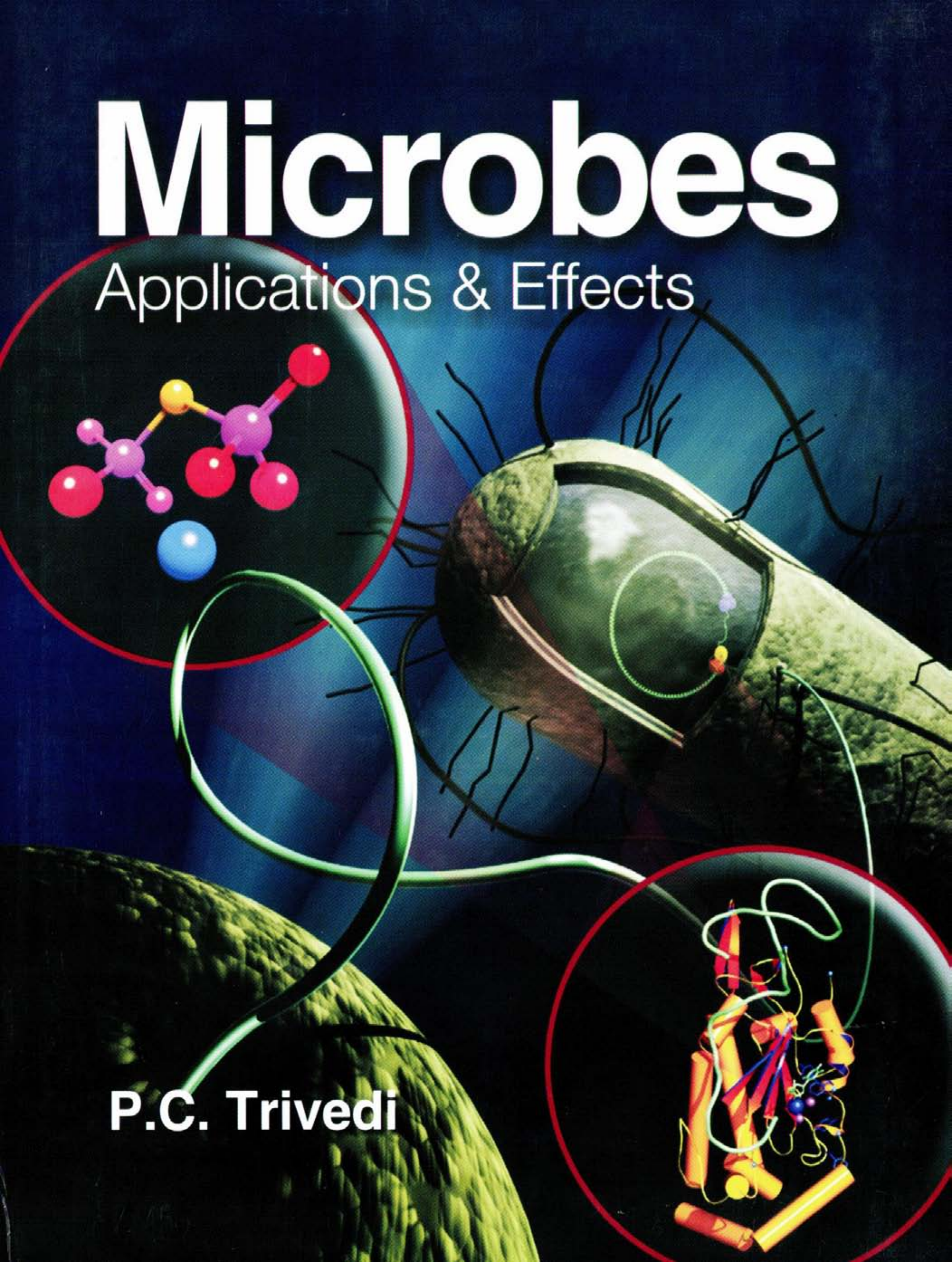

Microbes

Applications & Effects

P.C. Trivedi

MICROBES

APPLICATIONS AND EFFECTS

Editor

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FACTS AND MYTHS OF PROBIOTICS IN NEW MILLENNIUM

S.S. KANWAR, ADITI SOURABH AND M.K. GUPTA

The concept of probiotics evolved around 1900, when Nobel Prize-winning Elie Metchnikoff hypothesized that the long, healthy lives of Bulgarian peasants were the result of their consumption of fermented milk products and later he was convinced that yogurt contained the organisms necessary to protect the intestine from the damaging effects of other harmful bacteria. In the late 19th century, microbiologists identified microflora in the gastrointestinal (GI) tracts of healthy individuals that differed from those found in diseased individuals. These beneficial microflora found in the GI tract were termed probiotics.

The modern term 'probiotic' was first used by Fuller (1989), describing a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance. Later it was demonstrated that heat-inactivated bacteria or fragments of bacterial DNA have positive effects as well. Marteau et al. (2002) defined probiotics as 'microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being. Probiotics are also defined as non pathogenic microorganisms that when administered in adequate amounts confer beneficial physiological effect on host (Schrezenmeir and de Vrese, 2001; Reid *et al.*, 2003; and Bujalance *et al.*, 2007). Although viable probiotics are generally recommended, health-promoting effects of inactive probiotics have also been observed (Ouwehand and Salminen, 1999). The various microorganisms used as probiotics are summarized in Table 1.

These organisms are non pathogenic, bile tolerant, technologically suitable for industrial processes and produce antimicrobial substances like organic acid, hydrogen peroxide, carbon dioxide, bacteriocins etc (Dunne *et al.*, 1999). They have antioxidative activity (Kullisaar *et al.*, 2002), ability to adhere to gut tissue and to modulate immune responses (Saarela *et al.*, 2000). The selected strains of these microorganisms used in various products are generally regarded as safe (GRAS) and mainly belong to *Lactobacillus* and *Bifidobacterium* genera (Salvodori, 1998; and Sanders and in't Veld, 1999) which exhibit various positive attributes in order to be called as probiotic strains.

TABLE 1

Microorganisms reported as Probiotics (Holzapfel *et al.*, 2001)

Lactobacillus species	*Bifidobacterium* species	Other lactic acid bacteria	Non lactic acid microorganisms
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*Bacillus cereus* var. *toyo*
L. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli* strain nissle
L. casei	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freudenreichii*
L. crispatus	*B. breve*	*Leuconstoc mesenteroides*	*Saccharomyces cerevisiae*
L. delbrueckii subsp.	*B. infantis*	*Pediococcus acidilactici*	*Saccharomyces boulardii*
			bulgaricus
L. gallinarum	*B. lactis*	*Sporolactobacillus inulinus*	
L. plantarum	*B. longum*	*Streptococcus thermophilus*	
L. johnsonii			
L. paracasei			
L. gasseri			
L. reuteri			
L. rhamnosus			

FEATURES OF PROBIOTICS

A group of requirements and physiological effects for selection of an effective probiotic are (i) human origin (ii) safety (iii) ability to adhere to intestinal cells and thereby exclude or reduce pathogens adherence; (iv) ability to produce organic acids, hydrogen peroxide, bacteriocins etc. (iii) ability to resist microbicides (v) ability to be safe, noninvasive, noncarcinogenic, and nonpathogenic; (vi) to co-aggregate and form normal, balanced flora (vii) to reduce gut pH; (viii) to produce some of the digestive enzymes and vitamins (ix) to reduce cholesterol and bile salt deconjugation (x) to stimulate host immune response (xi) be present as viable cells, preferably in large numbers (xii) antioxidative activity (xiii) be stable and capable of remaining viable for periods under storage and field conditions or in delivery vehicles (xiv) be capable of surviving e.g. resistance to low pH, organic acids and influencing metabolic activities in the gut environment such as vitamin production, cholesterol assimilation etc. (xv) be associated with health effects (Fuller, 1989; Reid, 1999; Holzapfel and Schillinger, 2002; Grajek *et al.*, 2005 and Nguyen, *et al.*, 2007). Thus probiotics have multiple mechanisms of action, including prevention of pathogenic bacterial growth, binding to or penetration of pathogens to mucosal surfaces, stimulation of mucosal barrier function, production of antimicrobial agents or altering immunoregulation, decreasing proinflammatory and promoting protective molecules (Sartor, 2005; Novak and Katz, 2006).

The above properties have consequently become an important selection criteria for new probiotic strains. Recently, genes induced upon exposure to acid (Azcarate-Peril *et al.*, 2004; Azcarate-Peril *et al.*, 2005) and bile salts (Bron *et al.*, 2004) have been identified that might confer important roles in GI tract survival to these microorganisms.

An essential determinant in the choice of a probiotic microorganism is its ability to reach, survive, and persist in the environment in which it is intended to act. According to the generally accepted definition of a probiotic, the probiotic microorganism should be viable at the time of ingestion to confer a health benefit (Gilliland *et al.*, 2001). Although not explicitly stated, this definition implies that a probiotic should survive GI tract passage and, according to some, colonize the host epithelium. A possible exception to this rule is the increasing number of examples in which probiotic cell components (e.g. CpG DNA), rather than the living cells have been shown to exert sufficient measurable effect (Dotan and Rachmilewitz, 2005). Thus not only viable or dormant bacteria administered to the intestinal tract but also probiotic DNA is active, even if injected subcutaneously (Meier and Steuerwald, 2005). Nonetheless, the requirement for viable cells also remains an important factor for probiotic efficacy.

PROBIOTIC FORMULATIONS

Probiotics can be compounded in various ways depending on the sort of use intended. Probiotics can be in powder form, liquid form, gel, paste, granules or available in the form of capsules, sachets, etc. A probiotic may be made out of a single bacterial strain or it may be a consortium as well. The advantage of multiple strain preparations is that they are active against a wide range of conditions and in a wider range of animal species.

Probiotic cultures for food applications are frequently supplied in frozen, or dried form, either as freeze-dried or spray-dried powders. Most probiotic lactobacilli do not survive well, during the temperature and osmotic extremes to which they are exposed during the spray-drying process which leads to destruction of the properties and performance characteristics of these cultures. One approach used by a number of workers to improve probiotic performance in food systems is the addition of protectants for example thermoprotectants such as trehalose, growth-promoting factors including various probiotic/ prebiotic combinations (Desmond *et al.*, 2002; Corcoran *et al.*, 2004) and granular starch (Crittenden *et al.*, 2001) to the media prior to drying to improve culture viability during drying, storage and/or gastric transit. Encapsulation, as a means of protecting live cells from extremes of heat or moisture, such as those experienced during drying and storage is another technique that is increasingly used in the probiotic food industry.

For successful delivery in foods, probiotics must survive food processing and storage during product maturation and shelf-life. It is recommended that the probiotic culture must be present in the product at minimum numbers of 10^7 CFU ml^{-1} and even higher numbers have been recommended (Ishibashi and Shimamura, 1993; Lee and Salminen, 1995). In addition, the probiotic food product should be regularly consumed in sufficient quantities to deliver the relevant dose of live bacteria to the gut, keeping in mind the losses in cell viability typically encountered during gastric transit (Ross *et al.*, 2005).

CONCEPT OF PREBIOTICS

The concept of prebiotics is relatively new. A prebiotic is defined as "nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon that can improve the host health". In order to classify a food ingredient as prebiotic, it must (Gibson and Roberfroid, 1995):

- not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract;
- be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated;
- be able to alter the colonic flora in favor of a healthier composition
- be able to induce luminal or systemic effects that are beneficial to the host health.

A number of poorly digested carbohydrates fall into the category of prebiotics including certain fibres and resistant starches, but the most widely described prebiotics are non-digestible oligosaccharides (NDOs). Prebiotics may be oligosaccharides; fructooligosaccharides (FOS); galactooligosaccharides (GOS); raffinose, stachynose and soybean oligosaccharides (SOS); and xylooligosacchrides (XOS) (Srivastava and Goyal, 2007). These are low molecular weight carbohydrates with 2-10 degrees of polymerisation, which are poorly digested in the small intestine thus reaching the colon largely unaltered and can act as a substrate for the colonic microflora (Burns and Rowland, 2000). A prebiotic aims selectively to feed probiotic, indigenous to the human colon. One significant advantage with prebiotics is that they are non-viable food ingredients. Probiotic products are now available in different formulations in combination with prebiotic like fructooligosaccharides (FOS). The basic assumption is that, these indigestible ingredients reach the colon and can be utilized by the intestinal flora to stimulate those bacteria (specifically *Bifidobacteria*) that are naturally part of the ecosystem in the colon (Rastall *et al.*, 2005). These dietary oligosaccharides are known to have intestinal effects, generation of SCFAs (short chain fatty acids), improvement in defecation, reduction of putrefaction, prevention of colon cancer and improvement in mineral absorption.

The combination of probiotics with synergistically acting components such as prebiotics that can promote their growth and enhance their survivability through the GI tract, is termed as synbiotic. Alternatively, synbiotics are defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare.

HEALTH BENEFITS AND THERAPEUTIC EFFECTS OF PROBIOTICS

The consumption of probiotic products is helpful in maintaining good health, restoring body vigour, and in combating intestinal and other disease disorders. Probiotics can enhance intestinal health which includes stimulation of immunity, competition for limited nutrients with pathogenic micro-organisms, competitive blocking of adhesion sites at epithelial and mucosal surfaces, and inhibition of epithelial invasion. General reported health benefits of probiotics and their putative mechanisms (Sanders and in't Veld, 1999; Sanders, 2003) are summarized as below in Table 2.

TABLE 2

Summary of Health Benefits along with Postulated Mechanisms

Target Health Benefit	Postulated Mechanism
Aid in lactose digestion	Bacterial lactase hydrolyses lactose
Resistance to enteric pathogens	Secretory immune effect; Colonization resistance; Alteration of intestinal conditions to be less favorable for pathogenicity (pH, short chain fatty acids, bacteriocins); Alteration of toxin binding sites; Influence on gut flora populations; Adherence to intestinal mucosa, interfering with pathogen adherence; Upregulation of intestinal mucin production, interfering with pathogen attachment to intestinal epithelial cells
Anti-colon cancer effect	Mutagen binding; Carcinogen deactivation; Inhibition of carcinogen-producing enzymes of colonic microbes; Immune response; Influence on secondary bile salt concentration
Small bowel bacterial overgrowth	Influence on activity of overgrowth flora, decreasing toxic metabolite production; Alteration of intestinal conditions to be less favorable to overgrowth flora activities or populations
Immune system modulation	Strengthening of non-specific defense against infection and tumors; Adjuvant effect in antigen-specific immune responses; Enhancement of secretory IgA production
Allergy	Prevention of antigen translocation into blood stream
Blood lipids, heart disease	Assimilation of cholesterol within bacterial cell; Increased excretion of bile salts due to deconjugation by bile salt hydrolase; Antioxidative effect

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Target Health Benefit	Postulated Mechanism
Antihypertensive effect	Peptidase action on milk protein yields tripeptides which inhibit angiotensin 1 converting enzyme; Cell wall components act as angiotensin converting enzyme inhibitors
Urogenital infections	Adhesion to urinary and vaginal tract cells; Colonization resistance Inhibitor production (H_2O_2, biosurfactants)
Infection caused by *H. pylori*	Production of inhibitors of (lactic acid and others) *Helicobacter pylori*
Diarrhea linked to antibiotics, diarrhea caused by Rotavirus, colitis caused by *C. diffi cile*, nosocomial diarrhea	Competetive exclusion, translocation/barrier effect, immune response promoted
Hepatic encephalopathy	Inhibition of urease-producing gut flora
Intestinal inflammation, colitis, Crohn's disease, pouchitis	Ulcerative Immune response downregulated
Lowering of blood cholesterol	Deconjugation of the bile acids
Renal calculi	Changes in the digestive flora influencing the breakdown of oxalate

Probiotics exhibit a direct effect in the gut in the treatment of inflammatory and functional bowel disorders (Camilleri, 2006). In one of the most common functional bowel disorders such as irritable bowel syndrome, *L. plantarum* 299v and DSM 9843 strains have been reported in clinical trials to reduce abdominal pain, bloating, flatulence, and constipation (Motta *et al.*, 1991; MacFarlane and Cummings, 2002).The summary of the strain specific therapeutic effects of current probiotic bacteria are tabulated in the Table 3.

TABLE 3

Strain Specific Therapeutic Effects of Probiotics as Reported by Various Workers

Strain	Reported effects in clinical studies	References
L. johnsonii LA1	Adherence to human intestinal cells, balances intestinal microbiota, immune enhancement, adjuvant in *H. pylori* treatment	MacFarland, 2000; Salminen *et al.*, 1998
L.acidophilus NCFB 1748	Lowering of fecal enzyme activity, decreasing fecal mutagenicity, prevention of radiotherapy-related diarrhea, improvement of constipation	Fonden *et al.*, 2000; Salminen and Gueimonde, 2004

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Strain	Reported effects in clinical studies	References
L. rhamnosus GG (ATCC 53013)	Treatment and prevention of rotavirus diarrhea, prevention of antibiotic associated diarrhea, treatment of relapsing *C. difficile* diarrhea, reduction of cystic fibrosis symptoms.	MacFarland, 2000; De Roos and Katan, 2000; Fonden *et al.*, 2000
L. acidophilus NFCM	Lowering of fecal enzyme activity, high lactase activity, treatment of lactose intolerance, production of bacteriocins.	Fonden *et al.*, 2000
L. casei Shirota	Prevention of intestinal microbiota disturbances, balancing intestinal bacteria, lowering fecal enzyme activities, positive effects on reducing the recurrence of superficial bladder cancer	Salminen *et al.*, 1998; MacFarland, 2000; De Roos and Katan, 2000;
S. thermophilus; L. bulgaricus	No effect on rotavirus diarrhea, no immune enhancing effect during rotavirus diarrhea, no effect on fecal enzymes, strain-dependent improvement of lactose intolerance symptoms	Fonden *et al.*, 2000
L. acidophilus La-5	Balancing intestinal microbiota, protection against traveller's diarrhea, immune enhancement;	Fonden *et al.*, 2000
B. lactis	Treatment of viral diarrhea including rotavirus diarrhea, balancing intestinal microbiota	Fonden *et al.*, 2000; Macfarland, 2000
Lactobacillus gasseri	Fecal enzyme reduction, survival in the intestinal tract;	Fonden *et al.*, 2000
	Carcinogenic-associated enzyme reduction	Salminen *et al.*, 1998;
L. reuteri	Colonizing the intestinal tract, shortening of rotavirus diarrhea;	De Roos and Katan, 2000; Fonden *et al.*, 2000
	Shortened the duration of acute gastroenteritis;	Marteau *et al.*, 2001
	Shortened acute diarrhoea;	Shornikova *et al.*, 1997a, 1997b
Lactobacillus acidophilus	Significant decrease of diarrhoea in patients receiving pelvic irradiation;	Marteau *et al.*,2001
	Decreased polyps, adenomas and colon cancer in experimental animals;	Gorbach *et al.*, 1987; Parvez *et al.*, 2006;
	Prevented urogenital infection with subsequent exposure to three uropathogens *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa;* Lowered serum cholesterol levels	Sanders and Klaenhammer, 2001; Ouwehand *et al.*, 2002

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Strain	Reported effects in clinical studies	References
Lactobacillus acidophilus SDC 2012, 2013	Beneficial Effect reported in patients with Irritable Bowel Syndrome	Sinn *et al.*, 2008
Lactobacillus plantarum	Reduced incidence of diarrhoea in daycare centres when administered to only half of the children;	Vanderhoof, 2000
	Especially effective in reducing inflammation in inflammatory bowel; e.g., enterocolitis in rats, small bowel bacterial overgrowth in children, pouchitis;	Schultz and Sartor 2000; Vanderhoof, 2000
	Reduced pain and constipation in irritable bowel syndrome;	Vanderhoof, 2000
	Reduced bloating, flatulence, and pain in irritable bowel syndrome in controlled trial;	Nobaek *et al.*, 2000
	Positive effect on immunity in HIV+ children	Walker, 2000
L. helveticus and *Saccharomyces cerevisiae*	Reductions in systolic and diastolic blood pressure	Hata *et al.*, 1996
L. casei	Decreases in systolic and diastolic blood pressure and heart rate of hypertensive patients on administering powdered probiotic cell extracts	Sawada *et al.*, 1990; Nakamura *et al.*, 1995
Lactobacillus rhamnosus	Enhanced cellular immunity in healthy adults in controlled trial	Tomioka *et al.*, 1992
Lactobacillus salivarius	Suppressed and eradicated *Helicobacter pylori* in tissue cultures and animal models by lactic acid secretion	Aiba *et al.*, 1998; MacFarlane and Cummings, 2002
Saccharomyces boulardii (yeast)	Reduced recurrence of *Clostridium difficile* diarrhoea;	Pochapin, 2000;
	Shortened the duration of acute diarrhea, accelerated recovery and reduced the risk of prolonged diarrhoea;	Villarruel *et al.*, 2007
	Effects on *C. difficile* and *Klebsiella oxytoca* resulted in decreased risk and/or shortened duration of antibiotic-associated diarrhea;	Marteau *et al.*, 2001; MacFarland, 2000

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Strain	Reported effects in clinical studies	References
	Decreased only functional diarrhoea, but not any other symptoms of irritable bowel syndrome;	
	Interferes with *H. pylori* in colonized individuals	Gotteland *et al.*, 2005
Bacteroides species	Effective in chronic colitis, gastritis, arthritis	Vanderhoof, 2000
Bifidobacteria species	Reduced incidence of neonatal necrotizing enterocolitis;	Caplan and Jilling, 2000;
	Prevention of viral diarrhoea	Salminen *et al.*, 1998
Bifidobacterium longum	Prevents antigen-induced Th2 immune responses *in vivo* suggesting the role of probiotics in preventing allergic disease	Takahashi *et al.*, 2006
Enterococcus faecium	Decreased duration of acute diarrhoea from gastroenteritis	Marteau *et al.*, 2001

NUTRIENT SYNTHESIS AND BIOAVAILABILITY

Probiotics antagonize pathogens through production of antimicrobial and antibacterial compounds such as cytokines and butyric acid; reduce gut pH by stimulating the lactic acid producing microflora; compete for binding and receptor sites that pathogens occupy; improve immune function and stimulate immunomodulatory cells; compete with pathogens for available nutrients; other growth factors and modify toxins produced by pathogens or toxin receptors found in the gut wall; or produce lactase which aids in lactose digestion. Lactic acid bacteria are known to release various enzymes and vitamins into the intestinal lumen. This exerts synergistic effects on digestion, alleviates symptoms of intestinal malabsorption, lowers the pH of the intestinal content and helps to inhibit the development of invasive pathogens such as *Salmonella spp.* or strains of *E. coli* (Mack *et al.*, 1999). *Saccharomyces boulardii* has been reported to synthesize a phosphatase that can dephosphorylate endotoxins such as LPS from *E. coli* O55B5 and can partially inactivate its cytotoxic effects (Buts *et al.*, 2006). This mechanism may account for the protection afforded in cases of sepsis (Czerucka *et al.*, 2007). Bacterial enzymatic hydrolysis may enhance the bioavailability of proteins and fats and increase the production of free amino acids, short chain fatty acids (SCFA), lactic acid, propionic acid and butyric acid by lactic acid bacteria. When absorbed, these SCFAs contribute to the available energy pool of the host and may also protect against pathological changes in the colonic mucosa. The SCFAs lowers the pH of the contents of the large intestine, which creates an environment that prevents the growth of harmful bacteria (Topping & Clifton, 2001) and also aids the absorption of minerals, such as calcium and magnesium (Miles, 2007).

IMMUNE FUNCTIONS AND PROBIOTICS

The immune system is extremely complex, involving both cell-based and antibody-based responses to potential infectious agents. Immunodeficiency can result from certain diseases (e.g. cancer, AIDS and leukaemia) or to a lesser extent from more normal conditions such as old age, pregnancy or stress and; autoimmune diseases (e.g. allergies and rheumatoid arthritis) can also occur due to misdirected immune system activity. The majority of evidences reported from *in vitro* systems, animal models, and humans suggest that immune effects caused by probiotics are mediated through activating macrophages, increasing the levels of cytokine production (such as Tumour Necrosis Factor-a, Interleukin-12, Interleukin -18, and Interferon-g in human peripheral blood mononuclear cells), increasing natural killer cell activity and/or increasing levels of immunoglobulins (Perdigon and Alvarez, 1992; Ouwehand *et al.*, 2002). Innate immune responses are induced upon recognition of the conserved molecules produced by the probiotic microorganisms. These molecules, known as microbial pathogen-associated molecular patterns (PAMPs), are recognized by specific host pattern recognition receptors, which include the toll -like receptors (TLRs) and nucleotide binding oligomerization domain (Nod) proteins such as Nod1 and Nod2 (Inohara, and Nunez, 2003). TLRs sense extracellular PAMPs, whereas Nods recognize intracellular PAMPs (Girardin *et al.*, 2002). These receptors initiate NF-kB and MAPK (mitogen activated protein kinase) cascades, which are defense-related transcriptional factors for the production of innate cytokines. Consequently, NK cells are recruited to the site of infection and produce IFN-γ that promotes the activation of macrophages. Therefore, it is likely that the stimulation of TLRs and/or other receptors of host cells by *L. casei* has important consequences in the generation of an immune response that functions to protect the host (Kim *et al.*, 2006).

Furthermore, mechanistic events underlying these effects are now beginning to be understood from *in vitro* studies of host intestinal epithelial or immune cell responses to probiotic strains. Dendritic cells (DCs) play a key role in early bacterial recognition and, consequently, in shaping T-cell responses (Marco *et al.*, 2006). Several bacterial species including known probiotics (VSL#3 and *L. plantarum* 299v) have been reported to differentially induce *in vitro* maturation and cytokine expression of murine DCs, with the possibility to favor T helper-1, T helper-2 or T helper-3 immune responses (Christensen *et al.*, 2002; Hart *et al.*, 2004). These interactions with DCs appear to be at least partly mediated by *Lactobacillus* binding to the pattern-recognition receptor (PRR) DC-SIGN (DC-specific intercellular adhesion molecule 3-grabbing non-integrin) (Smits *et al.*, 2005). *Lactobacillus* interaction with DC-SIGN results in the development of regulatory T-cell populations involved in the production of interleukin-10 (IL-10). These findings clearly suggest that specific *Lactobacillus* strains can activate host immune components, however, the exact mechanism by which host innate immunity is activated remains obscure. Clinical testing has focused mostly on animal studies and that too at immune cell level, and not on actual incidence of disease, therefore it is difficult to extrapolate the findings of immune function studies of probiotics on human health.

LAB-containing functional foods are often included in the diet of immunocompromised patients, and it is known that agents with immunomodulatory activity may induce contrasting effects in normal and immunosuppressed individuals. Animal models and human studies (Table 4 below) provide a baseline understanding of the degree and type of probiotic-induced immune responses (Sanders, 1999).

TABLE 4

Immune Effects Evoked by Probiotic Bacteria in Immunocompetent Humans

Test product	Effect	Reference
Fermented milk (ST) with Donnet-*Lactobacillus johnsonii* La1 (10^8/d or 10^9/d)	↑ phagocytic activity + respiratory burst of peripheral blood leukocytes no effect seen at 10^8/d	Hughes *et al.*, 1999
Yogurt (10^{11} each ST with LB/d) (control: milk)	↑ 2′-5′ A synthetase activity in BMC (as more stable indicator of IFN) in) yogurt group; no effect on IFN-g, IL-1b, or TNF-a	Solis-Pereyra *et al.*, 1997
Lactobacillus GG powder capsules (5 x 10^{10}); placebo: microcrystalline cellulose	↑ IgM secreting cells against: rotavirus vaccine	Isolauri *et al.*, 1995
Fermented milk (10^{11}/d *Lactobacillus casei* Shirota) (control: milk)	No effect on natural killer cell activity, phagocytosis or cytokine production	Spanhaak et al., 1998
Fermented milk with *L. johnsonii* La1 (7 x 10^{10}) or *Bifidobacterium bifidum* Bb12 (10^{10}); no placebo	No effect on lymphocyte subsets, but ↑ phagocytosis of *Escherichia coli* compared to pre-feeding levels	Schiffrin *et al.*, 1995
Lactobacillus brevis subsp. *coagulans* (Labre) tablet, live, and heat-killed, 1.5, 3, and 6 x 10^8/d	↑ a-IFN (as measured by 2′-5′ A synthetase activity) in 3 and 6 x 10^8/d test groups	Kishi *et al.*, 1996
Yogurt (10^{11} each ST + LB/d) (control: milk)	↑ 2′-5′ A synthetase activity in BMC	Solis-Pereyra and Lemonnier, 1993
Fermented milk with 4 x $10^{9\text{-}10}$/d *L. johnsoni* La1 and *Bifidobacterium;* control: diet with no fermented foods	↑ serum IgA response to *Salmonella typhi* vaccine (adjuvant effect)	Link-Amster *et al.*, 1994
Yogurt with 3 x 10^{12}/d ST and LB	↑ serum IFN-g, B lymphocytes and NK cell subset	De Simone *et al.*, 1993

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Test product	Effect	Reference
Lactobacillus GG (2 x 10^{10-11})	↑ rotavirus specific IgA antibody secreting cells in rotavirus-infected infants	Kaila *et al.*, 1992, 1995
2x10^{10} CFU/day dried *Lactobacillus* GG	↑ IgA secreting cells; no change in clinical status of Crohn's disease patients.	Malin *et al.*, 1996
Bifidobacterium lactis formula	↑ IgA	Fukushima *et al.*, 1998

ST, *Streptococcus thermophilus*; LB, *Lactobacillus delbrueckii* subsp. *bulgaricus*; LA, *Lactobacillus acidophilus*; B, *Bifidobacterium* spp.; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; Ig, immunoglobulin; BMC, blood mononuclear cells.

Probiotic bacteria are also involved in down regulating inflammation associated with hypersensitivity reactions in patients with atopic eczema and food allergy (Majamaa and Isolauri, 1997; Murch, 2001; Isolauri, 2004). Probiotics enhances endogenous barrier mechanisms of the gut and alleviate intestinal inflammation, providing a useful tool for treating food allergy (Kalliomaki and Isolauri, 2004; del Miraglia and De Luca, 2004).

The presence of immunomodulatory properties and the pattern of immunomodulation, depends on the bacterial species involved, the individual strain, the use of viable or killed bacteria, and the bacterial dose. Children with HIV infections have episodes of diarrhea and frequently experience malabsorption associated with possible bacterial overgrowth. However, administration of *L. plantarum* 299v strain to immunocompromised hosts has shown a positive effect on immune response and its growth and development (Parvez *et al.*, 2006). The immune response may further be enhanced when one or more probiotics are consumed together which work synergistically, for example the administration of *Lactobacillus* in conjunction with *Bifidobacteria*.

CANCER AND PROBIOTICS

There are some evidences of primary studies reported by various workers that probiotic bacteria reduce cancer risk possibly by counteracting mutagenic and genotoxic effects (Sanders, 1999). In general, cancer is caused by mutation or activation of abnormal genes that control cell growth and division. Most of these abnormal cells do not result in cancer as normal cells usually out-compete abnormal ones. Also, the immune system recognizes and destroys most of the abnormal cells. Many processes or exposures can increase the occurrence of abnormal cells and among many potentially risky exposures, chemical exposures are important one. Cancer-causing chemicals (carcinogens) can be ingested or generated by metabolic activity of microbes that live in the GI system. It has been hypothesized (Parvez *et al.*, 2006) that probiotic cultures might decrease the exposure to chemical carcinogens by:

(i) detoxifying ingested carcinogens;

(ii) altering the environment of the intestine and thereby decreasing populations or metabolic activities of bacteria that may generate carcinogenic compounds;

(iii) producing metabolic products (e.g. butyrate) which improve a cell's ability to die when it should die (a process known as apoptosis or programmed cell death);

(iv) producing compounds that inhibit the growth of tumour cells; or

(v) stimulating the immune system to better defend against cancer cell proliferation.

It is reported that probiotics might be helpful in suppressing the growth of bacteria that convert procarcinogens into carcinogens, thereby reducing the amount of carcinogens in the intestine. The conversion of procarcinogens into carcinogens is mediated by enzymes like beta glucuronidase, azoreductase and nitroreductase etc., the activity of which have been shown to be reduced in the presence of a probiotic strain like *Lactobacillus acidophilus* both in humans and animals. One hypothesis for the prevention or delay of tumour development by lactobacilli is that they might bind to mutagenic compounds in the intestine (Isolauri, 2004), thereby decreasing the absorption of these mutagens.

RISKS OF PROBIOTICS

Treatment with probiotics is relatively safe, but not risk free. A recent report describes 3 patients with fungemia after intake of *S. boulardii* whose probiotic origin has been tested by DNA fingerprinting (Munoz *et al.*, 2005). Reports of infections of probiotic origin emphasize the fact that these patients are usually immunosuppressed with multiple ports of entry, such as venous and urinary catheter. Therefore, it is not recommended to give probiotics to those patients who are at increased risk of translocation-related problems (e.g. central venous catheters, artificial heart valves), those at high risk of developing sepsis (e.g. low white blood cell count), very young infants or those with bowel immotility problems (e.g. using D-lactic acid producing probiotics). The ability of specific probiotic strains to survive gastric conditions and to adhere strongly to the intestinal mucosa following oral administration may entail a risk of bacterial translocation, bacteraemia and sepsis (Table 5) (Isolauri *et al.*, 2004).

STATUS OF PROBIOTICS IN INDIA

In India, probiotics are often used as animal feed supplements for cattle, poultry and piggery. This requirement is also met by importing probiotics from other countries. It is rarely used for human beings. Sporolac, *Saccharomyces boulardii, L. bulgaricus and L. thermophillus* are the most commonly tested probiotics. Sporolac is manufactured using *Sporolactobacilli*. Lactobacilli solution is an example of a probiotic, usually given to paediatric patients in India. The latest and recent addition to the list of probiotics in India is ViBact (which is made up of genetically modified *Bacillus mesentricus*), which acts as an alternate to B-complex capsules. In India, only sporulating lactobacilli are produced and they are sold with some of the antibiotic preparations (Suvarna and Boby, 2005). Some of the Indian companies have started incorporating probiotics in certain food products like ice-cream (Amul-Prolife), fruit juices, drinks etc.

TABLE 5

Potential Clinical Targets of Probiotic Intervention

Effect	Potential mechanism	Potential risks
Nutritional management of acute diarrhoea	Reduction in the duration of rotavirus shedding, normalization of gut	Permeability and microbiota Risk related to host and strain characteristics
Nutritional management of allergic disease Inflammatory bowel disease	Degradation/structural modification of enteral antigens, normalization of the properties of aberrant indigenous microbiota and of gut barrier functions, local and systemic inflammatory response, increase in the expression mucins	Strains with proinflammatory effects/adverse effects on innate immunity Translocation/infection
Reducing the risk of infectious disease	Increase in IgA-secreting cells against rotavirus, the expression of mucins	Risk related to host and strain characteristics
Reducing the risk of allergic/inflammatory disease	Promotion of gut barrier functions, anti-inflammatory potential, regulation of the secretion of inflammatory mediators, and promotion of development of the immune system	Directing the microbiota towards other adverse outcomes/directing the immune responder type to other adverse outcomes

TRENDS AND FORECAST

The increased worldwide interest in probiotics has set the stage for expanded marketing of these products, even though much research remains to be done. It is known that gut bacteria, including probiotic bacteria, play a pivotal role in determining both quantitative and qualitative characteristics of mucosal immunity. To continue to use the term 'probiotic' for those bacteria which promote health by 'restoring mucosal T cell balance' would appear outmoded, out of step with contemporary thinking, and potentially confusing when communicating about immune-regulating bacterial species. It is suggested that a new term 'immunobiotics', identifying those bacteria that promote health through activation of the mucosal immune apparatus, is an appropriate evolutionary step as our knowledge base expands regarding host-parasite relationships and their outcomes, as they relate to health and disease. Recognition of bacteria that promote mucosal T cell function as 'immunobiotics' may move probiotic biology forward by focusing on a mechanism of outcome, i.e. Immunomodulation at distant mucosal sites. It does not, however, diminish the need to heed longstanding concerns regarding the biology and the accreditation of these bacteria as health enhancing agents.

The field of clinical research on probiotics is currently expanding to include studies of effects on the skin, joints, liver disease and obesity. In the latter condition, for example, very recent basic studies have shown that the conventional gut microbiota may induce a number of changes in gene expression relating to body fat accumulation through interaction with the epithelial expression of a fasting-induced adipocyte factor, e.g. angiopoietin like protein. This new identified factor inhibits lipoprotein lipase activity and triglyceride storage in adipocytes and interferes with insulin sensitivity (Walker *et al.*, 2006). Much work remains to be done.

In addition to a therapeutic role, certain probiotics have also been shown to function as effective prophylactic agents, being specifically engineered to function as novel vaccine delivery vehicles. Stimulating both innate and acquired immunity, these strains lack the possibility of reversion to virulence, which exists with the more conventional pathogenic platforms currently in development. For instance, Guimaraes et al. (2005) described the construction of a *L. lactis* strain which expresses inlA gene meant for coding internalin A, a surface protein related to invasion in *L. monocytogenes*. This may act as a safe and attractive alternative to attenuated *L. monocytogenes* which is being used as an antigen-delivery vehicle in the intestine. Probiotic vaccine carriers administered by the mucosal route mimic the immune response elicited by natural infection and can lead to long-lasting protective mucosal and systemic responses (Holmgren and Czerkinsky, 2005). Mucosal vaccine delivery (those administered orally, anally or by nasal spray) also offers significant technological and commercial advantages over traditional formulations, including reduced pain and the possibility of cross-contamination associated with intramuscular injection and the lack of a requirement for medically trained personnel to administer the vaccine

One exciting area of current research is chromosome sequencing of probiotics such as *Lactobacillus* species, including *L. johnsonii* La1 and *L. acidophilus*. The information gleaned from sequence data will provide opportunity to improve probiotic functionality and expand understanding of mechanisms. A knowledge of the mechanism by which defined probiotic strains contribute to health, allows the rational design of probiotic micro-organisms through genetic engineering to improve probiotic efficacy by enhancing a strain's ability to cope with stress at the genetic level. In addition to improving probiotic stress tolerance, recent studies have led to the development of 'designer probiotics' - strains tailored to target specific pathogens and / or toxins. The most significant application of designer probiotics to-date include the treatment of HIV (AIDS) and enteric infections (Sleator and Hill, 2008). The design of the new probiotic should, therefore, be such that safety is guaranteed often in its use. Furthermore, recent developments in synthetic and systems biology, based on the rapidly advancing 'omics' technologies, may continue to lead to the emergence of an ever-increasing number of novel genetic loci with defined additional functions. This coupled with computer aided bioinformatics and novel tools for genetic modification, may ultimately lead to the development of artificial micro-organisms (Lartigue *et al.*, 2007) and eventually to a new class of probiotics assembled from the components of various origins and tailored to fulfill all the requirements of an ideal therapeutic agent.

CONCLUSIONS

Today probiotics are available in a variety of food products and supplements. Probiotics are gaining importance because of the innumerable benefits, e.g. treating lactose intolerance, hypercholesterol problem, cardiac diseases and managing cardiac problems like atherosclerosis and arteriosclerosis. With the current focus on disease prevention and the quest for optimal health at all ages, the probiotics market potential is enormous. Health professionals are in an ideal position to help and guide their clients toward appropriate prophylactic and therapeutic uses of probiotics that deliver the desired beneficial health effects. There are many probiotic products at the market place and most have supporting evidence behind the advertised health claims. However, if the positive evidence looks promising, it has to admitted that there are some negative aspects that should not be forgotten. Key message that could possibly be communicated to consumers on probiotic products is: 'probiotic bacteria contribute to the normal functioning and healthy microbial balance of the digestive system'. However, different strains of probiotic bacteria exert different effects, and this is likely to pose a challenge for the development of more specific health claims. It is difficult to draw firm conclusions to substantiate health claims, because the strains and dosages used in studies vary greatly (Santosa *et al.*, 2006). Excessive intake, apart from leading to slight intestinal upsets may pose a problem in exceptional circumstances. Different strains have different actions in different clinical situations. Probiotics play a definite role in a number of clinical situations, namely rotavirus diarrhoea, post antibiotic diarrhoea and pouchitis. Their role in other clinical situations is yet to be defined. There is a need for refinement of *in vitro* tests to predict the ability of probiotics to function in humans. Many studies investigating the effects of probiotics have been conducted *in vitro* or in animals, and it does not necessarily follow that the same results will be found in humans. There is therefore a need for more randomised controlled trials (blinded) in this area, particularly looking at dose-dependency and duration of effect of specific probiotic strains. Compared with the efficacy of probiotics in human trials, the work on mechanisms through which probiotics can work is better understood. As probiotics work via multiple mechanisms, it is unlikely that resistance against probiotics will develop, and they are unlikely to increase the incidence of antibiotic resistance. New legislation governing the labelling of probiotics, such as indicating the species, strain and number of bacteria present is likely to come into force in the near future. Probiotics should not be considered a panacea for health, but can be incorporated into a balanced and varied diet to maximize good health.

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BIOTECHNOLOGY FOR IMPROVED RUMEN MICROBIAL ECOSYSTEM AND RUMINANT PRODUCTIVITY: SOME APPLICATIONS

RAVINDER NAGPAL, ANIL KUMAR PUNIA AND KISHAN SINGH

As the human population increases, a large variety and quantity of waste products is produced which can be consumed by animals but consequently the land available for both housing and agriculture is reduced. The nutrient energy in most of these by-products is not readily available and is not sufficient, even for the ruminant digestive system. This is especially the case for cellulosic materials which have slower rates of digestion because the cellulose is in a highly organized and complex form. Researchers are now trying to manipulate the rate of digestion of various feeds to favour both humans and ruminants, and to accelerate those rates that are slow, while delaying those with a high initial rate of nutrient release which can cause digestive disturbances (McSweeney *et al*, 1999). Theoretically, it is conceivable that up to 90% of ruminant feed could be made up of by-products which have been modified to improve digestibility. In practical terms, the benefits from the successful attainment of the objectives would be (i) to make underutilized by-products available as ruminant feed, (ii) to improve the digestibility and efficiency of utilization of these by-products for animal feed, and (iii) to reduce the requirement for hay and animal feed products, thus freeing land for more valuable crops. In India, ruminants are kept by small farmers on a small scale and the production systems are closely integrated with farming systems. One of the limiting factors for the expansion of production is the available feed resources, as under such farming systems, the farmers do not have adequate feed resources or the capacity to increase available feed. Usually agricultural by-products are utilized for feeding domestic ruminants. Given the present decline in the area available for forage production, ruminants have to depend more and more on fibrous residues and by-products for their energy sources. These by-products still have problems to be solved and many of them cannot be used without some modifying/ detoxifying treatments. However, considering the quantities of the by-products and the fact that tropical ruminants are better converters of low quality feed into milk and meat (Abdullah 1987), the by-products are likely to play an important role as potential feed sources in ruminant nutrition. Hence, an increase in understanding of rumen microbial ecology has the potential to make a major impact on feed utilisation in the parts of the world where cellulosic roughages are likely to be the most important feed for ruminants and in areas where higher quality supplements, e.g. brans and oilseed cakes, are scarce. A better understanding of rumen ecology can lead to: i) a better strategic use of scarce supplements so that they can be utilized not only as supplements in their own right but also provide support for basal feeds; and ii) greater production of rumen microbial proteins so that animals can grow better on basal feeds alone without a need for protein supplements. Gene-based technologies have the potential to improve the nutritive value of ruminant feedstuffs that are fibrous, low in nitrogen and contain antinutritive factors. Until recently, our knowledge of rumen microbiology was primarily based on classical culture based techniques (isolation, enumeration and nutritional characterization) which probably only account for 10 to 20% of the rumen microbial population. Conventional culture-based methods of enumerating rumen bacteria are being rapidly replaced by the development of nucleic acid based techniques which can be used to characterise complex microbial communities. The foundation of these techniques is rDNA (eg. 16S rRNA sequences) sequence analysis which has provided a phylogenetically based classification scheme for enumeration and identification of microbial community members. The 16S rRNA sequences in DNA extracted from a mixed digesta sample can be amplified by polymerase chain reaction (PCR) and the diversity and identity of the amplified DNA can be further analysed by several molecular techniques including: 1) restriction enzyme analysis of amplified polymorphic DNA (RFLP); 2) 16S rDNA based cloning, sequencing and probing; and 3) denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and single strand conformation polymorphism (SSCP). Quantitative estimates of microbial populations can also be performed by amplification of rDNA with specific primers using real time PCR.

RUMEN AND ITS FIBROLYTIC MICROBES

Ruminants posses a complex stomach system, in which the stomach is divided into four compartments, the first and the largest of which is the rumen followed by reticulum, omasum and abomasum. The rumen is the dominant feature of the digestive tract of cattle. This maintains a medium that supports a dense and varied population of microorganisms. These organisms ferment feed materials to produce mainly short-chain organic acids or volatile fatty acids, methane and carbon dioxide and the process provides substrate (feed) and energy (ATP) for the growth of micro-organisms. It is here that continuous anaerobic fermentation takes place by a complex community of microorganisms. The rumen is an extremely complex community of many microorganisms, protozoa, bacteria, fungi and probably other unknown microorganisms. The microbial mix in the rumen is complex and highly dependent on diet. Although a definite reason has not been found, it is known that some ruminants can utilize poor quality feed more efficiently. The main agents that break down fibre, sugars, starches and proteins in the rumen are all anaerobic and include bacteria, protozoa and fungi. The bacteria are the principal organisms that ferment plant cell-wall carbohydrates (Hungate, 1966) but the anaerobic phycomycetous fungi may at times be extremely important (Bauchop and Mountfort, 1981).

The rumen harbours various types of bacteria which are active in degradation of plant cell-wall components of the feeds. Some predominant ruminal bacterial species are *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens, Eubacterium ruminantium, Ruminobacter amylophilus, Streptococcus bovis, Succinomonas amylolytica, Prevotella ruminocola, Prevotella albensis, Prevotella brevis, Prevotella bryantii, Butyrivibrio fibrisolvens, Selenomonas ruminantium, Megasphaera elsdenii, Lachnospira multiparus, Succinivibrio dextrinosolvens, Anaerovibrio lipolytica, Peptostreptococcus anaerobius, Clostridium aminophilum, Clostridium sticklandii, Wolinella succinogenes,* and *Methanobrevibacter ruminantium.* It is natural to assume that fibre-digesting bacteria in the rumen are of primary importance when ruminants feed on poor quality material of which cellulose and hemicellulose can comprise about 80% in roughly equal proportions (Kamra, 2005). The cellulolytic bacteria provide fermentable energy sources both for themselves and other bacteria. These rumen bacteria can ferment soluble and even poorly soluble hydrolysis products of cellulose (Russel, 1985), thus demonstrating the reliance of the non-fibre digesting bacteria on fibrolytic ones. *Fibrobacter succinogenes* and the ruminococci are considered to be the most active in cellulose digestion. The hemicellulolytic activity of *F. succinogenes* and *Ruminococcus flavefaciens* appears to be directed mainly towards exposing cellulose through removal of hemicellulose because the mono- and oligosaccharides of this fibre component cannot be utilised by these bacteria and are available for others. In particular, *F. succinogenes* appears to occur in high numbers (10% or more of 'total culturable' bacteria) in the rumen of animals feeding on material containing highly crystalline forms of cellulose such as wheat straw or maize straw (VanGylswyk and Van der Toorn, 1986). *Butyrivibrio fibrisolvens* can comprise more than half of the more active hemicellulolytic bacteria in the case of the latter diet (Russel, 1985).

The rumen protozoa, first observed by Gruby and Delafond, (1843), belong to the class ciliatea and are of two types: Entodiniomorphic and Holotrichous. The majority of ciliate protozoa present in the rumen belong to entodiniomorphid group. Some of the major genera are *Entodinium, Eodinium, Metadinium, Epidinium, Diploplastron, Polyplastron* etc. They have a firm pellicle, and cilia are situated only on peristome and sometimes other parts of the cell. However, holotrichous protozoa such as *Isotricha, Dasytricha, Charonina, Polymorphella* etc., have cilia all over their body, but their numbers are very small. These protozoa are widely distributed in the rumen of domestic and wild ruminants and hind gut fermentors (Dehority and Orpin, 1996). However, only eight genera of ciliate protozoa have been commonly observed in the rumen of cattle and buffalo fed on wheat straw and concentrate mixture, e.g. *Isotricha, Dasytricha, Metadinium, Diplodinium, Eudiplodinium, Ophryoscolex, Entodinium,* and *Epidinium* (Sahu and Kamra, 2001; Kamra, 2005). The enzymatic profile of holotrich protozoa indicates that they have amylase, invertase, pectin esterase and polygalacturonase in sufficiently large quantities for using starch, pectin and soluble sugars as energy source. The enzymes responsible for cellulose and hemicellulose degradation have also been reported in the holotrich protozoa but the levels are very low compared to those present in the entodiniomorphid protozoa (Franzolin and Dehority, 1999).

Rumen anaerobic fungi, discovered for the first time by Orpin (1975), also contribute significantly to the prime function of the rumen, which is the digestion of plant cell walls to provide fermentation products for the nutrition of the host animal (Nagpal *et al*, 2007a, b, c). It is now well established that anaerobic fungi are extremely important for the voluntary feed intake of poor quality, mature herbage by sheep (Gordon and Phillips, 1993). All the species of rumen fungi so far isolated are capable of fermenting structural carbohydrates of plant cell walls. One of the characteristics of fungi is their rhizoidal penetration into the plant material, colonising the schlerenchyma and vascular tissues, eventually degrading the schlerenchyma (Ho *et al*, 1988). The rhizoidal system attaches to the more recalcitrant vascular tissue, resulting in the fungus remaining attached to this tissue despite the degradation of surrounding cells. In this way, the fungus remains attached to the tissue and is not washed out of the rumen with the liquid phase of the rumen contents. Together with the anaerobic bacteria and ciliate protozoa, they are responsible for the production of hydrolytic enzymes which degrade dietary polysaccharides and other carbohydrates and the fermentation of the resulting monosaccharides. An important role for these unusual microorganisms in ruminant nutrition has been proposed over the course of the past decade or so. The significance of this role is based largely on *in-vitro* studies of anaerobic fungi (Lee *et al*, 2004; Thareja *et al*, 2006; Tripathi *et al*, 2007a). However, there is very little direct evidence from studies with animals to support this position until recently (Dey *et al*, 2004; Paul *et al*, 2004; Tripathi *et al*, 2007b). Hence, a considerable potential exists for the selection and intra-ruminal inoculation of superior strains of anaerobic fungi obtained from either domesticated or wild herbivorous mammals.

FACTORS AFFECTING RUMINAL DIGESTION OF FEEDS

Animal production is very reliant on natural pastures as well as on other plant material often of poor quality. The role of fibre-digesting flora in the rumen of animals feeding on such material is particularly important in releasing fermentable carbohydrate both for themselves and other microbes. In many parts of India, natural grazing is often the most important or even the sole source of nutrition for ruminants. Where available, cereal straws are used as well as any type of plant material (shrubs and trees) including crop residues. For large parts of the year, the grazing can be dry and of very poor quality, characterized by high fibre content of low digestibility and very low protein content. Such material cannot maintain the condition of ruminants. In periods of drought, even this material is in short supply. Therefore, there is a need to look at some ways by which such low quality material could be upgraded and related to the rumen ecology. An optimal rumen environment generally ensures maximal microbial yield and energy utilization (Orskov and Viglizzo, 1994). Since most of the ruminant livestock in developing countries feed mainly on cellulosic crop residues, pasture, trees and bushes, it is important that the fermentation process be unimpeded. Forages differ greatly in their composition and structure. Temperate and tropical forages are covered on the outer surface by a thin waxy cuticle, which contains a tough basal polyester layer. Immediately below this is the plant cell, in which the cell walls account for the bulk of the aerial portion of the plant. The cell wall is composed primarily of cellulose and hemicellulose, with a small portion of lignin (Van Soest, 1982). The cuticular surface layer presents a formidable barrier to invasion by rumen microorganisms. The cutin layer appears to be totally resistant to microbial digestion within the rumen, except for some rumen fungi which may penetrate it (Ho *et al,* 1988). The ester linkage of cutin is hydrolyzed by some fungi and some aerobic bacteria. Most of the usable plant nutrients are internal. External plant tissues are only poorly colonized by rumen microorganisms while the inner tissues are heavily colonized. However, physical disruption (e.g. chewing) is necessary to allow optimal microbial access to the inner tissues which are then avidly colonized (Cheng *et al,* 1990).

For the digestion of cellulose and starch, attachment to these insoluble substrates is a pre-condition for rumen microbial population, if digestion is to proceed (McAllister *et al,* 1990). When these substrates are placed in the rumen, they attach to substrates very rapidly. Cheng and Costerton, (1980) postulated that rumen microorganisms can be classified into three groups: (i) microorganisms attaching to the rumen wall; (ii) microorganisms living freely in the rumen; and (iii) microorganisms attaching to feed particles. In the rumen, as much as 75% of these microorganisms are attached to feed particles. Microorganisms in the rumen have a variety of surfaces to which they may attach, and a distinct population of microorganisms adheres to each different surface. From an ecological viewpoint, bacteria with the ability to attach to feed particles have a great advantage over non-attaching microorganisms, which can flow more quickly from the rumen. The importance of the adhesion of microorganisms to substrates suggests that there is a possibility for better feed efficiency if we can increase the attachment. Pure cultures of cellulolytic bacteria and fungi digest cellulose *in-vitro* but digestion does not proceed at a similar rate to that seen in the rumen unless consortia are formed with non-cellulolytic *Treponema bryantii, Butyrivibrio fibrisolvens* and methanogenic bacteria. Cellulolytic bacteria interact with non-cellulolytic bacteria to promote the digestion of cellulosic materials (Kudo *et al,* 1987). Both protozoa and fungi may be active members of consortia (Kudo *et al,* 1990). Thus, the microbial ecology of the rumen is obviously very complex, including many interactions of microbial species and even though primary cellulolytic organisms may be present in a system, many other factors may be required to facilitate actual cellulose digestion.

SCOPE OF BIOTECHNOLOGY FOR BETTER RUMEN PERFORMANCE

Biotechnology is finding its way into many areas of agriculture and animal production through feed and crop plants, through the genetic pool of animals, and through manipulation of microbes that influence the growth and development of both animals and plants. Agricultural biotechnology offers a collection of technologies that involve the application of biologic processes and living organisms, or parts thereof (tissues, cells or enzymes) in the livestock, pharmaceutical and chemical industries, as well as for environment control (Falk *et al,* 2002). Rumen biotechnology applies the available knowledge on forestomach fermentation and the use and management of both natural and recombinant microorganisms to improve the efficiency of digestion of fibrous feedstuffs by ruminants (McSweeney *et al,* 1999). Methods for manipulating ruminal fermentation by biotechnology involve: microbial and anti-microbial feed additives (e.g., ionophores, antibiotics and microbial feed additives), introduction of genetically modified microorganisms in the rumen (cloning of genes from ruminal microorganisms, expression of foreign DNA in ruminal bacteria and establishment of new organisms in the rumen), implementing molecular techniques, rumen defaunation, and the introduction of foreign microbial species (Dominguez-Bello and Escobar, 1997). The main applications of biotechnology in ruminant nutrition have been for enhancing the nutritive value and quality of feeds, and to optimize the nutrients in the intestinal tract by using enzymes, silage inoculants, minerals, amino acids, hormones, and direct-fed microbials (DFM). Applications are being developed for improving the performance of animals through better nutrition. Enzymes can improve the nutrient availability from feedstuffs, lower feed costs and reduce output of waste into the environment. Prebiotics and probiotics or immune supplements can inhibit pathogenic gut microorganisms or make the animal more resistant to them (Nagpal *et al*, 2007d). The direct genetic manipulation of microbes that inhabit the rumen may be proposed as a possible mechanism for enhancing animal production. Nevertheless, the application of molecular biological techniques is beginning to contribute to a better understanding of the rumen through the precise methods now available for making *in vivo* observations. Molecular techniques now allow individual micro-organisms to be tracked in the rumen, and identification of organisms by genetic criteria is rapidly becoming an essential part of taxonomy, and has greatly facilitated studies of their populations *in vivo* (Gregg *et al*, 1993). Techniques are now available for addition of novel genes to rumen bacteria, using plasmid vectors and electroporation. Hence, expectations for the application of biotechnology are now considerably more confident. As studies of ruminal ecology become more precise, the contribution of biotechnology methods to animal production will become continually more significant. However, in the specific area of rumen bacterial genetic manipulation, the most significant developments are very recent and have so far made little impact upon how this technology is regarded.

BIOTECHNOLOGY FOR ENHANCED ANIMAL NUTRITION AND PRODUCTIVITY: SOME APPLICATIONS REVIEWED

Limitations to ruminant production include poorly digestible fibrous plant material, nitrogen losses and toxic compounds present in the feed. In tropical regions, these limitations can be so great as to become critical to the survival of ruminant animals in that environment. The fibre content of many tropical grasses is high and digestibility of the feed is low (McMeniman *et al*, 1981). Feed supplements are often unavailable or expensive. Anti-nutritive components are also very prevalent in tropical plants and these restrict the forage range for domestic livestock. Many of these limitations may be overcome, or at least alleviated by manipulation of rumen microbial populations. The rumen can be manipulated in two fundamental ways: firstly by the application of genetic techniques to modify the functional capacity of specific bacterial species; and secondly, through modification of rumen ecology by introducing novel bacterial species or by selectively enhancing populations of existing species.

Direct-fed Microbials/Microbial Feed Additives

In recent past, the use of live microbial feed additives to improve the efficiency of ruminant's production has increased in light of demands for natural growth-promoting agents. On these lines, direct-fed microbials (DFM) have been used to improve animal performance either by manipulation of rumen environment or by directly altering the metabolic activities of microbes in the rumen microenvironment. Research on the effects of DFM in young ruminants has mainly involved addition of *Lactobacillus* and *Streptococcus,* as intestinal inoculants to suppress diarrhea and to improve the growth rate. The efficacy of bacterial DFM has been extensively studied in the neonatal dairy calf. Bacteria based DFM i.e. *Lactobacillus, Enterococcus, Streptococcus,* and *Bifidobacterium,* have been studied in young calves, (Newman and Jacques, 1995) and the importance of these when fed to young calves has been well established in maintaining normal rumen microflora. Jaquette *et al,* (1988) and Ware *et al,* (1988) reported increased milk production from cows when fed *L. acidophilus* @ 1 x 10^9 cfu animal^{-1} day^{-1}. They also reported that cows fed *Lactobacilli* during transition period produced more milk, and had lower non-esterified fatty acids and higher glucose in blood than controls. Ghorbani *et al,* (2002) reported that steers receiving both lactate-utilizing *Propionibacterium* and *Enterococcus* had higher concentrations of ruminal acetate indicating a reduced risk of metabolic acidosis. However, most of the research work on DFM with lactating dairy cows has involved dietary supplementation with either *Aspergillus oryzae* or *Saccharomyces cerevisiae* (Newbold *et al,* 1995). *A. oryzae* mainly increases cellulolytic bacteria, changes volatile fatty acids fermentation favorably and stabilizes rumen pH. *S. cerevisiae* reduces rumen ammonia and increases growth of rumen bacteria. Recently, experiments with feeding of anaerobic fungi have also been shown to improve the fibre degradation (Paul *et al,* 2004; Dey *et al,* 2004; Lee *et al,* 2004; Thareja *et al,* 2006; Tripathy *et al,* 2007a, b; Nagpal *et al,* 2007a, b, c). There were significant increases in dry matter intake, body weight, growth rate, milk production, and milk fat/ protein percentage in some trials. The cause of the variable response may be related to the type of DFM fed; and more research is needed to understand the exact mode of action of these microbials.

Genetically Modified Microorganisms in the Rumen

There has been considerable reservation about the contributions that can be made by manipulating the genetics of rumen bacteria (Beard *et al,* 1995), and the basis for this reservation includes at least four areas of concern: i) Genetic manipulation of rumen bacteria has not been demonstrated as a reproducible and reliable technique, ii) bacteria grown in the laboratory are unlikely to retain their competitive features and are unlikely to return successfully to the rumen, iii) genetic changes to bacteria are likely to impair their competitive fitness, particularly if the changes do not directly benefit the bacterium itself, and iv) addition of an artificially manipulated bacterium to the rumen may upset the balance of the rumen, with detrimental effects on the host animal. However, data are now being accumulated to indicate that, at least in some cases, these difficulties may have been overestimated. There are now several systems by which new genetic material can be introduced into rumen bacteria as plasmids, and many different approaches have been made to the transformation of rumen bacteria, and most have shown very limited success. The technique that has proven generally useful for inserting new DNA is that of electroporation using an electrical pulse to generate openings in the cell wall, through which DNA can enter (Dower *et al,* 1988). Combining this with plasmids originating from the bacterial species to be modified has led to successful processes reported (Beard *et al,* 1995). The best example of the successful introduction of a new (but not genetically modified) organism in the rumen was the introduction of bacteria that degraded 3-

hydroxy-4(1H)-pyridone (DHP) into Australian ruminants (Dawson and Allison, 1988). These animals were unable to use *Leucaena leucocephala* because the non-protein amino acid, mimosine, was converted to DHP, but no further. The DHP then had toxic effects in the host. However, when the animals were inoculated with ruminal fluid from goats adapted to consume *L. leucocephala*, the DHP was further metabolized, thus avoiding the toxicity of effects of DHP. Also of interest are reports that certain species of ruminal organism can be used to inoculate the rumen to enhance rumen function. For example, when *Megasphaera elsdenii* was added intraruminally, a large inoculum decreased the severity of acute acidosis in cattle and smaller doses enhanced recovery of ruminal pH (Robinson *et al*, 1992). Inoculation of lambs with cultures of the rumen fungus, *Neocallimastix frontalis*, promoted rumen function and lead to earlier weaning (Theodorou *et al.*, 1990). In a study by Gregg *et al*, (1998), *Butyrivibrio fibrisolvens*, transformed with a gene encoding fluoroacetate dehalogenase, was inoculated in the rumen of sheep, and the animals showed markedly reduced toxicological symptoms of fluoroacetate poisoning.

With regard to fibre degradation in the rumen, much effort has been made in developing genetically modified bacteria that would have better fibre-degrding abilities. The construction of genetically modified bacteria has proceeded under the supposition that the rumen microbiota does not produce the precise mixture of enzymes to maximize plant cell-wall degradation. For e.g. *Ruminococcus* and *Fibrobacter* do not produce exocellulases that are active against crystalline cellulose, so adding this activity would make them more potent (Teather *et al*, 1997). An alternative approach could be to create recombinant becteria that can degrade fibre at low pHs. It is well known that fibre digestion declines in animals on high-grain diets because the pH of the rumen drops below 6.5 (Goad *et al*, 1998) and *Ruminococcus* and *Fibrobacter* are sensitive to even mildly acidic pH (Russel and Dombrowshi, 1980). Hence, addition of fibrolytic activity to an acid resistant species such as *Prevotella* would create an organism which would be far more competitive because it would be filling an acidic niche which autochthonous cellulolytic bacteria are unable to fill (Russel and Wilson, 1996). There have been many reports of genes being cloned from ruminal microorganisms into other expression systems. Genes have been cloned from many of the predominant species of ruminal bacteria as well as from the ruminal anaerobic fungi, by Gilbert *et al*, (1992) giving fascinating insight into the variety of enzyme activities necessary to digest plant fiber. It does seem, however, that there are factors governing the survival of bacteria in ruminal fluid that has not been understood yet. Furthermore, genetic modifications may themselves impose a metabolic burden on the host organism, making it less competitive than the wild type. The presence of bacteriophages, mycoplasmas, and bacteriocin-like toxins is well known (Klieve and Bauchop, 1988). What is much less clear is the effect these have on the survivability of different strains and on the metabolic inefficiency this may cause in ruminal fermentation. It may also be possible to devise a selection strategy whereby an ecological niche is effectively created for the new organism *in vivo* by adding certain materials to the feed. A further problem of using indigenous ruminal bacteria as vehicles for manipulating ruminal fermentation is their obligately anaerobic physiology. Growth and inoculation with a suitable culture, while straightforward in the anaerobic microbiology laboratory, would not easily lend itself to farm practices, particularly if repeated inoculation proved necessary. Thus, there are several problems to be solved associated with introducing and establishing modified ruminal organisms that will grow and produce new gene products *in vivo*.

Exploitation of Rumen Microbial Enzymes to Benefit Animal Production

The rumen is increasingly being recognized as a particularly promising source of superior fibrolytic enzymes. Cellulases and xylanases produced by ruminal fungi are among the most active fibrolytic enzymes described to date (Trinci *et al*, 1994; Thareja *et al*, 2006; Tripathi *et al*, 2007a). The quest to elucidate the mechanisms of fibre digestion and to find more efficacious enzymes for industrial applications, and technological developments which now allow the genetic manipulation of rumen microorganisms have inspired the cloning of a growing number of genes from ruminal bacteria and fungi. At least 75 different genes, the majority of which encode enzymes with a role in fibre digestion, have been cloned thus far from ruminal microorganisms. Most of these have been isolated from a small number of bacterial species, including *R. albus*, *R. flavefaciens*, *F. succinogenes*, *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* (Wallace, 1994). Researchers have only recently turned to the study of the genetics of anaerobic fungi isolated from the rumen. Enticed by their powerful fibrolytic activity and ability to utilise the most recalcitrant of plant cell wall polymers, researchers have cloned at least 27 genes from four fungal species. The cloned genes include five cellulases (Black *et al*, 1994; Xue *et al*, 1992a, 1992b; Zhou *et al*, 1994) and three xylanases (Black *et al*, 1994; Gilbert *et al*, 1992; Tamblyn Lee *et al*, 1993) from *Neocallimastix patriciarum*, an endoglucanase from *Orpinomyces joyonii*, eight cellulases, four xylanases and five mannanases from *Piromyces* sp. (Ali *et al*, 1995) and a phosphoenolpyruvate carboxykinase from *N. frontalis* (Reymond *et al*, 1992). As competition in the livestock industry increases, producers are actively seeking technological advances through which to increase their production efficiency. Supplementation of animal diets with fibrolytic enzymes, such as cellulases, xylanases and glucanases, increases their feed conversion efficiency and growth rate. These enzymes enhance the release of sugars from plant cell wall polymers, thereby increasing their availability to the animal and eliminating some of their naturally occurring anti-nutritional effects. Consequently, such enzyme supplementation can produce significant improvements in ruminant production, even though the rumen microbial population is known to produce an extensive array of potent endogenous fibrolytic enzymes. Over the last decade, advances in recombinant DNA technology have significantly improved microbial production systems. In addition, the rumen has been recognised as a rich, alternative source of genes for industrially useful enzymes and novel strategies are being developed for effective delivery of these gene products. Thus, the biotechnological framework is in place to achieve substantial improvements in animal production through enzyme supplementation. There is a growing trend in the livestock industry to supplement diets with fibrolytic enzymes. Amending rations with cellulases and/or xylanases increases the feed efficiency of livestock. Enzymatic hydrolysis of cellulose and xylan to simple sugars (e.g. glucose and xylose) provides the animal with carbon sources that are normally not made available by intestinal enzymes. Furthermore, enzymes eliminate certain forms of these polymers (e.g. arabinoxylan, found in wheat and rye; _-glucan, in barley and oats) that may have deleterious effects on nutrient absorption and promote intestinal disturbances by pathogenic enteric microorganisms. Enzymes produced by other microorganisms, including aerobic fungi and protozoa, also contribute to fibre degradation.

Introduction of Transgenic Plants and Animals

Recent advances in plant biotechnology may revolutionise the commercial enzyme industry by offering alternative, cost-effective methods of enzyme production. Large quantities of plant biomass can be produced inexpensively through the use of existing agricultural infrastructure. Expression of enzymes in plant species commonly used for animal feed will minimise downstream processing as the whole or parts of the producing plants are fed directly to livestock. The expression of a xylanases in tobacco plants has been reported, as has the expression of a phytase in tobacco and soybean (Russell, 1994). Herbers *et al,* (1995) achieved the constitutive expression of a truncated *Clostridium thermocellum xynZ* gene encoding a thermostable, high specificity xylanase in tobacco via the cauliflower mosaic virus (CaMV) 35S promoter. In similar experiments, transgenic tobacco plants were made to express phytase encoded by the *Aspergillus niger phyA* gene (Pen, 1994). Feeding trials demonstrated that transgenic tobacco seeds were as effective at promoting growth of broilers as was a commercial *A. niger* phytase product or inorganic phosphorus (Pen, 1994). In addition to providing an efficient alternative to traditional microbial systems, transgenic plants offer the added advantage of a safe and stable formulation system in the form of seeds.

Moreover, technological developments enabling introduction of genetic material into domestic animals (Briskin *et al,* 1991) have validated the concept of direct expression of these glucanases and xylanases in the animal itself as an option to adding microbial enzymes to the feed. To be of benefit to the animal, the enzymes should be expressed in the appropriate tissue, secreted into the lumen of the gastrointestinal tract, resistant to proteases, and active in environmental conditions (e.g. pH, temperature, osmolarity) prevailing in the lumen (Hall *et al,* 1993). Expression of fibrolytic enzymes in a non-ruminant animal was first demonstrated by Hall *et al,* (1993). A truncated endoglucanase gene from *Clostridium thermocellum* was expressed in the exocrine pancreas of transgenic mice. Carboxymethylcellulase activity was detectable in small intestinal contents, demonstrating that the recombinant enzyme was secreted from the pancreas. The obvious potential of this approach is a powerful incentive for continued efforts in this field of research. The potential for improvement of the efficiency of ruminant livestock feed utilisation by enzyme supplementation is widely recognised. However, the cost of this technology has inhibited its widespread application in the industry. Reducing the cost of feed enzymes through improved enzymes and more efficient production systems is the focus of much research in this area. Microbial isolates from the rumen produce a wide array of enzymes and comprise an immense and under-utilised gene pool. As exploration of this enzymological and genetic resource progresses from domestic ruminants to those adapted for survival in diverse habitats worldwide, the isolation of even more potent enzymes is certain.

Improving Ruminant Diets

In the 1960s, several studies were conducted to examine the efficacy of adding enzyme preparations to diets for ruminants. Clark *et al,* (1961) found that enzyme supplementation improved average daily gains by as much as 20%. Improvements in fermentation technology and the biotechnological development of more defined enzyme preparations have prompted a renewed interest in the use of enzymes in ruminant diets. More recent studies have attempted to define the conditions in a given feed that are most likely to result in a favourable animal response to enzyme supplementation (Feng *et al,* 1992). Factors such as substrate specificity of the enzymes, moisture level of the feed, the time required for enzyme-substrate interaction and temperature at time of treatment are all likely to influence the extent to which enzymes enhance the utilisation of feeds by ruminants. The binding of enzymes to their appropriate substrates is an absolute prerequisite for the digestion of plant cell walls in the rumen (McAllister *et al,* 1994). It follows, then, that treatment methods enabling adequate interaction between enzyme and substrate prior to feeding are most likely to improve animal performance. The enhanced degradation in enzyme-treated forages may be related to the increase in passage rate and reduction in the retention time of forage particles in the rumen of cattle as observed by Feng *et al,* (1992). Developments in biotechnology make it feasible to engineer enzyme cocktails for xylanase and _-glucanase, but the technology for specific production of many of the other enzymes (e.g. ferulic acid esterase, acetylxylan esterase, arabinofuranosidase) required for cell wall digestion is lacking. In the short term, then, improvements in ruminant performance arising from enzyme treatment of forages will most likely be the result of treatment of the forage with broad spectrum crude enzyme extracts from cellulolytic microorganisms (e.g. *Aspergillus* spp., *Trichoderma reesei).*

Improving Fibre Digestion through Genetic Assortment of Forage Crops

Fibre digestion in the rumen is constrained by retention time, digestion rate and the magnitude of the indigestible fraction (Orskov, 1991). These constraints could be alleviated by improving the environmental conditions in the rumen or increasing the fibrolytic capacity of the rumen microbes. The constraints to fibre digestion in the rumen are determined by the chemical and physical nature of the fibre, which in turn depends on the genetic make-up of the plant and the environment where it grows. Therefore the selection of cultivars that produce high levels of grain and better quality residues offer possibilities for improving fibre breakdown in the rumen (Orskov, 1991). Three possible avenues for improving fibre digestion through plant selection may be, (i) the exploitation of variation across varieties in cereal crops and forage legumes, (ii) introduction of genetically controlled quality related traits, and (iii) identification of geographic locations that could produce higher quality trees.

Biotechnology in Methane Mitigation from Rumen

A large proportion of the global ruminant population are located in tropical environments, where animals feed predominantly on low quality highly fibrous forages. Recent studies in respiration chambers have confirmed that methane emissions from ruminants fed on fibrous diets are higher than outputs from better quality temperate forages. The excretion of methane from the rumen can represent a loss of up to 15% of the digestible energy depending on the type of diet. Therefore, reducing methane production could benefit the ruminant energetically, provided the efficiency of ruminal metabolism is not compromised. Animal trials involving agents that specifically inhibit microbial enzymes associated with methane production probably provide the most reliable data for interpretation of the effects of inhibition of methanogenesis on digestive and animal performance parameters, indicating that a reduction in methanogenesis in the rumen can be associated with improvements in feed conversion efficiency without affecting intake (Cheryl *et al*, 2003). Furthermore, any attempt to reduce methane emissions from livestock is unlikely to be adopted unless production efficiency is at least maintained if not enhanced. The challenge therefore is to devise strategies, which reduce methane emissions from ruminants and improve production efficiency. By recombinant DNA technology, a derivative of wild type *E. coli* W3110 was constructed by replacing self promoter of nirBD operon encoding nitrate reductase by tac promoter which makes it constitutive and shows higher expression of nirBD. By using *E. coli* nir Ptac, the methane production was markedly decreased by addition of nitrate and nitrite as the nitrite reductase activity was approximately twice in *E. coli* nirPtac as compared to wild type. Thus it has been concluded that anaerobic cultures of *E. coli* W3110 and *E. coli* nir Ptac may decrease methane production in rumen (Sar *et al*, 2005). Therefore RT-PCR and oligonucleotide probes targeting the respective 16S rRNA of the methanogenic archaea, and the major fibrolytic bacteria (*Fibrobacter succinogenes, Ruminococcus albus* and *R. flavefaciens*) in the rumen would be a robust approach to quantifying the effect of reduced methanogenesis on important functional microbial groups. The molecular based ecology techniques are also likely to provide better insight into the interactions between methanogens and the other rumen microorganisms. All this information should assist in the development of strategies for improving production by reducing methanogenesis.

Silage and Silage Inoculants

Some commonly ensiled plants (e.g. grasses) are low in soluble carbohydrate and therefore often ensile poorly. The inclusion of cellulase and endoxylanase enzyme mixtures from a variety of fungi or bacteria during ensiling increases the amount of free soluble carbohydrate available for fermentation (van Vuuren *et al*, 1989). Consequently, conversion of carbohydrate to lactic acid is more extensive, resulting in a lower pH in enzyme-treated silage as compared to untreated silage (Chen *et al*, 1994). In some instances, enzyme treatment has increased the intake (DM basis) of silage by dairy cows and improved milk production, but these responses are less pronounced for cereal silages or silages with a high dry matter content (Chen *et al*, 1994). Enzyme treatment of grass silages has increased, decreased and had no effect on the apparent whole-tract digestibility of organic matter (Jacobs and McAllan, 1992). A novel ensiling process using a silage additive containing *Lactobacillus plantarum*, commercial cellulase enzyme and molasses, also resulted in rapid lactic acid production and degradation of cellulose (Singh *et al*, 1990). In a single study, different enzyme preparations were found to increase and decrease the digestibility of organic matter in sheep (Chamberlain and Robertson, 1989). These inconsistencies emphasise the need for more precise definitions of enzyme preparations and of the forage properties for which a given enzyme treatment is most likely to elicit a positive response in animal performance. The cost and inconvenience of adding enzymes prior to the ensiling of forages low in carbohydrates could be avoided if the bacterial species in the inoculant were also capable of producing the enzymes required to release soluble sugars from plant cell walls. Genetically modified *Lactobacillus* strains have now been developed which express cellulase and xylanase genes isolated from other organisms (Baik and Pack, 1990) and which have, in at least one case, been shown to exhibit competitive growth in silage (Sharp *et al*, 1994). Persistence of newly developed recombinant lactobacilli beyond their target environments is an important, but under-studied phenomenon. In a single study, Sharp *et al*, (1994) demonstrated the rapid loss of both unmodified and recombinant *L. plantarum* silage inoculants from the rumen. Elimination of these strains was attributed to predation by protozoa and other undefined factors (e.g. turnover rate, cell death). These findings are also relevant to the use of these genetically engineered microorganisms (GEMs) as probiotic feed additives, in which case it is essential that they establish and persist in the gastrointestinal tract. Factors influencing environmental persistence must be identified and taken into consideration during selection of microbial strains for genetic manipulation.

Producing Food from Ruminants through Biotechnology Interventions

Several methods are available to alter end-products of the rumen fermentation. These include glycopeptides which increase propionate and amino acids and decrease methane and ammonia nitrogen production. Monensin increases propionate and decreases methane and acetate production. Isoacids (e.g. isobutyrate) increase acetate and decrease ammonia production. Virginiamycin increases propionate production and, in some studies, decreases butyrate, acetate and lactate. The changes in rumen fermentation products brought about by these chemicals can have positive effects on animal production depending on whether growth, fattening or milk production is the desired product. The effect of these chemicals at the molecular level on the rumen ecosystem is poorly understood. Understanding their mechanism of action can lead to better methods for controlling rumen fermentation. Of equal importance in improving food production by ruminants is a knowledge of utilisation of rumen fermentation products at the molecular level. It is critical to understand gene regulation in adipose, muscle and the mammary gland. Identifying the important genes regulating metabolism in food producing animals is in its infancy. It may be that the activity of some genes should be increased while for others it should be lowered. Some current genes of interest are fatty acid synthase (fat deposition) and calpastatin (muscle metabolism).

FUTURE PROSPECTS AND CONCLUSION

While the efficiency and viability of ruminant production is important to the economies of some industrialised countries, the importance for developing nations like India with less overseas purchasing power are more far-reaching. Where ruminants fill essential needs for food, fibre and draught power, improvements in production and maintenance efficiency contribute directly to the living standards of a high proportion of the population. Much of the nutritional improvement possible through supplementation or processing of animal feed may be difficult to implement in these situations. It is vital, therefore, that improvements in ruminant production should make maximum use of the feed sources currently or potentially available, rather than depend on adding new elements that need to be purchased. Where a high proportion of animal feed is the residue from grain or other crops, digestibility is frequently the limiting factor in ruminant production. In those cases where crops leave residues with feed potential, there may be serious limitations created by the secondary compounds present in that material. There may be considerable value, therefore, in systems that help in fibre breakdown or that detoxify anti-nutritive factors. The capability of genetic manipulation to make rumen microbes digest plant fibres more efficiently remains to be proven. For this aim, there may be greater and more immediate gain in examining how different feed plants can be combined to supplement each other and optimise microbial growth and nutrient yield within the rumen. Microbes isolated from the fermentative organs of non-domestic animals (e.g. giraffes, camels, monkeys etc.) may be directly adaptable if they are able to colonise domestic ruminants. However, attempts to transfer microbes between animal species will be facilitated by the precise molecular methods now available to track individual organisms within complex mixtures. The development of molecular mechanisms for ruminant studies, including methods for microbial strain identification, estimation of population numbers and measurement of changes, has now reached a stage where less developed nations should be able to benefit from them. Future research will indicate which of the possible applications for genetic manipulation of rumen microbes are likely to be successful.

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EXPLOITATION OF ENVIRONMENTAL RESOURCES FOR ANTIBIOTIC PRODUCTION

VANDANA PRAVEEN AND C.K.M. TRIPATHI

INTRODUCTION

Antibiotics are defined as low-molecular-weight organic natural products of micro-organisms that are active at low concentrations against other micro-organisms (Demain, 1981). Antibiotics are one class of antimicrobials, a larger group which also includes anticancer, arti-viral, anti-fungal, and anti-parasitic drugs (Vandamme, 1984). But today the definition has been expanded to include the natural/synthetic compounds with obscure functions like anti-tumor, immunosuppressant, hypocholestrimic and antihelmintic etc (Berdy, 2005).

The selective action exerted by microbial secondary metabolite (penicillin) on pathogenic bacteria ushered in the antibiotic era. The "golden era" in discovery of new antibiotics of microbial origin (1950–1965) saw the discovery and introduction of penicillins, cephalosporins, rifamycins, streptomycin, chloramphenicol, tetracycline kannamycin, theinamycins, amphotericin B, nystatin, ksugamycins, bleomycins, mitomycin C, actinomycins, sakomycin, pristinamycins, virginiamycins and many more antibiotics and the age of chemotherapy came into full being (Kumazawa and Yogisawa, 2002). Two Nobel prizes for the discovery of antibiotics were given to Alexander Fleming, Chain and Florey in the 1940s for discovery and synthesis of penicillin and to S.A. Waksman in 1944 for the discovery of Streptomycin. (Peleczar *et al.*, 1993).

However, it was realized (for review, see Demain, 1983) that compounds which possess antibiotic activity also possess other activities, that some of these had been quietly exploited in the past, and that such broadening of scope should be exploited and expanded in the future. Much of this emphasis was brought about by Patil *et al.*, 2000, who pointed out the potential importance of chitinases as drugs.

NEED FOR THE SEARCH FOR NEW ANTIBIOTICS

The need for new and useful compounds to provide assistance and relief in all aspects of human condition is ever growing. Today, only one third of all diseases can be treated efficiently (Muller *et al.*, 2000). There is an urgent need for new drug entities due to:

1. Indiscriminate and overuse of antibiotics for human and veterinary use resulted into appearance of MDR micro-organisms. The dramatic appearance of antibiotic resistance in pathogenic *Shigella* sp. during epidemics of intestinal infections in Japan in the 1950s is yet another example of the rapid response of bacteria to the threat of antibiotic use. Clinical isolates of penicillin-resistant *Streptococcus pneumoniae,* the most common cause of bacterial pneumonia, increased in the US from 1987 to 1992 by 60 folds. MRSA infections have increased to an alarming extent throughout the world. About 90% of natural antibiotics fail to inhibit gram negative organisms such as *E. coli.*

Emergence of resistances against some important antibiotics has been given in Table 1. First significant pathogen, that influenced the use of streptomycin against *M. tuberculosis,* developed resistance as the result of mutations affecting the target site of the antibiotic. One of the first consequences of antibiotic usage in agriculture was the transfer of resistance genes from animal isolates (e.g., *Salmonella typhimurium*) to human pathogens. Microbes have the genetic flexibility to develop resistance (or tolerance) to any antimicrobial agent; this applies to bacteria, fungi, protozoans, and viruses also. Cross-resistance to tinidazole has been demonstrated with metronidazole- resistant *Giardia* strains (Upcroft and Upcroft, 2001). Cancer cell lines have also been found to show resistance for anthracycline antibiotics e.g. doxorubicin, once considered to be miraculous for cancer treatments (Lown, 1993).

2. Appearance of new diseases like SARS (Severe Acute Respiratory Syndrome), Ebola and Hanta viruses, *Cryptosporidium,* Leigonaries' disease, Lyme disease etc.

3. Other factors are the toxicity and side effects of the available drugs or low therapeutic index i.e. the ratio of the toxic dose to the therapeutic dose (Mazela and Davies, 1999). The toxicity of some of the current compounds limits their usage in chemotherapy. Fungal pathogens are becoming resistant to most of the antifungal antibiotics (Polyenes and azoles). More over fungal cells are complex organisms that share many biochemical targets with other eukaryotic cells. Therefore, new agents that interact with fungal targets not found in eukaryotic cells are needed (Randhawa and Sharma, 2004).

4. The recurring problems with disease in persons with organ transplants.

5. The existence of naturally resistant bacteria (e.g. *Pseudomonas aeruginosa* in cystic fibrosis), (Demain, 1999).

In last ten years, in spite of the discovery of the great numbers of new compounds, less totally new type of structures were discovered than in the previous decades. After 1970, the rate of discovery of useful compounds declined progressively, although several important agents nevertheless came to light, including the antihelmintic avermectin, the immunosuppressants, rapamycin and tacrolimus (FK506), and the natural herbicide bialaphos. In fact, the total number of known biologically active molecules continued to grow steadily after the end of the Golden Age (Demain & Fang, 2000).

NATURE AN INEXHAUSTIBLE SOURCE OF BIOACTIVES

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity and have least impact on environment quality. New bioactive drug lead compounds may be prepared by chemical synthesis or may be isolated from the nature. In early antibiotic era scientists started working as per their field of interest preferably in two branches. One which leads to chemical synthesis of these agents in laboratory and second which leads to isolation of these compounds from micro-organism (natural compounds). The largest users of natural medicines are the Chinese, with more than 5,000 plants and plant products in their pharmacopoeia (Bensky and Gamble, 1993).

TABLE 1

Dates of Deployment of Representative Antibiotics, and the Evolution of Resistance (Palumbi, 2001)

Antibiotic	Year deployed	Resistance observed
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	late 1960s

The pharmacological industry until now has synthesized several millions (according to some estimations about 3 to 4 millions) new organic chemical structures, but only a negligible part of them (no more than one from several ten-thousands, ~ 0.001%) became accepted drugs. From the ten thousands of known microbial metabolites about 150 ~ 160 (~ 0.2 ~ 0.3%) compounds were practically utilized as therapeutic agents (Berdy, 2005). Natural products usually show new mechanisms of action and exhibit novel therapeutic activities with higher probability than the synthetic or combinatorial compounds. In fact, the world's best known and most universally used medicine is aspirin (salicylic acid), which has its natural origins from the glycoside salicin which is found in many species of the plant genera *Salix* and *Populus* (Grabley and Thierick, 1999).

These plant products, in general, enhanced the quality of life, reduced pain and suffering, and provided relief, even though an understanding of the chemical nature of bioactive compounds in these complex mixtures and how they functioned remained a mystery. It was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microbes as a source for bioactive natural products. Then, scientific serendipity and the power of observation provided the impetus to Fleming to usher in the antibiotic era via the discovery of penicillin from the fungus *Penicillium notatum*. Since then, people have been engaged in the discovery and application of microbial metabolites with activity against both plant and human pathogens. In fact, the world's first billion-dollar anticancer drug, paclitaxel (Taxol), is a natural product derived from the yew tree (Wani *et al.*, 1971).

Natural products have been proved to serve as promising entities for chemotherapy because of following advantages:

- Natural products offer unmatched chemical diversity with structural complexity and biological potency (Verdine 1996).
- Natural products have been selected by nature for specific biological interactions. They have evolved to bind to proteins and have drug-like properties (Nisbet and Moore 1997).
- Natural products are the main source of pharmacophores having multiple activities. Cyclosporins, such as cyclosporin A and FK-506, are active not only as immuno-suppressants but also as antiviral and anti-parasitic agents (Bram *et al.*, 1993).
- Natural product resources are largely unexplored and novel discovery strategies will lead to the development of novel bioactive compounds. Natural product extracts are complementary to synthetic and combinatorial libraries. About 40% of the natural product diversity is not represented in synthetic compounds libraries (Henkel *et al.*, 1999).
- Research on natural products has led to the discovery of novel mechanisms of action, for example, the discovery of the role of guggulsterone (Urizar *et al.*, 2002).
- Natural products are powerful biochemical tools, serving as "pathfinders" for molecular biology and chemistry and in the investigation of cellular functions (Hung *et al.*, 1996).
- Natural products can guide the design of synthetic compounds (Breinbauer *et al.*, 2002).

Natural products and their derivatives have historically been invaluable as a source of therapeutic agents. It is true that the structural variations, the unique combinations of rare moieties and skeletons of natural products due to the extremely versatile biosynthetic capacity (extensive branching, series of alternative reactions, isomerization, condensations, polymerization, oxidation, alkylation) of the microbes, especially actinomycetes, are inexhaustible. Natural products usually show new mechanisms of action and exhibit novel therapeutic activities with higher probability than the synthetic or combinatorial compounds. It is important to realize that the primary purpose of combinatorial chemistry should be to complement and assist the efforts of natural-product drug discovery and development, not to supersede it. The natural product often serves as a lead molecule whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry. Natural products have been the traditional pathfinder compounds, offering an untold diversity of chemical structures unparalleled by even the largest combinatorial databases.

In 1977, Ist case of vancomycin resistant *S. aureus was* reported from Japan and it's repeated emergence requires some novel compound to treat such resistant strains. Teicoplanin, produced by *Streptomyces* sp., is a drug of choice for vancomycin resistant *Staphalococcus aureus* (VRSA) but till now not licensed in U.S.A. (Bardone *et al.*, 1978). Ramoplanin, a fermentation product of *Actinoplanes* spp., is a promising clinical candidate for treatment of vancomycin resistant *Enterococcus* (VRE) and methicillin resistant *Staphalococcus aureus* (MRSA) infections (Chen *et al.*, 2000; Mazela and Davies, 1999). This is an excellent example of the fact that natural products are the ultimate solution of the problem of drug resistant pathogens. A renaissance of natural products-based drug discovery is coming because of the trend of combining the power of diversified but low-redundancy natural products with systems biology and novel assays. Increasing the quality and quantity of different chemical compounds tested in diverse biological systems should increase the chances of finding new leads for therapeutic agents.

Most of the important antitumor compounds used for chemotherapy of tumors are microbially produced antibiotics. These include actinomycin D, mitomycin, bleomycins, and the anthracyclines, daunorubicin and doxorubicin. The recent successful molecule taxol (=paclitaxel) was originally discovered in plants, but has also been found to be a microbial metabolite. It is approved for breast and ovarian cancer and is the only antitumor drug known to act by blocking depolymerization of microtubules. Due to the development of novel target- directed screening procedures at Merck, the discoveries of new effective antibiotics active against bacterial cell-wall biosynthesis became commercial successes in the 1970s and 1980s. These include cephamycins, fosfomycin, and thienamycin. Thienamycin is the most potent and broadest in spectrum of all antibiotics known today.The unique activity of thienamycin is due to several factors: (1) It permeates the gram-negative outer cell membrane through porin channels at 10±20 times the rate of classical â-lactams. (2) It is not destroyed by the â-lactamases of the periplasmic space. (3) It binds to and inhibits all penicillin-binding proteins (PBPs) but is principally active against PBPs-2 and -1b rather than PBP-3, which is attacked by conventional penicillins and cephalosporins (Demain, 1999).

WHY STILL TARGET MICROBES IN THE SEARCH FOR ANTIBIOTICS?

Prokaryotes are a well known source of bioactive molecules. Over 17,000 active molecules have been described from prokaryotes. Only 1% of bacteria in the environment have been cultured. The remaining 99% of uncultured bacteria are thought to make many more novel secondary metabolites. Molecular tools are now available to access to genes of the environmental microbes which are difficult to cultivate in laboratories. In nature, all organisms need to compete in order to survive in their habitat. This biological task can be achieved by the development of competitive mechanisms such as the production of toxins, enzymes and antimicrobial agents like antibiotics. The most well known examples of natural product are microbial secondary metabolites, the antibiotics (Demain, 1999). Research has demonstrated that these metabolites are most likely produced by fungi and bacteria. The actinomycetes are a group of prokaryotic filamentous soil microorganisms which are known to be the top producers of antimicrobial agents, especially *Streptomyces* (Demain, 1999). Microbial natural products have also been developed as anti-diabetic drugs, hormone (ion-channel or receptor) antagonists, anti-cancer drugs, and agricultural and pharmaceutical agents. According to a study, 84 of a representative 150 prescription drugs in the United States fell into the category of natural products and related drugs (Grifo *et al.*, 1997). By the end of 2002 over 150 natural compounds were in clinical use. Out of which bacteria, actinomycetes and fungi produced 10, 70, and 35 % respectively (Berdy, 2005). Few microbial natural products which are under clinical trials are given in Table 2.

TABLE 2

New Drugs in Clinical Trials (Chin *et al.*, 2006 and Butler, 2005)

Drug	Natural/semisynthetic or synthetic	Producer organism	Bioactivity against
Tigecycline 2005, Wyeth	Semisynthetic product of tetracycline	*Streptomyces aureofaciens*	tetracycline-resistant bacteria
Telithromycin 2004 (Aventis)	Semisynthetic product of erythromycin A	*Saccharopolyspora erythraea*	macrolide resistant G+ve bacteria
Cefditoren 2001, (Spectracef TAP) bacteria	Semisynthetic derivative of cephalosporin	*Cephalosporium* species	â-lactamase producing G+ve and G-ve
Ertapenem, 2001 (Merck)	Analog of thienamycin	*Streptomyces cattleya*	Enterobacteria
Biapenem, 2002, (Wyeth Lederle) bacteria	Analog of thienamycin	*Streptomyces cattleya*	â-lactamase producing G+ve and G-ve
Daptomycin, 2003, (Cubist)	Natural lipopeptie	*Streptomyces roseosporus*	G+ve bacteria (MRSA, VRE)
Pimecrolimus, 2001, (Novartis)	Novel analog of ascomycin	*Streptomyces hygroscopicus* var *ascomyceticus*	Filamenous fungi
Caspofungin acetate, 2001 (Merck)	Derivative of pneumocandin B	*Glarea lozoyensis*	Unicellular and filamentous fungi
Micafungin sodium, 2005 (Fujisawa)	Natural echinocandin type	*Coleophoma empetri*	Azole-resistant *Candida* sp., and *Aspergillus* sp.
Everolimus, 2004 (Novartis)	Derivative of rapamycin	*Streptomyces hygroscopicus*	Immunosuppressive
Amrubicin hydrochloride, 2002 (Sumitomo Pharm. Co)	Derivative of doxorubicin	*Streptomyces peucetius* var *caesius*	Tumor xenografts of breast, lung and gastric cancer.
Miglustat, 2003 (Zavesca, Actelion)	analog of nojirimycin	*Streptomyces lavendulae*	type I Gaucher disease

NOVEL ANTIMICROBIAL AGENTS ACTING ON DIVERSE NEW TARGETS OF PATHOGENS

Advances in deciphering the genome of a variety of microbes will further help the development of agents against them by providing a wider selection of potential targets (Chin *et al.*, 2006). Specific microbial target enzymes will continue to attract attention for novel antimicrobial discovery. The fungal cell wall is a unique target that fulfills the criteria for selective toxicity. Cell wall acting agents are a new class of antifungal antibiotics with a novel mechanism of action and are inherently selective and fungicidal in nature. Fungal cell wall consists of fibrillar layer, mannoprotein, β-glucans, chitin and plasma membrane (Wills et al, 2000). Accordingly antifungal agents can be grouped as:

(a) **β-1,3 glucan synthetase inhibitors**: The β-1, 3-glucan polymer, an essential component of the fungal cell wall, is not present in mammalian cells. Echinocandins, β-1, 3 glucan synthetase inhibitors are produced by various fungal and *Streptomyces* cultures.

(b) **Chitin Synthase inhibitors:** Chitin is a major structural component of the cell walls of fungi. It is a (1-4)-β-linked homopolymer of N-acetyl-D-glucosamine, and is produced by chitin synthase from the nucleotide UDP-GlcNAc. Polyoxins and nikkomycins produced by *Bacillus* and *Streptomyces* spp. are two structurally related groups of secondary metabolites that act as specific inhibitors of chitin synthases.

(c) **Chitin hydrolyzing enzymes, chitinases** : Chitinases are produced by various plants, bacteria and fungi. These are hydrolyzing enzymes which degrade the chitin homopolymer into its N-acetyl-D-glucosamine units. Bacteria produce chitinase to digest chitin, primarily to utilize it as a carbon and energy source. Several chitinolytic enzymes have been identified in various Bacteria, *Streptomyces* and fungi including *Bacillus circulans, Alcaligens* sp., *Pseudomonas* sp., *Serratia* sp., *S. antibioticus, S. griseus, S. plicatus, S. lividans, Streptomyces venezuelae, Streptomyces halstedii* and *Trichothecium roseum* (Patil *et al.*, 2000).

(d) **Mannan binding antifungals, inhbitors of mannoprotein synthesis :** Mannose constitutes a major portion of the cell wall of many fungi, as well as the glycoproteins that form the protective capsule in *C. neoformans*. Mannopeptimycins are mannose binding antibiotics produced by *Streptomyces hygroscopicus* LL-AC98 (Wills *et al.*, 2000).

(e) **Sphingolipid inhibitors:** Sphingolipids are essential components of all eukaryotic plasma membranes and modulation of them exerts a deep impact on cell viability. Several natural products including sphingofungins, myriocin, lipoxamycin, viridiofungins, khafrefungin and aureobasidin A which inhibit the Spt enzyme (serine palmitoyltransferase) involved in sphingolipid biosysnthesis and possess in vitro antifungal activity have been discovered from microorganisms (Wills *et al.*, 2000).

(f) **Plasma membrane ATPase inhibitors :** Bafilomycin A1 is a macrolide antibiotic that inhibits the vacuolar H+-ATPase (V-ATPase) in several organisms (Wills *et al.*, 2000).

(g) **Protein synthesis inhibitors:** Several well characterised compounds are known to inhibit the RNA polymerases and elongation factors required for transcription and protein synthesis. Sordarin blocks the elongation cycle at the initial steps.

(h) **Inhibitors of signal transduction pathways**: Cyclosporin A (CsA), rapamycin, wortmannin and geldanamycin are inhibitors of signalling cascade.

(i) **Inhibitors of electron transport and membrane integrity:** are produced by microorganisms. With respect to mitochondrial electron transport inhibitors, a series of related antifungal agents UK2A, UK3A, and the structurally related antimycin A have been isolated from different species of *Streptomyces* (Ueki *et al.*, 1996).

(j) **Peptide deformylase inhibitors:** Peptide deformylase is a critical bacterial enzyme discovered almost 30 years ago, but it has not been possible to target it effectively until recently because it is an unstable enzyme. Peptide deformylase removes the formyl group from the amino-terminal formylmethionyl residue in bacterial proteins. It is a metallopeptidase that is an essential gene product in many bacteria and for which potent inhibitors have recently been described (Chen *et al.*, 2000).

DISTRIBUTION OF BIOACTIVE METABOLITES IN NATURE

The diversity of microbial life is enormous and the niches in which microbes live are truly amazing, ranging from simple rainforests, agricultural fields, rhizosphere, toxic industrial effluents, highly contaminated (sewage and hospitals) waters with pathogenic multiple drug resistant (MDR) organisms to extreme environments like polar ice, geothermal vents, dark caves, deep sea. Microbes must have evolved mechanisms to acclimatize to these specific unfavourable conditions leading to generation of metabolic pathways and primary & secondary metabolites. Along with these diverse habitats with unique ecosystems such as tropical forests soils, the deep sea, sites of extreme temperature, salinity or pH should also be explored (Waterman, 1998).

Such habitats often harbour novel microorganisms with unique metabolic pathways and many provide potential bioactive compounds. Microbes isolated from thermophilic/ hyperthermophilic environments offer an amazing array of metabolisms involving unique enzymes (Chin et al, 2006). Recently, thermostable polymerases e.g. Taq DNA polymerase, which laid the foundation for molecular biology were discovered from a thermophilic bacterium *Thermus aquaticus*. This bacterium has evolved to grow in hot springs at temperatures which kill most other species. These enzymes allow the amplification of as little as one molecule of DNA into a large amount by means of repeated cycles of melting, primer annealing & extension by the enzyme which is not destroyed by the high temperatures used in this process (Harvey, 2000).

Endophytes, symbionts, marine microbes & extremophiles such as acidophiles (from acidic sulfurous hot springs); alkalophiles (from alkaline lakes); halophiles (from salt lakes); baro- and thermophiles (from deep-sea vents); and psychrophiles (from arctic and antarctic waters, alpine lakes) are continually reported as sources of bioactives.

Symbionts

Atine ants are known to colonize an ascomycetus fungus of the genus *Escovopsis* (Ascomycotina). Third mutualist in this symbiosis, a filamentous bacterium (actinomycete) of the genus *Streptomyces* that produces antibiotics specifically targeted to suppress the growth of the specialized garden-parasite *Escovopsis* (Cameron, 1999).

Endophytes

Endophytes, include bacteria, actinomycetes, and fungi that spend part or all of their life cycle colonizing either inter or intra cellularly, the healthy tissue of a plant, are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture, and industry. It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes. Only a few these plants have ever been completely studied relative to their endophytic biology.

In addition, it is noteworthy that some plants generating bioactive natural products have associated endophytes that produce the same natural products. Such is the case with paclitaxel, a highly functionalized diterpenoid and famed anticancer agent that is found in each of the world's yew tree species (*Taxus* spp.). In 1993, a novel paclitaxel-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia* was isolated and characterized (Strobel *et al.*, 1993).

Endophytic microbes have produced novel secondary metabolites, e.g. cryptocandins, coronamycins, munumbicins, pseudomycins, kakduamycins active against MDR bacterial, fungal mycobacteria, *Plasmodium* sp. and even cancer cell lines (Cragg and Newman, 2000). A potent antifungal strain of *Serratia marcescens* was recovered from *Rhyncholacis pedicillata* and was shown to produce oocydin A, a novel antioomycetous compound having the properties of a chlorinated macrocyclic lactone. Currently, oocydin A is being considered for agriculture use to control the ever-threatening presence of oomyceteous fungi such as *Pythium* and *Phytophthora* (Strobel *et al.*, 1999).

A sample of the snakevine, *Kennedia nigriscans*, from the Northern Territory of Australia was selected for study since its sap has traditionally been used as medicine for many years. In fact, the plant contained a novel endophyte, *Streptomyces* sp. strain NRRL 30562, that produces wide-spectrum novel peptide antibiotics called munumbicins, active against gram-positive bacteria such as *Bacillus anthracis* and multidrug-resistant *Mycobacterium tuberculosis* as well as a number of other drug-resistant bacteria (Castillo et al., 2002). *Cryptosporiopsis quercina* was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia produced Cryptocin, a unique tetramic acid, possesses potent activity against *Pyricularia oryzae* and is being examined as a natural chemical control agent for rice blast. The ecomycins are produced by *Pseudomonas viridiflava* (Li *et al.*, 2000), a member of a group of plant-associated fluorescent bacteria. The ecomycins represent a family of novel lipopeptides and have molecular weights of 1,153 and 1,181 (Miller *et al.*, 1998).

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring and activity against *Bacillus subtilis, Salmonella enterica serovar gallinarum* and *Staphylococcus aureus* (Horn *et al.*, 1995). Another *Colletotrichum* sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed varied antimicrobial activity as well. *A. annua* is a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas (Lu *et al.*, 2000). Two novel human cytomegalo virus protease inhibitors, cytonic acids A and B, have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema* sp. (Guo *et al.*, 2000)·

A nonpeptidal fungal metabolite (L-783,281) was isolated from an endophytic fungus (*Pseudomassaria* sp.) collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo. This compound acts as insulin mimetic and, unlike insulin, is not destroyed in the digestive tract and may be given orally (Zhang *et al.*, 1999). A hugely beneficial immunosuppressant, cyclosporine was isolated from the fungus *Tolypocladium inflatum* (Borel *et al.*, 1991).

Marine Microorganisms

With more than 70% of the planet's surface covered by water, the oceans are probably the most promising habitat to explore for novel microbial biodiversity. It has been estimated that the biological diversity in marine ecosystems, such as the deep sea floor, is higher than in tropical rain forests. Since the 1970's more than 15,000 NPs have been isolated from marine microbes, algae and invertebrates. A team, including Prof. Russell Hill of the University Of Maryland Biotechnology Institute (UMBI), Australia and Nicole Webster of the Australian Institute of Marine Science (AIMS) used molecular techniques to identify the actinomycetes found living in coral reef sponges and sediments from the Great Barrier Reef. Surprisingly, approximately 25 percent of the bacterial gene pieces found was from newly discovered actinomycetes. This is an unexpectedly high proportion and indicates that marine sponges may be a good source of novel actinomycetes. Specific populations of marine adapted actinomycetes such as *Salinospora* spp. and *Marinophilus* spp. have been discovered and described recently. Sokoloff *et al* (1982), have isolated two antibacterial antibiotic, prianicins A & B from red sea sponges which were active (10 times more potent than tetracyclines) against beta hemolytic *Streptococcus*.

However, at the same time temperate ecosystems should not be excluded especially if novel isolation strategies such as metagenome cloning are used. Among others the cyclosporins and penicillins were isolated from fungi collected in temperate regions. Even cold regions can be rich in fungal diversity leading to a high hit-rate of novel psychrophilic or psychrotolerant species. A number of these species have recently been investigated and found to produce several bioactive cyclic peptides. These findings support the hypothesis that fungi from colder climates may be just as chemically prolific (and perhaps just as diverse) as those from tropical climates, the latter which are much more often cited as targets for biodiversity sought in screening programs (Chin *et al.*, 2006).

Soil

Soil still remains an important source because it carries a higher population of microbes than any other habitat In one study, approximately 10^7 bacteria were counted in 1 g soil, but as few as 0.1% were cultivable, using standard culture techniques. The other 99.9% of the population may represent novel genetic diversity (Bubnoff, 2003). Soil is a complex ecosystem. In soil, where a large variety of antagonistic organisms are present, competition has probably selected for strains able to promote their own survival by excreting toxic metabolites. Over 5 decades, soil micro-organisms have been utilized as a rich source of therapeutically important molecules. By the end of 2002 over 22000 bioactive secondary metabolites were published in the scientific and patent literature

(Berdy, 2005). Nearly 75 % of the world's antibiotics are produced by the *Streptomyces* spp. (=actinomycetes belonging to order actinomycetales). Soil is the most common habitat of *Streptomyces. Streptomyces* comprise 1-20 % of the total viable count and 64-97 % of the cultivable actinomycetes. Soil-based actinomycetes produce over 70 percent of naturally occurring antibiotics (Berdy, 2005).

Common characteristics of the *Streptomyces* spp are formation of substrate and aerial mycelium on solid media, their Gram-positive character, presence of spores, and a high G+C content of the DNA (60-70 mol%). *Streptomyces* spp are undoubtedly the largest producers of bacterial secondary metabolites (Anderson and Wellington, 2002). They are common in soil, but also found in sediments, composts and fodder, aquatic habitats, and buildings. Genomic studies have confirmed it: the ability to produce secondary metabolites is not uniformly distributed within the bacteria. The diversity of secondary metabolites from *Streptomyces* spp. is unrivalled and unmatched in medical significance (Anderson and Wellington, 2002). Among the antibiotics produced exclusively from actinomycetes are: glycopeptides antibiotics, macrolides, polyene antibiotics, aminoglycosides, anthracylines, polyether antibiotics; the novobiocin related glycosidic antibiotics, streptothricins, actinomycins and echinomycin; elfamycins, glutarimides, orthosomycins. The predominant part of the large, 18 to 60 membered macrocyclic lactone derivatives (including over 1000 compounds), ansa lactones (~150), benzanthraquinone derivatives (~200), thiostrepton-line thiazolyl peptides (140), cyclopolylactones (~40) are also derived from various actinomycetales species (Berdy, 2005).

MANIPULATING MICROBIAL PHYSIOLOGY TO ACTIVATE MICROBIAL PRODUCT FORMATION MACHINERY

The biochemistry involved in the production of antimicrobial agents is complex. All environmental factors such as pH, nutrient availability, soil chemistry and biological populations influence their production. In order to exploit the true potential of microorganisms, the physiological growth conditions used for generating extracts need to be diversified. The physiology of secondary metabolism has often been neglected. As antibiotics are not needed by the microbe to complete its life cycle, secondary metabolites (antibiotics) are produced in very low concentrations.

Secondary metabolites, including antibiotics, are produced in nature and serve survival functions for the organisms producing them. According to one definition, microbial secondary metabolites are substances that are not needed for the growth or other essential processes in the cell. The production of secondary metabolites is controlled by the availability of nutrients. Deregulation is brought about by nutritional as well as classical and molecular/genetic manipulations to by pass/or remove negative regulatory mechanisms and to enhance positive regulatory mechanisms. Secondary metabolism is brought on by exhaustion of a nutrient, biosynthesis or addition of an inducer and/or when growth rate decreases. These events generate signals that affect a cascade of regulatory events resulting in chemical differentiation (secondary metabolism) and morphological differentiation (Morphogenesis). The signal is often low molecular weight inducer which exerts negative control, i.e. by binding to and inactivating a regulatory protein (repressor protein) which normally prevents secondary metabolism and morphogenesis during rapid growth and nutrient sufficiency. Nutrient/ inducer presumably activate a "master gene" which either acts at the level of translation by encoding a t-RNA or by encoding a positive transcription factor. Feedback control also regulates metabolism with the accumulation of fermentation products (Bubnoff, 2003).

Carbon Regulation

In general, biosynthesis of different secondary metabolites (antibiotics, etc.) was inhibited by rapidly fermentable carbon sources. In fermentation experiments, the production of antibiotics is increased by the presence of a non preferred carbon source, or by phosphate starvation. *Streptomyces* species produce extracellular enzymes such as agarase (*S. coelicolor*), á-amylase (*S. rimosus, S. kannamyceticus, S. venezuelae*), protease, cellulase, chitinase, xylosidase, endosidase and â-galactosidase (*S. peucetius* var. caesius). Their production is also regulated by Carbon Catabolic Repression (CCR). An additional way to increase the production of a compound subject to CCR is by limiting the repressive carbon source concentration supplied to the fermentation (Sanchez and Demain, 2002). Since many of the secondary metabolites produced by microbes are extremely toxic, it seems sensible that they should suppress the formation of such compounds until rapid growth is nearing completion. Catabolic regulation of secondary metabolite synthesis could offer the cells a survival advantage. Perhaps for this reason, glucose suppresses the production of a wide range of secondary metabolites and secondary metabolic processes (Praveen *et al.*, 2007).

Nitrogen Regulation

The exact mechanism of nitrogen regulation of secondary metabolism is little understood but one reason for such control could be that many secondary metabolites, especially antibiotics contain at least one nitrogen atom, indicating that precursor molecules must include amino acids and/or nucleotides. Production of some aminoglycoside antibiotics e.g. neomycin and kanamycin is unfavouably affected by ammonium. Among the mineral nitrogen sources, ammonium was known to be needed in catabolite regulation of the secondary metabolism (Sanchez and Demain, 2002). Catabolite repression by the ammonium ions can regulate the secondary metabolites by inhibiting or by repressing enzymes, thereby allowing or not the biosynthesis of the precursors.

Phosphate Regulation

In natural environments, inorganic phosphorus is commonly the major growth limiting nutrient. In *B. subtilis*, phosphate starvation has been shown to stimulate 10 to 30 folds extracellular enzyme production in chemostat cultures, notably alkaline phosphodiesterase and acid phosphatases. Regulation by phosphorus sources includes both specific and general controls. A specific negative effect of inorganic phosphate arises from its ability to inhibit and/or repress phosphatases. Phosphate has been shown to regulate the synthesis of antibiotics belonging to widely differing biosynthetic classes including peptide antibiotics, polyene macrolides, tetracyclines and biosynthetically complex antibiotics. Industrial fermentation of these antibiotics is carried out at growth limiting concentrations of phosphate (Sanchez and Demain, 2002).

MEDIUM OPTIMIZATION FOR MAXIMUM ANTIBIOTIC PRODUCTION

For an economic production of antibiotics, the production and growth parameters e.g. temperature, agitation, aeration and concentration of nutrients can be optimized to give maximum yields out of minimum investment of time, energy and money. In order to access the effect of media components on the antibiotic yield, one has to design the experiment in such a way that each media component is uniformly distributed through out the sample space. There are a large number of techniques available to design culture media. They can vary from the traditional one-variable-at-a time method to more complex statistical and mathematical techniques involving experimental designs such as full and partial factorials, Plackett-Burman Designs (PBD) and Central Composite Designs (CCD). Each technique has advantages and disadvantages, and specific situations where they are best applied. No one 'magic bullet' technique exists for all situations. However, considerable advantage can be gained by logical application of the techniques, combined with good experimental design (reviewed by Kennedy and Krouse, 1999).

Plackett Burman Design

Application of statistical methods (CCD) requires information about the factors that are suitable for maximum antibiotic production. This idea can be achieved by classical method or with the help of PBD design, which is also a statistical design but it does not give any idea about the interaction effects among the medium components. It gives an idea about positive or negative effect of fermentation parameters. The PBD proposed by Plackett and Burman, is a two-level multifactorial design. These authors give a series of designs upto 100 experiments using an experimental rationale known as balanced incomplete blocks. The key to this technique is forming various combinations (assemblies) of the medium components with varying concentrations. With help of this design, up to N-1 components can be studied in N assemblies, where N must be a multiple of four (Plackett and Burman, 1946).

Central Composite Designs

The next stage in medium optimization would be to determine the optimum level of each key independent variable which has been identified by the PBD. This may be done by using surface optimization techniques which were introduced by Box and Wilson (Box and Wilson, 1951). Central composite designs are popular types of designs for fitting second order models. There are a number of reports in which the PBD and RSM have been used to screen the factors that have a crucial role in product formation (Chhatpar etal, 2003; Sarra etal 1993), product could be an enzyme, biomass or antibiotic. Production of penicillin by *Penicillium chrysogenum* was maximized using RSM and low concentrations of sucrose (~4g/L) and high concentration of corn steep liquor (~57.4 mg/L) were found to give higher yields (Oprime and Suazo,1997). Production of actinomycin-D, by an isolate, *Streptomyces sindenensis,* was enhanced by 3.56 folds (80 to 365 mg/ L) using statistical designs of PBD and CCD (Praveen *et al.*, 2008).

STRAIN IMPROVEMENT FOR HYPER-PRODUCTION OF ANTIBIOTICS

Although micro organisms are extremely good in presenting us with an amazing array of valuable products, they usually produce them only in amounts that they need for their own benefit; thus, they tend not to overproduce their metabolites. In strain improvement programs, development of a stable strain producing high titer of the metabolite in question is usually the desired goal. Micro organisms can generate new genetic characters ('genotypes') by two means: mutation and genetic recombination. Mutagenesis may be directed or random mutagenesis. In random mutagenesis, a gene is modified either existentially (spontaneous mutation) or intentionally (induced mutation). Applications of mutation have improved the productivity of industrial cultures (Diez *et al.*, 1997).

Techniques have been developed for enhancing the mutation rate of specific genes; these techniques are collectively known as directed mutagenesis. A prerequisite for using these techniques is, of course, knowledge of which genes to specifically mutate, and it is also necessary to have means of directing the mutagenesis at the genes controlling product biosynthesis. It has been reported that actively transcribing genes are more mutable. So mutagenesis under conditions of maximum biosynthetic activity should increase the probability of obtaining improved production mutants. At least five different classes of genes control metabolite production: (i) structural genes coding for product synthases, (ii) regulatory genes determining the onset and expression of structural genes, (iii) resistance genes determining the resistance of the producer to its own antibiotic, (iv) permeability genes regulating entry, exclusion and excretion of the product, and (v) regulatory genes controlling pathways providing precursors and cofactors. Overproduction of microbial metabolites is effected by (i) increasing precursor pools, (ii) adding, modifying or deleting regulatory genes, (iii) altering promoter, terminator and/or regulatory sequences, (iv) increasing copy number of genes encoding enzymes catalyzing bottleneck reactions, and (v) removing competing unnecessary pathways. Mutations leading to change in these genes can shift the metabolic profile of microbes (Diez et al, 1997).

It is now over 60 years since the first superior penicillin producing mutant, *Penicillium chrysogenum* X-1612, was isolated after X-ray mutagenesis. This heralded the beginning of a long and successful relationship between mutational genetics and industrial microbiology. Increased levels of mRNA corresponding to the three enzymes of penicillin G biosynthesis were found in high-penicillin producing strains of *P. chrysogenum* as compared to wild-type strains. High-producing strains contained an amplified region; a 106-kb region amplified five to six times as tandem repeats was detected in a high-producing strain, whereas wild-type *P. chrysogenum* and Fleming's original strain of *P. notatum* contained only a single copy. *Streptomyces* species produce a wide variety of secondary metabolites, including the avermectins, which comprise a series of eight related sixteen-membered macrocyclic lactones with potent antihelmintic and insecticidal activity. The eight distinct but closely related compounds are referred to as A1a, A1b, A2a, A2b, B1a, B1b, B2a, and B2b among the related avermectins, the B1 type of avermectin is recognized as having the most effective antiparasitic and pesticidal activity, and is therefore the most commercially desirable avermectin. Exclusive production of avermectin B1 was attained by directed mutagenesis of aveR1 gene or the aveR2 gene, or both the aveR1 and aveR2 genes of *S. avermitilis* (Adrio and Demain, 2006).

Random Mutagenesis

The most common method used to obtain high yielding mutants is to treat a population with a mutagenic agent until a certain 'desired' kill is obtained, plate out the survivors and test each resulting colony or a randomly selected group of colonies for product formation in flasks. The optimum level of kill for increased production of antibiotics is thought to be in the range 70-95% (Adrio and Demain, 2006).

Mutagens are classically divided into two types: physical, e.g. ultraviolet, gamma and X-irradiation, and chemical, e.g. ethyl methane sulphonate (EMS), nitroso-methyl-guanidine (NTG) and mustards such as ICR 170. Much more important is the distinction in the type of mutations induced, which depends on two factors: the type of DNA damage caused by the mutagen, and the action of cellular DNA repair pathways on this damage. Thus, for example, far UV gives a high proportion of pyrimidine dimmers, ionizing radiation gives a high degree of chromosome breakage and EMS and NTG are alkylating agents. DNA repair pathways themselves can be non-mutagenic (error free) or mutagenic, depending on the enzyme mechanism involved. The non-mutagenic repair pathways include photo reactivation, excision repair and recombination repair.

Strain improvement has been the main factor involved in the achievement of impressive titers of industrial metabolites. The production titer of tetracycline as far back as 1979 was reported to be over 20 g/ L, mainly due to strain improvement. Later, titers of 30-35 g /L were reached for chlortetracycline and tetracycline. The productivity of penicillin is 70 g/ L and that of cephalosporin C is over 30 g /L. High productivities of tylosin and salinomycin have been achieved by mutagenesis (15 and 60 g/l). Mutant isolation can be done by random and rational methods. Random methods involve picking up the survival colonies from agar surface randomly with out any defined criteria. In Rational screening mutants are isolated on the basis of the specific characteristics of the survivors; accordingly mutants can be of following types; morphological, auxotrophic, revertants of non-producing mutants: Mutants resistant to inhibition by antibiotics, mutants growing on phosphate limited media and metabolic mutants showing qualitative changes in the profile of metabolic products or mix of fermentation products (Adrio and Demain, 2006).

Mutational Biosynthesis

Mutational biosynthesis has been used for the discovery of many new secondary metabolites. The most well-known is the commercial antihelmintic agent doramectin, the production of which employed a mutant of the avermectin producer *S. avermitilis*. New anthracyclines and aglycones have been isolated from blocked mutants of the daunorubicin and doxorubicin producers. A further use of mutants has been the elucidation of metabolic pathways. Mutational biosynthesis has been exploited for the biosynthesis of tetracyclines, novobiocin, erythromycin, neomycin, tylosin, other aminoglycosides, rosaramicin, daunorubicin, other anthracyclines, actinomycin, carbapenems, ansamycins, patulin and phenazines (Adrio and Demain, 2006).

SCALE-UP OF BIOPROCESS

Scale-up is the study off production in large bioreactor vessels (fermentors). It is an essential process in industrial strain improvement. This is because most of the production processes operate at a much larger scale than that found in various levels of screening. One of the biggest unknown during the screening is if the improved mutant will perform well on scale-up. Often the correlation between titre performance on different types of screens (plates, tubes, shake flasks, fermentors and pilot plan) can be rather poor.

Both physical (agitation, aeration, oxygen mass transfer, shear stress, rheology of the vessel etc.) and biological factors (metabolism, growth etc.) are quite different in fermentors and in shake flasks. Moreover, controls on the reaction in the shake flasks are extremely limited while in the fermentor such controls are almost limitless. Although different types of bioreacters are available e.g. Air lift, fixed bed, fluidized bed, stirred tank reactors (Fermentors), most of the antibiotics have been produced by stirred tank bioreactors. For example, the specific production rate of penicillin fermentation (gram per cell dry weight) was over 50% less in a 6-L CSTR (Continuous Stirred Tank Reactors) when the rushton turbine was operated at 1000 rpm compared with 600 rpm (Justen et al., 1998). Rifamycin production was enhanced to significant levels by optimizing aeration and agitation of CSTR (El-Tayeb *et al.,* 2004).

NEW APPROACHES FOR THE ISOLATION OF NOVEL DRUG LEADS

Uncultivable Soil Microbes: Metagenomics

It is estimated that we are able to culture only about 1% of all the microorganisms present in a soil sample and often can not grow the microbial symbionts especially those found in marine organisms, which are probably responsible for making most of the natural products isolated from the marine world. The direct isolation of DNA should allow access to these untapped resources of molecular diversity. The term metagenome can be defined as the total genomic DNA present in a given environment. When trying to isolate new antimicrobial producing microbes, standard microbiological techniques may lead to the isolation and identification of previously identified antibiotics. By analyzing the soil metagenome, the probabilities of finding new genes encoding for novel chemotherapeutic agents are much higher, since there is no need for culturing the microflora present in the soil. This technique has no limitation since it can be used to isolate genes encoding for enzymes, proteins, antifungal agents, and antimicrobial agents.

Recently, scientists from the University of Wisconsin have isolated the antibiotics Turbomycin A and Turbomycin B from a soil DNA metagenomic library, both antibiotics exhibiting a broad-spectrum activity against gram-negative and gram-positive organisms (Gillespie et al., 2002). Platensimycin, a member of a new class of antibiotics that can kill MRSA in vitro (Abraham, 1986) were isolated from a South African soil sample using metagenomics. The discrepancy between the number of microbes detected by molecular methods and the number of strains in culture, demonstrate that there remains a relatively untapped source of novel strains in all ecosystems (Harvey, 2000).

Most of the antibiotics are reported to be produced by at least more than one microbial species. The cases of compounds restricted to single strains/species or to a few strains of a given biological species are rare in comparison. The genomes of actinomycetes (8 Mb), fungi (13–42 Mb) and myxobacteria (12 Mb) are much larger than needed for all basic functions. Therefore, it is widely supposed that part of the genome may encode genes for alternative metabolic pathways leading to the production of primary and secondary metabolites of our interest. Hence these organisms can produce an array of metabolites depending upon the fermentation conditions available (Knight *et al.*, 2003). For example, *Streptomyces coelicolor* A (3) 2 is designated as a potent producer of secondary metabolites. It produces methylenomycin, prodigiosin, actinorhodin, and other calcium-dependent antibiotics. In addition, several formerly unknown gene clusters (polyketide syntheses type I and II, non-ribosomal peptide syntheses) have been found in its genome. In *Streptomyces avermitilis* ATCC 31267, the producer of avermectin, 24 additional gene clusters have been sequenced (Omura et al, 2001). New producer organisms for Enfumafungin, Actinomycin-D and Rifamycins have been recently identified (Pelaez, 2000; Praveen *et al.*, 2008; Kim *et al.*, 2006).

Combinatorial Biosynthesis

As mentioned earlier, only a tiny proportion of microbial diversity has been cultured and many micro-organisms, particularly the marine micro-organisms, are difficult to maintain under laboratory conditions. One potential solution to the problem of accessing the chemical diversity of such inaccessible species is to remove the appropriate genetic material that codes for the secondary metabolic pathways and to incorporate it into more convenient organisms, such as *Streptomyces.* This strategy is being applied by Galilaeus Oy (Kaarina, Finland) for novel anthracyclines and by Terragen (Vancouver, BC, Canada) to access secondary metabolites from lichens and marine organisms. It is also being used by Kosan Biosciences (Hayward, CA, USA) in attempts to produce large quantities of epothilone (Harvey, 2000). The method has been particularly successful with polyketide synthase (PKS) genes: derivatives of medically important macroiide antibiotics and unusual polycyclic aromatic compounds have been produced by novel combinations of the type I and type II PKS genes, respectively. This work was facilitated by the finding, first discovered for the biosynthesis pathway of actinorhodin by *Streptomyces coelicolor,* that in both bacteria and fungi the genes involved in antibiotic biosynthesis are organized into compact clusters containing the structural genes specifying the numerous enzymes that make up a secondary metabolic pathway, the genes providing self-resistance and resistance to exogenous antibiotics, and very often the regulatory genes that coordinate the expression of the other genes in the cluster.

Polyketides, from organisms such as actinomycetes, have been isolated and studied for more than a 100 years and have produced some commonly used antibiotics (such as erythromycin, oleandomycin and spiramycin), immunosuppressant (such as rapamycin and FK506), the veterinary antiparasitic, avermectin, and the antifungal drug, candicidin. The discovery of the enzyme systems responsible for their bio-construction has led to a rejuvenation of the field. Many of the PKSs are multi-enzyme complexes that can be manipulated to act as 'factories' for the production of new synthetic polyketides. Daptomycin, a cyclic lipopeptide produced by *Streptomyces roseosporus,* is the active ingredient of Cubicin (daptomycin-forinjection), a first-in-class antibiotic approved for treatment of skin and skin-structure infections caused by Gram-positive pathogens and bacteremia and endocarditis caused by *Staphylococcus aureus,* including methicillin-resistant strains. Genetic engineering of the nonribosomal peptide synthetase (NRPS) in the daptomycin biosynthetic pathway was exploited for the biosynthesis of novel analogs of daptomycin. One compound was more potent against an *Escherichia coli imp* mutant which had increased outer membrane permeability (Kien *et al.*, 2006).

Gene Shuffling

Many natural product genes are modular and produce multifunctional enzymes. They have a high degree of plasticity. By interchanging and moving genes around within these clusters, hybrid enzymes can be produced that are capable of synthesizing an unlimited set of new molecules. Novel compounds were produced by gene transfer between strains producing the isochromanquinone antibiotics actinorhodin, granaticin, and medermycin. At a molecular level, DNA shuffling mimics, yet accelerates, evolutionary processes, and allows the improvement of individual genes and sub-genomic DNA fragments. An example of gene shuffling with four individual cephalosporine genes gave a yield improvement of between 270 and 540-fold. The best clone had eight segments from three of the four genes and 33 point mutations (Knight *et al.*, 2006).

Transformation of *S. galilaeus* ATCC 31133 with plasmid pMC213 containing the aklavinone 11-hydroxylase gene (dnrF, *Streptomyces peucetius* subsp. *caesius* ATCC 27952) resulted in the production of hybrid aclacinomycin A, which showed highly specific *in-vitro* cytotoxicity against leukemia and melanoma cell lines (Hwang *et al.*, 1995).

CONCLUSIONS AND FUTURE PERSPECTIVES

However, only a small percentage of known microbial secondary metabolites have been tested as natural-product drugs. Natural-product programs need to become more efficient, starting with the collection of environmental samples, selection of strains, metabolic expression, genetic exploitation, sample preparation and chemical dereplication. A renaissance of natural products-based drug discovery is coming because of the trend of combining the power of diversified but low-redundancy natural products with systems biology and novel assays. Increasing the quality and quantity of different chemical compounds tested in diverse biological systems should increase the chances of finding new leads for therapeutic agents. The major growth seen in the biotechnology industry in recent decades has largely been driven by the exploitation of genetic engineering techniques. The initial benefits have been predominantly in the biomedical area, with products such as vaccines and hormones that have received broad public approval. In the environmental biotechnology and industrial ecology sectors, biotechnology has the potential to reduce agri-chemical usage or remediate polluted environments.

One prerequisite to natural-product discovery that remains paramount is the range and novelty of molecular diversity. Currently, natural product chemistry is going through a phase of reduced interest in the drug discovery field. However, new developments may turn around this negative perception. Following points will be helpful to make the task easier:

> The systematic exploitation of selected ecosystems combined with the development of new techniques and media for isolation of novel micro organisms will allow the collection of representative strains from large parts of the micro-population. This maximized biodiversity will deliver chemical diversity for a given ecosystem.

- The direct expression of environmental DNA in heterologous surrogate hosts is progressing. There is a need for rapid and sensitive detection and characterization of new metabolites as well as their corresponding gene clusters.
- Manipulation of physiology should be based on experimental design and measurement of secondary metabolism.
- Gene-shuffling coupled with the genetic engineering power of, for example, PKS, will allow the generation of hybrid or unnatural microbial natural products.
- Total synthesis of natural products with interesting biological activities is paving the way for the preparation of new and improved analogs.
- In natural product chemistry, for rapid and accurate differentiation of chemical compound profiles an on-line measurement by LC-ELSD, DAD, MS, and NMR is essential.

Today, more than 30,000 diseases are clinically described, but less than one-third of these can be treated symptomatically, and only a few can be cured (Schultz and Tsaklakidis 1997). New chemical entities as therapeutic agents and for agricultural applications are urgently needed. Natural products can continue to play a major role in drug discovery. New strategies to natural-product based drug discovery will increase chemical diversity and reduce redundancy. Maximizing the discovery of new compounds and minimizing the re-evaluation of already known natural products will be crucial.

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4

FUNGI AS A SOURCE OF NOVEL BIOACTIVE METABOLITES

APARNA DAS AND SARDUL SINGH SANDHU

A conservative of the number of fungi on our planet is 1.5 million (Hawksworth, 2001). Due to their pharmaceutical potential, secondary metabolites of fungi have been studied for more than seventy years.

Synthesis of secondary metabolites are rare in animals but abundant in green plants, fungi and bacteria. They are produced in nature and serve survival function for their producer organisms. The secondary metabolites can be toxins, pheromones, antibiotics etc. The functions of secondary metabolites are: (1) Cleanse the immediate environment of competing microorganisms. (2) Protect the dominant and initiated spore from consumption by other microorganisms. (3) Slow down germination of spores until a less competitive and more favourable conditions for growth exist (Demain and Fang, 2004).

Identification of microorganisms and evaluation of its potential to produce bioactive compounds is of great interest in development of new molecules to fight against many pathogens (Hara Kishore *et al.*, 2007). A wide range of secondary metabolites are produced by fungi which has high therapeutic value as antibiotics, cytotoxic substances, insecticides, repellant etc (Demain, 1999). Secondary metabolites produced from fungi vary in production, function and specificity to a particular fungus (Keller *et al.*, 2002). These metabolites are being exploited in different fields of medicine and industries (Huisman and Ggray, 2002; Straathof *et al.*, 2002). The search for new drugs from fungi started with the discovery of penicillin by Alexander Fleming in 1929. It was produced by *Penicillium notatum* and functioned as a potent antibiotic against gram +ve bacteria. The assumption is that certain physical and biological situations in natural environment favour the production of diverse range of secondary metabolites (Dreyfuss and Chapela, 1994). This indicates that it might be more useful to investigate fungal isolates from other ecological niches than soil in order to take more direct approach towards creative and novel fungal groups. Some relatively sparsely explored fungal groups derived from such ecosystems are; fresh water fungi, marine fungi and endophytic fungi.

The secondary metabolites of marine fungus drives much interest due to repertoire of chemical defense systems. These poisons often exhibited potent pharmacological activities in assay systems used for search of new pharmaceuticals. They had anticancerous and antifungal activities.

The search for secondary metabolites from filamentous fungi has led to the discovery of many bioactive compounds. The versatility of microbial biosynthesis is conspicuous and are capable of producing novel biologically active metabolites (Baker and Alvi, 2004).

Many antibiotics in clinical use were developed from fungal metabolites. Of the various secondary metabolites produced, the most vital and explored are the antibiotics which confer antimicrobial and biocontrol property to the producer.

The structural novelty and diversity of fungal metabolites make them a logical source of new leads for drug discovery and development. The antibiotic era began with the discovery of significant antibiotics viz. penicillin, actinomycin and tyrothricin in 1939. Within the first 18 years of this era nearly 30 antimicrobials had come into use, as cited by Selman Waksman, the discoverer of streptomycin. During the last decade several pathogenic microbes developed resistance to available antibiotics. Infections by multidrug resistant isolates of *Candida* sp., *Staphylococcus epidermis*, *Staphylococcus aureus*, *Streptococcus* sp., *Enterococcus* sp. and *Escherichia coli* became more and more frequent stimulating the search for new antibiotics with novel mechanisms of action (Kotra and Mobashery, 1998; Morschhauser *et al.*, 2000; Sandven, 2000; Thomson and Moland, 2000).

Since antibiotics are produced by bacteria as well as fungi, the latter holds more promising results, as humans and fungi share common antagonists and humans can benefit from the natural defensive strategies of fungi that produce antibiotics to fight infections. Fungi are well known to show antibacterial, antifungal, larvicidal, molluscicidal, antioxidant and free radical scavenging activities (Keller *et al.*, 2002). A vast number of fungi have been utilized for biotransformation process and many more to be explored for isolation of some potential compounds.

Entomopathogenic fungi are rich sources of bioactive compounds. Screening results in selection of certain strains secreting high levels of extracellular antibiotics. These substances are active against some bacteria and fungi.

Endophytic fungi are known to contribute to their host plants by producing a plethora of substances that provide protection and survival values to the plant. These compounds when isolated and characterized are known to be potential for use in modern medicine, agriculture and industry. Novel antibiotics, antimycotics, immunosuppressants and anti cancer compounds are few examples (Strobel *et al.*, 2004).

Mushrooms are known to be rich source of antibiotics. They are defined as the fruiting body of a macrofungus (Soo, 2002). The number of mushrooms on earth is estimated to be 1,40,000, yet only 10% are known (Wasser, 2004). They are rich in vitamins viz., niacin, thiamine, riboflavin, biotin and ascorbic acid. They also contain a wide variety of bioactive molecules including terpenoids, steroids, phenols, nucleotides and their derivatives (Borchers, 1999). They are known to exhibit immune enhancing properties and can combat bacteria (Kupra *et al.*, 1979; Benedict and Brady, 1972), viruses (Suzuki *et al.*, 1990) and protozoa *Plasmodium falciparum*. Mushrooms are claimed to exhibit antitumor, antiviral, antibiotic, anti-inflammatory, hypoglycemic, hypocholesteromic and hypotensive activities (Chang and Miles, 1989; Breene, 1990; Miles and Chang, 1997). Zhou *et al.* (2006), reported that triterpenes isolated from *Ganoderma* mushroom exhibited anticancerous, antiviral, antioxidant, hepatoprotection and cholesterol synthesis inhibition activities.

ANTIBACTERIAL PROPERTY OF FUNGI

Entomopathogenic fungi are known to inhibit pests and insects of crops. Their antimicrobial property have also been reported by certain strains of *Beauveria bassiana.* The screening of 15 *B. bassiana* mutants resulted in the selection of one isolate which produced highest level of extracellular antibiotic activity. It was active against gram +ve organisms and certain gram -ve organisms also proved sensitive (Champlin, 1981). Kransoff *et al.* (2006) reported that metabolites NG-391 and NG393 produced by mutant strain of entomopathogenic fungi, *Metarrhizium anisopliae* exhibited potent S9 dependent mutagenicity against *Salmonella* sp.

47 typical entomopathogenic fungi were tested for their ability to produce antibiotic activity and to establish cultivation conditions which helped to establish production of bioactive compounds. 81% and 64% of these fungi produced either anti-*bacillus* or anti-*Staphylococcus* compounds, indicating that the majority of the entomopathogenic strains possessed the ability to produce antibacterial compounds. At 26°C, *Metarrhizium anisopliae* HF 293, *Nomuraea rileyi* HF588 and *Verticillium* HF 238 strains produced clear antibiotic activity against *Bacillus* and *Saccharomyces,* but only in presence of insect derived materials (Lee *et al.*, 2005).

An asexual strain of entomopathogenic fungi, CY-8202 isolated from *Cordyceps sinensis* showed strong inhibition of gram +ve bacteria including *Bacillus subtilis, Micrococcus tetragenus* and *Staphylococcus albus* and gram-ve bacteria including *Proteus vulgaris, Salmonella typhi, Aerobacter aerogenes* and *Salmonella* sp. (Wang *et al.*, 2002).

Yeon *et al.* (2007) reported growth inhibiting property of cordycepsin derived from fruiting body of *Cordyceps militaris* cultured on *Bombyx mori* pupae (CM-1) and pupae separated from the culture (CM-2). Methanolic extracts from CM-1 and CM-2 strongly inhibited growth of *Clostridium difficile* ATCC 9689, *Clostridium paraputrificum* ATCC 25780 and *Clostridium perfringes* ATCC 13124. The growth inhibiting principle of cordycepsin was characterized by spectroscopic analysis and it may be produced from fruiting body of *C. militaris* cultured on *B. mori* pupae and then transferred to host insect and accumulated. Natural cordycepsin and its two analogues, 2'deoxyadenosine and tubercidin functioned as potent antimicrobial agent against various diseases caused by harmful intestinal bacteria such as *Clostridia* sp.

An endophytic fungus known as *Muscudor albus* obtained from small limbs of cinnamon tree effectively killed certain human pathogenic bacteria by virtue of a mixture of volatile compounds that it produced. The most effective class of inhibitory compound was the esters, of which 1-butanol, 3-methyl acetate was the most active biologically (Strobel *et al.*, 2001).

Growth inhibition of pathogenic bacteria such as *E. coli, B. subtilis* and *P. fluorescens* was shown by endophytic fungus isolated from *Quercus variabilis*. 20.9% of strains showed strong inhibition to pathogenic bacteria and most active fungal strain was *Cladosporium* sp. (Wang *et al.*, 2007).

The secondary metabolites of fungus *Cephalosporium* sp. AL031 had broad spectrum antibiotic activity against *Shigella sonnei, Mycobacterium tuberculosis* and *Streptococcus pneumoniae* (Yunmei *et al.*, 2004).

Aspergillus niveus LU 9575 was investigated regarding the diversity of secondary metabolites during screening programme for detection of novel bioactive metabolites derived from endosymbiotic microbes isolated from arthropod hosts (Gebhardt *et al.*, 2002). This fungus was isolated form the gut of woodlouse. Methanol-acetone extract of mycelium were named as Aspochalamins A-D and Aspochalasins D and Z. Aspochalamins showed weak antibiotic activity towards gram +ve bacteria and compound A was the most active. Aspochalasin D was active against several gram +ve bacteria (Gebhardt *et al.*, 2004).

Mygind *et al.*(2005) reported that saprophytic ascomycete fungus *Pseudoplectania nigrella* produced a kind of defensin known as plectasin which is small cysteine rich peptide and showed activity against bacteria, fungi and viruses. Its recombinant peptide was produced with higher yield and purity. In vitro it inhibited *S. pneumoniae* and its strains which were resistant to conventional antibiotics. Plectasin cured mice of experimental peritonitis and pneumonia and showed extremely low toxicity in them.

Lentinula edodes or shiitake mushroom and its products are highly used in traditional Chinese medicines (Soo, 2002) and is the second most commonly produced edible mushroom in world. Shiitake's immunomodulatory property includes imparting increase in resistance of the host to bacterial infections. Lenthionine and Bis[(methyl sulphonyl) methyl] from shiitake has antibacterial activity against *S. aureus, B. subtilis* and *E. coli* (Hatvani, 2001). Antibacterial activity has also been seen in extracts of dried mushroom against *Streptococcus mutans* and *Prevotella intermedia* (Shouji *et al.*, 1999) and even liquid cultures (Hatvani, 2001; Ishikawa *et al.*, 2001) and chloroform, ethyl acetate or other extracts of dried mushrooms (Hassegawa *et al.*, 2005).

Lycoperdons and puffballs, members of phylum basidiomycota are large edible mushrooms and are producers of antibiotics which can cure sores, abrasions, deep cuts, haemorrhage and urinary tract infections as reported by Buswell and Chang in 1993. Its strains viz. *L. pusilum* and *L. giganteum* exhibited activity against *E .coli, S. aureus* and *Proteus vulgaris*. This indicated their possession of broad spectrum antimicrobial property against clinical isolates (Jonathan and Fasidi, 2003).

Extracts and derivatives from mushrooms are promising medicines in modern times as there is an increasing number of bacteria developing resistance to conventional antibiotics like MSRA and *Pseudomonas.* Studies have shown that *Ganoderma* demonstrated antimicrobial activity against gram-ve *E. coli* and *Pseudomonas aeruginosa* (Smania *et al.,* 2001).

ANTIMYCOTIC POTENTIAL OF FUNGI

Antimicrobial metabolites from selected strains of entomopathogenic fungi, such as *Paecilomyces gunii* RCEF0866 and RCEF0857, *Paecilomyces* sp. RCEF0189, *Beauveria bassiana* RCEF0285 and *Nomuraea prasina* RCEF0879 showed inhibition of *Candida albicans* (Fenglin *et al.,* 2005), the causative agent of candidiasis. It is an opportunistic infection caused by fungi particularly in patients with compromised immune systems eg. patients receiving organ transplant and cancer chemotherapy or those infected by HIV are prone to such infections.

Fukuda *et al.* (2004) discovered fungal funicone related compounds named actofunicone of fungal origin as potentiators of antifungal miconazole activity. The compound reinforced miconazole activity against *Candida albicans.* Five beauvericins were isolated from culture broth of fungal strain *Beauveria* sp. FKI-1366. They were found to be new, designated beauvericins D, E and F (Fukuda *et al.,* 2004), although two compounds were identified as beauvericin and beauvericin A (Nilanonta *et al.,* 2002).

Antimycotic activity was shown by extracts of secondary metabolites produced by *Aspergillus fumigatus* CY018, an endophytic fungus isolated for the first time from the leaves of *Cynodon dactylon.* The extracts were subjected to in vitro bioactive assays against human pathogenic fungi and *C. albicans* was inhibited (Liu *et al.,* 2004).

Ascomycetes have been reported as active producers of antimicrobial compounds with high therapeutic values (Quang, 2002). Among these, *Glomerella cingulata* has been reported for the potential of biotransformation and production of many bioactive compounds (Miyazawa *et al.,* 1998; Miyazawa *et al.,* 1995; Nankai *et al.,* 1998). Potent antifungal activities of crude extract of *G. cingulata* were found against *Rhizopus oryzae, Chyrosporium tropicum* and *Beauveria bassiana* (Hara kishore *et al.,* 2007).

Biologically active substances obtained from endophytic *Pezicula* strains, isolated from living branches of deciduous and coniferous trees showed strong fungicidal effect (Schulz *et al.,* 1995). The compounds were identified as (R)-mellein, (-)-mycorrhizinA, 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde, (+)-cryptosporiopsin and 4-epi-ethiosolide.

Mycelial growth of *Botrytis cinerea* was inhibited by metabolite produced by effective antagonist *Trichoderma.* The metabolite 6-pentyl-_-pyrone (6PAP) was determined quantitatively by HPLC (Pezet *et al.,* 1999).

Muscodor albus, a novel endophytic fungus discovered in rain forests of Central America was reported to produce volatile compounds, that are inhibitory to wide range of microbes. Exposure to gases of *M.albus* killed fungi like *Rhizoctonia solani* and *Xylaria* sp. (Strobel *et al.,* 2001). The range of microbes affected were oomycetes, basidiomycetes, ascomycetes and deuteromycetes.

The first instance of antimicrobial potential of endophytic fungi residing in *Q.variabilis* was reported by Wang *et al.* (2007). An active antifungal strain I(R)9-2 *Cladosporium* sp., isolated from *Quercus variabilis*, displayed growth inhibition of atleast one pathogenic fungi such *as Trichophyton rubrum, Candida albicans, Aspergillus niger, Epidermophyton floccosum* and *Microsporum canis.* From the broth of selected *Cladosporium* sp., a metabolite called brefeldin A was obtained.

An unique lipopeptide antimycotic, termed cryptocandin, was described from *Cryptocoriöpsis cf. quercina,* an endophytic fungus (Strobel *et al.*, 1999). Cryptocandin had inhibitory effect against isolates of *Candida albicans, Trichophyton mentagrophytes* and *T. rubrum.*

The first investigations on potential of basidiomycetes as sources of antibiotics were performed by Anchel, Hervey, Wilkins in 1941 (Sandven, 2000), when they prepared extracts of fruiting bodies and mycelia culture from over 200 species. They succeeded in the isolation and identification of pleuromutulin, a diterpene that is especially useful for the treatment of mycoplasm infection in animals (Brizuela *et al.*, 1998) and served for the development of first commercial antibiotics of basidiomycete origin. Several compounds that inhibit the growth of a large spectrum of saprophytes, phytopathogenic fungi, bacteria, actinomycetes and other fungi were isolated from basidiomycetes (Anke, 1989, 1995 and 1997).

Similar results were obtained by Rosa *et al.* (2003), while they isolated several cultures of Brazilian basidiomycetes and screened their antimicrobial activity against broad spectrum of organisms.

Some endophytic fungi from Garcinia plants are a potential source of antimicrobial agents (Phongpaichit *et al.*, 2006). 18.6% of 377 isolated fungi inhibited atleast one pathogenic organism in agar diffusion method such as *C. albicans, Microsporum gypseum* and some bacteria. Fungal molecular determination demonstrated that potent isolates D15, M76 and N24 represented *Phomopsis* sp., *Botryosphaeria* sp. and an unidentified fungal isolate respectively.

Jonathon and Fasidi (2003), reported that strains of *Lycoperdons* viz., *L. pusilum* and *L. giganteum* showed antifungal activity against *Microsporum boulardii, Candida albicans, Aspergillus flavus, Aspergillus niger* and *Trichophyton concentrum.*

Polypore mushrooms provide a protective immunological shield against a variety of microbes (Chihara, 1992; Mizuno *et al.*, 1995). Even the gilled mushrooms possess intensive antifungal activities. The mycelium of the submerged gilled oyster mushroom, *Pleurotus ostreatus* had shown effectiveness against *Aspergillus niger* (Gerasimenya *et al.*, 2001), one of the most aggressive of all molds and causative agent of aspergillosis, that can pose serious threat to patients with compromised immune systems. The extracellular metabolites and the heavy molecular weight cell wall polysaccharides of mushrooms play a dual role in antimicrobial activity. The polysaccharide lentinan from shiitake inhibits *Candida albicans* (Wasser and Weis, 1999).

ANTIVIRAL POTENTIAL OF FUNGI

Keeping in view the resistance shown by strains and the toxicity by drugs, new substances whether synthetic or natural with antiviral activities are being developed.

Novel anti-viral agents can be explored from marine derived fungus as they are known to be tremendous sources of new biologically active metabolites (Dreyfuss and Chapela, 1994). They represent an under explored resource for discovery of novel anti-viral agents. Rowley *et al.* (2003) described a series of peptides designated halovirus A-E(1-5) that were produced during the saline fermentation of marine derived fungus of genus *Scytalidium.* These linear peptides were potent in vitro inhibitors of *Herpes Simplex Virus* 1 and 2. Evidence presented that the halovirus directly inactivated herpes virus and this mechanism of action can be applicable in the prevention of HSV transmission.

The aqueous extract of *Agaricus blazei* showed antiviral effect against *Herpes Simplex Virus* type-1(HSV-1) and *Bovine Herpes Virus* type-1 (BoHV-1) in HEp-2 cell cultures (Bruggemani *et al.*, 2006). The aqueous extract was more effective regarding to virucidal activity for both viruses, than therapeutic action. HSV-1 is associated to human's oro-facial, ocular infection and encephalitis. BoHV-1 is responsible for infection in bovines, such as those involved with the upper respiratory and genital tracts (Hinkley *et al.*, 1998; Nakamichi *et al.*, 2002; Roizman *et al.*, 2001). Roizman *et al.* (2001) showed that the aqueous extract and fractions obtained from *A. blazei* Murill were able to inhibit the cytopathic effect of *Western Equine Encephalitis* (WEE) virus, poliovirus and HSV in cultures of Vero cells (Sorimachi *et al.*, 2001).

Compounds from basidiomycetes, *Ganoderma pfeifferi* and *Rozites caperata* demonstrated antiviral activity for HSV (Mothana *et al.*, 2003; Piraino and Brandt, 1999).

The valuable biological activity of edible mushroom was shown by the addition of a composition of alcohol precipitated hot water extracts of *Lentinula edodes, Agaricus blazei, Grifola frondosa, Coriolus versicolor, Ganoderma lucidum* and two types of *Cordyceps sinensis* extracts to conventional therapy in order to achieve greater effectiveness against hepatitis infection in nine months, rather than by conventional therapy alone in more than one year.

A study demonstrated that the human immunodeficiency virus showed some weakness to shiitake extracts. Lentinan in combination with 3'-azide-3'-deoxythymidine (AZT) suppressed the surface expression of HIV antigen more strongly than AZT alone in vitro. It also enhanced the effect of AZT on replication of HIV in various human hematopoietic cell lines in vitro (Jong and Birmingham, 1993). In another study several fractions of LEM (an aqueous extract of shiitake and its solid culture medium) caused inhibition of the infectivity and cytopathic effects of HIV.

A highly water soluble, low cytotoxic polysaccharopeptide (PSP) was isolated from *Trametes versicolor* (turkey tail) mushroom and exhibited anti-viral activity by inhibiting HIV replication (Collins and Ng, 1997).

ANTICANCEROUS POTENTIAL OF FUNGI

Takahashi *et al.* (1994) isolated nine(9) leptosins (A-I) from *Leptosphaeria* sp., an epiphyte on the marine algae *Sargassum tortile.* Leptosins A and C exhibited significant anti-tumor activity against sarcoma 180 ascites. Leptosins G, G_1, G_2, H, I, J showed cytotoxic activity towards cultured P388 cells.

In an experiment by Gebhardt *et al.* (2004), metabolites Aspochalamins A-D and Aspochalasin Z, produced by fungus *Aspergillus niveus* LU9575 showed cytostatic effects in tumor cell lines. Aspochalamins Band C showed moderate cytostatic effects in all cell lines tested viz., HM02, MCF7, Huh7 and HepG2. Aspochalamin A displayed a moderate inhibitory potency on growth of HM02 and MCF7 cells. Aspochalasin Z showed weak cytostatic effect in HepG2, MCF7 and HM02 cells.

In an experiment by Borchers *et al.* (1999), shiitake mushroom's ability of anti-tumor activity was explored. He treated ICR mice with N-butyl-N'-butanolnitrosoamine (BBN) and fed them with dried powdered shiitake increased the levels of macrophage activity and mitogenic response of lymphocytes to concanavalin A to almost normal levels, which were severely suppressed by BBN treatment. It also increased the cytotoxic effect of lymphokine activated killer cells and natural killer cells. These accompanying immunomodulatory effects may be the mechanism for anti-tumor effect.

The methanol extract of culinary medicinal mushroom, *Pleurotus pufmonanus* showed significant regression of solid tumor (Jose *et al.*, 2000).

Kawagishi *et al.* (1995) reported that Yamabushiitake (*Hericium erinaceus*) mushroom showed effectiveness against aggressive He La cells. Similarly *Agaricus blazei* and *Cordyceps sinensis* reported anti-tumor activity.

The anti-tumor effects of shiitake feed in murine system have been paralleled by the effect of lentinan, which has been reported to prevent both chemical and viral carcinogenesis. Lentinan has been reported to potentiate the effect of other drugs such as 5-fluorouracil (5-FU) (Ogawa *et al.*, 1999) and cis-diaminedichloroplatinum (II) (CDDP) in cancer treatment. Lentinan has been described as host mediated anti cancer drug and has been put through numerous clinical studies in Japan (Soo, 2002).

A medicinal mushroom called *Coriolus versicolor* (CV) has been widely prescribed for prophylaxis and treatment of cancer and infection in China (Chu *et al.*, 2002). Wide spectrum of biological activity was demonstrated by aqueous extract of CV, like inhibition of cancer growth and stimulation of immune system. The aqueous extract has gained popularity over conventional cancer therapies. There has been a substantial increase in interest in developing these extracts into efficacious oral proprietary products.

HYPOCHOLESTEREMIC AND HYPOTENSIVE ACTION

Shiitake mushroom's ability to lower blood cholesterol was first reported in 1960's. The main active compound was defined and named as eritadenine. It lowered all lipid components of serum lipoprotein in both animals and humans (Soo, 2002). It proved as a boon for many people suffering from cardiovascular disease, as it is a big cause of mortality worldwide and high blood cholesterol levels are an important risk factor in development of cardio vascular problems.

Blood pressure increase was prevented in hypertensive rats by feeding them dried shiitake. A decrease in both VLDL and HDL cholesterol levels were observed. Same observations were exhibited in human testing models too (Soo, 2002).

ANTIOXIDANT EFFECT

In a study conducted by Mau *et al.* (2002), three medicinal mushrooms viz., *Ganoderma lucidum, Ganoderma tsugae* and *Ganoderma lucidum* antler showed excellent anti-oxidant activity. Total phenols were the major naturally occurring antioxidant compounds found in methanolic extracts from medicinal mushroom.

Anti oxidant property was also shown by methanolic extracts of mushroom *Pleurotus pulmonnrius* (Jose *et al.*, 2002). The extract showed significant hydroxyl radical scavenging and lipid peroxidation inhibiting activities.

INDUCTION OF APOPTOSIS IN CANCEROUS CELLS

Sphingoid bases from lactic yeast, *Kluyveromyces lactis* were found to induce apoptosis in Caco-2-human colon cancer cells (Aida *et al.*, 2004).

CONCLUSION

A rich fungal genome is an essential component of our natural heritage as natural products are a huge resource for medicine as shown with the use of hundreds of plant and microbial species in thousands of different pharmaceutical products. Therefore the investigation of the medicinal value of fungi has become a matter of great significance as they are society's greatest protection against microbial diseases and other serious health conditions.

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5

MICROBIAL GENOMICS

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INTRODUCTION

Genomics is the study of the molecular organization of genomics, their information content, and products they encode. It is a broad discipline, which may be divided into at least three general areas :

- **Structural genomics** : It is the study of the physical nature of genomes. Its primary goal is to determine and analyze the DNA sequence of the genome.
- **Functional genomics** : It is concerned with the way in which the genome functions. That is it examines the transcripts produced by the genome and the array of proteins they encode.
- **Comparative genomics** : It is a area in which genomes from different organisms are compared to look for significant differences and similarities. This helps identify important, conserved portions of the genome and discern patterns in function and regulation.

The data also provide much information about microbial evolution, particularly with respect to phenomena such a horizontal gene transfer. It should be emphasized at the beginning that whole-genome sequence information provides an entirely new starting point for biological research. In the future microbiologists will not have to spend as much time cloning bones because they will be able to generate new questions and hypotheses from computer analyses of genome data, then they can test their hypotheses in the laboratory.

Microorganisms are the predominant form of life on the planet in terms of their diversity, total mass and absolute numbers. As a collective population, microorganisms are representative of all of the diversity that is found in higher eukaryotes and more, and have served as models for number of biological processes. We interact with microorganisms on a continuous basis with both positive and negative outcomes. Extending beyond their direct effects on our lives in terms of the foods that we eat, disease that we might succumb to and the probiotic effects of certain microorganisms, it have also interactions with all forms of life. Plants and animals also are engaged in a variety of relationships with microorganisms.

Why Study Microbial Genomes?

Studies on whole genome sequences give us a complete genomic blue print for an organism. We can examine how all the parts operate cooperatively to influence the activities and behavior of an entire organism–a complete understanding of the biology of an organism. Microbes provide an excellent starting point for studies of this type as they have a relatively simple genomic structure compared to higher, multicellular organisms. Studies on microbial genomes may provide crucial starting points for the understanding of the genomics of higher organisms. Analysis of whole microbial genomes also provides insight into microbial evolution and diversity beyond single protein or gene phylogenies. Analysis of whole microbial genomes is also a powerful tool in identifying new applications for biotechnology and new approaches to the treatment and control of pathogenic organisms.

Microbial genome sequencing strategy

"Shotgun" sequencing

- shear DNA into small fragments.
- insert into vector
- sequence in from vector.
- computer aligns & assembles sequences based on overlap.
- ordering of contigs.
- primer walking to complete sequence.

Genome Size (prokaryotes)

- Bacterial genome: 6 x 105 ~ more than 107

  Smallest known: *Mycoplasma genitalium* (470 protein coding genes, 3 rRNA genes, 33 tRNA genes.)
- Prokaryotes genome sizes are roughly proportional to gene numbers.
- Processes affect bacterial genome size.

  Gene duplication, small-scale deletions and insertions, transpositions, horizontal transfer, loss of genes in parasitic lines, etc.

Advantages of using microbial genomes

- Procaryotic genomes are much smaller than eukaryotic ones.
- No introns
- Little non-coding region between genes
- Most genes & gene function known

- Comparative genomics can be done with many very similar genomes.
- Large numbers of sequenced microbial genomes available.

Genomic Insights

- Prevalence of gene clusters and gene islands (genomic islands). Horizontal gene transfer between microbes, mediated by phage or phage-like elements, appears to be common.
- Closely related bacteria can have significant differences in genome content and structure.
- Intra cellular bacterial genomes have reduced genome size e.g., Buchnera
- Endosymbiont of aphids
- 50 million years of genetic isolation
- only observe gene loss

e.g., Rickettsia-25% non coding (vs. 10% for most other bacteria)-evidence of decay e.g., *Mycobacterium leprae*-massive decay.

E. coli **O157:H7**

- *E. coli* O157:H7 is a rare but virulent strain of *E. coli,* which lives in the intestinal tracts of mammals and man and causes serious and potentially fatal diseases.
- O157 can survive refrigeration and freezer storage. The major food sources carrying this organism are undercooked hamburger and roast beef, raw milk, improperly processed cider.
- Since 1982, there have been at least 16 major outbreaks in the US. Some 22 deaths have been recorded. CDC experts estimate there may be as many as 20,000 cases per year.

What makes *E. coli* **O157:H7 so dangerous?**

The pathogenicity (ability to cause damage) and virulence (degree of pathogenicity) of O157:H7 depend on :

1. The genes encoding the so-called Shiga toxin, such as stx 1 and stx 2;
2. The small, circular DNA molecules that encode "virulence factors".
3. Pathogenicity island-a section of chromosomal DNA containing many genes that contribute to pathogenicity.

Genomics, which explores the biology of organisms through their genetic blue prints, has profoundly affected the discipline of microbiology. It has led us to revise our definitions of microbial entities, reconsider their capabilities and re-evaluate the microbiological tool box of methods and approaches. In the breadth of its influence on various sub disciplines of microbiology (e.g. metabolism, physiology, ecology, host-pathogen relationships and industrial microbiology), and its interaction with other disciplines (e.g. human and veterinary medicine, agriculture, evolutionary biology and structural biology), the impact of genomics on microbiology is arguably unrivaled in this century. In this review, we discuss recent work in genomics that supports, challenges, expands or otherwise affects some key concepts in microbiology.

Prokaryotic, Eukaryotic or Viral Microbe

Recent genomic studies have cast an interesting light on our division of the microbial world into four groups : Bacteria, Archaea, Eukarya and viruses. Some of the ultra structural and biochemical bulwarks that neatly separated these groups for so many years- for example, the overall level of cellular complexity, cell size, genome size, composition of ribosomes (where present), lipid type and behavioral complexity-began to be chipped away in the pre-genomic era. But genomics has amplified the effect, and has provided a deeper understanding of the rules and exceptions that characterize the four microbial groups.

Giant Viruses

A good example is provided by recently reported genomic analysis of DNA viruses. Mimivirus is a DNA virus that infects amoebae. It is an extremely large (400 nm) particle that stains gram-positive and has a genome size of 1.2 Mb. The genome contains genes not previously reported in viruses, encoding proteins involved in translation of proteins and components of metabolic pathways. Thus in its cell size, genome size and gene content, this mini virus apex a small bacterium. In a recent review, Desjardins *et al.* argue that the expansion in the viral definition provided by mini virus is quantitative, but not qualitative, in nature. They attribute the latter trait to the genome of the polydnavirus. Cotesia congregate bracovirus (CcBv). The minivirus genome encodes proteins involved in transcription, translation and DNA replication; very few of these are found in the genome of CcBv. In addition, many 'typical' viral genes are absent from the CcBv genome. The fascinating life cycle of CcBv is tightly associated with two eukaryotic species- parasitic bracovid wasps and their caterpillar prey.

Expression of viral genes in the caterpillar obstructs the immune response and helps ensure survival of the parasitic wasp. Genomic analysis of CcBv revealed oddities such as extremely low coding density, the absence of genes predicted to encode DNA replication proteins, and the high percentage (70%) of genes that appear to contain introns. Collectively, these features resemble a segment of eukaryotic genomic DNA, rather than a viral genome. However, these gene models have not yet been tested in vitro (for example, by examination of cDNA sequences), and so their significance cannot be fully evaluated.

Overlapping Genome Size and Content

These shifting boundaries between the microbial domains extend findings that have emerged over the past 15 or so years that similarly complicated our microbial definitions. In the realm of cell size, these findings included the discovery of very large (e.g. Epulopiscium) and very small (e.g. ultramicrobacteria and Nanoarchaeum) bacterial cells. This has been paralleled by the report of bacterial genome sizes that range from smaller than that of minivirus to larger than some fungal genomes. In an analysis of the functional content of these larger prokaryotic genomes, konstantinidis and Tiedje demonstrated an over-representation of some categories of genes, such as regulation of transcription and secondary metabolism. Conversely, other gene categories such as translation and DNA processing contained relatively fewer members in larger genomes. The lack of correlation with non-coding DNA and hypothetical open reading frame content led the authors to conclude that genome expansion favors specific functional classes. Some phylogenetic groups are over presented in this analysis, therefore it will be interesting to observe whether or not these trends hold true as a more diverse set of genomes becomes available.

Recent analysis of several fungi has revealed that they have minimized stream lined genomes, some of which appear to have adopted elements of bacterial metabolism. When the genome of the filamentous fungus *Ashbya gossypii* was published in 2004 it was the smallest (9.2 Mb) free-living eukaryotic genome to have been reported. It appeared to be a very compact genome, exhibiting a short distance between genes and only 221 introns. Its high level of homology and synteny with the genome of *Saccharomyces cerevisiae* and the presence of a similar number of proteins suggested that A. gossypii represented a minimal genome size for a free-living fungus, and that evolution of *S. cerevisiae* involved duplication or fusion of two related species.

Two cryptosporidium genomes also exhibited very streamlined metabolic pathways, with a reliance on glycolysis for energy production. They were notably furnished with enzymes that showed high similarities to bacterial counter parts, including nucleotide conversion enzymes, fatty acid synthases and polyketide synthases. Finally, the much larger (40 Mb) genome of *Neurospora crassa* was predicted to encode elements of red light photo biology, including two putative phytochrome homologs that showed most similarity to bacteriophytochromes. Eukaryal acquisition of bacterial metabolic components is apparently not restricted to the fungi.

The recently published genome of *Entamoeba histolytica,* in common with the other amito chondiral protist lineages represented by Giardia and Trichomonas, appears to have reduced or eliminated most mitochondrial metabolic pathways and uses anaerobic pathways associated with anaerobic prokaryotes. The authors speculate that lateral transfer of 96 genes from bacteria was the most probable mechanism for acquisition of bacteria-like fermentation enzymes and glucose transporters, as well as oxidative and nitrosative stress resistance proteins. On a more global scale, the 13.8 Mb genome of *Schizosaccharomyces pombe* provided an early glimpse into the transition from prokaryote to eukaryote, which have required more new genes than needed for the transition from unicellular to multicellular. It was proposed that the distinction between prokaryote and eukaryote depends not only on the number of genes but also on their type and regulation.

Ever some prokaryotes clearly exhibit an affinity for 'exclusively' eukaryal or rchaeal proteins, for example bacteriorhodopsins (light-driven proton pumps) were previously known only in halophilic archaea but have been detected in a planktonic gamma-proteobacterium, and were later found also in planktonic alpha proteo-bacteria. Another example is provided by tubulins protein constituents of the eukaryotic microtubule cytoskeleton that are predicted to be present in the genome sequence of

Prostheocobacter dejongeii. For these, in vitro assembly and GTP hydrolysis have been demonstrated. Tubulins were previously unknown in the Bacteria and Archaea of 347 eukaryotic signature proteins derived by Hartman and Fedorov, 10 were identified in the genome of *Prosthecobacter dejongeii* and 17 in the genome of *Gemmata* sp. Wa-1 which suggest that ancestral members of these phyla might have given rise to a proto-eukaryotic organism.

The Nature of the Minimal Genome

Considerations of genome size naturally lead to questions of genome redundancy, in other words, how much of the genome is absolutely required for life? A recent high density mutagensis study suggests that the essential structural and metabolic requirements of *Mycoplasma tuberculosis* are quite different from those of *Haemophilus influenzae*. Some of these differences make sense in the context of the unique mycobacterial cell wall type (e.g. the presence of rhamnose and lipid metabolism machinery in *M. tuberculosis*) and the ability of H. influenzae to transport amino acids that must be synthesized de novo by *M. tuberculosis*. In the genomes of aphid endosymbionts, the theoretical minimal genome does not reflect the genetic complement of any naturally occurring cell. These findings challenge the concept of a 'universal' minimal genome, and have led to the suggestion that shared orthologs do not define the minimal gene set. The ability of genomically limited organisms to produce relatively complex cell structures is also under investigation. *Mycoplasma pneumoniae* synthesizes a terminal organelle that binds to the host epithelium. The polar nature of this structure and its possession of cytoskeletal elements raise interesting questions about how its replication and migration within the cell is accomplished using a sparse gene complement.

Microbe-microbe Relationship

Recent reports have extended the field of symbiont genomics, previously firmly entrenched in the bacterial and eukaryal domains, to archaea and viruses. The archaeal genome of *Nanoarchaeum equitans* (a parasite of another archaeal organism) has the smallest complete prokaryote genome (490 kb) published to date; this appears to have undergone reductive evolution in the same manner as reported for bacterial parasites. The genome seems to be relatively stable, without pseudogenes and non- coding region. From this evidence, it is infer that the divergence of this parasite from other archaeal lineages was an ancient event. Less amicable microbial relationships have also been examined, for example the genome of *Bdellovibrio bacteriovorus*, which preys upon other Gram-negative bacteria in a tightly coordinated attack.

An ingenious study of *Escherichia coli* bacteriophages has given us a glimpse into how microbes interact with each other to adapt to two opposing selection pressures: cooperation and competition. The scientist forced the phages to adopt a life cycle that contained stages of both conflict and cooperation, by engineering each phage with a separate antibiotic resistance gene and then imposing growth under selection of both antibiotics, to promote the evolution of rapid conflict resolution. In a short time, mediation was achieved as one of the phages drastically reduced its genome size through loss of genes encoding the virion, and the genomes of both phages were packaged into the same protein coat. The co-packaging ties the fitness interests of the phages together, the experimental demonstration of this conflict resolution mechanism demonstrated that cooperation, needed for organism transition to a higher level of biological complexity, can evolve rapidly.

Genomics has led us to revise our concepts not only of what a microbe is and what it does, but also the best way in which to approach the study of these organisms. The cultivation of microbes in pure culture, for decades the only path to microbial identification and characterization, was somewhat eclipsed by the bright new star of cultivation-independent methodology that began in the early 1990s. This approach, which analyzed (principally) the small-subunit ribosomal RNA (rRNA) genes of mixed microbial populations from environmental samples, revealed the diversity of the previously uncultured prokaryotic world and provided the basis for early studies of microbial biogeography. But exciting as this new field clearly was, it provided only a set of labels for the members of a microbial community, not an indication of their activities and hence their ecological role in that community (with the exception of a few taxa for which function can be extrapolated from taxonomy).

Functional prediction required access to the genome rather than a single gene, and the technological advances of high throughout put sequencing provided that access. This gave rise to the field of metagenomics (also known as community genomics, ecogenomics or environmental genomics), the direct genomic analysis of microbial communities that inhabit an environmental sample, without the need for prior separation of an organism from its habitat, and maintenance in either pure or mixed culture on artificial substrates.

This approach has been the subject of several recent reviews. Rather, we can draw attention to a consequence or side-effect of metagenomic analysis, namely that with the exception of studies that concern low complexity samples or in which the authors have the resources to generate vast amounts of sequence data, assembly of a complete microbial genome from metagenomic data remains a challenge. In part, because of this current limitation, microbiologists are displaying renewed interest in the art of microbial cultivation for many years. The scientist succeeded in growing this through a combination of high throughput (microtiter plate) cultivation techniques using dilute media, and rapid and sensitive screening using fluorescent probes specific for the SAR11 cluster. Availability of these isolates will allow genome sequencing to be performed, and therefore the genomic basis of their biology to be investigated. This is of special interest given the extremely small cell size (0.01 cubic micrometers) of these free-living organisms. This high-impact report was followed or accompanied by several others in which members of ubiquitous microbial groups poorly represented in our culture collections were isolated by tweaking of cultivation conditions even by simple adjustments such as the use of solid vs liquid formulations of the same growth medium or increasing incubation time.

Genomics might thus hold the distinction of being the only molecular methodology to have facilitated or encouraged the development of both cultivation-dependent and cultivation-independent approaches to microbiology-approaches that were often regarded by their proponents as rivals rather than complementary strategies. As genomic data continue to accumulate from studies of both cultivated isolates and environmental mixtures, it will be interesting to see how each approach could be used to in form the other.

Bacterial Species

This question has vexed microbiologists and confounded biologists of 'higher organisms' for years owing to the paucity of phenotypic characters available for taxonomic use, the inability of our workhorse taxonomic molecule (rRNA) to adequately resolve highly related groups, and the laborious nature and lack of reproducibility of DNA:DNA hybridization- the gold standard for delineation of prokaryotic species. The growing availability of genome data from multiple strains of a species promises to provide a quantitative measure of genome relatedness and hence species status, as has been recommended in the recent past and has recently been attempted. At the other end of the taxonomic spectrum, analysis of representative members of poorly represented phyla promises to provide a clearer picture of the true diversity of microbial genomes and will allow us to better resolve phylogenetic relationships between the phyla. The interface between microbial systematics, evolution and genomics also provides an opportunity to re-evaluate the genome as a static entity, particularly its susceptibility to acquire and lose genes by lateral gene transfer (LGT) within and across the species boundary. Phylogenetic analysis of multiple strains of *Staphylococcus aureus* indicated that diversification of the highly variable RD13 region, which encodes putative pathogenesis-related proteins, probably occurred by LGT and recombination. It has been suggested as Thermotoga maritime strains.

Some authors have proposed that such exchanges occur between even more distantly related phylogenetic entities (up to phyla) and even between bacteria and fungi or protests whereas other have urged caution in the use of compositional factors to determine the phylogenetic origin of laterally transferred genes, arguing that these factors do not reflect the genomic context of the original source, that rather an association with mobile elements such as phages. The relative impact of LGT on microbial evolution and diversification remains a fiercely debated topic, to the extent that steps towards achieving consensus have been recommended.

It is probable that with the accumulation of more genome data, both from cultivated isolates and from metagenomic sources, we will continue to observe fascinating exceptions to the 'rules' that govern the identities and capabilities of microbes. We can expect more complicated and interesting relationships between the four microbial domain-level groups (Bacteria, Archaea, Eukarya and viruses), but also in other realms, such as the autotroph-heterotroph, aerobe-anaerobe, and free living-symbiont distinctions, which might come to more closely resemble spectra than dichotomies. We can also look forward optimistically to a more quantitative and rigorous genome-based taxonomy that can be achieved today with our handful of molecular chronometers and phenotypic traits. It is doubtless that other genomic challenges and enhancements to the concepts of microbiology remain to be discovered.

This article reports the genome sequencing of a giant virus found in amoebae; both the particle size and genome size of this virus distinguish it from other viruses, together with the unusual presence of genes that encode components of the protein translation apparatus and DNA repair functions. The size and composition of the mini virus genome raise interesting questions about how we distinguish viruses from cellular parasites.

Analysis of this 0.6 Mb genome, which originated from a virus transferred by a parasitoid wasp to its leptidopteran prey, revealed a segmented structural organization and features unusual in viral genomes, such as a wide range of coding density and the presence of introns. As in the case of the minivirus genome, the polydnavirus genome challenges our assumptions of what constitutes a virus and a viral genome.

First report of the use of shotgun sequencing to reconstruct the genomes of uncultivated microbes. Two almost-complete genomes and three partial genomes were assembled from an acidic biofilm that harbors a relatively low-diversity microbial community.

Microbial Metabolomics

First attempt at extremely high-volume sequencing (of more than one billion base pairs) from an environmental sample. More than 1.2 million genes were predicted from the sequence data, estimated to originate from at least 1800 genomic species. This study provided a large and important data set that continues to be mined for new findings. First attempt at using quantitative genome data to establish a genome based species definition for prokaryotes. Integrating metabolomics into systems biology framework to exploit metabolic complexity: strategies and applications in microorganisms.

As an important functional genomic tool, metabolomics has been illustrated in detail in recent years, especially in plant science. However, the microbial category also has the potential to benefit from integration of metabolmics into system frameworks. The application cases of metabolomics in microorganisms answer what the metabolomics can do in strain improvements.

Since the first sequencing organism, *Haemophilus influenzae*, in 1995, genome sequencing initiatives have been performed in over 200 organisms, including 23 archaeal, 236 bacterial, and 39 eukaryotic genomes. This genome information has greatly facilitated the identification of open reading frames (ORFs) by combining other technologies such as analyses at the level of gene expression (transcriptomics), protein translation (proteomics), and bioinformatics. However, these applications are still not enough to assign functions to the orphan genes and thereby understand the complete and complex metabolic and regulatory networks in microorganisms. Metabolomics, as the complement to transcriptomics and proteomics, has its own advantages. One of the major advantages of metabolomics is that there are fewer metabolite types than genes or proteins. Such advantages reduce the processing complexities. Moreover, with the improved combined analytical platforms, the individual metabolites in the complex mixtures can be identified with certainty. Such improvements will make the metabolic comparatives analysis more precise and efficient because the raw data of genome and proteome must be explained based on the homology or homeology.

Finally, the dynamics metabolite levels in organisms must reflect the exact metabolic phenotypes under different cultural and genetic conditions fundamentally. Although we have not yet acquired concrete evidences on this, the current developments are exciting. For example, it has been recently recognized that several cell functions are mediated by and acted upon at the metabolome and metabolic network level (Fell 2001; Raamsdonk *et al.* 2001; Even *et al.* 2003; Mandal *et al.* 2003). Although some environmental perturbations or genetic manipulations may not cause the changes in transcriptome and proteome levels, they can and do have significant effects on the concentrations of numerous individual metabolites. The origin and past metabolomic research mainly focused on plant and biomedical science. The research group at Taylor *et al.* (2002) has developed the analytical and the data processing method by analyzing 433 metabolites in *Arabidopsis*. Compared with metabolomics in plant and biomedical science, microbial metabolomics had its own advantages in systems biology frameworks despite less effort being put in the microbial field, especially in strain improvements.

We actually know much more information in microorganisms than plants regarding the genome information, regulation information, metabolic networks and other system information. Microorganism such as *Escherichia coli* and *Saccharomyces cerevisiae* had about 600 metabolites (Oliver *et al.* 1998) compared with 200,000 metabolites of plant (Fiehn 2002). This will accelerate the processing and applications of metabolomic data in system biotechnology to improve microbial phenotypes the metabolomic data in strain improvements are still big challenges faced by microbiologists and biochemical engineers. Recently, many researchers had identified and illustrated this issue from different researching perspectives (Lee *et al.* 2005, Werf 2005).

Metabolomic Research Developments

Definitions and concepts in the field of metabolomics, there are many emerging widely used terms. However, the meanings of many of them are not always clear. In particular, the term "metabolomics" had been widely used with different meanings or scopes. According to the existing publications (Nicholson and Wilson 2003: Oliver 2002; Fiehn 2003); the clear concepts and main characteristics were classified in Table 1. Currently, it is not possible to quantify all the metabolites in one cell because of the broad diverse chemical characters of metabolites. So, the term metabolomics is usually used broadly to cover the researchers, the aim of which is to analyze the global metabolism in the metabolite level with general experiment protocols. However, the essence is performing metabolic target analysis or metabolite profiling. Research developments and representative publications. The metabolic profiling research originated from the 1970s, which is long before Stephen Oliver first suggested the word metabolome in 1998 (Oliver *et al.* 1998). Soon afterwards, with the help of combined analytical platforms, it developed to be one hot subject, and many scholars had made great contributions to this field.

Understanding Microbial Genomes

Genome sequencing projects completed over the past few years have revealed unimagined genetic diversity in the microbial world. To capitalize on its investments in these projects, the Department of Energy (DOE) in 2002 created the genomes to life program- now renamed genomics: GTL- to link genomics and computational science in the formidable task of deciphering the fundamental biology underlying all microbial life. In a road map released in October 2005, DOE outlined plans to merge microbial biological studies and computational models.

Genomics: GTL' ultimate goal is to master microbial biochemistry and physiology so well that scientists will be able to predict microbial behavior in different environments based solely on genome sequence. With this knowledge, they will be able to develop microbe based strategies for DOE interests like clean energy, bioremediation, and climate change control, says Aristides Patrinos, associate director of DOE's office of Biological and Environmental Research.

"Computational science is the hope for integrating across knowledge in a way that you say something predictive," says Grant Heffelfinger, deputy director for materials science and technology at Sandia National Laboratories in Albuquerque, New Mexico Heffel finger uses computational methods to study how protein complexes bring ocean carbon dioxide into *Synechococcus cyanobacteria* cells and convert it into sugar. Understanding this fundamental part of Earth's carbon cycle may help scientists devise ways to control atmospheric carbon dioxide released during fossil fuel consumption, he says. Many GTL-funded scientists study microbial species that hold potential for bioremediation.

They study gene expression, protein dynamics, and metabolic creations in the lab, and then use this information to design computational models that predict how microbes will function in varying environments- for example, with a different pH, salinity, temperature, or nutrient concentration. Derek Lovley, distinguished professor of microbiology at the University of Massachusetts-Amherst, is studying species of Geobacter that convert soluble uranium into an insoluble form that precipitates out of soil. "We can't possibly go to every site before deciding on a bioremediation strategy", Lovley says, so he and his coworkers are creating computational models that will eventually tell them how to alter conditions or genetically engineer the bacteria to convert uranium more efficiently. Geobacter can also produce energy from waste by oxidizing organic matter and transferring electrons onto an electrode. The bacteria create energy extremely efficiently, but also extremely slowly, so lovely is feeding high-through put data into computer models that will predict how to get the microbes to work faster. Models that allow this level of understanding will rely on development of technologies in biology and computing that don't yet exist, Patrinos says, and the Genomics: GTL program has laid out four "very ambitious but very strategic goals" for developing these technologies. The first three involve basic microbiology: characterizing the genes, proteins, and multiprotein complexes that govern microbial life; figuring out how these complexes interact in regulatory networks; and determining the molecular basis of interactions in communities such as biofilms.

The fourth goal is to develop computational methods to analyze these data and to predict unknown microbial functions. Much of genomics: GTL research and development will take place at four corner research facilities funded by DOE. Patrinos says that DOE will soon issuer quests for funding proposals for the first facility, which will produce and characterize proteins. The other facilities will focus on molecular complexes within cells, proteomics, and cellular systems and community dynamics. Eventually, these facilities will lead high-throughput study of microbial systems in much the same way that designated sequencing facilities have led high-through put genome sequencing, Patrinos says. All labs involved in Genomics: GT Ladhere to strict protocols that ensure quality control and reproducibility, says Adam Arkin, assistant professor of bioengineering and chemistry at the University of California-Berkeley, which means that biologists will be able to compare results across different types of data. Arkin's lab is combining data on gene expression, protein interactions, and metabolism in the sulfate reducer *Desulfovibrio vulgaris* to try to "get some idea of the cross-talk between pathways".

Creating open-access online data bases is also a big part of the project, says Michelle Buchanan, associate laboratory director for physical sciences at Oak Ridge National Laboratory in Oak Ridge, Tennessee. She is developing high through put methods to analyze protein-protein interactions in microbial cells, and her results will be deposited in databases that other scientists can access and annotate with their own research results. Other GTL scientists will create similar databases for metabolic pathways, regulatory networks, and community interactions. The time line for realizing specific biological or computational goals is "very sketchy [since] this is a basic science program," says Patrinos, but "we have already accomplished some early successes". Microbes' potential to contribute to technological innovation in the 21st century is indeed considerable, says Heffel finger. "We can learn a tremendous amount by a comparative knowledge of the diversity of microbes".

Metagenomics: Application of Genomics to Uncultured Microorganisms

Microbiology has explained a transformation during the last 25 years that has altered microbiologists' view of microorganisms and how to study them. The realization that most microorganisms cannot be grown readily in pure culture forced microbiologists to question their belief that the microbial world has been conquered. We were forced to replace this belief with an acknowledgment of the extend of our ignorance about the range of metabolic and organismal diversity.

This change fomented a revolution in microbiological thought. At the heart of this revolution was the convincing demonstration that the uncultured microbial world far outsized the cultured world and that this unseen world could be studied (105-108). This change in thinking was prompted by another, equally important realization: microorganisms under pin most of the geochemical cycles and many human health conditions that were previously thought to be driven by inorganic processes and stress, respectively. The glimmers of insight into the influence that microorganisms exert on the world propelled microbiologists to pursue the uncultured world. In 1931, Waksman optimistically believed that "a large body of information has accumulated that enables us to construct a clear picture of the microscopic population of the soil", and in 1923 Bergey's Manual stated categorically that no organism could be classified without being cultured.

By the mid-1980s, however, microbiologists have lost this confidence, and the language and practice of microbiology changed to accommodate the vast unknown of uncultured life. Concepts, assumptions, images, and words needed to be replaced when it became evident that they were based upon the premise that microorganisms did not exist unless they could be cultured. Pace and colleagues highlighted the need for nontraditional techniques to understand the microbial world: "The simple morphology of most microbes provides few clues for their identification; physiological traits are often ambiguous. The microbial ecologist is particularly impeded by these constraints, since so many organisms resist cultivation, which is an essential prelude to characterization in the laboratory."

In the ensuring years, microbiologists dedicated intense effort to describing the phylogenetic diversity of exotic and ordinary environments- ocean surfaces, deep sea vents, hot springs, soil, animal rumen and gut, human oral cavity and intestine.

Many new lineages were classified based on their molecular signatures alone. The next challenge was to elucidate the functions of these new phylotypes and determine whether they represented new species, genera, or phyla of prokaryotic life. This challenge spawned various techniques, including metagenomics, the genome analysis of assemblages of organisms. In a few years, the study of uncultured microorganisms has expanded question "What are they doing?" The outcomes of the recognition of uncultured microorganisms are worthy of examination. One of these outcomes, metagenomics, is further shaping microbiology. Metagenomics has already opened new avenues of research by enabling unprecedented analyses of genome heterogeneity and evolution in environmental contexts and providing access to far more microbial diversity than has been viewed in the Petri dish.

HISTORY OF THE CULTURE DIVIDE

The current excitement about the uncultured world may make students of modern microbiology wonder why this aspect of microbiology was largely ignored for so long. It is worth tracing the origins of microbiology, which did not rely on culturing, and examine the reasons for the shift to culturing and the subsequent discoveries that rekindled interest in the uncultured world. This article will use the term uncultured microorganisms to capture the entire spectrum of organisms that are not cultured in a specific experiment. These may include microorganisms that we have not attempted to culture and those that have been resistant to culturing efforts but may submit to culturing in the future.

Early Microbiology and the Microscope the roots of microbiology are firmly associated with the microscope. The first record of a human being's seeing a bacterial cell is in 1663. Antonie van Leeuwenhoek watched bacteria that he recovered from his own teeth through his homemade microscope. He was a keen observer and an outstanding maker of microscopes, and his observations and detailed illustrations of microbial life prompted many other observers (both scientists and nonscientists) to take an interest in the microscopic world. His colorful descriptions of bacteria made their study compelling; in his descriptions of the many shapes of the bacteria he sampled from his teeth, he marveled that one "shot through the water like a pike does through water", firmly establishing that these tiny objects were, indeed, alive. For 200 years, microscopy enabled microbiologists to view heterotrophs, autotrophs, and obligate parasites alike.

Among the advances during this period of microbiology was the work of botanist Ferdinand Cohn, who classified many bacteria and described the life cycle of Bacillus subtilis based on his microscopic observations. Although mycologists such as Franz Unger had understood the concept of pure cultures as early as the 1850s, it was in large part the emphasis on disease causality that solidified pure culture as the standard bacteriological technique for laboratory microbiology.

Robert Koch's postulates and his own innovation in developing culture media were instrumental in this shift, and from the 1880s forward, the microbiological world was divided into the cultured and the uncultured. Microbiologists were attracted to the power and precision of studies of bacteria in pure culture, and as a result, most of the knowledge that fills modern microbiology textbooks is derived from organisms maintained in pure culture.

Modern Microbiology- a pure culture is not enough because culturing provided the platform for building the depth and detail of modern microbiological knowledge, for a long time microbiologists ignored the challenge to identify and characterized uncultured organisms. They focused instead on the rich source of discovery found in the readily cultured model organisms, and this contributed to the explosion of knowledge in microbial physiology and genetics in the 1960s to mid-1980s. Mean while, the study of uncultured microorganisms remained in the hands of a few persistent scientists who began to accumulate hints that fitted at the edge of the microbiological consciousness, suggesting that culturing did not capture the full spectrum of microbial diversity.

One of the indicators that cultured microorganisms did not represent much of the microbial world was the oft-observed "great plate count anomaly" the discrepancy between the sizes of populations estimated by dilution plating and by microscopy. This discrepancy is particularly dramatic in some aquatic environments, in which plate counts and viable cells estimated by acridine orange staining can differ by four to six orders of magnitude and in soil, in which 0.1 to 1% of bacteria are readily culturable on common media under standard conditions.

Brock and colleagues encountered microorganisms in yellowstone hot springs that could not be cultured and others whose behavior in culture did not reflect their activities in situ. Many of the organisms could not be cultured on agar medium because their temperature requirements exceed the melting point of the agar. Therefore, elucidating the physiological function of microorganisms without culturing them required ingenuity. Brock's central technique involved the immersion of microscope slides in the spring for 1 to 7 days, followed by microscopic examination and often staining with fluorescent antibodies raised against cultured members of the taxonomic groups suspected to inhabit the environment. This approach estimated in situ population sizes and growth rates, which indicated, for example, that certain strains of Sulfolobus grew in the hot springs at temperatures well below the optima in pure culture (103). The expanding body of evidence indicating that it was imperative to study physiology in the environment led Brock's group to determine which organisms in the hot spring were responsible for photosynthesis. To do so, they placed an opaque cover over the spring for a week. The spring lost its pink color, leading them to infer that the genus Synechococcus, typically pink in culture, was a major contributor to photosynthesis.

Further evidence that drew attention to the uncultured world accumulated during the 1970s and 1980s. A study of oligotrophs indicated that incubation times longer than 25 days enhanced the recovery of certain organisms in culture.

The food industry generated intense interest in "injured bacteria" in food-live organisms that cannot be cultured following stressful treatments such as heat, chilling, or desiccation but represent a significant risk to human health. The concept of organisms that were viable but not culturable emerged from the work of Colwell and colleagues, who showed that strains of *Vibrio cholerae* were indeed alive and virulent when isolated from aquatic environments but did not grow in culture until after passage through a mouse or human intestine.

The confluence of these and many other scientific and technical advances steadily drew attention to the unculturable microbial world, but two discoveries figured significantly in the sharpened focus. The first was work on the diversity of soil bacteria, which demonstrated with DNA-DNA reassociation techniques that the complexity of the bacterial DNA in the soil was at least 100-fold greater than could be accounted for by culturing. This work suggested that the diversity of the uncultured world exceeded previous estimates. The second discovery was the demonstration that *Helicobacter pylori* causes gastric ulcers and cancer. Although spiral bacteria had been observed in the gastric mucosa of dogs in 1893 and in humans in 1996, and correlations between the appearance of the bacteria and peptic ulcers were noted in 1938, it was not until *H. pylori* was cultured that its role in disease was accepted. Culturing was accompanied by the satisfaction of koch's postulates on a human volunteer, providing definitive evidence for the causal relationship between the bacterium and ulcers.

Ironically, culturing was not that difficult. Plates accidentally incubated for 5 days instead of 3 revealed colonies later shown to be *H. pylori*. The fact that strong microscopic evidence for the role of *H. pylori* long preceded culturing and might have served as the basis for successful treatment decades earlier, perhaps reducing human suffering and mortality due to ulcers and cancer, did not escape the notice of microbiologists, medical practitioners, and the public. Whereas the studies of the complexity of the soil DNA demonstrated the diversity of the unknown world, the connection of uncultured bacteria and ulcers provided a striking example of the power of the undetected organisms. These discoveries provided compelling evidence that drew microbiologists to wrestle with the daunting challenge of devising strategies to access these organisms.

THE PARADIGM SHIFT

In 1985, an experimental advance radically changed the way we visualize the microbial world. Building upon the pioneering work of Carl Woese, which showed that rRNA genes provide evolutionary chronometers, Pace and colleagues created a new branch of microbial ecology. They used direct analysis of 5S and 16S rRNA gene sequences in the environment to describe the diversity of microorganisms in an environmental sample without culturing. The early studies were technically challenging, relying on direct sequencing of RNA or sequencing of reverse transcription-generated DNA copies. The next technical breakthrough arrived with the development of PCR technology and the design of primers that can be used to amplify almost the entire gene. This accelerated the discovery of diverse taxa as habitats across the earth were surveyed by the new technique.

The application of PCR technology provided a view of microbial diversity that was not distorted by the culturing bias and revealed that the uncultured majority is highly diverse and contains members that diverge deeply from the readily culturable minority. Today, 52 phyla have been delineated, and most are dominated by uncultured organisms. The application of phylogenetic strains-nucleic acid probes with fluorescent labels that facilitate visualization of single cells in situ-led to a recrudescence of microscopy as a central tool of microbiology and microbial phylogeny. Whereas traditional microscopy provides little phylogenetic information and fluorescent antibody studies require prior knowledge and culturing of an organism or one closely related to it to raise antibodies, phylogenetic stains require only an rRNA sequence, which can be derived from an environmental sample without culturing. Phylogenetic strains corroborated evidence from PCR-based studies but provided quantitative information as well, because the findings are based on direct observation that is not subject to the skewing of organism abundance potentially observed with PCR.

rRNA Analysis and Culturing

In addition to providing a universal culture-independent means to assess diversity, 16S rRNA sequences also provided an aid to culturing efforts. Bacteria may be recalcitrant to culturing for diverse reasons-lack of necessary symbionts, nutrients, or surfaces, excess inhibitory compounds, incorrect combinations of temperatures, pressure, or atmospheric gas composition, accumulation of toxic waste products from their own metabolism, accumulation of toxic waste products from their own metabolism, and intrinsically slow growth rate or rapid dispersion from colonies. Testing myriad conditions requires focus on the critical variables, is challenging and laborious, and can only succeed if there is a sufficiently quantitative assay available to determine whether the organism of interest is enriched under a specific set of conditions.

Nucleic acid probes labeled with fluorescent tags provide such as assay, facilitating quantitative assessment of enrichment and growth. As a result, culturing efforts have intensified recently, and successes have included pure cultures of members of the SAR11 clade, now termed the genus *Pelagibacter*, which represents more than one-third of the prokaryotic cells in the surface of the ocean but was known only by its 16S rRNA signature until 2002. The corollary to SAR11 in terrestrial environments is the *Acidobacteria* phylum. *Acidobacteria* are abundant in soil, typically representing 20 to 30% of the 16S rRNA sequences amplified by PCR from soil DNA, but until recently only three members had been cultured.

Once again, the culture-independent indications that it was prevalent in the environment led to intensive efforts to culture members of the Acidobacteria phylum. The current efforts to culture new microorganisms will be advanced by the information that metagenomics can reveal about uncultured organisms. Given that many organisms will not be coaxed readily into pure culture, a critical advance is to extend the understanding of the uncultured world beyond cataloging 16S rRNA gene sequences, and microbiologists have striven to devise methods to analyze the physiology and ecology of these diverse, uncultured organisms.

METAGENOMICS-CULTURE-INDEPENDENT INSIGHT

Among the methods designed to gain access to the physiology and genetics of uncultured organisms, metagenomics, the genomic analysis of a population of microorganisms, has emerged as a powerful centerpiece. Direct isolation of genomic DNA from an environment circumvents culturing the organisms under study, and cloning of it into a cultured organism captures it for study and preservation. Advances have derived from sequence-based and functional analysis in samples from water and soil and associated with eukaryotic hosts.

The word metagenomics was coined to capture the notion of analysis of a collection of similar but not identical items, as in a meta-analysis, which is an analysis of analyses. (Community genomics, environmental genomics, and population genomics are synonyms for the same approach). The idea of cloning DNA directly from environmental samples was first proposed by Pace, and in 1991, the first such cloning in a phage vector was reported. The next advance was the construction of a metagenomic library with DNA derived from a mixture of organisms enriched on dried grasses in the laboratory. Clones expressing cellulolytic activity were found in these libraries, which were referred to as zoolibraries, a term that has not been used widely in the field. The work of DeLong's group defined the field when they reported libraries constructed from prokaryotes in sea water. They identified a 40-kb clone that contained a 16S rRNA gene indicating that the clone was derived from an archaeon that had never been cultured. Construction of libraries with DNA extracted from soil lagged due to difficulties associated with maintaining the integrity of DNA during its extraction and purification from a soil matrix but eventually produced analyses analogous to those from sea water.

APPROACHES TO METAGENOMIC ANALYSIS

Metagenomic analysis involves isolating DNA from an environmental sample, cloning the DNA into a suitable vector. Phylogenetic stains. Fluorescent in situ hybridization biofilm samples from Iron Mountain Mine, Calif. Nucleic acid probes were labeled with indodicarbocyanine, and DNA was stained non specifically with 4, 6, diamidino-2-phenylindole. The nucleic acid probes are specific for (top left) Sulfobacillus spp., (top right) Archaea, (bottom left) Archaea on fungal filaments, and (bottom right) Eukarya.

Functional Genomics-based Studies of the Microbial Ecology of Hyperthermophilic Micro-organisms

Although much attention has been paid to the genetic, biochemical and physiological aspects of individual hyperthermophiles, how these unique micro-organisms relate to each other and to their natural habitat must be addressed in order to develop a comprehensive understanding of life at high temperatures. Phylogenetic 16S rRNA-based profiling of samples from various geothermal sites has provided insights into community structure, but this must be complemented with efforts to relate metabolic strategies to biotic and abiotic characteristics in high-temperature habitats. Described here are functional genomics based approaches, using cDNA micro arrays, to gain insight into how ecological features such as biofilm formation, species interaction, and possibly even gene transfer may occur in native environments, as well as to determine what genes or sets of genes may be tied to environmental functionality.

Microbial life as we know it depends on complex chemical, physical and biological interactions, factors that are often ignored in determining the salient metabolic and physiological features of individual micro-organisms. It can be challenging enough to understand how various metabolic pathways in an individual organism affect one another, but even more so when considering the possibility that intra and inter species interactions and other biological/abiological factors play a significant role. However, as the post-genomic era unfurls, and a myriad of powerful functional genomic tools have become available, holistic frameworks for complex biological phenomena can be envisioned. Among the opportunities presenting themselves is the prospect of examining comprehensive transcriptional and translational response patterns in a single organism or in a microbial community to various environment-based biological and abiological stimuli. While analyses based on 16S rRNA sequences have enabled the detection of species in a particular environment, functional assessments must be used to understand how species interact and what role specific micro organisms play in their particular niche. Genetic systems for hyperthermophiles have yet to be developed, but genomic based tools such as DNA micro arrays offer an alternative approach for deciphering novel physiological and ecological phenomena underlying life at high temperature.

In order to understand how species interact in a natural environment, it is important to look past the limitations of pure culture studies and instead examine how mixed populations respond to stimuli. Among the issues that need to be examined in high-temperature microbial ecology are why perthermophiles sense their biological environment, how they interact with each other, and how they fulfil specific roles in their environment. The ways in which specific phenotypes are regulated in hyperthermophiles are not known, and this prompts a number of interesting questions concerning intra-and inter species interactions in geothermal environments. What trigger biofilm formation in hyperthermophiles and how does this relate to species interactions within the sessile consortia? What mechanisms enable interspecies transfer of metabolites and what synergistic interactions are formed to enhance such exchanges?

It has been suggested through analysis of sequencing data that massive lateral gene transfer has occurred between certain hyperthermophilic species. If so, to what extent is genetic material exchanged among currently extant hyperthermophiles and how does such a process proceed? If in fact evolution occurs as a diverse biological unit, the nature of interactions within that unit must be examined at the cellular and, ultimately, the community level. If quorum sensing is used by hyperthermophiles, by what mechanisms does communication take place? A challenge in conducting mixed culture studies is that no tall species are culturable, and relatively few species have been isolated and grown successfully under laboratory conditions. one approach is to select representative model organisms to cultivate together to begin to understand the interaction taking place. one classical example of hyperthermophilic symbiosis is the pairing of a heterotroph with a methanogen.

Many hyperthermophilic heterotrophs produce hydrogen, and hyperthermophilic methanogens utilize this hydrogen as an energy source for growth. Hydrogen is a potent growth inhibitor for certain hyperthermophilic heterotrophs, so when the two physiological types are paired together the heterotroph benefits from the abatement of growth inhibition, while the methanogen secures a supply of its limiting growth reagent. This defined coculture is an initial step towards gaining an understanding of how species interact. While not a true representation of ecology in a natural setting, it enables the observation of species interactions in a controlled environment, a necessity when utilizing molecular tools such as micro arrays where cross contamination between species can be problematic.

The result of such a simple pairing can be dramatic, yielding such results a 10-fold gain in maximum cell density for the mixed species when compared with the pure culture, and high lighting drastic differences in global gene-expression patterns (M.R. Johnson and R.M. Kelly, unpublished work). In addition, microbial interactions in hyperthermophiles are not limited to synergistic relationships. Recently, an example of the first parasitic relationship between a hyperthermophile with agenome size of only 0.5 Mb and an archaeal host has been described, opening up new questions as to how the species perceives the presence of the host and subsequently attaches to it.

Biofilms

Biofilms found in hydrothermal systems contain microorganisms that can form a matrix and adhere to a surface as a community. In general, biofilms often allow co-operation among individuals and enhanced defence mechanisms. However, the specific functions of biofilms in hyperthermophilic niches have yet to be understood. In the better characterized mesophiles, biofilm cells have been shown to be involved in cell-to-cell signaling, pathogenesis, communal feeding and the formation of a complex biofilms secondary structure. Many questions remain about the roles, composition and structures of hyperthermophilic biofilms as well as possible signaling processes that may contribute to their formation. While biochemical analysis can yield information about biofilm composition, it offers little insight into the pathways used to form the biofilm, or how the biofilm is degraded or maintained. actively growing mesophilic biofilms first develop a matrix of growing cells, then expand the colony size, and finally detach portions of the colony from the biofilm in order to form new bio films. If a similar feature pattern of growth occurs in hyperthermophilic biofilms, by what methods do they detach? If the biofilm is formed with a scaffold of polysaccharides, then the gene-expression pattern of the cell's hydrolase inventory could prove very interesting. This information would not only offer insight into the genes needed for biofilm sloughing, but also information on the types of chemical linkage present in the biofilm material. Hyperthermophile-based biofilms have been observed for *Pyrococcus furiosus* and *Thermotoga maritime* and inco-cultures of *T. maritime* and *Methanococcus jannaschii.* The use of continuous culture (chemostat) is the method of choice for the efficient and consistent growth of hyperthermophilic biofilms. This method allows for the production of sufficient biofilm material for RNA extractions and has been used in micro array maritime cultures (M.A. Pysz and R.M. Kelly, unpublished work). Polycarbonate filters have provided a useful surface in hyperthermophilic continuous cultures for imaging biofilm growth by fluorescent microscopy, confocal laser scanning microscopy, scanning electron microscopy, and environmental scanning electron microscopy. Pairings such biochemical and image-based analysis of biofilm matrix with transcriptional information offers great potential for characterizing the structures formed by a hyperthermophilic biofilm community.

Genetic Transfer

With the complete genome sequences now available for a number of hyperthermophilic micro-organisms, it has become clear that gene transfer has played an important part in their evolution. As seen 4-24% of genes within genomes of sequenced hyperthermophilics species are predicted to be the direct result of lateral gene transfer. The mechanism underlying these gene-transfer events is unknown. Could the transfer be via pili formed by the cells to transfer DNA (conjugation), bacteriophage mediate transduction or transformation through direct DNA uptake? In the example of *T. maritime,* which has an astonishingly large number of predicted laterally transferred genes, no bacteriophage or conjugation-related genes have been identified from genome sequence analysis. There is possibility that transformation could occur via competence due to the presence of putative competence genes (drp A, com M, com E, com EA, com FC) similar to a type II secretion pathway and a type IV pilin-related protein, both of which are systems used in mesophiles for competence. However, such a system has yet to be confirmed in a functional setting. One way to begin to probe for the mechanisms of gene transfer in hyperthermophiles is to stress cultures and look for the induction of putative competence-related genes. Common competence-inducing events such as osmotic shock, heat shock and entrance into stationary growth phase can be examined for the ability to induce the expression of these putative competence genes. Ideally, the experiments should be conducted utilizing a large-scale cultivation approach. This methodology offers optimal control over growth conditions, and allows for the sampling of multiple time points over the course of a stimulation experiment from the same population, in essence building the statistical power of the study. In the mesophile *Streptococcus pneumoniae,* sampling many time points has proven to be important.

THE FUTURE OF MICROBIAL GENOMICS

Since the first complete microbial genome was published in 1995, more than 100 microbial genomes have been completely sequenced and published, and another 300 microbial genome sequencing projects are estimated to be in progress world wide. The significance of the information that has been derived from these complete individual genomes cannot be under estimated. Sequencing technologies have improved considerably, and the overall costs for sequencing have reduced to the point that sequencing a complete microbial genome, although still sometimes accompanied by various difficulties, is now almost routine. The field of microbial genomics has moved away from the primary initial focus on pathogen genomes to include the sequencing of diverse prokaryotes that occupy a range of environmental niches, and which are responsible for an array of environmental processes. Every genome that has been sequenced to date has provided new insight into biological processes, activities, and potential of these species that was not evident before the availability of the genome sequence. We have gained unprecedented insight into gene transfer (Nelson *et al.* 1999; Perna *et al.,* 2001). Environmental applications (Nelson *et al.,* 2002), and the virulence mechanisms in many of these species (Tettelin *et al.,* 2000; Tetteline *et al.,* 2001, Tettelin *et al.,* 2002).

Sequence data bases and comparative tools are now more easily accessible and allow for successful comparisons of different genomes, the identification of metabolic pathways and the analysis of transporter profiles across various species. Most significantly, the tremendous success of genome sequencing has allowed us to pursue other avenues where we can now derive genomic information from the multitudes of uncultivable prokaryotic species and complex microbial populations that exist in nature.

Accompanying the improved technologies that have resulted in lowered costs and improved bioinformatics tools, are new technologies that have bolstered our abilities to work with non-traditional species.

Organisms for which limited quantities of DNA are available can now be sequenced due to the availability of reagents that allow for exponential DNA amplifications. For example, *Epulopiscium,* the Institute is, currently sequencing the largest known heterotrophic bacterium (600 μm by 60 μm), for genomic research (TIGR) in Rockville, Maryland. Even though we are still unable to culture this bacterium in the laboratory, the genome sequence stands to provide important information on the unusual ability of *Epulopiscium* to produce live multiple offspring internally, as well as the changes that occurred early in the transition from the prokaryotic cell to eukaryotic cell. In addition, we anticipate that metabolic reconstruction and successful growth of this organism based on the functionally annotated genome will be possible. Generation of sufficient DNA for sequencing of *Epulopiscium* has been made possible, in part, by the development of bacteriophage φ 29 DNA polymerase as a tool for multiply primed rolling circle amplification of DNA directly from cells or plaques (Dean *et al.,* 2001; Dean *et al.,* 2002; Repli-GTM, Molecular Staging; TempliPhi TM, Amersham Biosciences).

The anticipated success of this project suggests that many uncultivable species for which we can sort a limited number of cells and generate small quantities of DNA can now be sequenced to completion. Although we are making progress in working with uncultivable species, it is evident that we will have to develop large-scale technologies to handle the cultivation of the large number of microbial species in nature. Zengler and workers have described a high-throughout cultivation method that employs the encapsulation of cells in gel micro droplets under low nutrient flux conditions followed by flow cytometry, to detect micro droplets that contain micro colonies. (Zengler *et al.,* 2002).

By trying to mimic the concentrations of these nutrients in their natural environments, some species can be successfully cultivated where they could not previously. They have shown that this technique can successfully be applied to multiple environments. The authors correctly point out that although the use of bacterial artificial chromosomes (BACs) allow for the identification of novel metabolic processes, cultivation will ultimately be necessary if a comprehensive understanding of these species is desired. The improvement of tools for sequencing and assembly, accompanied by a reduction in costs, has also been a significant boost to the field of environmental genomics. We know that the associations of micro organisms in the environment are significant, and many play major roles in elemental recycling, the conversion of biomass and the onset of disease. Molecular techniques such as 16S rDNA sequencing and phylogenetic analysis, restriction fragment length polymorphism (RFLP) and fluorescent *In situ* hybridization (FISH) analyses have allowed us to make tremendous advances in terms of being able to identify the extent of microbial diversity in various environments.

These techniques, however, have revealed limited to no information on the genetic diversity contained within these environments. For example, the physiological role that is being played by individual species identified by 16S rDNA sequencing cannot be defined. Some level of analogy may be drawn by extrapolation to available genetic information from related species, but it is apparent from whole genome sequencing that species that appear to be closely related from 16S rDNA sequences may have tremendous differences in genome composition (Perna *et al.,* 2001). Without being able to cultivate these organisms, the option of sequencing and analysing large DNA fragments retrieved directly from the environment becomes more attractive in an attempt to increase our understanding on how communities control various processes.

Deciphering the genetic information of uncultured species can currently be achieved by sequencing genomic libraries that are created directly from environmental DNA (Beja *et al.,* 2000a; Beja *et al.,* 2002; Quaiseret *et al.,* 2002. Liles *et al.,* 2003). This development has taken advantage primarily of the successful construction of large insert BAC and fosmid libraries (up to 120 kb in size). Initial surveys have demonstrated an unanticipated level of microbial diversity that remains to be explored (Beja *et al.,* 2002a; 2002b). Currently, this technique of sequencing and analysing genetic information obtained directly from the environment has been extended to the analysis of soils, the human oral cavity and gastrointestinal tract (GIT), and the Sargasso Sea. Although different styles of libraries may be used, whether a combination of small and medium insert libraries of BACs and fosmids, one major challenge in working with some of these environments is anticipated to be the successful assembly of all the sequence data so that large contiguous pieces of DNA which contain operons or links to phylogenetic markers can be generated. Micro-heterogeneity in these environments may also be an issue. The ideal would be to regenerate extremely large contiguous pieces of DNA (overlapping fosmids for example) or, if possible, complete microbial genomes of previously uncultivated species from these samples.

It remains to be seen however, if current assembly software can handle this challenge. One can envision that, with detailed bioinformatics analysis, an entire microbial community could be handled in a manner similar to the pipeline developed for annotating a microbial genome. As such, a single database could contain all of the predicted ORFs, their putative annotation, and biological role categories from a particular environment sample at a particular point in time. By examining the role assignments, it then becomes possible to identify all the biochemical pathways, and characterize the metabolic capacity of the entire microbial community that may be present. Just as a photorhodopsin was identified in the study by Delong and co-workers (Beja *et al.*, 2000b) based on the analysis of a single fosmid clone, an unprecedented amount of biochemical and physiochemical data undoubtedly will be obtained from the sequencing of other complex environments. The soil metagenome definitely will yield new antibiotics, as well as catabolic pathways for ring-based compounds and pathways for the synthesis of other secondary metabolites. The human GIT metagenome will yield significant information on the metabolic potential of the species that inhabit the GIT, their fermentation potential and the end products that they are capable of producing. This in turn will have implications for human GIT function, human health and the potential for these species to cause diseases. By having the metagenome of a healthy individual, we will be in a position to compare with the metagenome of a diseased individual allowing for the identification of possible microbial species and factors that are responsible for the onset of various diseases.

The first metagenomics meeting was held in Darmstadt Germany. This timely meeting was the innovation of groups at Darmstadt University of Technology and BRAIN Biotechnology Research and Information Network in Darmstadt, Germany, and the VAAM Functional Genomics Group in Feldafin, Germany. The meeting brought together some of the most respected leaders in the field including Ed De Long from the Moneterey Bay Aquarium Research Institute (California, USA), Oded Beja (Technicon-Israel Institute for Technology), Michael Wagner (University of Vienna, Austria) and Bill Martin (University of Dusseldorf, Germany). Representatives from a number of American and European funding agencies were also present. The discussions and presentations highlighted progress in the field of metagenomics, studies which have spread to include a range of soil ecosystems, biofilms, and sites from a number of oceans and seas. Soils in particular are being heavily studied for important enzyme activities, industrial biocatalysts and novel antibiotics. It is anticipated that this meeting is only the fore runner of many more of its kind that will be held on both sides of the Atlantic. Regardless of all the progress that we are making as environmental microbiologists, we are still presented with the situation where at the individual microbe level, close to 40% of each genome remains as hypothetical or conserved hypothetical proteins. In addition, it is humbling to realize that no single prokaryote has been studied to the point that all the gene functions within that organism are known. This demands that high-throughput methodologies be developed for the efficient analysis of these large data sets, including high-throughput proteomics, gene expression and protein-protein interaction studies. It is anticipated that these types of methodologies will further extend into an analysis of microbial communities where new techniques for studying the complex communities need to be developed, such that the associations between the previously cultivated and the estimated greater than 99% of uncultivated species can be evaluated. Micro arrays are rapidly becoming standard laboratory tools for investigating gene expression under different conditions, as well as for looking at the presence and absence of genes in different strains or species that are related to a reference genome. It is encouraging to note that some recent studies have demonstrated the success of using micro arrays in detecting gene expression in very complex microbial communities. Suppressive subtractive hybridization (SSH) has also been successfully to identify differences in community composition from environmental samples such as the rumen (B. White, pers. Comm.), and this methodology also holds significant promise as a tool for environmental genomics studies.

Each approach has strengths and limitations, together these approaches have enriched our understanding of the uncultured world, providing insight into groups of prokaryotes that are otherwise entirely unknown.

Sequenced-based analysis can involve complete sequencing of clones containing phylogenetic anchors that indicate the taxonomic group that is the probable source of the DNA fragment.

Alternatively, random sequencing can be conducted, and once a gene of interest is identified, phylogenetic anchors can be sought in the flanking DNA to provide a link of phylogeny with the functional gene. Sequence analysis guided by the identification of phylogenetic markers is a powerful approach first proposed by the DeLong group, which produced the first genomic sequence linked to a 16S rRNA gene of an uncultured archaeon. Subsequently, they identified as insert from sea water bacteria containing a 16S rRNA gene that affiliated with the Proteobacteria. The sequence of flanking DNA revealed a bacteriorhodopsin-like gene. Its gene product was shown to be an authentic photoreceptor, leading to the insight that bacteriorhodopsin genes are not limited to Archaea but are in fact abundant among the Proteobacteria of the ocean.

A promising application of phylogenetic anchor-guided sequencing is to collect and sequence many genomic fragments from one taxon. In more complex environments and taxa, reassembly of a genome may not be feasible, but inference about the physiology and ecology of the members of the groups can be gleaned from sequence data. This approach has been initiated with clones from diverse coils carrying 16S rRNA genes that affiliate with the Acidobacteria phylum, which is abundant in soil and highly diverse and about which little is known. Complete sequencing of the estimated 500 kb of Acidobacterium DNA in metagenomic libraries may provide insight into the subgroups of bacteria in this phylum that have never been cultured.

The alternative to a phylogenetic marker-driven approach is to sequence random clones, which has produced dramatic insights, especially when conducted on a massive scale. The distribution and redundancy of functions in a community, linkage of traits, genomic organization, and horizontal gene transfer can all be inferred from sequence-based analysis. The recent monumental sequencing efforts, which include reconstruction of the genomes of uncultured organisms in a community in acid mine drainage and the Sargasso Sea, illustrate the power of large-scale sequencing efforts to enrich our understanding of uncultured communities. These studies have made new linkages between phylogeny and function, indicated the surprising abundance of certain types of genes, and reconstructed the genomes of organisms that have not been cultured.

Biogeochemical Cycles

The use of phylogenetic markers either as the initial identifiers of DNA fragments to study or as indicators of taxonomic affiliation for DNA fragments carrying genes of interest because of their functions is limited by the small number of available markers that provide reliable placement in the Tree of Life. If a fragment of DNA that is of interest for other reasons does not carry a dependable marker, its organism of origin remains unknown. The collection of phylogenetic markers is growing, and as the diversity of markers increases, the power of this approach will also increase, making it possible to assign more fragments of anonymous DNA to the organisms from which they were isolated. Moreover, as more genomes are reconstructed, more genes will be linked to phylogenetic markers even though they were not cloned initially on the same fragment.

Functional Metagenomics Heterologous Expression

A powerful yet challenging approach to metagenomic analysis is to identify clones that express a function. Success requires faithful transcription and translation of the gene or genes of interest and secretion of the gene product, if the screen or assay requires it to be extracellular.

Functional analysis has identified novel antibiotics, antibiotic resistance genes Na (Li)/H transporters, and degradative enzymes. The power of the approach is that it does not require that the genes of interest be recognizable by sequence analysis, making it the only approach to metagenomics that has the potential to identify entirely new classes of genes for new or known functions. The significant limitation is that many genes, perhaps most, will not be expressed in any particular host bacterium selected for cloning. In fact, there is an inherent contradiction in this approach-genes are cloned from exotic organisms to discover new motifs in biology, and yet these genes are required to be expressed in Escherichia coli or another domesticated bacterium in order to be detected. The diversity of the organisms whose DNA has been successfully expressed in *E. coli* is surprising, but heterologous expression remains a barrier to extracting the maximum information from functional metagenomics analyses.

Identifying active clones- screens, selections, and functional anchors. The frequency of metagenomic clones that express any given activity is low. For example, in a search for lipolytic clones derived from German soil, only 1 in 730,000 clones showed activity. In a library of DNA from North American soil, 29 of a total of 25,000 clones expressed hemolytic activity. The scarcity of active clones therefore necessitates development of efficient screens and selections for discovery of new activities or molecules. Just as bacterial genetics elies on selections to detect low-frequency events, metagenomics will be advanced by seeking selectable phenotypes to increase the collection of active clones that can be compared, analyzed, and used to build a conceptual framework for functional analysis.

Several selections have proved to be fruitful. For example, the Daniel group designed a clever selection for Na (Li)/H antiporters that requires complementation of an *E. coli* mutant deficient in the three Na/H antiporters (nhaA, nhaB, and chaA) enabling growth on medium containing 7.5 mM LiCl. This powerful selection facilitated the discovery of two novel antiporter proteins in a library of 1,480,000 clones containing DNA isolated from soil. Another selection strategy involved complementation of an *E. coli* mutant deficient in biotin production, which led to the isolation of seven new operons for biotin synthesis from enrichment cultures derived from samples of soil or horse excrement. Selection for antibiotic resistance led to the isolation of a tetracycline resistance determinant from samples of the microbiota from the human mouth and aminoglycoside resistance determinants from soil. The selection for aminoglycoside resistance identified nine clones, six of which encoded 6-acetyl transferases that formed a new cluster based on sequence analysis. These genes were discovered in libraries containing a total of 4 Gb of DNA, or approximately 1 million genes, and thus their infrequent representation would have made it prohibitively laborious to discover them by a screen without a selection. This example illustrates the power of functional metagenomics-genes that are expressed in an ordinary host such as *E. coli* may be extraordinary and novel.

High-throughout screens can substitute when the functions of interest do not provide the basis for selection. For example, on certain indicator media, active clones display a characteristic and easily distinguishable appearance even when plated at high density. With the indicator dye tetrazolium chloride, Henne *et al.* detected clones that utilize 4-hydroxybutyrate in libraries of DNA from agricultural or river valley soil. Very rare lipolytic clones in the same libraries were detected by production of clear halos on media containing rhodamine and either triolein or tributaryin. The discovery of new biological motifs will depend in part on functional analysis of metagenomic clones. Functional screens of metagenomic libraries have led to the assignment of functions to numerous "hypothetical proteins" in the databases.

Innovation will be required to identify and overcome the barriers to heterologous genes expression and to detect rare clones efficiently in the immense libraries that are needed to represent all of the genomes in complex environments, such as soil. An emerging and powerful direction for metagenomic analysis is the use of functional anchors, which are the functional analogs of phylogenetic anchors. Functional anchors are functions that can be assessed rapidly in all of the clones in a library. When a collection of clones with a common function is assembled, they can be sequenced to find phylogenetic anchors and genomic structure in the flanking DNA. Such as analysis can provide a slice of the metagenome that cuts across clones with a different selective tool, determining the diversity of genomes that contain a particular function that can be expressed in the host carrying the library. Technological developments that promote functional expression and screening will advance this new frontier of functional genomics.

ECOLOGICAL INFERENCE FROM METAGENOMICS

The exigent questions in microbial ecology focus on how microorganisms form symbioses with eukaryotes, compete and communicate with other microorganisms, and acquire nutrients and produce energy. Thus far, metagenomics has provide insights into each of these areas, but in each instance, the challenge is to link the genomic information with the organism or ecosystem from which the DNA was isolated. Expression of a gene in a cultured host can establish gene functions, but without the appropriate biological context, circumspection is required in drawing ecological inferences. Future technical innovations are needed to extend in sights from metagenomics from inference to mechanistic analyses.

Many bacterial symbionts that have highly specialized and ancient relationships with their hosts do not grow readily in culture. Many of them live in specialized structures, often in pure or highly enriched culture, in host tissues, making them ideal candidates for metagenomic analysis because the bacteria can be separated readily from host tissue and other microorganisms. This type of analysis has been conducted with *Cenarchaeum symbiosum*, a symbiont of a marine sponge, a Pseudomonas-like bacterium that is a symbiont of Paederus beetles, *Buchnera aphidicola*, an obligate symbiont of aphids, the Actinobacterium Tropheryma whipplei, the causal agent of the rare chronic infection of the intestinal wall, and the Proteobacterium symbiont of the deep sea tube worm *Riftia pachyptila*. These systems provide good models for metagenomic analysis of more complex communities and thus warrant further attention in this review, although the term metagenomics typically connotes the study of multispecies communities. Therefore, the following section focuses on two of these obligate symbionts and the insight into their life styles offered by metagenomic analysis.

Buchneara-aphid symbiosis : The first genome reconstruction of an uncultured organism was that of *Buchnera aphidicola*, the endosymbiont of aphids. The relationship between the bacterium and the insect is ancient, leaving each partner unable to function independently of the other, as is reflected in the genomic analysis. Moran's group isolated bacterial DNA from the insect and sequenced and reassembled the bacterial genome. The genus *Buchnera* contains a "reduced" genome of 564 open reading frames. Upon comparison with a reconstructed ancestral genome, 1,906 genes appear to have been lost. Most of the functions are associated with biosynthetic pathways contributed by the host, suggesting that the genome shrinkage is the result of the symbiotic life style, which has become obligate because of gene loss. The reconstruction of *B. aphidicola's* genome provided insights into the evolution of the symbiosis between the insect and bacterium, the biochemical mutual dependence that they have developed, and the mechanisms of genome shrinkage and rearrangement. The success of genome reconstruction with a single uncultured species provided part of the impetus needed to propose sequencing and reconstructing genomes in more complex assemblages.

Proteobacterium-tube worm symbiosis : *Riftia pachyptila,* the deep sea tube worm, lives 2,600 m below the ocean surface, near the thermal vents that are rich in sulfide and reach temperatures near 400^{0}C. The tube worm does not have a mouth or digestive tract, and therefore it is entirely dependent on its symbiotic bacteria, which provide the worm with food. The bacteria live in the trophosome, a specialized feeding sac inside the worm. The bacteria and trophosome constitute more than half of the animal's body mass. The bacteria oxidize hydrogen sulfide, thereby producing the energy required to fix carbon from CO_2, providing sugars and amino acids (predominantly as glutamate) that nourish the worm.

The worm contributes to the symbiosis by collecting hydrogen sulfide, oxygen, and carbon dioxide and transporting them to the bacteria on hemoglobin-like molecules. The bacterium is a member of the Proteobacteria, as identified by 16S rRNA gene sequence. The bacteria have not been grown in pure culture in laboratory media, but they provide an excellent substrate for metagenomics because they reach high population density in the trophosome and exist there as a single species. Hughes *et al.* isolated DNA from the bacterial symbiont and constructed fosmid libraries from it that were used to understand the physiology of the bacteria. Robinson *et al.* identified a gene with similarity to ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubis CO) from the same fosmid library.

All of the residues associated with the active site are conserved in the protein sequence deduced from the DNA sequence, and it has highest similarity with the Rubis CO from *Rhodospirillum rubrum.* The characterization of this gene lends further support to the premise that the chemoautotrophic bacterial symbiont in *R. pachyptila* fixes carbon for its host. The libraries were also screened for two-component regulators with a labeled histidine kinase gene as a probe. They identified as two-component system whose components complemented an env Z and a pho R cre C double mutant, respectively. The discovery of a functional env Z homologue indicates that the symbiont carries a response regulator that is typical of Proteobacteria, although the signals eliciting responses from these proteins have not yet been identified. Genomic analysis of the symbiont also led to the identification of a gene encoding flagellin, which was expressed in *E. coli* and shown to direct the synthesis of flagella that are immunologically cross-reactive with *Salmonella* flagella. The presence of genes for flagella suggested to the that the endosymbiont has a free-living stage in its life cycle and may infect each generation of tube worms rather than being passed maternally.

COMPETITION AND COMMUNICATION

What can metagenomics tell us about microbial competition and communication? Competition for resources among community members selects for diverse survival mechanisms, including antagonism and mutualism among the members. Understanding these mechanisms is central to advancing the definition of principles that govern microbial community structure, function, and robustness. Historically, genetics has provided the most convincing evidence for traits contributing to microbial 1 fitness.

Classic mutant analysis has revealed genes required for microbial competition antagonism and mutualism. Mutant analyses have provided the greatest advances in knowledge because screening mutants containing random mutations for effects on fitness has led to the identification of genes that would not have been predicted to play a role in microbial competition or mutualism.

Genes for competition and cooperation are hard to recognize based on sequence alone because the utility of their functions is entirely dependent on ecosystem context and the nature of the resources that are limiting. Therefore, genomics by itself does not provide a means to test ecological hypotheses or identify genes that confer fitness, but it can provide the basis for forming hypotheses. Ecological hypotheses are difficult to test in microorganisms that cannot be cultured or for which there are no genetic tools; however, functional genomics coupled with chemical ecology can yield informative answers.

Chemical ecology involves the identification of small molecules with biological activity and proposed ecological function. These compounds can be identified through a variety of methods, including metagenomics. The addition of these molecules to communities can provide the basis for postulating their ecological roles in the community by measuring perturbations of community function. The following sections explore the discovery of small molecules in metagenomic libraries and postulate the ecological functions of these molecules in the organisms producing them. Small-molecule discovery by functional metagenomics has concentrated on antibiotics, which are of interest for their pharmaceutical applications as well as for their roles in ecosystem function. Traditional antibiotic screens for molecules that inhibit bacterial growth have led to the discovery of antibiotics in metagenomic libraries. They have not been a rich source of novel antibiotics, likely because of the experimental limitations associated with the search. In studies that report frequencies, antibiotic-producing clones are detected at a frequency of approximately 1 producer per 104 clones. This low frequency hinders discovery because space and labor are required to conduct typical antimicrobial screens.

With standard inhibition assays, a Mycobacterium-inhibiting antibiotic, terragine, was discovered from a soil metagenomic clone maintained in *Streptomyces lividans* and acyltyrosines from a clone maintained in *E. coli*. Colored antibiotics represent a disproportionate share of those discovered because they can be identified visually. For instance, a clone noticed for its brown pigment was found to produce melanin, which masked orange and red pigments, two novel antibiotics, turbomycin A and turbomycin B. A purple pigmented clone produced violace in, previously shown to be an antibiotic made by the soil bacterium *Chromobacterium violaceum*. The sequence of the genes on the metagenomic clone diverged substantially from the *C. visolaceum* violacein biosynthetic operon despite similar genetic organization, suggesting that the pathway on the metagenomic clone was derived from an organism other than *C. violaceum*. Osburne's group identified structurally related compounds, indirubin and indigo blue, in a soil metagenomic DNA library based on their blue color. Sequence-based screening for small molecules. The first polyketide synthases, enzymes involved in synthesis of polyketides, the broad class of antibiotics that includes erythromycin, epithilone, and rifamycin, were first cloned from soil with a PCR based approach. Seow *et al.* designed primers that hybridize with the highly conserved region of polyketide synthase genes and amplified novel polyketide synthase homologues directly from soil. This approach was adapted for screening metagenomic libraries by Osburne's group, who screened a 5,000-member metagenomic library for conserved regions of genes encoding type 1 polyketide synthase.

Primers directed a conserved region of polyketide synthase I genes that flanks the active site of the ketoacyl synthetase domain were used to screen pools of 96 clones. The screen yielded 11 new polyketide synthase homologues that contained significant sequence similarity to polyketide synthase genes from cultured organisms. In addition, screening clones in both *E. coli* and *Streptomyces lividans* by chemical means revealed two novel compounds, fatty dienic alcohol isomers antibiotics as signal molecules.

If antibiotics evolved as mediators of functions other than warfare, such as communication, antibiotic discovery will be expedited by screening metagenomic clones for signaling compounds as well as inhibitory compounds. The challenge is to develop assays that detect signaling by many compounds. A surprising result from the Davies group indicated that sub inhibitory concentrations of many antibiotics induce quorum sensing despite no resemblance in structure to the acylated homoserine lactones that appear to be the natural inducers. This result presents a propitious opportunity- a single screen might capture molecules that are quorum-sensing inducers as well as antibiotics. This opportunity was investigated by designing a high through put screen to identify compounds that induce the expression of genes under the control of a quorum-sensing promoter. The screen is intracellular, meaning the metagenomic DNA is in the same cell as the sensor for quorum-sensing induction. The sensor is comprised of the LuxR promoter, which is induced by acylated homoserine lactones, linked top gfp, and residues on a plasmid in an *E. coli* strain that did not induce quorum sensing itself.

If an inducer of the lux R-mediated transcription of gfp is expressed from the metagenomic DNA, the cell fluoresces and can be captured by fluorescence-activated cell sorting or as a colony observed by fluorescence microscopy. Conversely, this sensor system can detect inhibitors of quorum sensing if acylated homoserine lactone is added to the medium and fluorescence-activated cell sorting is set to collect the non fluorescent cells. Metagenomic libraries from micro biota of the soil and from the midgut of the gypsy moth have been subjected to this screen, and an array of genes have been identified. Their products are under analysis, and some appear to differ from previously described quorum sensing inducers (L. Williamson, c. Guan, B. Borlee, and J. Handelsman, unpublished data).

POPULATION GENETICS AND MICRO HETEROGENEITY

Metagenomic analysis has revealed that even apparently uniform populations contain substantial micro heterogeneity. Metagenomics-based model of biogeochemical cycles mediated by prokaryotes in acid mine drainage. Cell metabolic cartoons constructed from the annotation of 2,180 open reading frames identified 'in the Leptospirillum group II genome and 1,931 open reading frames in the Ferroplasma type II genome. The cells are shown within a biofilm that is attached to the surface of an acid mine drainage stream.

Within the population of *Cenarchaeum symbiosum* associated with the marine sponge, the rRNA genes are highly conserved, showing 99.2% identity, which indicates that the population comprises a single species. In the genomic regions flanking the rRNA genes, however, the DNA sequence identity drops to 87.8%. Similarly, Tyson *et al.* found that the genomes of the species or groups in acid mine drainage varied in their uniformity.

They found a high frequency of single- nucleotide polymorphisms among strains of the same species. The Ferroplasma type II group appears to contain a composite genome, with segments derived from three sources. In contrast, the Leptospirillum group II genome contained very few single-nucleotide or large-scale genome polymorphisms. These studies point to the importance of conducting genomic analysis on mixtures of strains to obtain a portrait of the heterogeneity within the species. In fact, metagenomics may provide insight into genome variation of organisms that can be readily cultured. If genetic variation in the environmental population is of interest, it may be more productive to clone the genome from the natural population than analyze the genomes of individuals cultured from it.

CONCLUSIONS AND FUTURE DIRECTIONS

Metagenomics has changed the way microbiologists approach many problems, redefined the concept of a genome, and accelerated the rate of gene discovery. The potential for application of metagenomics to biotechnology seems endless. Functional screens have identified new enzymes and antibiotics and other reagents in libraries from diverse environments. A number of barriers have limited the discovery of new genes that provide insight into microbial community structure and function or that can be used to solve medical, agricultural, or industrial problems.

Realizing the potential for discovery from metagenomics is dependent on the advancement of methods that are central to library construction and analysis. For sequence-based approaches, the speed and cost of nucleotide sequencing will be a barrier of rapidly diminishing significance as sequencing technology continues to improve. Sequence-based assignment of function will also benefit from advances in detection of homology, which will increasingly rely on the tertiary structures of predicted proteins rather than simply on primary sequence.

Advances that will facilitate the management and analysis of large libraries include bioinformatics tools to analyze vast sequence data bases and reassemble multiple genomes rapidly and affordable gene chips for library profiling or that readily distinguish clones that are expressing genes from those clones that are silent. Functional analysis will require more innovation in method development. Most important among these are strategies to improve heterologous gene expression and approaches for efficient screening of large libraries. Microbiology has long relied on diverse methods for analysis, and metagenomics can provide the tools to balance the abundance of knowledge attained from culturing with an understanding of the uncultured majority of microbial life. Myriad environments on Earth have not been studied with culture-independent methods other than PCR-based 16S rRNA gene analysis, and they invite further analysis.

Metagenomics may further add our understanding of many of the exotic and familiar habitats that are attracting the attention of microbial ecologists, including deep sea thermal vents; acidic hot springs; permafrost, temperate, desert, and cold soils; Antarctic frozen lakes; and eukaryotic host organs- the human mouth and gut, termite and caterpillar guts, plant rhizospheres and phyllospheres, and fungi in lichen symbioses. With improved methods for analysis, funding stimulated by recent triumps in the field, and attraction of diverse scientists to identify new problems and solve old ones, metagenomics will expand and continue to enrich our understanding of microorganisms.

DATA PROCESSING METHODS

The data processing methods were principal component analysis (PCA), hierarchial cluster analysis (HCA), artificial neural networks (ANNs), discriminate function analysis (DFA), partial least squares (PLS) regression, evolutionary computation (EC), and partial least squares discriminant analysis (PLS-DA).

The data output from the analytical platforms must be pre processed for noise reduction, baseline and time-shift correction, and spectrum extraction. Unfortunately, the automated deconvolution software such as automated mass pectral deconvolution and identification system (AMDIS) (Stein 1999; Halket *et al.* 1999) and standard libraries are only available for GC-MS. One the metabolites had been identified and quantified, any kind of multivariate data analysis method can be applied for data analysis. These methods can be classified into unsupervised and supervised methods (Goodacre *et al.* 2004). Fortunately, there are many softwares which can be applied to assist the process automation from the raw data pre processing to rapid visualization and interrogation of metabolomic data (Duran *et al.* 2003). According to the existing reviews (Jenkins *et al.* 2004: Stein 1999; Duran *et al.* 2003), the metabolomic standards (including data, method, and library) and integration of metabolomic data with other omic data will be the two key steps for future metabolomic development. The discussions on data classification (Dandekar *et al.* 2003), database construction and maintenance (Hardy and Fuell 2003), and new data analytical tools (Smilde *et al.* 2005) would soon proliferate. According to the existing publications, the applications or the functions of the metabolite data in micro organisms can be summarized as the following.

Comparative comprehensive metabolite studies because of the difficulties to quantify the whole cellmetabolome, the comparative metabolite studies are the main focus now. In an analysis of *E. coli* mutants, a genetic algorithm analysis of direct injection mass spectral entified just two or three peaks that served to pinpoint the nature of the mutation involved (Kaderbhai *et al.* 2003).

Others had used metabolic foot printing analysis with rapid and reproducible low-resolution mass spectra and Fourier transform frared spectroscopy (FT-IR) to distinguish between different physiological states of wild-type yeast and between yeast single-gene deletion mutants (Allen *et al.* 2003; Kaderbhai *et al.* 2003). Also, the differences of *S. cerevisiae* in concentrations of some of the intracellular metabolites were used to identify the phenotypes of several silent mutants (Raamsdonk *et al.* 2001). Comparative comprehensive metabolite studies were also used in authentication of other strains (de Nijs *et al.* 1997). Recently, the use of metabolite profiling is discussed for the identification and classification of yeasts and three other filamentous fungi (Smedsgaard and Nielsen 2005). Fermentation control the detection of microbial cross-contamination is one crucial step in fermentation. Elmroth *et al.* 1992 described a metabolomic method applying three different GC-MS methods for the analysis of fatty acids, amino acids, and carbohydrates in combination with the multivariate data analysis (MVDA) tool to monitor microbial contamination during the fermentative production of dextran by Leuconostocmesenteroides. Some other detections of microbial contamination had been reported before (Wilkinson *et al.* 1995). Dalluge *et al.* 2004 has developed a method based on rapid quantification of underivatized amino acids using liquid chromatography–electrospray tandem mass spectrometry (LC-MS-MS) to monitor the metabolism of 20 amino acids during microbial fermentation. They demonstrated that amino acid profiles, which detected LC-MSMS generally, correlate with observed growth profiles. Further, cellular growth events such as lag time and cell lyses can be detected using this method.

Metabolic control analysis (MCA) and metabolic flux studies (MFA) are the key components of metabolic engineering. Quantified intracellular metabolites can be used to address the in vivo reaction kinetics of one reaction, the reactions in one pathway, or the global regulatory metabolic control mechanism directly. The basic approach used here is to quantify exactly the metabolite differences in microorganism under different phenotypes (usually genetic or other perturbations). Comprehensive profiling of all the metabolites with very rapid sampling frequency is indispensable for such purpose. As illustrated before, mathematic models and standard data set can also greatly facilitate this process.

The most interesting practical results come from Oldiges and Takors (2005) and Buchholz *et al.* (2002). They advised one stimulus-response experiment, which investigates the enzyme kinetics of metabolism after a certain pulse. Applying this method is based on the detection of intracellular metabolites. With the aid of a rapid sampling and quenching routine, it is possible to take four to five samples per second during this process in vivo. They also indicate that stimulus-response experiments should also be applied to analyze pathway dynamics in anabolic routes. It actually provides one good example for how to use the metabolome data in reaction kinetics and pathway analysis to guide stain improvement. Forster *et al.* (2002) has also illustrated how to use the metabolome data in silico pathway analysis to identify gene functions.

Another example is the use of metabolite profiling to determine flux distributions in C. *glutamicum* to improve the lysine production (Hans *et al.* 2001); the metabolite profile of intermediates in the pentose phosphate pathway and key glyclytic intermediates were determined and used in industrial xylose-fermenting *S. cerevisiae* (Zaldivar *et al.* 2002). Although there are many applications, the real power of metabolome lies in the integration with other omic data. Although we had sequenced many model organisms in the past, the functions of many ORFs are still unknown even for *E. coli* and *S. cerevisiae*. Past efforts had revealed the disparities between mRNA abundance (transcriptome) and protein expression or enzyme activities (proteome) in yeast (Gygi *et al.* 1999); *E. coli* (Lee *et al.* 2003) *C. glutamicum* (Glanemann *et al.* 2003), and *B. subtilis* (Soga *et al.* 2002).

Further studies proved the stain physiological behaviors were not only dependent on translation level but also on metabolic level. All the research had raised doubts for the sufficiency of transcriptome and proteome in accessing the whole cell metabolism. In other aspects, Metabolite's regulation importance in metabolism had also been identified, particularly in central carbon metabolism (Yang *et al.* 2003), signal transduction (Martinez-Antonio and Collado-Vides 2003), and secondary metabolic pathways (Wang *et al.* 2004). Now, metabolomics had emerged as a functional genomic methodology that contributes to our understanding of the compel molecular interactions in biological systems. The principle contributions of metabolomics to this system include identification of regulating mechanisms in metabolic network and orphan gene function investigation.

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HOSPITAL WASTE : AT A GLANCE

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INTRODUCTION

Waste means "any thing which is not intended for further use". Hospital waste means "any solid, fluid, or liquid waste materials including its container and other product generated during short term and long term healthcare consisting observational, diagnostic, therapeutic and rehabilitative services for a person suffering from diseases or injury and during research testing and immunization of human beings. Hospital waste includes garbage, rubbish and biomedical waste. With industrialization and increasing healthcare services voluminous amount of waste is generated by hospital, nursing homes and other healthcare institutions". Hospital waste contains body parts, organs, tissues, blood and body fluids along with soiled linen, cotton, bandage, and plaster casts from infected and contaminated areas along with used needles, syringes and other sharps. It contains pathogens in mass, in their invisible forms. Therefore, its proper management is essential to maintain hygienic, aesthetics, cleanliness, and control of environmental pollution. If this substantial amount of waste is not properly managed it can pollute soil, air and water. Further, it can cause deadly diseases, either in endemic, sporadic or epidemic forms. Proper management means proper collection, segregation, storage, transportation and treatment of waste in safer manner to prevent nosocomial or hospital acquired infection. Diseases of modern era like hepatitis-B, AIDS are also drawing attention for proper management of hospital wastes as persons who are in touch of these materials during discharge of their services to mankind are also at the risk. This can be achieved by public awareness about hospital waste hazards and by making mandatory to officials of the institutions to follow the guidelines of Supreme Court and Ministry of Environment Forest, Government of India notification for biomedical.

With increasing health facilities in society the amount of hospital waste generated is also increasing substantially. This needs its proper disposal and management. Since it is highly infectious in nature, it is categorized as hazardous waste. Hospital waste is generated during diagnosis, treatment or immunization of human beings or animals or during research activities.

TYPES OF HOSPITAL WASTE

Hospital waste can broadly be classified as:

- **Biodegradable waste:** Biodegradable waste is the component which is a biological product. This waste which is capable of undergoing anaerobic or aerobic decomposition e.g. food and kitchen waste, paper and paper board etc. Paper and food waste of hospital because of its nature and composition is similar to biodegradable waste from house holds. This component of hospital waste can be safely mineralized using the biological systems.
- **Non biodegradable waste:** Non- biodegradable waste includes all such wastes, which have not been produced through the biological process. These wastes can not be acted upon by any kind of biological system. Further, their mineralisation is highly expensive and it is a useless process. This category of waste can be recycled and reused till the limit of safety. Non-biodegradable wastes include glass bottle, tin, plastics etc.
- **Infectious waste:** These are the waste which are infectious in nature and are potent to cause diseases. These wastes can neither be subjected to normal degradation processes nor can they be categorized as recyclable. We have to just get rid of them safely. The best thing about it is that, generally it is generate in less quantity and normally it is only 5-10 percent of the total hospital waste generated.
- **Chemicals or Medicinal waste:** These wastes are the medicines which have crossed their expiry dates or the medicines released due to leakage from containers or the pathological waste. Usually, the amount of this type of waste is very little but their proper disposal is essential. Mostly, the branded firms take their expired medicines back; however, this is not in practice for most of the time. Under this situation the medicines are directly drained into the sewage system. This ultimately, reaches to water bodies causing hazards to surface as well as ground water.

The quantum of hospital waste that is generated in India is about one to two kg per bed per day in hospitals and 600 gram per day per bed in the clinic of general practitioners. So, a hospital with hundred beds will generate approximately 100-200 kg. waste per day of which only 5-10% is hazardous or infectious which estimates to be at most five to ten kg per day (1). Only this very amount of the total hospital waste is deadly. Its proper handling is necessary because the pathogens can multiply their quantity and potential for infection.

CONSEQUENCES

Usually, in most of the developing and under developed cities in India, no proper norms are in practice for proper management of hospital waste. Hospital waste, as well as the surgical wastes can be seen flowing smoothly through open drainage system even in the exteriors of metropolitan cities. Following are some of the hazards, which can be caused by improper management of hospital waste:

- Injuries from sharps to all categories of personnel and waste handlers.
- Increase risk of infection to medical, nursing and other hospital staff.
- Improper and poor infection control can lead to super infection in patients having poor immunity level (HIV, hepatitis B).
- Persons handling wastes may be exposed to the hazard of chemicals and drugs.
- Improper waste management encourages unscrupulous persons to recycle disposables for repacking and reselling, which is deadly.
- Resistant strains of microorganisms may develop.
- Organic portion of the biomedical waste ferments and provide good media for flies breeding.

CATEGORY OF HOSPITAL WASTE

In light of the above, the proper disposal and management of biomedical wastes is of paramount importance. In order to combat the hazards of improper disposal of hospital waste certain guidelines have been recommended. Further, the hazardous hospital waste has been divided into various categories, such that each category should contain different type of waste, also requiring different treatment procedures, according to nature of component. The categories are discussed below:

- **Category 1:** This category includes anatomical wastes such as Human tissues, organs, body parts etc., their treatment and disposal options available presently are incineration or deep burial.
- **Category 2:** Category 2 includes, animal wastes such as Animal tissues, organs, body parts carcasses, bleeding parts, blood and experimental animals used in research etc. Their treatment and disposal options are also incineration or deep burial like category 1.
- **Category 3:** Microbiological and biotechnological wastes, such as waste from laboratory culture, specimens from micro organisms, vaccines, cell cultures, toxins, dishes, devices used to transfer cultures are included under the category 3. Their treatment and disposal options are- local autoclaving or micro waving or incineration.
- **Category 4:** This includes, waste sharps such as needles, syringes, scalpels, blades, glass, which are treated by chemical disinfection, autoclaving, micro waving or mutilation and shredding.
- **Category 5:** Category 5 includes discarded medicines and cytotoxic drugs such

as outdated, contaminated, discarded drugs. The treatment and disposal options are incineration, destruction or by disposal through the process of land fillings.

- **Category 6:** This includes soiled wastes, contaminated with blood and body fluids including cotton, dressing, soiled plasters, linen etc. Their treatment and disposal options are autoclaving, micro waving or incineration.
- **Category 7:** This category of hospital waste consists of solid wastes such as tubes, catheters, IV sets etc. Their recommended disposal is by means of chemical disinfection, autoclaving, micro waving, mutilation and shredding.
- **Category 8:** Category 8 includes liquid wastes such as waste generated from laboratory and washing, cleaning, disinfection etc. Their treatment and disposal options being followed presently are disinfection by chemical treatment and / or discharge into the drains.
- **Category 9:** This category includes incineration ash, and presently their disposal is only through land fillings.
- **Category 10:** Now in the last, category 10 includes chemical wastes which are treated either by another chemical or discharge of directly into the drains.

COLOUR CODES FOR HOSPITAL WASTE

To ease the separation of hazardous waste and to facilitate their separation and disposal, certain colour codes have been recommended (2). Such that, plastic bags or puncture proof plastic container of different colours be used for keeping the different category of hospital waste separately. The colour codes are as follows:

- **Yellow container:** *Yellow* coloured bags are to be used for the disposal of human and animal wastes, microbial and biological wastes and the soiled wastes. Waste of category 1, 2, 3 and 6 are to be kept together in yellow container.
- **Red container:** *Red* coloured disinfected containers or bags are to be used for the disposal of microbial and biological wastes and the soiled wastes. Waste of category 3, 6 and 7 are to be kept together in red container.
- **Blue; white and transparent containers:** *Blue, white* or *transparent* bags, puncture proof containers are to be used for the disposal of waste sharps and the solid wastes. Waste of category 4 and 7 are to be kept together in blue, white or transparent containers.
- **Black container:** *Black* coloured bags are used for the disposal of discarded medicines, cytotoxic drugs, incineration ashes and the chemical wastes. Waste of category 5, 9 and 10 are to be kept together in black container.
- **Green container:** *Green* coloured containers are to be used for the disposal of general wastes such as office wastes, food wastes and garden wastes.

ENVIRONMENTAL HAZARDS

Poor and improper management of biomedical waste, besides a great risk for hospital personnel's and supporting staff, can cause serious environmental problems through air, water and land pollution. Environmental problem can arise due to generation of biomedical waste and through the process of handling, treatment and disposal. The extent of environmental degradation by hospital waste is discussed below, briefly -

- **Air pollution:** Biological waste is a potent polluter; this can badly affect the air. Air polluted by biomedical waste can be biological, chemical, as well as radioactive in nature. Biological waste can pollute both indoor and outdoor environment.

(a) ***Indoor air pollution:*** Polluted air especially in indoors contains pathogens in the form of spores or pathogen itself which are present in contaminated waste, may live long. The patients and their attendants may acquire infection by these pathogens or their spores (Nosochomial infection), (2). Very little statistical data is available in this field. Research in this area is needed. Indoor air pollution can also be caused by poor ventilation, use of chemicals for disinfection, fumigation etc. which gives out acidic or noxious gases.

(b) *Outdoor air pollution:* Outdoor air pollution can be caused by pathogens which infect through water, food stuffs, soil etc. when waste is not pretreated or dumped openly outside the institution. It can be reduced by proper waste management practices. Chemical pollution of outdoor could be due to open burning of biomedical waste. The plastics and hazardous material in waste will generate harmful gases such as- oxides of sulphur, nitrogen and carbon etc. Gases like dioxins and furans which are generated during open burning as well as also during incineration are carcinogenic. It is necessary that open burning should be avoided. The laid down guidelines in this connection should be practiced as per prescribed standard.

(c) *Radioactive air pollution:* Use of 85 Kr and 133 Xe is the principal source of gaseous radioactive waste in hospitals. In the absence of special exhaust system an activated carbon trapping device may be used to keep away the emitting gases from polluting the air.

- **Water pollution:** The biomedical waste dumped into low lying areas, lakes and water bodies can cause severe water pollution. Liquid waste if not treated and let into sewers can also lead to water pollution. Water pollution can also be caused due to biological, chemical and radioactive substances. Leaching out of pathogens can pollute the water and may cause diseases. Heavy metals present in the chemical waste can also cause water pollution. They can enter into biological systems through the process of biological magnification. Pollution of water bodies can alter parameters such as, pH, BOD, DO, COD etc. and may further become toxic for the flora and fauna of the water body. Proper waste management can reduce water pollution markedly.

Radioactive material shipment is mostly in the form of liquid. Principal part of radioactive waste is of the residue of this shipment. Radioactive substances are found in the urine of patients and scintillation liquids used in radioimmunoassay.

- **Land pollution:** Land pollution by biomedical waste is inevitable as the final disposal of all biomedical waste including treated liquid effluent, is on the land. However through proper treatment it can be minimized. Soil pollution from biomedical waste is due to infectious waste discarded medicines, chemical used during treatment processes. Heavy metals present in the waste get enter into the ecosystem by being absorbed by plants, contaminating the food chain. Trace nutrient elements if present in considerable amount, are harmful to crops, animals and also to the human beings ultimately.

Radioactive waste which causes soil pollution includes, cadavers, protective clothing, absorbent paper, used in nuclear medicine imaging laboratory etc. Proper treatment and minimum generation of this kind of pollutant is the only way to reduce this kind of pollution.

CONCLUSION

In most of the cities in India, only a few large hospitals can be spared, but most of other smaller hospitals and nursing homes have no any effective system to safely dispose off their wastes. Even government and municipal hospitals are no better than these private nursing homes and hospitals regarding disposal of their wastes. Thus, an unauthorized reuse of medical wastes by rag pickers is being promoted by irresponsible dumping of these dangerous wastes in open bins.

Biomedical waste, because of its infectious nature and serious health hazards need care for its proper collection, segregation and disposal to minimize the pollution of air, water and soil. For this, the government of India has laid down certain rules which should be enforced by the officials and should be strictly followed by institutions and clinics in the welfare of mankind and animals.

Central Pollution Control Board and the State Pollution Control Boards, the agencies responsible to enforce these rules in hospitals are on one hand lacking adequate power and on the other hand there is no commitment, as a result most of the large hospitals have not complied with these rules even after expiry of new deadlines. In such situation NGO's and voluntary bodies have to come forward and compel the hospitals and nursing homes to fall in the line with the rules.

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CYANOBACTERIAL BIOFERTILIZER : PRESENT STATUS AND FUTURE PROSPECTS

SURENDRA SINGH, SHIPRA DAS AND ANURADHA TIWARI

INTRODUCTION

The population growth in the world is outstripping food supplies and agricultural productivity. The world population is expected to reach 7 billion within 25 years and over 10 billion in the year 2050, while agricultural production is growing at slower rate of about 1.8% annually. All human beings depend on agriculture that is required to produce food of the appropriate quality and in required quantities. Agriculture in India is the mean of livelihood of almost two third of the work force of the country. It has always been India's most important economic sector.

Modern agriculture depends on high input of chemical fertilizer and pesticide for crop production. Although such technology based agricultural practice has increased agricultural productivity and abundance, the resulting ecological and economical impacts have not always been positive. Environmental pollution and food safety due to chemical contamination have become a great concern all over the world. Application of high input technologies has resulting in significant increase in agricultural productivity. There is, however, a growing concern about the adverse effects of indiscriminate use of chemical fertilizers on soil productivity and environmental quality (Mishra and Pabbi, 2004). Rice and wheat occupy 75% of the total cultivated area under food grains and consume major share of the chemical fertilizers. The widespread application of single element fertilizers (especially N) in South East Asian countries. In the cultivation of major crops has led to accelerated exhaustion of other major and minor nutrients leading to nutrient imbalance and poor soil fertility. With the growing realization that chemical based agriculture is unsustainable and is slowly leading to ecological imbalance, the later part of the last century witnessed the emergence of the concept of "organic agriculture" advocating minimum use of chemical fertilizer and increasing dependence on biological inputs like compost, farm yard manure, green manure and biofertilizers (Karthikeyan, 2006). In the current scenerio therefore, an urgent need has been felt to deploy microbial biofertilizers which are multifaceted i.e. besides being diazotrophs, they can promote growth through production of auxins, bioactive molecules, produce secondary metabolites linked to biocontrol of bacterial, fungal diseases or improve soil structure, porosity through secretion of mucilage polysaccharides aiding in soil aggregation. Hence serious attention was paid by scientists to exploit microorganisms which are directly responsible to replenish the soil with certain nutrients like fixation of atmospheric nitrogen to ammonia, solubilization of phosphorus as well as addition of organic carbon. Hence this paper is intended to focus on the cyanobacterial potential as an efficient biofertilizers.

THE DISADVANTAGES OF USING CHEMICAL FERTILIZER

(1) Over application can result in negative effects such as leaching, pollution of water resources, destruction of microorganisms and friendly insects, crop susceptibility to disease attack, acidification or alkalization of the soil or reduction of soil fertility, thus causing irreversible damage to overall system.

(2) Oversupply of N leads to softening of plant tissue resulting in plant that are more sensitive to disease and pest.

(3) They reduce the colonization of plant roots with mycorihizae and inhibit symbiotic nitrogen fixation by rhizobia due to high nitrogen fertilization.

(4) They enhance the decomposition of soil, which leads to degradation of soil structure.

(5) Nutrients are easily lost from soil through fixation, leaching or gas emmision and lead to reduced fertility efficiency.

ADVANTAGES OF USING ORGANIC FERTILIZERS

(1) The nutrient supplies is more balanced, which helps to keep plants healthy.

(2) They enhance the colonization of mycorrihizae, which improves P supply.

(3) They enhance soil biological activity which improves nutrients mobilization from and chemical sources and decomposition of toxic substances.

(4) They enhance root growth due to better soil structure.

(5) They increase the organic matter content of the soil, therefore improving the exchange capacity nutrients, increasing soil water retention, promoting soil aggregation and buffering the soil against acidity, alkalinity, salinity, pesticides and toxic heavy metals.

(6) They release nutrients slowly and hence contribute to the residual pool of organic N and P in the soil, reducing N leaching loss and P fixation, they can also supply micronutrients.

(7) They supply food and encourage the growth of beneficial microorganisms and earthworms.

(8) They help to suppress certain plant diseases, soil born diseases and parasites.

BIOFERTILIZERS

Biofertilizer is defined preparation or product containing living or latent cells of microorganisms that activate the biological process render to form a fertilizer compound or make the unavailable form of elements to be available for plants. Under the scope of this terminology the biofertilizers encompass the formulation of nitrogen fixing microorganisms, phosphate solubilizing microorganisms and cellulolytic microorganisms.

TYPES OF BIOFERTILIZERS

Rhizobium

Rhizobia are symbiotic bacteria that fix atmospheric N_2 gas in plant root nodules and have a mutually helpful relationship with their host plants because of their N_2 fixing ability, legumes are less reliant on inorganic nitrogenous fertilizers than many other non legumes crops such as cereals and pasture grasses. N_2 fixation by legumes can also maintain soil fertility and can be beneficial to the following crops.

Azotobacter and Azospirillium

These are free living bacteria that fix atmospheric N_2 in cereals without any symbiosis and they do not need a specific host plant. Azotobacter are abundant in well drained neutral soil. They can fix 15-20 kg/ ha N/y. Azotobacter can also produce antifungal compounds to fight against many plant pathogens.

Phosphate Solubilizing Bacteria (PSB)

Under acidic or calcareous soil conditions large amount of phosphorus are fixed in the soil but are unavailable to the plants. Phosphobacterians, mainly bacteria and fungi can make insoluble phosphorus available to the plants. The solubilization effect of phosphobacterians is generally due to the production of organic acids that lowers the soil pH and bring about the desolution of bound forms of phosphate.

Vesicular Arbuscular Mycorrhiza (VAM)

Mycorrhizae are mutually beneficial (symbiotic) relationships between fungi and plant roots.VAM fungi infect and spread inside the root. They possess special structures known as vesicles and arbuscules. The plant roots transmit substances (some supplied by exudation) to the fungi, and the fungi aid in transmitting nutrients and water to the plant roots. The fungal hyphae may extend the root lengths 100-fold. The hyphae reach into additional and wetter soil areas and help plants absorb many nutrients, particularly the less available mineral nutrients such as phosphorus, zinc, molybdenum and copper. Some VAM fungiform a kind of sheath around the root, sometimes giving it a hairy, cottony appearance. Because they provide a protective cover, mycorrhizae increase seedling tolerance to drought, to high temperatures, to infection by disease fungi and even to extreme soil acidity.

Plant Growth Promoting Rhizobacteria (PGPR)

PGPR represent a wide variety of soil bacteria which, when grown in association with a host plant, result in stimulation of host growth. PGPR modes include fixing N_2, increasing the availability of nutrients in the rhizosphere, positively influencing root growth and morphology and promoting other beneficial plant-microbe symbiosis.

Cyanobacteria (BGA)

The cyanobacteria are ubiquitous in nature and are next to bacteria in distribution. They grow wherever water, sunlight and limited number of inorganic elements available and rice field ecosystem in the ideal niche for the growth and proliferation of cyanobacteria. Due to their wider occurrence in rice field ecosystem, these organisms have been explored as a mean to produce biologically fixed nitrogen.

Azolla

The beneficial effect of N_2- fixation for crop production is even more apparent in paddy fields which are populated by the water fern *Azolla* during the waterlogged period. The utility of Azolla as an efficient biofertilizer of paddy field has been known in North Vietnam for centuries. It is cultivated there over 400, 000 hectares of land as a specific crop for green compost and forage.

CYANOBACTERIA POTENTIAL AS BIOFERTILIZER

Blue green algae (cyanobacteria) are distributed world wide and contribute to the fertility of many agricultural ecosystem either as free living organisms with the water fern *Azolla* (Fay, 1983).The nitrogen fixing ability of many species is the principle but by no means the only reason for this increase in fertility.

Many tropical paddy fields receive no chemical fertilizers or natural manure yet they may remain productive and capable of supporting large population with basic food. The fertility of paddy soil is maintained by the activities of heterocystous blue green algae, which grows spontaneously and often luxuriantly in the water logged field. They provide fixed nitrogen to rice plants through both secretion of nitrogenous substances and on their decay and subsequent mineralization of organic substances in the soil.

Field trails in India indicate that blue green algal application results in about 10 to 15% increase in crop yield under optimum conditions. In India blue greens are also employed for the reclamation of alkaline and saline wastelands. The wasteland is enclosed by an earth embankment about 0.5m high, which promotes waterlogging during the rainy periods. The rapid growth of blue greens in the water logged plots decreases soil alkalinity and increase the nitrogen and organic content of the soil. Ina few years time the watelands can be converted to fertile fields.

Among the array of biofertilizers developed for different crops cyanobacteria constitute the most important inputs in rice cultivation. Cyanobacteria are able to generate their own photosynthate from CO_2 and water. This trophic independence makes BGA especially attractive as biofertilizers (Roger, 1985). As early as De in 1939, first paper appeared that the fertility of Indian rice field is maintained due to the presence of blue green algae. Since then large amount of work has been done to exploit this renewable biological resource as N input in rice cultivation. Cyanobacteria are both unicellular and multicellular filamentous. Some of the forms have terminally differentiated specialized structures called heterocysts. All heterocyst-bearing cyanobacteria are aerobic photodiazotrophs. However, the non-heterocystous forms fix much less nitrogen aerobically than they could fix anaerobically or microaerobically. The aerobic filamentous heterocystous as well as colonial forms represent the common flora of arable lands and are considered to be the important in nitrogen economy of rice cultivation. The most common filamentous heterocystous species include *Anabaena, Anabaenopsis, Aulosira, Calothrix, Cylindrospermum, Fischerella, Hapalosiphon, Mastigocladus, Nostoc, Scytonema, Scytonematopsis, Stigonema, Tolypothrix, Westiella* and *Westiellopsis* (Stewart et al, 1979; Prasanna and Kaushik, 1996) and the unicellular or colonial are the species of *Aphanothece, Chroococcidiopsis, Cyanothece, Dermocarpa, Gloeothece, Myxosarcina, Synechoococcus, Synechocystis and Xenococcus* (Ripkka et al, 1979; Prasanna and Kaushik, 1994). The non-heterocystous filamentous forms capable of nitrogen fixation are species of *Oscillatoria, Trichodesmium, Microcoleus, Pseudanabaena, Plectonema* and *Lyngbya* (Wyatt and Silvey, 1969; Stal and Krukbein, 1985).

NITROGEN METABOLISM

Most Blue Green algae can utilize various forms of nitrogen for growth. Among the inorganic sources of N, ammonia is most readily incorporated into cells. Its assimilation proceeds via the glutamine synthetase-glutamate synthase (glutamine oxoglutarate aminotransferase, GOGAT) pathways.

NH_4^+ + glutamate+ ATP$^{\text{glutamine synthetase.}}$ Glutamine +ADP+Pi+H_2O (1)

Glutamine + α-oxoglutarate + NADPH

Other ammonia assimilating routes may operate at higher ammonia concentrations. Enzymes involved in these pathways (glutamate dehydrogenase, alanine dehydrogenase, alanine dehydrogenase, aspartate dehydrogenase) have also been detected in extracts from several cyanobacteria.

Nitrate is the commonest source of combined nitrogen for cyanobacteria in nature. Its assimilation follows a sequence of reduction to ammonia, catalyzed by the enzymes nitrate reductase and nitrite reductase, similar to those established for green algae and higher plants.

Ferredoxin acts as the physiological electron donor in the two reactions. Both nitrate reductase and nitrite reductase are induced by their respective substrate, although the additional presence of ammonia repress the synthesis of nitrate reductase.

Among the organic sources of nitrogen, urea has been found to support growth of certain cyanobacteria in culture. Various amino acids mixtures can serve as a source on nitrogen for other strains. A few amino acids can acts as sole source of carbon and nitrogen for the slow growth in the dark of certain blue greens.

NITROGEN FIXATION

Biological N_2 fixation is the reduction of elemental nitrogen to ammonia, catalyzed by the complex enzyme system nitrogenase.This is one of the fundamental biological processes, essential for the maintenance of the nitrogen status of the whole biosphere. The reaction require a low potential reductant (ferredoxin or flavodoxin), magnesium ions (Mg^{2+}) and a considerable input of metabolic energy in the form of ATP.12 to 15 molecules of ATP are consumed for each molecule of nitrogen reduced. Nitrogenase is extremely sensitive to free O_2 and can function only under anaerobic conditions. Direct exposure of the enzyme to air results in the inactivitation and even the destructions of the component proteins. Nitrogen fixing cyanobacteria have the simplest nutritional requirements of all living organism. However, The extent to which blue green can profit from their remarkable autotrophic potential growth depends on their ability to reconcile the presence of an O_2 sensitive nitrogenase side by side with an O_2 evolving photosynthetic apparatus. It is noteworthy that in general cyanobacteria are more sensitive to high O_2 concentration and to high quantam dose of light (which could result in increased O_2 evolution) when they are grown under nitrogen fixing conditions.

Heterocyst provide an ideally suited microenvironment for nitrogen fixation in that they maintain the reducing conditions essential for nitrogenase activity. This results from the combination of several factors, like the absence of O_2 evolving photosynthesis, an active oxidative metabolism and the presence of an elaborate envelope which reduces the diffusion of atmospheric gases through the surface of the heterocyst to a level which is adequate to saturate N_2 the O_2 which seeps in can be dealt with by means of respiration. The protection of nitrogenase against the oxygen damage is further enforced by nitrogenase catalyzed H_2 evolution. H_2 is a by-product of the nitrogenase reaction, and account for about 20 to 30% of the total electron flux by nitrogenase. H2 may help to exclude O_2 from the site of nitrogenase; furthermore the oxidation the oxidation of H2 in the so-called oxyhydrogen reaction, catalyzed by an "uptake hydrogenase", may contribute significantly to the removal of free O_2 from the heterocyst.

Heterocyst s possess adequate mechanisms to meet the requirements of N_2 fixation for the reductant and energy. By retaining an active Photosystem I, they are capable of generating reducing power and ATP in cyclic photoelectron transport coupled with photophosphorylation. They are also able to provide the same requirements, though less efficiently, in an O_2 dependent dark respiratory metabolism.

A consequence of the inability of heterocyst to perform photosynthetic CO_2 fixation is that they must rely on the vegetative cells for the supply of carbon and a source of reductant. Carbon (possibly in the form of a disaccharide sugar) is translocated presumably through the fine channel (microplasmodesmata) transversing the small septum between vegetative cells and heterocyst. Fixed nitrogen (probably in the form of glutamine) may be exported through the same route but in an opposite direction from the heterocyst to the vegetative cells. The accumulation of cyanophycin polypeptide and the deposition of "plug" material in the heterocyst appear to have an important role in the regulation of supply fixed nitrogen to vegetative cells. Enzyme involved in both the synthesis and degradation of cyanophycin are several times more active in heterocyst than in vegetative cells. Cyanophycin in heterocyst may function as reservoir between N_2 fixation and the export of fixed nitrogen.

Among the Nitrogen fixing non-heterocystous cyanobacteria one can observe a variety of apparently less efficient and probably more ancient adaptations of the nitrogen fixing system to the prevailing oxygenic environment which enable N_2-fixation to proceed under microaerobic conditions.

The first discovery of N_2 fixing in a non-heterocystous species was made in 1961 with bloom-forming planktonic populations of the marine *Oscillatoria erythraea,* commom in tropical oceans. The trichomes are mostly aggregated in bundles, and N_2 fixing activity is associated with this typical bundled morphology. The individual trichomes within the bundle display a distinct morphological and physiological differentiation through segregation of photosynthetic and non photosynthetic regions The former appear granulated, contain carboxysomes and have been shown to incorporate ^{14}C- labeled CO_2. The latter lacks these features but show strong reduction potential, harbor attached bacteria and seems to provide favorable environment for the maintenance of nitrogenase.It is thought that O_2 tensions in the bundle center could become sufficiently decreased to permit N_2-fixation taking place in the non photosynthesizing cells. Thus in *O.erthraea* the contrasting physiological activities are restricted to separate regions of trichome.

Another case of a aerobic N_2 fixation is that carried out by a few closely related strains of the pleurocapsalean genus *Gleothece* (*Gleocapsa*), which displays a typical colonial morphology. The cell aggregate is surrounded by a lamellate envelope which encloses two or three cell generations produced in subsequent cell divisions. Although *Gleocapsa* can fix N2 in air, optimum rates of nitrogenase activity have been observed under subatmospheric O2 tension (10Pa), at low CO_2 concentrations. All these seem to indicate that the organism is relativelyO_2-sensitive under N_2-fixing conditions. Several suggestions have been put forward to explain how nitrogenase might be protected in *Gleocapsa* against inactivation of oxygen. Some refers to the protective role of the elaborated envelope. Other to the possibility of a temporal or spatial segregation of photosynthesis and N_2 fixing activities.

There are a few other, not fully confirmed, reports of aerobic N_2- fixation by non heterocystous forms while maintained under strict anaerobic conditions, as provided by continuous sparging of cultures with an O_2 free gas mixture (N_2 + CO_2). This was first demonstrated in *Plectonema boryanum* and later in several other non heterocystous strains. The latent ability (genetic information) to fix N2 is expressed only in the absence of free oxygen.

PADDY FIELD ECOLOGY

Paddy field ecosystem provides a favourable environment for the growth of BGA with respect to their requirements for light water high temperature end nutrient availability. This could be the reason BGA grows in higher abundance in paddy field soil than in upland soil (Watanabe and Yanamotu, 1971) as reported in the widely different climatic conditions of India (Mitra, 1951) and Japan (Okuela and Yamaguchi; 1952).

In paddy field, growth of BGA and algal successions are governed by climatic, physiochemical and biotic factors.

CLIMATIC CONDITIONS

(i) Light : BGA are generally sensitive to high light intensities. They develop various protective mechanisms like vertical migration in the water of submerged soil; preferential growth in more shaded zones like embankments, under or inside decaying plant material, or a few mm below the soil surface, photophobotaxis, photokinesis and stratification of the strain in algal mats. Resistance to high light intensities has been observed where N fixing cyanobacterial strains grow under a layer of eukaryotic algae. On the other hand light deficiencies may also be a limiting factor.

(ii) Temperature : The optimal temperature for BGA is about 30-35°C, temperature is rarely a limiting factor for BGA in paddy field because the range of temperature permitting their growth is larger than that required by rice; however, temperature influences the composition of algal biomass and the productivity. Low temperatures decreases the productivity and favors eukaryotic algae. High temperatures favors BGA and increases the algal productivity (Roger and Kulasooriya, 1980).

SOIL PROPERTIES

(i) pH : Among the soil properties pH is the most important factor determining the algal flora composition. Under natural conditions BGA grows preferentially in environments that are neutral to alkaline. This explains the positive relatioship occurs in the rice field between, water pH and BGA number, Soil pH and BG spores, soil pH and the N fixing algal biomass in samples homogenous for rice development (Roger and Reynaud, 1982).

BIOTIC FACTORS

Organisms that limit BGA growth are: pathogens, antagonistic organisms and grazers. Of these only grazers have been documented. The development of zooplanktons populations, especially Cladoceras, Copepods, Ostracods and mosquito larvae prevented the establishment of algal blooms within 1no or two weeks.

ALGALIZATION TECHNIQUE

The term "Algalization" was introduced by Venkataraman (1961.) and has been reported to have a beneficial effect on grain yield in China, Egypt, India, Japan, Philippines and Russia. However, there are also reports indicating failure of algalization. Algalization in rice field has proceeded beyond the stage of fundamental research and attempts have been made to popularize this technique, mostly among Indian farmers. In algalization two types of production are distinguished, that under controlled conditions and that in open-air soil cultures.

ARTIFICIALLY CONTROLLED CONDITIONS

Two main methods are used for controlled conditions. In first method algae is initially grown in liquid culture, then mixed with an inert material and dried. In the second method, algae are grown directly on the support. A mass culture procedure described by Watanabe (1959) adopted three consecutive steps :

(a) Preliminary culture of the unialgal strain is grown in ordinary flask in the laboratory.

(b) Culture is then transferred in a large aseptic tank and is subjected to continuous stirring under controlled laboratory conditions.

(c) Then the culture from the tank is then transferred in large bags made up of polyvinyl sheeting consisting a closed circulation system with bubbling of 5% CO_2 in air.

Then to conserve algae and facilitate their transportation, various inert supports are used. Pumice stone prove to be an efficient material (Hosoda and Takata, 1955). Mixing algal suspensions with sand and drying under the Sun permits the algae to retain its capacity for growth unimpaired for about two years. But due to its disadvantage of being heavier, sinking tendency in mud adhering the algal growth, growing the blue green on porous gravel made of volcanic earth and soaked in N free medium gave a material suitable for direct usage in field (Watanba, 1958). Synthetic sponge cut into blocks (2-4cm) are also used for algal growth and conservation (Venkataraman, 1972).

OPEN AIR SOIL CULTURE

Algae can be grown either in galvanized iron tray (Venkataraman, 1972) and shallow tanks made up of bricks and mortars (Mishra, 1979) to which a few kgs of soil is added, or in small field enclosed by earth enbankment (Agarwal, 1979; Kananaiyan, 1979; Pantastico and Jgonzales, 1976; Srinivasan, 1979; Venkataraman, 1968; Venkataraman, 1972). Starter culture are *Aulosira, Tolypothrix, Scytonema, Nostoc, Anabaena, Plectonema* which are recommended by All India Coordinate project on algae.

Method of Inoculation

The method of field application have been reviewed by Venkataraman (1961). When rice is transplanted, the algal inoculum is generally applied one week after transplanting either as a liquid algal suspension (Jha *et al.*, 1965) that can be supplemented with sodium molybdate (0.5kg ha^{-1}) (Venkataraman, 1961) or as a powdered material mixed with lime (Romanonko, 1956) or sand (Watanabe and Yamamoto, 1970, 1971). When rice is sown, seeds can be coated with a mixture of the algal suspension and 2-3kg $CaCO_3$ 10-20 kg seeds, and air dried in the shade (Venkataraman, 1961). The efficiency of the different methods have been tested and concluded that soil application and seed inoculation are preferred.

Effect of Algalization on the Rice Field

Algalization may effect plant size, N content and the number of tillers, ears, spikelets and filled grains per panicle. The most frequently used criterions for assessing the effect of algalization has been a better grain yield. The effect of algalization used with N fertilizers is controversial. Since biological N fixation is known to be inhibited by inorganic N the beneficial effect of algalization in the presence of N fertilizers was frequently interpretended as resulting from growth promoting substances produced by algae or also by a temporary immobilization of added N followed by a slow release through subsequent algal decomposition permitting a more efficient utilization of N by the crop.

EFFECT OF ALGALIZATION ON SOIL PROPERTIES AND MICROFLORA

Grain yield measurement suggest that algalization produce both cumulative and residual effect. This was attributed to a build up of both organic N content and the number of BGA propagules in the soil, facilitating the reestablishment of the BGA biomass. Several reports indicate an increase in organic matter amd organic nitrogen. Algalization has also been reported to increase aggregation status of the soil (Shield and Durrel, 1964), water holding capacity (Singh, 1961), available P and total microflora i.e. Azobacter, Clostridium and Nitrifiers (Ibrahim *et al.*, 1971).

LIMITING FACTORS FOR ALGALIZATION

Among the possible limiting factors responsible for the failure of algalization only pH and available P content of the soil have been studied. Since in soil algalization is insufficient inspite of the addition of lime and phosphate (Okuda and Yamaguch, 1952). Available P content is probably not the only factor limiting the effect of algalization. On the other hand, texture, organic matter content and total N are not probably important limiting factors (Subramanian *et al.*, 1965). Among the biotic factors that can possiblylimit BGA inoculum growth, grazing by the zooplanktons has already been mentioned. Other possible mechanism involves such as antagonism, competition etc have been cited but their role is not clear. Low temperature, heavy rainfall and cloudy weather have also been reported to limit the establishment of inoculum.

ECONOMICS OF ALGALIZATION

Inoculum Production

In 1979 the cost of inoculum production in a field plot of 40 m^2 was calculated to range from US $ 0.16 -0.02 Kg^{-1} depending the number of harvest and productivity. The cost of production in BGA factories was reported in 1978 by Rao.

Raw material required	Amount (kg)	Cost (US $)
BG culture	8.25	10.06
Super phosphate	8.25	0.61
Saw dust	6.5	0.095
Carbofuran	0.812	1.32
Soil transport		0.12
Harvest		1.22
		$13.425

Product obtained per kg harvest 36.58 US$

Net profit per 100kg of harvest 23.15 US$

In a year of normal conditions the farm can have profit of 623.05 US$

Cost of material for algal production per kg is 13.425 US$

PAY OFF OF ALGAL TECHNOLOGY

In trials conducted in five stations in India (1978), it was shown that by adding 10 kg algal cultures per hactere, costing of about Rs 30, extra yields of paddy worth Rs 500-Rs 700 can be obtained on the average (Rao, 1978). The above reports indicates that the algalization technique is easily adoptable by farmers of India. This technology tested in field trials in experimental farms has given positive results in both grain yield and economics. According to several reports such trials are still confined in India, and least information on the use of this technology is available in other countries.

TECHNOLOGY DEVELOPMENT

Refinement of technology for mass multiplication of strains has been always under consideration. Choice of suitable strains that can withstand wide variations of environmental conditions as well as survive the adverse ecological conditions without loosing physiological activity and nitrogen fixing ability has been of major concern. To overcome the problem of establishment, a composite inoculum have to be envisaged and strains have to be selected based on the existing data on occurrence and predominance of different species of BGA in rice field soils of the entire country.

CYANOBACTERIAL BIOTECHNOLOGY

Development of Herbicide Resistant Strain

Herbicides are used to kill or control weeds while as far as possible, leaving crop plants unharmed. Herbicides are applied to the soil as (1) pre-planting treatments and (2) pre-emergence treatments. The length of time that a herbicide remain active or persists in the soil is extremely important because it determines the time that weed control may be expected and also the time that a chemical is present in the environment. A certain amount of persistence is usually desirable for adequate weed control because it relates to phytotoxic effects that may prove injuries to subsequent crops or planting. In such cases, excessive persistence may restrict crop rotation option available to the farmer and could cause ground water contamination or other environmental problems. A factor that effects that persistence of herbicide in the soil are classified as either degradation process or transfer processes. In agricultural practices, uses of herbicides (pesticides) are important factors for maintaining high crop productivity (Day, 1987). But the emphasis on chemical control of plant insects and diseases has caused serious imbalance in the agro ecosystem. The small amount of the active ingredients, the formulation against and transformation products may both water and soil (Hill and Wright, 1978). These are habitats populated by cyanobacteria and other microorganisms. The herbicide effect on cyanobacteria has gained greater attention than eukaryotic algae. This shift in focus appears to have been promoted by the acknowledgement of the important role of many cyanobacteria as nitrogen fixer in soil (Da Silva *et al.*, 1975; Bold and Wynne, 1978; Irisarri *et al.*, 2001). Studies have been made extensively documenting the positive role of N_2 fixing cyanobacteria as biofertilizer in rice fields (Venkataraman, 1981; Mohapatra and Jee 1991; Kaushik, 1994; Jha *et al.*, 1999). Besides this cyanobacteria are reported to excrete plant growth promoting and soil binding substances (Whitton, 1965; Jones, 1988; Roger and Burns, 1994; Jha *et al.*, 1999).This reflects the justifiable concern for the fate of N_2-fixing cyanobacteria in herbicide treated paddy fields (Mishra *et al.*, 1989).

Generally toxicity of herbicides in cyanobacteria results in lethal activity and the most among these effects are changes in growth rate by inhibiting vital process and specific metabolic reaction of photosynthesis, respiration and N_2- fixation (Singh *et al.*, 2000c).

Biological action of herbicides on cyanobacteria includes inhibition of growth, macromolecular synthesis (Vaishampayan *et al.*, 1978; Philips *et al.*, 1992; Pandher *et al.*, 1994; Kaur *et al.*, 1997 ;Singh, 1973; Lal and Saxena, 1980; Padhy, 1985; Kolte and Goyal, 1992; Fairchild *et al.*, 1998; Kapoor and Arora, 2000 a, b; Irisarri *et al.*, 2001), photosynthetic and nitrogenase activity (Leganes and Fernandez, 1992; Singh and Tiwari, 1988), amino acid synthesis pathways (Steinrucken, *et al.*, 1980; Schulz *et al.*, 1984; Fisher *et al.*, 1986), mutagenic actions (Shirasu *et al.*, 1976; Singh *et al.*, 1979; Singh and Vaishampayan, 1978) or even complete elimination (Khan and Vaishya, 1995). These works relate to studies on toxicity of herbicides to cyanobacteria in culture, mostly on finding out the tolerance limits and on accumulation and metabolism of these herbicides. Certain cyanobacteria are reported to posses natural tolerance against herbicides (Lea *et al.*, 1984; Freidberg and Seijffers, 1988). In order to make the cyanobacterial biofertilizer programme successful, it is essential to use cyanobacterial strain to be used as successful inoculant for improving soil fertility and crop productivity require development of such potential diazotrophic strain capable of tolerating or resisting toxic actions of herbicides in their recommended or even higher dosages also with functional metabolic activities under field conditions. Such promising strains of cyanobacteria of local rice fields need to be screened for their use as biofertilizer, as agroclimatic conditions of any region plays an important role in the establishment and productive performance of microbial inoculant (Goyal, 1997).

Immobilization of Cyanobacteria

Immobilized biocatalysts (isolated enzymes, microorganisms, cell organelles, plant cell, animal cell and membrane) offers numerous prospects for biotechnology and form the basis of some traditional and new industries and services (Codd, 1987). Immobilization of biocatalyst refers to the confinement in a definite region of space, restricting its gross movement by attachment or entrapment on or in an insoluble and inert matrix or carrier. This separates the biocatalyst into a distinct phase, which is different from the bulk. However, research on the immobilization of micro-algae and cyanobacteria for biotechnological purpose is at an early stage, although several potential uses for immobilized photosynthetic microbes are already apparent. Cells may be used in immobilized form for the following purposes:

(1) Production of useful substance or energy by *de novo* biosynthesis or biotransformation.

(2) Removal of harmful or waste substances from potable water or effluent.

(3) Recovery of useful/valuable material from primary sources and effluent.

(4) As measuring devices

(5) Successful operation against environmental stresses/purturbances.

(6) Model system for research in cell physiology, biochemistry and recombinant DNA technology.

(7) Inocula delivery system and strain preservation.

There are large numbers of techniques now available for microbial cell immobilization (Woodward, 1985) on different supports. The selection of techniques for immobilization depend upon the nature of cells, nature of chemical conversion and its ultimate application in bulk phase surrounding geometry (Mattiasson, 1983;Tampion and Tampion, 1987). Normally immobilization of microbial cells is achieved through entrapment in organic polymer and of these alginates represent the most used polyanions (Guisely, 1989) and is specially suitable for photoautotrophs. Entrapment in polmeric matrix anchors the cell in a network providing microhabitat for their controlled growth and free diffusion of substrate and products. Much of the existing work on microbial immobilization has been done on potential heterotrophic microorganisms like yeast and bacteria (D'Souza, 1989; Iborra *et al.*, 1994; Strotman and Windecker, 1997; Canoras *et al.*, 1998). Early reports that have demonstrated potentials and prospects of cyanobacteria under immobilized state are H_2 production by *Anabaena, Anacystis, Mastigocladus, Nostoc muscorum* and *Oscillatoria* sp. (Lambert *et al.*, 1979; Kumar *et al.*, 1990; Markov *et* al., 1995a, b) production of ammonia and amino acids using parent and mutant strain (Kerby *et al.*, 1986, Brouers *et al.*, 1986; Mahesh and Kannaiyan, 1993; Balchander *et al.*, 1995; Kannayan, 1996). Further roles highlighted were in environmental depollution, in tertiary treatment of waste water in order to remove eutrophic nutrients i.e. nitrate and phosphate (Hall *et al.*, 1945; Garbisu *et al.*, 1991, 1993). Other contributory works in immobilized forms includes continuous antibiotic production by *Scytonema* (Chetsumon *et al.*, 1995), glutamate production by *Synechococcus* (Matsunaga *et al.*, 1996), NADP production by *Nostoc muscorum* (Bender *et al.*, 1995), CO_2 uptake by *Anabaena* (Markov *et al.*, 1995a), oil degradation (Sorkholv *et al.*, 1995), uptake and removal of phosphates, nitrite uptake (Jeanfills and Loudeche, 1986) and nitrate uptake (Garbisu *et al.*, 1991).

Application of cyanobacteria in agriculture and waste treatment is well documented, where they are used either in field conditions exposed to natural environment or in semicontrolled or controlled bioreactor system. In this multitude of environmental, agricultural or industrial applications, the cyanobacteria inocula encounters diverse macro and micro environmental stresses. These processes and applications include use of cyanobacteria as biofertilizer in water logged rice field, where they interact with agrochemical such as herbicides, insecticides fungicides and chemical fertilizers and soil reactions such as fluctuating pH, salinity, desiccation and other biotic stresses. Kannayan (1998) have shown that the cyanobacterial cultures immobilized in polyurethane (PU) foam increases the quality and viability of cyanobacteria and could survive for a period of 2-3 years. The cyanobacterial cultures is well protected inside the pores of the PU foam and also increases the nitrogen use efficiency of rice owing to its continuous photo-production of ammonia in rice field. Since immobilization of microbial cells are reported to provide protection against environmental stresses upto certain extent (Venkatasubramanian and Veith, 1979; Codd, 1987; Thakur, 1998; Singh *et al.*, 2000d) it can improve the survival strategy of biofertilizer strains of diazotrophic cyanobacteria against toxic actions of common rice field pesticides.

SALINITY STRESS

Coastal salinity and accumulation of salts in irrigated land are primary factors depressing yield in rice crop. Salinity can affect germination, metabolism, the size of plants, branching, leaf size and overall plant anatomy. Salt also affects photosynthetic components such as enzymes, chlorophyll and carotenoid contents [5] as well as the activity of ALA-D [6], second enzyme of porphyrin biosynthetic pathway that produce heme group substancesand chlorophyll in plants. The inhibitory effect of salt stress on plant growth is exhibited at several levels and involves an array of cellular processes such as cell division and expansion. These cellular processes are regulated by hormones for which homeostasis may be altered by salt. Several reports have indicated that application on crops of growth regulators, such as GA3 and cytokinin, produced some benefit in alleviating the adverse effects of salt stress[7]. GA3 reduced NaCl-induced growth inhibition of shoot rice seedlings [8], and kinetin lightened the influence of salinity on growth and production of plant growth regulators in Vigna sinensis and Zea mays [6]. Salt stress is one of the most serious factors limiting the productivity of rice, the staple diet in many countries. Gibberellic acid has been reported to reduce NaCl-induced growth inhibition in some plants including rice. Most paddy soils have a natural population of Cyanobacteria, prokaryotic photosynthethic microorganisms, which synthesize and liberate plant growth regulators such as gibberellins that could exert a natural beneficial effect on salt stressed rice plants. The cyanobacterial EP would contain gibberellin-like substances which may be responsible of the alleviation of salt stress adverse effect on hormone homeostasis. Cyanobacteria reacts to salt stress either through osmotic adjustment or by seceration of excess polysaccharides (Saxena and Kaushik, 1991). Salt tolerance in cyanobacteria is mediated by an osmoregulatory mechanism that involves the accumulation of carbohydrates, amino acids and quaternary ammonium compounds (Reed and Stewart, 1998). The salt adaptation strategy of cyanobacteria includes two main processes, namely

1. Maintanence of low internal contents of inorganic ions by active export mechanisms.
2. Synthesis and accumulation of osmoprotective compounds corresponding to the osmotic potential of the surrounding medium.

The first process is achieved by Na^+/H^+ antiporters that are coupled to cytochrome oxidase. Several compounds such as sucrose, trehalose, glucosyl, glycerol and glycine acting in the second process were identified in salt-loaded cyanobacteria (Reed and Stewart, 1988).

However, it would be important to confirm the presence of gibberellins in S. hofmanni extracellular products as well as the alteration of the hormonal homeostasis in this rice cultivar under salt stress.

BIOFERTILIZER PRODUCTION IN INDIA

The present production capacity of different biofertilizer production units in the country is about 4500 t/annum. Based upon the cultivated areas under rice present requirement is 18, 000 tonnes of straw based BGA biofertilizer. Total potential of BGA biofertilizer is very high and existing production units do not have the capacity to meet the requirement at the right time. Most of the production units lack adequate instruments, production techniques and skilled staff for production of quality based bio-inoculants. The result is the production of poor quality products, which are directly responsible for the failureso there is a need for upliftment of biofertilizer industry in India.

POSSIBLE BIOFERTILIZER PRODUCTION UNITS (AS ON 31.3.99)

1. Bharat Laboratory & Biological House for Agriculture, Dhule, Maharashtra, Capacity-9 tones along with other Biofertilizers
2. Biological Nitrogen Fixation Scheme, Agricultural Bacteriology Section, College of Agriculture, Pune (Maharashtra), Capacity - 36 tones along with other Biofertilizers
3. National Biofertiliser Development Centre, Ghaziabad (UP), Regional Biofertiliser Development Centre at Bangalore, Bhubaneshwar, Hissar, Imphal, Jabalpur, and Nagpur Capacity - 376 tones along with other Biofertilizers

CONSTRAINTS ON THE USE OF BIOFERTILIZERS

Reluctance among the farmers to adopt a BGA technology could be due to difficulty in its use, unavailability of the inputs required, market uncertainty, price fluctuations or preferences for very low management crop technology. Following may be the possible constraints:

1. Lack of moisture or assured irrigation, loss of material due to flooding,
2. Non-availability of enough quality material.
3. Lack of awareness.
4. Presence of native population so effects are marginalized.
5. Excessive predation by small animals.
6. Region wise unavailability of soil specific strains, which limit the use of bio-inoculants.
7. Lack of adequate mass production units.
8. Lack of quality standards
9. Poor production facilities.
10. Soil acidity, alkalinity, pesticide application and high nitrate levels
11. Soil nutritional deficiencies.
12. Lack of the publicity is one major constraint in popularizing biofertilizers among the farming community.

IMPORTANCE OF QUALITY CONTROL

Most of the world Government Programmes for the regulation of inoculant producers either do not exist or are inadequately funded or motivated (Olsen et al., 1996). However, the manufacturers should have a conscious on their product and without a quality control programme, he is unlikely to have any better information about the quality of his product than the consumer (Thompson 1984). To promote inoculant utilization in a developing country, it is very necessary to introduce high quality inoculant to the farmers along with proper recommendation. It is very important to set the standards based upon research findings.

BUREAU OF INDIAN STANDARDS FOR BIOFERTILIZERS

No standards are yet fixed up for BGA biofertilizers. For the quality control, State Governments have been requested to ensure checking of the quality of biofertilizers produced and marketed. Quality factors include incorporation of superior strains, the use of good quality carrier, survival in the carrier, free from contamination, adequate shelf life, proper and easy packing formulations which is easy to supply and highest number of live cells. A good policy and protocol including the availability of appropriate standards and establishment of proper testing and reporting agency is necessary for inoculant quality control (Thompson 1980). But there is no system in India where self-regulation by manufactures has proved to be unsatisfactory procedure (Thomson, 1999).

PROMOTIONAL ACTIVITIES

For successful launching of a biofertilizer programme, it is essential to equip the extension machinery with full technical backup of production and application. There is a need to give emphasis that biofertilizers are supplementary, renewable and non-polluting sources of nutrients.

- Training Programmes on Quality Control of Biofertilizer
- Training Course on Biofertilizer production technology
- Training to Extension Officials
- Awareness among farmers
- Supply of efficient strains to the manufacturers.
- Rendering technical advice
- Promotion of biofertilizer entrepreneurship and encouragement

GOVERNMENT'S FUTURE PLANNING FOR PROMOTION OF BIOFERTILIZERS

1. Encouragement to private industries, state governments, volunteers organizations for Production, distribution and marketing of biofertilizers.
2. Financial and technical support to such manufacturers.
3. Organization of massive field trials on different crops by the manufacturers and agriculture department of state governments.

4. Organization of training for farmers, extension workers, producers and traders on various aspects of technology.
5. Publicity programmers through mass media and other media.
6. Establishment of new Regional Biofertilizer Development Centers in different parts of the country and
7. To bring all production units under the control of single umbrella and recognition of DAC as nodal agency.
8. Creation of quality testing and quality control units and formulation of "biofertilizer Regulatory Act" (Fertilizer News April 1994)
9. Involvement of an independent agency (Biotech Consortium India Limited) to assess the quality of microbial inoculants. Such an agency should be able to obtain samples of microbial inoculant's packets from the dealer or market and assess the quality by performing blind tests using a coding system, which will not divulge the identity of the manufacturer to the technicians.

The algalisation technology certainly holds promise and economics are also favourable, but large-scale production and harnessing of maximum benefits from BGA biofertilizer will be possible only through proper analysis and resolution of major constraints. According to an estimate, 240 million tones of food grains will be required to feed about one billion expected populations by 2000AD and to achieve these milestone 20 million tones of mineral fertilizers will be required. The problem is so acute that it is beyond any single type of nutrient source to accept the challenges of appropriate nutrient supply. Integrated use of all the sources such as mineral fertilizers, organic manures, biofertilizer etc. is the only alternate for improving soil fertility. The use of organic manures and mineral fertilizers is in practice but use of biofertilizer in agriculture is not very popular.

CONCLUSION

In Asia, 24% of the increase in rice production from 1965-1980 was attributed to better crop nutrition, due primarily to the increased use of N fertilizers. Today, more than 20% of all N fertilizers produced world wide are applied to the rice fields of Asia. The densely populated and land scarce nations of Asia, where more than half of the earth's people live and rice is a staple food, are dependent on petroleum based fertilizers. Any reduction in the consumption of this fertilizers without effecting productivity as well as introduction of a cyclic nutrient supply system through biological sources would be ecologically and economically advantageous.The challenge is to increase our knowledge about limiting factors for cyanobacterial contribution to the N status of rice ecosystem. It is of no use to transfer technologies from one site to another, if the set of ecological interaction associated with such techniques can not be replicated. In the context of biological management of soil fertility we must consider that the advantages of slow N release might not be apparent in the first crop after inoculation and that the feasibility of the technique is not assured. There is still a lot to investigate about the multiple effects of natural source management, some of which may even be undesirable. Perhaps biofertilizers may not be an alternative to synthetic fertilizers, but they can enhances the availability of plant nutrients from the soil.

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8

MYCORRHIZA : THE SAVIOUR OF PLANTS FROM PHYTONEMATODES

AAKANSHA GUPTA AND RAKESH PANDEY

Nature is a home to innumerable organisms, a place where they interact in a coordinated fashion and form a complex environment. A variety of beneficial as well as harmful organisms are associated with plants and they exert different qualitative and quantitative impact on plant production and yield. In field, a plant is exposed to various pathogens and their association with the plant may not necessary end up in causing disease. The population of one major pathogen dominates all others and becomes the main disease causing agent resulting in reduction of crop yield. This complex association has been shown to cause distinct biological changes in both, the host and the pathogen. Plants known to be resistant to one particular pathogen become susceptible to it when attacked by some other pathogen. Furthermore, the presence of one organism may inhibit or stimulate the growth of other organism (Smith, 1987; Sikora, 1992; Sharma and Johri, 2002; Smith *et al.*, 2003).

Plant parasitic nematodes are most important pests of a large number of agricultural crops worldwide, especially in tropical and subtropical countries, where environmental conditions favor their multiplication, survival and dispers*al.*, Variety of deleterious nematodes feed on plant roots, buds, stem, crown, leaves, seeds, rhizome, sucker, seedlings and tuber. Nematode infested root can not efficiently uptake the nutrients and moisture present in soil thus creating different disease symptoms in plants. The general underground symptoms caused by plant parasitic nematodes are root galling, root lesions, root pruning and cessation of root growth whereas chlorosis, stunting and wilting are few above ground symptoms.

Loss caused by plant parasitic nematodes on a variety of agricultural crops is estimated to be more than $100 billion annually. The crop loss caused by different plant parasitic nematodes depends on various factors like crop species, soil types, nematode types, load of inoculums and environment. The damage is more pronounced when high levels of nematode inoculums with susceptible host plants are planted in fields. This results in low crop yield and poor quality product. Crops like vegetables, legume, pulses, sugar, fiber, fruits, cereals, and medicinal and aromatic plants are soft targets of these hidden and notorious pests (Luc *et al.*, 2005). The damage caused by nematodes sometimes becomes several folds when they interact with plant pathogenic fungi, bacteria and viruses. Some of the plant parasitic nematodes have been reported as predisposing agents for fungal and bacterial diseases whereas other nematodes act as vectors of plant viruses. In the developing countries the damage caused by plant parasitic nematodes has increased significantly because of lack of awareness in poor farmers about these hidden enemies.

Traditionally, in intensive farming system, synthetic chemical nematicides are used to control nematode pest. However, continuous use of nematicides results in decline in their efficacy and their use further threatens the environment, human health as well as the non-target organisms in the soil. Nowadays, most of the effective nematicides are banned for use in agricultural crops all around the world. Environmental and health concern has brought attention to alternative control methods such as biocontrol agents to manage the nematode diseases with the aim of maintaining the high standards of crop quality and production. Recently, interest has increased to discover new, ecofriendly and safe plant based nematicides (Gommers, 1973, Chitwood, 1992, 2002; Pandey *et al.*, 2003; Pandey and Bhargava, 2004; Li *et al.*, 2005) or bio-organics (Hoitink and Boehm, 1999; Starling, 1991; Sikora, 1992; Sharma *et al.*, 1994; McSorley and Gallaher, 1995; Rao *et al.*, 1996; Oka and Yermiyahu, 2002; Suarez *et al.*, 2004; Pandey 2005a; Padgham and Sikora, 2007; Siddiqui and Akhtar, 2008) to manage the nematode problem and remarkable success has been achieved. Use of bio-organics has become the first choice of most of the researchers due to their ecofriendlness, cheapness, availability and its potentiality. Researchers have come up with wide options of bio-organics like nematophagus fungi, endophytes, egg parasitic fungi, arbuscular mycorrhizal fungi (AMF), bacteria (Barron, 1977; Oostendrop and Sikora, 1990; Starling, 1991; Oyekanmi *et al.*, 2007) etc.

Mycorrhiza and phytonematodes, both are inhabitants of plant root system; they derive their food and nutrition from the plant root. The major difference lies in the kind of association they have with the plant. Mycorrhiza have obligate, mutualistic symbiosis with the plant system by taking carbohydrates and photosynthates from the plant and in turn supplying plant with water and nutrients from the soil especially phosphorus (P) ((Li *et al.*, 2006a), whereas phytonematodes are parasitic to the plant. Mycorrhiza not only provide the plant with nutrients, but also makes the associated plant more tolerant to heavy metals, drought and to various biotic and abiotic stresses and also make the soil healthier. Mycorrhiza usually compensates for the amount of nematode damage sustained by non- mycorrhizal plants. Mycorrhizal fungi and nematodes are often mutually inhibitory, each reducing the population of the other. The major research between mycorrhiza and nematode has been carried out on specific nematode genera like root-knot nematode, root lesion nematode and reniform as they belong to endoparasitic and semiendoparasitic group of nematodes. No doubt these nematodes are economically important but few other nematode species like *Tylenchorhynchus* spp, *Xiphinema* spp., *Longidorus* spp. are also important on few crops like grasses, fruits and corn (Luc *et al.*, 2005,). The interaction between ectoparasitic nematode and VA mycorrhizal fungi may be weak as compared to AM fungi and endoparasitic nematodes (Gera Hol and Cook, 2005).

The presence of vesicles and arbuscules is indicative of presence of mycorrhizal fungi in a root. Comprehensive research has been published on association of mycorrhizal fungi with large number of crops (Schönbeck, 1979; Harly and Smith, 1983; Bagyaraj, 1991; Linderman, 1994; Smith and Read 2002; Oyekanmi *et al.*, 2007). Mycorrhizal fungi are grouped in a single family, the Endogonaceae. The family is considered to be in an order of its own, the Endogonales, tentatively placed in the class Zygomycetes in the subdivision Zygomycotina (Benjamin, 1979).

Since AMF are obligate biotrophs, their *in vitro* culture is rather difficult. This limits the practical exploitation and understanding of their physiological, biochemical and genetical aspects. The mutualistic association between AM fungi and plant root provides the fungus with relatively constant and direct access to mono or dimeric carbohydrates, which is translocated from leaves to the root tissue and later to the AM fungi (Harrison, 2005). In return, the plant gains from the mycelium which covers a very large surface area in the root to absorb water and mineral nutrients from the soil, thus improving the mineral absorption capabilities of the plant roots. Plant roots alone may be incapable of taking up phosphate ions that are immobilized. The mechanisms of increased absorption are both physical and chemical., Mycorrhizal mycelia are much smaller in diameter than the smallest root, and can explore a greater volume of soil, providing a larger surface area for absorption. Also, the cell membrane chemistry of fungi is different from that of plants. Mycorrhizae are especially beneficial for the plant partner in poor nutrient soils. Recent advancement in science and technology has given a new impetus to the culture production of these obligate symbionts, so that they can be used to their fullest for the benefit of environmental and human health. In general the root-associated mycorrhiza is divided in three broad categories viz., ectotrophic mycorrhiza, endotrophic mycorrhiza and ecto-endotrophic mycorrhiza.

ECTOMYCORRHIZA

This is an important group of fungi where fungus grows predominantly intercellular in root tissues especially in the cortical region. The fungus by penetrating the epidermis secretes proteolytic enzymes and develops extensively outside the root, forming a network of hyphae, which is commonly known as harting net or fungus mantle. This fungus mantle or harting net saves the plant feeder roots from the attack of different soil borne pathogens including plant parasitic nematodes. Such AM fungi also produce some growth promoting substance like cytokinins which enhances the different growth parameters like root/shoot plant weight, length, yield. Such type of fungal association is commonly found in few forest trees belonging to families

Fragaceae, Butalaceae and Salicaceae. The ectomycorrhizae generally form a cover on root surfaces as well as in between the root's cortical cells. It has been reported that ectomycorrhizae are found in 10% of plant families, mostly the woody plant species.

ENDOMYCORRHIZA

Endomycorrhiza are associated with most of the plants belonging to family Graminae. Initially this type of mycorrhizal fungi grows between the cortical cells and later penetrates the cells forming arbuscules and vesicle. The hyphae of these mycorrhizal fungi produce glomalin, which may be one of the major stores of carbon in the soil. The most interesting part of this fungus is that its hyphae are restricted to the cortical region of the root and never penetrate the stele. Arbuscules in the cortical cells are supposed to be a major site of material exchange between host and fungus as they provide intimate contact between the plasmalemmae of the two symbiotic partners. Arbuscules are transient in nature and live for 1-15 days. Some of the species (*Gigaspora & Scutellospora*) of this group form vesicles within the root cells. Vesicles are lipid filled, terminal swelling of the hypha with storage function.

ECTO-ENDO MYCORRHIZA

This is an association of the fungus and roots of the plant representing a condition where typical ectotrophic intercellular infection is accompanied with intracellular penetration of the hyphae. This kind of mycorrhizal association is considered to be transitional between ectotrophic and endotrophic forms, where infection is typically ectotrophic along with endotrophic penetration of hyphae.

MAJOR ACTIVITIES OF MYCORRHIZAL FUNGI

Nutrient Uptake and Exchange

It is well established that arbuscular mycorrhizal fungi are obligate symbionts as they require the living root tissues for their proliferation and development. Root tissues are the major source of carbon and nitrogen for mycorrhizal fungi. There is complex type of interaction between plant and mycorrhizal fungi as AM fungi take up the plant host's photosynthesis products in the form of \o "Hexose"hexoses. The transfer of carbon from the plant to the fungi may occur through the arbuscules or hyphae (Pfeffer, 1999). AM fungi are mostly dependent on the plant for their nutrition (Harley and Smith, 1983, 1988). Inside the mycorrhizal fungi, hexose is converted to \o "Trehalose"trehalose and \o "Glycogen"glycogen and they further get degraded in the form sugar resulting in the enhancement of sugar concentration in root tissues (Pfeffer, 1999). The hexose in the root tissues' enters the oxidative \o "Pentose phosphate (not yet written)"pentose phosphate pathway which produces \o "Pentose"pentose for nucleic acids synthesis. Further the breakdown of lipids into hexoses, known as gluconeogenesis, occurs in the extraradical mycorrhizal fungi (Pfeffer, 1999). The benefit of mycorrhiza to plants is mainly attributed to increased uptake of nutrients, especially phosphorus. This increase in uptake of nutrient from the soil may be due to increase in surface area of mycorrhizal fungi in (Bolan, N.S., 1991). The available phosphorus concentration in the root zone can be increased by mycorrhizal activity. Mycorrhiza lower the rhizosphere \o "PH"pH due to the selective uptake of NH_4^+ (ammonium ions) and release of H^+ ions. Decreased soil pH increases the solubility of phosphorus precipitates. The hyphal uptake of NH_4^+ also increases the flow of nitrogen to the plant as NH_4^+ is adsorbed to the soil's inner surfaces and must be taken up by diffusion. A recent study has reported the transfer of nitrogen by AM fungi, which is an encouraging aspect for agriculturists all over the world (Cruz *et al.*, 2007; Govindarajulu *et al.*, 2005). Mycorrhiza modifies the chemistry and biology of the rhizosphere in a way that alters the nutrient cycling and suppresses the activity of the root pathogen.

Stress Tolerance

There is evidence that the plants with mycorrhiza are more tolerant to stress such as soil salinity, alkalinity, acidity and drought conditions. Moreover, due to their exploitation of larger soil volume, extended root growth and increased absorptive area, the mycorrhizal plants exhibit better growth than the non-mycorrhizal ones especially in the arid and semi arid region where low moisture and high temperature are very critical for survival and growth of the plants. Mycorrhizal plants are also more tolerant to toxic heavy metals than the non-mycorrhizal plants (Gonzalez-Chavez *et al.*, 2004; Hildebrandt *et al.*, 2007) .Recently glomalin related soil protein (GRSP) is shown to have high binding capacity for some heavy metals like Cu,Cd and Pb. (Gonzalez-Chavez *et al.*, 2004; Vodnik *et al.*, 2008; Xu *et al.*, 2008).

Drought stress tolerance may result not only from direct water supply from extra radical mycorrhizal hyphae, but also from indirect mycorrhizal effects, such as better nutritional status, hormonal regulation of stomata, good osmotic regulation, antioxidant level is also increased in AM plants. (Faber *et al.*, 1991; Wu *et al.*, 2006 a, b). The improved plant water relation leads to good plant health such as shoot dry weight, root dry weight and plant dry weight. Extra radical hyphae development and soil aggregation of pepper (*Capsicum annuum*) plants inoculated with *G.deserticola* were enhanced by drought condition (Davies *et al.*, 1992). It has been reported that mycorrhizal tomato plants had higher content of phosphorus than non mycorrhizal plants, leading to the conclusion that drought stress and phosporus content has a close relation. Due to this, the yield of tomato fruits were also increased, as P in the form of monocalcium phosphate neutralized the acidity in the fruits and thereby lessened the deterioration of the tomato fruits (Subramanium *et al.*, 2006).

Increased Soil Quality

In general, the mycorrhizal fungi effectively increase the availability of soil nutrients and bind the soil effectively. There is evidence that this enhancement of soil aggregate stability is due to the production of a soil protein is commonly known as glomalin. Glomalin contains tightly bound iron from 0.04%-8.8% (Rillig *et al.*, 2001: Nichols, 2003). Glomalin has been identified by monoclonal antibodies which had been raised by crushing the spores of AM fungi. This glomalin is referred as glomalin related soil protein (GRSP). Glomalin is hypothesized to improve soil aggregate water stability and decrease soil erosion. A strong correlation has been found between GRSP and soil aggregate water stability in a wide variety of soils where organic material is the main binding agent. (Rillig, 2001, 2004). Mycorrhizal soils maintained better soil structure, especially soil water stable aggregates and soil proteins, which are important for maintaining soil porosity, stability against wind and soil erosion, increased storage of carbon obtained from microbial degradation. According to recent research findings AM fungus inoculation could increase soil peroxide (POD), decrease Catalase (CAT), and not affect polyphenol oxidase (PPO) level in the soil, suggesting that reactive oxygen groups produced in the soil are in negligible amount when colonized by AM fungi. (Wu *et al.*, 2008)

MANAGEMENT OF PATHOGEN THROUGH USE OF AM FUNGI

Rhizosphere is a complex environment, where several microorganisms interact with each other. The association of AM fungi with root brings about several changes in the plants as these fungal organisms absorb nutrients and induce resistance against several plant pathogens (Dehne *et al.*, 1978; Dehne, 1982; Jalali, and Jalali, 1991). The AM fungi may produce metabolites when interact with roots and may be toxic to different plant pathogens including plant parasitic nematodes (Pandey *et al.*, 1999). The AMF colonizes the root system and make a thick fungal mat around the root therefore alter the pathogen to infest the colonized root system. These fungi may change the physiology of the root system or compete with other organisms for root colonization. There are strong evidences that there is accumulation of defensive plant compounds upon mycorrhization. Presence of reactive oxygen species, activation of phenyl propanoid metabolisms and accumulation of hydrolytic enzymes such as chitinases and glucanases have been reported in mycorrhizal roots. Only mycorrhizal plants form a papilla-like structure around the sites of pathogen infection through deposition of non- sterified pectins and callose, preventing the pathogen from spreading further and besides that accumulates more PR proteins and glucanases than non- mycorrhizal plants upon *Phytophthora* infection. (Cordier *et al.*, 1998; Pozo *et al.*, 1999, 2002) Mycorrhization makes the plant defence system stronger by inducing resistance, the mechanism specifically known as MIR (Mycorrhiza Induced Resistance, Pozo et. *al.*, 2007).

MYCORRHIZA AND PHYTONEMATODE MANAGEMENT

Mycorrhizal plants are often more resistant to diseases, such as those caused by soil-borne pathogens including plant parasitic nematodes. These effects are perhaps due to the improved water and mineral uptake in mycorrhizal plants. Arbuscular mycorrhizal fungi do have the potential to be a biocontrol agent for nematode management when both groups of organisms occur simultaneously in the root and rhizosphere of the same plant (Jain and Sethi, 1988). The plants heavily colonised with AM fungi are able to grow well in spite of the presence of damaging level of nematodes, thus promoting tolerance to nematodes (Pandey *et al.*, 1999). The favourable effect of mycorrhiza in decreasing the nematode disease intensity has been demonstrated in various crops (Ingham 1988; Jain and Hasan, 1998; Cooper and Grandison, 1986, Pandey, 2005a, b; Oyekanmi *et al.*, 2007)

MYCORRHIZA AND NEMATODE INTERACTION

Nematodes and arbuscular mycorrhizal (AM) fungi often occur in the root rhizosphere plants and therefore frequently encounter each other. Mycorrhizal fungi benefit the plant by increasing the absorption of nutrients and water and by protecting the root from soil-borne diseases. Plant parasitic nematodes are generally interfere with these functions. Generally arbuscular mycorrhizal fungi do not colonize the root tissues infected by endoparasitic nematodes, and nematodes avoid to infect root tissues which are colonized by VA fungi. Nematode - mycorrhizal interactions appear to be very specific and highly dependent on the particular association of plant cultivar, fungal and nematode species. Thus, the nature of interaction varies to neutral, positive or negative. However, the plant growth has found to improve irrespective of the kind of interaction (Sikora and Schonbeck, 1975; Hussey and Roncardori, 1982; Sitaramaiah and Sikora, 1982; Grandison and Cooper, 1986; Jain and Hasan, 1998a,b; Kassab and Taha, 1990; Sharma *et al.*, 1994; Sharma and Trivedi, 1994, Nagesh and Reddy, 1999; Pandey, 2005b).

On the other hand, early colonization by mycorrhiza is not always beneficial for plant growth in the presence of nematodes (Campubri *et al.*, 1993; Pinochet *et al.*, 1995) and mycorrhizal establishment can be influenced by nematode invasion of cortical tissues (Smith and Kaplan, 1988; Campubri *et al.*, 1993; Pinochet *et al.*, 1995). It seems that there is no single response in the interaction mycorrhiza-nematode and that the response depends on various factors like availability of nutrient in soil, soil types, mycorrhization, nematode infection, host plant etc.

The major constraint in using mycorrhizal fungi as a biocontrol agent associated is the production of mycorrhizal inoculums, which has prevented large-scale use of these bio-protective agents. Through research and development such constraint has been removed and nowadays AM products are now commercially available as bio-fertilizers throughout Europe and the U.S.A. and a new strain of AM fungi viz. *Glomus* sp. R10 ATCC-74311, with an increased capacity to colonize vegetable roots even under high phosphoric acid levels, has been patented and commercialized recently in Japan by Idemitsu Kosan Co., Ltd.

Mechanisms that underlay the beneficial effects of mycorrhiza against nematode attack could involve competition for space and anatomical changes due to mycorrhization that could affect nematode penetration (Azcón-Aguilar and Barea, 1996; Oyekanmi *et al.*, 2007). Both root knot and cyst nematodes bring about some physiological changes in root cells, but differ in their invasion strategies. The feeding cells induced within the vascular cylinder may breach the endodermis, spreading into the cortex where they compete for space with AMF. It has been reported that AMF can alter the root morphology and thus prevent the invasion and penetration of migratory nematodes.

Ectoparasitic nematodes are affected by mycorrhizal fungi indirectly due to root colonization by mycorrhizal fungi as their chitinase activity become higher in plant roots (Jothi and Sunderbabu, 2002, Li *et al.*, 2006b), increase amino acids in leaves like arginine (Gioyannetti *et al.*, 1991) and an increase in soluble sugars and phenols in plant roots (Umesh *et al.*, 1988), which directly or indirectly inhibit the nematode infection and population development. Early mycorrhizal inoculation of tomato plants is highly beneficial to plant development in soils infested with *M. incognita* by increasing the host tolerance to this noxious nematode pathogen. Pre-colonization with mycorrhiza in plants that are transplanted as seedlings could benefit from this increased tolerance to root-knot nematodes. Simultaneous mycorrhizal inoculation with nematodes was not effective in protecting tomato plants against *M. incognita* but conferred some protection to carrot infected with *P. penetrans* (Talavera *et al.*, 2001). Direct application of mycorrhizal spores to the soil is effective in protecting against nematode damage depending on the virulence of nematode species, host plant and bio-protective potential of the mycorrhizal strain (Talavera *et al.*, 2001). Similarly the presence of three AM fungus viz. *G. mosseae, G. fasiculatum and G. aggregatum* on *Mentha arvensis* reduces the nematode multiplication and enhances the oil yield of this essential oil bearing plants (Pandey *et al.*, 1997). But there were cases where researchers have reported that the nematode population was unaffected by mycorrhizal fungi (O'Bannon *et al.*, 1979; Cason *et al.*, 1983; Hasan and Jain, 1987).

Nematode feeding habit also play an important role as the association between mycorrhizal fungi and ectoparasitic nematode is weak than with endoparasitic nematode. Mycorrhizal association is less effective on some species of nematodes like dorlamoid group, as they generally feed on root tips where the mycorrhizal colonization is very less, therefore such groups of nematodes are difficult to be managed by the application of AM fungi.

It is suspected that when AMF colonize the roots, there may be some changes in gene expression which may lead to the formation of some toxic protein which may directly inhibit the nematode penetration in plants or nematode mobility. It is also possible that when AM fungi colonize the root there may be secretion of some useful metabolites which may induce resistance in plants against the nematode infection.

MOLECULAR APPROACHES IN MYCORRHIZAL RESEARCH

Molecular techniques have been used to further understand the signalling pathways which occur between arbuscular mycorrhizae and the plant roots. In the presence of plant root exudates, the mycorrhizal fungi undergo some physiological changes which allow it to colonize the host root. The colonized plant root exudates generally triggered and up regulate the expression of gene responsible for transcription of some antinematode protein. In experiments there was an increase in the transcription rate of 10 genes after 0.5h of exposure of root to the exudates and an even greater rate after 1h exposure. In the mycorrhizal fungi the genes were isolated and found to be involved in mitochondrial activity and enzyme production. The mycorrhizal fungal respiration rate was measured by O_2 consumption rate and increased by 30% three hours after exposure to root exudates. This indicates that AMF spore mitochondrial activity is positively stimulated by host plant root exudates. This may be part of a fungal regulatory mechanism that conserves spore energy for efficient growth and the hyphal branching upon receiving signals from a potential host plant (Tamasloukht, 2003).

Recent research has shown that AM fungi release a diffusional factor, known as the myc factor, which activates the nodulation factor's inducible gene mtENOD11. This is the same gene involved in establishing symbiosis with the nitrogen fixing bacteria, Rhizobium. (Kosuta *et al.,* 2003). When rhizobium bacteria are present in the soil, mycorrhizal colonization is increased due to an increase in the concentration of chemical signals involved in the establishment of symbiosis (Xie *et al.,* 1995).

Progress in the development of inoculums of AM fungi has been severely hampered by inability to grow them in the axenic culture. This might be due to obligate biotroph nature of the fungus, therefore they have to be grown in the presence of living root system. The most remarkable breakthrough was achieved by the use of *Agrobacterium rhizogenes.* Genetically transformed roots by Ri (root inducing) plasmid of *Agrobacterium rhizogenes* are quite helpful in the culture production of these obligate symbionts. *A. rhizogenes* is a soil inhabiting, gram negative bacterium which produces a neoplastic plant disease syndrome known as "hairy root" which has the ability to grow rapidly showing two fold multiplications within 45-48 hrs. This characteristic feature is due to presence of integrated copies of transfer DNA (T-DNA) which occur in a large plasmid of *A. rhizogenes,* the Ri (root inducing) plasmid. The TR- DNA locus of T-DNA of Ri plasmid also carry genes of certain opine, which are indicative of transformation. The rapid and stable root growth due to modification by *A. rhizogenes* infection is very important and favourable for the mass production of arbuscular mycorrhizal culture.

Ways by which AMF Helps Its Plant Partner from Nematode Pest

- Competition for food and space for their growth and proliferation
- AMF causes physiological changes in plant root, thus making them unfavourable for nematode multiplication
- AMF enhances the nutritional status of plant roots mainly by phosphorus and other essential elements like N, K, Ca and Mg which may induce resistance against nematode pest.
- AMF induces hardening of roots end and exodermis by increased lignin level, which inhibit the nematode penetration.
- Root exudation adversely affects the nematode hatching and movement.
- Increased level of amino acid and sugar production in AMF colonized roots induces resistance in plant against nematode.
- AMF stimulates the production of phenylalanine and tyrosine checking growth and reproduction of plant parasitic nematode.

CONCLUSION AND FUTURE PROSPECTIVE

It can be concluded that future research programmes need to be more inclined towards sustainable and ecofriendly approaches for management of plant parasitic nematode in agri crops including medicinal and aromatic crops. Indiscriminate use of pesticides to protect crops against noxious nematode has caused a global concern on bio-degradation, groundwater contamination, human and animal health hazard. To mitigate such a collosal loss, genuine efforts are need to be made in proper and fullest utilization of the potentiality of mycorrhiza as a biocontrol agent. The present thrust on research on biological control of nematode need to be intensified. The obligate nature of these symbionts is a major bottle neck to its mass culture production. Nowadays, emphasis has been given on bulk production and utilization of arbuscular mycorrhizal fungi on large scale and mass production of AMF by root-organ culture technique is one of the upcoming methods. Inhibition of nematode attack in plant by inducing the ability to produce some kind of toxin or by activating the nematicidal gene products may be one of the successful approach to combat with nematode problem in agri crops.

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9

BIOLOGICAL CONTROL OF SHEATH BLIGHT OF RICE WITH *PSEUDOMONAS FLUORESCENS*

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Sheath blight (ShB) of rice caused by *Rhizoctonia solani* is a potential threat to rice ultivation, causing severe yield losses to the crop. The disease appears in nursery as vell as in transplanted rice. Intensive cultivation of modern high yielding, early maturing, nd high tillering rice varieties with double cropping and high doses of nitrogenous ertilizers lead to enhance severity of the disease. (Krishnamurthy and Gnanmanickam, 998). Losses due to ShB usually range from 1.2 to 69% (Naidu, 1992). Lee and Rush 1983) reported that grain yield losses between 20 to 50%, when all the leaf sheaths were fected, while Roy (1979) observed yield loss between 10 to 36% depending upon the age of the crop infection. In Tarai region of Uttaranchal/Uttar Pradesh the disease has used yield losses up to 48.7%, in different rice cultivars (Sharma, 1996). The loss in ield was closely correlated with the number of hills affected in the field. There is also ose relationship between disease severity and yield reduction which varies among ltivars (Mascheth, 1983).

The pathogen is soil-borne and remains viable in soil for several months (Ou, 1985). he sclerotia of *R. solani* in soil play a significant role in perpetuation and spread of the isease in rice fields under water logged conditions (Kannaiyan and Prasad, 1978). The anagement of ShB by the use of resistant cultivars has not been successful because dequate level of resistance has not been found. Application of fungicides such as alidamycin, benomyl, Tilt, Contaf and Iprodione are effective, but most farmers in tropics not have access to these chemicals nor can they afford the cost of treatment. In recent ears, increasing use of fungicides in agriculture has been the subject of growing concern both environmentalists and public health authorities. Besides, their non-target effects d hazardous nature, they are becoming more expensive and some are loosing their fectiveness, because of development of resistant strains.

Biological control has emerged an alternative and most promising means of the management of plant pathogens. Several fungal (*Trichoderma* and *Aspergillus* spp.) and bacterial (*Pseudomonas* and *Bacillus* spp.) antagonists have emerged as alternative most promising means fór sheath ShB management (Elad *et al.*, 1980). Among the several bacterial antagonists *Pseudomonas fluorescens* have been considered as ideal biocontrol agent against several plant diseases because of their ability to colonize the rhizosphere, their short generation time and mobility as well as their potential to produce an array of compounds inhibiting growth and development of pathogens (Johri *et al.*, 1997). It also stimulates plant growth, even if there is no disease, which results in better yield (Mishra and Sinha, 2000).

Bacterial antagonists have emerged as an effective most promising means for ShB management. However, no effective and economic management strategies have so far been developed. Reports are on hand indicating effectiveness of *P. fluorescens* against *R. solani* (ShB pathogen), under *in vitro* conditions. However, such organism fails to control the pathogen in the field, in most of the cases due to various reasons. It is because of the fact that biological control recommendation may hold promise under certain set of conditions only (especially under control conditions). Time, rate, mode of application and formulations of bioagent, pathogen inoculum level, plant growth stages, soil factors etc. play important role in biocontrol strategy. This review examines the current successes and problems of biological control of soil borne plant pathogens particularly ShB of rice with *P. fluorescens*. This article also discusses possible reasons for inconsistent performance of biological control agents in the field approaches to help realize the full potential bacterial bioagents in plant disease control.

BACTERIAL BIOCONTROL AGENTS

The use of bacterial flora for management of plant disease and yield improvement started in the early part of 20th century (Cook and Baker, 1983). However, intensified research on biocontrol aspects accelerated in mid 1960's. Importance of the subject is fully reflected in the number of published reviews (Weller, 1988; Handelsman and Parke, 1989; de Weger and Lugtenburg , 1990; Chet *et al.*, 1991; Lugtenburg *et al.*, 1991; O'Sullivan and O'Gara, 1992; Lam and Gaffney, 1993; Whipps and Mequilken, 1993; Dube, 1995; Pankhurst and Lynch, 1995).

Recently, great interest has been developed in the plant associated pseudomonads, which affect plant growth divergently or indirectly. The application of these microorganisms as agents for biocontrol of plant disease in agriculture is becoming an import ant alternative to the use of chemical fungicides (Shahnaz and Ahmad, 2003). A number of bacterial species have so far been tried as biocontrol agents. These are *Agrobacterium, Actinoplanes, Alcaligenes, Amorphosprangium, Arthrobacter, Azatobacter, Bacillus, Cellulomons, Enterobacter, Erwinia, Flavobacterium, Micromonospora, Hafnia, Pseudomonas, Pasteuria, Rhizobium, Bradyrhizobium, Serratia, Streptomyces* and *Xanthomonas* (Weller, 1988). A number of commercial preparations of bacterial biocontrol agents for *R. solani* are available in the market (Table 1).

TABLE 1

Commercial Formulations of *Pseudomonas fluorescens*

Commercial products	Biocontrol agents	Pathogen
Becure	*P. fluorescens*	-
Biomass	*P. fluorescens*	*R. solani* etc.
Biospriny	*P. fluorescens*	*R. solani* etc.
Biosheld	*P. fluorescens*	*R. solani* etc.
Cingaurd	*P. fluorescens*	*R. solani* etc.
Dagger-G	*Pseudomonas fluorescens* (G-105)	*R. solani* and *Pythium ultimum*
Pant Biocontrol agent 1	*P. fluorescens*	*R. solani* etc.
Pant Biocontrol agent 3	*T. harzianum* + *P. fluorescens*	*R. solani* etc.
PfALR2	*P. fluorescens*	*R. solani* (Rice sheath blight)
PfMDU2	*P. fluorescens*	*R. solani* (Rice sheath blight)

Pseudomonads

These bacteria are characterized by straight or curved rods, which are motile by polar flagella. They are gram negative in strain reaction and chemo-organotrophs. Cell size is generally 0.5-1.0 × 1.5-4.0 µm, no spore formation and no resting stage is known. The genus *Pseudomonas* consists of a very diverse assemblage of bacteria in which some are pathogen to most of the plants, and some are non-pathogenic. The non-pathogenic pseudomonads in which most of the species have the ability of plant growth promotion and biological control of plant pathogens.

Fluorescent psudomonads are efficient root colonizers. They have been used in the control of disease caused by fungi, bacteria and even virus (Shahnaz and Ahmad, 2003). Rhizosphere bacteria fluoresecent pseudomonas spp. has been established as plant disease suppressers and plant growth promoters (Ryder *et al.*, 1994). Isolation of rhizosphere bacteria is easy as they are ubiquitous but selection is difficult since only a few of them will be diseases suppressors. It has been rightly pointed out by Lugtenberg and Dekrers (1999) that selection of potential strains of rhizosphere bacteria must be based on rhizopshrere competence, antagonistic ability and root colonizing ability.

Groups (non-pathogenic *Pseudomonads*)

(i) Fluorescent

- *Pseudomonas fluorescens*
- *P. putida*
- *P. aeruginosa*

- *P. chlororaphis*
- *P. aereofaciens*

(ii) Non-fluorescent

- *P. stulzeri*
- *P. alcaligenes*
- *P. medocina*
- *P. pseudoalcaligens*

Fluorescent pseudomonads

Fluorescent pseudomonads make up a diverse group of bacteria that can generally be distinguished from other pseudomonads by their ability to produce water-soluble yellow green pigment (Krieg and Holt, 1984). They are typically gram negative, chemo heterotrophic, motile rods with polar flagella and are grouped in r-RNA homology group-I (Palleroni *et al.*, 1973). They are known to produce a large number of secondary metabolites, which may affect the growth, and health of plants (Dube, 2001). Fluorescent pseudomonads have simple nutritional requirements, and this is reflected by the relative abundance of these organisms in nature. Mostly fluorescent pseudomonas and some non-fluroscent are having the biocontrol potential and plant growth promotion capacity, amongst which, most important are *P. fluorescens* and *P. putida* (Lam and Gaffney, 1993; Dube, 1995).

In the last two decades, particularly after their role in suppression of take-all disease of wheat, intensive research has been done on biocontrol potential of fluorescent pseudomonads. Several reviews have so far been published (Weller, 1988; Defago and Hass, 1990; Kloepper, 1992; O'Sullivan ad O'Gara, 1992; Dube, 1995). *Pseudomonas fluorescens* has been attempted in biocontrol against various diseases because of the properties like its ability to colonize the rhizosphere of plants. short generation time, ability of utilize a large number of organic substrates commonly found in root and seed exudates (Rao and Johri, 1999), relatively easy cultivation under laboratory conditions, mobility as well as potential to produce an array of compounds inhibiting growth of fungal pathogens, compatibility with commonly used pesticides and other biocontrol agents (Hass *et al.*, 1991 and Tripathi, 1999). They can be manipulated by current genetic techniques. Some strains of fluorescent pseudomonads induce systemic resistance and reduce plant diseases (Van Peer *et al.*, 1991).

MASS PRODUCTION OF *P. FLUORESCENS*

Cost effective large scale production along with shelf life and establishment of biological control agent in a targeted niche determine the success of biological control (Tewari *et al.*, 2003). One of the greatest obstacles to biological control of introduced antagonists has been the lack or scarcity of methods for mass culturing and delivering the biocontrol agents (Mukhopadhyay, 1996). The unique problem in developing biopesticides is that it represents a living system, which must be able to stand the process of formulation and should remain sufficiently viable for a period until it reaches the farmers (Vidhyasekaran and Muthamilan, 1995). Despite the limited progress, scientists are engaged in developing effective experimental system for growth and delivery of antagonists. For successful formulation, development of the bioagent, there is an urgent need to standardize the optimum conditions for growth and development (Tewari *et al.*, 2003). The bacterial biocontrol agent *Pseudomonas* has been used as cell-suspension for experimental purpose. For commercial production of these antagonists' different technologies has been adopted on industrial scale (Mukhopadhyay and Mukherjee, 1998; Vidhyasekaran and Muthamilan, 1999). Commercial formulation of *P. fluorescens* was developed using a mixture of carboxy methyl cellulose and talc (1:100, w/w) (Vidhyasekaran and Muthamilan, 1995; Vidhyasekaran *et al.*, 1997; Mishra *et al.*, 2001). Krishnamurthy and Gnanamanikam (1998) tested eight different combinations of methyl cellulose ether, talc and $CaCO_3$ in different proportions, the MC: Talc (1:4) is the most satisfactory on the basis of its ability to preserve the bacteria in viable state for the longest period of time. Pertinent reports of base materials/carriers used for mass production of Fluorescent pseudomonads are summarized in Table 2.

TABLE 2

List of Base Materials/carriers Used for Mass Production of Fluorescent Pseudomonads

Base material	Biocontrol agent	References
Peat	*P. fluorescens*	Vidhyasekaran and Muthamilan, 1995; Rabindran and Vidhyasekaran, 1996; Shivakumar *et al.*, 2000
Hydrogenated vegetable oil, monohydrate, lactose polyvinyl pyrrolidone and cross linked sodium carboxymethyl cellulose	*P. fluorescens*	Kanjanamaneesathian *et al.*, 1998
Methyl cellulose : talc (1 : 4)	*P. putida*	Krishnamurthy and Gnanamanickam, 1998
Talc	*P. fluorescens*	Kamala *et al.*, 1998; Mishra and Sinha 1998; Vidhyasekaran and Muthamilan, 1999; Rangeshwaran and Prasad, 2000; Loganathan *et al.*, 2001; Nand Kumar *et al.*, 2001; Babu and Seetharaman, 2002; Commare *et al.*, 2002; Ramanathan *et al.*, 2002
Dried xanthin gum (XG)	Fluorescent pseudomonads	Demir *et al.*, 1999
Talc, $CaCO_3$, CMC, Talc + CMC (4 : 1)	*P. fluorescens*	Singh and Sinha, 2005

MODE OF APPLICATION

One of the important areas of biocontrol research has been the delivery system. It is rather necessary to have an efficient, economic and ecologically viable mode of application of biocontrol agents in soil ecosystem. A good amount of work has been done on this aspect under different conditions. Based on experiments, Papavizas and Lewis (1981) concluded that the method of inoculum preparation of biocontrol agents, their mode of application, use of different food bases and an integrated application of other chemicals or cultural practices can improve the effectiveness of biocontrol agents. The application of biocontrol agents by broadcast, sprays, or by direct application in seed furrow at the time of sowing has the greatest potential for use in commercial glasshouse operations or small scale field experiments, for biological control of root pathogens (Cook and Baker, 1983). This method gives the antagonist an opportunity to possess a great "competitive advantage" when introduced on seed rather than soil because in the soil the roots must be within convenient reach of the antagonist in order for the antagonist to colonize on the roots before the pathogen (Table 3).

TABLE 3

Application of Bacterial Antagonists(s) by Different Methods against Sheath Blight of Rice

Biocontrol agents	Application method	Reference
Fluorescent and non fluorecent	ST	Mew and Rosales, 1986
Pseudomonas fluorescens	ST	Sakthivel *et al.*, 1988
Fluorescent and non fluorescent	ST	Devi *et al.*, 1989
P. fluorescens	ST	Gnanamanickam and Mew, 1990
P. fluorescens, P. putida, P. aureofaciens	ST	Lee *et al.*, 1990
P. fluorescens,	ST	Gnanamanickam *et al.*, 1992
P. fluorescens	FS	Lin *et al.*, 1992
Pseudomonas strain	ST, FS	Karkar *et al.*, 1992
Fluorescent and non fluorescent	ST, RD, FS	Thara and Gnanamanickam, 1994
P. fluorescens	ST, RD	Kanjanmaneesathian *et al.*, 1995
P. fluorescens	ST, RD, FS	Rabindran and Vidyasekaran, 1996
P. putida	ST, FS	Mageswari and Gnanamanickam, 1997
P. fluorescens	ST, SLT, RD, FS	Mishra and Sinha 1998
P. fluorescens	ST, RD, FS	Kamala *et al.*, 1998

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Biocontrol agents	Application method	Reference
P. putida	ST, RD, FS	Krishnamurty and Ganamanickam, 1998
P. fluorescens	ST, RD, FS	Meena and Muthusami, 1998
P. fluorescens	ST	Shiv Kumar and Narayanswamy, 1998
P. fluorescens	ST, SLT, RD, FS	Vidhyasekaran and Muthamilan, 1999
P. fluorescens	ST, SLT, RD, FS	Nand Kumar *et al.*, 2001
P. fluorescens	ST	Sivalingun, 2001
P. fluorescens	ST, SLT, RD, FS	Commare *et al.*, 2002
P. fluorescens	ST, RD	Pathak *et al.*, 2004
P. fluorescens	ST, SLT, RD, FS	Singh and Sinha 2005b

ST = Seed treatment, FS = Foliar Spray, RD = Root dip, SLT = Soil treatment

(i) Seed treatment

Inoculation of seeds with antagonists prevents seed decay and seedling blight. This also takes care of seed borne inoculum, particularly externally seed borne diseases affecting above ground plant parts. Even internally seed borne disease like loose smut of wheat is reported to be suppressed by seed treatment with bioagents (Aggarwal, 2000, Singh, 2000). Biological seed treatment has tremendous potential to make biological control a great success especially for seed and seedling diseases in vegetables, fruits, forest and other plantation crop nurseries (Mukhopadhyay, 1994).

Cell suspension as well as dry powder has been used to coat the seeds with potential antagonists (Chao *et al.*, 1986; Mukhopadhyay *et al.*, 1992). The widely used practice for experimental purpose is to harvest the bacterial colony from a fully grown culture plate, making suspension of desired concentration with some sticker, soaking the seeds in the suspension and air drying before sowing (Mukherjee, 1991; Pandey, 1996). Propagulus of biocontrol agents multiply on the seed surface and colonize roots of germinated seedlings (Tiwari, 1996). For commercial purpose, dry powder of antagonists was used @ 3 to 8 gm powder per kg seed, based on seed size and formulation of antagonist (Mukherjee and Mukhopadhyay, 1995; Vidhyasekaran and Muthamilan, 1995). There are several reports on hand indicating control of ShB by seed treatment with the bacterial antagonists (Table 3).

(ii) Normal seed treatment vs. seed bio-priming

Bio-priming treating of seed with biocontrol agents and then incubating under warm and moist conditions until just prior to radicle emergence has potential advantages over simple coating of seeds as it result in rapid and uniform seedling emergence (Callan *et al.*, 1997; Mathre *et al.*, 1999). Emergence from bio-primed seeds is better (Mathre *et al.*, 1994). Bio-priming could also reduce the amount of biocontrol agents that must be applied to the seed. Vidhyasekaran and Muthamilan (1995) showed that when chickpea seeds were given bio-priming treatment with *P. fluorescnes*, the population of biocontrol agent was significantly higher in the rhizosphere than those without bio-priming. Callan *et al.* (1997) demonstrated that a minimum of 10^7 cfu per seed was needed for protection of sweet corn seed from *Pythium* seed rot, but the rapid rate of multiplication of *P. aureofaciens* AB 254 on seed during bio-priming enabled an initial population of 10^4 to 10^5 cfu per seed to increase to 10^7 cfu per seed during bio-priming.

(iii) Soil Treatment

The beneficial effect of soil treatments with biocontrol agents in plant disease management lies in their ability to suppress pathogen populations. There are several reports on the application of *P. fluorescens* to the soil and other growing media either before or at the time of planting for control of a wide range of soil-borne fungal pathogens (Baby and Manibhushanrao, 1993, 1996). Such applications are ideally suited for green house and nursery, but because of the bulk requirement, cost and problem of uniform distribution, feasibility of field application is less. Published reports on ShB control by soil treatment are given in Table 3.

(iv) Seedling treatment

Treatment of planting materials with beneficial organism is becoming increasingly important. Seedling roots can be treated with cell suspension of bacterial antagonists. Few reports are available on the seedling dip method of incorporating the biocontrol agents to control of ShB disease (Table 3). Root dipping in antagonists suspension not only reduces disease severity but also enhances seedling growth (Mishra and Sinha, 2000; Mishra and Sinha, 2001; Singh, 2003).

(v) Foliar application

Existence of epiphytic micro-flora on plant surface including leaves and flowers is a natural phenomenon. There are many studies where phyllosphere microflora has been implicated in reduction of foliar diseases (Chattopadhyay and Nandy, 1982; Bora *et al.*, 1993; Haware *et al.*, 1996). Antagonists isolated from physlosphere can be introduced on the host surface by spraying their propagules. Bacteria are better colonizer than fungi in this respect (Parasher *et al.*, 1992). There are several reports showing effectiveness of biocontrol agents applied as foliar spray against ShB (Table 3). However, success of any antagonists on leaf/sheath surface depends largely on its ability to colonize these surfaces. Even environmental factors (humidity, temperature and sunlight) affecting the colonization affect bioefficacy of antagonists. Because of these reasons, foliar application is preferred during evening hours and it is more successful in rainy season crops.

SURVIVAL OF *PSEUDOMONAS* SPP. IN RHIZOSPHERE

In order to protect seeds and roots from pathogen infection, a biocontrol agent should essentially be able to rapidly colonize the rhizosphere. Many contradictory reports are available regarding the ability of biocontrol agents to colonize rhizosphere and spermosphere. It is well known that different *Pseudomonads* (fluorescent) have different abilities to colonize a particular root-niche (Loper *et al.*, 1985; deWeger *et al.* 1987). It has been suggested that introduced bacteria, in general get distributed along the roots in natural soil, propagate and survive for several weeks even if there is competition from indigenous rhizosphere microflora (Weller, 1988). There was good colonization of roots of rice seedlings, when *P. fluorescens* was applied as seed treatment (Kamala *et al.*, 1998; Vidhyasekaran and Muthamilan, 1999; Trane *et al.*, 2001).

MECHANISM INVOLVED IN BIOLOGICAL CONTROL BY *PSEUDOMONAS FLUORESCENS* AGAINST SHEATH BLIGHT OF RICE

(a) Direct action of biocontrol agents

(i) Antibiosis

The antagonistic organisms release antibiotics and other metabolites that are harmful to the pathogen and inhibit their growth (Table 4). Many antibiotics have been isolated and characterized from *P. fluorescens*. Antagonists produce antibiosis that may play a part in biocontrol of plant pathogens (Mukherjee, 1991; Weller, 1988). Agrocin 84 has antibiotic and mediates suppression of *Agrobacterium tumifaciens* by *A. radiobacter* strain 84 (Kerr, 1980; Thompson, 1987), phenazines produced by some fluorescent pseudomonads are suppressive to take-all of wheat disease (Weller and Cook, 1983). Purified antibiotics pyoluteorin and pyrrolnitrin produced by *P. fluorescens* strains Pf-5 protected cotton seedlings against *Pythium ultimum* and *R. solani*. Species of fluorescent pseudomonads produce secondary metabolites with antibiotic activities many of which have been implicated in suppression of soil-borne diseases like phenazine-l-carboxylic acid (PCA), 2,4-diacetylphlroglucinol (DAPG), oomycin-A, pyocyanine, pyoluteorin and pyrrolnitrin (Weller, 1988). Viscosin an active compound resembled the lip opeptide was extracted from the culture of *P. fluorescens* by 1H-NMR and HPLC analysis method given by Nielsen *et al.* (1998). Boruah (1991) reported that *P. fluorescens* produced two antibiotics phenazine (PHE) and 2,4-diacetyl phloroglucinol (PHL).

TABLE 4

Antagonism Between *P. fluorescens* and *R. solani* in Rice

Biocontrol agents	Mechanism	References
P. fluorescens	Antibiosis	Mew and Rosales, 1986
P. fluorescens	Antibiosis and siderophore	Sakthivel *et al.*, 1988
P. fluorescens	Antibiosis	Sakthivel *et al.*, 1988
P. fluorescens	Antibiosis, siderophore and competetion	Devi *et al.*, 1989
P. fluorescens	Antibiosis, siderophore and competetion	Gnanamanickam and Mew, 1990

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Biocontrol agents	Mechanism	References
P. putida	Antibiosis and siderophore	Lee *et al.*, 1990
P. aureofaciens	Antibiosis	Lee *et al.*, 1990
P. fluorescens	Antibiosis and siderophore	Gnanamanickam *et al.*, 1992
Pseudomonas sp.	Antibiosis	Lin *et al.*, 1992
P. fluorescens	Antibiosis and siderophore	Wijisundera and Herath, 1994
P. fluorescens	Antibiosis and sierophore	Homma, 1995
P. fluorescens	Antibiosis and siderophore	Kanjanamaneesathian *et al.*, 1995
P. fluorescens Strain PfALR2	Antibiosis	Rabindran and Vidhyasekaran, 1996
P. fluorescens	Antibiosis	Mishra and Sinha, 1997
P. fluorescens	Antibiosis	Kanjanamaneesathian *et al.*, 1998
P. fluorescens	Antibiosis	Mishra and Sinha 1998
P. fluorescens	Antibiosis	Velazhahan *et al.*, 1999
P. fluorescens	Antibiosis	Iigon *et al.*, 2000
P. fluorescens	Antibiosis	Singh, 2003
P. fluorescens strain PfMDU2	Antibiotics and siderophore	Nagarajkumar *et al.*, 2004

(ii) Substrate competition and niche exclusion

Competition is considered as a 'classical' mechanism of biological control. Microorganisms compete for limited available nutrient (Elad *et al.*, 1980; Harman, 2000). It involves competition between antagonist and plant pathogen for space and nutrients (Chet, 1987). It seems to be an important mechanism of biocontrol, but it is difficult to assess its actual contribution in biological control. This mechanism is assumed to be involved if no evidence for antibiosis is found in a particular interaction (Alexander, 1982; Cook and Baker, 1983). Competition for nutrients secreted by seed and root exudates occurs in most interactions between bacteria and pathogens on the root and hence the introduction of bacteria is responsible at least to some small degree for the biocontrol (Elad and Chet, 1987). Pseudomonads catabolize diverse nutrients and have fast generation times in the root zone and they are logical candidates for biological control by competition for nutrients, especially against the slow growing pathogenic fungi (Weller, 1988). Large populations of bacteria established on planting materials and roots become a partial sink for nutrients in the rhizosphere, thus reducing the amount of carbon and nitrogen available to stimulate spores of fungal pathogens for subsequent colonization of roots (Elad and Chet, 1987).

Fluorescent pseudomonads are especially suited to "mopping up" nutrients, as they are nutritionally versatile and grow rapidly in the rhizosphere (Weller, 1985). It has been reported that niche exclusion is potentially an important mechanism of antagonism by plant growth promoting rhizobacteria (PGPR).

(iii) Siderophores

Siderophores (Gr. "iron bearers") are defined as 'low molecular weight, virtually ferric specific legends, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell' (Weller, 1988). The structural diversity among the different siderophores is quite considerable and depends on the producing microorganisms. However, a common feature of all siderophores is that they form six coordinate octahedral complexes with ferric ion (Raymond *et al.*, 1984).

Soil pseudomonads generally produce fluorescent, yellow green, water soluble siderophores with both hydroxamate and phenolate group. Siderophores from different fluorescent pseudomonads strains have shown that the principal difference is the composition, number and configuration of the amino acids in the peptide backbone (Neilands and Leong, 1986). The production of these siderophores has been linked to the disease suppression ability of certain fluorescent pseudomonas spp. (Loper and Buyer, 1991). Altman (1970) first suggested the involvement of these compounds in plant growth promotion and disease suppression by these strains. However, the first real substantiation of this concept was studied by Kloepper *et al.* (1980) who isolated fluorescent siderophorus from B-10 showed its disease suppression ability. Boruah, (1991) isolated pyoverdin (PYN) siderophore from *P. fluorescens.*

Iron is generally present in the microbial environment as the ferric ion (Fe^{+3}), which is virtually insoluble in the presence of O_2 and therefore, is not available for microbial growth. Siderophores chelate Fe (III) and microbial membrane receptor proteins specifically recognize and take-up the siderophore-Fe-complex (Hemming, 1986; Leong, 1986; Neilands and Leong, 1986). This result in making iron unavailable to rhizospheric microorganisms, including plant pathogens, which produce less siderophores or different siderophores with lower binding coefficients. The result is less pathogen infection and biological control. The mechanisms of antagonism between bacterial biocontrol agent and *R. solani* affecting various crops are presented in Table 3.

(b) Indirect action of biocontrol agents

(i) Induction of systemic resistance (ISR)

Elicitation of resistance in plants by biocontrol agents has been subject of few research papers and is becoming a more researched topic amongest the scientists (Bailey and Lumsden, 1998). Some strains of bacterial biocontrol agent like *Pseudomonas* clearly showed potent inducers of systemic resistance like responses in plants (VanWees *et al.*, 1997; Meena *et al.*, 2000). In the systemic protection of carnation against Fusarium wilt by *P. fluorescens* WCS 417, heat killed bacteria or the purified bacterial outer membrane LPS were as effective in inducing resistance as live bacteria, indicating that the bacterial LPS acts as determinants of resistance by WCS 417 in carnation (VanLoon *et al.*, 1998). Cell wall extracts of *P. fluorescens* WCS 374 or WCS 4217 or purified LPS were as effective as live bacteria when applied to radish roots (Leeman *et al.*, 1996). Siderophores may induce resistance to the same level as the bacterial LPS (Leeman *et al.*, 1996). However, these siderophores trigger ISR in a bacterial strains and plant species specific way is unclear at present. In the studies on protection of rice plant against ShB pathogen (*R. solani*) by strains of *P. fluorescens* 7-14 and *P. putida* V-14i, there was upto 18% reduction in disease severity as compared to untreated control (Krisnamurthy and Gnanamanickam, 1997). Meena *et al.* (2000) reported that seed inoculation of *P. fluorescens* induce systemic resistance against leaf spot fungus (*Cercosporidium personatum*) in groundnut. Maximum disease protection was observed after 30 days of inoculation and there was increase in the activity of phenylalanine ammonia-lyase (PAL), phenolic content and lytic enzymes (defense related compounds) in the treated plants. Kempe and Sequeira (1983) suggested that resistance to a virulent strain of *P. solanacearum* (bacterial wilt pathogen) was induced in potato by treatment of seed pieces with *Pseudomonas fluorescens*. The production of HCN by *P. fluorescens* CHAO has been reported as another potential inducer of plant defense mechanism by generation of cyanide stress in root tissue. Hydrogen cyanide on or in the root is considered an inducer of resistance in tobacco to *Thielaviopsis basicola* (Defago and Hass 1990).

Several strains of *P. fluorescens* have been reported to induce systemic resistance against many diseases of rice (Rabindran and Vidyasekaran, 1996; Kirshnamurthy and Gnanamanickam, 1997; Vidyasekaran *et al.*, 1997),

(ii) Solubilization and sequestration of inorganic plant nutrients

In soil, various plant nutrients under go complex transition from soluble to insoluble forms that strongly influence their accessibility and absorption by roots. Microorganisms may strongly influence these transitions (Sen, 2000). Iron and manganese in particular, have been investigated with regard to both microbial solubilization of oxidized forms of these elements and their influence on plant disease (Graham and Webb, 1991).

Pseudomonas spp. produces some compounds (siderophores) with very high affinities for iron (Pandey, 1996; Varshney, 1997). Iron chelated with these siderophores is unavailable to plant pathogens, so their activity is thereby reduced, but plant roots can take up iron in this form either directly or after reduction of Fe^{3+} to Fe^{2+} (Pandey, 1996).

FACTORS AFFECTING EFFICACY OF BACTERIAL BIOAGENTS

There are various factors which affect the efficacy of biocontrol agents.

(i) Isolates of bacterial bioagents

The application of beneficial strains as agents for biological control of plant disease in agriculture is emerging as a potential alternative to the use of chemical fungicides (Weller, 1988). Several antagonistic microbes have been reported to be effective against rice ShB pathogen (Dasgupta, 1992). Many bacteria isolated from rice field soil, healthy and diseased plant parts, sclerotia of *R. solani* and paddy field water were found antagonistic to ShB pathogen (Table 5). Several report are on hand indicating biological control of ShB of rice by different bacterial biocontrol agents assessed in greenhouse as well as field conditions, respectively (Table 6&7). Several workers have isolated bacterial strains from rhizosphere, phyllosphere of diseased and healthy plants of rice, sclerotia

TABLE 5

Screening of Fluroscent Pseudomonads *in vitro* against *R. solani* for their Potential as Biocontrol Agents

No. of bacterial isolates isolated from various sources	Remark	References
23 isolates were selected after isolation	Among these 3 were identified as *P. fluorescens* which inhibit *R. solani*	IRRI, 1986
925 bacterial isolates form rice rhzosphere, rice plant, sclerotia and paddy water	Of the 56 isolates fluorescent, 869 isolates non-fluorescent, 91% fluorescent and 32.8% non-fluorescent were inhibitory	Mew and Rosales, 1986
323 bacterial isolates were isolated from paddy rice field	Three bacterial isolates (16, 26 and 29) proved the greatest inhibition of sclerotial germination and mycelial growth of *R. solani.* isolate 26 and 29 were used for formulation development	Kanjanamaneesathian *et al.,* 1988
125 bacterial isolates from rice rhizosphere and other crops	Of the 117 fluorescent, 8 non-fluorescent, 16 fluorescent and 6 non-fluorescent inhibited mycelial growth of *R. solani*	Devi *et al.,* 1989
400 isolates of bacteria from rice field	Three strains of *P. fluorescens, Bacillus* spp. one *Enterobactor* sp. were inhibitory	Gnanamanickam and Mew, 1990
288 bacterial isolates from rice rhizosphere, healthy roots, sclerotia and paddy water	*P. fluorescens, P. putida* and *P. aureofaciens* inhibited mycelial growth of *R. solani*	Lee *et al.,* 1990
49 *Pseudomonas* and *Bacillus* spp. isolated from healthy or infected rice plants, rhizosphare, soils, water in the paddy field or from sclerotia	89% from healthy rice plants and from rhizosphere soil and 73% from paddy field water inhibited mycelial growth *in vitro,* 4 of 49 isolates gave good disease control and 1 of the volatile promoted seedling growth	Lin *et al.,* 1992
523 bacterial isolates from rice field	19.3% were antagonistic to varying degrees against *R. solani.* Among these 15% were fluorescent and 85% non-fluorescent	Sarkar *et al.,* 1992
23 isolate from rice filed	All strain inhibited mycelial growth of *R. solani.* Among the 5 were found *Pseudomonas* spp.	Rosales *et al.,* 1993

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No. of bacterial isolates isolated from various sources	Remark	References
1757 isolates from roots and other tissues of rice plants	31% fluorescent and 12% strains inhibitory	Thara and Gnanamanickam, 1994
P. fluorescens isolates from the rhizosphere of different crop	One of the most effective strains Pf ALR2 inhibited growth of *R. solani* was developed as an antibiotic resistant strain and beat based formulation developed for this bacterium	Rabindran and Vidhyasekaran, 1996
700 bacterial isolates from diseased and healthy rice plant	46% bacterial strains antagonistic, 20 isolates possessed stronger antagonistic activity, these were distributed in 7 genera and 12 species including 5 sp. of pseudomonas	Chen *et al.*, 1998
Various strains of *P. fluorescens* were isolated from the rhizosphere of rice plants	Among the 8 isolates tested *in vitro*, 5 isolates inhibited the mycelia growth of *R. solani*. Among the 5 isolates, PF-1 was the most effective fungal inhibitor *in vitro*	Velazhahan *et al.*, 1999.
1274 bacterial isolates from paddy soil, sclerotia of *R. solani*, sheath blight lesion and healthy plants	61 isolates were strong antagonistic, 727 moderate and weak and 486 non-antagonistic strains were found	Chen *et al.*, 2000
105 isolates from paddy soil, water, sclerotia of *R. solani*, sheath blight lesion, healthy plants, rhizosphere, and phyllosphere were isolated	Among them *Pf*1, *Pf*5, FLP 19, FLP 82 and FLP 90 were most antagonist to *R. solani*	Singh, 2003
14 strains of Pf isolated from rhizosphere	PfMDU 2 was most effective in inhibiting the growth of *R. solani*	Nagarajkumar *et al.*, 2004
22 isolates isolated from rhizosphere of rice plant	Isolate Pf 1and FP 7 were most promising against *R. solani*	Radjacommare *et al.*, 2004
1000 isolates from different culture pattern of rice was isolated	13 were strong antagonist to *R. solani*	Ren *et al.*, 2005

of *R. solani* and paddy field water and tested against *R. solani* the causal agent of ShB of rice for their potentiality as biocontrol agents. Nagarajkumar *et al.*; (2004) evaluated the production of chitinase, beta-1-3-glucanase, siderophores, salicylic acid and hydrohen cyanide in 14 strains of *P. fluorescens* isolated from rhizosphere soil of rice. The highest beta-1-3-glucanase activity, siderophore, salicylic acid and hydrohen cyanide production were recorded with PfMDU2 strain. A significant relationship between the antagonistic potential of *P. fluorescens* against ShB pathogen and its level of beta-1-3-glucanase, siderophores, salicylic acid and hydrohen cyanide was observed. (Singh and Sinha 2004) tested different isolates of *P. fluorescens* against ShB and found that isolate Pf1 exhibited maximum reduction in disease severity and incidence and increase the grain yield and 1000- grain weight. The information pertaining to this aspect is summarized in Table 5.

TABLE 6

Biological Control of Rice Sheath Blight with Fluorescent Pseudomonads in Greenhouse

Isolate tested	Efficient isolate	Per cent disease suppression	Per cent yield increase	Reference
5 fluorescent and	F in-b-24	40.3	22.0	
4 non fluorescent	NF-in-b-17	58.2	20.1	Mew and Rosales, 1986
2 fluorescent and 1 non fluorescent	P Fcp	42-58	-	Devi *et al.*, 1989
1 fluorescent and 3 non fluorescent	In-b-591	22-89	Increase	Gnanmanickam *et al.*, 1992
6 isolates of *P. fluorescens* and *B. subtilis*	Is-241 Is-135	3.8 40.7	- -	Sarkar *et al.*, 1992
40 isolates of fluorescent and non fluorescent	F-110	0-76.9	-	Thara *et al.*, 1992
90 isolates of fluorescent and non fluorescent	NF-227	36-85	-	Thara and Gnanamanickam, 1994
P. fluorescens	Pf AL R2	Significant suppressed	-	Ravindran and vidaysekaran, 1996
P. fluorescens	-	75.0	-	Meena and Muthusamy, 1998
P. fluorescens	Isolate 1 & 13	50-83	-	Shivakumar and Narayanswamy, 1998
Pseudomonas and *Bacillus* spp.	4 isolates from 49	-	-	Lin *et al.*, 1992
P. fluorescens and *P. putida*	Isolate 7-14 and V14i	18	-	Krishnamurthy and Gnanamanickam, 1997
P. fluorescens	PF 1, PF 7, PB 2	29.2-45.1	17.7-25.9	Nandkumar *et al.*, 2001
P. fluorescens	PF 1, PF 7	62.1	12-21	Commare *et al.*,2002
Pseudomonas sp.	GRP-3	Reduced lesion length	-	Pathak *et al.*, 2004
P. fluorescens	Pf 1, Pf 5, FLP 19 FLP and FLP 90	32.5-48.4	8.6-10.3	Singh and Sinha 2005

TABLE 7

Biological Control of Sheath Blight of Rice with Fluorescent Pseudomonads under Field Conditions

Isolates tested	Efficient isolate	Percent disease suppression	Per cent yield increase	Reference
One fluorescent and one non-fluorescent	Pf Cp NF-2	65-72	13.63	Devi *et al.*, 1989
2 fluorescent, 2 non fluorescent and 1 mixed isolates	IN-b-33 1-14-1 7-14	66-98	-	Gnanamanickam *et al.*, 1992
6 isolates of *P. fluorescens* and *B. subtilis*	IS-241 IS-135	40	-	Sarkar *et al.*, 1992
40 isolates of fluorescent and non-fluorescent	F-6	0-34.2	-	Thara *et al.*, 1992
6 fluorescent isolates	V 14i	26.5 - 67.6	-	Thara and Gnanamanickam, 1994
P. fluorescens	$PfALR_2$	Significant suppressed	Increased	Ravindran and Vidyaskaran, 1996
P. fluorescens	P-91	11.7-35.0	-	Chen *et al.*, 1998
P. putida	Pp V14L	7.9-59.9	-	Krishnamurthy and Gnanamanickam, 1998
P. fluorescens	Pf-1	74-91	10.8 to 17.8	Shiv Kumar and Narayanswamy, 1998
P. fluorescens	Pf 1	-	Increase	Vidhyasekaran and Muthamilan, 1999
P. fluorescens	P 1	-	Increase	Kamala *et al.*, 1998
1274 isolates of antagonistic bacteria	B-196	50.0-81.9	Increased	Chen *et al.*, 2000
50 isolates of endophytic and epiphytic bacteria	S-18	-	-	Yi *et al.*, 2000
P. fluorescens	PF 1, PF 7	62.1	12-21	Commare *et al.*, 2002
P. fluorescens	Pf 1, Pf 5, FLP 19 FLP and FLP 90	35.2-43.7	7.8-11.2	Singh and Sinha 2005

(ii) Soil factors

Growth and stability of population of PGPR vary greatly, depending upon the nature of the strain, soil type, soil moisture, pH and temperature (Bhame *et al.*, 1987; Howie *et al.*, 1987 and Bahme *et al.*, 1988). Soil micro fauna are important in the dissemination of bacteria (Cayrol *et al.*, 1987). DeWeger *et al.* (1987) claimed that motility was required for colonization of potato by *P. fluorescens*. On other hand, micro fauna (Clarhrolm, 1984) and bacteriophges (Stephens *et al.*, 1987) may reduce population of bacteria on roots. Gamlie and Katan (1991) reported that regression analysis showed a significant, inverse relationship between soil pH and increased growth and between soil pH and population densities of fluorescent Pseudomonads in rhizosphere. Chen *et al.* (1998) found density of the rhizosphere population was higher and more root segments were colonized in autoclaved soil than in non-autoclaved soil. Rhizospheric colonization was greater at 20°C than at 28°C. Colonization of FLPs 10 (a good rhizosphere colonizer) in the rhizosphere of tomatoes growing in 4 of 5 soils tested was similar, but in an acidic Tachi loam soil, the population was lower and decreased more rapidly than in the other soils. Adjustment of pH in the Tachi soil from the original 4.1 to a range of 5.3-7.1 increased rhizosphere population. Matsuguchi and Sakai (1995) observed that soil salinity markedly modified the population of fluorescent pseudomonads in the soil root system, such that the population of *P. putida* group becomes dormant in the rhizosphere and replaced those of the *P. fluorescens* group which appeared to be a major group of fluorescent pseudomonads in the soil-root system which was not subjected to salinity. Soil application of *P. fluorescens* formulation in soil having Ph 7.0 enhanced the effectivity of the ioagents and increasing seedling emergence and reducing ShB severity and incidence than alkaline and acidic Ph (Singh, 2003).

After extensive experimentation following conclusions have been drawn in the field of biological control of plant pathogens, i) biological control of plant pathogens occurs, but its general utility was skeptically regarded (Garret, 1965; Baker and Schwinn, 1993), ii) both the resident and introduced antagonists are able to diminish plant disease (Dubey, 1995), iii) the ecological balance of soil microorganisms can be manipulated by modifying the organic matter content, temperature or Ph (Weller, 1998), iv) microorganisms compete for limited available nutrients (Elad *et al.*, 1998; Harman, 2000), v) some microorganisms may parasitize other microorganism (Sharan *et al.*, 2001), vi) antagonists produce antibiotics that may play a part in biocontrol of plant pathogens (Weller, 1988), vii) some soils are naturally suppressive to same soil borne plant pathogens or became so following prolonged occurrence biological agents.

(iii) Cultural practices

Fluorescent pseudomonads may be relatively host specific, cultivar specific or non-specific in root colonizing ability and in plant growth promotion. Gerhardson *et al.* (1985) and Astrom and Gerhardson (1988) reported that plant genotypes affected the activity of bacteria. It has been observed different cropping systems had effect on the occurrence of fluorescent pseudomonads. In comparison with the total aerobic bacteria the population of fluorescent pseudomonads and of the strong siderophore producing bacteria changed in a characteristic way. Lucas and Collet (1988) showed the effect of nitrogen as fertilizers on the population of fluorescent pseudomonads and found the fluorescent pseudomonads spp. were 4 times more numerous in soils with ammonium sulphate than in those with ammonium nitrate. Singh and Sinha, (2005d) tested different combinations of NPK fertilizers on the effectivity of *P. fluorescens* on ShB, maximum reduction in disease was observed with N60P60K40 however, the effectivity was minimum with treatment N0P0K0. Application of *P. fluorescans* 7 days before inoculation of *R. solani* was found highly effective in increasing seedling emergence, reducing disease severity and incidence than simultaneous application (Singh and Sinha 2006). Of the three rates viz., 2, 4 and 8 g/ litre of the *P. fluoresceans* tested against ShB on transplanted rice , higher rate (8 g/litre) was found highly effective in reducing disease severity and incidence and increasing grain yield and 1000-grain weight (Singh and Sinha 2005b). Organic amendments such as green manures, stable manures and compost can provide the food base and has long been recognized to facilitate the biological control if applied well ahead of planting (Weller, 1988).

There is considerable interest in manipulating the soil microbial community to achieve the biological control of soil borne plant pathogens (Cook and Baker, 1983; Papavizas, 1985). Edaphic microorganisms stimulated by amendment contribute to the suppressive activity of the amended soils through all four principal mechanisms of biological control: a) competition, b) antibiosis, c) parasitism/predation and systemic induce resistance (Harman, 2000). This type of control is based on activities of biological control agents with in the context of microbial communities and their response to soil and plant introduced energy reserves. The concentration and availability of nutrients (carbohydrates in lignocelluloses substances, chitin, lipids, etc.) within the soil organic matter play a critical role in regulating these activities (Weller, 1988). High doses of nitrogenous fertilizers increased the severity of disease. However, slow release nitrogenous fertilizers reduce its attack (Roy, 1984). Application of potash and silica reduced the disease (Deren *et al.*, 1994).

The concentration of Zn^{2+} upto 1 Mm did not significantly inhibit the growth of fungi. However 10 Mm Zn^{2+} significantly decreased the mycelial growth of *F. solani, A. niger* and *T. viride* and completely inhibited the growth of *R. solani* (Babich and Stozky, 1978). The addition of Zn to Zinc deficient soils resulted in reduced yield loss in the presence of *R. solani,* a reduction in disease score and no changes in the concentration of nutrients in the shoots. However, under Zinc deficiency, increasing level of added *R. solani* resulted in significant yield loss, an increase in disease score (Streeter *et al.*, 2001). Soil application of the *P. fluorescens* in zinc amended soil showed 15.9% disease severity and 30.1% incidence. However, application of *P. fluorescens* in zinc deficient soil resulted in 20.3 and 32.2% disease severity and incidence, respectively. Soil application of neem cake enhanced the effectivity of the *P. fluorescens* in increasing seedling emergence and reducing ShB severity and incidence (Singh, 2003).

GROWTH PROMOTION BY THE *PSEUDOMONAS FLUORESCENS*

Bacterial biocontrol agents are reported to induce the growth of various crops. Reports are on hand which indicate that bacterial biocontrol agents also induce the growth of rice plants. Singh (2003) reported that *P. fluorescens* increased germination, root and shoot length and fresh root and shoot weight and dry root and shoot weight in rice when applied as seed treatment and soil treatment. Promotion of plant growth, increase of plant height, numbers of tillers and yield have been observed in *P. fluorescens* treated rice seedlings by Mishra and Sinha (1999, 2000) and Nandkumar *et al.*, (2001). These responses may be due to altered root growth because of N production (Baker and Schippers, 1987; Voisard *et al.*, 1989), hormonal effect on plant growth (Imam *et al.*, 1977), increased root exudation (Prikryl and Vancura, 1980), induced host resistance (Kempe and Sequeria, 1983). Activity of biocontrol agents could also reduce the concentration of substances in soil that are inhibitory to plant growth (Windham *et al.*, 1986). Enhancement of nutrient uptake by the plant and enzyme synthesis to regulate the level of plant ethylene have been observed (Krishamurthy and Gnanamanickam, 1997). There is production of plant hormone like substances or increase in the uptake of nutrient such as N, P, Zn, Fe, Mn, etc. either by changing physiology of plant or by converting nutrients into available form as well as synergetic effect on plant growth, yield and dinitrogen (Vidyasekaran and Muthamilan, 1995). These bacteria are ideally suited as soil inoculants because of their potential of rapid and aggressive colonization. Direct growth promotion occurs when a rhizobacterium produces metabolites that directly promote plant growth without interactions with native soil micro-flora (Dube, 2001). In contrast, antibiotics, siderophores and HCN, which decreased activities of pathogen or deleterious microorganisms and thereby, increased plant growth, are examples of indirect growth promotion by biocontrol agents. Pertinent reports of plant growth promotion by *Pseudomonas* are summarized in Table 8.

TABLE 8

Plant Growth Promotion by Fluorescent Pseudomonads in Rice

Bacterial isolates	Plant growth promoting activity	References
P. fluorescens	Increased plant growth	Sakthivel *et al.*, 1988
Pseudomonas sp.	Increased seedling growth	Lin *et al.*, 1992
P. fluorescens	Promoted, shoot and root growth	Mishra and Sinha, 1998
P. fluorescens	Increase in plant growth	Mishra and Sinha, 1997,1998, 1999, 2000
P. fluorescens (Strains FP 1, FP 7 and PB 2)	Promotion of plant height, number of tillers and yield	Nandkumar *et al.*, 2001
P. fluorescens	Increase germination root and shoot length and fresh root and shoot weight and dry root and shoot weight	Singh and Sinha, 2003
Pseudomonas *sp.*	Shown growth promoting effect	Radjacommare *et al.*, 2004
P. fluorescens (T 300363, S 300265 and S 90132)	Promotion of plant growth	Ren *et al.*, 2005

STRAIN IMPROVEMENT AND FUTURE STRATEGIES

Biological control activity is a complete process involving a variety of mechanisms which are likely to act in concert, which may differ in individual biocontrol strains. Papavizas (1987) suggested some genetic manipulation to improve the effectiveness of biocontrol agents for plant disease control, such as exposure to fungicides, mutagenesis, selection and hybridization by using biocontrol agents and fungicides. Xu, *et al.*; (2004) conducted a study to improve the biological control efficiency of *P. fluorescens* strain P5 by the inserting a chitinase gene 6.5 kb DNA fragment in vector pDSK519 to construct a new plasmid, pDSK51965. The new plasmid was introduced in strain P5, forming a new strain P5-1 contained a functional chitinase gene. P5-1 had higher effect than P5 against ShB.

Genetic engineering offers an exciting approach to strain improvement. The cloning of genes from biocontrol agent, whose products are essential for pathogencity or which contributes quantitatively to virulence, should provide the rational basis for strain improvement. More information is needed about the basic ecology of *P. fluorescens* interactions in the natural environment such as new techniques of genetic manipulation and strain improvement that can be utilized to enhance the performance of chosen agents. It is also important that successful examples of the use of biocontrol agent are introduced in to agricultural practices to set precedents for industry and crop producers to show that this approaches is worthy of the efforts to register and commercialize such products.

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10

TRYPSIN INHIBITOR : EMERGING STRATEGY OF PLANT DEFENSE AGAINST INSECT AND PATHOGENS

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INTRODUCTION

Plants have evolved elegant and complex chemical systems to defend themselves against the pathogens and herbivores that attempt to consume them. The current necessity of disease resistance in plants provided the motivation to hold this review article. Plants have complex defensive strategies to protect themselves against insects and pathogens. They posses many genes encoding defense-related proteins including resistance genes involved in gene-for-gene interactions with insects, genes encoding signal transduction proteins and downstream effector genes encoding proteins that protect plants against insect feeding. In order to combat the various modes of attack by the co-evolving microbial pathogens, pests plants use their rich and diverse arsenal of chemical weaponry which includes phytoalexins hydrolytic enzymes and the low molecular weight necrogenic proteins, commonly called pathogenesis related proteins. Some macromolecules active in defense have primary roles in the plant example, proteinase inhibitor (Trypsin inhibitor), lectins, alkaloids, lipoxygenase, chitinases and β-glucanases

Using these naturally occurring genes (the presence of trypsin inhibitor (TI) gene in plants) via genetic engineering various defense strategies have been planned out. The present review deals with compilation of the recent advances made in this field and a general outlook on the various methods and strategies used for conferring resistance in plants against insects and pathogens.

With ever expanding population, through out the world the need for food has been increasing continuously. Thus, the need for disease resistant varieties is a pre-requisite. Today, breeders all over the world are developing new hybrid cultivars but success in breeding for resistance remains localized because of location specific races. Thus, new methods and strategies are being used to combat disease. Insects and plant diseases are responsible for annual crop losses in excess of 10 to 15 percent worldwide. This represents not only an economic loss of more than $100 billion, but a significant loss in food production as well (Brears & Ryals, 1994).

Plant Responses to Disease Caused by Microbes and Insect Attack

Harmful physiological disorders produced in the plant body is called a "Disease". Physiological changes in the plant body cause structural abnormalities which either reduce the economic value of its product or cause its death. Living pathogens produce disease if, they get favorable environment for survival and multiplication, for example, spores of many fungi are present in the soil but they are unable to produce infection, because of the lack of favorable temperature, humidity etc. on which they are dependent. Plant infections are influenced by many factors such as genetic response of the plant to the activity of the pathogen and inherent resistance or susceptibility of the host (Singh, 1980). Genetic response of the host to the pathogen is an innate factor, which determines the success of infection. However, genetic character breaks down under the influence of several factors such as tissue condition (seedling and adult plant reaction), nutritional status of host, climate and presence of aggravating or synergistic biotic agencies such as nematode/fungus/bacterium complex (Singh, 1980).

Plants also respond to insect attack with a bewildering array of responses, which are broadly categorized as direct, indirect defenses and tolerance. Direct defenses constitute many plant traits (e.g. thorns, silica trichomes, primary and secondary metabolites) that by themselves affect the susceptibility to and/or the performance of attacking arthropods and thus increase plants fitness in environments with herbivores. (Duffey & Stout, 1996).

Indirect defenses are plant traits that attract predators and parasitoids of herbivores and increase the carnivore's foraging success and thereby facilitate top down control of herbivore populations (Karban & Baldwin, 1997). Herbivore attacked plant release volatile organic compounds (VOCs) which are highly specific, attract arthropod predators, and function as a direct defense by repelling the ovipositing herbivores (De Moraes *et al.*, 2001; Kessler & Baldwin, 2001).

"Tolerance" indicates the sustainability of plant genotype in tissue loss with little or no decrease in fitness relative to the undamaged state (Stowe *et al.*, 2000).

Plant Responses to Pathogens and Herbivores Differ

Enormous studies have been done over the past decade to understand the highly complex molecular events, which occur in plant-pathogen interactions. Starting from mutual recognition of host and pathogen, the first step, to the systemic acquired resistance (SAR), the final step (Grover & Gowthaman, 2003). Defenses against microbes are highly effective; in this the cells surrounding the infection site rapidly die and fill themselves with antimicrobial compounds so to prevent the spread of the pathogen, to the neighboring cells. In contrast, the herbivore attack is associated with recognition and wounding. The physiological and behavioral autonomy of herbivores allows plants to use defenses that are effective against pathogens. For example, plants use secondary metabolites that specifically target the nervous, digestive, endocrine organ systems unique to herbivores and use higher trophic-level interactions defensively by providing information or nutritional encouragement to the predators of herbivores.

Plants also evolve certain mechanisms to counter-balance the attacks of herbivores e.g., producing new active proteinase inhibitors (PIs). Some are able to retain defensive action even in an ever changing chemical environments of a plant as it undergoes a cycle of maturation and senescence. Several strategies have been developed to engineer disease resistance into plants. One strategy relies on the natural defense system using enzyme inhibitors, which have the ability to inhibit the activity of the enzymes. Plants have a wide array of defense proteins including the proteinaceous proteinase inhibitors and lectins induced in response to insect attack. Most of the enzyme inhibitors have been found in the seeds of the various plants. Enzyme inhibitors generally serve as storage or reserve proteins, as regulators of endogenous enzymes and as defensive agents against attacks by animal predators and insects or microbial pest. Protease inhibitors are present in the plant and are the inhibitors of different enzymes-trypsin, chymotrypsin, papain, elastase, carboxypeptidase A and B etc. Proteinase inhibitors (PIs) of plants inhibit proteolytic enzymes of animal and fungal origin and are therefore, thought to be protective agents against insects and fungi (Green & Ryan, 1972). PIs represent the most well studied class of plant defense proteins and are abundantly present in the storage organs/seeds and tubers.

All the four classes of proteinases are found in different plant organs or tissues (Ryan & Walker-Simmons, 1981). Presently, eight families of serine proteinase inhibitors are known. These are the Bowman-Birk, Kunitz, Potato I, Potato II, Cucurbit, Cereal super family, Ragi AI and Thaumatin-PR like families (Richardson, 1991).

PROTEASE INHIBITORS

PIs are highly stable defensive proteins of plant tissues that are both developmentally regulated and induced in response to insect and pathogen attack (Ryan, 1990). Their properties include a high stability at low pH and a remarkable resistance to endogenous and exogenous proteinases, these two characteristics make these proteins well adapted to the harsh conditions prevailing in the cellular compartments where they occur-the vacuole, the cell wall and/or apoplast. Pathogenesis related proteins (PRs) were first observed as a new protein component induced by Tobacco mosaic virus (TMV) in hypersensitively reactive tobacco (Van Loon & Van Kammen, 1970). PR proteins were isolated from soluble extracts of tobacco leaves reacting hyper sensitively to TMV, 10 major acidic proteins were isolated from this material and grouped into five families on the basis of their relative mobility on native alkaline polyacrylamide gels and on their serological relationships (Van Loon *et al.*, 1987). These five major groups occurred in many plant species, including dicots and monocots. They are well studied, particularly in three families Fabiaceace, Poaceae, and Solanaceae.

The possible role of PIs in plant protection was investigated as early as 1947 when, Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products. Plants, microorganisms and animals contain proteins that exhibit the peculiar property of forming complexes with proteolytic enzymes, promoting the inhibition of their activity by competing for the catalytic site. These proteins are also known as "Protease inhibitors". The term "protease" includes both "endopeptidases" and "exopeptidases" whereas; the term "proteinase" is used to describe only "endopeptidases" (Ryan, 1990). These inhibitors are present in storage organs like seeds and tubers, do not inhibit endogenous plant proteases, but have specificity for animal or microbial enzymes. The primary site of action of protease inhibitors is the digestive system of pathogens (Richardson, 1991). These proteins serve an important function in storage protein metabolism by regulating the level of proteinase activity during seed development (Ryan, 1988). It has been shown that they also play an important role against pathogen attack by acting as endogenous insecticides (Hilder *et al.*, 1987). Several non-homologous families of proteinase inhibitors are recognized among the animal, microorganisms and plant kingdom. Majority of proteinase inhibitors studied in plant kingdom originates from three main families namely Leguminosae, Solanaceae and Gramineae (Richardson, 1991).

More recently novel PIs with defensive capabilities were described in many other plant families. In 1994, the sixth family of PR proteins was created along with five new families, and the category was allotted to a well-characterized family of PIs first described by Green and Ryan (1972). In soybean (*Glycine max*), among others, two important families of proteinase inhibitors have been characterized: the Kunitz inhibitor, which shows specificity for trypsin (Kim *et al.*, 1985), and the Bowman-Birk, which inhibits trypsin, chymotrypsin, and elastase (Ryan, 1988 and Tan-Wilson, 1988). Pathogenic organisms colonizing plant tissues rely on a set of proteinases as part of their virulence factor. These proteinases belong to the four widespread mechanistic classes of proteolytic enzymes i.e. serine-, cysteine-, aspartic-, and metallo-proteinases. In parallel, plants have evolved genes encoding inhibitors that inactivate some of these proteinases and thus may reduce the ability of the aggressor to digest host proteins and therefore limit the availability of aminoacid source for the invader. Most of the PIs known and characterized in plants belong to the group of the serine-proteinase inhibitors, which inhibits trypsin. Trypsin is involved in developmental processes such as molting and synthesis of neuropeptides, thus Trypsin inhibitors (TI) can disrupt these processes leading to major damage to the growth and development of the larvae (Lipke *et al.*, 1954).

Serine proteinase inhibitors are widely distributed in plants and are obtained from many sources. Seeds are especially rich in inhibitors; the inhibitors are present in about 6% of the total soybean protein (Rackis and Anderson, 1964) and up to 10% of the soluble proteins of potato tubers (Ryan *et al.*, 1967). In Solanaceae family, proteinase inhibitors are found in the foliage of plants, they are normally expressed in the foliage at low levels, but following an attack by insects, the levels of proteinase inhibitors increases dramatically (Park and Thornburg, 1991). The classification of inhibitors is based on homology of amino acid sequences, active center structure, position of disulfide bonds, and mechanism of inhibition. Mostly plant proteinase inhibitors which are known and studied so far interact with serine proteinases (trypsin, chymotrypsin, subtilisin) (Dunaevskii, 1990). Although the biological role of proteinase inhibitors is still not sufficiently clear. It has been suggested that they may perform three main functions- serving as storage proteins, being regulators of activity of endogenous proteinases and as agents protecting plants against insects and pathogenic microflora (Tremacoldi, 2002). Inhibitors of plant proteases can regulate the hydrolysis of proteins inside the cells and participate in the mechanisms of plant defense against herbivore insects and pathogens (Richardson, 1991). Plant, microorganisms and animals contain proteins that exhibit the peculiar property of forming complexes with proteolytic enzymes, promoting the inhibition of their activity by competing for the catalytic site. Their molecular mass varies from 10 to 90 kDa, and they exhibit high or no specificity to the target enzyme (Tremacoldi, 2002). Protein inhibitors of serine (e.g., trypsin and chymotrypsin) and cysteine proteases have emerged as a class of antifungal proteins that have potent activity against plant and animal pathogens.

DEFENSIVE ROLE OF PROTEASE INHIBITOR AGAINST PATHOGENS

Evolutionary pressures of various kinds have been hypothesized to cause active and rapid evolutionary change. Various lines of evidence suggest that a major function of proteinase inhibitors is to combat the proteinases of pests and pathogens. The secreted proteinases of the latter organisms are key components of invasive cocktails, required for entry in to the host and rapid utilization of its constituent proteins. In these situations, there is clearly evolutionary pressure for the host to respond by evolving new and effective inhibitors (Bush *et al.*, 2001). The role of proteinase inhibitors in resistance to invasion is the observation that massive accumulation of proteinase inhibitors occurs in certain tissues and organs that are likely sites of attack. First are those tissues whose nutritional value presents to a pest or pathogen the best possible site for attack, for example, seeds (Birk, 1996), other plant storage organs such as plant tubers (Pouvreau *et al.*, 2001). The chemistry and biochemistry of proteinase inhibitors have been studied in detail not only because of their biological importance but also because they provide excellent models for specific interactions between macromolecules. Protease inhibitors are found in most plants and several of them have been purified and extensively investigated. Extensive studies have been carried out on protease inhibitors purified from soybean *(Glycine max)*, lima bean *(Phaseolus lunatus)*, navy bean *(Phaseolus vulgaris)*, broad bean *(Vicia faba)*, runner bean *(Phaseolus coccineus)*, garden bean *(P. vulgaris)*, mung bean *(Phaseolus aureus)*, bush bean *(P. vulgaris* var. *nanus)* and lentils *(Lens culinaris)* (Birk, 1996).

Pusztai (1966) reported the isolation of a trypsin inhibitor from kidney bean *(P. vulgaris)*. These proteins are bi-functional, inhibiting enzymes as well as inhibiting insect and fungal growth. Blanco-Labra *et al.* (1995) identified a bi-functional α-amlyase/trypsin inhibitor from corn. Similarly, Peng and Black (1976) showed that TI and chymotrypsin inhibitor levels in tomato plant increased following infection by the pathogenic fungus, *Phytophthora infestans*, but this host response only occurred in resistant tomato varieties to the fungus and not in susceptible varieties. Levitskii and Pogoreletskaya (1985) showed the presence of components which inhibited non-trypsin type proteinases of the pathogen *Botrytis cinerea* and inhibited the development of *Helicoverpa armigera* larvae.

Enzyme inhibitors found in seeds have received particular attention because of their potentially deleterious effects in animal and human nutrition and their possible role in the defense of plants against microbial and insect pests. Some of these inhibitors have a double function against proteases and amylases (Richardson *et al.*, 1987). The possible involvement of TI in plant defense against fungal pathogens has also been implicated by their antifungal activities. It has been proposed that these proteinaceous inhibitors may be storage proteins that play a role in masking the activity of preexisting plant proteases, and they protect the seeds against pathogens and phytophagous insects (Liener & Kakade, 1969). However, the mode of action of many of these proteins against fungi has not been clearly demonstrated. A 14 kDa protein from corn kernels showed inhibitor activity against bovine trypsin and α-amylase from the red flour beetle (*Tribolium castaneum*) and the yellow mealworm (*Tenebrio molitor*) respectively. It has been recently demonstrated that the 14 kDa TI was associated with resistance to *Aspergillus flavus* infection and aflatoxin production in 11 corn genotypes (Chen *et al.*, 1999). The purified TI inhibited *in-vitro* conidia germination and hyphal growth of *A. flavus*, the major aflatoxin-producing fungus in crops such as corn, cotton, peanut, and tree nuts. The ability of TI to inhibit the growth of *A. flavus* and some other fungal pathogens suggests its potential importance in the control of aflatoxin contamination of corn and other crops as well as of some other fungal diseases. TIs were described to be active only against a very limited group of fungi. A mixture of trypsin and chymotrypsin from tobacco leaves inhibit spore germination and germ tube growth of *Botrytis* and *Fusarium* (Lorito *et al.* 1994). Chen *et al.* (1999) reported a 14 kDa TI from maize seed as a potent inhibitor of trypsin.

It has been studied that the protein composition varies among genotypes (Pesic *et al.*, 2007) as well as trypsin inhibitors level (Tan-Wilson, 1986) similarly, trypsin inhibitor activity (TIA) is also affected by the genotype (Vollmann *et al.*, 2003). Trypsin inhibitor activity has also been studied in wheat. This inhibitor is found to be heat labile protein, and has been extensively purified. However the specific activities of the purest preparations are rather low (Mikola & Suolinna, 1969)

The amino acid sequences of the inhibitors of chymotrypsin and trypsin from soybean (Koide *et al.*, 1972), lima bean (Tan & Stevens, 1971), maize and peanuts (Hochstrasser & Werle, 1971) have been reported. Although a large number of different proteinase inhibitors have been isolated from potatoes (Iwasaki *et al.*, 1971 and Kaiser & Belitz, 1972), there are as yet no details of the amino acid sequences of any of these inhibitors (Richardson, 1974). Their broad spectrum activity includes suppression of pathogenic nematodes, inhibition of spore germination and mycelium growth (Dunaevskii *et al.*, 1997).

Soybean trypsin inhibitor (SBTI) has been found more effective than cowpea trypsin inhibitor (CPTI) in reducing the proteolytic activity of gut extracts obtained from *Helicoverpa armigera*. Antifungal activities have also been reported for TI proteins from several crops including TI from barley and trypsin and chymotrypsin inhibitors from cabbage as well as the 22 kDa TI from corn and the 24 kDa cysteine protease inhibitor from pearl millet.

Differences in the accumulation of PIs and proteases in compatible and incompatible interactions between plant and microorganisms associate these groups of proteins to the resistance and susceptibility concepts, generating perspectives of understanding of the biochemical bases that govern the plant-pathogen interactions (Harsulkar *et al.*, 1998).

Several molecular studies provides evidence that genes involved in pathogen recognition (i.e. R-genes) are subject to selection (Parniske *et al.*, 1997). PIs are among the best-studied plant defenses not encoded by R-genes (Ryan, 1990). These inhibitors are thought to be involved primarily in defense against herbivores, which rely on proteases to digest the proteins they consume (Koiwa *et al.*, 1997). However, because pathogens rely on proteases to facilitate infection and spread within hosts, PIs may also limit the severity of pathogen infection (Ryan, 1990). PI genes have practical advantages over genes encoding for complex pathways i.e. by transferring single defensive gene from one plant species to another and expressing them from their own wound inducible or constitutive promoters thereby imparting resistance against pests. Hence, Protease inhibitors are one of the prime candidates with highly proven inhibitory activity against pest. Therefore a detailed study on the Protease inhibitor specifically trypsin inhibitor is necessary as it blocks the activity of the prime serine proteinase.

TRYPSIN INHIBITOR

Plants developed defense systems to combat various pathogens through out their life cycle, from the seed stage until senescence, and particularly important, the embryonic stage which is kept free from infection. Trypsin belongs to serine protease class, which is responsible for the initial digestion of protein in the gut of higher animals. There are several embryonic defense mechanisms, including the production of plant lectins and pathogen-related proteins in response to attacks by pathogens or insects (Swegle *et al.*, 1992). Plant seeds contain a large number of different types of serine proteinase inhibitors, which block trypsin and chymotrypsin from animal, fungal, and bacterial origins.

Among many types of protease inhibitors from plants, Kunitz-type and Bowman-Birk type inhibitors have been most extensively studied. Members of the Kunitz-type inhibitor family have a molecular mass of about 20 kDa and two disulphide bridges, whereas those of the Bowman-Birk family are smaller (typically ~8 kDa or 16 kDa) and richer in disulphide bonds (Birk, 1987). Bowman-Birk protease inhibitors (BBIs) from dicotyledonous seeds such as soybean are small (~8 kDa) double headed proteins i.e. ~8 kDa inhibitor contains two reactive sites, each of which specifically inhibits trypsin and chymotrypsin.

KUNITZ TRYPSIN INHIBITOR (KTI) : Kunitz Trypsin inhibitor (KTI) is characterized by a molecular mass of 21 kDa and four cysteines.

BOWMAN-BIRK INHIBITOR (BBIs) : Bowman-Birk inhibitors are of relatively low molecular mass of 10 kDa with four cysteines linked to seven disulfide bridges, and two reactive sites (Onesti *et al.*, 1991). Generally, they inhibit trypsin like proteases on one site and chymotrypsin on the second one. The Bowman-Birk inhibitors (BBI) from dicotyledonous seeds such as soybean are 8 kDa double-headed proteins i.e., both trypsin and chymotrypsin are inhibited by the two reactive sites in a single inhibitor molecule. In contrast, the 8 kDa inhibitors from monocotyledonous seeds are single headed. The presence of various BBIs in most species studied (Ishikawa *et al.*, 1985) is attributed either to the expression by distinct genes and/or to the post-translational proteolytic cleavage of few aminoacids at the N- and C-terminal end of the inhibitor. Monocots also have a 16 kDa double-headed inhibitor (Prakash *et al.*, 1996). A 16 kDa TI purified from the barley rootlet was characterized as a member of BBI family.

Despite wide diversity, some TIs share sequence homology with seed storage proteins. Proteinase inhibitors and many seed storage proteins are encoded by families of polymorphic genes that probably arose by a complicated process of gene duplication, DNA exchange, and exon reshuffling. Like many other seed proteins, TIs are synthesized as large precursor proteins. After co-translational cleavage of the signal (pre) peptide in the endoplasmic reticulum, they undergo limited proteolytic processing in vacuoles. Here short pro-peptides (N-terminal, internal, and C-terminal sequences) are cleaved off. Final assembly and deposition of the inhibitor take place in compartmentalized storage organelles along with the active storage proteins (Mandal *et al.,* 2002). Trypsin inhibitors have been purified and characterized from several cultivars of dry beans over a period of years by many investigators. The number of trypsin isoinhibitors present in dry beans has been in question, along with the reported physical and chemical characteristics of the isolated inhibitors.

DISTRIBUTION OF TI IN PLANTS

Trypsin inhibitors are the most studied, of the serine proteinase inhibitors and are thought to participate in the array of defensive substances that plant synthesize for protection against pest and pathogens. These were firstly discovered in reserve organs of plants which are sources of proteins.

A. Seeds and Tubers

Seeds and tubers of the many plants assayed for their presence were found to contain TI. Seeds of leguminous plants such as the soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), the cowpea (*Vigna unguiculata*), the Brazilian carolina tree (*Adenanthera pavonina*) and many others were found to be rich sources of TIs.

Inhibitors from soybean

The most well known of the plant serine proteinase inhibitors is the Soybean Kunitz Trypsin Inhibitor [SBTI] (Laskowski & Kato 1980). It is 21 kDa that complexes with the enzyme with a very high association constant (ca 10^{10} M^{-1}). SBTI is a seed specific protein that is expressed in high amounts during development (Vodkin, 1981). The inhibitor was originally isolated by Kunitz and is one of the three active isoforms (Kim *et al.,* 1985). Soybean also contain another serine proteinase inhibitor; it is the Bowman-Birk proteinase inhibitor (BBI) that inhibits trypsin and chymotrypsin at independent reactive sites. Thus, it is called double-headed inhibitor. It is a small protein molecule of ca 10 kDa that also forms strong complexes with the two enzymes (association constants on the order of 10^{10} M^{-1}). As the name implies it can form ternary complexes in which two different enzymes strongly associate with the inhibitor molecule (Birk, 1985).

Inhibitors from other legume seeds

The cowpea (*Vigna unguiculata*) is a legume whose seeds contain several trypsin and chymotrypsin inhibitors. Some of these were isolated in pure form and their aminoacid sequences were determined (Xavier- Filho & Ventura, 1988).

Seeds of *Vigna unguiculata, Vigna angularis, Phaseolus vulgaris, Arachis hypogaea, Macrotyloma axillare, Vicia angustifolia, Canavalia brasilense, Dioclea grandiflora, Clitoria ternatea,* among others belong to the Bowman-Birk family of small (ca 10 kDa) double-headed inhibitors. (Richardson, 1990). Many other seeds of leguminous plants were found to accumulate serine proteinase inhibitors during development. The seeds of the Brazilian carolina tree for example, were found to be one of the richest sources of inhibitors: 20% of the soluble protein of the seeds is formed by the Kunitz type TI (Richardson *et al.*, 1986).

Inhibitors from Cucurbit seeds

Seeds of plants of the family Cucurbitaceae like the squash, cucumber and zucchini, contain inhibitors of trypsin and Hageman factor that are characterized by their low molecular weight (Filho, 1992). They are small homologous proteins of ca 30 aminoacids that form complexes with trypsin. These are among the strongest complexes ever known, with association constants of the order $10^{10}M^{-1}$ (Wieczorek *et al.*, 1985).

Inhibitors from roots and corms

TIs are found in roots of the sweet potato (*Ipomea batatas*) as well as in the arrowroot (*Sagitaria sagitofolia*) (Xavier-Filho *et al.*, 1989). Corms of several species of the aroids (*Alocasia, Colocasia* and *Cytosperma*) contain TIs of the Kunitz family (Hammer *et al.*, 1989).

B. Leaves and Fruits

Trypsin inhibitory activity has been detected in leaves of several plants of the legume family such as soybean, alfalfa and Ladino clover (Kendall 1951). Alfalfa leaves have also shown to synthesize a TI; the inhibitor was identified as a member of the Bowman-Birk family (Brown and Ryan, 1984). Win gate *et al.*, 1989 isolated and characterized TI from unripe fruits of *Lycopersicon esculentum* that help in the protection of these organs against herbivory (Filho, 1992).

MODE OF ACTION

To colonize host plant tissue, microbial pathogens rely on a set of hydrolytic enzymes that are coordinately expressed during the course of infection. The specific involvement of proteinases in these processes is difficult to evaluate, since the relative importance of proteolytic activity in pathogenicity may be highly variable and when required, proteinase activity may be involved at specific steps of the infection cycle. Bacterial and fungal pathogens secrete proteinases that are generally not individually required for growth on synthetic media, but contribute to their aggressiveness when colonizing plant tissues. These inhibitors play a decisive role in the regulation of protein metabolism. This is supported by the observations made by Haynes and Feeney, 1967 that the inhibitor concentration is relatively high in young and growing tissues but low in the older tissue. The well characterized soybean KTI and BBIs were found to inhibit proteinases from nonpathogenic fungi and bacteria, in addition of being active on trypsin, chymotrypsin, and elastase (Marchetti *et al.*, 1995).

Besides regulating the hydrolysis of proteins inside the cell, they participate in defense mechanisms of plants against herbivore insects and/or pathogens. For example, in interactions between plants and fungi, during the penetration and colonization processes, extra cellular proteases of a pathogen promote the hydrolysis of proteins of the cell wall. Thus, protease inhibitors of the host plant act directly against these enzymes, delaying the proteolysis of cell walls and membranes, and reducing the cellular disorganization (Ryan, 1990).

Protease inhibitors are polypeptides present in many plants, which in addition to their physiological function provide a natural defense against insect attack (Malehorn *et al.*, 1994). PIs are induced as components of many defense cascades under various stress prone conditions such as insect attack, mechanical wounding, pathogen attack and UV exposure. PIs inhibit the gut proteinases of the insect that adversely affects the protein digestion in the gut and force the insect to synthesize alternative proteases to compensate for the inhibited activity. This leads to deficiency of essential aminoacids and exerts physiological stress on the insects leading to growth retardation. This mechanism of action minimizes the possibility of developing resistance in the insects and reduces crop damages.

The defensive action of TI gene was first demonstrated by Hilder *et al.*, 1987 by transferring TI gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of insect pests including Lepidopterans such as *Heliothis* and *Spodotera*, coleopterans such as *Diabrotica, Anthonomnous* and *Orthoptera* such as Locusts. Many insects, particularly Lepidopteras, depend on serine proteases (trypsin, chymotrypsin and elastase endo protease) as their primary protein digestive enzymes. Disruption of aminoacid metabolism by inhibition of protein digestion has been a key target for use in insect control. SBTI has been reported to be inhibitory to the brown plant hopper in transgenic rice plants (Lee *et al.*, 1999).

Trypsin inhibitor belong to a common protein super family and like elastase, chymotrypsin are responsible for the initial digestion of proteins in the gut of most higher animals (Garcia Olmedo *et al.*, 1987). *In vivo* they are used to cleave long essentially intact polypeptide chains into short peptides, which are then acted upon by exopeptidases to generate amino acids, the end products of protein digestion. The enzyme reacts with the inhibitor molecule as if it were a proper substrate. Instead of dissociation of the cleaved molecule, the partners remain bound in a strong covalent complex through the amino acid residue at the N-terminal side of a so-called reactive site.

The mechanism of binding of the plant protease inhibitors to the insect proteases appears to be similar with all the four classes of inhibitors. The inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant (10^7 to 10^{14} M at neutral pH values) thus effectively blocking the active site. A binding loop on the inhibitor usually "locked into conformation" by a disulphide bond, projects from the surface of the molecule and contains a peptide bond (reactive site) cleavable by the enzyme (Walker *et al.*, 1998). This peptide bond may be cleaved in the enzyme inhibitor complex, but cleavage does not affect the interaction, so that a hydrolyzed inhibitor molecule is bound similar to an unhydrolyzed one. The inhibitor thus directly mimics a normal substrate for the enzyme; but does not allow the normal enzyme mechanism of peptide cleavage to proceed to completion i.e. dissociation of the product (Walker *et al.*, 1998). All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with similar standard mechanism (Laskowski and Kato 1980).

CURRENT STUDIES ON TRYPSIN INHIBITOR AS DEFENSE PROTEINS

World wide crop losses without the use of pesticides and other non-chemical control strategies is estimated to be about 70% of crop production; the world wide pre-harvest losses due to insect pests, despite, the use of insecticides is 15% of total production representing over US $100 billion (Krattiger, 1997). This problem is more acute in tropics and subtropics, where the climate provides conducive environment for a wide range of insects and necessitates massive efforts to suppress the population densities of different pests in order to achieve an adequate supply of food crop losses by the different diseases caused by the pathogens.

PIs are presently a potential tool for crop improvement targeting plant protection and human nutrition. Utilizing trypsin inhibitor gene(s) of plant origin as bio-insecticide for developing transgenic crop plant has been an accepted line of approach in crop biotechnological program. It has been reported that transgenic plants with suitable trypsin inhibitor gene can resist insect (Johnston *et al.*, 1989, Xu *et al.*, 1996).

Lingling and co-workers (2005) transferred cowpea trypsin inhibitor gene into cauliflower by *Agrobacterium*-mediated transformation method and produced 14 transgenic cauliflower plants. Ceci *et al.*, 1995 reported several serine proteinase inhibitors in *Brassica napus*. Out of the three distinct TIs, one (rapeseed trypsin inhibitor-RTI) shows identical properties to the MTI-2 [MTI-mustard trypsin inhibitor] protein, including a high amino acid sequence similarity of about 70%. The RTI protein being more active than MTI-2 has been successfully transferred to tobacco and *A. thaliana* plants (De Leo *et al.*, 1998)

Proteinase inhibitor gene from a native cowpea has been isolated and cloned into a λ ZAP II genomic library. The genomic clone shared 86% homology with a cowpea trypsin inhibitor of IV mRNA and 81% with Bowman-Birk protease inhibitor genes of soybean. Further studies showed, that the cowpea protease inhibitor has a signal peptide cleavage site between 69-70 amino acid residues i.e. TTA-AM, equivalent to alfalfa trypsin inhibitor (ATI). This signal peptide (44-55 amino acid residues) target the ATI to the vacuole of the cell (Mc Gurl *et al.*, 1995).

With the advent of genetic transformation techniques, it has become possible to clone and insert genes into the crop plants to confer resistance to insect pests. Resistance to insects has been demonstrated in transgenic plants expressing genes for δ-endotoxin from *Bacillus thuringiensis* (Bt), protease inhibitors, enzymes and plant lectins. Most of the plant derived genes produce chronic rather toxic effects and some insect pests are not sensitive to some of these factors. Genes conferring resistance to insects have been inserted into crop plants such as maize, cotton, potato, tobacco, rice, broccoli, lettuce, walnuts, apples, alfalfa and soybean. Many insects, particularly lepidoptera depend on serine proteases (trypsin, chymotrypsin and elastase endoproteases) as their primary protein digestive enzymes. Genes encoding members of various serine protease inhibitors (SPIs) have been cloned and introduced into crops, which are involved in regulating the digestive enzymes of the insects/herbivores.

Transgenic tobacco has also been shown to enhance protection against *Spodoptera littoralis* and *M. sexta*. Similarly, TI gene containing sweet potato cultivar "Tainong 57" and tobacoo cultivar "W38" retarded the larval growth of *S. litura* as compared to control plants (Yeh *et al.*, 1997).

Deployment of protease inhibitors for insect control requires a detailed analysis of the particular crop insect interactions. The range of dissociation constants (K_d) for different PIs with specific proteases is large and can be used in selecting the most effective inhibitor for gene transfer in a particular situation, e.g. transgenic tobacco expressing high levels of Kunitz type of TI from soybean (SBTI) performs better than the tobacco plants expressing (cowpea trypsin inhibitor) CpTI against *H. virescens*. Proteolysis by gut extracts is 40 fold more susceptible to inhibition by SBTI than the CpTI (Gatehouse *et al.*, 1993). However, CpTI is considered to be more useful for transfer, because it is not deleterious to mammals (Puzstai *et. al.*, 1992). As many SPIs are toxic to beneficial insects such as honey bees (Malone *et al.*, 1995; Burgers *et al.*, 1996). CpTI containing transgenic cotton lines have been found to be highly resistant to cotton bollworm (Li *et al.*, 1998). Similarly it has been observed that the expression of potato TI gene in rice confers resistance to insects (Duan *et al.*, 1996). A gene encoding a CpTI in transgenic rice significantly increased resistance to *C. suppressalis* and *Sesamia inferens* thus suggesting the usefulness of CPTI in controlling insect pests (Xu *et al.*, 1996). CpTI transformed *Brassica oleracea* var. *capitata* cultivars 'Yingchun' and 'Jingfeng' showed resistance to *P. rapae* in laboratory tests (Fang *et al.*, 1997).

Sasikiran and coworkers (2002) have studied changes in expression of trypsin, chymotrypsin and -amylase inhibitors in sweet potato during growth phase, sprouting and wounding. They observed a steep increase in TI activity in the tuberous roots of sweet potato during the tuber-bulking phase indicating the possible role of the inhibitor in regulating the activity of plant proteases. Similar effects with trypsin and chymotrypsin inhibitor content was reported in the endosperm of sorghum, cereals and legumes seeds (Mulimani *et al.*, 2002). It was found that TI activity decrease during emergence of sprouts. Zhang and Corke (2001) carried out similar studies in the vegetative tissues of sweet potato. Lin and Tsai (1992) reported that during water deficiency there is an increase in biosynthesis of TI by exogenous plant regulators. Hou and Lin (1997) reported the existence of TI as complex with polyamines and it played a vital regulatory role in sweet potato. Various researchers all over the world have found that wounding also lead to accumulation of proteinase inhibitor. Artificial wounding by heat or even crushing with a file mimic the action of an insect bite and stimulate the production of the inhibitor (Hilder *et al.*, 1987).

In the literature, as far is known, there is only one report of a legume gene that encode a Kunitz-type protease inhibitor produced during nodulation (Manen *et al.*, 1991). Lievens *et al.*, 2004 has identified a novel marker which encode a protease inhibitor (SrPI 1) in *Sesbania rostrata*. It was found that its expression is enhanced during nodulation and was not up regulated by wounding or upon infection with wide host range pathogens. These results showed that SrPI 1 has a N-terminal signal peptide of the soybean trypsin inhibitor (Kunitz) family of protease inhibitors (Richardson, 1991). It has been proposed that SrPI 1 has the property to inhibit plant protease that facilitates bacterial invasion in *S. rostrota* tissues or cells. Further studies by Goormachtig and coworkers (1999) shows that Srchi 13 encodes a class III chitinase that play a role in controlling the spread of bacteria and their Nod factors. Similarly, Srchr 1 encodes an early nodulin that is similar to chalcone reductase. Studies showed that this gene is expressed in the uninfected cells of the central tissue where it is involved in the synthesis of antibiotic phytoalexins that prevent bacterial entry.

The efficacy of PIs was studied on various plants like chickpea (*Cicer arietinun*), pigeon pea (*Cajanus cajan*), cotton (*Gossypium arboreum*), groundnut (*Arachis hypogaea*), winged bean (*Psophocarpus tetragonolobus*) and potato (*Solanum tuberosum*). Harsulkar and coworkers (1999) reported retardation in the growth of *Helicoverpa armigera*, a devastating pest of important crop plants that claims a major share in crop losses every year. The work evaluated PIs potential against *H. armigera* gut proteinase (HGP) through a series of *in-vitro* and *in-vivo* experiments. Specific inhibitors of insect gut proteinases were also identified.

The studies performed by Harsulkar and his coworkers revealed a number of isoforms of HGP differing in intensity and mobility. It indicated a dichotomy between the TIs and HGPIs, as not all of the trypsin PIs, inhibit HGP, although most of the HGP activity is trypsin like (Johnston *et al.*, 1991; Harsulkar *et al.*, 1998). Proteinaceous inhibitors of proteinases are constitutively expressed at high levels in storage organs and seeds; whereas others are induced in leaves by wounding. Mechanisms involved in wound inducibility of PIs in leaves of tomato and potato are well-documented (Pena-Cortes *et al.*, 1995). Reports by Schaller and Ryan (1995) showed an induction of PI in species of Solanaceae, Fabaceae, Cucurbitaceae, Poaceae. Work carried out by Giri and his coworkers (1998) showed that synthesis of PI in chickpea begins at 24 d after flowering and are accumulated until seed matures i.e. 60 d after flowering. PI is produced at early stages of germination, prior to the accumulation of storage proteins. *In-vivo* studies indicated that the larvae degraded inhibitors efficiently thus determining the fate of chickpea PI in *H. armigera*.

Various strategies have been cantered for improving plant defenses to plant parasitic nematodes by using PIs. Cysteine proteinase inhibitor (termed as cystatin) and cowpea trypsin inhibitor are used in defense mechanism as they represent the predominant proteinase activity in plant parasitic nematodes. Urwin *et al.*, 2000 studied the role of proteinase inhibitor genes as transgenes to control *Rotylenchulus reniformis*. The engineered cystatin Oc-IDD86 expressed ectopically in transgenic plants showing considerable effect by reducing parasite density and fecundity. Similar results for cowpea trypsin inhibitor were obtained except no reduction in fecundity was observed.

Due to physiological independence of herbivores from their host plants, plants respond to herbivore attack with defense responses that decrease herbivore performance, not only in attacked tissues but also systemically in plant parts of high fitness value which the herbivore might relocate. Moreover, plants attract predators to the attacking herbivores with systemically released volatile signals and hence the relevant spatial scale for most plant-herbivore interactions is minimally whole plant and frequently extends beyond the plant to include components of a plant community (Kessler & Baldwin, 2002).

Wound induced PIs have been shown to enhance plants' resistance to insects by inhibiting the proteolytic enzymes of the attacking insect. Recent work by Tamayo *et al.*, 2000 in maize describes a herbivore-induced PI that inhibits both elastase and chymotrypsin in the midgut of *Spodoptera littoralis* larvae. The defensive action of PIs mainly depend on their affinity and specificity for the midgut proteinases of the attacking insects and in the ability of the insect in altering proteinase and over expressing proteinases. As PIs and other defensive proteins are direct gene products, their defensive effects have been tested by genetic transformation in a number of plant species as illustrated above. Recently *A. thaliana* cysteine proteinase inhibitor gene (*Atcys*) was introduced in white poplar, which conferred resistance to *Chrysomela populi*, by inhibiting most of the digestive proteinase activity (Delledonne *et al.*, 2001). Studies have also shown that introduction of a PI gene in host DNA and its expression in positive (conferring resistance) way depends on the counter action of insects. TI is being used for developing insect resistant transgenic plants as listed in Table 1.

TABLE 1

Transgenic Plants for Enhanced Resistance Against Predators by Expression of Trypsin Inhibitor

Gene source	Transformed plant	Predators
Cowpea trypsin Inhibitor (CpTI)	Tobacco	*Manduca sexta*
	Tobacco	*Spodoptera litura*
	Rice	*Sesamia inferens,* *Chilo suppressalis*
	Potato	*Lacnobiaoleracea*
	Apple	Coleoptera,Lepidoptera
	Lettuce, Tomato	Coleoptera,Lepidoptera
	Sunflower	Coleoptera,Lepidoptera
	Sweet potato	Coleoptera,Lepidoptera
Sweet potato TI	Tobacco	*Chrysodeixis criosoma*
	Tobacco	*Spodoptera litura*
Barley TI	Wheat	*Sitophilus zeamatis*
Mustard TI	*Arabidopsis*, Tobacco	Lepidoptera
Soybean Kunitz inhibitor	Rice, Potato, Tobacco	*Nilaparvata lugens* Lepidoptera

PERSPECTIVE AND FUTURE DIRECTIONS

Much of the foundation for the future successful manipulation of plants by genetic engineering is now in place. Concerns have been expressed about the potential adverse ecological effects of their release and widespread cultivation. In the case of plants expressing pesticidal genes, these concerns have focused on the impact of the crops on non-target organisms. Inhibitors of all four dominant classes of protease (serine, cysteine, aspartic, and metalloproteases) occur in plants. They often accumulate in tissues in response to wounding or herbivory and are an important element of natural plant defense strategies (Ryan, 1990).

Ingestion of serine PIs affect lepidopteran species whilst cysteine PIs, also referred to as cystatins affect members of coleopteran families, as well as some Homoptera. The expression of PIs in GM plants as an insect defense has been widely investigated and there is evidence that PIs represent a broad based resistance strategy for control of plant-parasitic nematodes. The technology for developing insect resistant transgenic plants is expanding very rapidly. Transgenic crops expressing PIs are going to play an important role in future. In the studies mentioned above, the efficiency of the expression of introduced PI gene was found to be sufficient to impart resistance to the challenged insects. Recent field trials carried out in the US showed that the expression of CpTI in tobacco provided significant protection in the field against *Helicoverpa zea* (Hoffmann *et al.*, 1992).

Plants respond to herbivore attack with a bewildering array of responses, thus the major emphasis should be given in understanding the interaction of plant with pathogen or plant with pest and *vice versa*. By integration, the understanding with the techniques of molecular biology researchers should be able to increase the wide range of methods available for the control of insect pests. Thus, in future genetically engineered insect-resistant crops could make a major contribution to the development of resistant varieties.

Many studies are in progress to identify new strategies, which could increase the efficiency of TI or PI defense mechanism. Understanding protein-protein interactions and new techniques like phage display could open new frontiers in the use of TI for insect or pathogen resistance.

CONCLUSION

Reliable and efficient bioassay systems with better understanding of plant-pathogen interaction or plant-insect herbivory will be needed to aid the use of TI as defense mechanism. It can be anticipated that useful information emerging from global genomics efforts in crop species, including model legumes will help in improving plants through genetic engineering. Our knowledge of TI and other related PIs occurring in plants has expanded significantly in the last ten years. With the advent of biological measures used to combat diseases caused by pathogens or crop losses caused by pests the use of non-chemicals in agricultural field has increased. Based on this knowledge, several strategies have emerged for developing crop varieties resistant to pathogens and insects. The manipulation of host plant genome by introducing TI gene led to resistance in transgenic plant. Several studies carried out in wheat, rice, soybean, tobacco and barley has shown a protective effect against insects.

The current biotechnology-based approach of insect pest management is to transfer selected genes that condition resistance to target insect pests from one species of plant to another. Constitutive expression of single defensive molecules imposes increased selection pressure, and as a result, can lead to rapid development of new insect biotypes that are insensitive to the transgenic products. The use of TI may enhance the resistance durability of transgenic plants. A good understanding of the natural defense regulation mechanisms would facilitate pest control in modern agriculture. The coming years will undoubtedly witness an increasing application of TI or other PIs for the genetic improvement which would in turn help in achieving higher insecticidal and pathogen resistance activity in plants.

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11

GENETICS OF *ASPERGILLUS NIDULANS*

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INTRODUCTION

Many reviews on the genetics of ***Aspergillus nidulans*** have been published, viz., Pontecorvo *et al.* (1953), Pontecorvo and Käfer (1958), Käfer (1977), Käfer *et.al.* (1982), Roper (1966, 1971), Clutterbuck (1974, 1980, 1994), Clutterbuck & Arst (1995), Birkett & Roper (1977), Sinha & Tiwari (1985), Moustacchi, *et.al.* (1986), Sinha & Jha (1985), Jha and Jha (1991^a), Arst (1994), Crebelli *et al.* (1995, 2002), Bos (1996), Osmani & Mirabito (2004), Goldman & Käfer (2004), Benigni *et al.* (2004), Scazzocchio (2006) and Becker & de Castro-Prado (2007). Goldman & Käfer (2004) recommended *A. nidulans* as a model system to characterize the DNA damage response mainly with respect to mitotic recombination, inducible responses to detrimental environmental changes and genetic control of the cell-cycle.

In this intensive and extensive review, primarily intended to elucidate the manipulation and exploitation of this experimental organism as a genetic test-system has been dealt with exhaustively. There are various test-systems for the assay and detection of different kinds of genetic end-points. Every test-system has unique inherent properties that make them fit for the study of a particular genetic damage, e.g., micronuclei test in *Allium cepa* (Scott *et al., 1982),* gene conversion in *Saccharomyces cerevisae*(Fogel *et al., 1983),* mitotic crossing over in *A. nidulans* (Crebelli *et al.*, 2002), recombination analysis in *Neurospora crassa* (Suzuki *et. al*, 2005), study of recombinant DNA in *Escherichia coli* (Primerose *et al.*, 2006*)*, resistant mutants in *Botrytis cineria* (Ziogas & Kalamarakis, 2001), intragenic suppressors in *Ascobolus immersus* (Decaris *et al.*, 1978), intragenic complementation in *Cryptococcus neoformans* (Kraus *et al.*, 2005), intragenic pseudorevertants in *Chlamydomonas reinhardtii* (James & Lefebvre, 1992).

Almost all the test systems are directly or indirectly exposed to the environmental factors, i.e., physical or chemical. These factors may have direct or indirect impact on the genetic material and may or may not manifest it phenotypically. The test-system may display genetical alteration either in one stroke or may have cumulative effect. Whatever be the mechanism of the mode of action of the factor, the primary purpose is to find out the ultimate induced genetical changes. Equally important is the fact that the environmental factors are undergoing changes in quantity and quality continuously as a result of natural variation or man made activities. A large number of novel chemicals, eg., insecticides, pesticides, rodenticides, herbicides, antibiotics, xenobiotics, synthetics and polymers are being synthesized or are found naturally. In addition, the reckless use of these chemicals without an exhaustive test of the genetic damage being caused by them, and release to the environment without meticulous and sensitive test, makes the biosphere prone to genetical alteration, including human being (Sinha and Jha, 1986[b]). Therefore, it is important to spot the variant genetic abnormalities in an apt test-system or in a group of test-systems to validate the use of any new chemical.

A. nidulans is preferred and widely used as a genetic test-system, as it appears to be a well tested, tried and suitable experimental organism for the exploration of a wide range of genetic variations including chromosomal abnormalities (Käfer, 1965[a,b]). Therefore, the advantages, peculiarities and the superiorities of *A. nidulans* as a test-system are to be outlined. Broadly speaking, these may be categorized into morphological, genetical and its advantages in culture. The inherent attributes of *A. nidulans* from genetical and biological point of views, make it a suitable test-system for the in-depth study of the detection of various kinds of genetic damages. Mainly, there are three kinds of coloured condia in *A. nidulans* (i.e., white, green and yellow) and these may form points, patches or sectors of the respective colonies. The visual inspection either by the naked eye or microscopic examination by the stereo-microscope leads to rapid visualization of the putative genetic variation. The advantages and disadvantages of *A. nidulans* are discussed in brief hereunder.

MORPHOLOGICAL ADVANTAGES

The conidia produced by asexual cycle in *A. nidulans* are darkly coloured. The spotting of the coloured sectors, suggestive of some kind of genetic damage leads to quick detection of the anomaly. It grows fastly at 37°C. It has a compact, radial, colonial growth facilitating visual inspection of the colonial morphology and a large number of conidia is examined on a single plate. *A. nidulans* forms heterokaryon between nuclei of different genotypic constitution in the same cytoplasm. Thus the study of the interaction between the two different nuclei in the same cell (i.e., in the heterokaryon) is carried out. Thus the genetical interaction between the two nuclei or consequence of fusion of the two nuclei is analysed easily.

GENETICAL ADVANTAGES

A. nidulans has unique features for the detection of cryptic or large hereditary alterations. Hence experimental organism is manipulated for the study of a wide range of genetic phenomena, e.g., different kinds of mutations, somatic recombinations, and chromosomal aberrations. For the detection of the mitotic recombination and mutation caused by the environmental mutagens, various well-marked strains of *A. nidulans* have been introduced (Käfer, *et al.*, 1976, 1982; Käfer, 1984).

The detection and visualization of complicated chromosomal variations like reciprocal and non-reciprocal translocations either in mitotic or meiotic test-systems become evident in *A. nidulans,* due to availability of the well marked haploid as well as diploid strains of *A. nidulans.*

ADVANTAGES IN CULTURE

It grows on specific synthetic medium over a wide range of temperature (25 to 42°C). The genetic map of *A. nidulans* is well known (Käfer, 1958; Clutterbuck, 1980, 1981, 1994; Clutterbuck & Arst, 1995). A list of mutants isolated so far has already been published by Clutterbuck with gene symbols and genotypes of the respective strains. Thus stable heterozygous diploids of the desired combination are obtained conveniently with a range of phenotypes of choice. The design of experiment is manipulated extensively and intensively depending upon the purpose of investigation.

CYTOLOGICAL DISADVANTAGE

A. nidulans is not amenable to cytological analysis (Robinow & Caten, 1969). The cytological abnormalities like chromosomal aberrations, mitotic and meiotic crossing-overs, sister chromatid exchanges, clastogenicities and other small or large chromosomal variations cannot be studied conveniently and distinctly in *A. nidulans.* The inability is ascribed to its small size of the chromosome, i.e., less than 0.2 μ. As the resolving power of light microscope is 0.2μ, the spontaneous and induced changes in the chromosomes cannot be probed decisively by cytological techniques because of ambiguous and indistinct chromosomal configurations. Therefore *A. nidulans* is exploited genetically.

1. MITOTIC CROSSING-OVER

Introduction

The essence of sexuality is genetic recombination. The production of genetic recombinants leads to genetic diversity. The consequent selection and fixation cause evolution of new strains. Stern (1936) found a parallel of meiotic recombination in *Drosophila melanogaster* in mitosis and the phenomenon was designated as mitotic crossing-over. The pairing and genetic exchange of the chromatinic segments occur during ordinary somatic mitosis. Later on, this event was also observed in *A. nidulans* by Pontecorvo *et al.* (1954) and elaborated in detail by Pontecorvo and Käfer (1958). The two propositions were advanced to account for the genetic process, i.e., somatic mutation or somatic recombination (Käfer, 1958). But the experimental data in case of *Drosophila melanogaster* as well as *A. nidulans* suggested the latter hypothesis and it also explained the incidence of "twin-spots" (Käfer, 1960, 1961). The emergence of "twin-spots" would require the occurrence of simultaneous mutations of the two loci of adjacent cell, whereas a single crossing-over in the appropriate region would explain the origin of "twin-spots", i.e., yellow, white and green sectors in the adjacent region. The experimental observations corroborated the latter mechanism.

Distinction with Meiotic Crossing-over

The main points of distinction between mitotic and meiotic crossing-over is with respect to the frequency. Unlike meiotic crossing-over, the frequency of mitotic crossing-over is very low. The overall incidence of the mitotic crossing-over in *A. nidulans* is nearly 2% taking into consideration at least 14 chromosomal arms in a haploid organism and assuming equal frequency of both the types of centromere. In another word, one in every 50 mitotic divisions shows such an event (Käfer, 1961). The spontaneous reciprocal crossing-over producing the "twin-sectors" occurs with the frequency of about 1 in 10,000 (Wood & Käfer, 1967; Shanfield & Käfer, 1971).

"Twin-spots"

When crossing-over occurs during mitosis between non-sister strands in the appropriate region, small or large variant colored sectors are produced, i.e., yellow and white or green. These are known as "twin-spots". According to the mutation theory, such "twin-spots" would demand simultaneous mutation at the two loci in adjacent cells, while according to recombination theory these could be explained by the occurrence of crossing-over in the appropriate gene centromeric region. The relative high frequency of "twin-spots" even greater than that for single yellow spots, therefore indicates a recombinational explanation.

Selection

The selection of somatic recombinants is critical, as the frequency of mitotic crossing-over is very low. Therefore, recovery requires ingenuous design of experiment. Previously selection was done for various kinds of markers, e.g., visual markers, nutritional markers and resistance markers and for multiple auxotrophy accompanied with starvation. Selection can also be done for the cis-heterozygote from a trans-heterozygote. The selection of markers plays a crucial role in the selection of somatic recombinants from the test-system. The homozygosis occurs in all the markers distal to the point of crossing-over and the recessive traits are expressed, facilitating quick detection of the point of exchange and the phenomenon of recombination.

Diploids of *A. nidulans* are genetically unstable and produce very infrequently mitotic recombinants in the form of fan shaped sectors or "twin-spots". This event is usually confined to the recombination of markers in a single chromosome arm but in case of *A. nidulans* there are 14 long chromosome arms. Hence, a test-system could be synthesized having suitable markers on all the possible arms for the study of recombinational events. Very close to this proposition (Käfer *et al.*, 1976) synthesized a diploid aptly suited for the exploration of this phenomenon.

Cytological Explanation

Mitotic crossing-over like meiotic ones occurs at the four strand stage during mitosis. If the chromatinic exchange takes place in a suitably synthesized diploid heterozygous strain, having the recessive markers in repulsion, the homozygosis for the markers distal to the point of exchange ensues and results into the appearance of corresponding phenotypic sectors. In contrast, markers proximal to the centromere, i.e., markers before the point of exchange, as well as the markers on the other arm will remain unaffected. The two parental or two non-recombinant strands and two non-parental or two recombinant strands are formed. Out of these four, if one parental and one recombinant strands go to one pole giving rise to the two daughter nuclei, each one of which is homozygous, one for the dominant and another for the recessive ones. Movement of both the recombinant strands to one pole or parental strands to another pole cause heterozygosity in each nucleus and do not manifest genetic diversity. The mitotic crossing-over reflects the sequence of genes or order of genes in the region showing mitotic recombination. The maximum frequency of mitotic crossing-over is found in the region adjacent to the centromere. Thus mitotic maps are easily prepared on the basis of the frequencies of mitotic crossing-over.

Cell-cycle

Cell-cycle regulation has a profound impact on mitotic recombination (Osmani and Mirabito, 2004) in *A. nidulans*. Morris (1976[a,b]) specifically, targeted at the nuclear division and did a pioneering research with respect to the understanding of the regulation of mitosis. Quantum-leaps were made from biochemical analysis (Felden *et al.*, 1976; Sheir-Neiss *et al.*, 1978) of the genetic systems of *A. nidulans*. Many biological mutants,. e.g., bim (blocked in mitosis), nim (never in mitosis), sep (septation mutants), nud mutants (distribution of nuclei within the cell) and their respective genes were identified by different groups of scientists (Osmani & Ye, 1996, 1997; Harris, 2001; Morris, 2000). Many of the mutants were found to be readily reversible (Oakley & Morris, 1981, 1983; Bergen & Morris, 1983; Bergen *et al.*, 1984). The gene encoding protein kinase plays a key role\ in regulation (Osmani *et al.*, 1994). The arrest of cell-cycle at G_1, S and G_2 was studied by Bergen *et al.*, 1984, Cdc_2 protein kinase or cyclin dependent kinase was isolated in *A. nidulans* (Osmani *et al.*, 1994).

Mechanism of Crossing-over

The physical, chemical and biological agents induce crossing-over in the diploid either by inhibiting or by interfering with nucleic acid synthesis, e.g., actinomycin D, Sulfadrugs and bleomycin or binding or cross-linking to DNA molecules, e.g., nitrogen mustard or by chromosomal breaks, e.g., by various kinds of irradiation or by DNA single and double strand breaks and abnormal DNA protein cross-links.

These damages are caused by either intrinsic or extrinsic causes. If left unrepaired, DNA damage can result in cell-cycle arrest, cell death (Goldman & Käfer, 2004), if repaired incorrectly leads to crossing-over. Incorrectly or completely unrepaired DNA facilitates chromatinic exchange and ultimately manifests the crossing-over. The role of DNA replication, gene transcription, DNA repair and cell-cycle check-points must co-ordinate with each other to promote survival of normal cellular function, following DNA damage (Levitt & Hickson, 2002). The genetic control of the cell-cycle and its relation to mitotic-recombination has been reported by Käfer & May (1997, 1998). The role of nuclear event and regulatory check-points have been emphasized (Morris, 1976[a,b]; Morris and Enos, 1992; Morris *et al.*, 1998). They have dealt with effects of blocks in the cell-cycle progression, e.g., mutants blocked in mitosis or in the formation of condensed chromosome or in the appearance of mitotic-spindle apparatus. All these events have also played a major role in DNA recombination. Nucleotide excision repair (Goldman *et al.*, 2002), any other genes (Griffith & Carr, 1998; Goldman *et al.*, 2002) inactivate gene in DNA repair leading to mitotic crossing-over. Many mutants defective in DNA repair have been detected *A. nidulans.*

Amin *et al.* (2003) and Cheng *et al.* (2003), found apoptosis like effect in *A. fumigatus.* These events were induced by dihydrosphingosin and phytoshingosin, inducers of apoptosis. In transit, a few cells which did not undergo apoptosis and showed large scale DNA fragmentation, and having least genetic load underwent recombination. Mainly the three kinds of agents induced mitotic crossing-over in *A. nidulans.* The agents and their respective references are tabulated below:

TABLE 1

The Intergenic Mitotic Crossing-over (MCO) Inducing Factors

Inducing agents	References
(A) Physical factors	
(i) Ultra-Violet (UV) light	Fratello *et al.* (1960); Kwiatowski (1962); Kwiatowski & Grad (1965); Wood & Käfer (1967, 1969); Käfer (1969); Shanfield & Käfer (1969); Jansen (1972); Osman *et al.* (1993); Goldman & Käfer (2004)
(ii) X-ray	Morpurgo (1962); Goldman & Käfer (2004)
(iii) _-irradiation	Kwiatoski (1962); Käfer (1963^{b})
(iv) Decay of 32p	Strigini (1962); Strigini *et al.* (1963)
(B) Chemical factors	
(i) Antibiotics (Bleomycin)	Demopoulos *et al.* (1982); Käfer (1990)
(ii) Alkylating agents	
(a) Diepoxybutane	Morpurgo (1963); Putrament (1966)
(b) Methyl-bis-chloro ethylamine	Morpurgo (1963)
(c) Methyl methane sulfonate (MMS)	Bertoldi & Griselli (1980); Käfer and Mayor (1986); Kress Fagundes *et al.* (2003)
(d) N-methyl-N'-nitrosoguanidine (MMNG)	Swirsky *et al.* (1988); Osman *et al.* (1993); Käfer and May (1998)
(iii) Chelating agents	Kwiatowski (1965)

Contd...

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Inducing agents	References
(iv) Fluorodeoxyuridine and fluorouracil	Beccari *et al.* (1967); Bandierra *et al.* (1970)
(v) Flurodeoxy uridine (FUDR) and nitrosoguanidine	Shanfield & Käfer (1971)
(vi) Formaldehyde	Fratallo *et al.* (1960)
(vii) Fungicides	
(a) Actinomycin D	Kappas (1978)
(b) Botran	Kappas (1978); Käfer (1990)
(c) Chloroneb	Kappas (1978)
(viii) Pesticides	
(a) Aminotriazole	
(b) Dichlorovos	Bignami *et al.* (1977)
(ix) Pharmaceutical drugs belonging to sulpha group	Bignami *et al.* (1977)
(a) Sulfalene	Bignami *et al.* (1974); Aulcino *et al.* ($1975^{a,b}$)
(b) Sulfamethiozole	Bignami *et al.* (1974); Aulcino *et al.* ($1975^{a,b}$)
(c) Sulfanilamine	Bignami *et al.* (1974); Aulcino *et al.* ($1975^{a,b}$)
(x) Other Pharmaceutical drugs	
(a) Econazole	Crebeli *et al.* (2002)
(b) Hydroxyquinone	Crebeli *et al.* (2002)
(C) Genetical factors	
(i) bimD	Danison *et al.* (1992); van Heemst *et al.* (2001)
(ii) $bimD_6$	Holt & May (1996)
(iii) homologous and non homologous recombination (uvsC and uvsE)	Chu (1997)
(iv) mus81, nuclease	Hishida , (2001)
(v) musN mutants	Käfer & Chae (1994); Hofmann & Harris (2001)

Contd...

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Inducing agents	References
(vi) mre11	Semighini *et al.* (2003)
(vii) mis-match excision repair protein deficient	Eisen & Hanawalt (1999)
(viii) nimO	James *et al.* (1999)
(ix) nuv mutants	Osman *et al.* (1993)
(x) nimX2	Mc Guire *et al.* (2000)
(xi) non-removal of small alkyl groups	Baker *et al.* (1992); wood *et al.* (2001)
(xii) nucleotide excision repair deficient	Eisen & Hanawalt (1999)
(xiii) nudA1	Goldman & Morris (1995)
(xiv) Rad6	Goldman & Käfer (2004)
(xv) Rad51 and Nb31(scaA1)	van Heemst *et al* (1997); Bruischi *et al.* (2001)
(xvi) scaA	Bruischi *et al.* (2001)
(xvii) scsA1	Kress fagundes *et al.* (2003)
(xviii) uvsB	Jansen (1970^{a,b}); Shanfield & Käfer (1969)
(xix) uvsC	Jansen (1970^{a,b}); vanHeemst *et al* (1977); Käfer & Mayor (1986)
(xx) ufsl and uvsI	Han *et al.* (1998); Chae & Käfer (1993); Goldman *et al.* (2002)

Intragenic Crossing-over

The mitotic recombination within a gene or cistron is regarded as intragenic mitotic crossing-over. The intragenic recombination is observed during mitosis as well as meiosis. It is a non-reciprocal process producing either entirely non-reciprocal products or a mixture of non-reciprocal and reciprocal ones. In the former case, the term gene-conversion is applied to the event. In case of intracistronic mitotic crossing-over a strong interference is seen in the region undergoing the event. Thus there is a high degree of localized interference (Pritchard, 1954, 1960; Siddiqui, 1962^{a,b}). At the same time, polarity is also noticed (Siddiqui and Putrament, 1963).

The intragenic recombination has been investigated in detail in *A. nidulans* using a test-system, which is heteroallelic diploid for at least two mutations. The alleles chosen for study are adenine, i.e., ad (Putrament, 1964^{b}), para-aminobenzoic acid, i.e., paba alleles (Jansen, 1966; Putrament, 1964^{a}, 1965), and para-fluoro phenylalanine (fpa) resistance mutants (Beccari *et al.*, 1967, Morpurgo & Volterra, 1966[a,b], 1968; Petrelli & Ricci, 1966). The alteration in the structure of the cistron is known as a result of the occurrence of the event, which in many cases is unequal (Morpurgo & Volterra, 1966[a,b], 1968). The Two processes, i.e., mitotic and meiotic crossing-over have different mechanisms. The former is intragenic and the later is intergenic. The intragenic crossing over is never involved in the segregation of distal markers. The chemicals inducing the two different processes are different, corroborating the conclusion that the two processes do not have identical mechanism. For example FU (Fluorouracil) and FUDR (Fluorodeoxyuridine) increase the frequency of mitotic crossing-over but not of the meiotic crossing-over. Morpurgo & Volterra (1968) postulated that the intragenic recombination encompasses the physical chromatinic exchange between single DNA strands, followed by DNA damage repair in the locus involved in the process or near to it. The various factors causing intragenic crossing-over are listed in table 2.

TABLE 2

The MCO Inducing Agents (Intragenic)

Inducing factors	References
Physical factor	
Ultraviolet irradiation	Jansen, 1964; Jansen, 1965
Genetical factors	
(a) uvs B and uvs C	Jansen, 1967, 1970[a,b]
(b) uvs D and uvs E	Fortuin, 1971[a,b,c,d]; Jansen, 1969[a,b]
Nutritional factor	
Adenine or Para-aminobenzoic acid	Putrament, 1967[a,b]

2. MITOTIC NON-DISJUNCTION

The non-separation of the daughter centromere from the parental ones leads to mitotic non-disjunction. During mitotic anaphase, the centromere divides and the chromatids get detached from each other resulting into disjunction. If this process fails, the mitotic non-disjunction is caused (Sinha & Jha, 1983). Cytologically the two chromatids of the chromosome are not disjoined and shift towards the same pole. Genetically, the homozygosis appears not only on one arm of the chromosome like mitotic crossing-over but on both the arms of a linkage group (Sinha & Jha, 1986[a]).

The genotypic constitution of *A. nidulans* test system varies as per the requirement. The meticulous design of experiment for the detection of mitotic non-disjunction demands a heterozygous diploid strain with an appropriate number of markers on both the arms of a linkage group in order to differentiate it from the products of mitotic crossing-over, which displays homozygosity for a single marker or for a group of markers located on any one arm only. The agents known to induce mitotic non-disjunctions are listed in Table 3.

TABLE 3

The Agents Used in Inducing Mitotic Non-disjunction

Inducing agents	References
Physical agents	
(a) X-ray	Morpurgo, 1962
(b) UV-ray	Bertoldi and Griselli, 1980
(c) Ionizing radiations, e.g., electrons, X-rays and _-particles.	Normansell & Holt, 1979
Chemical agents	
(i) Actinomycin D	Kappas, 1978
(ii) Acridines	Ball & Roper, 1966; Upshall & Croft, 1967
(iii) Aniline	Kappas *et al.*, 1974
(iv) Aminotriazole and dichlorovos	Bignami *et al.*, 1977
(v) Benomyl	Bertoldi & Griselli, 1980; Bignami *et al.*, 1977; Kappas, 1981
(vi) Benzodiazepine (dipotassiumchlorazepate)	Kappas, 1978; Kappas *et al.*, 1974
(vii) Botran	Kappas, 1978
(viii) Chloral hydrate	Crebelli *et al.*, 2002; Sinha & Jha, 1983
(ix) Chloroneb	Kappas, 1978
(x) Formaline	Fratello *et al.*, 1960
(xi) FPA and MMS	Bertoldi & Griselli, 1980
(xii) Griseofulvin	Kappas & Georgopoulos, 1974; Sinha & Jha, 1986^a
(xiii) Hydroquinone	Crebelli *et al.*, 2002
(xiv) Pharmaceuticals:	
(a) quinolines (iodohydroxyquinoline and dichloro methyl hydroxyl quinoline).	Bignami *et al.*, 1974
(b) Pyrazolidines (sulfinopyrazone and hydroxyl phenyl butazone)	Bignami *et al.*, 1974
(xv) Thiabendazole	Crebelli *et al.*, 2002
(xvi) Thimerosal	Crebelli *et al.*, 2002

Contd...

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Inducing agents	References
(xvii) Various chemicals of different composition, viz., hydrocholorothiazide, phanquone, diethyl ammonium dihydroxy benzene sulfonate and N-nitrofurfurylidone aminohydantoin; benzimidazole fungicides, MBC and thiabendazole.	Bignami *et al.*, 1974

3. HAPLOIDISATION

The process of formation of haploids from the diploids of *A. nidulans* is known as haploidisation. Mitosis too leads to production of haploids but the mitotic haploidisation differs from meiotic haploidisation. The crossing-over is absent in mitotic haploidisation. In nature, the stable state of *A. nidulans* is the haploid ones. The vegetatively dividing diploid nuclei of *A. nidulans* are not perfectly stable and spontaneously (Jha & Sinha, 1990[a]) give rise to haploid sectors, at a very low frequency of 0.02% (Pontecorvo *et al.*, 1953). Haploidisation has been frequently exploited as a genetic tool for designating genes to their respective linkage-groups (Jha & Sinha, 1992[b]) and for the isolation and characterization of genetic processes like translocation (Käfer, 1962, 1963[a], 1965[a,b], 1975; Käfer & Chen, 1964; Käfer & Upshall, 1974) and genetic damages like recessive lethals (Azevedo, 1965) as well as for making isogenic strain and for knowing the phenotypes of the recessive traits.

Three mechanisms have been proposed for explaining the mechanism of haploidisation, i.e., selection or induction or a blend of selection and induction (Jha & Sinha, 1991). A haploidising chemical acting by means of selection, hinders the growth of the diploids, while favours the growth of haploids. In other words, the growth of the haploids is promoted and is kept at a selective advantage (Bonatelli & Azevedo, 1977). Consequently, preferentially the haploid colonies outgrow the diploid ones by deterring the growth of diploid colonies. Thus haploids have a competitive edge and outgrow the diploid ones and emerge in the form of fastly growing sectors. The ploidy level of *A. nidulans* has also been determined on the basis of differential effects of the chemical, methyl benzimidazole-2-yl-carbamate (MBC) on the haploid and diploid of *A. nidulans* colonies. The haploid colonies profoundly grow and diploid colonies are tiny ones. An inducing chemical does not make distinction between haploid and diploid colonies rather induce the production of haploid nuclei. The effects of chemical with respect to growth are identical. Thus in the presence of chemical, the two colonies look alike, but when transferred to complete medium without inducing chemical the segregating haploid sectors are visually identified and confirmed genetically. Singh and Sinha, (1979) found chloral hydrate to be an inducing chemical. The third kind of chemical has both the features of induction and selection, e.g., parafluoro-phenylalanine (Lhoas, 1961, 1967 1968). FPA interferes with spindle formation, tubulin assembly and microtubule arrangements, leading to disorganized migration of chromosomes to the two poles and ultimately induces the formation of haploids by mitotic non-disjunction. The chemical has additional property of selection, hence it inhibits the growth of diploid colonies and consequently the haploid ones are selected. The list of chemicals inducing haploidisation by various workers is mentioned in Table 4.

TABLE 4

Haploidisation Inducing Chemicals

Inducing chemicals	References
1. Arsenate	Van Arkel, 1963
2. Actinomycin D	Kappas, 1978
3. Aniline	Kappas *et al.*, 1974
4. Acridine yellow	Kinghorn & Pateman, 1975
5. Benlate	Hastie, 1970; Kappas, 1981; Kappas *et al.*, 1974
6. Botran	Kappas, 1978
7. Chloroneb	Azevedo & Santana, 1975
8. Chloral hydrate	Singh & Sinha, 1976, 1979, 1983, Sinha & Jha, 1983
9. Formalin	Fratello *et al.*, 1960
10. Griseofulvin	Kappas & Georgopoulos, 1974; Jha & Sinha, 1986^{a,b}
11. Nystatin	Ditchburn & Macdonald, 1971
12. n-glycosyl polifuigin	Bal *et al.*, 1975
13. Methyl benzimidazole-2-yl-Carbamate	Davidse, 1973; Kappas *et al.*, 1974
14. Methyl thiophanate and thioureido	Kappas *et al.*,1974
15. Thiabendazole and fungicide thiophanate	Kappas *et al.*, 1974
16. Para-fluorophenylalanine	Lhoas, 1961, 1967, 1968

4. ANEUPLOIDS

The spontaneous aneuploids are obtained either from asexual conidia having vegetatively recombining diploid nuclei (Käfer, 1961) or from conidial samples of haploid strains or from cleistothecia (Pritchard, 1954; Faulkner, 1967). The chemical induction of aneuploidy has been reviewed by Roper, 1971; Clutterbuck, 1974; Birkett & Roper, 1977; Scott & Käfer, 1982; Käfer, *et al.*, 1982; Käfer, 1984; Sinha & Jha, 1985; Bos, 1996; Crebelli; *et al.*, 2002; Benigni *et al.*, 2004). The biological factors leading to production of aneuploids are well documented in *A. nidulans* (Goldman & Käfer, 2004; Osmani & Mirabito, 2004). The chromosomal mis-segregation at anaphasic state in mitotic or meiotic test-system leads to origin of aneuploids (Jha & Sinha, 1992^{a}).

The colonies of aneuploids show morphological abnormality. These are anomalous, slow-growing, poorly conidiating and as a rule the unstable centres of the aneuploid colonies give rise to stable euploid sectors. For pinpointed diagnosis and spotting of different kinds of aneuploids, the test system must have markers situated on all the arms of various linkage groups (Jha & Sinha, 1992[a]). Such genotypes facilitate quick and correct identification (Käfer, 1960), by the technique called "sector analysis" (Käfer, 1961). This technique has been widely and exclusively used for pursuing the segregation of markers in the segregating euploids sectors.

The ratio of segregation of the wild type and recessive markers is 1:1 in the disomics and 2:1 in trisomics. The rule is only whole chromosome segregation. The trisomics have huge centre and rich conidiation (Jha & Sinha, 1990[b]). Four tests are mainly employed for deciding the ploidy level, i.e., the conidial diameter and the conidial volume, mycelial colour and segregation of markers. The sectors become darker and the size of conidial heads is more in case of trisomic. The spontaneous and induced segregation of markers is in harmony with "Benlate test" (Upshall, *et al.*, 1977). The colonial morphology of the aneuploids (i. e., disomics and trisomics) are specific for a particular linkage group and does not depend upon the genotype of the heterozygous diploids. All the eight possible disomics have distinct size of the center of the aneuploids (Jha, 1998[a]). The amount of condiation, the density and colour of the mycelial growth are typical. The phenotypic specificity of the aneuploids have nothing to do with the genetic background (Käfer, 1961; Pollard *et al.*, 1968; Upshall, 1971; Käfer & Upshall, 1973). They also extended the phenotypic specificity to Birmingham collection. The non-segregating disomic colonies in *A. nidulans* has been reported by Jha (1998[b]). The non-segregating disomic colonies (Jha, 1998[b]) and multiple trisomics (Jha, 1998[c]) have also been isolated, morphologically and genetically well characterised.

The frequency of aneuploids from Birmingham isolates, either from the cleistothecia or from the conidia ranges from. 0.1% to 2% (Käfer, 1961; Upshall, 1966, 1971; Pollard *et al.*, 1968; Shanfield & Käfer, 1971; Käfer & Upshall, 1974). In any case the frequency is perfectly low, allowing *A. nidulans* to be used as a test-system for the study of the effects of detrimental environmental factors. The consequent statistically significant increase in the frequency of the aneuploid colonies refers to induction.

INDUCTION OF ANEUPLOIDY

The maximum fidelity of replication, segregation and appropriate DNA damage repair response ensure the integrity of the genome (Osmani & Mirabito, 2004). Any deviation or lapse in the process/es may cause genomic instability (Käfer, 1988). DNA replication, gene transcription, DNA repair and cell-cycle checkpoints (Käfer & May, 1998), must all interlink properly to regulate DNA damage (Levitt & Hicksen, 2002). Griseofulvin (Crackower, 1972; Jha, 1985) and chloral hydrate (Mercer & Morris, 1975; Singh and Sinha, 1976; Sinha and Jha, 1983, 1986[a]), induce anomalous segregation of chromosomes (Sinha & Jha, 1983; Jha & Sinha, 1986). Sodium deoxycholate and many other chemicals (Bellincampi *et al.*, 1980; Morris, 1980) cause mitotic non-disjunction. Since the role of nuclear membrane is crucial in the orientation of polar-bodies during spindle formation, the disruption of the nuclear membrane leads to the disruption of the mitotic spindle apparatus and enhances chromosomal mis-segregation and finally generates aneuploids. The another kinds of chemicals, e.g., para-fluorophenylalanine (Morris & Oakley, 1979), Methyl-benzimidazole-2-yl-carbamate (MBC) (Davidse, 1973, 1975, Davidse & Flach, 1977) and thiabendazole (Davidse & Flach, 1978; Crebelli, *et al.*, 2002) directly interfere with polymerisation of tubulin, disturb microtubule's structure and function.

Azevedo & Santana (1975) and Kappas (1978) found breakage deletion of chromosomes in case of botran and chloroneb accountable for aneuploidy in *A. nidulans.* DNA replication, chromosome condensation, formation of mitotic spindle, separation of chromosomes at anaphase and inter alia interdependence were studied in detail by Enos & Morris, 1990 and Enos *et al.*, 1991 and they found linkage between the two processes, i.e., chromosome condensation and spindle formation in a coordinated way (Morris *et al.*, 1998). The simultaneous increase in mitotic index and spindle index referred to a common mechanism of control (Morris *et al.*, 1998). In addition, the cell defective in chromosome condensation and dysfunction in spindle assembly also reinforced the co-ordinated control.

The events in the upstream affect the downstream processes and chain of events do not follow biochemical pathway but exhibit the other regulatory concept (Osmani *et al.*, 1988[a,b]). The events of the cell-cycle are co-ordinated by check-point type regulation (Hartwell & Weinert, 1989). Enos & Morris (1990) linked kinesin function with mitosis and elucidated chemical events underlying spindle formation and chromosome movements leading to the production of aneuploids (Enos *et al.*, 1991). Effimov & Morris (1998) suggested a check-point dealing with microtubule assembly and formation.

A. nidulans is not a suitable material for cytological investigation because of its small chromosomes (Robinow & Caten, 1969). Therefore genetical exploration only deciphered many of the genetic end-points (Jha & Sinha, 1986). The simultaneous increase in the number of the diploid mitotic non-disjunctional, aneuploids and haploids indicate that all the three events are the manifestations of a unique independent event (Käfer, 1960) and are inter-connected (Käfer, 1961). One pair of homologous chromosomes at metaphase has four chromatids (Chromosomes). At anaphase in case of normal division the centromere divides and produces two daughter nuclei, each having only two of the four chromatids. But either spontaneously or as a result of induced spindle malfunction, if a pole receives one chromatid and the other pole receives three chromatids, the result would be formation of one (2n-1) nuclei, i.e., monosomic and the other would be (2n+1) a trisomic nuclei (Jha & Sinha, 1990[b]). The (2n-1) nuclei and (2n+1) nuclei by means of gradual sequential loss of chromosomes would be transformed into haploids and diploids (Sinha & Jha, 1985). In addition to previously described cell-cycle regulatory mechanism, any chemical affecting the function and structure of spindle or polymerization/depolymerization of tubulin or microtubule associated proteins (MAPs) or the integrity of the nuclear membrane causes production of aneuploids (Jha & Sinha, 1992[a]). The following factors are used as an inducers of aneuploids in *A. nidulans.*

TABLE 5

Aneuploidy Inducing Factors

Inducing agents	References
(A) Physical factors	
(i) Gamma irradiation	Käfer, 1963^{b}; Käfer, 1986
(ii) Ultraviolet light	Käfer, 1969
(iii) Decay of the incorporated 32p	Strigini, 1962; Strigini *et al.*, 1963
(B) Chemical factors	
(i) Acridines and acriflavin	Ball & Roper, 1966; Croft, 1966; Upshall & Croft, 1967
(ii) Amphotericin B	Bellincampi *et al.*, 1980
(iii) Alcohols	Harsanyi *et al.*, 1977; Käfer, 1984
(iv) Bromochloromethane	Benigni *et al.*, 2004
(v) Bromodi-chloromethane	Benigni *et al.*, 2004
(vi) Chloral hydrate	Sinha & Jha, 1983, Käfer, 1986;Crebelli *et al.*, 2002
(vii) Dibromochloroflurometħane	Benigni *et al.*, 2004
(viii) Econazole	Bellincampi *et al.*, 1980
(ix) Formalin	Fratello *et al.*, 1960
(x) Fenarimol	Bellincampi *et al.*, 1980
(xi) Griseofulin	Sinha & Jha, 1985
(xii) Miconazole	Bellincampi *et al.*1980
(xiii) MMS	Käfer, 1987, 1988
(xiv) N-methyl-N′-nitro-N-nitrosoguanidine (NTG)	Shanfield & Käfer, 1971
(xv) Piamaricin	Belincampi *et al.*, 1980
(xvi) P- fluorophenylalanine	Shanfield & Käfer, 1971
(xvii) Thiram	Upshall & Johnson, 1981
(xviii) Tallysomycin	Demopoulos & Kappas, 2005
(C) Biological factor :	
(i) Aberrations	Pollard *et al.*, 1968

CONCLUSION

The preference of *A. nidulans* as a genetic test-system relies on the factors such as, easy handling of the experimental organism, rapid detection, and unequivocal results. The genetic damages are not only restricted to a single or a few genetic processes but cover a wide range of genetic end-points. Apart from these advantages of the test-system, the study may be limited to a genetic test for a single genetic phenomenon or may be extended to simultaneous multidirectional genetic events in the same system. Extensive manipulation of *A. nidulans* as a test system would check the risk of reckless use of newly synthesized or unassayed drugs, chemicals and environmental mutagens, and prove as a boon for the health and welfare of human being.

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12

ROLE OF CEREAL CYST NEMATODE MANAGEMENT IN FOOD SECURITY AND FOOD SAFETY

S.P. BISHNOI AND S.M. YADAV

DISTRIBUTION

In India, *H. avenae* causes the disease locally known as 'Molya' (Molya in local language of Sikar and Jaipur denotes 'deformed' and disease gets in name due to characteristically deformed root system) and it was first reported from Sikar district of Rajasthan in 1958 by Vasudeva. But now it has been reported from Ajmer, Alwar, Bhilwara, Churu, Jaipur, Jhunjhunu, Pali, Sirohi, Udaipur, Hanumangarh, Bikaner, Bharatpur, Dausa districts of Rajasthan (Koshy & Swarup, 1971; Mathur *et al.*, 1975, Bishnoi *et al*); Ambala, Bhiwani, Faridabad, Gurgaon, Mahendergarh, Rohtak and Sirsa districts of Haryana (Bhatti *et al.*, 1980; Bajaj & Walia, 1985); Faridkot, Hoshiarpur, Jalandhar, Kapurthala, Ludhiana, Patiala and Sangrur districts of Punjab (Koshy & Swarup, 1971; Chhabra, 1973); Jammu and Kashmir (Singh *et al.*, 1976) and Himachal Pradesh (Koshy & Swarup, 1971); Delhi and Aligarh, Badaun, Bulandshahar, Ghaziabad districts of Uttar Pradesh (Swarup *et al.*, 1982; Siddiqui *et al.*, 1986). It is speculated that this nematode is continuing its spread slowly and gradually towards the Indo-Gangetic plains of U.P.

DIVERSITY

Up till now more than 11 pathotypes have been detected from various countries on the basis of International test assortment and grouped on the basis of International terminology. From India, Mathur *et al.* (1974) reported the occurrence of five pathotypes of this species in Rajasthan and observed that they were different from European pathotypes. Bhatti *et al.* (1977) found that the behaviour of Haryana population was different from Rajasthan population and speculated the possibility of *H. avenae* being a species complex. Swarup *et al.* (1979) reported that population of Rajasthan (Jaipur, Udaipur) belonged to pathotype-1, distinct from population of Punjab (Ludhiana, Hoshiarpur) which formed pathotype-2. Siddiqui and Hussain (1989) showed that Ghaziabad (U.P.) population was different from Aligarh (UP) population on the basis of reaction on some international host differentials. After scanning the literature Andersen and Andersen (1982) assigned Indian populations to pathotype Ha 21, Ha 31 and Ha 41. Bekal *et al.* (1998) attributed Nazafgarh, Delhi population to Ha 71 pathotype. Bishnoi & Bajaj (2004) concluded that Jaipur, Udaipur, Narnaul, Sirsa & Delhi population belong to pathotype Ha21 which Punjab (Ludhiana) and Ambala (Haryana) population belong to pathotype (Ha 41) and Himachal Pradesh population belong to Ha 31 of *Heterodera filipjevi.* On the basis of international host differential reaction, bio-chemical studies and morphometric studies of eight population of 4 states.

YIELD LOSSES

The extent of losses depends upon nematode inoculums present in the soil. Handa (1983) estimated yield losses upto 87 per cent in heavily infested nematode field in Rajasthan, but Mathur *et al.* (1986) reported avoidable loss in wheat ranging from 32.46 to 66.5 per cent with inoculum varying from 4.6 to 10.6 eggs/ml soil. Mukhopadhyay, (1972) reported that 10 cysts/kg soil caused 10 percent losses which may go upto 64 per cent with 1250 cysts in pots. Losses caused by this nematode may go upto 100 percent (Sheshadri, 1970). In term of rupees Van Berkum and Sheshadri (1970) estimated loss of 255 lakh rupees for barley in three districts of Rajasthan alone while Sharma & Sharma (2000) estimated a loss of about Rs. 11.25 crores in wheat in Rajasthan.

CONTROL

1. Crop Rotation

The use of crop rotation in nematode management is aimed to combat nematological population by growing non host crops after a host crop to bring down the nematode population to a level which may prevent the significant damage to cereal crops. Attempts were made to manage the population through fallowing or by growing non host crops because CCN being host specific to cereals. Handa (1983) found that nematode population decreased by 70 per cent with continued rotation of non host crops like mustard, carrot, fenugreek & gram or by following and increased yield by 87 per cent. Increase in barley yield by 56 per cent with two years rotation of non host crops was noted (Handa *et al.* 1975).

2. Manuring/Fertilizers

It has been known for the past several years that organic manuring influences the development of *Heterodera species.* On the basis of pot and field trials, Mathur (1969) reported that oil cakes, farm yard manure, compost and saw dust applications resulted in improved plant growth and subdued multiplication of the eelworm. He mentioned their efficiency when organic fertilizers provided through oil cakes, saw dust and farm yard manure/compost. Among inorganic fertilizers, the role of nitrogen application was significant. The application of nitrogen resulted in better plant growth and more nematode multiplication. He also reported that the role of phosphorus and potash was not significant, Handa (1983) while working on nitrogenous fertilizers reported that increase in nitrogen increased the plant height, ear length, and fodder and grain weight significantly in barley whereas increase in nematode count was non-significant but certainly there was significant increase over initial population. However, he mentioned that significant increase in plant growth and increase in final nematode count depends on the initial population. Better growth of plants can be obtained only in lower populations up to 5 L/ml soil but above it, results may be affected.

3. Irrigation

Mathur *et al.* (1981) reported higher multiplication of nematodes in well irrigated fields in wheat and barley as compared to soil with poor moisture. Handa (1983) found that sandy loam soil resulted in more yield of barley with reducing the irrigation gap i.e. 20 days with maximum post harvest population built up of nematode in question.

4. Mixed Cropping

"Gojra" (mixed cropping of wheat and barley) is common practice in Rajasthan. Handa *et al.*, (1980) reported the beneficial effect of resistant variety of barley (Rajkiran) with susceptible variety of wheat Kalyansona for increase in grain yield and decrease in nematode population as compared to susceptible crop of wheat/barley. They further, indicated the possibility of use of combination of different crops with varying susceptibility to nematode to decrease the population and obtaining optimum yield.

5. Summer Ploughings

While studying the survival of *Heterodera avenae* under controlled temperatures, a marked reduction in survival of cyst contents at 28°C by Mathur (1969) and Handa *et. al.*, (1975) observed decrease in population of cereal cyst nematode with summer ploughings and subsequent increase in the yield of cereals.

The above scientists reported that the soil temperatures during May and June generally remain more than 30°C and go up to 48°C. By ploughings, the soil is loosened and freshly formed cysts are disturbed and with the turning of the soil they also come up in the soil zone (0 to 20 cm) of higher temperature. The moisture of the soil evaporates and cysts are subjected to dry heat. The nematode is sensitive to desiccation, hence its contents start drying. The success of the ploughings depends on two aspects-high temperature and no rainfall during May and June. With the findings of Handa (1983), it is envisaged that 3 to 5 summer ploughings will bring down the initial population and subsequently better yield of wheat and barley crops could be obtained.

6. Date of Sowing

Normally wheat and barley are sown during second week of November to end of November. Mathur (1969) tried by sowing of wheat and barley from 18th Oct. to 26th Dec. at weekly intervals in pots and concluded that change in the date of sowing did not influence the incidence of CCN and eelworm multiplication.

7. Chemical/Nematicidal Treatments

Use of chemicals, though invariably costly or uneconomical, their efficacy allows their use where populations have reached at very high levels.

(A) Soil Application

Research work on control of CCN in cereals with soil incorporation of chemicals has been attempted by a large number of workers. Swarup et al. (1976) and Handa *et. al.* (1980) found DD, DBCP and granular formulations to be efficacious in decreasing the population of *H. avenae* and increasing the yield of barley crop. In wheat, similar results have been reported by many researchers Mathur *et al.* (1986) and Handa *et. al.* (1980) found drilling of granular formulations better than broadcast (Table-5). Granular formulations of carbofuran and aldicarb have been found very effective in increasing yield of barely and reducing the nematode population. Added advantage in barley is complete control of aphids.

Research works on different chemicals have been carried out to manage the CCN infestations on the farmer's fields. In early 1970-80's, experiments were conducted with DD, DBCP (EC), aldicarb and carbofuran on barley and wheat by Yadav & Singh (1975), Handa *et. al.* (1980), Mathur *et. al.* (1984, 1986), Handa and Mathur (1979), Singh and Yadav (1978), Handa *et al.* (1980, 1985) and found the effectiveness of these chemicals in increasing the fodder and grain yields of wheat and barley and reducing the nematode population in the fields. They observed that carbofuran and aldicarb when applied through drill @ 0.1 kag ai/ha were very effective.

Use of chemicals on field scale, has very limited scope under socio economic conditions of farmers of disease prone areas especially in cereals and the scientists suggested the use of these chemicals when the initial nematode population is very high (5 I/g sailor above) and other alternatives are not available.

(B) Seed Treatment

Use of seed dressing chemicals is quite common and practiced for control of many fungal diseases. The main advantage of seed dressers is (a) use of less quantity of chemical, (b) reduction in the cost of application & (c) easy to apply. Much work for management of CCN through seed treatment was not done as the availability of seed dressing nematicides is limited. Among some of the seed dressing nematicides tested at different doses, carbofuran SP as well as flow able and aldicarb sulfone @ 4 kg ai/ha were found effective in increasing grain yield of wheat and barley with no change in final population.

8. Integrated Nematode Management

Studies on integrated management of CCN on wheat and barley by integrating the different methods namely-summer ploughings + irrigation, summer ploughings + nitrogenous fertilizers + seed treatment or soil application of nematicides were carried out by Handa *et al.* 1981 & 1984, Handa, 1983 and Mathur *et al.*, 1984. They found that the integration of these methods has given encouraging results for increasing the crop yield and reducing nematode population.

Summer ploughings + nitrogenous fertilizers: Handa *et al.* (1981) and Mathur *et al.* (1984) found that a single factor, i.e. nitrogen at the highest dose (90 kg N/ ha or) increased grain yield in tall barley variety in ploughed plots but as the ploughings were combined with nitrogen, smaller doses of nitrogen gave equal or better results.

The additive effect of ploughings with nitrogen is clearly evident as the number of ploughings increased the yield.

9. Genetics and Source of Resistance

The production of resistant cultivars is the most promising method for managing *H. avenae*. Different sources of resistance found associated with a single dominant gene, are present in barley (Yadav *et al*, 1987) but breeding for disease resistance is complicated because of the presence of different pathotypes of *H. avenae* with different levels of virulence. Nevertheless, several cultivars of barley have been developed in India by incorporating resistance in the local popular cultivars.

Plant resistance is the cheapest, easy to recommend & most practical method of nematode management. Looking the seriousness of the CCN in lighter soils of state and availability of resistant gene in barley (Mathur, 1969) breeding programme was initiated at ARS, Durgapura. Breeding for nematode resistance was started in 1971 with well adapted varieties. RDB-1, RS-6 and DL-69 that were crossed with exotic resistance sources like Morocco, Marocaine and PI 253826. Out of hundreds lines showing resistance, one line later given number RD 387, was found agronomically superior in yield and showed complete resistance to prevailing pathotypes of CCN except Ambala population (now characterized as *H. filipjevi*, Bishnoi & Bajaj, 2000). This variety named as Rajkiran evolved from cross RDB-1 X Marocaine (Rha 3 gene) was later released in 1979 for cultivation in nematode infested fields. Only drawback in this variety is that it slightly late in maturity. Due to transfer of this undesirable character of late maturity (150 days) on this variety from exotic source of resistance, it became necessary to find out other indigenous source of resistance. The first indigenous source of resistance identified in barley was in genetic material PL-1 01, DL-69, C-164. Subsequently, one of these resistance sources (PL-101) was transferred in two local cultivars and two resistant cultivars RD 2052 & RD 2035 with good grain yield potential thus resulted in release in 1991 & 1993, respectively. These two varieties are at present cultivated largely in the state and are helping in reducing the area of CCN infestation. Recently, a malt cultivar RD 2508 was released for nematode infested field.

Programme for breeding resistance to CCN in barley is still being continued to obtain better quality and higher grain yield variety.

In wheat, a large number of exotic and indigenous varieties/genotypes were tested. One Australian genotypes namely AUS 15854 was found completely resistant to CCN at ARS, Durgapura. After a thorough tests for the resistance, breeding programme was initiated in 1991 by making crosses of AUS 15854 with local varieties and resulted to the development of seven genotypes i.e. CCNRV, (J-24 x AUS 15854), CCNRV, (Raj 2184 x AUS 15854), CCNRV, (Raj 3877 x AUS 15854), CCNRV, (Raj 2329 x AUS 15854), CCNRV, (Raj 2535 x AUS 15854), CCNRV. (Raj 2009 x AUS 15854), CCNRV, (Kalyan Sona x AUS 15854) out of these seven resistant lines CCNRV, has an average grain yield of 41.0 q/ha with good agronomical characters. This variety has complete resistance to CCN in field and was named Raj MR-1 & released in 2002 for cultivation in CCN infested area of the state.

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13

CHILLI LEAF CURL VIRAL DISEASE : A REVIEW

RISHI KESH MEENA, VIDYA PATNI AND D.K. ARORA

Spices are being used ever since the ancient civilizations of India, Mesopotamia and China. Spices denote all the aromatic or pungent substances of vegetable origin. The term spices and condiments apply to such natural plant or vegetable products or a mixture of these. They are used in original or grounded form for imparting flavour, aroma and pungency to food. Spices constitute an important group of agricultural commodity, which is virtually indispensable in culinary art. They are well known as appetizers or preservatives. Many of these have rich medicinal properties and are used in pharmaceutical industry especially in the preparation of ayurvedic medicines, perfumery, cosmetic products, religious rituals etc. Within the past one decade, the international trade in spices has grown by leaps and bounds. An estimated 500,000 tonnes of spices and herbs valued at US $1500 million are now imported/exported globally (Agrawal, 2003). India is the world's largest exporter of spice and spice products viz. ground spices, blended spices, spice pastes. Herbs and spices have been valued for ages not only for their culinary use but also for their medicinal properties. They provided the material used in various systems of alternative medicine such as Ayurveda, Siddha, Unani, and Chinese and Tibetian medicine as well as in Naturopathy, Aromapathy, Homeopathy, and remedies (Daswani *et al.*, 2003). Spices are used as food flavouring and as beneficial hypolipidemic food adjuncts (Srinivasan *et al.*, 2004; Srinivasan, 2005).

India has been regarded as the home of spice plants from ancient times (900 B.C.). With an annual output of two million tonnes, India enjoys a pre eminent position in spice production. But various micro organisms viz. fungi, bacteria, virus, mycoplasma, and nematodes affect the spice production and not only reduce the production per unit area, but also affect the quality of spices.

India is the largest consumer of spices with domestic consumption of 1.9 million tones, leaving about 1.3 lakh tonnes for export. Although nearly about 60 out of the recorded 107 spices are cultivated in India, only 16 are important, viz. black pepper, cardamom, ginger, turmeric, large cardamom, clove, chillies, garlic, saffron, celery, cumin, coriander, fennel, fenugreek, ajwain and suwa.

Among the spices, chilli (*Capsicum)* is an important spice. It is cultivated throughout the world. Modern day taxonomists, including C.B. Heiser (1969) have recognized five cultivated species of *Capsicum; C.annuum* L. *C.frutescencs* L, *C.chinense* Jacq, *C.pendulum* Willd., *C.pubescens* Ruiz and Pavon -the last three are of lesser economic importance and are grown only in the Western hemisphere. *C.annuum* is the most widely grown pepper all over the world and all the varieties cultivated in the United States of America and Europe belong to it.

Chilli had been widely used since ancient times for seasoning, in medicines and as spice (Kochhar, 2003). Katuvira (chilli) is carminative, nerve stimulant, increases thirst and inflammation. Fruit has a bitter sharp taste, analgesic, expectorant, stomachic, tonic, carminative, a powerful local irritant, heart and general stimulant. It is useful in atonic dyspepsia. It enriches blood, lessens inflammation and pain. It is a source of Vitamin C. It acts as an acid stimulant and externally as a rubefacient.

Red chilli is applied to the part affected by a dog bite; it minimizes the affect of the poison. It also acts as an antiseptic by preventing the formation of pus in the wound.

The fruits are used when green as well as in dried forms as condiment vegetable and pickles. Medicinally, chillies are used as tincture and juice of fruit of C. *annuum* is used in toothache, digestive disturbance, hoarseness, dyspepsia, yellow fever. Extracts of chillies are used in the manufacture of ginger beer and beverages.

Chilli vinegar is an excellent stimulant imparting a good flavour to fish and meat. The Indian diet contains chilli as the most important ingredient. Since antiquity, chilli has been used as food additives, preservatives and as herbal medicines for maladies ranging from itch, pain to constipation. Even though chilli itself has been used as a counter irritant for ages, by itself, it is an irritating compound and has a variety of toxic effects.

DISTRIBUTION, CULTIVATION AND PRODUCTION OF CHILLI

India is the largest producer and second largest exporter of chilli in the world. In India, chilli is mainly grown during the kharif (monsoon) season (June-October) as rain fed crop or with supplementary irrigations. The major chilli growing states in India are Andhra Pradesh, Rajasthan, Maharashtra, Karnataka, Orissa and Tamilnadu, which account for about 75% of the total area of chilli cultivation as well as production. Rajasthan is one of the major producers and it occupies the 7th position in chilli production in the country. The major chilli producing districts are Jodhpur, Ajmer, Bhilwara, Pali, Sikar, Bharatpur, Sawai-madhopur and Karauli.

DISEASES OF CHILLI

A large number of diseases affect the production of chillies adversely. Some of the important diseases are as follows :

(A) Bacterial diseases

(a) Bacterial soft rot of pepper caused by *Erwinia carotovora*
(b) Bacterial leaf spot caused by *Xanthomonas vasicartoria*
(c) Wilt disease caused by *Pseudomonas* spp.

(B) Fungal diseases

(a) Anthracnose and ripe fruit rot disease caused by *Colletotrichum capsici.*
(b) Damping off caused by *Pythium* spp., *Phytopthora* spp; *Rhizoctonia* spp. and *Fusarium* spp. etc
(c) Wilt caused by *Verticillium* spp. and *Pellicularia* spp.
(d) Powdery mildew caused by *Oidiopsis taurica.*

(C) Viral diseases

Various viral diseases are: chilli leaf curl disease tobacco mosaic disease, cucumber mosaic disease, tobacco etch disease, etc.

One of the main reasons for the low productivity of chilli is its susceptibility to various pests and disease (Paroda and Chadha, 1996) including the viral diseases, which not only reduce yield but also the quality. In India, several viruses have been reported to affect chilli (Rama Krishnan, 1961) (Table 1). These include *Cucumber mosaic virus* (CMV), *Pepper vein banding virus, Pepper vein mottle virus, Potato virus y* (PVY), *Tobacco etch virus* and *Tobacco mosaic virus* (TMV) (Rishi and Dhawan, 1989; Bidari and Reddy, 1990; Kiranmai *et al.,* 1998; Chitra *et al.,* 2002). Some other viruses have been transmitted artificially (Ramakrishnan, 1961).

In India, *Tomato Leaf Curl New Delhi Virus* (ToLCNDV) has recently been shown to be associated with chilli leaf curl disease (Khan *et al.,* 2005). Several other *begomovirus* species associated with chilli leaf curl such as *Cotton leaf curl multan virus* (Husain *et al.,* 2003), *Chilli leaf curl multan virus* (Shih *et al.,* 2003) and *Pepper yellow leaf curl Indonesian virus* (Tsai *et al.,* 2006) were reported from Pakistan and Indonesia.

TABLE 1

Viruses Reported to Cause Diseases in *Capsicum* spp.

Genus	Species		Diseases
Begomoviruss	*Pepper yellow leaf curl Indonesian virus*	PepYLCIV	Yellow leaf curl
	Cotton leaf curl multan virus	CLCUMV	Leaf curl
	Tomato leaf curl New Delhi Virus	ToLCNDV	Leaf curl

Contd...

Contd...

Genus	Species		Diseases
	Pepper huasteco virus	PHV	Mosaic
	Texas pepper virus	TPG	Mosaic
	Tomato dwarf leaf curl virus	TDLCV	Leaf curl and stunting
	Serrano golden mosaic virus	SGMV	Golden mosaic
	Tomato yellow leaf curl virus	TYLCV	Leaf curl and stunting
	Chilli leaf curl multan virus	Chilcuv (Mul)	Leaf curl
Carla virus	*Pea streak virus*	PeSV	Streak
	Red Clover vein mosaic virus	RCVMV	Mosaic
Como virus	*Broad bean stain virus*	BBSV	Mottle and Necrosis
	Broad bean true mosaic virus	BBTMV	Mosaic
	Pea green mottle virus	PGMV	Mottle
	Pea mild mosaic virus	PmiMV	Chlorosis
Cucumber virus	*Cucumber mosaic virus*	CMV	Severe mosaic
	Pepper veinal mottle virus	PVMV	Mottling
Enamovirus	*Bean leaf roll virus*	BLRV	Leaf roll
	Pea enation mosaic virus-1	PEMV-1	Leaf enation
Umbra virus	*Pea enation mosaic virus-2*	PEMV-2	Leaf enation
Faba virus	*Broad bean wilt virus*	BBWV	Severe mosaic
Potex virus	*White clover mosaic virus*	WCMV	Mosaic
Poty virus	*Bean yellow mosaic virus*	BYMV	Tip necrosis mosaic
	Clover yellow vein virus	CIYVV	Necrosis
	Lettuce mosaic virus	LMV	Vein leaning
	Passion fruit woodiness virus	PWV	Mosaic
	Pea seed-borne mosaic virus	PSbMV	Mosaic
Potato virus Y	PVY	Leaf rolling	
Tobamovirus	*Tobacco mosaic virus*	TMV	Mosaic
Tobravirus	*Pea early browning virus*	PEBV	Browning
Tospovirus	*Tomato spotted wilt virus*	TSWV	Browning
Tymovirus	*Plantago mottle virus*	PIMV	Mottle

LEAF CURL DISEASE OF CHILLI

Leaf curl disease in recent years has emerged as one of the most important and destructive diseases of chilli causing severe losses in yield in many chilli growing areas in India. The occurrence of leaf curl disease of chilli in India was reported in early sixties (Mishra *et al.,* 1963).The disease is transmitted by white fly, *Bemisia tabaci* Genn. Based on the transmission to tobacco, which developed leaf curl symptoms, the causal virus of chilli leaf curl disease was reported as *Tobacco leaf curl virus* (Mishra *et al.,* 1963; Dhanraj and Seth, 1968). Amin 1979 reported that the chilli leaf curl disease is the most destructive disease of chillies (*Capsicum annuum* L.) in Sri Lanka and India. The different types of foliage malformation seen in chillies have been described as leaf curl and are reported to be caused by virus mites and thrips (Wijegunasekara *et al.,* 1986). However, the leaf curl induced by mites and thrips are different from that caused by virus as the virus associated disease is irreversible and cannot be controlled by insecticidal application. The virus induced leaf curl symptoms are characterized by vein clearing, upward curling, deformation of leaves stunting of plants and abscission of flower buds.

Shukla and Shri Ram (1977) investigated viral diseases occuring naturally on chillies in and around Udaipur and Jodhpur and found that isolates collected from Udaipur region showing mosaic symptoms contained CMV, TMV and PVY and samples from Mathania harbored tobacco leaf curl virus. Sandhu and Chohan (1979) characterized a virus disease showing symptoms like mottling followed by leaf deformities in chilli. They found that the causal virus is transmitted by aphid and the host range is restricted to *Capsicum annuum, Cucurbita pepo, Datura metel, Physalis floridana, Nicotiana glutinosa, N. tabacum, Solanum nigrum and S. melongena.* They also found that the virus was serologically related to pepper mottle virus.

Bidari and Reddy (1990) studied the preservation and storage of eight viruses (PVY, PVMV, PVMV, TEV, CMV, TMV, TSWV and TRSV) isolates from chilli in Karnataka. Rishi and Dhawan (1989) conducted a study to characterize and identify viruses associated with mosaic disease of Capsicum and found five different types of viruses based on Ouchtrlony double diffusion test and reaction on differential hosts. They also found that the intensity of mosaic disease was greater as compared to leaf curl disease. They found that CMV, PVX, PVY, TMV were associated either singly or in combination with mosaic disease. Bidari and Reddy (1990) indicated that the average incidence of chilli mosaic was 53% in the commercial chilli growing areas of Karnataka. They also developed a set of seven differential host plants (*Chenopodium amaranticolor, Cucumis sativus, Nicandra hysaloides, Nicotiana gultinosa, Datura stramonium, Datura metel, Capsicum annuum* cv California wonder) for the identification of chilli viruses. Behl (1990) identified an unusual strain of PVY inciting severe mosaic and leaf distortion on bell pepper. The virus was found to be transmitted by sap and aphids *Myzus persicae* and *Aphis fabae* in a non persistent manner. Sharma *et al.* (2004) characterized at least two different *Poty virus* and *Cucumo virus* associated with mosaic disease complex of bell pepper based on host range, symptomatology, transmission and electron microscopy. Satya Prakash *et al.* (2002) observed a viral disease of chilli causing dark green mottling and distortion symptoms on leaves in chilli growing areas of eastern Uttar Pradesh in 1997 and 1998. The causal virus was identified as a poty virus related to CMV based on symptomatology, host range transmission, physical properties, electron microscopy and serology.

The gemini viruses are the most recently described group of the plant pathogenic viruses. The earliest record of the work on disease now included in this group dates back to the end of 19th century. It is likely that yet another disease caused by a gemini virus has a further distinction. In the year 752 A.D. a japanese empress described through a poem the beauty of the yellow leaves of the plant now called *Eupatorium chinense* and thus unwillingly contributed what is possibly the first report on symptoms caused by a plant virus and more specifically of the gemini virus type.

MORPHOLOGY OF THE PATHOGEN

Geminivirus

The geminivirus group consists of single stranded DNA (ssDNA) viruses infecting a large number of important crop plants. The name gemini virus is derived from the Latin word Gemini = twins. The genome is characterized by a circular single stranded DNA of 2.5-3.0 Kb, encapsidated in unique geminate quasi isometric virus particle of 15-18nm, (Goodman,1977; Harrison *et* al.,1977; Davies and Stanley,1989; Hanley-Bowdon *et al*, 1999; Harrison and Robinson,1999). Bock *et al.* (1974) isolated a geminivirus for the first time and Goodman (1977) demonstrated the ssDNA nature of the genome. In 1991, the fifth report of the International Committee for Taxonomy of Virus (ICTV) (Frincki *et al.*, 1991) described the taxonomic structure of the geminivirus group as three subgroups, namely subgroup I with the type member *Maize streak virus* (MSV), subgroup II with the type member *Beet curly top virus* (BCTV) and the subgroup III with the type member *Bean golden mosaic virus* (BGMV).

In 1995, the geminiviruses were classified under a family *Geminiviridae* (Murphy *et al.*, 1995) containing three genera belonging to I, II, III. In the seventh Report of the ICTV (Vanregenmortel *et al.*, 2000), four genera namely *Mastrevirus, Curtovirus, Begomovirus* and *Topocuvirus* under the family *Geminiviridae* were approved. The genus *Mastrivirus* (subgroup I) with MSV as the type species consisted of 12 species and two tentative species. The genus *Curtovirus* (subgroup II) with BCTV as type species contained three species and one tentative species. The *Begomovirus* (subgroup III) with BGMV as type species consists of 76 species and eight tentative species. The genus *Topocuvirus* (subgroup IV) has one member, *Tomato pseudo-top virus* (TPCTV), which is also the type species.

The criteria for demarcating genera in the family *Geminiviridae* are genome organization, insect vector, host range and sequence relatedness. The genus *Mastrivirus* consists of viruses having a single genomic component encoding for four proteins, which mainly infect monocotyledonous plants and are transmitted by leafhoppers. The members of the genus *Topocuvirus* have a single genomic component encoding six proteins, infects dicotyledonous (dicot) plants and is transmitted by leafhoppers. The members of the genus *Curtovirus* have a single genomic component encoding seven proteins and infect dicot plants and are transmitted by leafhoppers. The members of the genus *Begomovirus* infect dicots and are transmitted by whiteflies. The Begomoviruses have either one (monopartite) or two genomic components (bipartite, DNA-A, and DNA-B).The monopartite *Begomovirus* encode for six proteins and bipartite *Begomovirus* encode five to six proteins by DNA-A and two proteins by DNA-B (Varma and Malathi,2003).

Genus *Begomovirus*

This is the largest genus of family geminiviridae. The type of the genus is BGMV. The members of Begomovirus have a host range among dicotyledonous species. They are transmitted by whitefly, *Bemisia tabaci.* Most members of this genus have their genomes divided between two DNA components (bipartite); the genome of some member is monopartite. The important bipartite *Begomovirus* are BGMV, *African cassava mosaic virus* (ACMV) *Tomato golden mosaic virus* (TGMV), *Mungbean yellow mosaic virus* (MYMV), *Tomato leaf curl New Delhi virus* (ToLCNDV), etc. The *Tomato yellow leaf curl virus* (TYLCV) from Israel, Thailand, and Sardinia have monopartite genome (Rochester *et al.,* 1990; Kheyre-pour *et al.,* 1991). Padidam *et al.* (1995) subdivided whitefly transmitted Geminiviruses (WTGs) into two groups i.e. WTGs from the new world e.g. Brazilian isolate of BGMV California isolate of *Squash leaf curl virus* (SqLCV) and WTGs from the old world e.g. Indian Cassava mosaic virus (ICMV), Mungbean yellow mosaic virus from Thailand etc on the basis of sequence analysis of N- terminal region (960-70 amino acids) of the coat protein gene and biological properties.

LOCATION OF VIRUS IN HOST BODY

There seems to be the tendency of gemini viruses to be confined to the phloem where particles occur in the nuclei of the infected cells. These particles are simiiar to those of the animal Parvoviruses in having ssDNA and in inducing characteristic nucleopathic changes (Harrison *et al.,* 1977, Kim and Flores, 1979).

DETECTION OF VIRUS

A virus is a set of one or more nucleic acid template molecules encased in a protective coat or coats of protein or lipoprotein, which is able to organize its own replication only within suitable host cells. Within such cells virus production is (1) dependent on the host's protein synthesizing machinery, (2) organized from pools of the required materials rather than by binary fission, and (3) located at sites which are not separated from the host cell contents by lipoprotein bilayer membrane (Matthews,1981).

The ability to assay viruses is an essential requirement for most aspect of their isolation and study. Four general methods for assay are available: infectivity, serological, physical, and chemical. Of these, infectivity measurements are the most basic since it is only because of biological activity that we know we are dealing with a virus. Simultaneous application of two or more assay methods that depends on different properties of the virus is useful, and is often essential for many kinds of experiments. The problems of detecting viruses and of diagnosing virus diseases involve the use of assay techniques.

While electronic microscopy is undoubtedly the most reliable and direct method for locating the virus in the host tissue, these are used mainly with purified preparation. But this process is expensive, cumbersome and time consuming (Corbett, 1974).

To avoid these difficulties some quick and simple methods have been suggested, polymerase chain reaction (PCR) is one of them. This method besides being simple, can detect the presence of causal organism (virus) in the initial stages much before the symptoms are visible externally.

The polymerase chain reaction is used to amplify a segment of DNA that lies between the two regions of known sequence where two oligonucleotides (deoxy) as primers can bind the opposite strands of DNA due to the complementary nature of base sequences. It was first suggested by Mullis *et al.* (1986) and first demonstrated by Scharf *et al.* (1986) for the addition of a restriction enzymes site synthesized into the 5′-ends of PCR oligomer primers. Because this technique requires the use of custom synthesized oligomer primers for each DNA modification, it is reffered as custom-PCR (cPCR) engineering (Slightom, 1991).

The PCR method varies depending on the virus targeted and number of primers. These primers have been used by many workers in many virus infected plants (Navot *et al.*, 2004; Nagate *et al.*, 2004; Hassan *et al.*, 2005; Zitikaitae and Staniulis, 2006; Usta *et al.*, 2007).

The amplified DNA products produced by PCR are detected by agarose gel electrophoresis. It is the standard method for the separation, identification and purification of DNA and RNA fragments ranging in size from a few hundred nucleotides to 20 kb. Besides agarose, polyacrylamide gel electrophoresis is also used for the same purpose, but is preferred for small DNA fragments. The technique of agarose gel electrophoresis is simple, rapid to perform and capable of resolving DNA fragments that can't be separated adequately by other procedures such as density gradient centrifugation. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of fluorescent ethidium bromide dye under ultraviolet light. If necessary, these bands of DNA can be recovered from the gel and used for a variety of cloning purposes. Gel electrophoresis gives a rapid method for detecting characteristic viral coat protein (Paul, 1975).

Agarose is a linear polymer extracted from seaweed. Agarose gels are cast by melting the agarose in the presence of desired buffer until a clear transparent solution is obtained. The melted solution is poured into a mould and can be made into a variety of shapes, sizes, and porosities (4-10%) and the gel is allowed to harden. On hardening, the agarose forms a matrix, the density of which is determined by the concentration of agarose. When an electric field is applied across the gel, DNA which is negatively charged at neutral pH migrates towards the anode. The rate of migration depends on a number of parameters: (1) molecular size of DNA, (2) agarose concentration (3) conformation of DNA and (4) composition of electrophoresis buffer.

Larger molecules migrate slower than the smaller molecules because they have to find their way through the pores of the gel .By using gels of different concentrations it is possible to resolve a wide range of DNA molecules. The electrophoretic mobility of DNA is also affected by the composition and ionic strength of electrophoresis buffer. In the absence of ions, electrical conductivity is minimal and DNA migrates very slowly. In buffers of high ionic strength, electrical conductance is very efficient.

Lotrapul *et al.* (1998) isolated *Tomato yellow leaf curl geminivirus* hybridized with a 2.6 kb DNA band present in DNA extracts from sweet potato plant infected with Sweet potato leaf curl virus (SPLCV). *Prunus necrotic ring spot virus* (PNRSV) was detected by using double antibody sandwich (DAS) ELISA and RT-PCR by Ulubas and Ertunic (2004). Ansari *et al.*, (2006) and Meena *et al.* (2007) detected the chilli leaf curl virus by using AVF_9 and AVR_{10} primers for the amplification of coat protein gene of geminivirus.

DISEASE MANAGEMENT

Methods of disease control vary considerably from one disease to another, depending upon the kind of pathogen, the host and the interaction of these two. In controlling diseases of crops, plants are generally treated as populations rather than as individuals. Considering the regularity with which most serious diseases of crop plants appear in an area year after year, the rapid spread of most plant diseases and the difficulty of curing a disease after it has begun to develop, it is easy to understand why almost all control methods are aimed at protecting plants from becoming diseased rather than at curing them after they have become diseased. The various control methods can be classified as regulatory, cultural, biological, physical and chemical, depending on the nature of the control agents employed.

To prevent the epidemic diseases, all the host plants infected by or suspected of harbouring the pathogen may have to be removed and burnt. This eliminates the pathogen and prevents greater losses from the spread of the pathogen to additional plants. The physical agents commonly used in controlling plant diseases are temperature, dry air and unfavourable light wavelengths. Cultivation of resistant varieties not only reduce losses from disease but also reduce expenses of sprays and other methods of disease control and avoids pollution of the environment with toxic chemicals that would otherwise be used to control plant diseases. The protection of plant from pathogens include some biological controls (fungal and bacterial antagonists) but mainly the chemical control measures, that is, the use of chemicals for foliar sprays and dusts, seed treatments and control of post harvest diseases of the produce. Biological control involves protection of plants from pathogens by deployment of antagonistic microorganisms at the infection site before or after infection takes place.

DISEASE MANAGEMENT BY USING PLANT EXTRACTS AND INSECTICIDES

Chilli (*Capsicum annuum* L.) is one of the most popular crops grown in India. Chilli leaf curl virus is transmitted by the white fly, (*Bemisia tabaci* Genn.) and is the most dreaded disease of chilli. Yield losses due to this disease have been reported to be 100%. Management of chilli leaf curl disease has been attempted from time to time by several workers. In recent years the potential of plant extracts based virus inhibitory and resistance inducing chemicals extracted from higher plants is being explored for the management of viral diseases (Mann *et al.*, 2001; Kumar *et al.*, 2006 and Ansari *et al.*, 2007).

Use of chemicals and plant extracts to reduce the mosaic disease indirectly by controlling insect vectors is a common practice. For the management of other viral diseases different insecticides and plant extracts having antiviral properties were used by many workers (Peshney and Moghem, 1989; Cheema *et al* 1991; Othman *et al* 1991; Ekbote and Patil 2005).

Joshi and Dubey (1976) studied the efficiency of selected insecticides in controlling the chilli leaf curl disease and found that the rate of spread of the disease was slightly less in the plant treated with insecticides (Basudin, Ekatin and Folidol E-605) compared to that of untreated controls. Thekre *et al.* (1979) reported that the main cause of the chilli leaf curl appears to be the sap sucking pests like thrips and mites.They also evaluated some insecticides against thrips on chilli crops and found that Monocrotophos was highly effective in the control of chilli thrips. Datar (1980) reported chili leaf curl disease in Maharashtra caused by Tobacco leaf curl virus, transmitted by white fly *Bemisia tabaci.* He evaluated eight insecticides for the control of chilli leaf curl disease by reducing the population of white fly (vector) and found that Monocrotophos was highly effective in reducing the leaf curl incidence in chilli. Boghade *et al* (1985) evaluated nine insecticides in order to control the disease caused by thrips, mites and aphids and found Monocrotophos as highly effective.

Rajapaksha and Jaya sena (1989) reported that the chilli leaf curl symptoms in Srilanka is spread by thrips (*Scitothrips dorsalis*) and a virus transmitted by white fly in the dry zone of Srilanka and they found that white fly acquired the virus more efficiently from chilli than tomato.

Patnaik and Mohapatra (1997) carried out a study to evaluate the possible effects of nitrogen and potassium on the incidence of thrips on chilli leaf curl disease. They found that protection by Methomyl at 0.5 Kg a-1/ha at 50DAP and Monocrotophos 1/ha at 75 DAP irrespective of fertilizers level brought about substantial reduction in thrips population as well as the chilli leaf curl incidence.

Systemic insecticides and acaricides were effective in reducing mosaic incidence by reducing the transmission of virus (Khan and Mukhopadhyaya, 1985; Nandi halli and Thontandarya 1986 and Devi and Reddy, 1995; Shu *et al.*, 2003; Borovsky *et al.*, 2006).

MATERIALS AND METHOD

The experiments were laid out in randomized block design with plot 2_2 m and spacing 30cm between rows and 15cm between plant. Three replicates of each treatment were used. In the first experiment five plant species were viz. *Azardirachta indica* (Neem), *Datura innoxia, Calotropis procera, Lantana camara* and *Ricinus communis* were selected.

Chilli pots were sprayed with 10% concentration of leaf extracts and different concentration of insecticides separately viz. Endosulfan, Monocrotophos, Melathion

(0.1%, 0.2%, and 0.3% of each) and Methyl parathion (Folodol) (0.2%). Spray of plant extract /insecticides was repeated every 15 days. For control, plants were sprayed with distilled water.

CONCLUSION

10% Plant extract of *Azadirachta indica* and 0.2% concentration of Monocrotophos was effecting in reducing the chlli leaf curl disease.

Disease management by antiviral protein powder (AVP) of *Clerodendrum aculeatum*

Plant viral diseases are often a major limiting factor in Chilli production with severe incidence and yield reduction upto 70% (Ariyaratne *et al.*, 1996).Villalon (1981) reported that viruses are known to attack Chilli which causes yield loss to the extent of 42-80%. The cultivation of chilli is adversely affected by various viral diseases resulting in enormous losses in the form of reduced yield and deformed unmarketable fruits (Bidari and Reddy, 1990).The use of antiviral proteins (AVPs) from plant is one of the promising approaches to control viral diseases.

Some well characterized AVPs from *Phytolacca americana, Mirabilis jalapa, Dianthus caryophyllus, Clerodendrum aculeatum, Amaranthus viridis and Trichosanthes kilowii* has been reported by Guo *et al.* (1999). All these belong to a class of proteins called ribosome-inactivating proteins (RIPs) (Birbieri and Stirpe 1982). The RIPs usually show virus inhibitory activity when mixed with virus inoculum and incubated for some time before inoculation on test plants. RIPs are found to be localized extracellularly in the plants. Verma *et al.* (1994) found that the extract of a few exotic healthy plants induce strong systemic resistance and provide short term immunity to susceptible plants against viral infection.

Highly susceptible plants could be protected from naturally occurring viruses by prophylactic treatment with plants extracts like *Boerhaovia diffusa, Bougainvillea spectabilis, Clerodendrum fragrans, Peuderanthemum bicolor* and *Mirabilis jalapa* (Verma and Kumar, 1980; Verma and Dwivedi, 1983; Awasthi *et al.*, 1984; Verma *et al.*, 1985; Verma and Baranwal, 1988; Khan and verma 1990).

The phenomenon of systemic induced resistance is probably related to the activation of cryptic defence mechanism of the susceptible hosts. As a result of activation synthesis of a new translocatory virus inhibitory agent (VIA) plants accumulate significant amount of VIA, either escape infection or if infected, develop mild disease symptoms after a significant delay in time.

Verma and Varsha (1995) used *Clerodendrum* leaf extract for the prevention of tobacco leaf curl virus on the plant.

Management of viral diseases of vegetable crops through use of antiviral agents was carried out by Verma and Shrivastava (1993) and Awashti (2006). Exogenous application of small amounts of these RIPs to the surface of plant leaves prevent virus infection. They presumably inhibit virus infection by entering the cytoplasm along with virus particles and shutdown protein synthesis thus preventing early viral replication presumably translation of viral RNA.

The endogenously occurring systemic resistance inducers do not inactivate viruses *in vitro* but activate certain host defense genes, prompting the production of new virus inhibitors/neutralizing agent (VIA) in the susceptible host (Verma and Awasthi, 1980; Khan and Verma 1990). The VIA is translocated to the upper parts (non-infected) of the plant and induces short to long term immunity. The higher concentration of resistance inducers effectively stimulate the production of VIA.

MATERIALS AND METHOD

Seeds of chilli varieties- were soaked in 0.1% *Clerodendrum* powder (treated) and in distilled water overnight (control). Each time 3 replicates of each variety were taken. These were sprayed with 0.1% *Clerodendrum aculeatum* protein powder in distilled water periodically. A total of eight sprays were given, first 6 sprays were given at 8 days interval and last 2 sprays were made at 15 days interval and percentage disease incidence was calculated at 15 days interval.

The effect of *Clerodendrum aculeatum* is short lived hence, multiple sprayings are required. *Clerodendrum aculeatum* has many other advantages over other chemicals as it is easily biodegradable, does not leave any residue, is non-phytotoxic and is easily absorbed by the plant and is easy to use.

Hence this method can be used to control the disease. Not only virus incidence and severity was reduced but the growth of plants and yields were also increased.

B.3. Conclusion: Antiviral protein powder of *Clerodendrum aculeatum* gave the best result in controlling the chilli leaf curl disease, when compared with insecticides or plant extracts sprays.

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