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Review

Bioprospecting and antifungal potential of chitinolytic microorganisms

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Fungal plant diseases are one of the major concerns to agricultural food production world wide. Soil borne pathogenic fungi such as *Pythium*, *Fusarium*, *Rhizoctonia* and *Phytopthora* attack most of the economically important crop plants (either through seed root before germination or seedling after germination) resulting in loss of billions of dollars. Moreover, the management of chitinous waste is also pressing need today. Mycolytic enzymes (chitinases, proteases and glucanase) producing microorganisms may help in solving these problems. These microorganisms have ability to lyse the fungal cell wall and also have the potential to manage the chitinous waste by producing chitinases. Many chitinolytic microorganisms have potential to control fungal plant pathogens but they are not fully successful in all the cases due to different geological and environmental conditions. Thus, bioprospecting to find novel, highly chitinolytic microorganisms which help in developing potential biocontrol agent. Furthermore, to increase the survivability of biocontrol agents, a formulation may also be necessary. This review is focused on the progress of chitinase genes, chitinolytic microorganisms and their diversity as well as formulation of chitinolytic producers which have the potential to control fungal plant pathogens

Key words: Bioprospecting, biocontrol, chitinolytic enzymes, formulation.

INTRODUCTION

Fungal plant diseases are one of the major concerns to agricultural production. It has been estimated that total losses as a consequence of plant diseases reach 25% of the yield in western countries and almost 50% in developing countries. Of this, one third is due to fungal infections (Bowyer, 1999). So there is a pressing need to control fungal diseases that reduce the crop yield so as to ensure a steady and constant food supply to ever increasing world population. Conventional practice to overcome this problem has been the use of chemical fungicides which have adverse environmental effects causing health hazards to humans and other non-target organisms, including beneficial life forms. Hence there is increasing concern towards the toxicity and biomagnification potential of these chemicals in agriculture. Currently practices based on molecular biology techniques which involve development of transgenic plants which are resistant to plant pathogens, are being used. However there have been few reports of fungal infestations in resistant varieties (Schickiler and Chet, 1997).

Chitin (a homopolymer of β-1,4 linked N-acetylglucosamine) has broad spectrum distribution in the biosphere (like in the shells of crustaceans, such as crab, shrimp and lobster, exoskeleton of marine zoo-planktons like coral and jellyfish, insects such as butterflies and ladybugs. Therefore formulations based on chitinases, enzymes that hydrolyze chitin, offer potential biocontrol agents. Chitinases are reported to play a protective role against fungal pathogens (Boller, 1985). Besides its ability to attack the fungal cell wall directly, chitinases release

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oligo-*N*-acetyl glucosamines that function as elicitors for the activation of defense-related responses in plant cells (Ren and West, 1992).

The present review provides an overview of current knowledge concerning the biofungicidal potential of chitinolytic microorganism.

PHYLOGENETIC DISTRIBUTION OF CHITIN IN NATURE

Prokaryotes: Chitin is absent in prokaryotes despite its chemical similarity to the polysaccharide backbone of peptidoglycan. Gooday (1995) has reported chitin as a component of many *Streptomycete* spores and stalks of prosthecate bacteria.

Fungi: Most of the fungi contain chitin in the cell wall which ranges from 22-40% (Muzzarelli, 1977). Its presence together with that of other polysaccharides has been used as a criterion for fungal taxonomy. The polysaccharide forms fibrils of different lengths depending on the species and cellular location (Gow and Gooday, 1983).

Protista: Chitin is present in cysts, walls of some ciliates, amoebae, chrysophytes, algae and in the spines of diatoms, the purest form of chitin being isolated from the diatom, *Thalassiosira fluvitalis* (Bartnicki-Garcia and Lippman, 1982).

Animals: In animals this polysaccharide is found as a structural component and is limited to few organs, such as the integuments of arthropods, nematodes and mollusks, and in the gut lining and exoskeletons of insects. Insect exoskeletons are largely composed of chitin-protein complexes, whereas crustacean shells contain large proportions of $CaCO_3$ in addition to protein (Kramer and Muthukrishnan, 1997).

Chitin in nature exists between a fully acetylated chitin and fully deacetylated chitosan with very little at either extreme. The mucorale organism *Absidia coerulae* has been found to have chitosan as a cell wall component (Muzzarelli et al., 1994).

MANAGEMENT OF CHITINOUS WASTE

After cellulose, chitin is the most abundant biopolymer. In 1993, estimated world wide annual recovery of chitin from the processing of marine invertebrates was 37,000 tonnes (Shaikh and Deshpande, 1993), which has increased to 80, 000 tonnes in the year 2000 (Patil et al., 2000). In India alone 60,000 to 80,000 tonnes of chitinous wastes are produced annually, from which a lot of chitin can be recovered (Suresh and chandrasekaran, 1998).

Conventional method: Conventionally, these wastes are disposed either by burning or land filling but these methods are harmful to the environment since burning releases carbon dioxide and carbon monoxide to the environment, which adds to global warming while land filling is harmful as the degradation is very slow and one of the end product is ammonia which is a potent pollutant of ground water (Muzzarelli, 1997). The cost of transporting such waste, environmental pollution concern and ethical questions as to the morality of ignoring 70-80% of the dry weight of the catch have highlighted the necessity of finding alternative method (Simpson and Haard, 1985; Nicol, 1991; Vyas and Deshpande, 1991).

Chemical method: Alternatively, the chitin can be recovered from chitinous waste materials through chemical method that involves demineralization and deproteinization mediated by a strong acid or base. However, the use of these chemicals may cause partial deacetylation of the chitin and hydrolysis of the polymer. The chemical treatments also create waste disposal problems, because neutralization and detoxification of the discharged waste water are necessary. Furthermore, the value of the deproteinized liquid is diminished because of the presence of sodium hydroxide (Brine and Austin, 1981; Gagne and Simpson, 1993, Wang et al., 1997, Oh et al., 2000).

Biochemical method for deproteinization: To overcome these problems, alternative eco-friendly and economic methods like use of microorganisms or proteolytic enzymes for the deproteinization of crustacean waste have been used to produce useful products like chitin and chitosan (deacetylated form of chitin) that play vital role in sewage treatment, animal feed, food preservation and formulations of biofungicides (Broussignan, 1968; Gagne and Simpson, 1993; Bustos and Michael, 1994; Yang et al., 2000; Gohel et al., 2005b).

Biological method for degradation of chitinous waste: Moreover, the enzymatic degradation of chitinous waste involves chitinases, playing a dual role in utilization of chitinous wastes and decreasing the production cost of the microbial chitinases (Gohel et al., 2005a). Rattanakit et al. (2002) reported about chitinase formulation by using shrimp shellfish waste as a substrate for solid state cultivation of *Aspergillus* sp. SI-13. Wang et al. (2001) reported microbial reclamation of shellfish wastes for the production of chitinases where they prepared shrimp and crab shell powder by treating shellfish processing waste with boiling and crushing and it was used as a substrate for chitinolytic microorganisms. Labrie et al. (2001) reported effect of chitin waste-based composts on oomycete plant pathogens.

Table 1. Role of chitinases in different phyla.

Organism	Role of chitinases	References
Bacteria	Mineralization of chitin, also in nutrition and parasitism.	Flach et al., (1992); Connell et al., (1998).
Fungi	Physiological role in cell division, differentiation and nutritional role related to mycoparasitic activity (e.g. in *Trichoderma sp.*).	Kuranda et al. ,(1991); Gooday et al., (1992); Mellor et al. ,(1994); Alalam et al., (1995).
Plants	Defence against fungal and bacterial pathogens by degradation of their cell walls. Specific isoforms may play a role in embryo development, pollination and sexual reproduction.	Schlumbaum et al., (1986); De Jong et al., (1992); Leung (1992); Kim and Chung (2002).
Insects	Developmental process of cuticle degradation at different larval stages.	Karmer and Fukamiso, (1985); Merzendorfer and Zimoch (2003).
Protozoa	Malarial parasites produce sufficient quantities of chitinase to penetrate the chitin containing peritrophic matrix of the mosquito midgut.	Huber et al., (1991); Langer et al. (2002).
Human	Chitotriosidase activity helps in defence against nematodal infections. Moreover, its enzymatic activity is markedly elevated in serum of patients suffering from lysosomal lipid storage disorders, sarcoidosis and thalassemia.	Escott et al. ,(1996); Choi et al. ,(2001); Aguilera et al, (2003); Gianfrancesco and Musumeci (2004).
Animal	The high chitinase level in goat and bovine blood (serum) might be a function of slow renal secretion which keeps the enzyme level comparatively low in case of abnormal lysozyme production (monocytic-myelomonocytic leukemias and renal diseases).	Lundblad et al. (1974).
Yeast	α –subunit of toxin secreted by *Kluyveromyces lactis* has chitinase activity which is most likely required for the γ subunit to gain entry to the sensitive cell. Chitinases has an essential role in cell separation during budding of the chitinous yeast *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* chitinase also used as antifungal.	Butler et al. ,(1991); Kuranda and Robins, (1991); Smit et al, (2001); Carstens et al. ,(2003); David (2004)

Table 2. Nomenclature of chitinolytic enzymes.

Chitinolytic enzymes	EC No.	Mode of action	Reference
Endochitinase, poly[1,4-(*N*-acetyl-β-D-glucosaminide)] glycanohydrolase.	3.2.1.14	Random hydrolysis of *N*-acetyl-β-D-glucosaminide 1,4-β-linkages in chitin and chitodextrins.	Fischer and Stein (1960).
Exochitinase, chitobiase, β-*N*-acetylhexosaminidase, β-*N*-acetyl-D-hexosaminide *N*-acetylhexosaminohydrolase	3.2.1.52	Hydrolysis of terminal non-reducing *N*-acetyl-D-hexosamine residues in *N*-acetyl-β-D-hexosaminides	Cabezas (1989)

CHITINASES

Chitinases hydrolyzing chitin (Jeauniaux, 1966) have broad spectrum of distribution in nature including bacteria, fungi, nematodes, plants, insects, fish and human. The physiological functions of chitinases depend on their source (Table 1). Use of chitinolytic microorganisms take care of waste disposal and production of biofungicides for control of fungal plant pathogens.

Chitinolytic systems: The chitinolytic system comprises of an endochitinase, chitobiase and an exochitinase whose actions may be synergistic and consecutive in the degradation of chitin to free GlcNAc (*N*-acetyl glucosamine). Nomenclature of chitinolytic enzymes has been described in Table 2.

Characteristics of plant chitinases: Plant chitinases are known as pathogen related proteins (PRPs), because

Table 3. Substrates of chitinolytic enzymes and the products measured (Patil et al., 2000).

Product measured	Soluble substrates					Insoluble substrates		
	CMC[A] /EGC[B]	Chitool-igomers	4-MU-oligomers[C]	p-NP-oligomers[D]	Chitin	Colloidal chitin	[acetyl-^{3}H] chitin	CC-RBB[F]
Formation of reducing sugars	+	+			+	+	+	
Release of fluoro-/chromophore (electrophoresis)	+		+	+		+		+
Formation of oligomers (HPLC, NMR)	+	+			+	+		
Decrease in degree of polymerization (viscosity)	+							
Decrease in degree of polymerization (staining)	+				+	+		

[A] carboxy methyl chitin; [B] ethylene glycol chitin; [C]4-Methylumbelliferyl, [D] *p*-nitrophenyl, [E]remazol brilliant violet, [F]colloidal chitin with Remazol Brilliant Blue R.

chitinases along with β-1,3-glucanases are induced by pathogens (Wyatt et al., 1991; Abeles et al., 1971; Selitrennikoff, 2001). They also inhibit the mycelial growth of many pathogenic fungi *in vitro*. Most of the chitinases produced are endo-type which liberate N-acetylchitooligosaccharides from chitin. N-acetylchitooligosaccharides are considered to be elicitor, evoking various defense responses. These include the production of phytoalexins, induction of PRPs, generation of active oxygen species and lignification (Fanta et al., 2003). Plant chitinases can be subdivided into five classes based on their amino acid similarity and presence of signal peptide, a hevein (chain binding) domain, a hinge region, a catalytic domain and a C-terminal extension.

Microbial chitinases: Relatively little is known about the number, diversity and function of chitinase produced by microorganisms, even though chitin is one of the most abundant polymers in nature. Chitinases find application in biocontrol, offer an attractive alternative or supplement for the control of plant diseases.

Method for the isolation of chitinolytic microorganisms: Chitinase activity can be qualitatively assayed by determining the clearance zone developed around the colonies growing on the colloidal chitin agar medium (Cody, 1989; Wirth and Wolf, 1990). The potency of the isolates for chitinase production is determined on the basis of ratio of zone of clearance (CZ) to colony size (CS) (Cody, 1989). This procedure requires longer incubation time for about 5 to 6 days and is relatively less sensitive because of the poor visualization of the CZ. More sensitive, rapid and user friendly method for screening the chitinolytic microorganisms by incorporating calcofluor white M2R (0.001% w/v) in chitin agar has been developed (Vadiya et al., 2003a).

Detection and quantification of chitinases activity: The activity of chitinases can be qualitatively assayed by using chitin agar plate either with or without fluorescent dye. Activity staining method can also be used for qualitative assay. Activity staining can be done by incorporating ethylene glycol chitin in the gel (Trudel and Asselin, 1989). However, this method has limitations since the gel can not be further used for protein staining and there is problem of mobility of chitinase in the gel because of the presence of polysaccharide in the gel. These problems have been over come by running protein sample in the gel without incorporating chitin followed by diffusion on chitin agar plate containing fluorescent dye (Gohel et al., 2005c). Colloidal chitin with Remazol Brilliant Blue R was also used as a substrate for colorimetric assay of chitinase (Gómez Ramírez et al., 2004). Various substrates used and the products

Table 4. Comparison of chemical and biological fungicides.

Characteristic	Chemical	Biological
Spectrum	Broad	Narrow
Impact on target	Immediate	Gradual
Development of resistance	Development of resistance has been reported	No report on development of resistance
Impact on environment	Leave hazardous residues in food, feed and water	Biodegradable, no residues, non-polluting and ecofriendly
Effect on animal	Hazardous for man, mammals, fishes and birds	Safe for man, mammals, fishes and birds
Effect on human health	Toxic and carcinogenic Immunological changes and impaired reproductive functions.	No ill effects reported.

measured for the estimation of chitinases are listed in Table 3.

BIOPROSPECTING OF DIVERSE CHITINASES

Biodiversity prospecting or bioprospecting is the exploration of wild plants and animals for commercially valuable genetic and biochemical resources. In many cases bioprospecting is a search for unique bioactive compounds in microorganisms, plants and animal species that thrive in extreme environments, such as rainforests, deserts and hot springs, etc. Within the microbial world, a large majority of species are as yet unexplored and understanding the genetic diversity within microbial world is a first step in mining useful products of biological importance. A potentially significant untapped source is the ocean. It covers more than 70% of the earth and harbors a vast majority of plants, animals and microorganisms. The ocean thus represents a virtually unexplored resource for discovery of novel compounds with useful applications (De-Long, 2005).

Diversity of culturable and unculturable microorganisms: Majority of bacteria in environmental niche cannot be isolated or cultured using traditional cultivation methods. Amann et al., (1995) reported that the culturability of microorganisms in sea water is as low as 0.001% as compared to 0.3% in soil. (Amann et al., 1995).

Molecular approaches for characterizing microbial species and assemblages have significantly influenced our understanding of microbial diversity and ecology. These approaches have provided unique insights into the uncultured microbial communities of soils and waters because they avoid the biases inherent in the traditional culture based microbiological techniques (Hugenholtz et al., 1998). Coastal sediments play a significant role in remineralisation of organic matter (Wollast, 1991; Ravenschlag et al., 1999). Few cultivation independent studies of microbial diversity in marine sediments have been conducted (Gray et al., 1996). The sequences recovered in these studies of microbial diversity revealed the presence of mainly unknown organisms only distantly related to known isolates.

The metabolic capabilities of unculturable bacteria and the role of these microbes in specific biogeochemical processes may be different from their culturable counterparts. An approach for exploring the metabolic capabilities of uncultured bacteria is to examine the genes involved in specific biogeochemical processes. This approach is a step towards identifying microbial groups driving these processes and determining whether the metabolism of cultured bacteria adequately represents the metabolic capabilities of the uncultured bacteria. Studies have already examined several genes encoding enzymes mediating biogeochemical reactions in C, N, and S cycles and have compared these genes in cultured and uncultured bacteria in marine environments. These enzymes, genes include, the nitrogen fixing enzyme (nifH), nitrite reductase (nirK and nirS), methane monoxygenase (pmoA), dissimilatory bisulfite reductase (dsv) in sulphate reducing bacteria and nitrous oxide reductase (nosZ) in denitrifying bacteria (Cottrell and Kirchman, 1999). Genes of cultured and uncultured bacteria were not identical suggesting that cultured bacteria do not adequately model biogeochemical processes driven by uncultured bacteria.

Molecular methods for isolating chitinases from uncultivated microorganisms: Genes encoding chitinases may be particularly interesting examples of non- essential genes in uncultured bacteria since previous work has suggested that the evolution of these enzymes has been impacted by lateral gene transfer (Gracia-Vallve et al., 1999). Chitinases are probably not essential for heterotrophic bacteria living in most environments including oceans because of availability of alternate organic carbon sources (Kirchman and White, 1999).Many types of cultured bacteria and archaea are known to degrade chitin but the identity of uncultured bacteria degrading chitin in nature is unknown. Chitinase

Table 5. Summary of chitinase genes.

Source	Host	Genes cloned	Remarks	Reference
Aeromonas hydrophila, Pseudomonas maltophila	*Bacillus thuringiensis* var. *israelensis*	Chitinase (CTS)	Increase in biocontrol potential	Wiwat et al., (1996)
Aeromonas caviae	E. coli	Chitinase (Chi A)	Highly substrate specific	Lin et al. ,(1997).
Bacillus licheniformis	B. thuringiensis	Chitinase (TP-1)	Tested against Spodoptera exigua larva	Tantimavanich et al., (1997).
Bacillus circulans	E. coli	Chitiase (Chi AI)	*Bacillus circulans* promoter could be recognized by *E.coli* transciption system	Zheng et al.,(1998).
Vibrio furnissii	E. coli	N-Acetylglucosaminidase (Exo II)	Unique substrate specificity and shares similarity with 5 bacterial and one yeast β-glucosidase	Chitlaru et al., (1996).
		Chitodextrinase (Endo I)	Periplasmic, converts chitooligomers to dimer and monomer	Keyhani and Roseman (1996).
Xanthomonas spp.	E. coli	Chitinase (Chi A)	Four domains with a family 18 catalytic domains	Sakka et al., (1998).
Serratia marcescens	E. coli	N-acetylglucosaminidase (Chb)	Main catalytic domain is domain III with α / β barrel fold.	Tews et al., (1996).

Table 6. Chitinase gene transformed transgenic plants.

Transgenic plant	Gene	Source	Pathogen	Reference
Broccoli	Endochitinase	Trichoderma harzianum	*Alternaria sp.*	Mora and Earle (2001)
Cucumber	Chitinase (RCC2)	Oryza sativa	Botrytis cinerea	Tabei et al., (1998)
Elite indica rice	PR-3 chitinase (RC7)	*O. sativa*	R. solani	Datta et al., (2001)
Grapevine	Class-I chitinase (RCC2)	*O. sativa*	Uncinula necator	Yamamoto et al., (2000)
Indica rice	Classs-I chitinase (Chi11)	*O. sativa*	R. solani	Datta et al., (2000)
Japonica rice	Class-I chitinase (Cht-2, Cht-3)	*O. sativa*	*Magnaporthe grisea*	Nishizawa et al., (1999)
Potato	Endochitinase [ThEn-42 (chit42)]	*T. harzianum*	Alternaria alternata, A. solani, Botrytis cinerea, R. solani	Lorito et al., (1998)
Strawberry	Chitinase	*O. sativa*	*Sphaerotheca humuli*	Asao et al., (1997)
Tobacco	Endochitinase [ThEn-42 (chit42)]	*T. harzianum*	*Alternaria alternata, A. solani, Botrytis cinerea, R. solani*	Lorito et al., (1998)
Wheat	Chitinase (chi11)	*O. sativa*	*Fusarium graminearum*	Chen et al., (1999)

genes cloned directly from uncultured marine microorganisms suggested the presence of a large pool of uncultured chitin degrading bacteria in aquatic systems. Information on bacterial chitinase genes is largely restricted to cultured γ-proteobacteria or gram positive bacteria. Since γ-proteobacteria are widespread in the ocean (Giovannoni et al., 2000), comparing chitinase genes in cultured and uncultured bacteria in this phylogentic group will prove informative. Gram positive bacteria are quite rare in sea water (Giovannoni et al., 2000). To access chitinase genes in uncultured γ-proteobacteria and in other bacteria, it may be possible to use a PCR based approach with oligonucleotide primers patterned after conserved amino acid residues or after

conserved nucleotide sequences of chitinase genes in cultured bacteria (Suitil and Kirchman, 1998).

Molecular methods are needed to study chitinase producers without the isolation of bacteria in pure cultures. Methods that use nucleic acid probes and PCR primers cannot be designed solely with cultured bacteria because nucleotide sequences of chitinase genes from cultured bacteria so far characterized are very different suggesting that the chitinase sequences from uncultured bacteria will differ from the culturable ones (Cottrell et al., 1999). One alternative approach that does not rely on conserved nucleotide sequences is to construct genomic libraries to retrieve genes from natural bacterial communities without cultivation (Schimidt et al., 1991).

Bacterial chitinase genes have been retrieved from diverse terrestrial environments including alkaline soils, sandy soils, and pastures. However studies of chitinases from aquatic systems are quite rare.

Biogeographical distribution of functional chitinases: Abundant production of chitin in sea, suggests that most marine bacteria are able to degrade chitin. However, studies with cultures showed that relatively few marine bacteria degrade chitin, ranging from 0.4- 19 % of the total cultured bacteria (Okutani et al., 1975). Extensive studies have been carried out for chitinase genes from diverse terrestrial environment such as grassland (Krsek et al., 2001), alkaline soil (Tsujibo et al., 2003), and sandy soil (Williamson et al., 2000). However few reports are available for chitinases in aquatic system (kirchman et al., 1999). The diversity of chitinase genes from the community DNA isolated from diverse aquatic habitats has been studied. Such studies have attempted to attribute biogeographical distribution of functional chitinases. Comparison of chitinase gene sequences from environments with distinct chemical and physical characteristics may yield insight into how environmental conditions select for enzymes with novel properties. The culture independent recovery of microbial genomes from environmental samples can be used to explore the nature of microbial community network and to monitor community structure over time (Lecleir et al., 2004).

When chitin degradation by the soil bacterium *Serratia marcescens* was investigated, it was found that in addition to chitinases, the bacterium also makes a protein called CBP21 which binds to and disrupts the chitin polymer making it more accessible to degradation by chitinases. It have been reported that adding CBP21 dramatically speeds up the degradation of chitin by chitinases. CBP21 works by binding to chitin through highly specific interactions that disrupt the chitin structure making the individual sugar chains in the chitin polymer more amenable to enzymatic degradation (Suzuki et al., 1998; Vaaje-Kolstad et al., 2005).

The discovery of this new protein that participates in chitin degradation has many potential applications. For example, transgenic plants that express both chitinases and CBP21 would be able to combat fungi by degrading chitin in their cell walls. A better understanding of natural chitin turnover increases our ability to interfere with chitin metabolism in insects and other microorganisms (Broglie et al., 1991; Jach et al., 1995; Ding et al., 1995; Vaaje-Kolstad et al., 2005).

BIOCONTROL

Microbial ecology principles will be important in any future efforts to practice ecofriendly pest management.

Presently, we lack a complete understanding of a number of important issues relating to interactions between pests and crop plants as well as between pests and other microorganisms. A lack of understanding of these interactions limits our ability to understand the processes that occur during our efforts to implement ecologically crop management. Unless a more thorough understanding of the ecofriendly principles dictating microbial and plant interactions is obtained, efforts to restore ecological integrity will remain empirical and will lack transferability between systems. On the other hand, by understanding particular model systems in which ecological restoration can be successfully implemented, it should be possible to develop common strategies and practices by which pest management can be relied upon in the future. The important ecological principles that will need to be understood as well as recent attempts to obtain information in these areas, will now be presented.

The general mechanism of biological control can be divided into direct and indirect effects of the biocontrol agent (BCA) on the plant pathogen. Direct effects include competition for nutrients or space, production of antibiotic and lytic enzymes, inactivation of the pathogen's enzymes and parasitism. Indirect effects include all those aspects that produce morphological and biochemical changes in the host plant, such as tolerance to stress through enhanced root and plant development, solubilization or sequestration of inorganic nutrients, and induced resistance (Viterbo et al., 2002).

a) Competition for substrates and site exclusion: It is a common mechanism for control of fungi where the antagonist and the pathogen are closely related (Muslim et al., 2003). There are many reports for controlling fungal wilt caused by Fusarium using non- pathogenic Fusarium (Lauter et al., 1990). Since both are closely related both will compete for the same nutrient and site of infection .For example it has been shown that when used in field they compete for carbon source and for site of infection (Larkin et al., 1998; Muslim et al., 2003). Root

colonization by introducing a more efficient root colonizer such as fluorescent pseudomonas (Plant Growth Promoting Rhizobia, PGPR) has also been shown to reduce the population of major and minor pathogens. *Pseudomonas aeruginosa* PNA1, isolated from rhizosphere of chicken pea plants, has been shown to be effective against a number of phytopathogenic fungi and oomycetes (Anjaiah et al., 2003)

b) Antibiosis : It is one of the important mechanisms of control of fungal infection. A number of highly effective disease-suppressive agents are found among the fluorescent pseudomonads, making this group of bacteria the most widely studied group of antibiotic producers in the rhizosphere. The first antibiotic clearly implicated in biocontrol by fluorescent pseudomonads was the phenazine derivatives that contribute to disease suppression. Although bacilli have received less attention as potential biocontrol agents than pseudomonads, evidence indicating that they may promote effective disease suppression is accumulating. The bacilli are particularly attractive for practical use because they produce stable endospores, which can survive the heat and desiccation conditions that may be faced by biocontrol agents (Turner and Backman, 1991; Lumsden et al., 1995; Osburn et al., 1995; Handelsman and Stabb, 1996). *Trichoderma* and *Gliocladium* are closely related funga1 biocontrol agents. Each produces antimicrobial compounds and suppresses disease by diverse mechanisms (Howell et al., 1993).

c) Induced systemic response (ISR): It is defined as the process of active resistance dependent on the host plant's physical and chemical barriers, activated by biotic and abiotic agents (inducing agent) (Leeman et al., 1996). This response involves production of many pathogenesis related proteins (PR-proteins) which mainly include (a) phenol oxidases, peroxidases and polyphenoloxidases (Nicholson and Hammerschmidt, 1992; Wojtaszek, 1997) and (b) enzymes like β-1,3 glucanases, chitinases, β-1,4 glucosidases and N-acetlyglucosaminidases (Heil and Bostock, 2002). Changes that have been observed in plant roots exhibiting induced system resistance include: (1) strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics (Yedida et al.,1999); (2) increased levels of enzymes such as chitinases, peroxidase polyphenol oxidase, and phenylalanine ammonia lyase (Nicholson and Hammerschmidt, 1992; Wojtaszek, 1997); (3) enhanced phytoalexin production (Marley and Hillocks, 1993); and (4) enhanced expression of stress-related genes (Zhang et al., 2002). However, not all biochemical changes are found in all bacterial-plant combinations.

d) Parasitism and production of extracellular enzymes: This is also one of the important biocontrol mechanisms for plant disease control. The ability of bacteria, especially actinomycetes, to parasitize and degrade spores of fungal plant pathogens is well established (Nelson et al., 1986).

Considerable effort has gone into identifying cell wall degrading enzymes produced by biocontrol strains of bacteria even though relatively little direct evidence for their presence and activity in the rhizosphere has been obtained. Chitinolytic enzymes produced by both *Bacillus cereus* (Chang et al., 2003) and *Pantoea agglomerans* are involved in biocontrol of fungal pathogens (Bonaterra et al., 2003).

Strategies used in biocontrol: The most widely used strategy for the biocontrol involves the expression of heterologous chitinase and glucanase genes in plants to reinforce the primary defence responses. Studies have confirmed that these genes and their protein products are important players in the defence responses of plants. The levels of chitinase and glucanase increase dramatically as soon as a pathogen attack occurs (Ferraris et al., 1987). Both these enzymes are responsible for disrupting the fungal cell wall and/or prevention of hyphal growth (Vaidya et al., 2001; Gohel et al., 2004), and therefore preventing the pathogen from further colonising the plant tissue. Over expression of these genes will therefore cause higher levels of the enzymes on the plant cell surface, which might lead to a faster and more effective interaction and neutralisation of the invading pathogen.

Another strategy that is based on the same principle, involves the expression of antifungal peptides that show specific activity against a pathogen such as *Botrytis cinerea* (Monteiro et al., 2003). Antimicrobial peptides are found in most plant species and a specific peptide often provides resistance to one or more pathogens. The mechanism of their action often entails the prevention of hyphal growth, once again limiting the infecting pathogen to the initial point of infection.

Chitinases not only play an important role in the defence mechanisms of plants, but also in the mycoparasitic processes of fungi. Mycoparasitic and antagonistic fungi have been studied in order to develop a biological alternative to the chemical fungicides currently dominating agricultural practices (Lorito et al., 1998; Frankowski et al., 2001; Masih et al., 2000 and 2001).

The antagonistic activity of biological control agents towards phytopathogenesis is based on the secretion of

the extracellular lytic enzymes. The antifungal mechanism of *Trichoderma*, extensively studied and widely used biocontrol fungus, relies on cell wall degrading enzymes such as chitinases, proteases and glucanases (Lorito et al., 1998). These enzymes are strong inhibitors of many important plant pathogens. The chitinases are able to lyse the chitin of cell wall of the mature hyphae, conidia, chlamydospores, and sclerotia. *Trichoderma* chitinases are substantially more antifungal than any other chitinases purified thus far from any other source when assayed under the same conditions. They are more active than corresponding plant enzymes, effective on a much wider range of pathogens, and are nontoxic to plants at high concentrations. The *Trichoderma* chitinase genes are capable of producing chitinolytic enzymes which reach the antifungal activity level of some chemical fungicides and extensive testing *in vitro* has shown that there are virtually no chitinous pathogens resistant to *Trichoderma* chitinases and hence they have become excellent candidates for reinforcing plant defense hypersensitive reactions (Perez et al., 1994). Proof of this concept has been clearly demonstrated within the agricultural community with the use of the *Trichoderma harzianum* 42kD endochitinanse gene (Carsolio et al., 1999; Margolles et al., 1996); transgenic lines of various agricultural crops including apple have been established and pathogenicity trials have clearly demonstrated improved resistance when plants were challenged with disease causing fungal pathogens (http://www.glfc.cfs.nrcan.gc.ca).

OPTIMIZATION AND FORMULATION OF CHITINOLYTIC ENZYMES AND MICROORGANISMS

Biofungicides for control of seedling diseases of economical important crop plants are attacked by various soil borne pathogenic fungi such as *Pythium*, *Fusarium*, *Rhizoctonia*, *Phytopthora* and others which cause either seed root before germination or seedling after germination resulting in billions of dollars of cumulative crop losses (Hebbar and Lumsden, 1999). Pigeonpea (*Cajanus cajan*) is very important legume of India and *Fusarium* wilt of pigeonpea causes loss of several million US$ (Reddy et al., 1990). Apart from *Cajanus cajan* there are many important crops found globally as well as in India, which are infested by *Fusarium* wilt such as melon (Luo et al., 2001), citrus fruit, groundnut (Manjula et al., 2004) cotton (Emani et al., 2003), wheat (Sivan and Chet, 1986), spinach (Tsuda et al., 2001), barley (Schwarz et al., 2001), cucurbits (Freeman et al., 2002), banana (Pegg et al., 1996), cabbage, tomato (Pascual and Melgarejo, 1997), cucumber (Martinez et al., 2003) sweet-potato (Clark and Moyer, 1988) and peas (Armastrong and Armastrong, 1974). Thus there is need to control the *Fusarium* wilt and other pathogens. Moreover, comparatively chemical fungicides are not efficient when compared to the biological fungicides (Table 4). Thus, optimization of product and its formulation are most critical aspects to translate laboratory scale activity into adequate field performance for any crop-protection agent. The formulation must be user friendly which has to fulfill several criteria including allowing a microorganism to retain and express its fungicidal properties; providing a significant extension of shelf life. Moreover, the critical important step which is facing problem in industrial scale is to harness the organism to industrial process of mass production especially in fermentation as well as incorporation into user-friendly formulations (De-Vrije et al., 2001).

Different strategies for enhancing chitinolytic enzymes production and biomass: Before formulation of chitinolytic microorganisms, the favourable medium constituents are required for increasing the growth and production of chitinolytic enzymes. Moreover to attain a cost effective process, it is imperative to select a growth medium. There are large numbers of reports available on conventional or one-factor-at a time and statistical method for designing/selecting the media for enhancing the growth and production of chitinolytic enzymes (Vaidya et al., 2001; Felse and Panda, 1999; Madhavan-Nampoothiri et al., 2004). However, the conventional or one-factor-at a time approach becomes extremely time consuming, expensive and unmanageable when large numbers of variables have to be studied and does not depict the combined effect of all the factors involved. Moreover, the method requires large number of experiments to deter- mine optimum levels, which are unreliable (Halland, 1989; Vaidya et al., 2001). Optimizing all the affecting parameters by statistical experimental designs can eliminate these limitations of a single factor optimization process collectively (Halland, 1989; Montogomery, 2000). The statistical methodologies are preferred because of various advantages in their use such as rapid and reliable short-listing of nutrients, understanding the effect of the nutrients at varying concentrations and significant reduction in total number of experiments resulting in saving time, glassware, chemicals and manpower (Srinivas et al., 1994; Carvalho et al., 1997; Vaidya et al., 2003). There are many other techniques available for screening and optimization of process parameters including non-statistical and self-optimization techniques (Felse and Panda, 1999). Numbers of statistical softwares are commercially available to carry out optimization such as Analyse-It, STATGRAPHICS Plus 5, Statistica, JMP Statistical Discovery Software, Design-Expert and MINITAB Release 13.3 (Sekel, 2001). After statistically optimizing the medium constituents in flask level, the resulted medium will be further used in fermentation process to

scale up the fungicidal microorganisms. Several methodologies have been adopted for mass production, but only submerged fermentation is widely used for economical approach in terms of high yields in relatively short period of time.

Strain improvement of chitinase production: Although naturally occurring organisms provide a major source of chitinolytic enzymes, genetic improvement plays an important role in their biotechnological applications. There are number of different methods available for strain improvement for increasing the chitinase production. These involve:

1) Random mutagenesis through physical (UV, Gamma etc.) and chemical (EMS) agents which have been employed to obtain improved biological strains, including *Pantoea dispersa* (Gohel et al., 2004) and *Alcaligenes xylosoxydans* (Vaidya et al., 2003);
2) Site directed mutagenesis where antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 were increased by addition of a cellulose binding domain (Limon et al., 2004);
3) Transposon mutagenesis and deletion analysis revealed that chi D codes for a transacting repressor of chitinase and chitobiase expression, where as the chi E codes for an inducer. Suzuki et al., (1998) have reported the presence of CBP21 (chitin binding protein) a major protein in the culture supernatant when *S. marcescens* 2170 was grown in the presence of chitin. Comparison of the amino acid sequence with that of other proteins showed that, CBP21, is similar to CHB1 of Streptomyces *olivaceoviridis* suggesting a wide distribution of this type of chitin binding protein in chitinolytic microorganisms;
4) Fungal protoplast fusion was used to improve the strain of *Trichoderma sp.* Limon et al. (2001) have produced hybrid chitinases with stronger chitin-binding capacity by fusing to Chit42 a Ch B D from *Nicotiana tabacum* ChiA chitinase and the cellulose binding domain from cellobiohydrolase II of *Trichoderma reesei.* In addition to mutation and protoplast fusion, molecular cloning is being effectively used to achieve overproduction of chitinases, change in the induction pattern or change in the localization of chitinase. From studies in *Serratia liquefaciens* (Joshi et al., 1988) the genes chi B, chi C, chi D and chi E were found to be closely linked with chi A at a separate location on the chromosome. The chi A and chi B genes code for chitinases, and chi C codes for chitobiase. Connell et al. (1998) have conducted experiments to show that endochitinase encoded by chi A gene is an extracellular protein, secreted by the eps system, which is also responsible for secreting the cholera toxin. These findings indicate that the chi A and cholera toxin have functionally similar extracelluar transport signals that are essential for eps dependent secretion. The description of some chitinase genes reported has been summarized in Table 5.

Transgenic plants: The activity of chitinase against fungi makes it an attractive candidate for developing resistant varieties of agriculturally important crop plants. Number of transgenic plants have been created by transforming chitinase gene from various microbial and plant sources. Table 6 gives the information regarding chitinase based transgenic plants.

Induction of chitinases: Production of chitinase in microorganisms is controlled by a repressor-inducer system in which chitin or the products of its degradation serve as inducers (Monreal and Reese, 1969). Chitinolytic microorganisms have trace quantities of constitutive enzymes that are continuously released even under starvation conditions. Chitobiase is always found to be produced along with chitinase in all media and is not found to get affected by catabolite repression. It was also found to be very effective in degrading the dimers and trimers of N-acetyl glucosamine, thus contributing to the release of chitinase inducers (Felse and Panda, 1999).

Repression of chitinase synthesis by glucose in almost all organisms indicates that catabolite repression may be involved in the regulation of microbial chitinases. Saito et al., (1998) have detected the role of glk A encoding glucose kinase to play a role in glucose repression of chitinase production in *Streptomyces lividans*. When a DNA fragment carrying glk A was introduced into a *S. lividans* mutant defective for glucose repression, its ability to utilize glucose and glucose repression of chitinase production was restored. This finding indicated that glk A is involved in catabolite repression.

In *Pseudomonas fluorescens* BL 915, Gaffney et al., (1994) found that the expression of uncharacterized chitinolytic activity was regulated by a two-component system consisting of a transmembrane environmental sensor protein (Lem A) and cytoplasmic response regulator protein (Gac A). Cloning of the gac A regulatory region from the strain BL 915 in certain heterologous soil isolates of *Pseudomonas fluorescens* was found to stimulate expression of otherwise latent genes indicating that global regulation by a two component system may be a common feature of the regulation of chitinase expression.

FORMULATIONS

The formulations must be fundamentally compatible with the biocontrol agents and generally, the formulated materials have superior action as compared to

Table 7. Biocontrol agents available in market for control of fungal plant pathogens.

Agents	Target pathogens/Disease	Plant	Reference
Candida oleophila I-182	*Botrytis* spp., *Penicillium* spp.	citrus, pome fruit	Ecogen, Inc., (2005) Cabot Blvd. West, Langhorne, PA 19074
Trichoderma spp.	pathogenic fungi that cause wilt, take-all, root rot, and internal decay of wood products and decay in tree wounds	flowers, fruit, ornamentals, turf, and vegetables	Binab, Box 161, 546 22 Karlsbor, Sweden
Fusarium oxysporum	*Fusarium oxysporum, Fusarium moniliforme*	basil, carnation, cyclamen, tomato	www.biofox.com
Trichoderma spp.	*Sclerotinia, Phytophthora, Rhizoctonia solani, Pythium* spp., *Fusarium, Verticillium*	flowers, strawberries, trees, vegetables	www.bioplant.dk
Pseudomonas aureofaciens	Dollar spot, Anthracnose, *Pythium aphanidermatum*, Michrochium patch (pink snow mold)	turf	www.ecosoil.com
Pseudomonas syringae	*Botrytis cinerea, Penicillium* spp., *Mucor pyroformis, Geotrichum candidum*	fruit, citrus, cherries, and potatoes	www.villagefarms.com/biosave/index.html
Pseudomonas fluorescens A506	Frost damage, *Erwinia amylovora*, and russet-inducing bacteria	almond, apple, apricot, blueberry, cherry, peach, pear, potato, strawberry, tomato	www.nufarm.com
Pseudomonas chlororaphis strain	leaf stripe, net blotch, *Fusarium* sp., spot blotch, leaf spot, and others	barley and oats	www.bioagri.se
*Bacillus subtilis*GB03*, other B. subtilis,B. lichenformis, B. megaterium*	*Rhizoctonia, Pythium, Fusarium,*and *Phytophthora*	Greenhouse and nursery	www.growthproducts.com
*Burkholderia cepacia*type Wisconsin	*Rhizoctonia, Pythium, Fusarium*, and disease caused by lesion, spiral, lance, and sting nematodes	alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetable crops, and wheat	www.helenachemical.com
Myrothecium verrucaria	Parasitic nematodes	cole crops, grape, ornamentals, turf, trees	www.valent.com
Bacillus licheniformis SB3086	Dollar spot, low and moderate disease pressure	turf	www.novozymes.com/ecoguard
Fusarium oxysporum	*Fusarium oxysporum*	asparagus, basil, carnation, cyclamen, gerbera, tomato	Natural Plant Protection, Route d'Artix B.P. 80,64150, Nogueres, France
Agrobacterium radiobacter Strain 84	crown gall disease caused by *Agrobacterium tumefaciens*	fruit, nut, and ornamental nursery stock	www.crowngall.com
Bacillus subtilis MBI600 (rhizobia also in formulation)	*Fusarium* spp.*, Rhizoctonia* spp*, Aspergillus*	soybean, alfalfa, dry/snap beans, peanuts	www.beckerunderwood.com
Burkholderia cepacia	*Rhizoctonia solani, Fusarium* spp., *Pythium* spp.	vegetables, cotton	www.soiltechcorp.com
Bacillus subtilis GB03	*Rhizoctonia solani, Fusarium* spp., *Alternaria* spp., and *Aspergillus* spp. that attack roots	cotton, legumes	www.gustafson.com
Coniothyrium minitans	*Sclerotinia sclerotiorum* and *S. minor*	cucumber, lettuce, capsicum, tomato, and ornamental flowers in greenhouse production	BIOVED, Ltd., Ady Endre u. 10, 2310 Szigetszentmiklos, Hungary

Table 7. Contd

Streptomyces griseoviridis strain K61	*Fusarium* spp., *Alternaria brassicola, Phomopsis* spp., *Botrytis* spp., *Pythium* spp., and *Phytophthora* spp. that cause seed, root, and stem rot, and wilt disease	field, ornamental, and vegetable crops	www.agbio-inc.com
Agrobacterium radiobacter K1026	*Agrobacterium tumefaciens*	fruit and nut trees, caneberries, roses, and other ornamental nursery stock	www.bio-care.com.au
Pythium oligandrum	*Pythium* spp., *Fusarium* spp., *Botrytis* spp., *Phytophthora* spp., *Aphanomyces* spp., *Alternaria* spp., *Tilletia caries*, *Pseudocercosporella herpotrichoides, Gaeumannomyces graminis, Rhizoctonia solani, Sclerotium cepivorum*	vegetables (tomatoes, potatoes, pepper, cucumbers, Brassicaceae vegetables), fruits (grapes, strawberries, citrus), legumes, cereals, canola, forest nurseries and ornamental plants	biopreparaty@mbox.vol.cz
Gliocladium catenulatum	soilborne pathogens that cause seed, root and stem rot, and wilt disease	ornamental, vegetable, and tree crops	www.agbio-inc.com

unformulated materials (Rhodes, 1990). There are particular challenges to be faced, because the active ingredient is a living organism which must be kept relatively immobile and inactive in storage, but quickly resumes its normal metabolic processes once applied to target site. To achieve this some form of drying or lyophilizaion is required (Tariq et al., 1999).

Fungal antagonists can be formulated as wettable powder, granular powder, fluid-bed granules using dextrin as binder and a reduced content of alginate. Alginate gel has also been used to prepare bacterial and fungal formulations (Campbell, 1989; Desai et al., 2002; Hokkanen, 1990). Different forms of *Penicillium oxallicum* conidia such as freeze drying and spray drying were used for the control of *Fusarium* wilt in tomato (Larena et al., 2003).

Bacterial antagonists have been formulated in variety of ways to control plant pathogens. The sporulating, gram-positive bacteria offer a solution to the problem of stability and desiccation. Gram-positive microorganism offer heat and desiccation resistant spores that can be formulated into stable and dry powder products. Such formulations are then usually used as a wettable powder, water-dispersible granules or dust where proxel will generally be used to prevent the microbial contamination (Benitez et al., 1998; Hokkanen, 1990; Hornby, 1990).

Another approach is the suspension of organisms in oil, where the purpose is to exclude oxygen which prevents respiration (Honeycutt and Benson, 2001). Generally active ingredients are used which are encapsulated and then suspended in an oil base. Addition of silica gel to oil formulation has been found to prolong shelf life as has been reported for mutated conidia (Moore et al., 1995 and 1996; Tariq et al., 1999)

The non spore forming organisms are more difficult to formulate because they do not have the longer survival time and are readily killed by desiccation. These are traditionally formulated into various solid carriers such as wettable powder. Liquid formulations with either aqueous or mineral oil are user friendly which help in slow, continual growth of the organisms. However this problem still needs to be solved.

Chung et al., (2005) have developed formulation containing S*. padanus* + 1% (w/w) PBGG (A granulate biofungicide named PBGG which was developed by combining *Pseudomonas boreopolis* with *Brassica* seed pumice, glycerine and sodium alginate) which helped in controlling *Rhizoctonia solani* which cause damping-off of Chinese cabbage. Granular formulation of *T. virens* GL21 in combination with *B. cepacia* BC-1 or *B. ambifaria* BC-F was applied as a seed treatment of cucumber, where significantly improved suppression of damping-off caused by *R. solani* was reported (Roberts et al., 2005). Viswanathan and Samiyappan (2002) have used talc based formulation in *Pseudomonas* strains against red rot disease in sugarcane which played role in its management. Pesta and rice flour formulations of BNR fungi (binucleate *Rhizoctonia* sp.) provided effective control of preemergence damping-off of implants caused by *R. solani* (Honeycutt and Benson, 2001). Biocontrol of *Rhizoctonia* diseases by BNR fungi have been reported for bean (Cardoso and Echandi, 1987), cabbage (Ross et al., 1998), potato (Escandi and Echandi, 1991), and cucumber (Villajuan-Abgona, 1996). Harris et al. (1994) reported that isolates of BNR were effective for control of damping-off caused by *R. solani* in *Capsicum* spp. (Honeycutt and Benson, 2001). The list of biocontrol agents available in market are listed in Table 7 and rese-

Table 8. Examples of bacterial agents for biocontrol of fungal plant pathogens

Agents	Target pathogens/Disease	Plant	Reference
P. fluorescens VO61	Pythium ultimum	*Lotus corniculatus*	Bagnasco et al., (1998)
Pichia anomala	*Penicillium roquefortii*	Wheat, rye, barley and oats	Petersson and Schnurer (1998)
P. fluorescens VO61	Pythium ultimum	*Lotus corniculatus*	Bagnasco et al., (1998)
B. subtilis BACT-D	*Pythium aphanidermatum*	Tomato	Utkhede et al., (1999)
Paenibacillus sp. 300	*Fusarium oxysporum*	Cucumber	Singh et al., (1999)
Pseudomonas aureofaciences AB254	Pythium spp.	Sweet corn	Mathre et al., (1999)
P. aureofaciens AB244		Tomato	Warren and Bennett (1999)
P. fluorescens VO61	Rhizoctonia solani	Rice	Vidhyasekaran and Muthamilan, (1999)
P. fluorescens WCS358	F. oxysporum *f.* sp. *lini*	Flax	Duijff et al., (1999)
P. putida BTP1	Pythium aphanidermatum	Cucumber	Ongena et al., (1999)
Serratia plymuthica	*Pythium ultimum*	Cucumber	Benhamou et al., (2000)
Bacillus brevis	*Fusarium udum*	Pigeonpea	Bapat and Shah, (2000)
Pseudomonas putida B E2	*Verticillium dahliae* Kleb	Strawberry	Berg et al., (2001)
Alcaligenes xylosoxydans	*Fusarium udum*	Pigeonpea	Vaidya et al., (2003b)
P.dispersa	*Fusarium udum*	Pigeonpea	Gohel et al., (2004)

arch reports of available biocontrol agents are listed in Table 8.

The biocontrol developers faced a problem in developing the control of diseases because crops are grown under a multiplicity of climatic and environmental conditions which include temperature, rainfall, soil type, crop variety which change from farm to farm or even within one field (Tariq et al., 1999). Thus, factors such as soil texture, potentiality of biocontrol agent, biotic condition and type of pathogens need to be considered when attempting to develop new potential biocontrol agent for particular area (Powell et al., 1990).

FUNGAL PLANT PATHOGEN MANAGEMENT BY COMBINATION OF CHEMICAL AND BIOLOGICAL FUNGICIDES

Commercial production and application of biopesticides at farm level demands a few prerequisites such as viability for longer period, high tolerance to variable weather conditions and physiological stress associated with transportation, storage and application which are cost effective, easy to handle, have no adverse effects on seed germination and plant growth. Thus, technology must be developed to extend their range of effectiveness against multiple pathogens as well as formulations that provide suitable life, efficiency and user friendliness in the environment (De-Vrije et al., 2001). Moreover, replacing pesticides entirely by biological agent is not always an attainable goal (Benitez et al., 1998). Integrated biological and chemical control seems to be a very promising way of controlling pathogens with minimal interference with the biological equilibrium (Baek and Cook 1982; Lorito et al., 1993). Different fungicides and soil fumigants are widely used for controlling soil borne plant pathogens. Because of the concern regarding the toxicity of these compounds, there is a general trend to reduce the amounts applied to soil (Chet, 1987). One of the most attractive ways of reducing the amounts of fungicide is the integration of sub lethal doses of chemicals with the biocontrol agent which is resistant to the doses of the chemical fungicide. This method of control has been found to attain a degree of disease suppression equal to that with a full fungicide treatment in a number of cases. El-Tarabilly et al. (2000) reported biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. Benitez et al. (1998) used *Trichoderma harzianum* with Methyl bromide to control infection of *R..solani* and *F. solani* on tobacco. Chet et al. (1994) also used *T. harzianum* with captan to control *Verticillium dohliae* infection of potato. Chet (1987) reported that *T. virens* with metalaxyl helps to control the infection *P. ultimum* on cotton. Hader et al., (1979) used *T. harzianum* with PNCB to control *R..solani* infection on egg plant. Elad et al., (1983) also used same

mixture as described by Hader et al. (1979) to control *R..solani* infection of *S. rolfsii* infection on peanuts.

CONCLUSION AND FUTURE CONSIDERATION

Food security is one of the essential existential needs which can never be ignored by any society. The world population is expected to rise to around 10 billion mark by 2025. More people mean more food. To supplement the nutritional need, agriculture must become intensive and sustainable, to maintain and enhance, without destroying the environment on which it depends. The development of such a global system for sustainable food production is one of the greatest challenges faced by the humans.

The quantum of fertile soil is limited. Secondly the expansion of urban settlements, industrialization and provision for ever growing civic needs are exerting severe pressure on lands reserved for cropping. Most of the population explosion is witnessed in developing and under developed countries. It is speculated that global demand for cereals will increase from 1 billion tonnes to 2.7 billion tonnes and considering the losses in storage and processing, the real need may be about 3.4 billion tones. Most of this additional demand needs to be met either by improving the crop yield or by preventing microbial infection and the post-harvest losses till the produce reach the consumers.

Studies on chitinolytic microorganisms have yielded a large increase in knowledge regarding their role in inhibition of growth of fungal plant pathogens. Still this knowledge is not sufficient enough to formulate a preparation based on these agents that can work efficiently in all different environmental conditions. The biocontrol agents are also affected due to the geological and environmental conditions. Studying the environmental conditions of the area where biological control agent has been employed and statistical based formulation approach techniques will increase life span of microorganisms in different ecological conditions. Moreover, extensive studies are required on the maximum utilization of chitinous wastes for production of chitinases and biomass.

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