a symbiotic network (Kluge et al. 2002).

Molecular methods based on rDNA sequences have been used to explore AM fungal diversity and/or to identify the fungi in roots. Several PCR primers for *Geosiphon* were designed and tested on samples collected in the field. *Geosiphon* DNA could be amplified by nested PCR from plant roots and *Anthoceros* (unpubl. data). However, this is not necessarily evi-

dence for AM formation. In our experience, cleaning the surface of roots completely from attached hyphae appears to be hardly possible, and erroneous detection of DNA from externally attached hyphae cannot be ruled out. Nevertheless, our primers are highly specific and are presently being applied to evaluate the occurrence of *Geosiphon* in nature.

4 Culture Systems for the *Geosiphon* Symbiosis

4.1 Laboratory Culture Systems

The *Geosiphon* symbiosis has been cultured in our laboratory since 1994. The crucial factor for successful cultures seems to be phosphate limitation. We use three different substrates for the cultures, a slightly brownish 'aquarium quartz sand', chemically clean fine white quartz sand (Sigma), or sterilized soil from the natural habitat. The latter is still the most reliable way to grow the symbiosis, and these cultures usually can be maintained for 6–12 months. The substrate can be recycled several times. In the sand system, the symbiosis grows more slowly, but some cultures have been maintained for several years.

The culture microcosm is composed of a polypropylene flower pot with filter paper strips (or a roll) running through holes in the bottom (Fig. 13). For soil cultures, dry soil is sieved through a 2-mm sieve, washed three times with distilled water, dried, sieved again, and mixed with charcoal powder (1–2 $g\,kg^{-1}$). A layer of coarse sand overlaid by fine sand is filled into the pot, and the soil added on top. The substrate is soaked with distilled water from below, and put into the glass beaker containing distilled water.

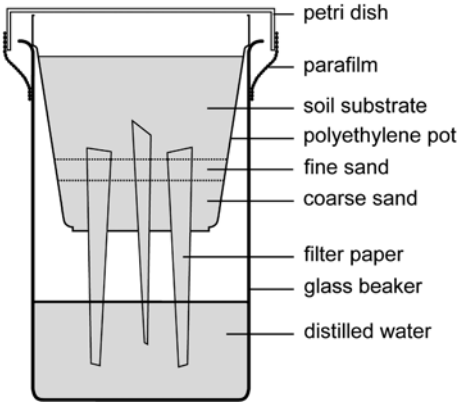


Fig. 13. Culture system for the *Geosiphon* symbiosis

A thin, irregular charcoal layer is scattered on the substrate surface. The setup is covered with aluminium foil and autoclaved three times at 2-day intervals. The pot is then covered with a 9-cm Petri plate lid and sealed with parafilm.

For the quartz sand cultures, the brownish 'aquarium sand' is acid-washed before use. Sand culture pots are soaked with a nutrient-poor '*Geosiphon* medium' (GM32): 0.1 mM $MgSO_4$, 0.2 mM $Ca(NO_3)_2$, 0.1 mM KCl, 1 µM K_2HPO_4, 12 µM $FeCl_3$, 12 µM Na_2-EDTA, 0.08 µM $ZnSO_4$, 0.9 µM $MnCl_2$, 16.2 µM H_3BO_3, 0.04 µM $CoCl_2$, 0.08 µM Na_2MoO_4, 0.01 µM $CuSO_4$, 0.01 µM $NaHSeO_3$, 0.01 µM $NiCl_2$, 0.01 µM Na_3VO_4, 0.005 µM $K_2Cr_2O_7$, 0.5 mM MES, pH 6.0 (KOH-titrated). We currently also add KBr (0.1 µM; 'GM33'). The sand cultures do not work without a thin charcoal layer scattered at the surface (Figs. 1, 2 and 3).

To inoculate the cultures, one to several clusters of 10–20 *Geosiphon* bladders from an established culture are placed onto the substrate. This always carries enough free-living cyanobacteria. Alternatively, *Geosiphon* spores isolated from cultures under sterile conditions are put on the surface, slightly covered with substrate, and a few 1-mm pieces of a *Nostoc* thallus are placed nearby. We normally use *Nostoc punctiforme* strain 1:1-26 (originally isolated from *Geosiphon* by D. Mollenhauer; =SAG 69.79, =PCC 9503), which is cultured on BG11 medium solidified with 2% agar and overlaid with cellophane foil. The spores formed are globose in fine substrates (Schüßler et al. 1994), but may be 'deformed' in coarse sand (Fig. 7). The cultures are grown at 20 °C with a 14-h light/10-h dark rhythm and illuminated with a photosynthetic photon flux density (PPFD) of 80–160 µmol m^{-2} s^{-1} (fluorescent tubes TDL 58 W/25 CE and L 58 W/77 Fluora).

The procedures above work well, but we sometimes modify details. e.g. some cultures may grow better when the pot in the beaker has direct contact with the water. However, by using the filter paper 'bridges', moisture conditions are more stable and culture success more predictable. Cultures on soil are possible, but less reliable, without the addition of charcoal. Some lots of the natural substrate lead to excessive growth of *Nostoc*. This might be due to higher phosphate content or accessibility (see Fig. 4), e.g. because of a higher pH. The substrate may be buffered to a pH of 5–6 but, when using organic buffers, this can have unforeseen effects on nutrient availability. Our methods can therefore probably be refined.

4.2 Culture Systems for Microscopic Investigations

Microscopic observations are made by inverted microscopy of liquid cultures (Fig. 14). Petri plates (35 mm) with holes in the lid and base are closed

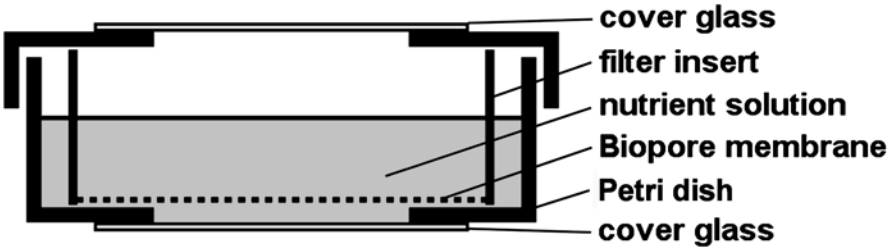


Fig. 14. The microscopic culture system for the *Geosiphon* symbiosis

by cover slips glued with nontoxic aquarium silicone. A culture plate insert (Millicell CM, Millipore) with a 0.4-µm pore size membrane (Biopore) is placed inside the Petri plate. The small spacers at the bottom of the inserts are removed to provide a closer space in between the coverslip and membrane.

Geosiphon bladders and ~ 1-mm pieces of a *Nostoc* thallus, washed two to three times in distilled water, are transferred into liquid medium on the bottom coverslip before the filter is inserted and more medium added (we add up to 3 ml in total). A limited vertical space (< 1 mm) increases the density of hyphae, which can be observed microscopically, in particular with higher-magnification lenses. The system prevents damage by liquid movement and has excellent optical properties, since the membrane becomes transparent when wet. It allows gentle medium exchange (the organisms stay in the small undisturbed volume below the membrane) and addition or removal of dyes or inhibitors. The symbiosis can be grown in such chambers for several weeks, before it starts to degenerate.

4.3 Synchronization of the *Nostoc* Life Cycle

Investigations of the partner recognition and the development of the symbiosis require a method to synchronize the developmental cycle of the cyanobacteria to stimulate the symbiosis-compatible *Nostoc* stage. Synchronization is achieved by illumination (PPFD of 20 $\mu mol\, m^{-2}\, s^{-1}$) with different light qualities, which are obtained with coloured foils transmitting ~ 490—550 nm ('green light') and > 600 nm ('red light'). *Geosiphon* bladders excised from all hyphae and small pieces of *Nostoc* are transferred into culture chambers and illuminated with red light for 30–36 h, resulting in the differentiation of motile hormogonia (Damerval et al. 1991; Schüßler et al. 1997), which spread over the coverslip. Simultaneously, new hyphae grow from the bladders. Illumination is then switched to permanent green light, inducing the differentiation to primordia and the remaining life-cycle

stages. This method allows the study of the symbiosis-compatible *Nostoc* stages, partner recognition, incorporation of cyanobacteria, and development of the symbiosis. If more growing hyphae are needed, *Geosiphon* bladders can be pre-germinated in green light (inhibiting hormogonia formation) before adding *Nostoc*.

5
Metabolic Aspects of the Symbiosis

5.1
C and N Metabolism

^{14}C tracer studies showed that the *Geosiphon* bladders fix CO_2 (Kluge et al. 1991). After short incubation (1 min) in light, mainly phosphate esters were labelled. After 30 min in light, the pattern changed in favour of sugars ($\sim$ 23%, including some trehalose and raffinose), amino acids (20%, more then half being alanine) and organic acids (13%, more then half malate); about 28% of the soluble labelled compounds were still phosphate esters.

In darkness, mainly malate ($>$ 40%), fumarate (9%) and some amino acids (mainly asparagine and glutamine) were labelled. This indicates PEPcase-mediated CO_2 fixation in the pentose phosphate pathway (PPP), which was considered to be due to the cyanobacteria (Kluge et al 1991). However, AM fungi also show PPP activity (Bago et al. 2002), and the BLOs (see Sect. 7.2) could contribute to the observed CO_2 fixation.

For *Geosiphon* bladders it was shown that only molecules with a radius $<$ 0.45 nm can pass the cell wall (Schüßler et al. 1995), which is too small for the effective uptake of glucose but allows permeation of, for example, phosphate, possibly reflecting natural conditions. ^{14}C glucose was not taken up or metabolized by *Geosiphon* bladders (Kluge, pers. comm.). It is possible that the cell walls of fine hyphae growing into the substrate are much more permeable, but the extraradical mycelium of AM fungi also does not take up hexoses (Bago et al. 2002).

The photosynthesis rate of the endosymbionts is roughly double that of free-living *Nostoc*, and they also tolerate higher light intensities before photoinhibition occurs (Bilger et al. 1994). The endosymbionts are thus shown to be photosynthetically very active, and the major role of *Nostoc* in this symbiosis is photosynthesis. The photosynthesis products are converted by the fungus into glycogen and lipids, the latter found in large amounts in the basal part of the bladders. Unfortunately, information on lipid metabolism in the *Geosiphon* symbiosis, central in the AM physiology (cf. Chap. 10), is still rare.

The symbiosis is not only C- but also N-autotroph. The capability of N_2 fixation is indicated by growth on nitrogen-free medium, nitrogenase activity (Kluge et al. 1992), and the occurrence of heterocysts – although BLOs (see Sect. 7.2) may also contribute to N_2 fixation. The *Nostoc* strain isolated from *Geosiphon* can release glutamate when free-living (Strasser and Falkner 1986), but there is no evidence that this occurs during symbiosis. In common with AM, matter exchange between the partners in this symbiosis is not well understood.

5.2 Uptake and Content of Inorganic Nutrients

Little is known about the element composition of AM fungi, although they mediate supply of inorganic nutrients, particularly phosphorus, to most vascular plants. The element composition of *Geosiphon* bladders and spores grown on sand cultures with GM32 medium (see Sect. 4.1) was measured by proton-induced X-ray emission (PIXE), revealing some evidence of acquisition and storage of inorganic nutrients (Maetz et al. 1999a, b). It was shown that the fungus accumulates high concentrations of P ($\sim$ 1.5%; all values as percent dry weight), whilst in the symbiosome P concentration was only 0.3%. Most of the P must be stored in the vacuoles (perhaps as polyphosphate). The same holds true for Cl ($\sim$ 2%) and K ($\sim$ 5%). Interestingly, Cl^- seems to play an important role as counter ion, and is also osmotically relevant.

Microelement measurements revealed that Se is present in low concentration ($<$ 1 ppm), close to the limit of detection. Fe and Cu show lower concentration in the symbiosome compared to the rest of the bladder. By contrast, Mn and Ni are present in $\sim$ 20- and $\sim$ 5-fold higher concentrations respectively within the symbiosome. This could be due to, amongst others, the Mn-protein of photosystem II and prokaryotic Ni-containing hydrogenases, which play a role in N_2 fixation or are coupled to photosynthesis for H_2 recycling. In some lichens, urease (a Ni-containing enzyme) is induced by a decreasing ammonium concentration (Pérez-Urria et al. 1993), as in many bacteria. Maybe *Nostoc* reacts similarly, due to loss of ammonium to the fungus. Some of these results could be useful when developing better-defined culture systems, e.g. Ni and/or Mn limitation could reduce the growth of the free-living photobiont and lead to conditions better suited for symbiosis establishment.

5.3 Heavy Metal Uptake and Resistance

AM fungi may be involved in heavy metal tolerance of plants (Hildebrandt et al. 1999). Some preliminary studies were performed on heavy metal uptake by *Geosiphon*, also with respect to future identification of heavy metal-induced genes (see Sect. 7.3).

Cu, Cd, Tl and Pb uptake over up to 122 h was measured by PIXE (Scheloske et al. 2001). Free-living *Nostoc* was strongly affected by all heavy metal treatments. The cells bleached completely and did not survive. By contrast, the endosymbiotic cyanobacteria did not bleach, apparently being protected against heavy metal toxicity. Two trends were revealed in the study: (1) the application of higher heavy metal concentrations (5 versus 1 µM) led to a higher accumulation, (2) Pb and Tl, but not Cu and Cd accumulate in higher concentrations when applied individually (1 µM), compared to a 1 µM (each) mix, perhaps due to induced resistance. We have also measured the uptake of Ni, Co, and Mo (unpubl. data). It is clear that *Geosiphon* accumulates heavy metals – e.g. after 8 h in 1 µM Pb, the concentration of Pb is $\sim$ 50 times higher (related to fresh weight) compared to the nutrient solution. Therefore, the metals probably are detoxified intracellularly and transported into the vacuoles, but an increased S content, which could be indicative for phytochelatins, was not found.

6 Phylogeny and Taxonomy of *Geosiphon* and AM Fungi

6.1 *Geosiphon* is an 'AM Fungus'

Little was known about the phylogenetic relationships of the fungus forming the *Geosiphon* symbiosis until recently. Gams (CBS, The Netherlands) first noted that *Geosiphon* might be related to AM fungi (Mollenhauer 1992). The spores of *Geosiphon* show distinct characters similar to those of AM fungi (Schüßler et al. 1994), and SSU rDNA analyses (Gehrig et al. 1996) showed that *Geosiphon* belongs to a basal branch within the AM fungi. Some misleading assumptions were published then, and *Geosiphon* was even placed into the '*Ascomycotina*' (for details and references, see Schüßler 2002). In fact, with respect to the AM fungi analysed, *Geosiphon* is most closely related to *Archaeospora leptoticha* (Kramadibrata et al. 2000; Schüßler et al. 2001a), commonly found in wild grasses in Japan (Sawaki et al. 2004). Therefore, *Geosiphon* undoubtedly belongs to the Archaeosporales (Schüßler et al. 2001b; Table 1).

Table 1. The new taxonomy for 'AM fungi'. (Schüßler et al. 2001b; for recent changes, see Walker and Schüßler 2004)

Glomeromycota Glomeromycetes	Families	Genera
Glomerales	Glomeraceae	*Glomus* ('*Glomus* group A' or 'B')
	fam. ined.	incertae sedis ('*Glomus* group A' or 'B')
Diversisporales	Gigasporaceae	*Gigaspora* and *Scutellospora*
	Acaulosporaceae	*Acaulospora* and *Entrophospora*
	Pacisporaceae	*Pacispora*
	Diversisporaceae	*Diversispora*
Paraglomerales	Paraglomeraceae	*Paraglomus*
Archaeosporales	Geosiphonaceae	*Geosiphon*
	Archaeosporaceae	*Archaeospora*

Since the description as phycomycetous lichen (Knapp 1933), the *Geosiphon* symbiosis was often treated as lichen, although this is no longer tenable. Hawksworth and Honegger (1994) gave a 'lichen' definition which excludes endosymbioses. They write, '*A lichen is an ecologically obligate, stable mutualism between an exhabitant fungal partner (the mycobiont) and an inhabitant population of extracellularly located unicellular or filamentous algal or cyanobacterial cells (the photobiont)*'. Moreover, the *Geosiphon* fungus does not belong to the lichen-forming Asco- and Basidiomycota. Although this is a matter of definition, in our opinion, the term 'lichen' for the *Geosiphon* symbiosis is misleading. In the emendation of *Geosiphon* and the Geosiphonaceae, the orthographically correct name *Geosiphon pyriformis* was introduced, and the species name now refers to the fungus only (Schüßler 2002).

Our studies on *Geosiphon*, and the finding that *Glomus* is not a monophyletic group (Schwarzott et al. 2001), resulted in investigation of the phylogeny of AM fungi in more detail. Based on comprehensive SSU rDNA sequence analyses, the AM fungi (together with *Geosiphon*) can clearly be separated in a monophyletic clade (Schüßler et al. 2001b), and recent studies have shown that the Zygomycota phylogeny is not well understood (Benny et al. 2001; O'Donnell et al. 2001). The classification of the AM fungi within the Zygomycota was artificial, and the 'AM and related fungi' (the latter meaning fungi such as *Geosiphon*) were placed into a new phylum, the Glomeromycota. The details of the higher ranking taxonomy (Table 1) are published elsewhere (Schüßler et al. 2001b; Schüßler 2002; Walker et al. 2004; Walker and Schüßler 2004).

The new classification is based on a robust molecular phylogeny which is open for additions without serious conflict with the proposed taxa. This is important, since one should be aware that the number of families and gen-

era will increase in future. Biodiversity and other studies already indicate yet unknown phylogenetic clades. As a reference for data of Glomeromycota phylogeny and taxonomy, we will further develop our web page at http://www.amf-phylogeny.com.

6.2 The Origin and Evolution of AM Fungi and the AM Symbiosis

It is frequently discussed whether AM fungi have played a crucial role in exploring resources like P and water for the first land plants. It was stated that AM probably was already formed by early bryophyte-like land plants, more than 460×10^6 years ago (Redecker et al. 2000; see also Remy et al. 1994). AM symbioses with 'lower' plants still exist (Read et al. 2000) and, for one fungus, *Glomus claroideum*, it is known that it forms symbioses with vascular plants as well as hornworts (Schüßler 2000). Another, more far-reaching idea was the proposal of a partnership between an aquatic alga and a 'phycomycetous' fungus as the initial step in land plant evolution (Pirozynsky and Malloch 1975). Our works about the *Geosiphon* symbiosis indicate that such a hypothetical association with a green alga appears rather probable (Schüßler 2002), since glomeromycotan symbioses show a very broad spectrum of photobionts.

An even earlier origin of 'AM-like' symbioses may be suggested. Molecular studies indicate that 'AM fungi' are much older than land plants (Heckman et al. 2001). In damp or semi-aquatic habitats, where 'ancestral AM fungi' began to colonize land, cyanobacteria would have been the prominent photoautotrophic organisms. Therefore, glomeromycotan fungi could have already lived symbiotically with cyanobacteria long before land plants evolved, and the *Geosiphon* symbiosis could mirror such an early stage of symbiosis. In future, microfossils might reveal whether such glomeromycotan symbioses played an ecologically important role for the colonization of the land habitat, as suggested for lichens (Heckman et al. 2001).

Doubtlessly, present knowledge shows that the symbiosis with AM fungi was an early step in land colonization by plants, perhaps driven by fungi already capable of symbiosis formation. This implies that land plants co-evolved with glomeromycotan fungi since their origin and, consequently, nutrient uptake (and other) mechanisms of plants evolved in close dependency on the fungi – and vice versa. This has important consequences, and scientists investigating plant nutrition or designing transgenic plants should keep this in mind.

7
The *Geosiphon* Symbiosis – a Model System for AM?

In the *Geosiphon* symbiosis, the bladders represent the 'symbiotic stage', and it is thus comparable to an active mycorrhiza with all the nutrient exchanges taking place (see Fig. 6). The symbiosis shows functional as well as structural similarities to the AM, raising the suggestion that it can serve as a model system for the AM symbiosis (Schüßler and Kluge 2001; Schüßler 2002). When compared to AM, the *Geosiphon* bladders have some advantages, e.g. they can be investigated microscopically in great detail (Figs. 8–11), all substances taken up by the symbiosis are transported across the fungal plasma membrane only, and the huge bladders are well suited for microinjection.

7.1
Partner Recognition and Symbiosis Establishment

The fungus–plant interaction leading to the establishment of AM has to be an 'unspecific' mechanism insofar as that a certain fungus can recognize vascular plants as well as hornworts to form the symbiosis (Schüßler 2000). At first glance, the idea of comparing the recognition of cyanobacteria with that of plant roots seems artificial. However, cyanobacterial extracellular polysaccharides (EPS) can be very similar to plant cell wall compounds (Hoiczyk and Hansel 2000), and may contain cellulose like homoglucans, pectin-like compounds, xyloglucans and complex EPS. It was even discussed whether today's plant carbohydrates have evolved from such 'ancient' cyanobacterial EPS.

Our methods allow a detailed, time-resolved characterization of the *Nostoc* stages capable of symbiosis establishment, and we have at least two in vivo markers to distinguish these stages microscopically: ConA labelling of the *Nostoc* surface (Schüßler et al. 1997), and pigment fluorescence spectra (Wolf and Schüßler, unpubl. data). We know the time period when the 'symbiosis-compatible' stages of *Nostoc* exist, and that mannose is not directly involved in the recognition but that nevertheless a lectin-mediated mechanism is indicated (unpubl. data).

7.2
Bacterial Endosymbionts (BLOs)

The role of 'BLOs' in glomeromycotan fungi is unknown. *Geosiphon* harbours BLOs of the same type as those found in diverse branches of the

Glomeromycota (Schüßler et al. 1994). These bacteria are not enclosed within a host membrane. They probably are ancient, obligate endosymbionts, which are horizontally transferred and are likely to have been symbionts of the fungi for many hundreds of million years.

Little is known about these bacteria, except for those found in the Gigasporaceae (see Bianciotto et al. 2003 and references therein) which have been recently described as '*Candidatus* Glomeribacter gigasporarum'. Several genes were characterized, e.g. *vac*B, a *pst* operon, and *nif* D/K. Particularly the *nif* genes are highly interesting, since this means that BLOs could fix N_2. However, these are not the 'typical' BLOs for AM fungi, because they have a different ultrastructure and are enclosed by a host membrane. Specific primers for *Glomeribacter* do not amplify SSU rDNA from non-gigasporacean BLOs.

We do not yet know the phylogeny of the *Geosiphon* BLOs. However, perhaps in the future their role in the AM can be uncovered by using *Geosiphon*. Therefore, we intend to investigate their relationships, in which context FISH could play an important role (Bertaux et al. 2003). *Geosiphon* bladders have the advantage that the big cells can easily be cut after fixation, resulting in direct access to the BLOs in the interior.

7.3 Identification of Differentially Expressed Fungal Genes

When compared to the AM, one of the main advantages of the *Geosiphon* symbiosis for gene expression studies is that only one eukaryote is present. In the symbiotic stage of the AM, the plant is always accompanying and fungal gene expression is difficult to investigate. By contrast, from the *Geosiphon* symbiosis fungal mRNA can be isolated specifically and easily by means of its poly(A) tail.

Studies on differential gene expression under certain environmental conditions were initiated. For example, we are trying by subtractive hybridization methods to gather indications about genes relevant for the uptake (and metabolism) of photosynthetic products by the fungus. Candidate genes are sugar transporters, C transfer to the fungal symbiosis partner being a key factor in the AM. Characterized gene fragments then could also be used for screening purposes, and the design of primers for the amplification of corresponding genes in other AM fungi. Other approaches include the investigation of the reaction to heavy metal stress (see Sect. 5.3). Methods for microinjection have already been established and, if project funding were available, these could be used to inject GFP-construct expression vectors suitable for AM fungi.

8 Conclusions

Geosiphon pyriformis is the only fungus known to form endosymbiosis with cyanobacteria, and undoubtedly belongs to the Glomeromycota. Uncovering the phylogenetic relationships within the 'AM fungi' is not only of taxonomic interest, but also important for the planning and interpretation of many studies. Moreover, evolutionary implications, referring to the origin of the AM symbiosis, were raised by the better understanding of the *Geosiphon* symbiosis.

Due to the structural and functional similarities to the AM, and the huge fungal cells formed, the *Geosiphon* symbiosis may be used as a model for AM. It offers considerable advantages for the study of, for example, nutrient transport processes, lipid metabolism and the recognition between the symbionts.

Principal advantages for research on AM(-like) symbioses are that fungal mRNA can be isolated easily, and that gene expression can be induced in liquid culture systems. In an active AM, such gene expression studies are complicated tasks. *Geosiphon* bladders could, once suitable expression vector systems are identified, even serve as a system for in vivo expression of GFP constructs. We hope that such work will be funded, and that the *Geosiphon* symbiosis can play its role as a model system for the AM symbiosis in future.

Acknowledgements. We are grateful to the DFG for financing our work on the *Geosiphon* symbiosis (GRK340) and molecular phylogeny (Schu 1203/1), and to Chris Walker for his long-lasting collaboration and help with the English.

References

Bago B, Pfeffer PE, Zipfel W, Lammers P, Shachar-Hill Y (2002) Tracking metabolism and imaging transport in arbuscular mycorrhizal fungi. Plant Soil 244:189–197

Benny GL (2001) Zygomycota: trichomycetes. In: McLaughlin DJ, McLaughlin EG, Lemke PA (eds) The Mycota VII(A). Springer, Berlin Heidelberg New York, pp 147–160

Bertaux J, Schmid M, Chemidlin Prevost-Boure N, Churin JL, Hartmann A, Garbaye J, Frey-Klett P (2003) In situ identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238 N. Appl Environ Microbiol 69:4243–4248

Bianciotto V, Lumini E, Bonfante P, Vandamme P (2003) '*Candidatus* Glomeribacter gigasporarum' gen. nov., sp. nov., an endosymbiont of arbuscular mycorrhizal fungi. Int J Syst Evol Microbiol 53:121–124

Bilger W, Büdel B, Mollenhauer R, Mollenhauer D (1994) Photosynthetic activity of two developmental stages of a *Nostoc* strain (cyanobacteria) isolated from *Geosiphon pyriforme* (Mycota). J Phycol 30:225–230

Damerval T, Guglielmi G, Houmard J, Tandeau de Marsac N (1991) Hormogonium differentiation in the cyanobacterium *Calothrix*: a photoregulated developmental process. Plant Cell 3:191–201

Gehrig H, Schüßler A, Kluge M (1996) *Geosiphon pyriforme*, a fungus forming endocytobiosis with *Nostoc* (Cyanobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis. J Mol Evol 43:71–81

Hawksworth DL, Honegger R (1994) The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. In: Williams MAJ (ed) Plant galls. Organisms, interactions, populations. Systematics Association, Special volume no 49. Clarendon Press, Oxford, pp 77–98

Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early colonization of land by fungi and plants. Science 293:1129–1133

Hildebrandt U, Kaldorf M, Bothe H (1999) The zinc violet and its colonization by arbuscular mycorrhizal fungi. J Plant Physiol 154:709–717

Hoiczyk E, Hansel A (2000) Cyanobacterial cell walls: news from an unusual prokaryotic envelope. J Bacteriol 182:1191–1199

Kluge M, Mollenhauer D, Mollenhauer R (1991) Photosynthetic carbon assimilation in *Geosiphon pyriforme* (Kützing) F.v. Wettstein, an endosymbiotic association of fungus and cyanobacterium. Planta 185:311–315

Kluge M, Mollenhauer D, Mollenhauer R, Kape R (1992) *Geosiphon pyriforme*, an endosymbiotic consortium of a fungus and a cyanobacterium (*Nostoc*), fixes nitrogen. Bot Acta 105:343–344

Kluge M, Mollenhauer D, Wolf E, Schüßler A (2002) The *Nostoc–Geosiphon* endocytobiosis. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 19–30

Knapp E (1933) Über *Geosiphon pyriforme* Fr. Wettst., eine intrazelluläre Pilz-Algen-Symbiose. Ber Deutsch Bot Ges 51:210–217

Kojima T, Sawaki H, Saito M (2004) Detection of arbuscular mycorrhizal fungi, *Archaeospora leptoticha*, and related species colonizing plant roots by specific PCR primers. Soil Sci Plant Nutr 50:95–101

Kramadibrata K, Walker C, Schwarzott D, Schüßler A (2000) A new species of *Scutellospora* with a coiled germination shield. Ann Bot 86:21–27

Maetz M, Schüßler A, Wallianos A, Traxel K (1999a) Subcellular trace element distribution in *Geosiphon pyriforme*. Nucl Instrum Meth B 150:200–207

Maetz M, Przybylowicz WJ, Mesjasz-Przybylowicz J, Schüßler A, Traxel K (1999b) Low-dose nuclear microscopy as a necessity for accurate quantitative microanalysis of biological samples. Nucl Instrum Meth B 158:292–298

Mollenhauer D (1992) *Geosiphon pyriforme*. In: Reisser W (ed) Algae and symbiosis: plants, animals, fungi, viruses, interactions explored. Biopress, Bristol, pp 339–351

Mollenhauer D, Mollenhauer R (1997) Endosymbiosis between *Nostoc* and *Geosiphon pyriforme*. Institut für Wissenschaftlichen Film (IWF) Göttingen, Germany, Film-No C1955

Mollenhauer D, Mollenhauer R, Kluge M (1996) Studies on initiation and development of the partner association in *Geosiphon pyriforme* (Kütz.) v. Wettstein, a unique endocytobiotic system of a fungus (Glomales) and the cyanobacterium *Nostoc punctiforme* (Kütz.) Hariot. Protoplasma 193:3–9

O'Donnell K, Lutzoni FM, Ward TJ, Benny GL (2001) Evolutionary relationships among mucoralean fungi (Zygomycota): evidence for family polyphyly on a large scale. Mycologia 93:286–296

Pérez-Urria E, Avalos A, Guzmán G, Vicente C (1993) Urease production and secretion by three Antarctic lichen species. Endocytobios Cell Res 9:239–243

Pirozynski KA, Malloch DW (1975) The origin of land plants: a matter of mycotrophism. BioSystems 6:153–164
Rai AN, Söderbäck E, Bergman B (2000) Cyanobacterium-plant symbioses. New Phytol 147:449–481
Read DJ, Duckett JG, Francis R, Ligrone R, Russel A (2000) Symbiotic fungal associations in 'lower' land plants. Philos Trans R Soc Lond B 355:815–831
Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921
Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843
Sawaki H, Sugawara K, Saito M (1998) Phylogenetic position of an arbuscular mycorrhizal fungus, *Acaulospora gerdemanii*, and its synanamorph *Glomus leptotichum*, based upon 18S rRNA gene sequence. Mycoscience 39:477–480
Scheloske S, Maetz M, Schüßler A (2001) Heavy metal uptake of *Geosiphon pyriforme*. Nucl Instrum Meth B 181:659–663
Schüßler A (2000) *Glomus claroideum* forms an arbuscular mycorrhiza-like symbiosis with the hornwort *Anthoceros punctatus*. Mycorrhiza 10:15–21
Schüßler A (2002) Molecular phylogeny, taxonomy, and evolution of *Geosiphon pyriformis* and arbuscular mycorrhizal fungi. Plant Soil 244:75–83
Schüßler A, Kluge M (2001) *Geosiphon pyriformis*, an endocytosymbiosis between fungus and cyanobacteria, and its meaning as a model system for arbuscular mycorrhizal research. In: Hock B (ed) The Mycota IX. Springer, Berlin Heidelberg New York, pp 151–161
Schüßler A, Mollenhauer D, Schnepf E, Kluge M (1994) *Geosiphon pyriforme*, an endosymbiotic association of fungus and cyanobacteria: the spore structure resembles that of arbuscular mycorrhizal (AM) fungi. Bot Acta 107:36–45
Schüßler A, Schnepf E, Mollenhauer D, Kluge M (1995) The fungal bladders of the endocyanosis *Geosiphon pyriforme*, a *Glomus*-related fungus: cell wall permeability indicates a limiting pore radius of only 0.5 nm. Protoplasma 185:131–139
Schüßler A, Bonfante P, Schnepf E, Mollenhauer D, Kluge M (1996) Characterization of the *Geosiphon pyriforme* symbiosome by affinity techniques: confocal laser scanning microscopy (CLSM) and electron microscopy. Protoplasma 190:53–67
Schüßler A, Meyer T, Gehrig H, Kluge M (1997) Variations of lectin binding sites in extracellular glycoconjugates during the life cycle of *Nostoc punctiforme*, a potentially endosymbiotic cyanobacterium. Eur J Phycol 32:233–239
Schüßler A, Gehrig H, Schwarzott D, Walker C (2001a) Analysis of partial Glomales SSU rRNA genes: implications for primer design and phylogeny. Mycol Res 105:5–15
Schüßler A, Schwarzott D, Walker C (2001b) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycol Res 105:1413–1421
Schwarzott D, Walker C, Schüßler A (2001) *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. Mol Phylogen Evol 21:190–197
Strasser P, Falkner G (1986) Characterisation of the glutamate/aspartate-transport system in a symbiontic *Nostoc* sp. Planta 168:381–385
Walker C, Schüßler A (2004) Nomenclatural clarifications and new taxa in the *Glomeromycota*. Mycol Res 108:981–982
Walker C, Blaskowsky L, Schwarzott D, Schüßler A (2004) *Gerdemannia* gen. nov., a genus separated from *Glomus*, and *Gerdemanniaceae* fam. nov., a new family in the *Glomeromycota*. Mycol Res 108:707–718

16 Sebacinaceae: Culturable Mycorrhiza-Like Endosymbiotic Fungi and Their Interaction with Non-Transformed and Transformed Roots

Ram Prasad[1], Huong Giang Pham[2], Rina Kumari[1], Anjana Singh[3], Vikas Yadav[3], Minu Sachdev[1], Amar Prakash Garg[1], Tatjana Peskan[4], Solveig Hehl[5], Irena Sherameti[4], Ralf Oelmuller[4], Ajit Varma[3]

1 Introduction

The term mycorrhiza refers to the association between fungi and roots of higher plants. This association is usually considered a mutualistic symbiosis because of the highly beneficial relationships established between both partners, in which the host plants receive mineral nutrients via the fungal mycelium (mycotrophism), while the heterotrophic fungi obtain carbon compounds from the host plants (Harley and Smith 1983; Varma et al. 1999, 2004).

Arbuscular mycorrhizal (AM) fungi belong to nine genera: *Gigaspora*, *Scutellospora*, *Glomus*, *Acaulospora*, *Entrophospora*, *Archaeospora*, *Gerdemannia*, *Paraglomus* and *Geosiphon*, the only known fungal endosymbiosis with cyanobacteria (see Chap. 15). The monoxenic cultivation of these micro-organisms in association with suitable root organ cultures has received increased interest in the last decades, and several fundamental research areas are now widely covered using this system.

[1]Department of Microbiology, Ch. Charan Singh University, Meerut, Uttar Pradesh, India
[2]International Center of Genetic Engineering & Biotechnology (UNO, Triesta, Italy), New Delhi, India
[3]School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India & Amity Institute of Herbal and Micriboal Studies, Sector 125, New Super Express Highway, Noida, Uttar Pradesh, India, Tel.: +91-120-2432400, Fax: +91-11-26704511, E-mail: ajitvarma@aihmr.amity.edu/ajitvarma73@hotmail.com
[4]Institute of General Botany and Plant Physiology, University of Jena, Dornburger Strasse 159, 07743 Jena, Germany
[5]Advanced Imaging Microscopy, Carl Zeiss Jena GmbH, Carl-Zeiss-Promenade 10, 07745 Jena, Germany

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

Scientists from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, have screened a novel endophytic fungus, *Piriformospora indica*, which mimics the functional capabilities of typical AM fungi (Pham et al. 2004a). Electron microscopy and genomic studies employing the analysis of a part of 18S and 28S rRNA placed it in the Hymenomycetes (Heterobasidiomycetes; Verma et al. 1998; Varma et al. 1999; Weiß et al. 2004). The properties of this fungus have been patented (Varma and Franken 1997, European Patent Office, München, Germany, Patent No. 97121440.8-2105, November 1998). The culture has been deposited at Braunschweig, Germany (DMS No. 11827), and the National Bureau of Agriculturally Important Microorganisms (NBAIM), Pusa, New Delhi, India.

Various controversial generic concepts have been introduced for this heterogeneous assemblage of Heterobasidiomycetes (McGuire 1941). After Schröter (1889) had formally separated Heterobasidiomycetes with transversely septate basidia ('Auriculariei', later mostly referred to as Auriculariaceae or Auriculariales) from those with longitudinally septate basidia ('Tremellinei', later Tremellaceae or Tremellales), there was a general agreement that the species of the *Sebacina* complex had to be classified in the Tremellales. Within this order, Wells and Oberwinkler (1982), emphasizing microscopic characters rather than basidiome morphology, erected the family of Sebacinaceae to include species with clamp-less hyphae and ovate to pyriform, longitudinally septate basidia. In their Sebacinaceae, they included *Sebacina incrustans*, the type species of *Sebacina*, and closely related Sebacinas (*Sebacina* s. str.), but also *Tremellodendron*, *Tremelloscypha* and *Efibulobasidium*, thus integrating resupinate, coralloid, infundibuliform and pustulate forms. The phylogenetic position of the Sebacinaceae within the Basidiomycota gives an overview of phylogenetic relationships inside this subgroup of Hymenomycetes, for which the new Sebacinales is proposed which includes *P. indica* (Garnica et al. 2003; Weiß et al. 2004).

In this chapter, efforts have been made to demonstrate the *in vitro* cultivation of members of the Sebacinaceae, and their interactions with transformed and non-transformed root systems.

2 Sebacinaceous Fungi

Bandoni (1984) revised the Tremellales and Auriculariales on the basis of ultrastructural, ontogenetic and ecological characters. Sebacinaceae were transferred to the new concept of Auriculariales which then included taxa with septate basidia and continuous parenthosomes. Weiß and Oberwinkler (2001), and Kottke et al. (2003) validated major parts of Bandoni's (1984) concept of Auriculariales in a molecular phylogenetic study using

nuclear rDNA coding for the D1/D2 region of the large ribosomal subunit (LSU). Their molecular analysis confirmed the monophyly of the Sebacinaceae (including also *Craterocolla cerasi*, which fits the micromorphological concept of Sebacinaceae). On the other hand, it also suggested that the Sebacinaceae form a separate lineage of Hymenomycetes, which must be excluded from the Auriculariales.

Sebacinaceae is a monophyletic group which occupies a basal position within Hymenomycetidae (Weiß and Oberwinkler 2001; Urban et al. 2003); *P. indica* also occupies the same taxonomic position. Based on 28S and internal transcribed spacer (ITS) data, the orchid *Neottia nidus-avis* was found to be closely related to conventionally known mycorrhizal fungi, described in the group of rhizoctonia (imperfect fungi). Using molecular analytical methods like PCR, molecular cloning and sequencing, members of Sebacinaceae have been shown to be involved in various mycorrhizal associations in the field (Berch et al. 2002; McKendrick et al. 2002; Selosse et al. 2002a, b; Urban et al. 2003). Recent studies have indicated that *P. indica* belongs to Sebacinaceae and is closely related to *Sebacina vermifera* sensu (Garnica et al. 2003; Weiß et al. 2004). Fungi included in this distinct group are *Sebacina incrustans*, *Sebacina epigaea*, *Sebacina aff. epigaea*, *Tremelloscypha gelatinosa*, *Sebacina dimitica*, *Efibulobasidium rolleyi*, *Craterocolla cerasi*, *Piriformospora indica*, *Sebacina vermifera* sensu (Warcup and Talbot 1967) and *Sebacina* sp.

Warcup and Talbot (1967) isolated Heterobasidiomycetes, which they identified from their sexual stages formed in axenic culture as *S. vermifera* from roots of Australian terrestrial orchids. Later, such fungi were also isolated from pot-cultured ectomycorrhizae and arbuscular mycorrhizae (Warcup 1988). Since the remaining taxa of Auriculariales (Bandoni 1984) are likely to be wood decomposers (Wells and Bandoni 2001), the mycorrhizal potential of Sebacinaceae seems a good ecological feature to separate

Table 1. Recognized members of Sebacinaceae

Fungi	Remarks
Sebacina incrustans	Non-culturable[a]
Sebacina epigaea	Non-culturable[a]
Sebacina aff. epigaea	Non-culturable[a]
Tremelloscypha gelatinosa	Non-culturable[a]
Sebacina dimitica	Non-culturable[a]
Efibulobasidium rolleyi	Non-culturable[a]
Craterocolla cerasi	Non-culturable[a]
Piriformospora indica	Culturable
Sebacina vermifera var. sensu	Culturable
Sebacina sp.	Culturable

[a]Scientists have failed to culture these fungi on defined synthetic media

members of this group from other, morphologically quite similar Heterobasidiomycetes belonging to the Auriculariales. However, sebacinoids have been demonstrated recently to be ectomycorrhiza (Selosse et al. 2002a). Observations on ectomycorrhizae and basidiomes suggest that species of Sebacinaceae are fairly common mycobionts in various ectomycorrhizal plant communities (Urban et al. 2003). Fungal strains included in Sebacinaceae are given in Table 1.

3 Host Range and Growth Promotion Effect of Sebacinaceous Fungi

Members of Sebacinaceae were observed to be associated with a large number of mono- and dicotyledonous plants, inducing pronounced growth promotional effects [(Table 2) Singh et al. 2001; Varma et al. 2001], with the exception of the plants belonging to the Cruciferae and some plants belonging to the Chenopodiaceae and Amaranthaceae [(Table 3) Read 1999; Varma et al. 1999; Varma et al. 2001; Singh et al. 2003b]. Literature suggests that the members of these groups normally do not form associations with AM fungi (Dension et al. 2003). Under *in vitro* conditions, *P. indica* Verma et al. and *S. vermifera* sensu Warcup and Talbot were demonstrated to interact with the root system of cruciferous and chenopodaceous plants, viz. mustard (*Brassica junaceae*), cabbage (*Brassica oleracea* var. *capitata*; Kumari et al. 2003), *Arabidopsis thaliana* (Pham et al. 2004a) and spinach (*Spinacia oleracea*). A report indicated the ability of *P. indica* to colonize the rhizoids of a liverwort (bryophyte), and the thalli failed to grow under *in situ* conditions in the absence of this fungus (Varma et al. 2000, 2001; Pham et al. 2004a). *P. indica* was further shown to form associations with terrestrial orchids such as *Dactylorhiza purpurella* (Stephs.) Soo, *D. incarnate* L. Soo, *D. majalis* (Rchb.F.) Hunt & Summerh and *D. fuchsia* (Druce) Soo (Blechert et al. 1999; Singh and Varma 2000; Singh et al. 2001; Varma et al. 2001; Pham et al. 2004a).

4 Eco-Functional Identity

Sebacinaceae members *P. indica* and *S. vermifera* colonize the root cortex and form inter- and intracellular hyphae. Within the cortical cells, the fungus often forms (intracellularly) dense hyphal coils or branched structures. These fungi also form chlamydospores or vesicle-like structures within or between the cortical cells. Like AM fungi, hyphae multiply within the host

Table 2. Plants tested for interaction with members of Sebacinaceae

Plant[a]
Acacia catechu (L.f.) Wild (black catechu)
Acacia nilotica (L.) Wild (gum)
Abrus precatorius L. rosary pea (precatory bean)
Adhatoda vasica L. syn. (malabar nut)
Aneura pinguis L. Dumort. (liverwort)
Arabidopsis thaliana L. Heynh. (mouse ear cress)
Artemisia annua L. (Chinese wormwood)
Azadirachta indica A. Juss (neem)
Bacopa monniera L.Wett. (brahmi)
Beta vulgaris Linn. (beetroot)
Brassica juncea L (mustard)
Brassica napus L. (Canadian turnip)
Brassica oleracea var. botrytis L. (Alif) (broccoli)
Brassica oleracea L. var. *capitata* (cabbage)
Cassia angustifolia Senna Patti (gallow grass hemp)
Chlorophytum borivillianum Baker (musli)
Ch. tuberosum Baker (Mexican orange)
D. purpurella (Steph's) Soo' (lady orchid)
Daucus carota L. Queen Anne's-lace (carrot)
Delbergia sisso Roxburg (rosewood)
Dianthus caryophyllus L. (carnation)
Eruca sativa L. (salad rocket, arugula)
Glycine max L. Merr. (soybean)
Myc^{-} *Glycine* max cv. Frisson (two strains)
Myc^{-} *Pisum* sativum L. (pea)
Nasturtium officinale R. Br. f. (watercress)
Nicotiana tabaccum L. (tobacco)
N. attenuata L. (mountain tobacco)
Oryza sativa L. (rice)
Petroselinum crispum L. (curly parsley)
Pisum sativum L. (pea)
Populus tremula L. (aspen)
P. tremuloides Michx. (clone Esch5) (quaking)
Prosopis chilensis Stuntz sys. (Chilean mesquite)
P. juliflora (Swartz) DC. (honey mesquite)
Quercus robur L. (clone DF 159) (oak)
Setaria italica L. (thumb millet)
Solanum melongena L. (eggplant)
Sorghum vulgare L. (millet)
Spilanthes calva DC (clove)
S. oleracea L. Pinaatti Ruokarnaatti
Tectona grandis Linn. f. (teak)
Terminalia arjuna L. (Arjun tree/stem bark)
Tephrosia purpurea L. Pers. (sarphunkha/purpurea)
Withania somnifera L. Dunal (winter cherry)
Zea mays var. white (maize)
Zizyphus nummularia Burm. fil. (jujube)
Zea mays var. white (maize)

[a] Data are based on root colonization analyses *in vivo* and *in vitro* (cf. Varma et al. 2001; Singh et al. 2003a, b)

Table 3. Typical non-hosts for AM fungi but interacted with members of Sebacinaceae

Plant[a]
Beta vulgaris Linn. (beetroot)
Brassica juncea L (mustard)
Brassica napus L. (Canadian turnip)
Brassica oleracea var. botrytis L. (Alif) (broccoli)
Brassica oleracea L. var. *capitata* (cabbage)
Dianthus caryophyllus L. (carnation)
Eruca sativa L. (salad rocket, arugula)
Myc$^-$ Glycine max cv. Frisson (two strains)[b]
Myc$^-$ *Pisum sativum* L. (pea)[b]
Nasturtium officinale R. Br. f. (watercress)
Spinacia oleracea L. Pinaatti Ruokarnaatti (spinach)

[a] Data are based on root colonization analyses of plants grown under in vitro conditions (Varma et al. 2001; Kumari et al. 2003; Singh et al. 2003a, and unpubl. data)
[b] Myc$^-$ mutants did not interact with AM fungi or *P. indica*

cortical tissues and never traverse through the endodermis. Likewise, they also do not invade the aerial portion of the plant (stem and leaves).

The characteristic features of *P. indica* are the following:

- axenically cultivable on synthetic media
- absence of clamp connections
- high frequency of anastomosis formation
- hypha–hypha aggregation often observed
- absence of hyphal knots
- dolipores septum with continuous and straight parenthosomes
- chlamydospores 16–25 µm in length and 10–17 µm in width
- 8–25 nuclei per spore

The fungus further presents some functional similarities with AM fungi, which are:

- broad and diverse host spectrum
- hyphae extramatrical, inter- and intracellular
- hyphae never invade the endodermis
- chlamydospores in soil and within cortical tissues
- sexual stages not reported
- positive phytopromotional effects on tested hosts
- phosphorus mobilizer

- phosphorus transporter
- efficient for biological hardening of micropropagated plantlets
- potent biological control agent against root pathogens

Table 4. Composition of media[a]

Constituent, pH	Concentration
Aspergillus medium (Hill and Kaefer 2001)	(g/l)
Glucose	20.0
Peptone	2.0
Yeast extract	1.0
Casamino acid	1.0
Vitamin stock solution	1.0 ml
Macroelements from stock	50 ml
Microelements from stock	2.5 ml
Agar	10
$CaCl_2$, 0.1 M	1.0 ml
$FeCl_3$, 0.1 M	1.0 ml
pH	6.5
Macroelements (major elements), stock	(g/l)
$NaNO_3$	120.0
KCl	10.4
$MgSO_4 \cdot 7H_2O$	10.4
KH_2PO_4	30.4
Microelements (trace elements), stock	(g/l)
$ZnSO_4 \cdot 7H_2O$	22.0
H_3BO_3	11.0
$MnCl_2 \cdot 4H_2O$	5.0
$FeSO_4 \cdot 7H_2O$	5.0
$CoCl_2 \cdot 6H_2O$	1.6
$CuSO_4 \cdot 5H_2O$	1.6
$(NH_4)_6Mo_7O_{27} \cdot 4H_2O$	1.1
Na_2EDTA	50.0
Vitamins	(%)
Biotin	0.05
Nicotinamide	0.5
Pyridoxal phosphate	0.1
Amino benzoic acid	0.1
Riboflavin	0.25

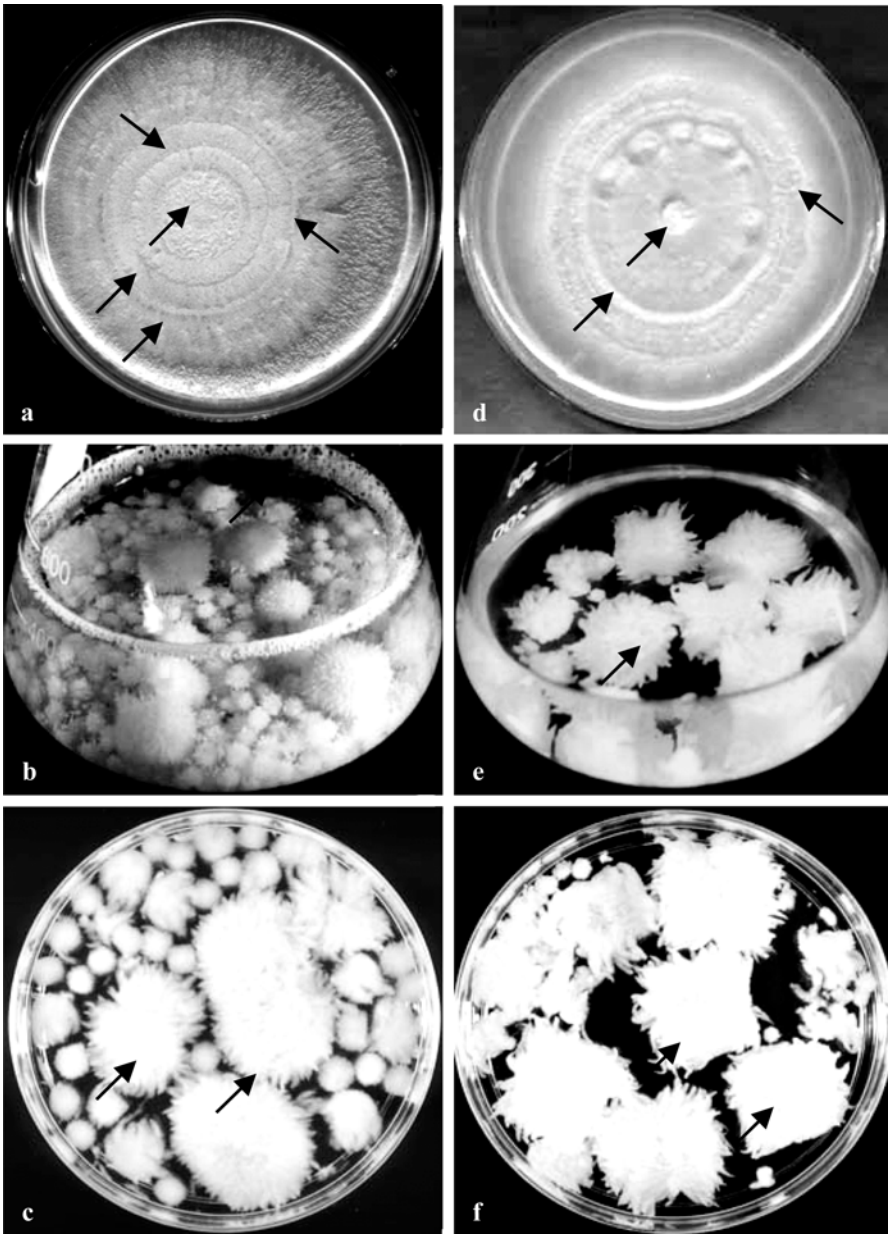
[a]The pH was adjusted to 6.5 with 1 N HCl. All the stocks were stored at 4 °C except vitamins, which were stored at –20 °C. In broth culture agar was excluded. Modified Aspergillus medium (Varma et al. 2001): the media composition was the same, except that the quantities of yeast extract, peptone and casein hydrolysate were reduced to one-tenth in quantity

5
Axenic Cultivation

Numerous media composition are described in the literature for the multiplication of soil fungi but almost no literature is available for the axenic cultivation of members belonging to Sebacinaceae. Table 4 describes medium compositions for successful mass cultivation of some of these symbiotic fungi. Information is lacking on the axenic cultivation of other members (Table 1). Some media have been suitably modified in our laboratory (Pham et al 2004b). Routinely, the medium used in our laboratory for the cultivation of these fungi and also to study their interactions with several plants was the Aspergillus medium described by Hill and Kaefer (2001) and suitably modified in our laboratory (Table 4). Other media used were glucose asparagine agar (Crook et al. 1950), malt extract medium (Gallowey and Burgess 1962), malt yeast extract medium (Varma and Bonfante 1994), MMN (modified Melin-Norkrans; Johnson et al. 1957), MMN1/10 medium (Herrmann et al. 1998), MMNC medium (Marx1969; Kottke et al. 1987), plate count agar (APHA 1978), MS (Murashige and Skoog 1962), potato dextrose agar (PDA; APHA 1978), White's medium (White and Braun 1941) and WPM medium (Ahuja et al. 1986). Aspergillus medium was also used to observe the interactions with transformed and non-transformed roots (Varma et al. 2001; Pham et al. 2004b)

Circular agar discs (about 4 mm in diameter), supporting spores and actively growing hyphae of members of the Sebacinaceae, were placed onto Petri dishes (90-mm diameter) containing solidified Aspergillus or any other medium. Inoculated Petri dishes (90-mm diameter) were incubated in an inverted position for 7 days at $3 \pm 2\,^{\circ}\mathrm{C}$ in the dark (Fig. 1). For broth cultivation, usually 4–5 fully grown fungus agar discs (4 mm in diameter) were inoculated into each 1,000-ml Erlenmeyer flask containing 600 ml of nutrient solution. Flasks were incubated at $30 \pm 2\,^{\circ}\mathrm{C}$, at constant shaking at 100 rpm on a rotary shaker (Fig. 2).

Fig. 1a–f. Morphological appearance of the Sebacinaceous fungus *P. indica*. **a–c** *P. indica*, **d–f** *S. vermifera* sensu. **a** Axenic culture on Aspergillus agar medium in a 90-mm Petri dish, photographed after 7-day incubation at $30 \pm 2\,^{\circ}\mathrm{C}$ in the dark. One disc of inoculum was placed at the centre (4-mm diameter). *Arrows* show the place of inoculum and rhythmic rings. **b** Growth in Erlenmeyer flask (500-ml capacity), containing Aspergillus broth (250 ml) incubated on a rotary shaker at 144 rpm at $30 \pm 2\,^{\circ}\mathrm{C}$ for 7 days in the dark. *Arrows* show the coral-like growth. **c** A magnified view of the coral-like growth from the broth culture. Morphological appearance of colonies of *Sebacina vermifera* sensu. The procedure and the medium composition for growth were identical to *P. indica*. **d** Axenic culture on Aspergillus agar medium. **e** Growth in Aspergillus broth medium. *Arrows* show the coral-like growth. **f** A magnified view of the coral-like growth from the broth culture ►


a
d
b
e
c
f

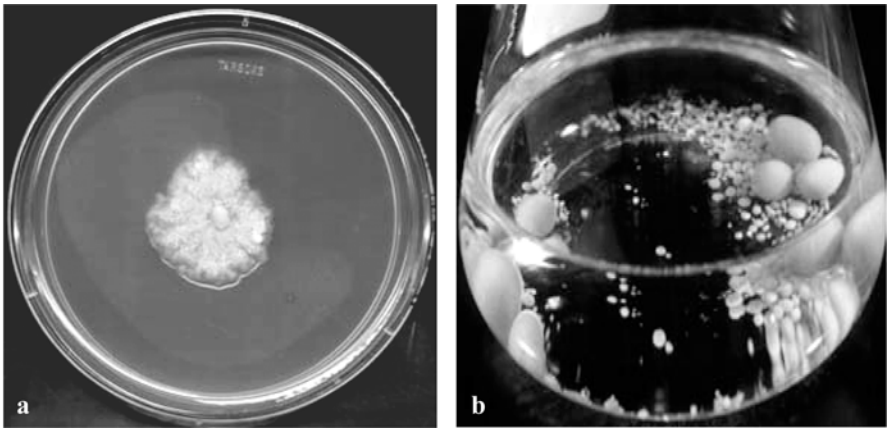


Fig. 2. a Axenic culture of *Sebacina* sp. on Aspergillus agar medium grown in a 90-mm Petri dish, photographed after 60 days of incubation at 30 ± 2 °C. One disc of inoculum was placed at the centre (4-mm diameter); a rough and hard surface was observed on the solid medium. **b** Growth in an Erlenmeyer flask containing Aspergillus broth, incubated on a rotary shaker at 144 rpm for 30 days in the dark

6 Monoxenic Culture

Various AM fungal species belonging to *Gigaspora*, *Glomus*, *Scutellospora* and *Acaulospora* are nowadays successfully cultured monoxenically in association with either transformed or non-transformed roots as plant partners (see Chap. 2). Verma (1996) was successful in transforming *Zea mays* roots on MS medium mediated by *Agrobacterium rhizogenes*, and achieved the co-culture of these organs with members of Sebacinaceae fungi. They observed extensive proliferation of the fungus within the root system, and completion of the fungal life cycle of *P. indica*. Different protocols can be used with other plant organs (stem, leaf, cotyledon) of different dicotyledonous plant species. The list is rapidly lengthening, especially since many laboratories are developing 'hairy root' technology for the production of plant secondary metabolites. Fungi belonging to Sebacinaceae also induce the proliferation of roots.

7 Interaction with Transformed Roots

Piriformospora indica was successfully associated with root organ cultures of transformed maize (*Zea mays* var. white L.; Fig. 3). This association

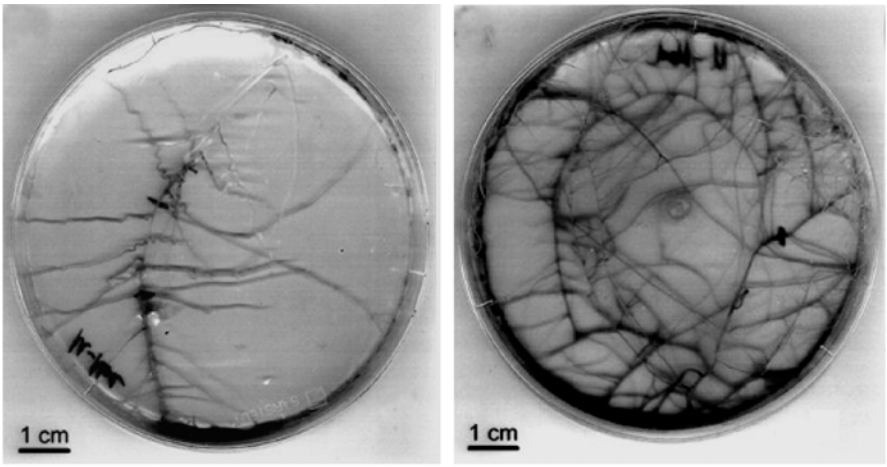


Fig. 3a, b. Effect of *P. indica* on transformed carrot roots after 20 days of inoculation. **a** Control (without fungus) showing poor development of root system. **b** Heavy root proliferation in the presence of the fungus

was materialized by a marked root surface colonization by the fungus and its establishment into the cortical tissues (inter- and intracellular; Figs. 4, 5, 6). Characteristic pear-shaped spore formation occurred in the cortical regions of the root as well as in the extramatrical environment.

The hyphae first colonized the root surface (Fig. 7) and produced structures similar to appressoria. Subsequently, they entered and traversed through the cortical cells and produced vesicles and also differentiated into intercellular, highly coiled structures. At maturity, external and internal spores were formed (Varma et al. 2001). The chlamydospores appeared isolated or in clusters and were distinctive due to their pear-shaped structure (Fig. 8). The chlamydospores were (14–)16–25(–33) µm in length and

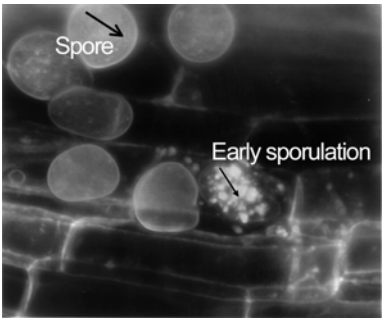


Fig. 4. Colonization of carrot root organ culture with *P. indica*. Cells are heavily colonized intracellularly. *Arrows* indicate the formation of chlamydospores

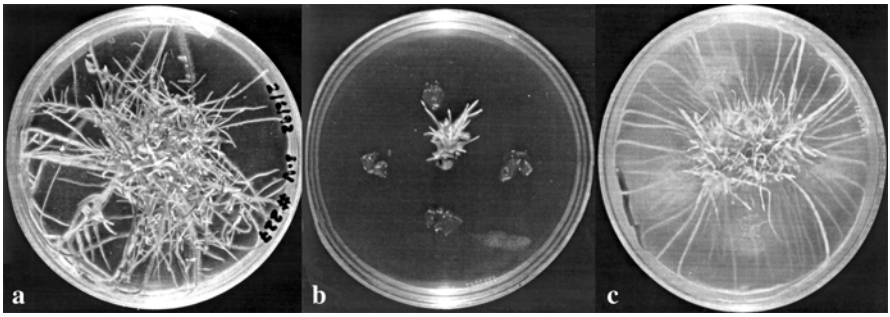


Fig. 5a–c. Root organ culture of *Zea mays* L. var. white strain grown on MS media. A 1-cm^2 agarose disc containing *P. indica* mycelium and spores was transferred onto a fresh minimal medium and incubated at 25 °C for 20 days. **a** Regenerated roots. **b** An early stage of establishment of dual culture. **c** Regenerated roots colonized with the fungus

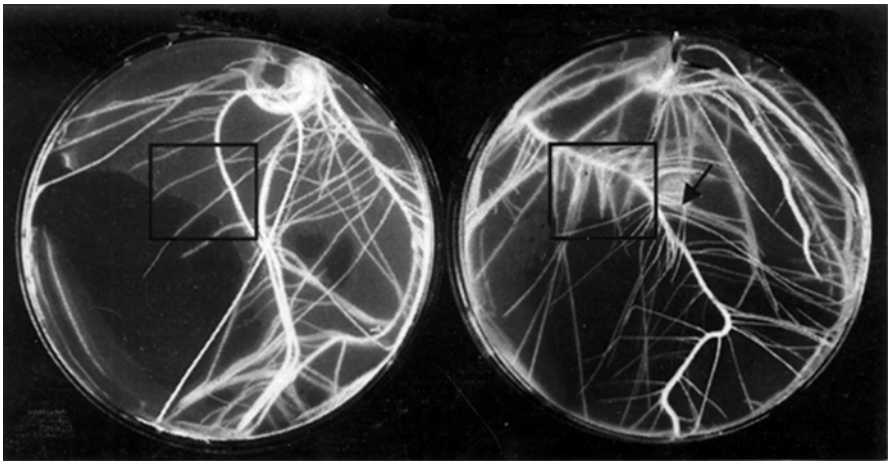


Fig. 6. Root colonization of *Zea mays* L. var. white strain with *Sebacina vermifera* sensu photographed after 20-day incubation on MS media at 25 °C. *Left* control, *right P. indica*

(9–)10–17(–20) µm in width. At maturity, these spores had walls up to 1.5 µm thick, which appeared two-layered, smooth and pale yellow (Fig. 9). The cytoplasm of the chlamydospores was densely packed with granular material and usually contained 8–25 nuclei. Chlamydospores were strongly autofluorescent (Fig. 10).

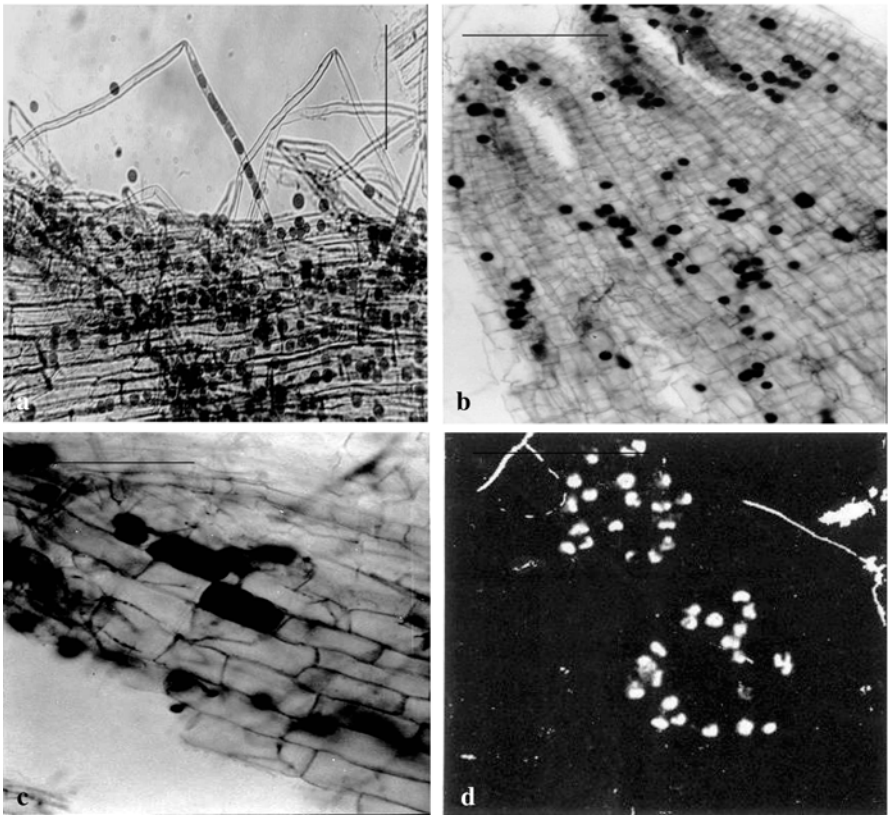


Fig. 7a–d. Surface sterile seeds of *Zea mays* L. var. white strain pre-germinated on water agar medium. They were transferred on solidified MS media without phosphate and carbon sources. After 1 week, *P. indica* inoculum was placed near the growing roots. Dual culture was maintained for 3 weeks at 25 °C. **a** Colonized root tip. **b** A view of the root tip showing extensive inter-/intracellular fungus colonization. **c** Chain of chlamydospores into the cells. **d** Nuclei in chlamydospores. They were stained with DAPI and observed in epifluorescence. Different optical planes were assembled into one picture using the Improvision software package (Improvision, Govenny, UK)

8
Interaction with Non-Transformed Roots

Nine-day-old *A. thaliana* plantlets were transferred to MMN1/10 medium (Peskan et al. 2004) and inoculated with *P. indica*. The fungal inoculum was placed 3 cm away from the roots to avoid initial physical contact. MMN1/10 medium was chosen since it contains low concentrations of phosphate and nitrate, and no carbon source conditions known to promote the interac-

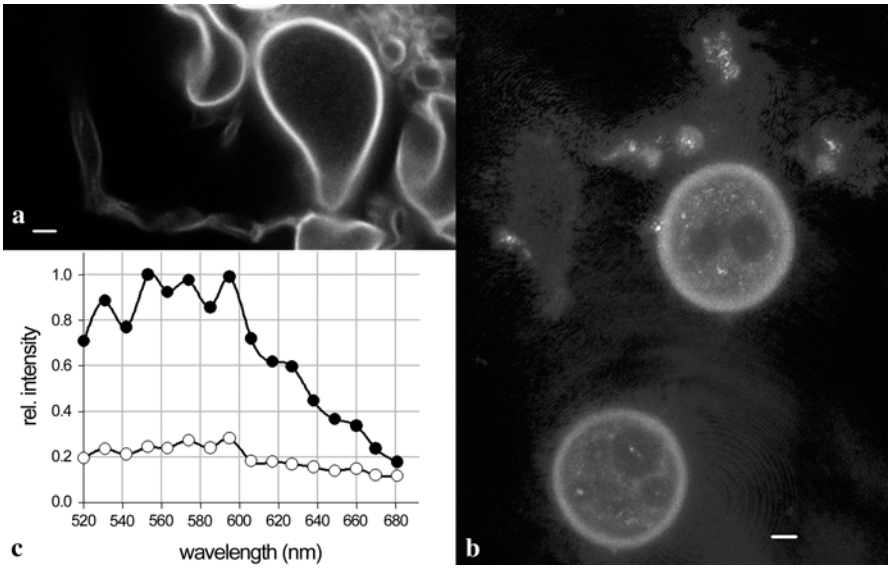


Fig. 8a–c. Chlamydospores of *P. indica* observed by LSM 510 META confocal microscope (Carl Zeiss, Germany). **a** Autofluorescent pear-shaped dormant chlamydospores (*bar* 40 µm). **b** After 48-h germination, the autofluorescent property was considerably reduced (*bar* 40 µm).**c** Relative autofluorescence curve monitored in green light. *Closed dots* at pre- and *open dots* after post-germination on MMN 1/10 medium at 25 °C

tion between plants and symbiotic fungi. The fungus grew slowly on the co-cultivation medium and produced only a few spores. No difference in root growth could be detected within the first 2 days after co-cultivation. After 3 days, stimulation of root growth became visible whereas after 7 days, intensive and uniform root proliferation in form of extended and branched lateral roots was detectable for all inoculated plants (Fig. 11). Inoculation of *A. thaliana* roots with *P. indica* was accompanied by changes in the morphology of the root hairs, which were visible even before the root proliferation became obvious (Fig. 12). Under the experimental growth conditions, the root hairs of inoculated plants grew longer and were thinner than those of the controls. After 2 weeks of co-cultivation, the fungal hyphae surrounded the root surface and grew inside the root hairs.

The strong autofluorescence property of dormant spores weakens soon after germination on the defined medium (Fig. 13). Normally, the growing roots of *A. thaliana* are non-fluorescent, but on co-cultivation with *P. indica* they acquired strong autofluorescence.

The observations that the interaction of these organisms exhibit features similar to those observed for AM fungi suggest that this might be a suitable model system to study plant–microbe interactions at a molecular level.

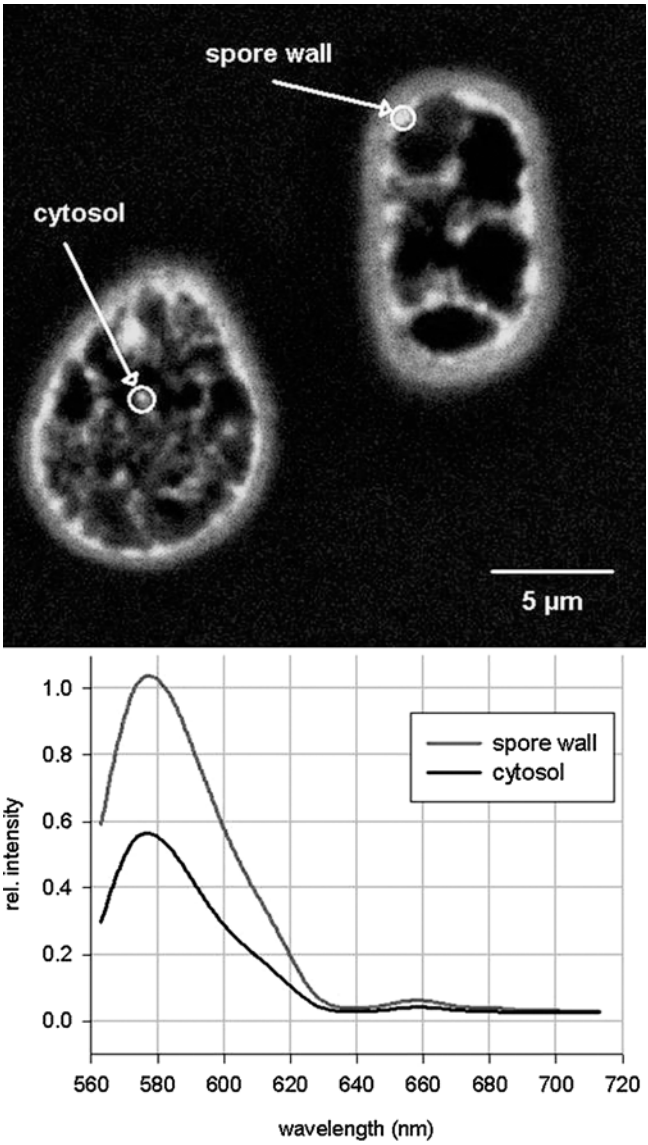


Fig. 9. A cross section of dormant chlamydospores of *P. indica*. Relative autofluorescence was measured by LSM 510 META confocal microscope (Carl Zeiss, Germany)

Co-cultivation of *A. thaliana* with *P. indica* promotes plant growth. Particularly striking are the effects of the fungus on root proliferation and the morphology of the root hairs. Growth promotion occurred before the fungal hyphae grew around or inside the roots. Therefore, this effect must be initiated by early signalling events from the fungus. Promotion of plant

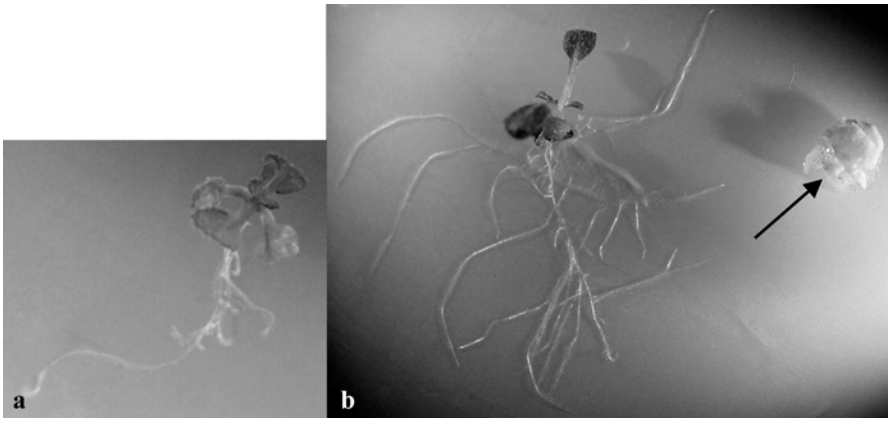


Fig. 10a, b. Root development in *A. thaliana* after 4 weeks of co-culture with *P. indica*. Two-week-old *A. thaliana* plantlets were transferred to MMN1/10 medium and cultivated at 22 °C under short day conditions. **a** The roots of the control plant, no fungus. **b** Inoculated plant with *P. indica*

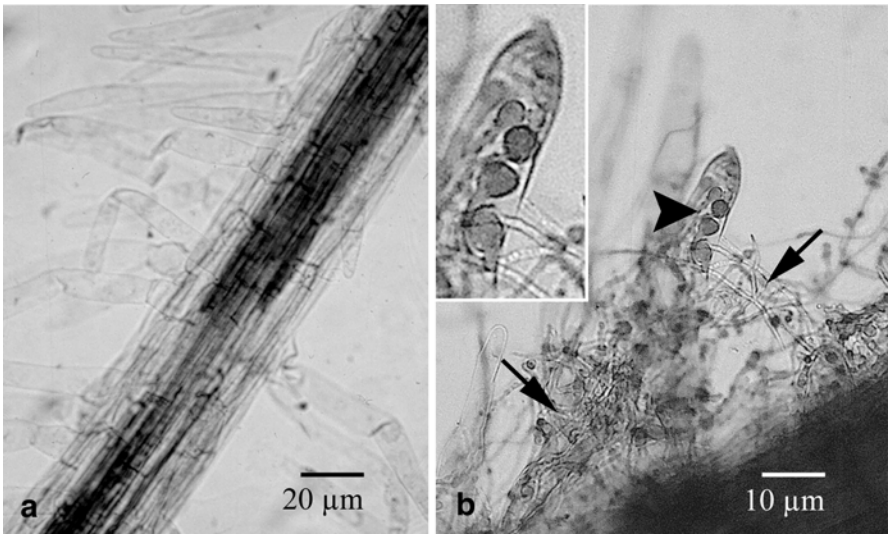


Fig. 11a, b. Root system (cf. Pham et al. 2004a). **a** Controlled with root hairs. **b** Excessive fungus root colonization. *Inset* shows a magnified view of a root tip hypha. Root segments were stained with cotton-blue and examined under the light microscope (Zeiss Axioplan model MC 100, Germany)

growth before the establishment of symbiosis is unusual for many arbuscular mycorrhiza systems, where the plant responds to the fungus only after the symbiosis is established, as result of a better supply of nutrients

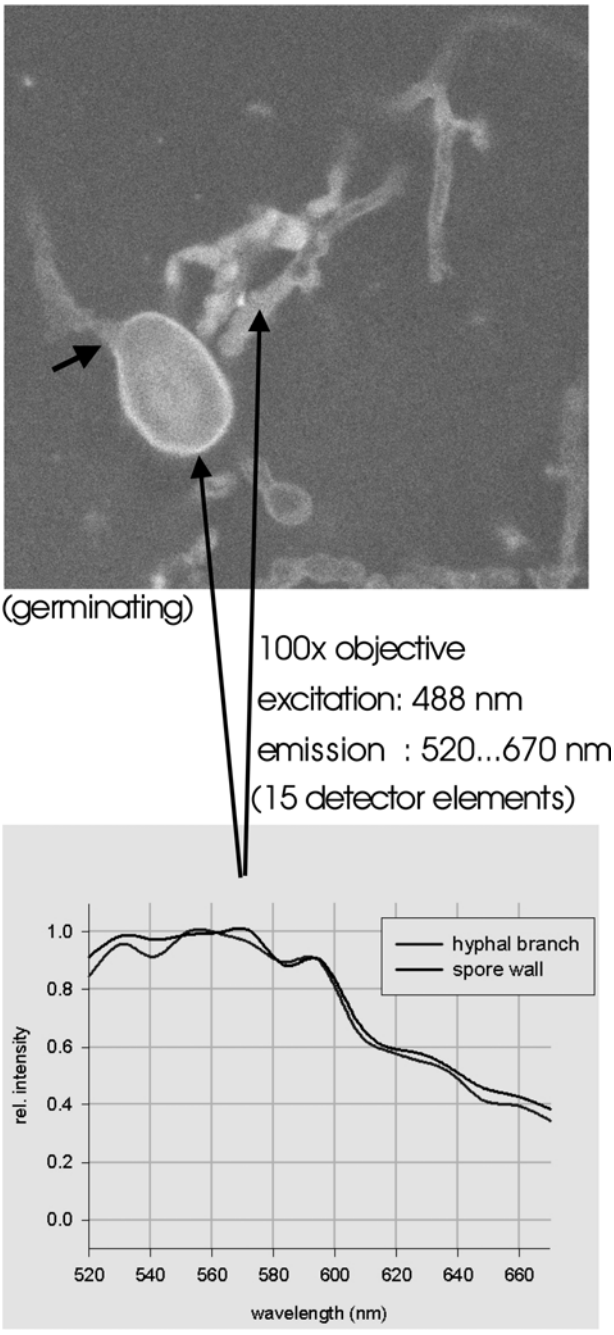


Fig. 12. Diminishing autofluorescence of *P. indica* dormant chlamydospores examined by laser confocal microscopy (LSM 510 META, Zeiss, Germany)

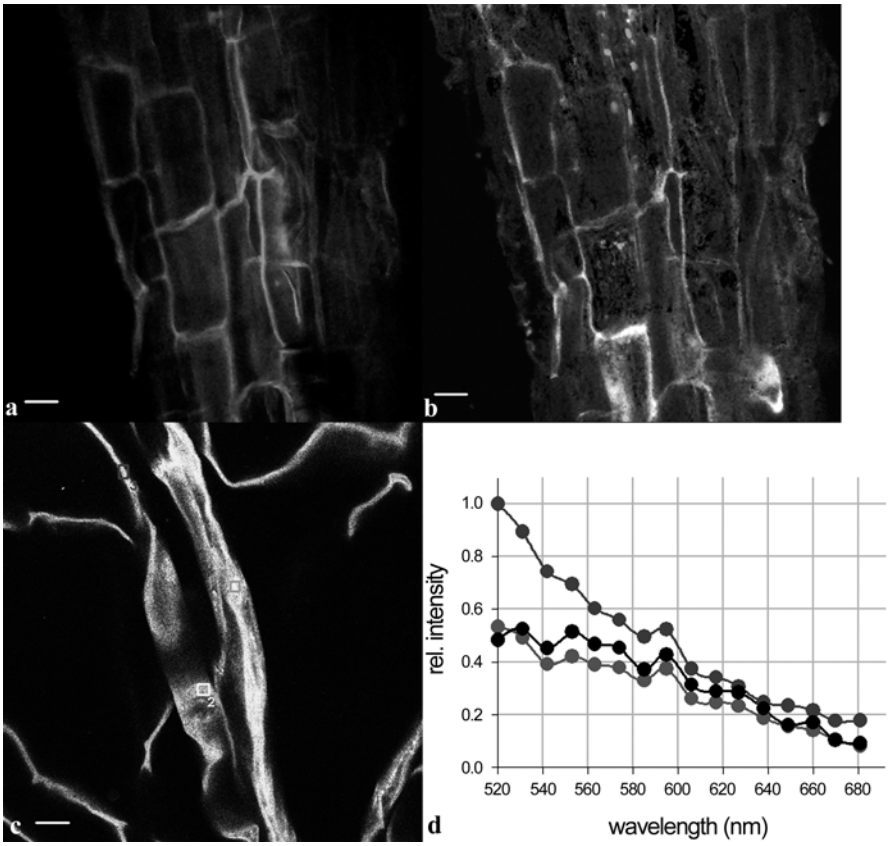


Fig. 13a–d. Autofluorescence in the developing root hairs as a result of co-culture with *P. indica*. *A. thaliana* plantlets were cultivated as described for Fig. 10. Root hairs were examined by laser confocal microscopy (LSM 510 META, Zeiss, Germany)

(Harrison 1999). It appears that the changes on the roots provoked by the micro-organisms ensure successful accommodation of the fungus and prevent its rejection by the plant. The modified morphology of the roots and root hairs suggest that the fungal signal(s) interferes with signalling pathways of plant hormones which control root growth and differentiation. One possible candidate is auxin. Mutation in the auxin signalling pathway prevents the extension of lateral roots, opposite to the effect of the cultivation of roots with the fungus. Moreover, it has been shown that both auxin and ethylene are required for root hair elongation in *A. thaliana* (Rahman et al. 2002; Takahashi et al. 2003). Auxin mutants have short root hairs, but the number and shape of the root hairs are not affected. We found that root hairs are one of the major target sites for *P. indica*, and that the overall

number of roots hairs and their length is substantially stimulated in the presence of the fungus. A stimulatory effect of *P. indica* on the number of root hairs is also observed for an auxin mutant, although they fail to grow longer. Thus, auxin is not the primary target site for *P. indica* action. However, the phytohormone appears to be required for the promoting effect of *P. indica*-mediated root hair elongation (Shahollari et al. 2005).

We isolated root hairs from co-cultivated and control seedlings and performed subtractive hybridizations. One of the earliest messages which responded to *P. indica* in *Arabidopsis* root hairs codes for a serine/threonine protein kinase (At3g25250). Twenty-four hours after co-cultivation, and before a physical contact between the two organisms can be detected at the microscopic level, the message level of the protein kinase is increased more than three-fold. The kinase exhibits strong sequence similarities to members of the AGC protein kinase family (cf. Bogre et al. 2003). The *Arabidopsis* AGC kinases contain sequence motifs for the docking of a protein kinase called PDK1, which becomes activated by 3-phosphoinositide. Thus, PDK1 could couple lipid signals to the activation of downstream protein kinases of the so-called AGC kinase family. Lipid-derived signals are central to regulating a multitude of cellular processes in plants, including growth (cf. Bogre et al. 2003 for detailed information). Since specific members of the AGC kinases appear to be involved in key growth signalling pathways, they might be good candidates for *P. indica*-induced root hair elongation.

9 Conclusion

The biotechnological applications of the monoxenic cultures of fungi appear a very promising field to further understanding of the molecular basis of fungus–plant symbiosis. So far, the work has been done with the non-axenically culturable AM fungi belonging to primitive members of Zygomycetes (Glomeromycota). Sebacinaceous fungi are unique and display some physiological functions common to AM fungi. However, members of Sebacinaceae belong to an advanced fungal community – Basidiomycetes. Interestingly, they are cultivable on defined and non-defined synthetic media, and preserve their characteristics to colonize living plant roots. These fungi not only promote plant growth and development but also extensively proliferate in transformed and non-transformed root systems. These fungi may also serve as an alternative to *Agrobacterium* for producing 'hairy roots' and in transformation studies.

Acknowledgements. Root organ culture mediated by AM fungi was supplied by Dr. Stéphane Declerck, Belgium. The Indian authors are thankful to DBT, DST, CSIR, UGC and the Government of India for partial financial assistance. We would also like to thank Bationa Shahollari for sharing her data with us.

References

Ahuja MR (1986) Aspen. In: Evans DA, Sharp WR, Ammirato PJ (eds) Handbook of plant cell culture 4, techniques and applications. Macmillan, New York, pp 626–651

APHA (1978) Standard methods for examination of dairy products, 14th edn. APHA, Washington, DC

Bandoni RJ (1984) The Tremellales and Auriculariales and alternative classification. Trans Mycol Soc Jpn 25:489–530

Berch SM, Allen TR, Berbee ML (2002) Molecular detection, community structure and phylogeny of ericoid mycorrhizal fungi. Plant Soil 244:55–66

Blechert O, Kost G, Hassel A, Rexer RH, Varma A (1999) First remarks on the symbiotic interactions between *Piriformospora indica* and terrestrial orchids. In: Varma A, Hock B (eds) Mycorrhiza, 2nd edn. Springer, Berlin Heidelberg New York, pp 683–688

Bogre L, Okresz L, Henriques R, Anthony RG (2003) Growth signaling pathways in *Arabidopsis* and the AGC protein kinases. Trends Plant Sci 8:424–31

Crook P, Carpenter CC, Klens PF (1950) The use of sodium propionate in isolating actinomycetes from soils. Science 112:656

Denison RF, Bledsoe C, Kahn ML, O'Gara F, Simms EL, Thomashow LS (2003) Cooperation in the rhizosphere and the "free rider" problem. Ecology 84:838–845

Gallowey LD, Burgess R (1962) Applied mycology and bacteriology, 3rd edn. Leonard Hill, London

Garnica S, Weiß M, Oertel B, Oberwinkler F (2003) Phylogenetic relationships of European *Phlegmacium* species (Cortinarius, Agaricales). Mycologia 95:1155–1170

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, New York

Harrison M (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 50:361–389

Herrmann S, Munch JC, Buscot F (1998) A gnotobiotic culture system with oak microcuttings to study specific effects of mycobionts on plant morphology before, and in the early phase of, ectomycorrhiza formation by *Paxillus involutus* and *Piloderma croceum*. New Phytol 138:203–212

Hill TW, Kaefer E (2001) Improved protocols for aspergillus medium: trace elements and minimum medium salt stock solutions. Fungal Genet News Letter 48:20–21

Johnson CN, Stout PR, Broyer RC, Carlpon AB (1957) Comparative chlorine requirements of different plant species. Plant Soil 8:337–353

Kaefer E (1977) Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genet 19:33–131

Kottke I, Guttenberger M, Hampp R, Oberwinkler F (1987) An in vitro method for establishing mycorrhizae on coniferous tree seedlings. Trees 1:191–194

Kottke I, Beiter A, Weiß M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: *Jungermanniales*have sebacinoid mycobionts while *Aneura pinguis*(*Metzgeriales)* is associated with a *Tulasnella*species. Mycol Res 107:957–968

Kumari R, Yadav HK, Bhoon YK, Varma A (2003) Colonization of Cruciferous plants by *Piriformospora indica*. Curr Sci 85:1672–1674

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

McGuire JM (1941) The species of Sebacina (Tremellales) of temperate North America. Lloydia 4:1–43

McKendrick SL, Leake JR, Taylor DL, Reed DJ (2002) Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. New Phytol 154:233–247

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays of tobacco tissue culture. Plant Physiol 15:473–497

Peskan T, Shahollari B, Pham H-G, Hehl S, Markent C, Blank V, Kost G, Varma A, Oelmueller R (2004) Association of Piriformospora indica with Arabidopsis thaliana roots represent a novel system to study beneficial plant-microbe interactions and involve in early plant protein modifications in the endocytoplasmic reticulum and in the plasma membrane. Physiol Plant 122:465–471

Pham GH, Singh An, Malla R, Kumari R, Prasad R, Sachdev M, Rexer KH, Kost G, Luis P, Kaldorf M, Buscot F, Harrmann S, Peskan T, Oelmüller R, Saxena AK, Declerck S, Mittag M, Stabentheiner E, Hehl S, Varma A (2004a) Interaction of *Piriformospora indica* with diverse microorganisms and plants. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin Heidelberg New York, pp 237–265

Pham GH, Kumari R, Singh A, Sachdev M, Prasad R, Kaldorf M, Buscot F, Oelmüller R, Peskan T, Weiss M, Hampp R, Varma A (2004b) Axenic cultures of *Piriformospora indica*. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology, Springer, Berlin Heidelberg New York, pp 593–616

Rahman A, Hosokawa S, Oono Y, Amakawa T, Goto N, Tsurumi S. (2002) Auxin and ethylene response interactions during Arabidopsis root hair development dissected by auxin influx modulators. Plant Physiol 130:1908–1917

Read DJ (1999) Mycorrhiza: the state of the art. In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology and biotechnology, 2nd edn. Springer, Berlin Heidelberg New York, pp 3–34

Schröter J (1889) Die Pilze Schlesiens. IJU Kern's, Breslau

Selosse MA, Bauer R, Moyersoen B (2002a) Basal hymenomycetes belonging to Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155:183–195

Selosse MA, Weiß M, Jany JC, Tillier A (2002b) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) LCM Rich and neighboring tree ectomycorrhizae. Mol Ecol 11:1831–1844

Shahollari B, Varma A, Oelmueller R (2005) Expression of receptor kinase in roots is stimulated by the basidiomycete Piriformospora indica and the protein accumulates in Triton-X-100 insoluble plasma membrane microdomains. J Plant Physiol (in Press)

Singh A (2001) Intergeneric hybridization between a yeast strain and a plant growth promoting endophytic fungus by isolation, fusion and regeneration of somatic protoplasts. PhD Thesis, Jawaharlal Nehru University, New Delhi

Singh A, Varma A (2000) Orchidaceous mycorrhizal fungi. In: Mukerji KG (ed) Mycorrhizal fungi. Kluwer, Amsterdam, pp 265–288

Singh An, Singh Ar, Rexer KH, Kost G, Varma A (2001) Root endosymbiont: *Piriformospora indica*-A boon for orchids. J Orchid Soc India 15:89–101

Singh An, Singh Ar, Kumari M, Rai MK, Varma A (2003a) Biotechnology importance of *Piriformaspora indica*-a novel symbiotic mycorrhiza-like fungus: an overview. National

Institute of Science Communication, CSIR, New Delhi, Plant Biotechnology Spec Issue Indian J Biotech 2:65–75

Singh An, Singh Ar, Kumari M, Kumar S, Rai MK, Sharma AP, Varma A (2003b) AMF-like fungus *Piriformaspora indica* – a boon for plant industry. In: Prasad BN (ed) Biotechnology in sustainable biodiversity and food security. Oxford & IBH, New Delhi, pp 101–124

Takahashi H, Kawahara A, Inoue Y (2003) Ethylene promotes the induction by auxin of the cortical microtubule randomization required for low-pH-induced root hair initiation in lettuce (*Lactuca sativa* L.) seedlings. Plant Cell Physiol 44:932–40

Urban A, Weiß M, Bauer R (2003) Ectomycorrhizae involving sebacinoid mycobionts. Mycol Res 107:3–14

Varma A and Bonfante P (1994) Utilization of cell wall related carbohydrates by ericoid mycorrhizal endophytes. Symbiosis 16:301–314

Varma A, Verma S, Sudha, Sahay NS, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Rai MK, Sudha N, Sahay N (2000) Microbial-biotechnology: new paradigms and role in sustainable agriculture. In: Rajak RC (ed) Microbial biotechnology for sustainable development and productivity. Scientific Publishers, India, pp 22–37

Varma A, Singh A, Sudha, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: an axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) The Mycota IX. Springer, Berlin Heidelberg New York, pp 125–150

Varma A, Abbott L, Werner D, Hampp R (2004) The-state-of-art. In: Varma A, Abbott L, WernerD, Hampp R (eds) Plant surface microbiology. Springer, Berlin Heidelberg New York, pp 1–12

Verma S (1996) Plant endomycorrhizal fungal interactions: a biotechnological approach. PhD Thesis, Barakatullah University, Bhopal

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarabhoy A, Bisen P, Bütenhorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root colonizing fungus. Mycologia 90:896–903

Warcup JH (1988) Mycorrhizal associations of isolates of *Sebacina vermifera*. New Phytol 110:227–231

Warcup JH, Talbot PHB (1967) Perfect states of rhizoctonias associated with orchids. New Phytol 66:631–641

Weiß M, Oberwinkler F (2001) Phylogenetic relationships in *Auriculariales* and related groups – hypotheses derived from nuclear ribosomal DNA sequences. Mycol Res 105:403–415

Weiß M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F (2004) *Sebacinales*: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1–8

Wells K, Bandoni RJ (2001) Heterobasidiomycetes. In: Mclaughlin DJ, McLaughlin EG, Lemke PA (eds) The Mycota VII. Systematics and evolution, part B. Springer, Berlin Heidelberg New York, pp 85–120

Wells K, Oberwinkler F (1982) *Tremelloscypha gelatinosa*, a species of a new family Sebacinaceae. Mycologia 74:325–331

White RC, Braun AC (1941) Crown gall production by bacteria-free tumor tissue. Science 94:239–241

Part VI
Biotechnology

17 Large-Scale Inoculum Production of Arbuscular Mycorrhizal Fungi on Root Organs and Inoculation Strategies

Alok Adholeya[1], Pragati Tiwari[1], Reena Singh[1]

1 Introduction

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs which, after root colonization, exert widely accepted benefits to a wide range of host-plant species. The fungi colonize the root cortex in a mutualistic association, resulting in a bi-directional transfer of carbon from the plant to the fungus and of minerals, especially phosphorus, from the fungus to the plant. Mass production of contaminant-free AM fungi remained a bottleneck for application in agriculture for decades. However, since the early work of Mosse and Hepper (1975), and subsequent development by Strullu and Romand (1986, 1987) and Bécard and Fortin (1988), the monoxenic cultivation system has become a valuable tool to produce contaminant-free AM fungi, allowing the realization of large-scale production under strictly controlled conditions. Bécard and Fortin (1988) developed an efficient technique to cultivate AM fungi in association with transformed host roots on synthetic growth medium. A number of AM fungal species (see Chap. 2) have been successfully cultivated on root organs and are used to conduct innovative, basic research (Fortin et al. 2002).

Monoxenic cultivation has several advantages over conventional pot cultivation systems regarding inoculum production. This technique offers pure, sterile, bulk, contaminant-free propagules, hitherto not practicable using conventional modes of pot culture, aeroponic or hydroponic techniques. In addition, this technique has a edge over other conventional modes of mass production, whereby a several-fold increase in spore/propagule production is achieved in less time and space. The technology involves the extraction of potential viable propagules from soils, surface sterilization and optimization of growth conditions for germination under aseptic conditions. This is followed by the association of the propagules with a suitable excised host root for propagule production and

[1]Centre for Mycorrhizal Research, Biotechnology and Management of Bioresources Division, The Energy and Resources Institute (TERI), DS Block, India Habitat Centre, Lodi Road, New Delhi 110003, India, Tel.: +91-11-24682111, Fax: +91-11-24682144, E-mail: aloka@teri.res.in

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

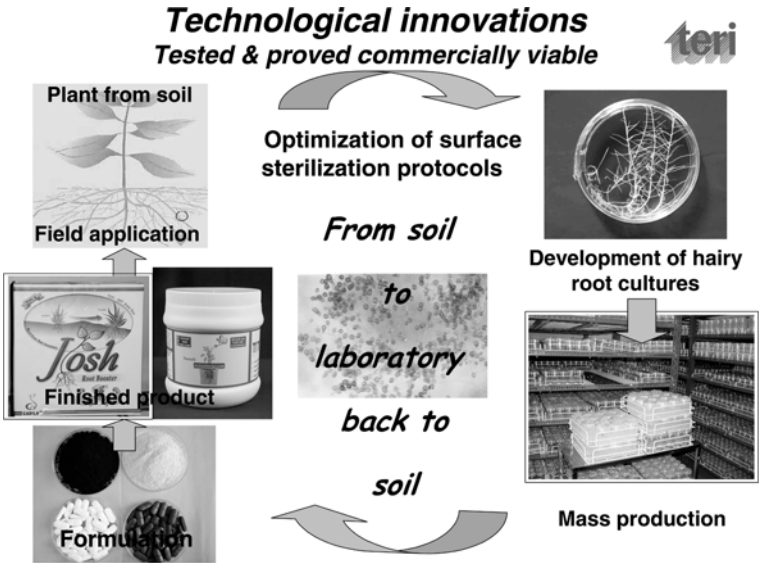


Fig. 1. Technological development from a root organ-based technique

recovery. Mass-produced propagules are then formulated in a utilizable form and stored before application to the target plant (Fig. 1). This sums up the long journey from the soil system to laboratory propagation, and subsequent application to land or potted plants, allowing practical exploitation of their potential.

Earlier reviews have compared the monoxenic culture system with conventional techniques of large-scale production and have proven the former's effectiveness (Verma and Adholeya 1996; Douds et al. 2000). The feasibility of commercializing mycorrhizal inocula has often been dependent on the ease and economics of mass production and the formulation of large amounts of viable, stable and highly efficacious mycorrhizal propagules.

2
Monoxenic-Based AM Inoculum Production

Mass production of AM fungi has been achieved with several species, but *G. intraradices* remains the most promising, with increased spore production obtained since the early investigations on monoxenic cultivation until today (Table 1). In 1992, Chabot et al. established cultures from surface-sterilized spores as starter material and produced 750 spores in 30 ml medium after a period of 4 months of growth in a mono-compartmental petri plate system. Using sheared roots as starter inoculum, Diop et al.

Table 1. Comparative account of AM fungal spore production achieved by different workers (per ml of media)

Author	Spore recovery (spore/ml)	Incubation time (months)
Chabot et al. (1992)	25	4
Diop et al. (1994)	30	3
St Arnaud et al. (1996)	1,000	3–4
Jolicoeur et al. (1999)	13	5
Douds (2002)	3,250	7
Adholeya (2003)	3,000	3

(1994) obtained approximately 890 spores after 3 months of incubation. An advanced mode of airlift bioreactor-based production was adopted by Jolicoeur et al. (1999). These authors recovered 12,400 spores per litre of medium. St Arnaud et al. (1996) obtained 15,000 spores in a bi-compartmental Petri plate in 3–4 months. This bi-compartmental system was improved by Douds (2002) by replacing the medium in the distal compartment by fresh medium at regular intervals. With this procedure, this author obtained 65,000 spores in the distal side of the bi-compartment in a period of 7 months. With the technology developed at the Centre for Mycorrhizal Research, The Energy and Resources Institute (TERI), New Delhi, India, the recovery of infective propagules approximated 250,000–300,000 spores in 3 months in 100 ml of medium. The TERI technology here adopts optimization at different levels, identifying the rate-limiting factors leading to the bulk production for commercial utilization. The AM fungi in genus *Glomus* provide the possibility of using colonized roots as inoculum material. This was also optimized in parallel to achieve higher root colonization, up to 70–80% (Tiwari and Adholeya 2003). The sub-cultivation of the root organ and its harvest have been attained at 4 and 12 weeks respectively. Such improvement allows higher spore and propagule recovery when compared with the unit volume of media in earlier published research (Table 1). This also facilitates the efficient utilization of space and energy in the production system, i.e. solid-state fermentation. Since the technology is more dependent on personnel, it lowers the number of man-days and achieves higher productivity. Many process controls were developed in order to reduce the levels of contamination (generally from 10–15% to 3–5%, common under tropical conditions).

As a whole, this process has made the technology viable and attractive for industries to assimilate and adopt. The technology has so far been transferred to two leading agriculturally and pharmacologically based industries in India.

3 Formulations

Formulation technologies largely take care of possible adverse environmental effects and factors that may render the inoculum ineffective. Formulation is essentially a blend of microbial propagules with a range of carriers or adjuvants, to produce a material that can be effectively delivered to the target application. Several mycorrhizal inoculum formulations have been proposed. At the research laboratory level, glass beads have been used (Redecker et al. 1995), and so has expanded clay (Plenchette et al. 1983) in the commercial sector. These formulations have the advantage of allowing the natural entrapment of mycorrhizal spores and roots during the growth phase, under greenhouse conditions. The beads have a porous texture with numerous air spaces into which the mycorrhizal propagules can establish. Mixing of the air-dried inoculum with inert carriers such as sand, vermiculite and soil-rite also has been documented (Millner and Kitt 1992). Mycorrhizal inoculum is available in the form of powdered inoculum, tablets/pellets or granules, gel beads and balls (Fig. 2). Intraradical spores/vesicles of *Glomus* spp. could also be entrapped in alginate beads, and used as such (Redecker et al. 1995). Isolated intraradical materials in

Fig. 2. Formulation types for AM fungal application

such beads were observed regenerating and colonizing fresh roots under controlled conditions (Strullu and Plenchette 1991). Entrapment in alginate beads was also shown to be effective with monoxenically produced spores (Declerck et al. 1996).

In commercial operations, where each step in the production process adds to the cost of the end product, carrier cost is an important criterion in the process development. A successful formulation carrier must be economically viable to produce (preferably made of a locally available inert material with non-toxic waste), with no deleterious effects on the mycorrhizal symbiosis. It should further be easy to handle, allowing effective dispersion during application. The formulation should permit early dissolution or dispersion (for tablets/pellets/granules) near the roots, in the case of potted plants, so that roots can easily invade the carrier for efficient mycorrhization. If the carrier is very strongly glued and does not dissolve during watering, the effect may be reduced, since roots and mycorrhizal propagules may not be able to establish contact.

During the distribution of inoculum, the issue of its dilution for localized and effective application needs to be addressed. Among other factors, organic matter is considered to encourage microbial activity, soil structure and enhance plant growth. It would also be useful to incorporate in mycorrhizal inoculum stimulatory compounds such as flavonoids (Bécard and Piché 1989; Gianinazzi-Pearson et al. 1989), as suggested in the bead system or even for synergistic micro-organisms (Hildebrant et al. 2002).

Another aspect of formulation development concerns the conditions under which the process is carried out. Such conditions are of importance because a living organism is used as biofertilizer. This implies that the viability and intactness of the organisms cannot be compromised at any stage of the process, beginning with incubation, monitoring of development and possible contamination, harvesting, drying or grinding, until the organism is finally mixed with the carrier and packaged. Strictly controlled growth conditions must be applied, with careful attention to maintain the effectiveness of inoculum. Even a short lapse could result in a loss of viability of the organism, and discourage the end user from applying these methods in agriculture.

4
Inoculation Strategies and Application Technology

Inoculation refers to the application of a commercially available inoculum of AM fungi to plant seeds or into soil ready for planting. The inoculum is a material which carries mycorrhizal fungi in a usable form, i.e. spores, hyphae and colonized root fragments. An inoculant product is best used

when there is reason to believe that indigenous soil populations of AM fungi are low or native AM fungi are no longer effective. It is particularly important to inoculate plants when out-planting to non-irrigated sites, or when dealing with disturbed soils where plants have difficulties to establish their root systems.

It should be stressed that the mere presence of an AM fungus does not imply benefits to a plant. With improvements in technology and AM fungal isolate selection, it should be possible to use novel and more efficient isolates of AM fungi to replace less efficient native isolates, already present in the soil. We think that introduced AM fungi may become diluted in their effect as they reside in the soil for extended periods, although they may still sporulate. Thus, we suggest that adding optimum levels of newly selected isolates of AM fungi at each planting could be beneficial, although perennial plants need inoculation only once.

In principle, symbiosis can be achieved from only one propagule which germinates and colonizes a root, but it may take a long time for the AM fungus to spread to a significant portion of the root system under such conditions. Therefore, it is better to initiate multiple infections to speed up the colonization process, as shown by infectivity assays of the inocula (Sharma et al. 1996). Typically, one refers to the number of fungal propagules delivered by each product onto each seed, or into the soil around each seed. In theory, the larger the number of AM fungal propagules delivered to the root zone at application, the faster the colonization of roots. How efficacious this is in practice will, of course, vary with product, environmental conditions, the delivery method, and a number of other variables. Calculations of the number of AM fungal propagules per plant, or per area, are based on various parameters such as (1) weight or volume of the package; (2) number of AM fungal propagules; (3) application rate of the inoculum to seed or soil; (4) extent of adhesion of the product to the seed; (5) seeding rate per acre.

5
Application Technology

For successful mycorrhizal colonization, fungal propagules should be in close contact with the plant roots (Fig. 3). Inoculum formulation may involve one or more AM fungal species or even other organisms, such as beneficial bacteria or fungi which together enhance desirable parameters in the target plant. There are five main application technologies: broadcasting, in-furrow application, seed dressing, root dipping and seedling inoculation.

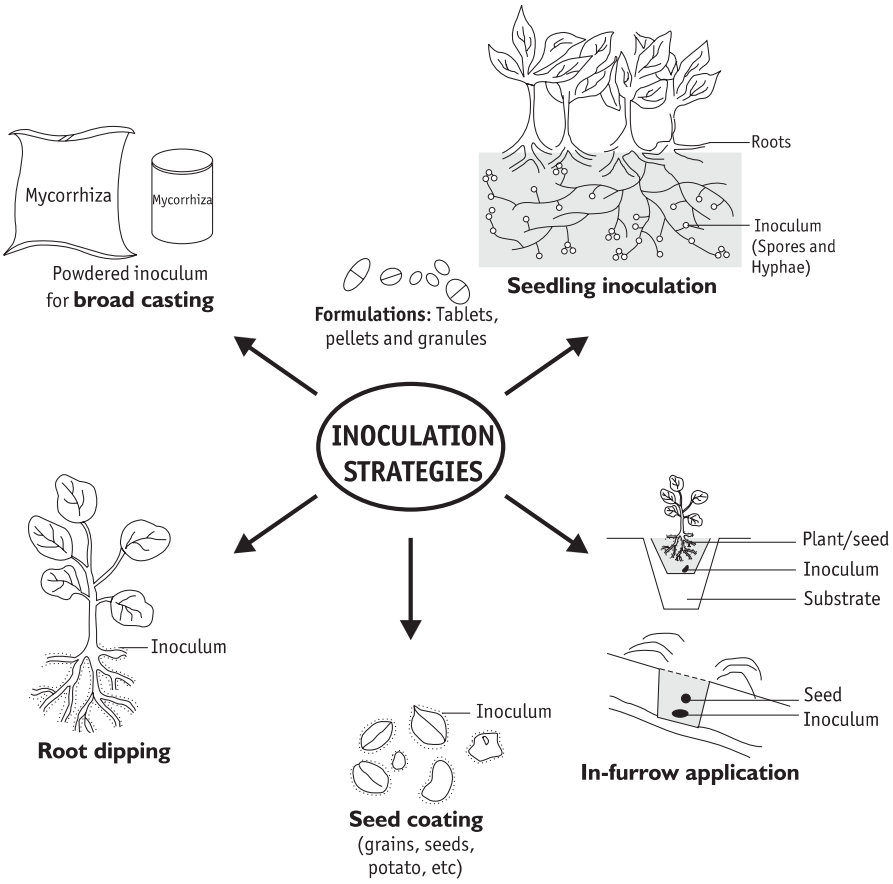


Fig. 3. Application technology and inoculation strategies for AM fungal products

5.1 Broadcasting

In this method, seeds and inoculum are broadcasted over the soil surface. They are then mixed with the top few centimetres of soil. Inoculum growing in the immediate vicinity of germinating seeds will colonize the seedling's roots. Broadcasting requires no special equipment, and can be done manually or mechanically by truck and tractor-pulled agricultural broadcasters. Small fertilizer spreaders, available at most hardware stores, can also be used. This method has the benefit of covering a large area with both seed and inoculum quickly. However, a large amount of inoculum is required to ensure colonization, since only inoculum immediately adjacent to seeds will colonize them.

5.2 In-Furrow Application

In this method, a small trench is made (just under the seed planting depth) and the inoculum is placed directly in the trench. Seeds are placed over the inoculum and covered with soil. The seeds germinate and their roots penetrate the inoculum layer. Hyphae can then colonize the young roots as they enter the inoculum layer. The trenching method works very well and produces high levels of mycorrhizal colonization.

5.3 Seed Dressing

Treating seeds – a practice known as dressing or coating – is another method of inoculation. Here, pellets of the inoculum are prepared by mixing them with a sticky carrier such as gum acacia before rolling them with seeds. The seed pellets are dried and planted over large areas using a seed drill. The advantage of this method is that the inoculum can readily colonize the germinating seed, because of direct contact. Less inoculum is required, and the labour requirements are minimal (Sieverding 1991).

5.4 Root Dipping

AM fungi and other beneficial microbes are usually absent in artificial potting mixes or substrates commercially used in nurseries, and transplants of *in vitro* raised plants are devoid of AM fungi. Inoculation of plantlets with appropriate AM fungal isolates gives excellent results with many ornamental plants. The root-dipping technique is generally recommended for inoculating micro-propagated and nursery-raised plants with AM fungi. These pot-raised and *in vitro* raised plantlets are first washed in water and the roots are then dipped into the inoculum (mixed with a carrier) for 5 min. The inoculum sticks to the roots, and the potting can then be carried out using standard methods. Successful AM fungal establishment has been reported in a variety of rooting media containing sand, gravel, peat, expanded clay, pumice, perlite, bark, sawdust, vermiculite or mixtures of these.

5.5 Seedling Inoculation

Producing inoculated seedlings for large-scale field plantations is more labour-intensive. Seedlings can easily be grown in nursery beds containing mycorrhizae where they will be colonized with a high degree of efficiency. The seedlings can then be transplanted in the field. This method is generally used to inoculate the seedlings of forest tree species, but it can also be used for some crops.

6 Field Evaluation

Mycorrhizal associations have evolved in complex and relatively stable natural environments which support mixed assemblages of plant species, but today man seeks to manage the symbiosis in simplified systems often involving monocultures of agricultural, horticultural or forest crops. Research on mycorrhizal functions has been carried out largely under standardized laboratory or glasshouse conditions specifically designed to exclude the complexities associated with natural soil as well as interspecific interactions between 'hosts' and fungal symbionts. This explains partly why we are able to manipulate the mycorrhiza of single fungal and 'host' species under sterile conditions, or in those soils lacking indigenous inocula, however, attempts to introduce or manage fungal symbionts in natural conditions have often failed in the past. In the 1990s, several reports in the area of plant propagation presented positive results demonstrating the value of AM fungi in the field (Table 2).

Before end users actually make use of AM fungi, the technology must be tested. The following issues need to be critically evaluated during the assessment of technology in the field:

1. Performance of plant species inoculated with AM fungi with respect to growth parameters, yield benefits, nutrient uptake and cost-economic feasibilities.
2. Nutrient budgeting, which tests the efficiency of AM fungi in improving the availability of nutrients to the plant.
3. Savings in costs on fertilizer purchase.
4. Improvement of soil conditions.

AM fungal inocula raised monoxenically at TERI, India, were tested in the field in different regions of the country with a variety of crop hosts, in fields

Table 2. Successful reports on field application of arbuscular mycorrhizal (AM) fungi on different crops (*continued on next page*)

Crops	AMF used for inoculation	Reference
Agricultural crops		
Acacia nilotica	*Glomus intraradices*	Sharma et al. (1996)
Allium cepa	Indigenous AMF	Gaur and Adholeya (2000)
Allium sativum	Indigenous AMF	Koch et al. (1997)
	Glomus etunicatum, *Glomus intraradices*	Matsubara et al. (1994)
Apium graveolens	*Glomus etunicatum*, *Glomus intraradices*	Matsubara et al. (1994)
Cajanus cajan	*Glomus* sp.	Ianson and Linderman (1993)
Calpogonium caeruleum	Nine AMF species	Ikram et al. (1992)
Capsicum annuum	*Glomus intraradices*, *Glomus mosseae*, *Glomus etunicatum*, *Gigaspora rosea*	Douds and Reider (2003)
Coriandrum sativum	*Glomus intraradices*	Gaur et al. (2000)
Cucumis sativus	*Glomus* spp.	Rosendahl and Rosendahl (1991)
Daucus carota	*Glomus intraradices*	Gaur et al. (2000)
Eleusine coracana	AMF species	Isobe and Tsuboki (1998)
Ervum lens	*Glomus clarum*	Xavier and Germida (2002)
Glycine max	AMF species	Isobe and Tsuboki (1998)
Hordeum vulgare	*Glomus etunicatum*	Mendoza and Bori (1998)
Linum-usitatissimum	Native AMF	Thompson (1994)
Manihot	*Glomus clarum*	Fagbola et al. (1998)
Oryza sativa	AMF species	Isobe and Tsuboki (1998)
Phaseolus vulgaris	AMF species	Isobe and Tsuboki (1998)
Pisum sativum	*Glomus etunicatum*, *Glomus intraradices*	Matsubara et al. (1994)
Solanum tuberosum	Indigenous AMF	Gaur and Adholeya (2000)
Sorghum bicolor	*Glomus* sp.	Isobe and Tsuboki (1998)
Trifolium repens	AMF species	Isobe and Tsuboki (1998)
Trigonella foenum-graecum	*Glomus intraradices*	Gaur et al. (2000)
Triticum aestivum	*Glomus* sp.	Ryan and Angus (2003)
Vigna spp.	AMF species	Isobe and Tsuboki (1998)
Zea mays	*Glomus mosseae*, *G. versiforme*	Bi et al. (2003)

where their potential was evaluated. The tested crop species included wheat, potato, mung bean, black gram, soybean, chickpea, chilli and sugarcane, all of which exhibited an average increase in yield of 0.4 t/ha (tonnes/hectare) and a decrease of approximately 25–50% in the expenditure on phosphate

Table 2. (*continued*)

Trees		
Coffea arabica	*Gigaspora margarita*, *Acaulospora laevis*	Bhattacharya and Bagyaraj (1998)
Gymnacranthera farquhariana	*Acaulospora laevis*, *Gigaspora gigantea*, *Glomus fasciculatum*, *G. geosporum*, *G. macrocarpum*	Bhat and Kaveriappa (1998)
Knema attenuata	*Acaulospora laevis*, *Gigaspora gigantea*, *Glomus fasciculatum*, *G. geosporum*, *G. macrocarpum*	Bhat and Kaveriappa (1998)
Liquidamber styraciflua	*Glomus etunicatum*	Brown et al. (1981)
Myristica fatua	*Acaulospora laevis*, *Gigaspora gigantea*, *Glomus fasciculatum*, *G. geosporum*, *G. macrocarpum*	Bhat and Kaveriappa (2000)
Poinsettia	*Gigaspora margarita*	Barrows and Roncadori (1977)
Sporobolus wrightii	AMF species	Richter and Stutz (2002)
Zephyranthes	*Glomus intraradices*	Scagel (2004)
Fruit crops		
Asparagus officinalis	*Gigaspora margarita*, *Glomus fasciculatum*, *G. mosseae*	Matsubara et al. (2000)
Bromus inermis	*Glomus fasciculatum*	Bildusas et al. (1986)
Citrullus lanatus	*Glomus clarum*	Kaya et al. (2003)
Citrus unshiu	*Glomus ambisporum*, *G. fasciculatum*, *G. mosseae*, *Gigaspora ramisporophora*	Shrestha et al. (1996)
Fragaria	*Glomus intraradices*	Werner et al. (1991)
Olea europia	*Glomus intraradices*, *G. mosseae*	Estaun et al. (2003)
Rubus	*Glomus clarum*, *G. etunicatum*, *G. intraradices*, *Gigaspora rosea*, *Gi. gigantea*, *Gi. margarita*, *Scutellospora calospora*, *S. heterogama*, *S. persica*	Taylor and Harrier (2000)

fertilizers. The use of such inocula allowed reclamation of several stressed systems such as fly ash overburdens, alkali chlorine sludge and distillery effluent loaded sites. AM fungal technology in India has evolved as a low-cost way to ensure an enhanced phosphorus supply, thereby ensuring an increased yield in an environmentally safe manner.

6.1 Factors Affecting the Field Inoculation

The success of AM fungal inoculation depends upon the crop species involved, the size and effectiveness of indigenous AM fungal populations, the fertility of the soil, and cultural practices. Recognition of the lack of (absolute) host-plant specificity by AM fungi (Mosse 1975) impeded progress in the selection of effective fungi for field applications. The assumption that any (non-host specific) isolate selected for 'effectiveness' based on arbitrary criteria, such as aggressive root colonization, copious sporulation, or growth enhancement in pot cultures, would improve any growth or yield parameter in the field under conditions not previously determined has led to inconclusive results in the past.

AM fungi lack absolute host-plant specificity, but conditions for their utilization are modified by ecological specificity (McGonigle and Fitter 1990) – colonization is multiple but appears to be preferential. The determination of preference by exotic fungi for potential crops prior to their introduction to new sites is therefore needed. Nutritional conditions of the soil play an important role in the survival and effectiveness of the inocula. The genus *Glomus* is more frequently found in fertile soils, with high nutrient levels (Hayman and Stovold 1979; Jeffries et al. 1988). In contrast, the genera *Scutellospora*, *Acaulospora* and *Gigaspora* are more abundant in low-nutrient or nutrient-binding soils (Koske 1987; Gemma et al. 1989). Some species, like *G. intraradices*, have a vast soil parameter range while others, like *Acaulospora* species, are restricted to acidic soils in the tropics. Thus, broad-range species like *G. intraradices* should be selected for inoculation purposes.

A range of soil chemical, physical and mycorrhizal characteristics determines the plant responses to mycorrhizal inoculation (Hamel 1996), phosphorus being the most determining factor. Phosphorus levels above 20 ppm can reduce the number of spores (Mårtenson and Carlgren 1994) and the amount of colonization by the mycorrhizal fungi (Jakobsen 1986). The complexity of the soil environment and biotic factors such as fungivorous insects (Finlay 1985), soil microflora (Azcon-Aguilar and Barea 1992; Linderman 1992) or even weather are likely to intervene and further obscure these relationships. Other soil conditions, such as pH (Young et al.

1985), temperature and texture, also govern the effect of inoculation. Mycorrhizal dependency also varies according to the crop species and the soil level of phosphorus availability (Plenchette et al. 1983).

Crop management strategies also affect the development and function of AM fungi (Jansa et al. 2002). Practices like tillage, monoculture, high fertilization and intensive agriculture detrimentally affect both colonization and mycorrhiza-mediated nutrient uptake (Perron et al. 2001). Use of a few agrochemicals like benomyl, benlate, aliette and ridomil (Sukarno et al. 1996) has some detrimental effect on the development of AM fungi.

7 Responsibility of the Scientific Community Towards Technology Development

The use of AM fungi inoculation for natural plant production is still in its infancy and demands basic inputs by researchers, commitment by industries, and adequate guidance to end users about its potential usage. It is imperative that academic, governmental and industrial scientists collaborate jointly to improve their knowledge and develop their use, with further efforts to tune the existing products for the market. Strict quality control measures need to be designed for the inoculum-producing industries be-

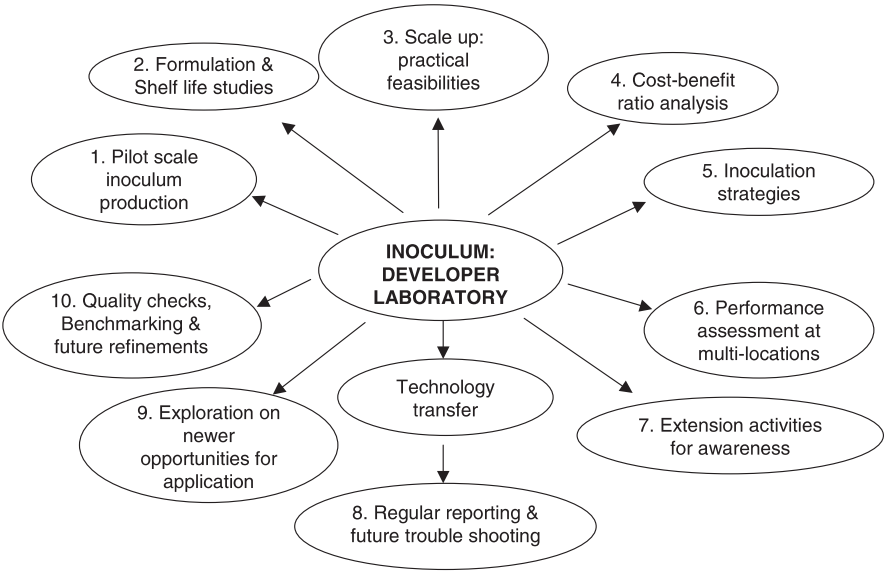


Fig. 4. Strategic responsibility of the technology developer laboratory – beginning to end

fore they release the product for sale and commercialization. The measures are the benchmarks (reference standards for quality assurance) which include tests of viability, shelf life, dilution possibility, and application and dispersion-associated tests (Fig. 4).

7.1 Quality Control and Benchmarking

Mycorrhizal inocula, as well as other microbial bio-inoculants and organic fertilizers available on the market, share a common problem: quality control and its regulation. It is very difficult to ensure that the products are of standard quality. In India and comparable countries, most commercial organic fertilizers are not covered by the type of national or international standards which govern the quality of chemical fertilizers.

Thus, specific protocols for quality control of AM fungal inocula need to be developed and standardized for application. This is essential not only as a guarantee for producers and users but also for the protection of ecosystems. Moreover, this would also help in quality management and assessment of inoculum potential with every batch of inocula produced. Quality control of commercial AM fungal inoculum is extremely important for developing faith among the user community, along with its effectively demonstrated potentials. Unless this is achieved, the potentials will remain unexplored among the other biofertilizers. It is important to evaluate the produced inoculum from commercial units with certain reference values to ensure the strict adherence to the protocols and methodologies recommended by recognized and independent laboratories. This is most vital, as several handling errors occur at the industrial level during technology adoption and implementation, causing subsequent problems in product quality, which may lead to the dissatisfaction of both the end users and producers.

For the mass production of AM fungi, critical benchmarks at all stages of inoculum development, covering all possible parameters desirable for ensured production, are identified. These include viability checks at processing stages until the formulation stage, ranging from the colonization of host roots, weight of dried inoculum at harvest, propagule estimations, infectivity potential of crude and formulated diluted inoculum, formulation conditions like temperature and suitable storage conditions. Such benchmarks also help institutionalized process efficiency at the production level.

Once the commercial launch of the formulation is achieved, both the developer of the technology and the distributing industries share equal responsibilities for the authenticity and performance of commercialized products, and must continue to work together to evaluate responses ob-

tained in the field by the end users. This would ensure confidence building and continuous use of these products over the years. It is important to regularly validate product performance, customer satisfaction and willingness for future use, to monitor the effectiveness of the inoculum.

The ethical responsibility of the laboratory developing AM fungal inoculum should consider the following features desired by the end user:

- Compatibility with local indigenous AM fungi for prompt and effective plant growth
- Ability to survive, and stability in the carrier system
- Ability to survive while seed-coated, even under adverse climatic conditions
- Wide-range of host applications
- Ability to maintain genetic stability
- Absence of harmful contaminants
- Prolonged shelf life.

8
Scopes and Applications of Monoxenically Based AM Fungal Technology

Some of the mechanisms to improve the monoxenically based AM fungal technology are identified in this section. These offer possibilities which need to be explored in the future to enhance the technology and efficiency of the monoxenic system of AM fungi.

8.1
Consortium Development of AM Fungi

Existing formulations in most commercial products deal with a single species of AM fungi or a consortium, mostly originating from pot-based multiplication (a detailed list is documented in review by Tiwari et al. 2002). Such products do not perform optimally when applied under certain agro-climatic conditions, or they grow together with interfering micro-organisms in the propagation phase. In nature, a variety of microbes, including AM fungi, interact in the plant rhizosphere and benefit the plant by enhancing the availability of nutrients and increasing resistance to disease-causing micro-organisms. Root systems are usually colonized by more than

one AM fungal species (Daft and Nicolson 1974; Abbott and Robson 1982; Merryweather and Fitter 1998; Jacquot et al. 2000). Most often, three to five and, occasionally, even nine AM fungal species, from different families and suborders occupying the same root system at the same time, have been reported (Morton et al. 1995). Daft (1983) suggested that multiple colonization, i.e. use of a consortium, rather than a single organism effective under different environmental conditions, was probably more beneficial to the plant, because a single endophyte may not be able to withstand certain environmental changes. The successful co-culture of the two AM fungal species *G. intraradices* and *Gigaspora margarita* (Fig. 5), reported by Tiwari and Adholeya (2002), has opened a new perspective in the development of consortium-based AM fungal inocula. It is obvious that a formulation containing a package of useful micro-organisms would have several advantages over single-isolate AM fungal inocula, and this aspect merits further investigation.

8.2 Host-Based Enhanced Productivity in AM Fungus Production

Another aspect of improving the monoxenic mass production protocol is the selection of appropriate Ri T-DNA transformed host roots. It has been reported that spore formation and extraradical mycelium development as well as root colonization and vesicle development (in the genus *Glomus*) are influenced by the host (Tiwari and Adholeya 2003). The growth medium is also a crucial factor in mass-production systems in which high propagule recovery is desirable. Tiwari and Adholeya (2003) showed that the spread of mycorrhizal roots and AM fungal propagule counts differ greatly between different kinds of transformed host roots. Douds (2002) also reported the importance of medium manipulation in enhancing the productivity of in vitro systems, however, the practicability and cost economics of the systems must be considered before attempting commercial production.

8.3 Mycorrhizae and Coupling Beneficial Organisms: Complete Package for Organic Systems

One key biotechnological goal is to use combined inoculation of selected rhizospheric micro-organisms to minimize fertilizer application and to maximize plant growth and nutrition (Linderman 1986; Barea and Jeffries 1995; Barea et al. 1998; Probanza et al. 2001). Literature reports confirm that selected combinations of microbial inocula enhance the positive effect

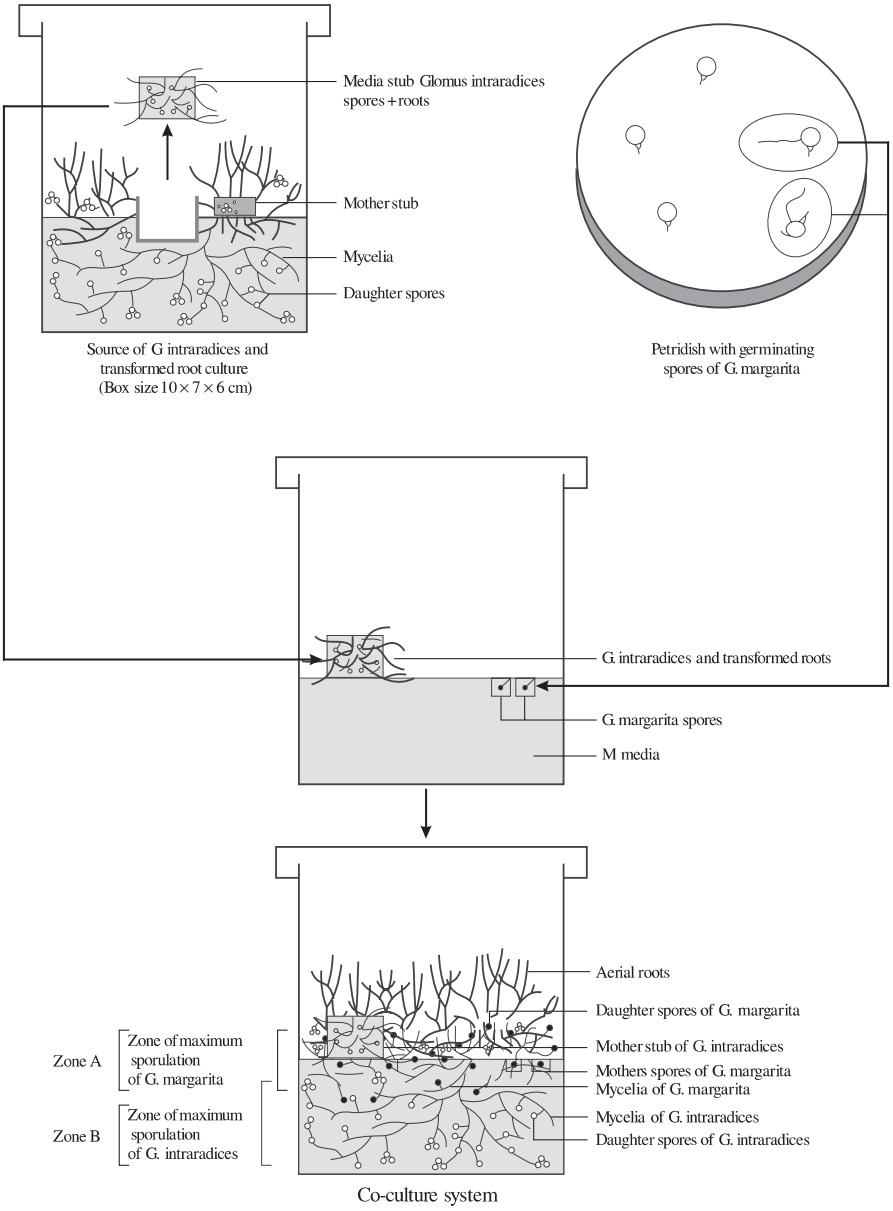


Fig. 5. Co-culture development process for multiple AM fungal cultivation (Tiwari P, Adholeya A 2002)

achieved by each microbial group considered separately (Toro et al. 1997, 1998). Also, free-living bacteria produce certain stimulatory metabolites that enhance plant development and growth, particularly when associated

with AM fungi (Azcon 1993; Ahmad 1995). Within microbial species, the wide genetic variation explains the high potential of the micro-organisms to adapt to different environments. Therefore, it is necessary to develop specific host-strain combinations of both bacteria and AM fungi with high effectiveness under a wide range of experimental conditions (Medina et al. 2003).

Thus, these suggestions may be taken into account while offering the AM fungus-based bio-inoculants for sustainable plant production.

9
Potential Tool for Organic Farming

In recent years, the world has seen a growing awareness of health and environmental issues, and sustainability has become a key word in discussions on economic development, particularly in relation to developing countries. The community is becoming more and more conscious of these issues globally, and government policies in industrialized as well as developing countries are increasingly being formulated to encourage organic and sustainable agriculture. Producers are turning to certified organic farming systems as a means of lowering input costs, decreasing reliance on non-renewable resources, capturing high-value markets and premium prices, and boosting farm income. Organic farming severely restricts the use of artificial chemical fertilizers and pesticides. Instead, it relies on developing a healthy, fertile soil and growing a mixture of crops. Supplementing the nutrient requirement of crops through organic composts/manures is essential for sustaining soil fertility and crop production. Many producers, manufacturers, distributors and retailers specialize themselves in growing, processing and marketing an ever-widening array of organic food and fibre products. In India, AM fungal inoculants are now widely used to the satisfaction of increasing numbers of organic gardeners and farmers.

10
Conclusions

Monoxenic cultivation technologies are now a reality, and provide significantly high and economically attractive options to chemical fertilizations. These systems gain more relevance to the tropical conditions wherein AM fungal counts are poor. This mode of mass production offers a several-fold increase in effective propagule production over conventional bulking techniques. This mode of AM production offers an edge due to its user-friendly and highly adoptable packaging. The transition of technique to technology

requires several attenuations and system approaches to make the technology cost-effective and economically attractive as well. This overall approach includes the identification of major rate-limiting factors, low-cost environmentally safe inputs and optimization of the scale of production. The changed scenario, in which bulk production is the need of the hour to meet the growing demand, is followed by several multi-location field demonstration trials on a wide range of plant hosts for extensive field evaluation. Any successful technology needs to have in-built components on quality control and process benchmarking for its efficient quality assurance. Both developing and developed countries have been successful in developing such technologies. However, there are still several bottlenecks and practical difficulties exist in its global acceptance, due to quality apprehensions of end users about the available inocula from various sources. Therefore, legislative intervention at governmental level is highly desirable for these bio-inoculants, for their more effective contribution in different growing systems towards a sustainable future in plant production.

With these findings and recognized effects, AM fungi thus appear to have a bright future, gaining tremendous importance in an effort towards reducing chemical application for sustainable agriculture, particularly among marginal farming communities.

A balanced but dynamic economy is needed from such technological innovations, to lead to a shared co-operative economic system for wider acceptability in the drive for sustainable agriculture.

Acknowledgements. The authors acknowledge partial financial support from the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, for the AMF mass production technology development. We sincerely thank Dr. RK Pachauri, Director General, The Energy and Resources Institute (TERI), New Delhi, India, for the infrastructure and environment for developing the technology and kind cooperation regarding its subsequent refinements. Dr. (Ms.) Nandini Kumar is duly acknowledged for evaluating the manuscript for editorial corrections.

References

Abbott LK, Robson AD (1982) Infectivity of vesicular-arbuscular mycorrhizal fungi in agricultural soils. Aust J Agric Res 33:1049

Abbott LK, Robson AD (1984) Colonization of the root systems of subterranean clover by three vesicular arbuscular mycorrhizal fungi. New Phytol 96:275–281

Abbott LK, Robson AD (1991) Field management of VA mycorrhizal fungi. In: Keister DL, Cregan PB (eds) The rhizosphere and plant growth. Kluwer, Dordrecht, pp 355–362

Adholeya A (2003) Commercial production of AMF through industrial mode and its large-scale application. In: Proc 4th Int Conf Mycorrhizae (ICOM4), Montréal

Ahmad MH (1995) Compatibility and co-selection of vesicular arbuscular mycorrhizal fungi. New Phytol 99:257–265

Allen EB (1988) Below ground spatial patterning: influence of root architecture, microorganisms and nutrients on plant survival in arid lands. In: Ellen EB (ed) The reconstruction of disturbed arid lands: an ecological approach. Westview Press, Boulder, pp 113–135

Alten von H, Blal B, Dodd JC, Feldmann F, Vosatka (2002) Quality control of arbuscular mycorrhizal fungi inoculum in Europe. In: Gianinazzi S, Barea JM, Haselwandter K (eds) Mycorrhiza technology in agriculture. Birkhauser, Basel, pp 281–296

Azcón R (1993) Growth and nutrition of a nodulated mycorrhizal and non-mycorrhizal *Hedysarum coroarium* as a result of treatments with fractions from a plant growth promoting rhizobacteria. Soil Biol Biochem 25:1037–1042

Azcón-Aguilar C, Barea JM (1992) Interactions between mycorrhizal fungi and other rhizosphere microorganisms In: Allen MF (ed) Mycorrhizal functioning. Chapman & Hall, New York, pp 163–198

Barea JM, Jeffries P (1995) Arbuscular mycorrhizas in sustainable soil plant systems. In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology and biotechnology. Springer, Berlin Heidelberg New York, pp 521–559

Barea JM, Andrade G, Biaciotto V, Dowling D, Lohrke S, Bonfonte P, O'Gara F, Azcón-Aguilar C (1998) Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for the biocontrol of soil-borne plant fungal pathogens. Appl Environ Microbiol 64:2304–2307

Barrows JB, Roncadori RW (1977) Endomycorrhizal synthesis by *Gigaspora margarita* in poinsettia. Mycologia 69:1173–1184

Bécard G, Fortin JA (1988) Early events of vesicular arbuscular mycorrhizal formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989) Fungal growth stimulation by CO2 and roots exudates in vesicular arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2230–2325

Bécard G, Piché Y (1992) Status of nuclear division in arbuscular mycorrhizal fungi during in vitro development. Protoplasma 174:62–68

Bhat P, Kaveriappa KM (1998) Effect of AM Fungi on growth and nutrient uptake in some endemic forest tree species of the Western Ghat Forests of Karnataka, India. In: Proc 2nd Int Conf Mycorrhizae (ICOM2), Uppsala

Bhattacharya S, Bagyaraj DJ (2002) Effectiveness of arbuscular mycorrhizal fungal isolates on Arabica coffee (*Coffea Arabica* L.). Biol Agric Hortic 20:125–131

Bi YL, Li XL, Christie P, Hu ZQ, Wong MH (2003) Growth and nutrient uptake of arbuscular mycorrhizal maize in different depths of soil overlying coal fly ash. Chemosphere 50:863–869

Bianciotto V, Bandi C, Minerde D, Sironi M, Tichy HV, Bonfante P (1996) An obligately endosymbiotic fungus itself harbors obligatory intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Bianciotto V, Perotto S, Ruiz-Lozano JM, Bonfonte P (2002) Arbuscular mycorrhizal fungi and soil bacteria: from cellular investigations to biotechnological perspectives. In: Gianinazzi S, Schuepp H, Barea JM, Haselwandker K (eds) Mycorrhizal technology in agriculture. Birkhauser, Basel, pp 19–31

Bildusas IJ, Pfleger FL, Stewart EL, Dixon RK (1986) Response of *Bromus inermis* inoculated with *Glomus fasciculatum* to potassium fertilization and drought stress. Plant Soil 95:441–444

Brown RW, Schultz RC, Kormanik PP (1981) Response of vesicular-arbuscular *Glomus etunicatus* endomycorrhizal sweetgum seedlings *Liquidambar styraciflua* to three nitrogen fertilizers – ammonium sulfate, ammonium nitrate, and potassium nitrate. For Sci 27:413–420

Daft MJ (1983) The influence of mixed inocula on endomycorrhizal development. Plant Soil 71:331–337

Daft MJ, Nicolson TH (1974) Arbuscular mycorrhizas in plants colonizing coal wastes in Scotland. New Phytol 73:1129–1132

Declerck S, Strullu DG, Plenchette C (1996) In vitro mass production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycol Res 100:1237–1242

Declerck S, D'Or D, Cranenbrouck S, Le Boulengé E (2001) Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. Mycorrhiza 11:225–230

Dehne HW (1982) Interaction between vesicular arbuscular mycorhizal fungi and plant pathogens. Phytopathology 72:1115–1119

De Souza FA, Berbara RLL (1999) Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia 91:343–350

Diop TA, Plenchette C, Strullu DG (1994) Dual axenic culture of sheared root inocula of vesicular arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Douds DD Jr (2002) Increased spore production by *Glomus intraradices* in the split-plate monoxenic culture system by repeated harvest, gel replacement, and re-supply of glucose to the mycorrhiza. Mycorrhiza 12:163–167

Douds DD, Reider C (2003) Inoculation with mycorrhizal fungi increases the yield of green peppers in a high P soil. Biol Agric Hort 21:91–102

Douds DD, Gadkar V, Adholeya A (2000) Mass production of VAM fungus biofertilizer. In: Mukerji KG, Chamola BP, Singh J (eds) Mycorrhizal biology. Kluwer, Dordrecht, pp 197–215

Estaun V, Camprubi A, Calvet C, Pinochet J (2003) Nursery and field response of olive trees inoculated with two arbuscular mycorrhizal fungi, *Glomus intraradices* and *Glomus mosseae* . J Am Soc Hortic Sci 128:767–775

Fagbola O, Osonubi O, Mulongoy K (1998) Growth of cassava cultivar TMS 30572 as affected by alley-cropping and mycorrhizal inoculation. Biol Fertil Soil 27:9–14

Filippi C, Bagnoli G, Citernesi AS, Giovannetti M (1998) Ultrastructural spatial distribution of bacteria associated with sprocarps of *Glomus mosseae*. Symbiosis 24:1–12

Finlay RD (1985) Interactions between soil micro-arthropods and endomycorrhizal associations of higher plants. In: Fitter AH, Atkinson D (eds) Ecological interactions in soil. British Ecological Society, Oxford, pp 319–31

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root organ cultures. Can J Bot 80:1–20

Gadkar V, Adholeya A (2000) Intraradical sporulation of AM *Gigaspora margarita*in long-term axenic cultivation in Ri T-DNA carrot root. Mycol Res 104:716–721

Gaur A, Adholeya A, Mukerji KG (2000) On-farm production of VAM inoculum and vegetable crops in marginal soil amended with organic matter. Trop Agric 77:21–26

Gemma JN, Koske RE, Carreiro M (1989) Seasonal dynamics of selected species of V-A mycorrhizal fungi in a sand dune. Mycol Res 92:317–321

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) In vitro enhancement of spore germination and early hyphal growth of vesicular arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243–255

Hamel C (1996) Prospects and problems pertaining to the management of arbuscular mycorrhizae in agriculture. Agric Ecosyst Environ 60:197–210

Hayman DS, Stovold GE (1979) Spore populations and infectivity of vesicular-arbuscular mycorrhizae fungi in New South Wales. Aust J Bot 27:227–234

Hilderbrandt U, Janetta K, Bothe H (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919–1924

Hodge A (2000) Microbial ecology of arbuscular mycorrhiza. FEMS Microbiol Ecol 32:91–96

Ianson DC, Linderman RG (1993) Variation in the response of nodulating pigeonpea (*Cajanus cajan*) to different isolates of mycorrhizal fungi. Symbiosis 15:105–119

Ikram A, Mahmud AW, Chong K, Faizah AW (1992) Growth-responses of *Calopogonium-Caeruleum* to dual inoculation with vesicular-arbuscular mycorrhizal fungi and bradyrhizobia. Field Crop Res 31:131–144

Isobe K, Tsuboki Y (1998) The relationship between growth promotion by arbuscular mycorrhizal fungi and root morphology and phosphorus absorption in gramineous and leguminous crops. Jpn J Crop Sci 67:347–352

Jacquot EV, Tuinen D, Gianinazzi S, Gianinazzi-Pearson V (2000) Monitoring species of arbuscular mycorrhizal fungi in plant and in soil by nested PCR: application to the study of the impact of sewage sludge. Plant Soil 226:179–188

Jakobsen I (1986) Vesicular-arbuscular mycorrhiza in field-grown crops. III. Mycorrhizal infection and rates of phosphorus inflow in pea plants. New Phytol 104:573–581

Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225–234

Jasper DA (1994) Bioremediation of agricultural and forestry soils with symbiotic microorganisms. Aust J Soil Res 32:1301–1319

Jeffries P, Spyropoulos T, Vardavarkis E (1988) Vesicular-arbuscular mycorrhizal status of various crops in different agricultural soils of northern Greece. Biol Fert Soil 5:333–337

Jolicoeur M, Williams RD, Chavarie C, Jortin JA, Archambault J (1999) Production of *Glomus intraradices* propagules, an arbuscular mycorrhizal fungus, in a airlift bioreactor. Biotechnol Bioeng 63:224–232

Kaya C, Higgs D, Kirnak H, Tas I (2003) Mycorrhizal colonisation improves fruit yield and water use efficiency in watermelon (*Citrullus lanatus* Thunb.) grown under well-watered and water-stressed conditions. Plant Soil 253:287–292

Kloepper JM, Schroth MN (1978) Plant growth promoting rhizobacteria on radishes. In: Gibert-Clarey T (ed) Proc 4th Int Conf Plant Pathogenic Bacteria, Station de Pathologie Végétale et Phytobactériologie, Angers, France, pp 879–882

Koch M, Tanami Z, Bodani H, Wininger S, Kapulnik Y (1997) Field application of vesicular-arbuscular mycorrhizal fungi improved garlic yield in disinfected soil. Mycorrhiza 7:47–50

Koske RE (1987) Distribution of VA mycorrhizal fungi along a latitudinal temperature gradient. Mycologia 79:55–68

Linderman RG (1986) Managing rhizosphere microorganisms in the production of horticultural crops. Hortic Sci 21:1299–1302

Linderman RG (1992) Vesicular-arbuscular mycorrhizae and soil microbial interactions. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in sustainable agriculture. ASM, CSSA, SSSA, Madison, WI, pp 45–70

Mårtensson AM, Carlgren K (1994) Impact of phosphorus fertilization on VAM diaspores in two Swedish long-term field experiments. Agric Ecosyst Environ 47:327–334

Matsubara Y, Harada T, Yakuwa T (1994) Effect of vesicular-arbuscular mycorrhizal fungi inoculation on seedling growth in several species of vegetable crops. J Jpn Soc Hortic Sci 63:619–628

Matsubara Y, Kayukawa Y, Yano M, Fukui H (2000) Tolerance of asparagus seedlings infected with arbuscular mycorrhizal fungus to violet root rot caused by *Helicobasidium mompa*. J Jpn Soc Hortic Sci 69:552–556

McGonigle TP, Fitter AH (1990) Ecological specificity of vesicular-arbuscular mycorrhizal associations. Mycol Res 94:120–122

Medina A, Probanza A, Gutierrez Manero, Azcon R (2003) Interactions of arbuscular mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). Appl Soil Ecol 22:15–28

Mendoza J, Borie F (1998) Effect of *Glomus etunicatum* inoculation on aluminum, phosphorus, calcium, and magnesium uptake of two barley genotypes with different aluminum tolerance. Comm Soil Sci Plant Anal 29:681–695

Merryweather J, Fetter AH (1998) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta*. I Diversity of fungal taxa. New Phytol 138:117–12

Millner PD, Kitt DG (1992) The Beltsville method for soilless production of vesicular-arbuscular mycorrhizal fungi. Mycorrhiza 2:9–15

Morton J, Bentivenga SP, Beever JD (1995) Discovery, measurement, and interpretation of diversity in arbuscular mycorrhizal fungi (Glomales, Zygomycetes). Can J Bot 73:S25-S32

Mosse B (1975) Specificity in VA mycorrhizas. In: Sanders FE, Mosse B, Tinker PB (eds) Endomycorrhizas. Academic Press, London, pp 469–484

Mosse B, Hepper CM (1975) Vesicular arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Perron F, Légère A, Tremblay G, Simard RR, Angers D, Hamel C (2001) Crop and weed response to nutrient source, tillage and weed control method in a corn-soybean rotation. Can J Plant Sci 81:561–571

Plenchette C, Fortin JA, FurlanV (1983a) Growth responses of several plant species to mycorrhizae in a soil of low fertility. I. Mycorrhizal dependency under field conditions. Plant Soil 70:199–209

Plenchette C, Furlan V, Fortin JA (1983b) Responses of endomycorrhizal plants grown in calcined montmorillonite clay to different levels of phosphorus. I. Effect on growth and mycorrhizal development. Can J Bot 61:1377–1383

Plenchette C, Declerck S, Diop TA, Strullu DG (1996) Infectivity of monoaxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri-T DNA transformed carrot root. Appl Microb Biotechnol 46:545–548

Probanza A, Mateos JL, Lucas JA, Ramos B, de Felipe MR, Gutierrez manero FJ (2001) Effects of inoculation with PGPR *bacillus* and *Pisolithus tinctorious* on *Pinus pinea* L. growth, bacteria rhizosphere colonization and mycorrhizal infection. Microb Ecol 41:140–148

Redecker D, Thierfelder H, Werner D (1995) A new cultivation system for arbuscular mycorrhizal fungi on glass beads. J Appl Bot Ang Bot 69:183–188

Richter BS, Stutz JC (2002) Mycorrhizal inoculation of big sacaton: implications for grassland restoration of abandoned agricultural fields. Restor Ecol 10:607–616

Rosendahl CN, Rosendahl S (1991) Influence of VAM fungi on the response of cucumber to salt stress. Environ Exp Bot 31:313–318

Ryan MH, Angus JF (2003) Arbuscular mycorrhizae in wheat and field pea crops on a low P soil: increased Zn-uptake but no increase in P-uptake or yield. Plant Soil 250:225–239

Scagel CF (2004) Soil pasteurization and inoculation with *Glomus intraradices* alters flower production and bulb composition of *Zephyranthes* spp. J Hortic Sci Biotech 78:798–812

Sharma MP, Gaur A, Bhatia NP, Adholeya A (1996) Growth responses and dependence of *Acacia nilotica* var. *cupriciformis* on the indigenous arbuscular mycorrhizal consortium of a marginal wasteland soil. Mycorrhiza 6:441–446

Shrestha YH, Ishii T, Matsumoto I, Kadoya K (1996) Effects of vesicular-arbuscular mycorrhizal fungi on satsuma mandarin tree growth and water stress tolerance and on fruit development and quality. J Jpn Soc Hortic Sci 64:801–807

Sieverding E (1991) Vesicular-arbuscular mycorrhiza management in tropical agrosystems. GTZ Technical Cooperation, Echborn, Germany

Soderberg KH, Olsson PA, Baath E (2002) Structure and activity of the bacterial community in the rhizosphere of different plant species and the effect of arbuscular mycorrhizal colonization. FEMS Microbiol Ecol 40:223–231

Strullu DG, Plenchette C (1991) The entrapment of *Glomus* sp in alginate beads and their use as root inoculum. Mycol Res 95:1194–1196

Strullu GD, Romand C (1986) Méthodes d'obtention d'endomycorrhizes à vésicules et arbuscules en conditions axéniques. C R Acad Sci Paris 303:245–250

Strullu GD, Romand C (1987) Culture axénique de vésicules isolées à partir d'endomycorrhizes et réassociation in vitro à des racines de tomate. C R Acad Sci Paris 305:15–19

Strullu DG, Diop TA, Plenchette C (1997) Réalisation de collections in vitro de *Glomus intraradices* (Schenck et Smith) et de *Glomus versiforme* (Karsten et Berch) et proposition d'un cycle développement. C R Acad Sci Paris 320:41–47

Sukarno N, Smith FA, Smith SE, Scott ES (1996) The effect of fungicides on vesicular-arbuscular mycorrhizal symbiosis II. The effects on area of interface and efficiency of P uptake and transfer to plant. New Phytol 25:139–147

Taylor J, Harrier L (2000) A comparison of nine species of arbuscular mycorrhizal fungi on the development and nutrition of micropropagated *Rubus idaeus* L. Cv. Glen Prosen (red raspberry). Plant Soil 225:53–61

Tepfer DA (1989) Ri T-DNA from *Agrobacterium rhizogenes*: a source of genes having applications in rhizosphere biology and plant development, ecology and evolution. In: Kousugg T, Nester EW (eds) Plant microbe interactions. Molecular and genetic perspectives. Mc Graw-Hill, New York, pp 294–342

Thompson JP (1994) Inoculation with vesicular-arbuscular mycorrhizal fungi from cropped soil overcomes long-fallow disorder of linseed *(Linum-Usitatissimum L)* by improving P and Zn uptake. Soil Biol Biochem 26:1133–1143

Tiwari P, Adholeya A (2002) In vitro co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. FEMS Microbiol Lett 206:39–43

Tiwari P, Adholeya A (2003) Host dependent differential spread of *Glomus intraradices* on various Ri T- DNA transformed roots in vitro.Mycol Prog 2:171–177

Tiwari P, Prakash A, Adholeya A (2002) Commercialization of arbuscular mycorrhizal fungi. In: Arora (ed) Handbook of fungal biotechnology. Marcel Dekker, New York

Toro M, Azcón R, Barea JM (1997) Improvement of arbuscular mycorrhizal development by inoculation with phosphate-solubilizing rhizobacteria to improve rock phosphate bio-availability (^{32}P) and nutrient cycling. Appl Environ Microbiol 63:4408–4412

Toro M, Azcón R, Barea JM (1998) The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotypes, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*. New Phytol 138:265–273

Utkhede RS, Li TSC, Smith EM (1992) The effect of *Glomus mosseae* and *Enterobacter-aerogenes* on apple seedlings grown in apple replant disease soil. J Phytopathol 135:281–288

Verma A, Adholeya A (1996) Cost-economics of existing methodologies for inoculum production of vesicular-arbuscular mycorrhizal fungi. In: Mukerji KG (ed) Concepts in mycorrhizal research. Kluwer, Dordrecht, pp 179–194

Werner MR, Kluson RA, Gliessman SR (1991) Colonization of strawberry roots by Va mycorrhizal fungi in agroecosystems under conventional and transitional organic management. Biol Agric Hort 7:139–151

Xavier LJC, Germida JJ (2002) Response of lentil under controlled conditions to co-inoculation with arbuscular mycorrhizal fungi and rhizobia varying in efficacy. Soil Biol Biochem 34:181–188

Young JL, Davis EA, Rose SL (1985) Endomycorrhizal fungi in breeder wheats and Triticale cultivars field-grown on fertile soil. Agron J 77:219–224

Part VII
Methodology

18 Methodologies for in Vitro Cultivation of Arbuscular Mycorrhizal Fungi with Root Organs

Sylvie Cranenbrouck[1], Liesbeth Voets[1], Céline Bivort[1], Laurent Renard[1], Désiré-Georges Strullu[2], Stéphane Declerck[3]

1 Introduction

The monoxenic culture of arbuscular mycorrhizal (AM) fungi has markedly improved our understanding of the symbiosis. In the past 15 years, increasing amounts of literature have been devoted to this intimate plant–fungal association using various AM fungi in vitro cultivation systems, with different hosts, AM fungal propagules and growth media. The proportion of papers published using either in vitro, axenic, monoxenic, root organ culture or ROC as keywords, relative to the overall literature dealing with AM fungi (ISI web of Science http://www.isinet.com)[4], was less than 1% in the years 1987–1989, increasing to approximately 5% in the subsequent 6 years (1990–1995), to reach a plateau at 8% from 1996 to present. The invariable proportion between papers using AM fungi in vitro systems and complete literature on AM fungi since 1996 until today suggests that the use of this system still remains in the hands of a limited number of researchers, and that new progress is necessary to reach a broader audience. If one agrees that the axenic culture of AM fungi remains the major challenge at the start of this new millennium, the present diffusion of clear protocols on AM fungi in vitro culture techniques may render this technology more widely accessible and secure its broad and reliable dissemination. Indeed, as stated by Bago and Cano (see Chap. 7) . . . "*AM (fungi) monoxenics are far*

[1]Université Catholique de Louvain, Unité de Microbiologie, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

[2]Laboratoire de Phytonique, Université d'Angers, 2 Bd. Lavoisier, 49045 Angers cedex, France

[3]Mycothèque de l'Université Catholique de Louvain (MUCL), Unité de Microbiologie, Université Catholique de Louvain, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium, Tel.: +32-10-474644, Fax: +32-10-451501, E-mail: declerck@mbla.ucl.ac.be

[4]Keywords used for the overall literature on endomycorrhizal fungi: 'AM fungi' OR 'AM symbiosis' OR 'VAM fungi' OR 'VAM symbiosis' OR 'endomycorrhiz*' OR 'vesicular-arbusc*' OR 'vesicular arbuscular' OR 'arbuscular' OR (*Glomus* AND mycorrhiz*). Keywords used for literature related to the endomycorrhizal fungi monoxenically cultured: ['AM fungi' OR 'AM symbiosis' OR 'VAM fungi' OR 'VAM symbiosis' OR 'endomycorrhiz*' OR 'vesicular-arbusc*' OR 'vesicular arbuscular' OR arbuscular OR (*Glomus* AND mycorrhiz*)] AND ('in vitro' OR 'monoxenic' OR 'axenic' OR 'root organ culture' OR 'transformed roots')

Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

more than just a routine technique . . . strict protocols should be followed to successfully achieve it and . . . some training/expertise on AM establishment, fungal colony development and hyphal morphogenesis under such conditions is mandatory for researchers aiming to use this technique, to be able to certify the quality of the material obtained and, consequently, the reliability and accurateness of the results obtained." This chapter is not aimed to propose a literature overview but rather to provide detailed protocols to succeed in the monoxenic culture of AM fungi. It is obvious that for every technique different protocols (disinfection process, growth medium, choice of propagule, etc.) have been published, and we cannot discuss all of these in detail. Therefore, we choose to describe the routine techniques used in GINCO (*G*lomeromycota IN Vitro COllection) with, at each step, references to other papers using other approaches, so giving the reader the broadest perception of the techniques that presently exist and which are the most appropriate for their research interest.

2 Process Description

The process of obtaining and maintaining monoxenic cultures of AM fungi can be separated into four main steps. These are the selection of the adequate AM fungal propagules (see Sect. 6.1), the sampling, disinfection and incubation of the propagules on a suitable growth medium (see Sect. 6.2), the association of the propagules with a suitable host root (see Sect. 7), and the subcultivation of the AM fungi (see Sect. 8). Prior to these four steps are the selection of the appropriate culture system (see Sect. 3), the preparation of the synthetic culture media (see Sect. 4), and the management of the host root, i.e. transformation and subcultivation (see Sect. 5; Fig. 1).

3 Selection of the Culture System

Basically, two culture systems are used: the mono-compartmental system in square or round Petri plates, and the bi-compartmental system in round Petri plates.

The first system consists of a mono-compartmental Petri plate filled with a growth medium (see Sect. 4), on which is placed a contaminant-free, actively growing excised root together with AM fungal propagules. This system was developed in the mid-1970s (Mosse and Hepper 1975) and since then has been applied with success to numerous *Glomus* species (Table 1). Recent reports have also demonstrated its applications to *Scutellospora*

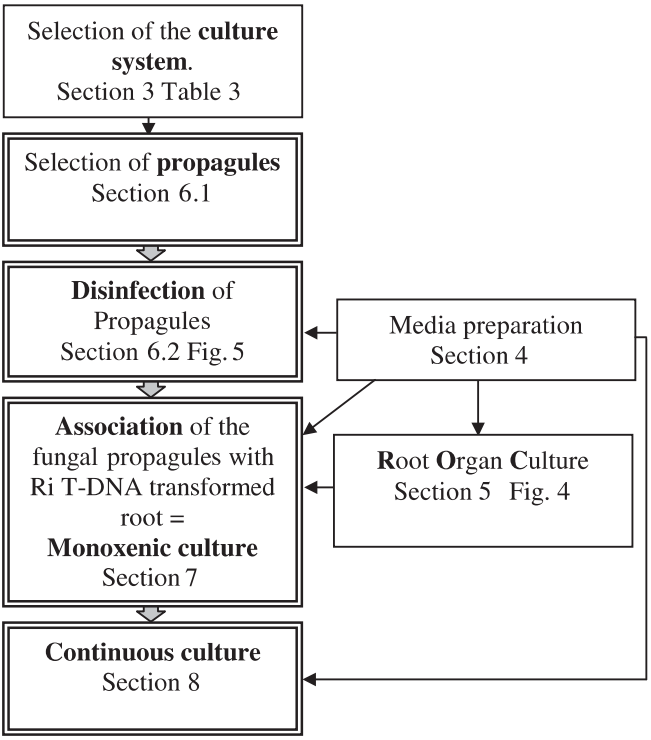


Fig. 1. General scheme of the monoxenic culture process

reticulata (de Souza and Declerck 2003) and *Acaulospora rehmii* (Dalpé and Declerck 2002).

This system was slightly modified for the cultivation of *Gigaspora* species (*Gi. margarita*, *Gi. roseae*, *Gi. gigantea*; Table 1) by placing a Ri T-DNA transformed carrot root – having negative geotropism – in the upper part of a square Petri plate set vertically, with the germinated spore just below (Fig. 7F; Bécard and Fortin 1988; Bécard and Piché 1992; Diop et al. 1992). Since the germ tube growth of these *Gigaspora* species is also negatively geotropic, the germinating hyphae are oriented towards the root, thus facilitating contact and colonization (see Sect. 7). The root growth is restricted to the upper part of the plate while after colonization the mycelium develops in the lower part. It was observed that the sporulation mainly occurs in the section with less root development.

The second system (also named split system) consists of a bi-compartmental Petri plate, with a proximal compartment in which the mycorrhizal root develops and containing a synthetic growth medium (see Sect. 4), and a distal compartment in which only the mycelium is allowed to grow

Table 1. General overview of the AM fungi cultured in the monoxenic system with the different options available for the medium, culture system, propagule type, etc. The mentioned citations refer to the first and most descriptive system with the different options mentioned (*continued on next page*)

Species	Starter propagules	Host root	Medium	Culturing model	Production of mature spores (size, µm)[a]	Continuous culture[b]	Citations
AMF species not producing vesicles							
Gigaspora gigantea (Nicolson & Gerd.) Gerd. & Trappe	Spores	T. *Daucus carota* L.[1]	M	Mono (incubated with angle)	Yes	No	Gadkar et al. (1997)
	Spores	T. *Daucus carota* L.	G+T (see citation)	Mono	Yes	No	Mosse (1988)
Gigaspora margarita Becker & Hall	Spores	NT. *Lycopersicon esculentum* Mill.[2]	MS	Mono	Yes	No	Miller-Wideman and Watrud (1984)
	Spores	T. *Daucus carota* L.	M	Mono (incubated with angle)	Yes	No	Gadkar et al. (1997)
	Spores	T. *Daucus carota* L. NT. *Linum usitatissimim* L.[3] NT. *Tagetes pastula* L.[4] NT *Althaea officinalis* L.[5]	M	Mono	Yes	No	Karandashov et al. (1999)
Gigaspora roseae Nicolson & Schenck	Spores	T. *Daucus carota* L.	M	Mono (incubated vertically; square Petri plates)	Yes (mean= 300 µm)	No	Bécard and Fortin (1988), Bécard and Piché (1992), Diop et al. (1992)
Scutellospora reticulata (Koske, Miller, Walker) Walker & Sanders	Spores	T. *Daucus carota* L.	MSR	Mono	Yes (280–500, mean=376)	Yes	de Souza and Declerck (2003)
AMF species producing vesicles							
Acaulospora rehmii (Sieverding & Toro)	Spores	T. *Daucus carota* L.	MSR	Mono	Yes (112–162)	No	Dalpé and Declerck (2002)
Glomus caledonium (Nicoson & Gerd.) Trappe & Gerd.	Spores	T. *Daucus carota* L.	M (modified)	Mono	Yes (180–300)	Yes	Karandashov et al. (2000)
Glomus cerebriforme McGee	MRF	T. *Daucus carota* L.	M	Bi	Yes	Not mentioned	Samson et al. (2000)

Glomus clarum Nicolson & Schenck	Spores	T. *Daucus carota* L. T. *Trifolium repens* L.[6]	M	Mono	Yes (100–180, mean=130)	Yes	de Souza and Berbara (1999)
Glomus constrictum Trappe	Spores	Plantlets of *Ziziphus mauritania*[7]	MS modified	Other	Not mentioned	No	Mathur and Vyas (1999)
Glomus deserticola Trappe, Bloss & Meng	MRF+spores	Plantlets of *Ziziphus mauritania*	MS modified	Other	Not mentioned		Mathur and Vyas (1995)
Glomus etunicatum Becker & Gerd	Spores	T. *Daucus carota* L.	M	Mono	Yes (40–130)	Yes	Pawlowska et al. (1999)
Glomus fasciculatum (Thaxter sensu Gerd.) Gerd. & Trappe emend. Walker & Koske	MRF	NT. *Fragaria x Ananassa* Duchesne.[8] NT. *Alium cepa* L.[9] NT. *Solanum lycopersicum* Mill[10]	SR	Mono	Yes	Yes	Strullu and Romand (1986)
	MRF	T. *Daucus carota* L.	MSR	Mono	(60–90)	Yes	Declerck et al. (1998)
Glomus fistulosum Skou & Jakobson	Spores	T. *Fragaria x Ananassa* Duchesne	M	Mono	No	No	Nuutila et al. (1995)
Glomus intraradices Schenck & Smith	Isolated vesicles from MRF	NT. *Solanum lycopersicum* L.	SR	Mono	Yes	Yes	Strullu and Romand (1987)
	Spores	T. *Daucus carota* L. NT. *Solanum lycopersicum* L.	M	Mono	Yes (85)	Yes	Chabot et al. (1992)
	MRF	NT. *Solanum lycopersicum* L.	M	Mono	Yes (65–80, mean=80)	Yes	Diop et al. (1994a, 1994b)
	Spores	T. *Daucus carota* L.	M	Bi	Yes	Yes	St-Arnaud et al. (1996)
	Spores+MRF	T. *Daucus carota* L.	M	Mono	Yes	Not mentioned	Karandashov et al. (1999)
	MRF	T. *Daucus carota* L.	MSR	Mono	Yes (80–100)	Yes	Declerck et al. (1998)
	Spores	T. *Medicago truncatula* Gaertn.[11]	M	Mono	Yes	Not mentioned	Boisson-Denier et al. (2001)
	MRF	T. *Daucus carota* L.	M	Bi	Yes	Yes[c]	Douds (2002)

Table 1. (*continued*)

Species	Starter propagules	Host root	Medium	Culturing model	Production of mature spores (size, µm)[a]	Continuous culture[b]	Citations
Glomus mosseae Nicolson & Gerdemann	Spores isolated from sporocarps	NT. *Lycopersicum esculentum* Mill. NT. *Trifolium pratense* L[12]	Modified White's medium	Mono	No	No	Mosse and Hepper (1975)
	Spores	T. *Daucus carota* L. T. *Convolvulus sepium* L.[13] T. *Solanum tuberosum* L.[14] T. *Vigna unguiculata* Walp.[15]	(Mugnier and Mosse 1987)	Bi (modified; see citation)	No	No	Mugnier and Mosse (1987)
	Spores+MRF	T. *Daucus carota* L.	M	Mono	No	No	Karandashov et al. (1999)
	Spores	T. *Daucus carota* L.	M	Mono	No	No	Douds (1997)
	Spores	*Saccharum officinarum*[16] *Sorghum vulgare*[17]	M	Mono	No	No	Raman et al. (2001)
Glomus macrocarpum Tulasne & Tulasne	MRF	T. *Daucus carota* L.	MSR	Mono	Yes (160–180)	Yes	Declerck et al. (1998)
Glomus proliferum Dalpé & Declerck	MRF	T. *Daucus carota* L.	MSR	Mono Bi	Yes (22, 40–66) (46)	Yes	Declerck et al. (2000)
Glomus versiforme (Karsten) Berch	MRF	NT *Solanum lycipersicum* L.	M	Mono	Yes (mean=65)	Yes	Diop et al. (1994a)
	MRF	T. *Daucus carota* L.	MSR	Mono	Yes (60–80)	Yes	Declerck et al. (1998)

MRF = Mycorrhizal root fragment. M = Minimal medium (Bécard and Fortin, 1988). MSR = Modified Strullu-Romand medium (Declerck et al. 1996). SR = Strullu-Romand medium (Strullu and Romand 1986). G+T = Modification of the Goford and Torrey medium (Mosse 1988). Mono = Mono-compartmental system. Bi = Bi-compartmental system. Other = Modified *in vitro* culture system in flask with plantlets as host. T = Ri T-DNA transformed root with *A. rhizogenes*. NT = Non-transformed excised root.
1 = Carrot, 2 = Tomato, 3 = Flax, 4 = Marigold, 5 = Marshmallow, 6 = Clover, 7 = Indian jujube, 8 = Strawberry, 9 = Onion, 10 = Tomato, 11 = Barrelclover, 12 = Red clover, 13 = Bindweed, 14 = Potato, 15 = Cowpea, 16 = Sugar cane, 17 = Sorghum.

[a] If mentioned in the publication; [b] Yes (No) = With (Without) production of mature spores after life cycle establishment; [c] = Which consist by extracting the distal compartment and refill it with fresh medium

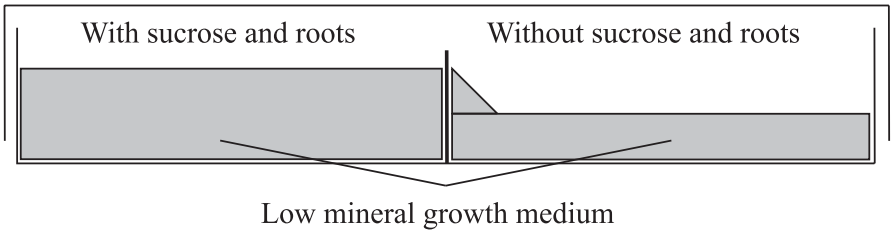


Fig. 2. Diagram of the bi-compartmental system from St-Arnaud et al. (1996). The mycorrhizal root grows in the proximal compartment and only the fungus is allowed to cross the separation wall and to develop in the distal compartment

on a similar synthetic medium, but lacking C source. Both compartments are physically separated by a plastic wall. Roots crossing the partition are trimmed at regular intervals. This system was developed by St-Arnaud et al. (1996; Fig. 2).

In the bi-compartmental system, the spore and mycelium density produced in the distal compartment is markedly higher in comparison to the proximal compartment, which is probably related to the absence of the root and the difference in availability of C (Fortin et al. 2002), making this system more productive than the mono-compartmental system. It is particularly adapted to the culture of *Glomus* species (Table 1). Recently, Douds (2002) has increased the spore production of a *G. intraradices* strain by repeated harvesting and gel replacement of the distal compartment.

Practical recommendations before starting a monoxenic culture:

Most of the steps described below are conducted under a laminar flow, under sterile conditions. A biohazard flow hood can also be suitable, but specific arrangements are necessary when a stereo microscope is used. The bench should be cleaned with ethanol. Similarly, all the material (needles, scalpels, forceps, etc.) necessary for monoxenic cultivation should be sterile or sterilized by flaming or in a bead sterilizer. Other materials (glassware, membranes, etc.) must be autoclaved.

4
Culture Media Preparation

The most widely used mineral media for monoxenic cultivation of AM fungi are the Minimal (M) medium (Bécard and Fortin 1988) and the Modified Strullu-Romand (MSR) medium (Declerck et al. 1996, from Diop 1995, modified from Strullu and Romand 1986). Both media result from the em-

Table 2. Comparison of the composition of minimal (M) medium and modified Strullu-Romand (MSR) medium (Fortin et al. 2002)

	M medium	MSR medium
$N(NO_3^-)$, µM	3,200	3,800
$N(NH_4^+)$, µM	–	180
P (µM)	30	30
K (µM)	1,735	1,650
Ca (µM)	1,200	1,520
Mg (µM)	3,000	3,000
S (µM)	3,000	3,013
Cl (µM)	870	870
Na (µM)	20	20
Fe (µM)	20	20
Mn (µM)	30	11
Zn (µM)	9	1
B (µM)	24	30
I (µM)	4.5	–
Mo (µM)	0.01	0.22
Cu (µM)	0.96	0.96
Ca panthotenate (µM)	–	1.88
Biotin (µM)	–	0.004
Pyridoxine (µM)	0.49	4.38
Thiamine (µM)	0.3	2.96
Cyanocobalamine (µM)	–	0.29
Nicotinic acid (µM)	4	8.10
Glycine (mg/l)	3	–
Myo-inositol (mg/l)	50	–
Sucrose (g/l)	10	10
pH (before sterilization)	5.5	5.5
Gelling agent (g/l)	5	3

piric modification of media usually used for in vitro plant culture and are equally successful for a range of AM fungi. The composition of M and MSR media is listed in Table 2. Differences between both media are discussed in Fortin et al. (2002). Similarly, a complete comparison of the successive media developed prior to the M and MSR media is given in Bécard and Piché (1992).

4.1 Material

Equipment

- Laminar flow hood
- Autoclave
- Balance (0.001 g)
- Magnetic stirrer
- pH-meter
- Glass bottles (50, 100, 500, 1000 ml)

Laboratory material

- Erlenmeyer flask
- Volumetric flask (50, 100, 500, 1000 ml)
- Petri plates
- Spoon
- Glass bottles (50, 100, 500, 1000 ml)

4.2 Stock Solutions for MSR Medium

The MSR medium routinely used in GINCO is detailed below. For the M medium preparation, refer to Bécard and Fortin (1988).

1. Solution 1: macroelements
   To prepare 1 l of solution, dissolve 73.9 g $MgSO_4 \cdot 7H_2O$, 7.6 g KNO_3, 6.5 g KCl and 0.41 g KH_2PO_4 in 500 ml of demineralized water. Adjust the volume to 1 l and store at 4 °C.
2. Solution 2: calcium nitrate
   To prepare 1 l of solution, dissolve 35.9 g $Ca(NO_3)_2 \cdot 4H_2O$ in 500 ml of demineralized water. Adjust the volume to 1 l and store at 4 °C.
3. Solution 3: vitamins
   To prepare 500 ml of solution, dissolve 0.09 g calcium panthotenate, 0.0001 g biotin, 0.1 g nicotinic acid, 0.09 g pyridoxine, 0.1 g thiamine and 0.04 g cyanocobalamine in 200 ml of demineralized water. Adjust the volume to 500 ml. Dispense in 5-ml fractions and store at −20 °C.
4. Solution 4: NaFeEDTA
   To prepare 500 ml of solution, dissolve 0.16 g NaFeEDTA in 200 ml of demineralized water. Adjust the volume to 500 ml, preserve from light and store at 4 °C.

5. Solution 5: microelements
   To prepare 500 ml of solution,

   a) Dissolve 1.225 g of $MnSO_4 \cdot 4H_2O$ (or 0.93 g of $MnSO_4 \cdot H_2O$) in 50 ml demineralized water. Adjust the volume to 100 ml.

   b) Dissolve 0.14 g of $ZnSO_4 \cdot 7H_2O$ in 50 ml of demineralized water. Adjust the volume to 100 ml.

   c) Dissolve 0.925 g of H_3BO_3 in 50 ml of demineralized water. Adjust the volume to 100 ml.

   d) Dissolve 1.1 g of $CuSO_4 \cdot 5H_2O$ in 30 ml of demineralized water. Adjust the volume to 50 ml.

   e) Dissolve 0.12 g of $Na_2MoO_4 \cdot 2H_2O$ in 50 ml of demineralized water. Adjust the volume to 100 ml.

   f) Dissolve 1.7 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 50 ml of demineralized water. Adjust the volume to 100 ml.

   g) Mix the 100 ml of the manganese sulphate (a), zinc sulphate (b) and boric acid (c) solutions.

   h) Add 5 ml of copper sulphate (d) solution, 1 ml of sodium molybdate (e) and 1 ml of ammonium molybdate solution (f). Adjust the volume to 500 ml and store at 4 °C.

4.3 Medium Preparation

1. To prepare 1 l of MSR medium, mix 10 ml of solution 1 (macroelements) with 10 ml of solution 2 (calcium nitrate), 5 ml of solution 3 (vitamins), 5 ml of solution 4 (NaFeEDTA), 1 ml of solution 5 (microelements) and 10 g of sucrose.
2. After complete dissolution of the sucrose, adjust the volume to 1 l with demineralized water.
3. Adjust the pH to 5.5 with NaOH 1 M (rectify with HCl 1 M if necessary).
4. Add 3 g/l Gelgro (ICN Biochemicals, Cleveland, OH) or 5 g/l Phytagel™ or 8 g/l agar.
5. Autoclave for 15 min at 121 °C under 1 bar pressure.
6. After autoclaving, maintain the medium at 60 °C in a water bath.

Table 3. Medium quantities required in the different monoxenic systems

	Proximal or root compartment	Slope	Distal-compartment	Used preferably for
Mono-compartmental Petri plates (9-cm diameter)	Fill with ~ 35 ml MSR	No	No	*Scutellospora*, *Glomus*
Mono-compartmental Petri plates (squared 12×12 cm)	Fill with ~ 100 ml MSR	No	No	*Gigaspora*
Bi-compartmental Petri plates (9-cm diameter)	Fill the proximal compartment with ~ 25 ml MSR until the top of the separation	Create a slope of 45 °C with ~ 5 ml MSR without sugar	Fill with ~12 ml of MSR without sugar	*Glomus*

7. Pour the medium in the Petri plates under laminar flow, as indicated in Table 3.

For bi-compartmental Petri plates, it is important to promote spreading of mycelium from the proximal to the distal compartment. Two procedures can be used. In the first procedure, the proximal compartment is filled with the synthetic growth medium to the top of the separation wall. In the distal compartment, a slope (~ 45°) is realized with the same medium but lacking C. After solidification, the distal compartment is half-filled horizontally with the same medium lacking C (Fig. 2). In the second procedure, both compartments are filled 2 mm above the partition wall (Rufyikiri et al. 2003). After solidification, the medium in the distal compartment is cut along the partition wall with a scalpel and removed by using a spatula. The distal compartment is further replaced with a new medium lacking C at the level of the partition wall.

5
Host Root

5.1
Choice of Host Root

Various plants have been used to establish monoxenic cultures with AM fungi (see Chap. 7, Table 1). The first host roots used in monoxenic cultures

were *Lycopersicon esculentum* Mill. (tomato) and *Trifolium pratense* L. (red clover) associated with *Glomus mosseae* Nicolson & Gerd. (Mosse and Hepper 1975), and *Fragaria x ananassa* Duchesne. (strawberry) and *Alium cepa* L. (onion) associated with several *Glomus* species (Strullu and Romand 1986; Table 1).

A natural genetic transformation of roots with the soil bacterium *Agrobacterium rhizogenes* Conn. was achieved decades ago (Riker et al. 1930; Ark and Thompson 1961), but only applied in mycorrhizal research since the mid-1980s (Mugnier and Mosse 1987). Since the development of the carrot hairy root line (*Daucus carota* L.), established by Bécard and Fortin (1988), this root has become the most widespread host for monoxenic cultivation of AM fungi. Transformed roots of strawberry (Nuutila et al. 1995) and tomato (Simoneau et al. 1994; Khaliq and Bagyaraj 2000) were also developed with fewer studies published. Recently, transformed roots of *Medicago truncatula* Gaern. have been obtained and associated with AM fungi (Boisson-Denier et al. 2001). It is probable that this association will receive increasing attention in the coming years, thanks to its usefulness as a legume model plant in molecular biology (Cook 1999; May and Dixon 2004). Transformed roots have several advantages over non-transformed roots for monoxenic cultivation. Their hormonal balance is modified, allowing profuse proliferation on synthetic media. It is generally accepted that this modification induces the production of growth hormones in the roots, thereby eliminating the necessity of incorporating of plant hormones into the culture medium. Stability of the transformation over time is dependent on the host cultivar and bacterial strain combination (Labour et al. 2003), but hairy roots used for further experiments have always tested positive.

In association with AM fungi, Ri T-DNA transformed roots show greater AM intraradical colonization and sustain higher extraradical hyphal development than non-transformed roots, which is an advantage for fungal production (Fortin et al. 2002). However, the pattern of colonization, distribution of vesicles, mycorrhizal spread and sporulation mechanisms – all the important growth stages of the fungus – can vary within different cultivars of the same host (Tiwari and Adholeya 2003). Furthermore, transformation of the same host cultivar with a different bacterial strain results in a different mycorrhizal susceptibility (Labour et al. 2003). More research is therefore needed to get a better insight into the physiological host preference/specificity of these fungi.

5.2 Host Root Transformation

Agrobacterium rhizogenes Conn., a gram-negative soil bacterium which induces hairy root disease of dicotyledonous plants, is used to induce hairy roots. In roots transformed with this bacterium, a segment of the bacterial DNA, named the region T (transferred DNA) of the plasmid Ri (root inducing) is incorporated into the host plant cells (Chilton et al. 1982). Integration and expression of this DNA in the plant genome lead to the development of the hairy root phenotype and synthesis of novel low molecular weight compounds called opines (Tepfer and Tempé, 1981). Depending on the strain of *A. rhizogenes* used for the transformation, different principal opines can be found in the tissues of the hairy root: agropine, mannopine, cucumopine or mikimopine (Dessaux et al. 1992). In addition, depending on the gene incorporated in the plant genome, the root can have a change of geotropism. Some hairy roots show a highly negative geotropic nature, some only have a slightly negative geotropic behaviour while others keep their positive geotropism. This is due to a change of auxin sensitivity and the redistribution of this hormone after root transformation (Legué et al. 1996). Roots with a negative geotropism should be incubated in inverted position, to make the roots grow inside the medium.

In this section, only the transformation procedure of carrot (*Daucus carota* L.) with *A. rhizogenes* A4 is described. For the transformation procedures of other plants used as hosts in monoxenic cultures, we refer to the references given in Table 1 of Chapter 7.

5.2.1 Material

Equipment

- Laminar flow hood
- Cooled heated incubator
- Bunsen burner or bead sterilizer

Laboratory material

- Forceps
- Scalpel
- Petri plates filled with 1% water agar
- Petri plates filled with modified White's (Bécard and Fortin 1988) medium supplemented with 500 mg/l carbenicillin
- Small plastic wrap roll

Disinfection solutions

- 95% (v/v) ethanol
- 1% NaOCl
- Distilled water

Bacterial strain

- Agrobaterium rhizogenes strain A_4

5.2.2 Procedure (Following Bécard and Fortin 1988)

1. Thoroughly wash the carrots in water, peel them, dip in 95% (v/v) ethanol for 10 s, and surface sterilize in 1% NaOCl for 15 min.
2. Rinse the carrots in sterile distilled water and cut transversely into 5-mm-thick slices with a sterilized scalpel.
3. Place the slices in Petri plates filled with 1% water agar and inoculate with the A_4 *A. rhizogenes* strain on the distal face of the sections. The bacterial suspension is taken from a 2-day-old culture grown on Difco Nutrient agar.
4. After 3 weeks, some transformed roots proliferate on the inoculated sections. Aseptically excise and transfer the roots to Petri plates containing modified White's medium, supplemented with 500 mg/l carbenicillin. Three successive sub-cultures are necessary to free the transformed roots of bacteria.
5. Finally, excise one root apex from the final subculture (minimum length 3 cm) and place it on fresh modified White's medium to initiate a clonal culture.
6. Seal the Petri plates with plastic wrap and incubate in an inverted position in the dark at 27 °C.
7. Maintain the excised root on MSR medium as described below.

5.3 Host Root Cultivation

5.3.1 Materials

Equipment

- Laminar flow hood

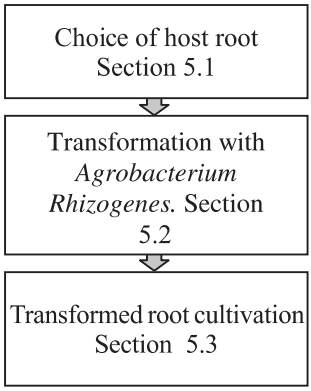


Fig. 3. General scheme of the transformed carrot root organ culture

- Cooled heated incubator
- Bunsen burner or bead sterilizer (cf. Fig. 3)

Laboratory material

- Petri plates filled with MSR medium
- Forceps
- Scalpel
- Small plastic wrap roll

5.3.2 Procedure

1. Every 3–4 weeks, carefully remove white, healthy, turgescent non-ramified apexes (with a minimum length of 3 cm) from the Petri plate.
2. With forceps, place two roots with actively growing apexes in a 9-cm-diameter Petri plate containing fresh MSR medium (the two apexes should be placed 'head to tail' to favour their growth in opposite directions, thereby covering the whole plate) (Fig. 4).
3. When the roots are placed on the medium, push lightly on the apexes to encourage them to intimately contact the gel. Proceed very carefully to avoid breaking the apex.
4. Seal the Petri plate with plastic wrap and incubate in a inverted position at 27 °C in the dark.

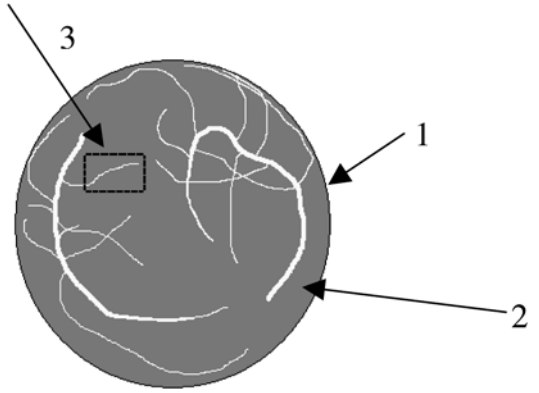


Fig. 4. Representation of a 4-week root organ culture. 1 Main root, 2 secondary root, 3 apex suitable for subcultivation

6
AM Fungal Propagules: Selection and Disinfection

6.1
Selection of Propagule

As described in Fortin et al. (2002), two types of propagule can be used to initiate monoxenic cultures: (1) spores or sporocarps, and (2) mycorrhizal root fragments containing vesicles or isolated vesicles. Depending on the AM fungi, often one type of propagule is better adapted to initiate a monoxenic culture (Table 1). For *Glomus* species producing small spores (diameter < 100 µm), disinfection of spores is often difficult without damage. The germination rate and hyphal re-growth are generally low or even absent. Species producing such spores habitually also produce a high number of vesicles within roots, i.e. *G. intraradices*, *G. proliferum*, which therefore should be considered the preferred propagules to initiate the monoxenic culture. Germination of vesicles either isolated or within the root segment is fast and efficient. In the case of mycorrhizal root segments, multiple germination arises from the root extremities (Diop et al. 1994, Declerck et al. 1996), increasing the infective potential.

For species producing few (some Glomeraceae species, *G. caledonium*, *G. mosseae*, etc.) or no vesicles (the Gigasporaceae) and/or big spores (diameter > 150 µm), spores should be used. When the fungus produces sporocarps, these are always the most appropriate propagules to initiate the monoxenic culture because of their multiple germination (Fig. 5).

Remark: When monoxenic monosporal cultures are required (in phylogeny studies, for example) from species established in monoxenic culture from mycorrhizal root fragments, it is necessary to subculture single iso-

lated daughter spores with a new host root. Only the first generation initiated with this daughter spore and subsequent generations will be adapted for such studies.

6.2 Disinfection Process

For a successful disinfection of spores and root pieces, a combination of antibiotic treatments should be applied on the extracted material (spores or root pieces). The successful combination of chloramine T and streptomycin was developed for *Glomus* species by Mosse (1962) and later a combination of chloramine T, streptomycin and gentamicin with the inclusion of the surfactant Tween 20 was reported by Mertz et al. (1979) for *Gigaspora* species. As an alternative, or as supplement to the use of antibiotics, sodium hypochlorite was used by Daniels and Menge (1981). Nowadays, sodium hypochlorite is replaced by calcium hypochlorite, since it is an instable product which degrades when remaining attached to the fungal propagules. The sodium hypochlorite solution, however, when not rinsed thoroughly, stays around the disinfected spores or root pieces where it can have a toxic effect. Besides this chemical treatment, Strullu and Romand (1986) adjusted a physical treatment, i.e. a treatment with sonications. With this treatment, even the microparticles which remain attached to the root pieces and spores are removed, assuring a thorough cleaning of the material.

6.2.1 General Considerations

Prepare all the material under laminar flow. The vacuum outlet and filtration system (Fig. 6F) should be assembled, the nylon membrane placed on the filter support, and the apparatus autoclaved at 121 °C for 15 min (1 bar pressure).

The disinfection occurs in two main steps (Fig. 5): a chloramine T treatment (step 1) and an antibiotic treatment (step 2). Prior to the disinfection process and depending on the propagule, i.e. spore or root fragment, and on the complexity of its structures, e.g. ornamented spores which influence the level of contamination, it is necessary to resort to pre-treatment steps (Fig. 5) or to adapt the duration of contact with each solution.

Some species like *Gi. margarita* and *Gi. gigantea* require a cold treatment prior to disinfection. In this case, the spores are placed at 4 °C (3 weeks) prior to isolation and disinfection (Fortin et al. 2002).

Species type	Inoculum	The desinfection occurs in	Pretreatment 1	Pretreatment 2	Step1 Chloramine T treatment	Step2 Antibiotics treatments
Species producing large spores eg. Gigasporaceae – Glomaceae eg. *Glomus caledonium, mosseae*, etc.	Spore	Vacuum filtration apparatus	None	None	10 min (2-10min)[1]	10 min (2-10 min)[1]
Species producing sporocarps : Glomaceae : *Glomus mosseae*	Sporocarp	Vacuum filtration apparatus	Ultrasonic 1 min	None	10 min (2-10min)[1]	10 min (2-10min)[1]
Species with high root colonization and Vesicles production : Acaulosporacea-Glomaceae eg. Gl*omus intraradices, fasciculatum, proliferum*, etc.	Root fragments	(Ultrasonic bath (half of the time))[2]	(Ethanol 98 % 10s)[2]	Ca-hypochlorite 6% 2 min	10 min (2-10min)[1]	10 min (2-10min)[1]

Fig. 5. Summary of the disinfection procedure. *1* Exposure time must be adapted to the contamination level of the starting material and the sensibility of the structures. *2* These steps can be suppressed if the root is not too contaminated and fragile

6.2.2 Material

Equipment

– Laminar flow hood

- Vacuum filtration apparatus (Whatman®)
- Sonicator
- Stereo microscope
- Cooled heated incubator
- Bunsen burner or bead sterilizer

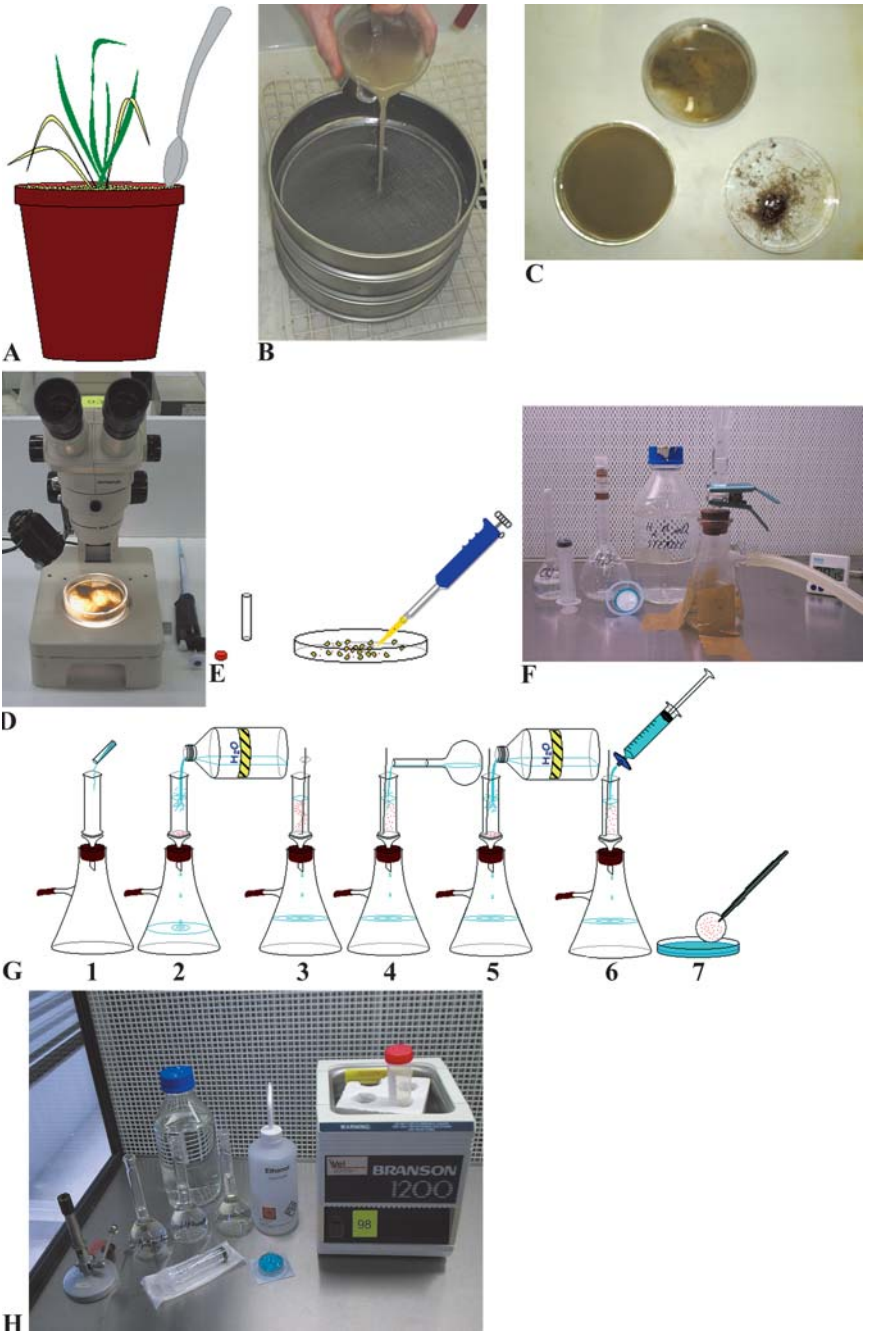
Laboratory material

- Sterile Petri plates (90 mm diameter)
- Petri plate filled with MSR medium
- 20-ml sterile syringe
- 0.2-µm sterile filter (Acrodisc GelmanSciences)
- Sterile nylon membrane with 0.45-µm pores, 25-mm diameter (adapted to the vacuum apparatus; GelmanSciences)
- Sterile Pasteur pipette
- Spoon
- Forceps
- Scalpel
- Needles
- Adjustable volume pipette and sterile tips
- Small plastic wrap roll
- 50-ml sterile centrifuge tube (falcon type) or sterilized small beaker
- 50-ml floating (polystyrene) support for centrifuge tube (or small beaker)
- Ground clay (Terragreen®)

6.2.3 Solutions for Disinfection

For about 100 spores, 30 sporocarps or 10 root fragments:

1. Sterile water 1 l.


A
B
C
D
E
F
G
1
2
3
4
5
6
7
H
BRANSON
1200

◀ **Fig. 6A–H.** Illustration of the disinfecting process. **A–G** refer to spores/sporocarps (see Sect. 6.2.4), and **H** refers to root fragments (see Sect. 6.2.5). **A** Sampling of spores from a pot culture. **B** Sieving of soil sample. **C** Soil fractions after sieving. **D**, **E** Selection of spores for the disinfection process. **F** Illustration of the disinfection material (spores/sporocarps). **G** Scheme of the disinfecting process for spores (sporocarps). 1 Transfer of the propagules to the filtration apparatus, 2–5 rinsing with water, 3 agitation, 4 chloramine T treatment (step 1), 6 antibiotic treatment (step 2), 7 transfer to antibiotics solution. **H** Illustration of the disinfection material (root fragments)

2. Ethanol 95–98% (100 ml) for pre-treatment 1 and disinfection of the material.
3. Calcium hypochlorite solution 2% (= 20 g/l, 100 ml) for pre-treatment 2.
   – *Remark*: When the calcium hypochlorite is not totally dissolved, the solution must be filtered through a Whatman® filter paper no. 1.
4. Chloramine T 2% solution (= 20 g/l) with two drops of Tween 20 (should be added before use; 100 ml; solution for step 1).
5. Antibiotics solution: Streptomycin sulphate 0.02% (20 mg/100 ml) and gentamicin sulphate 0.01% (10 mg/100 ml; 100 ml; solution for step 2).
   – *Remark*: This must be filtered with a 0.2-µm acrodisc filter (GelmanSciences) before use.

6.2.4
Spores, Sporocarps

Sampling of Inoculum.

Spores or sporocarps can be collected from a field soil sample or from a pot culture.

1. Sample approximately 100 ml of soil (growth substrate) with a spoon from a pot culture and transfer to a plastic container or a small beaker. The soil sample should be homogeneous, thus recovered from the complete volume of the pot culture. Refill the hole with fresh growth substrate (Terragreen®; Fig. 6A).
2. Transfer the soil to a 1-l beaker and add 500 ml of demineralized water. Mix gently and before complete settling transfer the supernatant to the top of a sequence of sieves with cutoffs 500, 106 and 38 µm (Fig. 6B). This combination is suitable to collect AM fungal spores; the upper fraction will retain soil and root debris but also mycorrhizal roots, while both other fractions will retain spores belonging to all the genera.

3. Transfer each fraction to a glass Petri plate with a small amount of water (Fig. 6C). Pick up the spores (∼ 100) or sporocarps (∼ 30) with an automatic pipette under a binocular microscope (Fig. 6D). Wash the spores several times in water until complete elimination of fine debris is achieved. The debris-free spores (or sporocarps) are transferred to a small tube containing water (Fig. 6E).

Remark: When few spores are present in the sample, it may be more convenient to use the centrifugation method based on the establishment of a gradient using a highly concentrated substance like sucrose, glycerol or Percoll etc. (Mertz et al. 1979; Furlan et al. 1980; Hosny et al. 1996).

Disinfection Procedure.

1. To eliminate soil debris from the peridium of the sporocarps, treat them in an ultrasonic bath for 1 min. Usually, such pre-treatment is not required for spores, unless they are ornamented.
2. Transfer the cleaned spores or sporocarps to the filtration apparatus connected to a vacuum outlet (Fig. 6F and G, image 1), rinse them twice with sterile water (Fig. 6G, image 2) and agitate gently with a Pasteur pipette (Fig. 6G, image 3). Water outflow should be constant and slowed down by the end of the process to eliminate the liquid, but avoiding complete drying of the membrane.
3. Treat the spores or sporocarps with chloramine T 2% solution (with 2–3 drops of Tween 20), and filter it very slowly through the spores or sporocarps for a 10-min period. Continue manual agitation with the Pasteur pipette during the treatment (step 1, Fig. 6G, image 4).
4. Wash the spores three times with sterile water (Fig. 6G, image 5).
5. Treat the spores with the antibiotic solution for 10 min by following the same process as described above (step 2, Fig. 6G, image 6).
6. When the solution is removed, interrupt the filtration carefully and transfer the membrane supporting the disinfected spores or sporocarps into a plastic Petri plate containing the filtered antibiotics solution (Fig. 6G, image 7).
   Remark: After the solution is removed, the membrane must be wet but without liquid film on its surface which can cause the loss of spores when the apparatus is disassembled.
7. Under a binocular microscope, select healthy-looking spores and place them in Petri plates containing the MSR medium (five spores or one sporocarp per plate; enough space should be left between the spores to facilitate their transfer after germination; Fig. 7A).

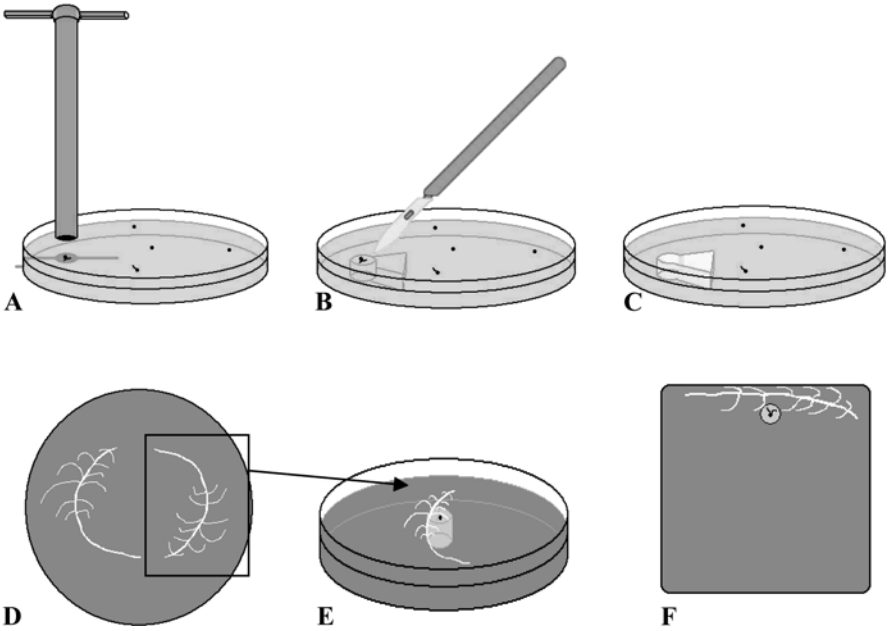


Fig. 7A–F. Establishment of a monoxenic culture from a disinfected spore. **A–C** Extraction of a disc of gel supporting the germinated spore. **D** Selection of a suitable host root. **E** Placement of the propagule and the host root in a round Petri plate. **F** Placement of the propagule and the host root into a square Petri plate

8. Seal the Petri plates with plastic wrap and incubate at 27 °C in the dark.

6.2.5 Root Fragments, Isolated Vesicles

Sampling of Inoculum.
The best results are obtained with young, healthy roots containing numerous vesicles. This type of material is not easy to collect under field conditions, and mycorrhizal roots from trap plants cultured in the greenhouse are more suitable to initiate a monoxenic culture. Leek plants are a convenient host because the roots are white and translucent, making the vesicles of the fungus more easily observable.

1. Sample roots from pot culture with a forceps.
2. Under a stereo-microscope, select between 10 and 20 root fragments (∼ 5 cm length) which containing numerous vesicles.

3. Rinse them with demineralized water and store in a sterile 50-ml tube (or in a sterile glass beaker) in sterile water (under laminar flow).

Disinfection Procedure.

The sterile tube is placed in an ultrasonic bath on a floating support. The apparatus is alternatively switched on half of the time for each disinfection step (Fig. 6H).

1. Clean the roots thoroughly with sterile (demineralized) water, three times.
2. Treat the roots with alcohol (95–98%) for 10 s (pre-treatment 1) (Fig. 5).
3. Rinse three times with sterile (demineralized) water.
4. Treat the roots with the calcium hypochlorite solution for 1–2 min (pre-treatment 2) (Fig. 5).
5. Rinse three times with sterile (demineralized) water.
6. Treat the roots with the solution of chloramine T 2% (20 g/l, with 2–3 drops of Tween® 20) for 10 min (step 1).
7. Rinse 3 times with sterile demineralized water.
8. Treat the roots with the antibiotic solutionsm for 10 min (step 2) (Fig. 5).
9. Remove the antibiotic solutions and transfer the roots to a Petri plate with a fresh antibiotic solution. Keep them in the antibiotic solution until use.
10. Place one root fragment of 1-cm length, containing numerous vesicles (selected under the binocular microscope), onto a Petri plate filled with MSR medium.
11. Seal the Petri plates with plastic wrap and incubate at 27 °C in the dark.

Enzymatic Treatment for Vesicle Isolation.

To obtain isolated vesicles, the incubated root fragments, disinfected following the method described above, are treated with an enzymatic solution. This solution degrades the root and, as a result, the mycorrhizal structures, i.e. vesicles, intraradical hyphae and arbuscules, are released. Vesicles can be selected under a binocular microscope and cultivated in vitro on synthetic medium.

Procedure (following Strullu and Romand 1987).

1. Prepare the enzymatic solution: macerozyme R10: 0.2 g/l, cellulase R10 : 1 g/l, driselase: 0.5 g/l in 1 l of liquid culture medium.
   – *Remark*: This must be filtered with a 0.2-µm acrodisc filter (GelmanSciences) before use.
2. Pour 5 ml of this solution into a Petri plate (45-mm diameter) and add one drop of streptomycin (5 ml/l).
3. Place about 10 root fragments of 1-cm length into each Petri plate.
4. After 24 h, the root fragments are transferred to Petri plates containing the same liquid culture medium without enzymes.

Different protocols for the liberation of mycorrhizal structures in root fragments are fully described in Saito (1995) and Solaiman and Saito (1997).

Remark: Isolated vesicles can also be obtained using a physical treatment. The disinfected root fragments are in this case pierced with a needle. When the root fragment is opened, the vesicles are released into the surrounding solution.

7
Monoxenic Culture Establishment

7.1
Germination of Disinfected Propagules

The germination of spores and sporocarps often occurs in a period varying between 2 and 30 days after disinfection. In general, spores and sporocarps do not require any specific conditions for germination. However, it has been observed for some AM fungal strains that germination was stimulated by the presence of a root or CO_2 (Bécard and Piché 1989). Therefore, for some strains, the propagules are placed in the vicinity of a root. It was also demonstrated for some *Gigaspora* species (e.g. *Gi. margarita* and *Gi. rosea*) that cold treatment is always required before isolation and disinfection (see Sect. 6.2.1).

For mycorrhizal root fragments, the hyphal re-growth from the extremities of mycorrhizal root segments is usually observed within 2–15 days. Germination of vesicles occurs 2–10 days after disinfection.

For both inoculum types, spores/sporocarps and mycorrhizal root segments or isolated vesicles, it is important to associate the germinated propagules with a host root shortly after germination to avoid damage of the newly formed hyphae during the association process and to increase colonization aptitude.

7.2 Material

Equipment

- Laminar flow hood
- Stereo-microscope
- Cooled heated incubator
- Bunsen burner or bead sterilizer

Laboratory material

- Sterile Petri plates
- Petri plate filled with MSR medium
- Forceps
- Scalpel
- Needles
- Cork borer (0.05 to 0.25 cm diameter)
- Adjustable volume pipette and sterile tips
- Small plastic wrap roll

7.3 Association Methods

1. Select a host root (transformed carrot root) of 8-cm length with few apexes forming a herringbone structure from a 2–3 week old root organ culture (see Fig. 7D).
2. If the inoculum is a spore (since five spores were placed in the same plate for germination):
   a) From a Petri plate filled with the MSR medium, remove a disc of agar with a cork borer. This disc should be identical in size to the disc of gel supporting the germinated spore.
   For *Glomus* and *Scutellospora*, the disc of gel should be removed from the centre of a mono-compartmental Petri plate (9-cm diameter) or from the centre of the proximal compartment of a bi-compartmental system (for *Glomus*).
   For *Gigaspora* species, the disc of gel should be removed from the centre of the upper part of a square mono-compartmental plate. The diameter of the disc should be large enough to include the spore and germinating hyphae (9-mm diameter is usually enough; Fig. 7A–C).

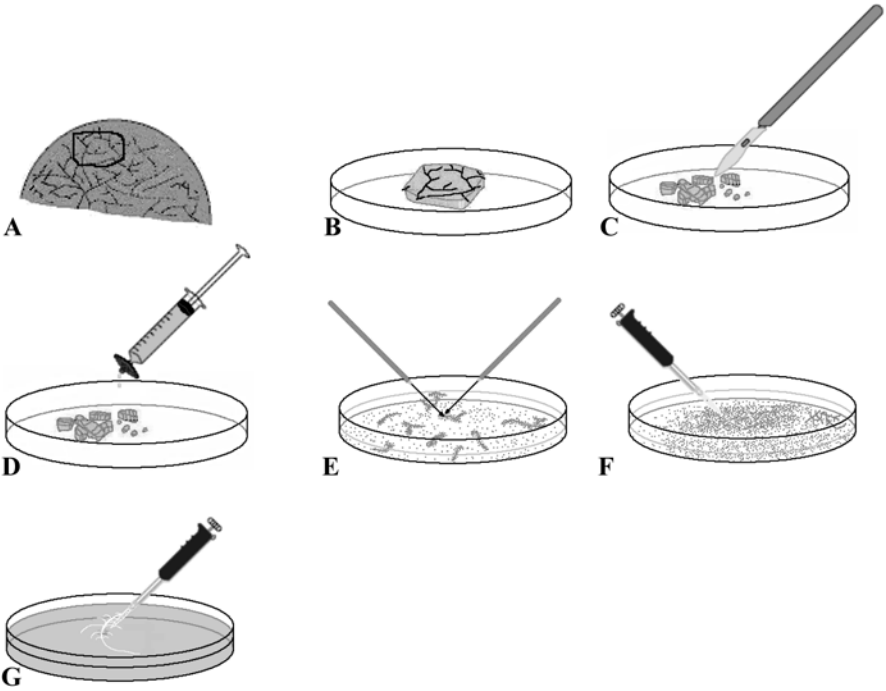


Fig. 8A–G. Illustration of subcultures from dissolved spores. **A** Selection of a piece of gel containing spores. **B–D** Addition of 10 mM citrate buffer to dissolve the gel. Spores can be better separated when the gel piece is fragmented. **E** Separation of spores in small clusters. **F, G** Host root inoculation

b) Transfer the disc of gel supporting the germinated spore of *Glomus* and *Scutellospora* (Fig. 7D, E) or *Gigaspora* (Fig. 7F) into the hole of the removed disc.

c. Continue with step 3.

3. Place the root carefully in the near vicinity of the propagule (spore, sporocarp or mycorrhized root fragment) into the Petri plate, so that the growing hyphae are perpendicular to a secondary root (see Fig. 7E, F).

4. Seal the Petri plates with plastic wrap and incubate in an inverted position at 27 °C in the dark for the *Glomus* and *Scutellospora* species in mono- or bi-compartmental systems. The *Gigaspora* cultivated in a square Petri plate has to be incubated vertically (root in the upper part) at 27 °C in the dark.

8
Continuous Culture

8.1
Association Establishment

For a *Glomus* species like *G. intraradices*, a mycelium appears after a few days and new spores are produced very quickly thereafter. The mycelium grows extensively, rapidly invading the complete volume of the Petri plate. In the bi-compartmental system, the sporulation is higher in the distal compartment containing no carbon (see Sect. 3). The sporulation capacity of the fungal strain and the development of mycelium depend on the strain. After 3 months, a culture of *G. intraradices* can produce more than 10,000 spores.

For other *Glomus* strains, for example, *G. caledonium*, which produces large spores and few vesicles, mycelium can grow rapidly and extensively but spores appear very slowly (after one or more months).

For Gigasporaceae (*Gi. rosea*, *S. reticulata*, etc.), the production of mycelium is less profuse, auxiliary cells appear rapidly, but it takes several weeks for spores to be produced (Fortin et al. 2002).

8.2
Continuous Culture

The first continuous culture (see definition in Chap. 1) of *Glomus* species was achieved by Strullu and Romand in 1986 using the intraradical form, and was thereafter extended to various AM fungi (Table 1). The continuous culture is obtained by associating monoxenic mycorrhizal roots and/or spores (Chabot et al. 1992), often attached to extraradical mycelia, to a new, actively growing non-mycorrhizal host root, onto a fresh medium. This first method is largely used and is effective for a range of *Glomus* species (Diop et al. 1994a; Strullu et al. 1997; Declerck et al. 1998, 2000) and for *Scutellospora reticulata* (de Souza and Declerck 2003; Table 1).

A second method has been used by St-Arnaud et al. (1996) and is effective for *Glomus* species having a well-developed intraradical phase, such as *G. intraradices*. In this method, apical segments of actively growing mycorrhizal roots with or without extraradical mycelium-supporting spores are transferred to a fresh medium. The root and associated fungus continue to proliferate across successive transfers onto fresh medium. This procedure requires the use of young, actively growing cultures, to allow the continuous growth of the host root.

For *Glomus* species producing few spores and vesicles, continuous cultures are difficult to obtain. For *Gigaspora* species they were only reported as unpublished results in Fortin et al. (2002). Hence, for this latter species, it is necessary to periodically start new monoxenic cultures with disinfected spores from pot cultures.

8.3 Material

Equipment

- Laminar flow hood
- Stereo-microscope
- Cooled heated incubator
- Bunsen burner or bead sterilizer
- Agitator (preferably rotating agitator)

Laboratory material

- Sterile Petri plates
- Petri plate filled with MSR
- 20-ml sterile syringe
- 0.2-µm sterile filter (Acrodisc GelmanSciences)
- 50-ml sterile centrifuge tube (falcon type)
- Forceps
- Scalpel
- Needles
- Adjustable volume pipette and sterile tips
- Small plastic wrap roll

8.4 Solutions

1. Citric acid stock solution: 0.1 M (autoclaved).
2. Sodium citrate stock solution: 0.1 M (autoclaved).

3. Citrate buffer (0.01 M): under laminar flow, mix 0.018 volume citric acid solution, 0.082 volume sodium citrate solution, complete the volume with sterile water, and adjust the pH to 6 if necessary with NaOH 1 M. The solution is filtered through an acrodisc 0.2 mm before use.
4. Sterile water.
5. Ethanol for material disinfection.

8.5 First Method: Propagule Re-Association

8.5.1 Isolation of Propagules

Propagules can be extracted from the gel in several ways:

1. If spores are relatively big (> 150 µm) and occur in small quantity (such as *Gigaspora*, *Scutellospora* and some *Glomus* species), it is easy to extract a single spore attached to the extraradical mycelium with a needle. Only one spore is necessary to start a new culture but, to increase the chances of association, around five spores can be use as inoculum.
2. If spores are numerous and of small to medium size, such as reported for the majority of the *Glomus* species cultured monoxenically, simply cut a small piece of gel containing large amount of spores (i.e. 1–5 cubes of 0.5 cm^3, ~ 200 spores) attached to the extraradical mycelium.
3. Alternatively, for species producing high numbers of spores, the gelling agent may be removed from the culture medium, to stimulate re-growth of the fungus. The gelling agent (=agar substitutes like Gelgro or Phytagel but not agar) can be dissolved by a citrate buffer (10 mM; Fig. 8) as follows:
    a. Extract a piece of gel containing approximately 200 spores from the mother culture and transfer it to an empty sterile Petri plate (diameter 9 cm; Fig. 8A–C).
    b. Add 10× the volume of citrate buffer filtered through a 0.2-µm acrodisc (for example, for a gel block of 0.5 g, add 5 ml of buffer) and seal the plate with plastic wrap.
    Remark: Do not put too much liquid in the Petri plate, to avoid any risk of contamination (for example, in a Petri plate of 9-cm diameter, put a maximum of 25 ml citrate buffer; Fig. 8C, D).

3. Agitate the Petri plate slowly on a rotating agitator (50 rotation/min) at 25–27 °C for 30 min to 1 h, depending on the volume of gel to dissolve.
4. Transfer the spores attached to the extraradical mycelium to a new Petri plate containing sterile water using a binocular microscope (under laminar flow), then separate the spores into individuals or little clusters attached to the extraradical mycelium (containing 15–30 spores, depending on spore size; Fig. 8E).

8.5.2 Association with a Root Host

1. Place a transformed carrot root suitable for association (8-cm length, with about six secondary roots of 1–2 cm length) in a Petri plate filled with the MSR medium. In the mono-compartmental culture system using square Petri plates (suitable for *Gigaspora* species), the root is placed in the upper part of the Petri plate; in the other models (round mono- and bi-compartmental), the root is placed in the centre of the root compartment.
2. Place the medium pieces containing spores attached to the extraradical mycelium, or the single spore (or spores clusters) and/or mycorrhizal root pieces homogeneously along the root, in the vicinity of secondary roots, to favour the association (Fig. 8F, G). Association generally occurs near the growing apex where the cellular wall is thinner and easier to penetrate.
3. Seal the Petri plate with plastic wrap and incubate at 27 °C in the dark in an inverted position (vertically for *Gigaspora* species).

Germination and association usually occur between the 3rd and the 15th day after association.

8.6 Second Method: Mycorrhized-Apex Transfer

For *Glomus* species showing a well-developed intraradical phase, the easiest way to obtain a continuous culture is to transfer a section (2 × 2 cm) of medium containing healthy root apexes with extraradical hyphae and spores onto a Petri plate containing MSR medium. The mycorrhizal root continues to grow and the fungus proliferates.

Remark: It is necessary to use a young culture (some weeks). When the culture is too old, the medium becomes exhausted and the root becomes necrotic.

9 Conclusion

The protocols presented here aim to give researchers the possibility to initiate and maintain their own AM fungal monoxenic cultures. They should encourage general use of such fungal materials, enlarging the diversity of species cultured in this system. The undisputable benefits derived from this culture system are highlighted in all chapters of this book. This considerable impact is not only restricted to the study of symbiotic interactions, but also permits the increase in knowledge in the morphology, taxonomy, phylogeny and biochemistry fields together with some aspects of their ecology.

Acknowledgements. This work was made possible by financial support of the Belgian Federal Science Policy Office (contract BCCM C3/10/003), the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA), and the EC project ICA4-CT-2002-100016. Many thanks are extended to José Murias for illustrations and to Maria-Genalin Angelo-Van Coppenolle.

References

Ark PA, Thompson JP (1961) Detection of hairy root pathogen, *Agrobacterium rhizogenes*, by the use of fleshy roots. Phytopathology 51:69–71

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation in Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 112:77–83

Bécard G, Piché Y (1992) Establishment of vesicular-arbuscular mycorrhiza in root organ culture: review and proposed methodology. Method Microbiol 24:89–108

Boisson-Denier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker DG (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal associations. Mol Plant Microb Interact 14:695–700

Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321

Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempé J (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genome of host plant root cells. Nature 295:432–434

Cook DR (1999) *Medicago truncatula* – a model in the making! Curr Opin Plant Biol 2:301–304

Dalpé Y, Declerck S (2002) Development of *Acaulospora rehmii* spore and hyphal swellings under root-organ culture. Mycologia 94:850–855

Daniels BA, Menge JA (1981) Evaluation of the commercial potential of the vesicular-arbuscular mycorrhizal fungus *Glomus epigaeus*. New Phytol 87:345–354

Declerck S, Strullu DG, Plenchette C (1996) *In vitro* mass-production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycol Res 100:1237–1242

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

de Souza FA, Berbara RLL (1999) Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia 91:343–350

de Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012

Dessaux Y, Petit A, Tempé J (1992) Opines in agrobacterium biology. In: Verma DPS (ed) Molecular signals in plant-microbe communications. CRC Press, Boca Raton, pp 109–136

Diop TA (1995) Ecophysiologie des champignons mycorhiziens à vésicules et arbuscules associés à *Acacia albida* Del. dans les zones Sahelienne et Soudano-Guineene du Sénégal. Thèse de Doctorat, Université d'Angers

Diop TA, Bécard G, Piché Y (1992) Long-term in vitro culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. Symbiosis 12:249–259

Diop TA, Plenchette C, Strullu DG (1994a) Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Diop TA, Plenchette C, Strullu DG (1994b) In vitro culture of sheared mycorrhizal roots. Symbiosis 17:214–227

Douds DD (1997) A procedure for the establishment of *Glomus mosseae* in dual culture with Ri T-DNA-transformed carrot roots. Mycorrhiza 7:57–61

Douds DD (2002) Increased spore production by *Glomus intraradices* in the split-plate monoxenic culture system by repeated harvest, gel replacement, and resupply of glucose to the mycorrhiza. Mycorrhiza 12:163–167

Fortin JA, Bécard G, Declerck S, Dalpé Y, St Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Furlan V, Bärtschi H, Fortin JA (1980) Media for density gradient extraction of endomycorrhizal spores. Trans Br Mycol Soc 75:336–338

Gadkar V, Adholeya A, Satyanarayana T (1997) Randomly amplified polymorphic DNA using the M13 core sequence of the vesicular-arbuscular mycorrhizal fungi *Gigaspora margarita* and *Gigaspora gigantea*. Can J Microbiol 43:795–798

Hosny MH, Dulieu H, Gianinazzi-Pearson V (1996) A simple and rapid method for collecting Glomales spores from soil. In: Azcon-Aguilar C, Barea JM (ed) Mycorrhizas in integrated systems: from genes to plant development. European Commission edn EUR16728, Luxembourg, pp 541–542

Karandashov VE, Kuzovkina IN, George E, Marschner H (1999) Monoxenic culture of arbuscular mycorrhizal fungi and plant hairy roots. Russian J Plant Physiol 46:87–92

Karandashov VE, Kuzovkina IN, Hawkins HJ, George E (2000) Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots. Mycorrhiza 10:23–28

Khaliq A, Bagyaraj DJ (2000) Colonization of arbuscular-mycorrhizal fungi on Ri T-DNA transformed roots in synthetic medium. Indian J Exp Biol 38:1147–1151

Labour K, Jolicoeur M, St-Arnaud M (2003) Arbuscular mycorrhizal responsiveness of in vitro tomato root lines is not related to growth and nutrient uptake rates. Can J Bot 81:645–656

Legué V, Driss-Ecole D, Maldiney, R, Perbal G (1996) The response to auxin of rapeseed (*Brassica napus* L.) roots displaying reduced gravitropism due to transformation by *Agrobacterium rhizogenes*. Planta 200:119–124

Mathur N, Vyas A (1995) In vitro production of *Glomus deserticola* in association with *Ziziphus nummularia*. Plant Cell Rep 14:735–737

Mathur N, Vyas A (1999) Improved biomass production, nutrient uptake and establishment of in vitro raised *Ziziphus mauritiana* by VA mycorrhiza. J Plant Physiol 155:129–132

May GD, Dixon RA (2004) *Medicago truncatula*. Curr Biol 14:R180-R181

Mertz SM Jr, Heithaus JJ, Bush RL (1979) Mass production of axenic spores of the endomycorrhizal fungus *Gigaspora margarita*. Trans Br Mycol Soc 72:167–169

Miller-Wideman MA, Watrud LS (1984) Sporulation of *Gigaspora margarita* on root cultures of tomato. Can J Microbiol 30:642–646

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mosse B (1988) Some studies relating to "independent" growth of vesicular-arbuscular endophytes. Can J Bot 66:2533–2540

Mosse B, Hepper C (1975) Vesicular arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Nuutila AM, Vestberg M, Kauppinen V (1995) Infection of hairy roots of strawberry (*Fragaria* × *Ananassa* Duch.) with arbuscular mycorrhizal fungus. Plant Cell Rep 14:505–509

Pawlowska TE, Douds DD, Charvat I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Raman N, Sahadevan C, Srinivaan V (2001) Growth of AM fungi on in vitro root organ culture of *Sorghum vulgare* and *Saccharum officinarum*. Indian J Exp Biol 39:1293–1298

Riker AJ, Banfield WM, Wright WH, Keitt GW, Sagen HE (1930) Studies on infectious hairy root of nursery apple trees. J Agric Res 41:507–540

Rufyikiri G, Thiry Y, Declerck S (2003) Contribution of hyphae and roots to uranium uptake and translocation by arbuscular mycorrhizal carrot roots under root-organ culture conditions. New Phytol 158:391–399

Saito M (1995) Enzyme activities of the internal hyphae and germinated spores of an arbuscular mycorrhizal fungus, *Gigaspora margarita* Becker & Hall. New Phytol 129:425–431

Samson J, Dalpé Y, Piché Y (2000) Isolement in vitro de deux nouvelles souches de *Glomus* en co-culture avec des racines de carotte transformées. In: Coll Mycorhizes 2000, Rivière-du-Loup, Québec

Simoneau P, Louisylouis N, Plenchette C, Strullu DG (1994) Accumulation of new polypeptides in Ri T-DNA transformed roots of tomato (*lycopersicon esculentum*) during the development of vesicular-arbuscular mycorrhizae. Appl Environ Microbiol 60:1810–1813

Solaiman MDZ, Saito M (1997) Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. New Phytol 136:533–538

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth an spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332

Strullu DG, Romand C (1986) Méthodes d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. C R Acad Sci Paris 303 :245–250

Strullu DG, Romand C (1987) Culture axénique de vésicules isolées à partir d'endomycorhizes et réassociation in vitro à des racines de tomate. C R Acad Sci Paris 305:15–18

Strullu DG, Diop TA, Plenchette C (1997) Réalisation de collections in vitro de *Glomus intraradices* (Schenck et Smith) et de *Glomus versiforme* (Karsten et Berch) et proposition d'un cycle de développement. C R Acad Sci Paris 320:41–47

Tepfer DA, Tempé J (1981) Production d'agropine par des racines formées sous l'action d'*Agrobacterium rhizogenes*, souche A4. C R Acad Sci Paris 292:153–156

Tiwari P, Adholeya A (2003) Host dependent differential spread of Glomus intraradices on various Ri T-DNA transformed roots in vitro. Mycol Orig 2:171–177.

Subject Index