Characterization of chitinolytic ability of *Bacillus subtilis* AF 1 and its use in development of improved formulation for plant growth promotion and disease control

A thesis submitted for the degree of
Doctor of Philosophy

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CERTIFICATE

This is to certify that **Ms. Manjula Karpurapu** has carried out the research work embodied in the present thesis entitled **"Characterization of chitinolytic ability of *Bacillus subtilis* AF 1 and its use in development of improved formulation for plant growth promotion and disease control"** for the degree of **Doctor of Philosophy** under my supervision in the Department of Plant Sciences. School of Life Science, University of Hyderabad.

This work has not been submitted for the award of any degree or diploma of any other University or Institute.

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DECLARATION

1 hereby declare that the work presented in this thesis entitled **"Characterization of chitinolytic ability of *Bacillus subtilis* AF 1 and its use in development of improved formulation for plant growth promotion and disease control"** has been carried out by me under the supervision of **Dr. Appa Rao Podile** in the Department of Plant Sciences, School of Life Sciences. University of Hyderabad, Hyderabad - 500 046, and that this work has not been submitted for any degree or diploma of any other University or Institute.

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Manjula Karpurapu

Contents

List of Abbreviations

CC	Colloidal Chitin
cm	Centi meter
CMC	Carboxy Methyl Cellulose
CMCase	Carboxy Methyl Cellulase
DW	Distilled Water
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immuno Sorbent Assay
endo-PG	Endopolygalacturonase
exo-PG	Exopolygalacturonase
glucanase	β-1,3-glucanase
h	hour
log CFU	$\log_{10}$ Colony Forming Units
mg	milli gram
min	minute
ml	milli liter
MM	Minimal Medium
NA	Nutrient Agar
NAGase	β-1,4-N-acetyl glucosaminidase
PAGE	Poly Acrylamide Gel Electrophoresis
PDB	Potato Dextrose Broth
PGPR	Plant Growth Promoting Rhizobacteria
PL	Pectate Lyase
PME	Pectin Methyl Esterase
PMSF	Phenyl Methyl Sulfonyl Fluoride
RM	Richards Medium
Tween 20	Sorbitan monolaurate
μg/μm	10^{-6}gram/10^{-6} meter
YEPD	Yeast Extract Peptone Dextrose Medium

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Fig.3.5c Shelf life of alginate-based formulation: Alginate beads prepared from mid log, late log and sporulating phase grown AF 1 cells and stored at 4°C and 30^0C were destabilized in 0.2 M KH_2PO_4 buffer. Aliquots were plated on NA-ampicillin plates after serial dilutions. The log CFU were recorded for alginate beads containing immobilized mid log, late log and sporulating phase AF 1 cells stored at 4^0C and 30°C.

Fig. 3.6a Shelf life of peat-based formulation: Mid log, late log and sporulating phase AF 1 culture was inoculated in to presterilized peat and stored at 4^0C and 30°C, separately. The log CFU of AF 1 cells in 1.0 g of peat was determined by vortexing the sample in saline and plating the aliquots on NA-ampicillin after serial dilution with saline. Log CFU of AF 1 were presented in formulations inoculated with mid log, late log and sporulating phase AF 1 cells and stored at 4^0C and 30°C.

Fig. 3.6b Shelf life of peat-based formulation prepared from diluted liquid cultures: 100-fold diluted mid log, late log and sporulating phase AF 1 culture with H_2O and PDB separately was inoculated in to presterilized peat and stored at 30^0C. The log CFU of AF 1 cells in 1.0 g of peat was presented in peat inoculated with mid log, late log and sporulating phase AF 1 cultures.

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treatments with freshly grown AF 1, alginate, peat, peat+chitin, peat+*A. niger* mycelium and spent compost.

Fig. 3.10 Biological control of Fusarial wilt in pigeon pea: Pigeon pea seeds [wilt susceptible (ICP 2376) and resistant (ICP 8863) genotypes] were treated with freshly grown AF 1 and different formulations stored for four months after preparation. Treated seeds were sown in native and *F. udum*-infested soil separately. Percent disease control was presented in a) susceptible (ICP 2376) and b) resistant (ICP 8863) genotypes.

Plant growth promoting activity of formulations under disease pressure was assessed in terms of percent increase in emergence (c & d), plant length (e & f) and dry weight (g & h) was presented in thiram, freshly grown AF 1 cells, alginate, peat+chitin and spent compost treatments.

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Plant growth promoting activity of formulations under disease pressure was assessed in terms of percent increase in emergence (c & d), plant length (e & f) and dry weight (g & h) was presented in thiram, freshly grown AF 1 cells, alginate, peat+chitin and spent compost treatments.

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Introduction

Microorganisms in the soil and root interface:

Plant root surface (rhizoplane) is a natural habitat which represents **a** heterogeneous population of microbes comprising of both pathogens and non-pathogens. Quantitative assessment indicates that these microbes comprise a wide range of organisms including yeasts, fungi, bacteria, actinomycetes etc., Different plant parts represent specific ecological niches depending upon the nature of their substrates and the microorganisms harboured by them.

The root-soil surface is a dynamic environment, where microorganisms, plant roots and soil constituents interact. Microbial colonization of either the root surface and/or the root tissues (root colonization) or the adjacent volume of soil under root influence (rhizosphere colonization) has a substantial impact on plant fitness and soil quality (Lynch, 1990). Microorganisms which are stimulated to grow around plant roots, where exudates and other forms of carbon substrates are being supplied by the plant consist of two main groups: saprophytes and syrnbionts. Both of them comprise detrimental, neutral and beneficial bacteria and fungi. Detrimental microbes include major plant pathogens and mono-parasitizing and non-parasitizing deleterious rhizosphere organisms, either bacteria or fungi (Weller and Thomashow, 1994; Nehl *et al.,* 1996). Beneficial microorganisms, which include saprophytes and mutualistic symbionts, arc known to play an important role in sustainability of natural ecosystems and agro-ecosystems. Some of them can be used as inoculants for beneficial plant growth and health.

Need **for biological control of plant** pathogens:

Modem agriculture is highly dependent on use of increasing amounts of chemical pesticides. Repeated use of such chemicals has polluted the environment and encouraged the development of resistance among target organisms. The exposure of human population and natural habitats to increasing levels of pesticides is becoming unacceptable, and prompted the search for new strategies of pest control that reduce or eliminate the dose of pesticide. Biological control became an attractive proposition and, as a method for controlling plant pathogens, has been studied extensively.

Biological control of plant pathogens involves the use *of* biological processes to reduce the inoculum density of a pathogen and maintains soil populations below disease threshold levels. This reduces crop losses interfering minimally with the ecosystem and without damaging the environment.

Successful biocontrol of fungal pathogens in field and greenhouse conditions have been reported. The biological control of crown gall by *Agrobacterium radiobacter* K84 is an outstanding example of an antagonist that has effectively and economically controlled a major plant disease which has been commercialized (Kerr, 1980; Ryder and Jones, 1990). Many other important plant diseases were controlled by using strains of *Pseudomonas* spp. A fluorescent Psuedomonad RD: 1 was able to suppress the black root rot caused by *Thielaviopsis basicola* in tobacco seedlings with an associated increase in growth (Reddy and Patric. 1990). Seed treatment with *Pseudomonas fluorescens* strain WCS37 was able to suppress Fusarial wilt of radishes and increased the number of marketable radishes (Leeman *et al.,* 1990). Fluorescent Pseudomonads were used as biocontrol agents against forage legume root pathogenic fungi (Bagnasco *et al.,* 1998).

Plant Growth Promoting Rhizobacteria (PCPR):

The term "PGPR" for plant growth promoting rhizobacteria or yield increasing bacteria (YIB) describes a subset of rhizosphere microbes which cause increased plant growth after inoculation on to seeds. The early reports documenting plant growth and yield increases with PGPR involved root crop hosts (Burr *et al.,* 1978; Kloepper and Schroth, 1978; Geels and Schippers, 1983). There are several ways in which different PGPR have been reported to directly facilitate the proliferation of their plant hosts (Glick, 1995). PGPR can fix atmospheric nitrogen and supply it to plants; they synthesize siderophores that can solubilize and sequestrate iron from the soil and provide it to plants; they synthesize several different phytohormones that act to enhance various stages of plant growth; they may have mechanisms for the solubilization of minerals such as phosphorous that can then become more readily available for plant growth; and they may synthesize some less well characterized low molecular mass compounds or

enzymes that can modulate plant growth and development. A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms. These PGPR are active in many key ecosystem processes such as those involved in the biological control of plant pathogens, nutrient cycling and seedling establishment (Kloepper *et al,* 1991; Schippers *et al.*, 1995;Glick, 1995).

Kloepper *et al.* (1980) treated seed pieces of potatoes with *Pseudomonas fluorescens* and *P. putida* which increased the potato yield. Similar results of increase in yield was also reported by Suslow and Schroth (1982) using *Pseudomonas* spp. for seed treatment of sugarbeets. Microbes with proven performance in field trials and produced on commercial scale include two fungi *(Gliocladium virens* G-21 and *Trichoderma harzianum* KRL-AG-2), three Gram negative bacteria (*Agrobacterium radiobacter* K84, *Pseudomonas fluorescens* EG 1053, and *Burkholderia cepacia* type Wisconson), and three Gram positive bacteria *(Bacillus subtilis* GB03, *B. subtilis* MBI 600, and *Streptomyces griseoviridis* K61) (Cook *et al.,* 1996).

Biological control of plant pathogens by PGPR:

The first reports of PGPR on potato noted that growth promotion was associated with a reduction of total fungal propagules on the rhizoplane (Kloepper and Sehroth, 1981). This suggested that the select PGPR strains could also be used to reduce pathogen populations in the root zone. Some potato PGPR were subsequently shown to reduce populations of soft rot causing *Erwinia carotovora* (Kloepper, 1983). Broadbent *et al.* (1971) isolated *Bacillus subtilis* A13 which exhibited antagonism against many soil born plant pathogens and when applied at the rate of 10^6 - 10^7 cells per seed of barley, controlled diseases caused by *Rhizoctonia solani, Pythium* spp. and *Fusarium* spp. A13 was also effective against fungal pathogens of other cereals and carrots. Seed treatments by antagonists like *Bacillus subtilis, Trichoderma* spp., *Penicillium* spp. and *Chaetomiun globosum* effective for protection of cow pea and soybean (Kommedahl *et al.,* 1981). This method equalled the treatments of the thiram and captan.

Mechanisms involved in biological control by PGPR:

Different mechanisms employed by biocontrol agents through which they prevent/lessen the population of pathogens include

1) Competition for nutrients
2) Production of antibiotics
3) Production of lytic enzymes
4) Induced resistance
5) Inhibition of pathogenecity-related enzymes

1) Competition for nutrients: PGPR can prevent the proliferation of phytopathogens, and thereby facilitate plant growth, is through the production and secretion of siderophores with a very high affinity for iron (Castignelli and Smarrelli, 1986). The secreted siderophore molecules bind most of the Fe^{++} available in the rhizosphere, and as a result effectively prevent the pathogens in this immediate vicinity from proliferating because of lack of iron (O'Sullivan and O'Gara, 1992). Evidence for this mechanism comes from a number of different studies including a report that a mutant strain of *Pseudomonas putida* overproduced siderophores and was more effective than wild type in controlling a strain *of Fusarium oxysporum* that is pathogenic to tomatoes (Vandenburgh and Gonzalez, 1984). In another report, a mutant strain of *P. aeruginosa* that was deficient in siderophore production no longer protected tomato plants against *Pythium*-damping off (Buysens *et al.,* 1994). Direct confirmation that PGPR in the rhizosphere actually synthesize siderophores in response to iron-limiting conditions came from a study in which monoclonal antibodies were used to develop an enzyme linked immunosorbent assay (ELISA) to quantify the amount of siderophore from a fluorescent Pseudomonad that was present in a barley rhizosphere (Buyer *et al,* 1993).

2) Production of antibiotics: One of the most effective mechanisms that PGPR employ to prevent proliferation of phytopathogens is the synthesis of antibiotics. Evidence for the direct involvement of antibiotic production in PGPR-mediated disease suppression has come from two different types of experiments:

a) Antibiotic non-producing mutants of several different disease-suppressive bacterial strains were no longer able to prevent phytopathogens causing damage to plants (Thomashow and Weller, 1988; Howie and Suslow, 1991; Keel *et al,* 1992).

b) When an antibiotic-producing (wild-type) strain of *Pseudomonas fluorescens* was genetically manipulated to overproduce the antibiotics pyoluteorin and 2,4-diacetyl phloroglucinol, the resultant strain protected cucumber plants against disease caused by *Pythium ultimum* more than did the wild type strain (Maurhofer *et al,* 1992; Schnider *et al,* 1994).

3) Production of lytic enzymes: Some PGPR strains have been found to produce enzymes that can lyse fungal cell walls. Cell walls of most of the phytopathogenic fungi (except Oomycetes) are made up of chitin, which is a homo polymer of N-acetyl glucosamine residues linked in β-1,4 linkage. Chitinases which hydrolyse this polymer are produced by various organisms and have been implicated in the control of fungal diseases. Chitinases are defined as enzymes cleaving a bond between C_1 and C_4 of two consecutive N-acetyl glucosamines of chitin. The mycolytic activity of fungal as well as bacterial antagonists is mainly due to the lytic enzymes like Chitinases and glucanases (Mitchell and Alexander, 1962; Henis and Chet, 1975). Although neither the *fi*- 1,3-glucanases nor Chitinases from numerous bacterial species or fungi function as antifungal agents, the two activities in combination act synergistically to provide effective control against several plant pathogenic fungi (Ogasawara and Tanaka, 1978; Tanaka and Watanabe, 1995).

Chitinase is an inducible enzyme secreted by many microorganisms in cultures containing chitin or its oligomers as sole carbon source (Monreal and Reese, 1969). Chitinolytic enzymes have been implicated as factors contributing to the ability of *Pseudomonas stutzeri* to act as biocontrol agent (Lim *et al,* 1991). The extracellular chitinase and laminarinase produced by *P. stutzeri* could digest and lyse *Fusarium solani* mycelia there by preventing fungus from causing crop loss owing to root rot. Reduction in the incidence of plant disease caused by *Rhizoctonia solani, Sclerotium rolfsii,* and *Pythium ultimum* by using a β-1,3-glucanase producing strain of

Pseudomonas cepacia that was able to damage fungal mycelia was achieved (Fridlender *et al.,* 1993). Evidence for the biological control of *Rhizoctonia solani* and *Fusarium oxysporum* by chitinase of *Aeromonas caviae* was documented by Inbar and Chet (1991). It is even shown that a genetically engineered *Escherichia coli* with cloned chitinase inhibits fungal growth by breaking the tips of fungal germ tube (Shapira *et al.,* 1989). *Serratia marcescens* (Horwitz *et al,* 1984), *S. liquefaciens* (Joshi *et al,* 1988), *Vibrio vulnificus* (Wortman *et al,* 1986), *Streptomyces* spp. (Ueno *et al,* 1990) represent some of the potent bacterial chitinase producers. Extracellular p-1,3-glucanase isolated from *Bacillus circulans* IAM1165 lysed fungal cell walls most effectively (Aono *et al,* 1995).

4. Induced resistance: Treatments with biocontol PGPR rapidly activate multiple mechanisms of disease resistance which include the accumulation of antimicrobial low molecular weight chemicals (phytoalexins and leaf surface diterpenes) and protective biopolymers (lignin, callose and hydroxy proline rich glycoproteins). Increase in activity of enzymes in the pathways leading to production of such products, and increase in the amount of Chitinases, β-1,3-glucanases, peroxidases, and other pathogenesis related (PR) proteins, has also been reported (Lawton and Lamb, 1987). The induction of systemic resistance to *P. tabacina* in tobacco, coincided with the accumulation of β-1,3-glucanases, Chitinases, and other PR-proteins (Tuzun *et al.,* 1989), and an anionic isozyme of peroxidase (Ye *et al,* 1990). Enhanced peroxidase activity (Hammerschmidt and Kuc, 1982) and induction of Chitinases (Metraux and Boiler, 1986) and β-1,3-glucanases (Rabenantoandro *et al,* 1976) was found in systemically protected cucumber plants. Chitinases and p-l,3-glucanases, which are biologically active against fungi by hydrolyzing fungal cell wall polymers, and peroxidases, which generate H_2O_2 and oxidize phenols, are important in lignin biosynthesis. Seed treatment with *Bacillus subtilis* AF 1 stimulated phenylalanine ammonia lyase (PAL) in bacterized seedlings suggesting that some PGPR strains applied to seeds can influence the host plant

susceptibility to the pathogen by inducing resistance (Podile and Laxmi, 1998).

5) Inhibition of pathogenecity-related enzymes: **Synthesis of hydrolytic** enzymes by fungal pathogens during the first phase of host pathogen interaction is crucial for the infection process (Elad, 1997; Elad and Evensen, 1995). These enzymes include cell wall degrading enzymes i.e., pectolytic enzymes (exo- and endo- polygalacturonases, pectin lyases, pectin methyl esterases) (Van Den Heuvel and Waterreus, 1985) and cutinase (hydrolyses ester linkages of cutin polymer) (Shishiyama *et al.,* 1970) which are important pathogenecity factors. Inhibition of pathogenecity factors of pathogen is one of the mechanisms by which the biocontrol agent affects/reduces disease severity (Kapat *et al.,* 1998).

In addition to the aforesaid mechanisms there are few different ways by which biocontrol agents inhibit phytopathogens. The ability of some Pseudomonads to synthesize hydrogen cyanide (to which these Pseudomonads are themselves resistant) has been linked to the ability of those strains to inhibit some pathogenic fungi, although the role of hydrogen cyanide in disease suppression is not firmly established (Voisard *et al.,* 1989). Several different microorganisms including strains of *Cladosporium werneckii, Burkholderia cepacia* and *Ralstonia solanacearum* hydrolyze fusaric acid (Toyoda and Utsumi, 1991), the damage causative agent to plants that occurs upon *Fusarium* infection, and prevent plant diseases caused by various species of *Fusarium.*

Formulations of PGPR:

Technical problems involved in the successful inoculation of agricultural crops with biocontrol agents/PGPR include the delivery of sufficient inoculum to the target, economical production of large quantities of microorganisms, promotion of extended shelf life, and the development of convenient formulation. Beneficial strains of microorganisms are delivered commercially as formulated inoculants. Production of microbial formulations generally consists of a carrier medium and other additives which provide an acceptable shelf life, easy application, and enhancement of the performance of the microorganism. In most cases, the microbes are

produced initially through fermentation and mixed with the carrier material to obtain the final products.

The most important criterion for success in formulating microbes is the ability to deliver a critical number of viable microorganisms consistently (Paau, 1988). For microbial inoculants, the type of chosen formulation and delivery is a key point for the efficacy of the inoculum. Liquid formulations include aqueous, oil- or polymer-based products. Combinations such as oil-in-water emulsions are also used. Microbes are fermented directly in the liquid medium, added to the liquid base, or dried and then resuspended in the liquid medium to obtain the formulation product.

Polysaccharides and derivatives of polyalcohols are polymers frequently used to alter the fluid properties of the formulation. Most oil- and polymer-based products contain dormant microbes, while the aqueous fomulations may contain either dormant or metabolically active microbes. Liquid formulations are most easily obtained but they are not always satisfactory because storage and preservation are difficult. Solid fomulations, dehydrated or lyophilized, such as powders, give occasionally better results and the shelf life of inoculum can be extended.

Dry powder form and dry granules arc most successfully produced with spore forming microbes, where drying processes such as spray-dry, freeze dry or air-dry are used for this purpose. The yield of microorganisms from the drying process varies with different microbes and with their physiological state. All dry formulations contain dormant microbes where as moist powder and granular fomulations usually contain metabolically active microbes.

With solid formulations one of the main problems encountered is that microorganisms, before becoming active, have to be rehydrated, which causes generally a high loss in viability due to osmotic shock. Gelled formulations resolve in part the drawbacks previously described and, in this case the used techniques consist directly entrapping the microorganisms in polymer gels (Bashan, 1986). The biopolymers in gelled formulations are remarkable for their rheological properties which limit heat transfer when inocula are spray dried. They are very stable when stored dry and recover

their viscosity immediately after being applied to soil. In the field, the microorganisms are protected until the polymer structure has been totally degraded. Rhizobial inoculants entrapped in polyacrylamide, alginates, or xanthanes showed good recovery of viable cells even after prolonged periods of storage (Mugnier and Jung, 1985).

Application of formulation products:

Formulated microbes can be pre-coated on to the seed, mixed with the seed at planting, applied to the seed furrow, or mixed in the seed bed. The choice is usually dictated by the users routine practice and equipment availability, to which the properties of the formulations have been tailored. Pre-coating/seed bacterization is best performed with a formulation containing dormant microbes since dessication and substances present on the seed coat are deleterious to metabolically active microbes upon prolonged exposure. Formulations that contain a high moisture level are not desirable since the moisture may swell the seed coat, weaken the physical strength of the seed and trigger premature seed germination.

The formulations of prime importance for precoating seeds include dry powder and oil/and polymer based liquid inoculants. Materials such as xanthan gum and gum arabic arc sometimes used to help adhesion of the inoculant to seed. Formulations that are often mixed with the seed at planting include liquid based and moist powder inoculants with or without the use of sticker materials. Very fine dry powder formulations arc dusted on to the seed at planting. Formulations applied to the seed at planting usually deliver a high number *of* viable microbes and allow users to apply the inoculants directly in the planter box. If the formulations contain metabolically active microbes, delayed planting will result in loss of microbial viability/activity.

Liquid formulations are applied in to the seed furrow by either spraying or dipping. Application of inoculants to the seed furrow or the seed bed usually requires more material since the microbes are applied to the general vicinity of the seed rather than specifically to it. In some instances, this may be advantageous since there may be more opportunity for the microbes to exert their beneficial effects as the seed germinates and its roots

grow through the soil. Indeed, by increasing the biological activity of the inoculant strain and improving the tolerance of the strains to chemicals and temperature extremes, the pressure on a formulation to deliver a very high number of viable microbes may be lightened.

Chitin/chitosan in improving plant health:

Apart from microbial inoculants, application of biopolymers like chitin and chitosan to farming soils have shown interesting results. Use of chitosan as seed/leaf coating resulted in yield increase. Hadwiger *et al.* (1984) found that chitosan treatment in coating seeds had many beneficial effects, such as inhibition of fungal pathogens in the vicinity of the seeds and enhancement of plant resistant responses against diseases. By using this method crop yields were increased from 10% to 30% in wheat, peas and lentils and therefore, has been accepted for wheat coating in eleven states in the United States (Li *et al.,* 1997). Yano and Tsugita (1988) treated soybean seeds with 1% colloidal chitosan in 0.25% lactic acid. This resulted in earlier germination of treated seeds compared to control seeds. Pospieszny and Atabekov (1989) sprayed a dilute chitosan solution on *Phaseolus vulgaris* leaves, which resulted in a significant reduction in the number of local lesions produced by the alfalfa mosaic virus.

In plants chitin, chitosan and their derivatives enhance the induction of various biological self defense compounds, including phyloalexins (pisatin, phaseollin, rishitin, orcinol. ipomeamarone etc.), PR- proteins, protein inhibitors and lignins (Hirano, 1997). In response to chitin, chitosan and their derivatives, plant cells produce extracellular chitinase and phenyl alanine ammonia lyase. These enzymes prevent microbial infections in plants. Chitin and chitosan fertilized in to farming soils improved microbial flora with increase in total number of useful *Actinomycetes* and decrease of harmful *Fusarium* spp. Chitosan inhibits the growth of both *Fusarium* and *Helminthosporium* in an *in vitro* test, because of cationic chitosan chains (Hirano and Nagao, 1989). Water soluble chitin oligomers, chitosan, chitosan sulfate, carboxy methyl chitosan have shown antibacterial activity against pathogenic species of *Clavibacter michiganense* subsp. *michiganense, C. michiganense* subsp. *insidiosum, Xanthomonas compestris*

pv. *pelargonii*, *X. compestris* pv. *phaseoli*, *Pseudomonas syringae* pv. *phaseolicola*, *P. syringae* pv. *tomato, Erwinia amylovora, E. carotovora* sub sp. *carotovora, Agrobacterium tumefaciens* and *Escherichia coli*. Chitin and its derivatives have also shown antiviral and antiphage properties (Struszczyk and Pospieszny, 1997). Chitin and its derivatives are biologically degradable just like alginate which do not cause environmental pollution and, therefore, would be the choice supplements in PGPR inocula.

Similarly there were reports on use of urban and agricultural wastes for use as mulches on avocado and citrus and delivery of microbial biocontrol agents like *Trichoderma harzianum, Gliocladium virens* and *Pseudomonas fluorescens* (Casale *et al,* 1995). Results have shown that the addition of some composts to soil increased the incidence of PGPR in the tomato rhizosphere exhibiting antagonism towards *Fusarium oxysporum* f.sp. *radicis-lycopercisi*, *Pyrenochaeta lycopercisi*, *Pythium ultimum* and *Rhizoctonia solani* (Alvarez *et al.,* 1995).

Bacillus spp. as **bio-control PGPR:**

Members of the genus *Bacillus* arc Gram V endospore forming bacteria that grow aerobically. They are appealing candidates for biocontrol because they produce endospores tolerant to heat and dessication. *Bacillus subtilis* has been used for many years in attempts to control plant pathogens and increase plant growth. Seed treatments with *B. subtilis* have since been shown to control various diseases in a variety of crops (Merriman *et al,* 1974: Turner and Backman, 1991; Handelsman *et al,* 1990). *B. subtilis* obtained from lysed mycelium of *Sclerotium rolfsii* has been particularly useful in increasing yields and stimulating plant growth (Broadbent *et al,* 1971). Several antifungal metabolites of *B. subtilis* have been identified as typical peptides with broad spectrum of activity (Beckhouse and Stewart, 1989). Fungal death was not, however, directly related to the initial toxic effects of the antibiotics. It followed rupture of the hyphal walls, when gross disruption of cell compartmentation would cause cytoplasmic coagulation. Three possible mechanisms were attributed to hyphal bursting *viz.* a) increase in turgor pressure b) weakening of walls from internal processes, and c) action of bacterial enzymes. *B. cereus* RB14 has shown suppressive

effect on *Rhizoctonia solani* causing damping-off in tomatoes. The culture filtrate, cell suspension and culture broth were effective in decreasing the disease incidence in pot trials in sterile and non-sterile soils (Shoda *et al.,* 1997). *Bacillus* spp. L324-92 controls three root diseases of wheat, namely take-all caused by *Gaeumannomyces graminis* var. *tritici,* root rot caused by *Rhizoctonia solani* AG8, *Pythium irregulare* and *P. ultimum* (Kim *et al,* 1997).

B. subtilis **AF 1 as biocontrol PGPR:**

B. *subtilis* AF 1 was isolated from pigeon pea *(Cajanus cajan)* wilt non-conducive soils and was a potential antagonist of plant pathogenic fungi (Podile and Dube, 1985). AF 1 also promotes emergence and nodulation (Podile, 1995; Podile and Dube, 1988). Seed bacterization with AF1 enhanced the levels of total phenols, phenyl alanine ammonia lyase and peroxidase in pigeon pea seedlings with a corresponding decrease in wilt disease incidence, indicating the possible involvement of induced host plant resistance in AF 1 mediated disease control (Podile *et al.,* 1995). *B. subtilis* AF 1 extensively colonizes the surface of the groundnut *(Arachis hypogaea)* crown rot pathogen *Aspergillus nigcr* and lyses the mycelium under *in vitro* conditions (Podile and Prakash. 1996). The decrease in crown rot incidence in AF 1 treated groundnut seedlings was attributed to early induction of lipoxygenase (Sailaja *et al.,* 1998).

Objectives of the present study:

In light of the available information on the potential of *B. subtilis* AF 1 as a biocontrol PGPR strain. an attempt has been made to further understand the mechanism of action against fungi and also to develop a suitable formulation. The ability of AF 1 to produce fungal cell wall degrading enzymes was used to improve the formulation with chitin supplements. The formulations have been assessed for their biological control and plant growth promoting efficiency. In addition, an attempt was also made to study the effect of *B subtilis* AF 1 on the production of cell wall hydrolytic enzymes of two pathogens. Two types of plant pathogen systems were used to check the biocontrol efficiency of *B. subtilis* AF 1 and its formulations.

Materials and Methods

Materials:

The details of the materials used in the study are presented in Table 2.1.

Table 2.1. Different organisms, seed and other materials, specifications and source of the materials.

S.No.	Material Category	Specification	Source	Comments
1.	Organisms	a) *Bacillus subtilis* AF 1	a) Isolated from rhizosphere of pigeon pea in wilt non-conducive soils. Resistant to 100µg ml^{-1} ampicillin.	a) Gram +ve, spore forming bacterium with broad spectrum antifungal activity against fungal pathogens of plants.
		b)*Aspergillus niger*	b) Isolated from crown rot infected groundnut plants	b) Causes crown rot infection in groundnut
		c) *Fusarium udum*	c) Isolated from wilt infected pigeon pea plants.	c) Causes wilt in pigeon pea.
2.	Seed Material	a) pigeon pea (*Cajanus cajan*) ICPL 85010 ICP 8863 ICP 2376	ICRISAT, Asia Centre.	a) ICPL 85010 & ICP 8863 are wilt resistant genotypes. ICP 2376 is a wilt susceptible genotype.
		b) groundnut/ peanut *(Arachis hypogaea)* TMV 2 FDRS 10	-do-	b) TMV 2 is crown rot susceptible & FDRS 10 is a resistant genotype.

Cont'd.

3.	Carrier materials	a) Peat	a) Velina Scientifics, Hyderabad.	a) Most commonly used carrier material for rhizobial formulations.
		b) Sodium alginate	b) SD-fine chemicals, India.	b) Commonly used polyanion for entrapment of cells and enzymes.
		c) Spent Compost	c) Premier Mushroom Farms Ltd. Hyderabad.	c) Compost after cultivation of *Agaricus bisporus*, rich in organic matter and mycelium of *A bisporus.*
4.	Supplements	a) Powdered chitin	a) Sigma Chemical Company, USA.	a) Powdered chitin prepared from crab shells is used in growth of chitinolytic organisms and as substrate for assay of Chitinases.
		b) *A. niger* mycelium	b) Prepared in laboratory.	b) log phase grown mycelium was extensively washed and dried at 120^{0}C for 24 h.
5.	Pesticides	a) bavistin [carbendizim] b) thiram fbis-(dimethyl thiocarbamoy 1) disulfide]	Local pesticides shop.	Routinely used fungicides.

cont'd

6.	Media	a) Yeast Extract Peptone Dextrose Medium (YF.PD) b) Yeast extract supplemented Minimal medium with chitin as carbon source (MM) (Monreal and Reese, 1969) c) Potato Dextrose Broth (PDB) d) Nutrient Agar medium (NA) e) Richard's Medium (RM)	Ingradients were purchased from Himedia Chemicals, India.	To grow the organisms at a specific pH and temperature.
7.	Distilled water	Tap water was first distilled using a steel distillation unit and second time using a glass distillation unit.	Prepared in our laboratory	This would be referred as distilled water (DW) through out the text.

Methods:

I. Direct antagonism of *B. subtilis* AF 1 towards fungal pathogens:

a) Fungal cell wall degrading enzymes of B. subtilis AF 1:

Preparation of inoculum: *B. subtilis* AF 1 was grown in YEPD medium for 12 h at 30°C and 180 rpm on a rotary shaker. One ml of this culture containing 10^7 cells was used to inoculate 50 ml of MM in 250 ml conical flasks.

Preparation of enzyme: *B. subtilis* AF 1 was grown in MM pH 8.5 with 0.5% chitin/laminarin as sole carbon source at 30^0C and 150 rpm on a rotary shaker for glucosaminidase/ glucanase production, respectively. The culture was grown for 8 days for p-l,4-N-acetyl glucosaminidase and 4 days for β-1,3-glucanase production unless mentioned specifically. The culture was centrifuged at 7500 rpm for 10 min at 4^0C and the supernatant was filtered through 0.45 μm filter. The cell free culture filtrate was added with 0.002% sodium azide and

stored at 4°C. Before using for enzyme assays the filtrate was brought to room temperature.

Reagents for β-1,4-N-acetyl glucosaminidase assay (NAGase):

Reagent A: 6.1 g of dipotassium tetraborate tetrahydrate was dissolved in 100 ml of distilled water.

Reagent B: 1.5 ml of distilled water was added to 11.0 ml of concentrated HC1 and made up to 100 ml with glacial acetic acid. 10.0 g of para dimethyl amino benzaldehyde (DMAB) was dissolved in this mixture. 10 ml of this solution was diluted to 100 ml with glacial acetic acid just before use.

Preparation of colloidal chitin (CC): Colloidal chitin was prepared according to the method of Berger and Reynolds (1988). Ten grams of chitin was slowly dissolved in 400 ml of concentrated HC1 with stirring at 4°C. The mixture was incubated in water bath at 37^0C until viscosity decreased. To this mixture 4.0 liters of sterile distilled water was added and left overnight at 4^0C. The supernatant was slowly decanted and the precipitate was collected on a filter paper and washed extensively with sterile distilled water to attain neutral pH. The colloidal chitin was dissolved in 250 ml sterile distilled water prior to use.

Assay of NAGase:

NAGase was assayed colorimetrically as described by Boiler and Mauch (1988) using crude filtrate as an enzyme source. The reaction mixture consisted of 0.5 ml of 1.5% colloidal chitin. 0.5 ml of cell free culture filtrate of *B. subtilis* AF 1 and 0.5 ml of sodium acetate buffer pH 5.2. The reaction mixture was incubated at 37^0C for 3 h and centrifuged at 3000Xg. Aminosugar estimation (Rcisslig *et al.*, 1955) was carried out using 0.5 ml of the supernatant by adding 50 µl of reagent A and boiled for 3 min. The mixture was immediately cooled to room temperature and 1.5 ml of reagent B was added and incubated at 37^0C for 45 min. The color developed was recorded with appropriate blanks. One unit of enzyme activity was defined as µmole of N-acetyl glucosamine released ml^{-1} h^{-1}. The specific activity was expressed as units of enzyme activity per mg protein where the protein concentration was determined as described by Bradford (1976).

Reagents for **β-1,3-glucanase** assay:

DNS Reagent: 10.6 g of 3,5-dinitrosalicylic acid (DNS) and 19.8 g of NaOH were dissolved in 1416 ml of distilled water. To this mixture 306.0 g of sodium potassium tartarate. 7.6 ml of molten phenol and 8.3 g of sodium metabisulfite were added and dissolved. 3.0 ml of this reagent was titrated with 0.1 N NaOH which usually takes 5.0-6.0 ml HC1. NaOH was added if required (2.0 g = 1.0 ml 0.1 N HC1).

Assay of **β-1,3-glucanase:**

β-1,3-glucanase was assayed colorimetrically by measuring the reducing groups released from enzyme substrate reaction with dinitro salycylic acid (DNS) reagent (Miller, 1959). The reaction mixture consisted of 50 µl of cell free culture filtrate of *B. subtilis* AF 1, 50 ul of 4% laminarin and 50 µl of sodium acetate buffer pH 5.2 which was incubated at 37 C for 60 minutes and reducing groups were estimated using DNS reagent. One unit of enzyme activity was defined as µmol of glucose released ml^{-1} h^{-1}. The specific activity was expressed as units of enzyme activity per mg protein .

Optimum conditions for NAGase/ **β-1,3-glucanase** production:

To improve the yield of NAGase and p-1.3-glucanase in the cell free filtrate. different parameters including fermentation time. culture conditions, pH of the medium. nature and concentration of carbon source. concentration of yeast extract. potassium phosphate. Tween 20, EDTA (Ethylene Diamine Tetra Acetic acid) and PMSF (Phenyl Methyl Sulfonyl Fluoride) were optimized in the growth medium. Also. the effect of trace metal ions on NAGase and β-1,3-glucanase production was determined by including $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $ZnSO_4.2H_2O$ and $Co(NO_3)_2.6H_2O$ in the growth medium (Table 2.2).

Table 2.2. Details of the initial and modified culture conditions to improve the yield of NAGase and β-1,3- glucanase.

S. No	Parameter	Initial condition/conc. NAGase	glucanase	Alteration range NAGase	glucanase
1.	Fermentation time (days)	10	4	2-20	0.5-3
2.	Specificity of carbon source	CC	laminarin	CC *A niger* cell walls chitobiose chitosan NAG	laminarin
3.	Carbon source (%)	0.5	0.5	0.5 - 5.0	0.2 - 2.0
4.	pH of the medium	8.5	8.5	5.0-9.0	5.0-9.0
5.	Yeast extract (%)	0.03	0.03	0.05 - 0.5	0.05 - 0.5
6.	EDTA (mM)	-nil-	-nil-	0.1-1.0	0.1-1.0
7.	PMSF (mM)	-nil-	-nil-	0.1-1.0	0.1-1.0
8.	Trace metals (mM)				
	$FeSO_4.7H_2O$	-nil-	-nil-	1.0	1.0
	$MnCl_2.4H_2O$	-nil-	-nil-	1.0	1.0
	$ZnSO_4.2H_2O$	-nil-	-nil-	1.0	1.0
	$Co(NO_3)_2.6H_2O$	-nil-	-nil-	1.0	1.0
9.	Tween 20 (%)	-nil-	-nil-	0.02-0.1	0.02-0.1

Determination of cell numbers: The enzyme units. in all cases, were correlated with the cell numbers. Serially diluted culture in individual experiment was plated on NA supplemented with 100 μg ml^{-1} ampicillin and cell numbers were expressed as log CFU.

Partial purification of NAGase: NAGase of *B. subtilis* AF 1 was partially purified in a single step by affinity chromatography on a chitin column. *B. subtilis* AF 1 was grown in MM at pH 7.0 containing 3.0% colloidal chitin as sole source of carbon supplemented with 0.3% yeast extract, 0.04% Tween 20 and 0.5 mM PMSF at 30° C for six days at 150 rpm. The culture was centrifuged at lO.OOOXg for 10 min at 4° C. The supernatant was passed through a 0.45 μm filter to obtain a cell free culture filtrate and lyophilized. The

resulting powder was dissolved in 100 mM sodium phosphate buffer (pH 7.0) at 10 mg ml" (w/v). Two mg of crude protein was loaded on to chitin column.

Packing of chitin column: Ten grams of powdered chitin was washed extensively with one liter each of 1 N NaOH and 1 N HC1. The resulting material was washed with sterile distilled water until it becomes neutral and finally washed with 10 mM sodium phosphate buffer pH 7.0. The washed chitin was packed into a column (15 X 1.2 cm) at 4°C and equilibrated with 10 mM sodium phosphate buffer pH 7.0.

Affinity chromatography: The crude culture filtrate protein was loaded on to the column at one unit activity per 200 mg of matrix and the flow rate was adjusted at 15 ml h⁻ to achieve complete binding of NAGase to chitin. Only proteins with affinity for chitin get bound to the matrix and the unbound proteins were eluted using the 100 mM sodium phosphate buffer at 4^0C. The bound NAGase was eluted at 37^0C in 2 ml fractions using the same buffer. The fractions were analysed for NAGase activity. The purity of the enzyme was checked by polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) followed by activity staining.

Activity staining of NAGase: Fractions with high NAGase activity were concentrated by lyophilization and electrophoresed on 7.5% native polyacrylamide gel at 4^0C. The gel was washed in 100 mM sodium phosphate buffer pH 7.0 at 4°C for 10 min and overlaid with another 7.5% polyacrylamide gel containing 0.1% colloidal chitin (CC) and 100 mM sodium phosphate buffer pH 7.0 in place of resolving gel buffer. The gel containing electrophoresed Chitinases overlaid with substrate containing gel was incubated at 37^0C under moist conditions for 4 h. The activity of NAGase was visualized as clear lysis zones against opaque colloidal chitin substrate background.

In vitro inhibition of *A. niger* by partially purified NAGase of ***B. subtilis*** **AF 1:**

Growth of *A. niger* was studied in microtiter plates in presence of cell free culture filtrate, partially purified NAGase of *B. subtilis* AF 1, casein and peptone separately in an enzyme linked immunosorbent assay (ELISA) reader at 405 nm as described by Podile and Prakash (1996). *A. niger* spore suspension was prepared in potato dextrose broth to achieve a concentration of 10^5 spores per ml. To 25 |il of *A. niger* spore suspension in potato dextrose broth, crude

culture filtrate proteins and partially purified NAGase were added at a concentration of 1.0 mg and 1.0 µg, respectively to a final volume of 200 µl per well. Every alternate well was kept empty. For each concentration quadruplicates were maintained and the experiment was repeated twice. As a control, 25 µl of *A. niger* spore suspension was added to 175 µl of potato dextrose broth. Similarly potato dextrose broth with casein or peptone at 1.0 mg ml" and 25 µl fungal spore suspension in 200 ul volume were also maintained. The absorbance of each well added with protein and spore suspension was recorded at 24 h intervals.

b) Effect of B.subtilis AF 1 on hydrolytic enzymes of fungal pathogens:

Mycelial discs of actively growing *A. niger* and *F. udum* were inoculated separately in to 25 ml of RM in 250 ml conical flasks and allowed to grow at 30°C and 150 rpm on a rotary shaker for 24 h. AF 1 culture was grown in 25 ml of MM pH 7.0 for 6 h (up to mid log phase, containing 10^7 cells ml^{-1}) and the cells were pelleted, washed and suspended in 2 ml of sterile distilled water. The cell suspension was inoculated in to the above flasks at the time of inoculation of mycelial discs. *B subtilis* AF 1 grown alone in MM, *A. niger* and *F. udum* grown alone in RM with appropriate carbon source served as controls. The carbon source varied depending upon the enzyme studied. Sodium polypectate for exo- and endo-polygalacturonase, pectin for pectate lyase and pectin methyl esterase and Carboxy methyl Cellulose for Carboxy Methyl Cellulase were included in the medium as carbon source. At the same time another set of controls were maintained where growing cultures of *A. niger/F. udum* were inoculated with autoclaved mid log phase culture of AF 1 grown in MM (cell debris was washed and suspended in 2 ml of DW). The dual cultures were incubated for 6 days at 30°C, and the cultures were centrifuged at 10,000Xg for 5 min at 4^0C. The supernatant was filtered through 0.45 urn filters to obtain cell free culture filtrates and 0.002% sodium azide was added and preserved at 4^0C. Plant cell wall hydrolytic enzymes were assayed using these culture filtrates.

Assay **of plant cell wall hydrolytic enzymes:**

Exo-polygalacturonase (exo-PG): Exo-PG activity was measured as the release of galacturonic acid from sodium polypectate using Miller's (1959) DNS method. The reaction mixture contained 400 µl of 0.25% sodium polypectate

dissolved in 50 mM sodium acetate buffer pH 5.2 and 100 µl of culture filtrate. The net increase in galacturonic acid in the reaction mixture was determined by comparing the measured optical densities with those on a standard curve prepared with galacturonic acid. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 µmol galacturonic acid min^{-1} ml^{-1} of the culture filtrate, under the assay conditions.

Endo-polygalacturonase (endo-PG): Endo-PG breaks down polygalacturonic acid randomly to form polymer fragments terminated by a reducing group. To assay endo-PG, the reducing groups released from polygalacturonate was measured as described earlier by Collmer *et al.* (1988). The reaction mixture contained 50 µl each of 2% polygalacturonic acid, 0.4 M NaCl, 0.2 M sodium acetate buffer pH 5.2, distilled water (1:1:1:1 ratio) and 50 µl of culture filtrate. One unit of enzyme activity was defined as the amount of enzyme which released 1 µmol of reducing groups min^{-1} ml^{-1} of the culture filtrate, under the assay conditions.

Pectate lyase (PL): PL activity was assayed by measuring the increase in absorbance at 232 nm, due to the production of unsaturated bonds during the depolymerisation of polygalacturonic acid. The reaction mixture contained 2.5 ml of substrate (0.5% sodium polypectate dissolved in 50 mM Tris-HCl buffer and 1 mM $CaCl_2$) and 0.5 ml culture filtrate. One unit of PL activity was defined as the amount of enzyme that caused absorbance at 232 nm to increase at a rate of 1.0 min^{-1} ml^{-1} of the culture filtrate, under the assay conditions.

Pectin methyl esterase (PME): PME was assayed by the continuous titration technique as described by Elad *et al.* (1994). The pH of the enzyme substrate reaction mixture was measured immediately after the addition of the culture filtrate to the substrate (t = 0 min). The reaction mixture was incubated for 60 min at 30^{0}C in a temperature controlled water bath. The final pH (at t – 60 min) was restored to the initial level (pH at t = 0 min) by the addition of 0.02 M NaOH, and the volume of 0.02 M NaOH needed for the pH adjustment was used for the calculation of enzyme activity. One unit of PME activity was defined as the amount of enzyme that, under the standard conditions, required the addition of 1 µg of NaOH min^{-1} ml^{-1} of the culture filtrate to maintain the initial pH.

Carboxy methyl cellulase (CMCase): CMCase was measured in terms of reducing groups released from CMC. The reaction mixture contained 300 ul of 1% CMC dissolved in 50 mM acetate buffer, pH 5.2 and 200 ul culture filtrate. One unit of enzyme activity was defined as the amount of enzyme which catalysed the release of 1 umol glucose min^{-1} ml^{-1} of the culture filtrate, under the assay conditions.

II. Formulation of *B. subtilis* AF 1 and application of formulation products:

a) Formulations of B. subtilis AF 1 (Plate Fig. 1):

Alginate formulation: *B subtilis* AF 1 was grown to three different growth phases i.e. mid log (16 h), late log (32 h) and sporulating phase (72 h) in PDB medium at 30^0C and 150 rpm on a rotary shaker. 2% (w/v) sodium alginate was added to the cell suspension after adjusting the cell numbers to 10^{10} ml^{-1} and allowed to mix on the same rotary shaker at 100 rpm for 20 min at 30^0C. The mixture was added, drop wise, with the aid of a 5.0 ml sterile disposable syringe fitted with a needle in to sterilized and gently stirred 0.1 M $CaCl_2$ solution under aseptic conditions. The droplets formed gel beads immediately entrapping the *B. subtilis* AF 1 cells. The beads were allowed to stabilize with in the same $CaCl_2$ solution. Average size of these beads was 2.5 mm. After stabilization the beads were washed twice with sterile distilled water and divided in to two equal halves. One half of the beads were stored at 4 C (hydratcd) and the other half dried in laminar flow hood at 30^0C for 72 h (dehydrated) before storing them in sterile screw cap vials at 30^0C.

Solubilization of **beads:** To dissolve the alginate beads for bacterial counts, potassium phosphate buffer pH 7.0 (0.1 M to 1.0 M) and citrate phosphate buffer pH 7.0 (0.1 M to 1.0 M) were used and the rate of release of AF 1 cells was determined. One ml of buffer was used to destabilize single alginate bead. A buffer which destabilizes these hydrated and dehydrated beads faster with moderate osmolarity was chosen for further use.

Survival of AF 1 after dissolution of alginate beads:

Survival of AP 1 cells after the dissolution of alginate bead in the same buffer was assessed for 12 h by plating the aliquots after serial dilution on NA supplemented with 100 μg ml^{-1} ampicillin.

Plate Fig. 1 Formulations of *B. subtilis* AF 1 in different carrier materials: AF 1 was either immobilized in 2% sodium alginate, or liquid culture was directly inoculated in to peat, peat-supplemented with chitin/ *A. niger* mycelium and spent compost under aseptic conditions. Different formulations were stored in screw cap vials or in plastic self sealing bags.

Top left: Peat-based formulation
Top right: Peat+ chitin based formulation
Centre: Alginate-based formulation
Bottom left: Peat+*A. niger* mycelium-based formulation
Bottom right: Spent compost-based formulation

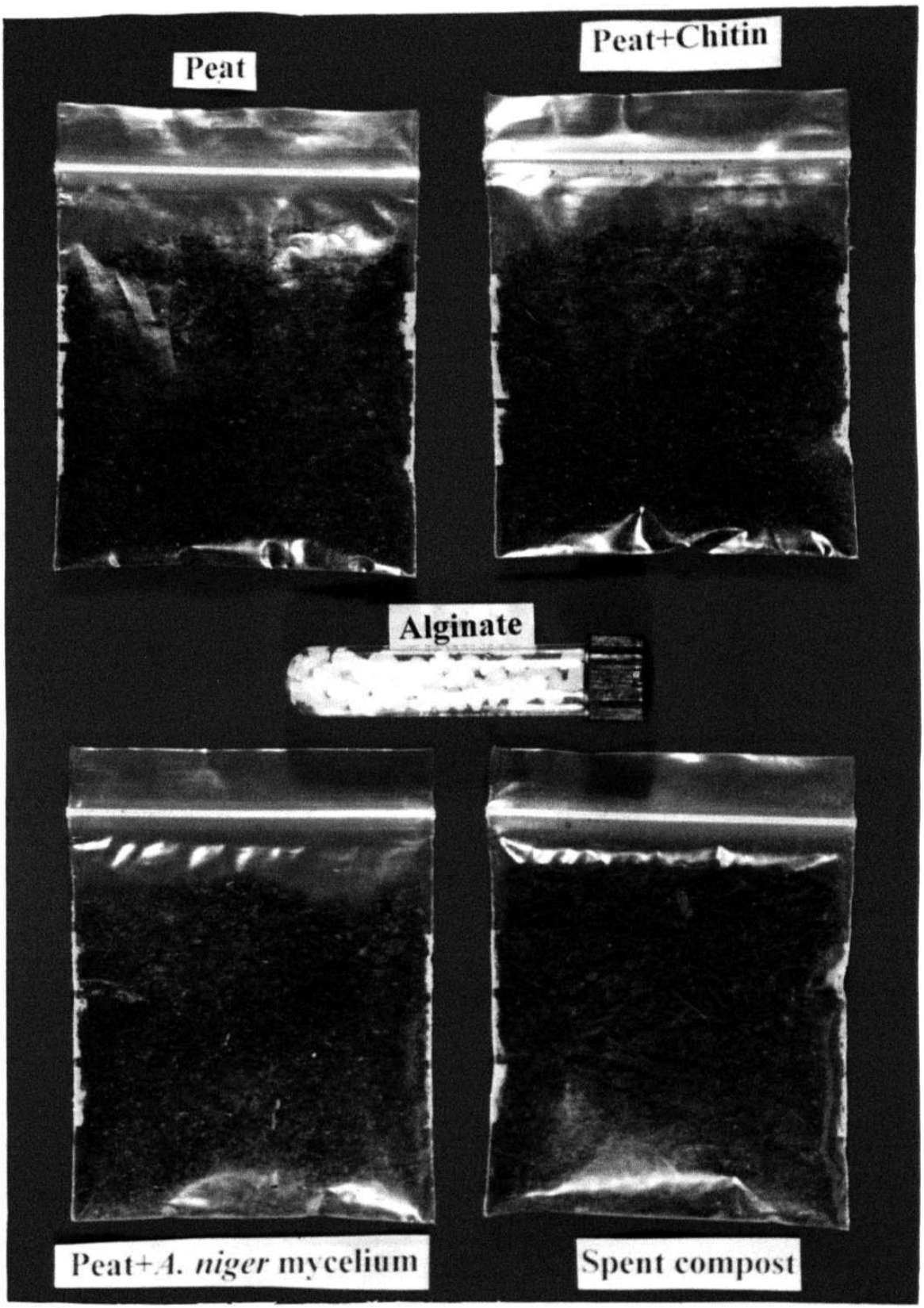

Peat
Peat+Chitin
Alginate
Peat+*A. niger* mycelium
Spent compost

Plate Fig 1

Assessment of shelf-life: Shelf life of alginate inoculant prepared from mid log phase, late log phase and sporulating phase cells was assessed by destabilizing and enumerating the number of cells per bead after every 10 days over a period of six months. The effect of inoculant storage temperature on survival of AF 1 cells was assessed by storing the dry and dehydrated beads at 30^0C temperature and hydrated beads at 4°C.

Peat-based formulation: Locally purchased commercial peat was used as carrier material. Initial pH of the peat (6.0) was adjusted to 7.0 by adding $CaCO_3$. The neutralized peat was sterilized and packed into polyethylene self sealing bags. One hundred gram packed peat was inoculated separately with 50 ml of AF 1 culture grown for 16, 32 and 72 h (cell numbers adjusted to 10^6 ml^{-1}) and mixed well. Inoculated peat packets were thoroughly kneaded to ensure absorption of the liquid culture into the peat. Peat-based formulations were stored at 4^0C and 30^0C, separately for a period of six months. Moisture loss from peat was compensated by adding sterile distilled water based on the differences in weight compared to initial weight at regular intervals. The viability of AF 1 was assessed by counting the log CPU in 1 g peat sample for every 10 day intervals by suspending the sample in saline and plating on NA supplemented with 100 μg ml^{-1} ampicillin.

Peat formulation with diluted liquid cultures of *B. subtilis* AF 1: Sterile distilled water and potato dextrose broth were used to dilute AF 1 culture grown to mid log phase, late log phase and sporulating phases. One hundred-fold diluted mid log, late log and sporulating phase cultures (cell numbers adjusted to 10^4 ml^{-1}) were used to inoculate peat to investigate the influence of the nutrient status of the diluent on the multiplication and survival of AF 1 in the peat. Viability of AF 1 was assessed as described earlier in 30 C stored products.

Chitin/*A. niger* mycelium supplemented peat formulation: AF 1 culture was inoculated in to peat supplemented with 0.5% powdered chitin. *A. niger* was grown in RM for 48 h (log phase) at 30°C and 150 rpm. The mycelium was harvested and dried at 120 C for 24 h. The dried mycelium was added to peat at 0.5% (w/w). AF 1 culture grown to mid log, late log and sporulating phase (cell numbers adjusted to 10^6 ml^{-1}) was inoculated in to the peat mixed with

mycelium. Survival of AF 1 was monitored over a period of six months in chitin and mycelium supplemented peat formulations separately.

Mushroom spent compost-based formulation: Spent compost from *Agaricus bisporus* cultivation was used as a carrier material, which had an initial pH of 7.6. The pH of carrier material was adjusted to 7.0 by adding 1 M citrate phosphate buffer pH 3.0 and kneading the material thoroughly. Spent compost was sterilized and packed into surface sterilized polyethylene self sealing bags under aseptic conditions. 50 ml of AF 1 culture grown to mid log. late log and sporulating phase (cell numbers adjusted to 10^6 ml^{-1}) was inoculated in to 100 g of spent compost packed in to these bags and survival was assessed for a period of six months in 1 g samples.

b) Application of formulation products:

Formulation products which showed good survival of AF 1 cells were used for seed bacterization to assess their ability to promote plant growth and control fungal diseases in pigeon pea and groundnut. Performance of formulation products was compared with fresh AF 1 culture seed bacterization.

Pigeon pea growth promoting **activity** of formulation products:

Five grams of peat/peat-supplemented with 0.5% chitin/A *niger* mycelium/spent compost was used for coating 100 pigeon pea (ICPL 85010) seeds using 0.5% CMC as an adhesive. One hundred sodium alginate beads containing immobilized *B. subtilis* AF 1 cells were destabilized in 10 ml of 0.2 M potassium phosphate buffer pH 7.0 and to this 10 ml of 1% CMC was added. The resulting suspension was used for coating 100 pigeon pea (ICPL 85010) seeds. The application methods were designed to achieve 6.4 to 6.6 log CFU of Bacilli per seed. Seeds coated with CMC alone served as controls.

In glass house: Plant growth promoting activity of formulation products at zero time of their preparation and after storage for three months and six months was tested on pigeon pea (ICPL 85010) in glass house. Percent emergence of seedlings was noted after 7 days. Dry weight and length of plants were recorded after 30 days.

In field: A set of field experiments were conducted using four month-old formulation products. The treated seeds were tested both in Kharif and Rabi seasons. All the treated seeds, as described above, were sown in 12 X 14' field

plots in triplicates. Increase in emergence, plant length and dry weight was recorded as mentioned earlier.

Biocontrol activity of formulations in glass house :

Control of pigeon pea wilt: *F. udum* was grown in potato dextrose broth for 48 h at 30^0C and 150 rpm speed on a rotary' shaker. The mycelium was harvested by filtering the culture through Whatmann No.1 filter paper. The mycelium was mixed at the rate of 2.0 g per one kg of soil (w/w) and filled in to the upper l/4th portion of 25 X 30 cm plastic pots. The lower 3/4th portion of pots was filled with native soil. Eight seeds were sown in each pot and each treatment represented 160 seeds in 20 pots. ICP 2376 (wilt susceptible) and 1CP 8863 (wilt resistant) seeds coated with CMC/formulation products were sown in pots filled with native and pathogen-infested soil separately. The seedlings with wilt infection i.e. external wilt symptoms and/or with internal vascular browning were counted, and disease incidence was calculated. Performance of biocontrol strain was compared with a commercial fungicide (thiram) in both susceptible and resistant genotypes of pigeon pea. Percent emergence, increase in plant length and dry weight in native and pathogen-infested soil were recorded. Results obtained were analysed by paired t-test.

Control of groundnut crown rot: The experimental set up was similar to that of above experiment except for the number of seeds sown in each pot was 5 and *A. niger* mycelium was mixed in soil in place of *F udum.* TMV 2 (crown rot susceptible) and FDRS 10 (crown rot resistant) seeds coated with CMC/formulation products were used. The seedlings which showed pre-emergence rot and post-emergence crown rot infection were counted, and % disease control was calculated. Performance formulation products was compared with a commercial fungicide (bavistin). Percent emergence, increase in plant length and dry weight were recorded in native and pathogen-infested soil. The population of AF 1 in the rhizoplane of formulation product treated plants was recorded over a period of 28 days at an interval of 7 days as described above.

Survival of AF 1 in the rhizoplane:

The population of AF 1 in the rhizoplane (per cm root length) of formulation product treated plants was recorded both in plant growth promoting (in glass house and field) and disease control experiments (in native and pathogen-

infested soil). Roots from treated plants were washed gently with sterile water, cut in to 1 cm length pieces and crushed gently in 0.2 M potassium phosphate buffer. The resulting aliquots were serially diluted with saline and plated on MM plates overlaid with 0.1% colloidal chitin and $100 \mu g\ ml^{-1}$ ampicillin. AF 1 populations were determined over a period of 28 days in glass house experiments and 100 days in field experiments.

Results

I. Direct antagonism of *B. subtilis* AF 1 towards fungal pathogens:

The antagonistic activity of *B subtilis* AF 1 towards fungal pathogens was studied in two parts. The first part deals with the production of fungal cell wall degrading enzymes and their role in growth inhibition of fungal pathogens. Second part deals with inhibition of plant cell wall degading enzymes of fungal pathogens in presence of *B. subtilis* AF 1.

a) Fungal cell wall degrading enzymes of B. subtilis AF 1:

Antagonism exhibited by various biocontrol agents against fungal pathogens is significantly contributed by fungal cell wall degrading enzymes of biocontrol agents. Ability to produce Chitinases and glucanases is likely to contribute to their success against fungal pathogens. Chitinolysis by biocontrol strains will be useful, if the carrier material is supplemented with chitin and its related substances for slow release of nutrients for the organisms and also to impart additional benefits to the plant.

B. subtilis AF 1 was grown in MM containing 0.5% CC/laminarin and the cell free culture filtrate was assayed for chitinases/β-1,3-glucanase. NAGase and β-1,3-glucanase were detectable from 24 h and 12 h, respectively. Endochitinase activity was not detectable in the culture supernatant. The experiment was repeated three times and confirmed that *B. subtilis* AF 1 was able to use chitin/laminarin in the medium by producing extracellular NAGase/β-1,3-glucanase.

Optimum conditions for **NAGase/β-1,3-glucanase** assay:

Optimum conditions for the assay of NAGase/β-1,3-glucanase were studied in preliminary experiments which included pH and molarity of buffer, nature and concentration of substrate and temperature. 10 mM to 500 mM citrate phosphate buffer pH 2.6 to 7.0, sodium phosphate buffer pH 5.8 to 8.0, sodium acetate buffer pH 3.8 to 5.6, and Tris HC1 buffer pH 8.1 to 8.8 were used for the NAGase assay. 100 mM sodium phosphate buffer pH 7.0 and colloidal chitin (1.5%) supported maximum NAGase activity. Over a range from 20 to 50°C, the optimum temperature for NAGase activity was 37°C. The enzyme was stable in the pH range from 6.5 to 8.0, and up to 40°C temperature. The enzyme retained >95% activity in presence of $CaCl_2$, KCl,

$MgCl_2$, $MnCl_2$, NaN_3, and EDTA and lost 67 to 80 % in presence of $ZnSO_4$, $FeSO_4$ and $CuCl_2$. Subsequently, NAGase was assayed in 100 mM sodium phosphate buffer pH 7.0, at 37^0C with CC (1.5%) as substrate.

Citrate phosphate buffer (50 mM, pH 6.8) and 2.0% laminarin supported maximum β-1,3-glucanase activity. The optimum temperature for the β-1,3-glucanase was 37^oC. β-1,3-glucanase was stable in the pH range from 5.8 to 7.0, and also up to 40^oC. The enzyme retained about 95% activity in presence of NaCl, $CuCl_2$, KC1, $MgCl_2$, Mn Cl_2 and NaN_3, while the activity was completely lost in presence of $FeCl_3$, $AgNO_3$, $HgCl_2$ and EDTA. Therefore, β-1.3-glucanase was assayed using 50 mM citrate phosphate buffer pH 6.8, at 37^0C, with laminarin (2.0%) as substrate.

Optimum conditions for **NAGase/β-1,3-glucanase** production:

To increase the yield of both NAGase and β-1,3-glucanase in the cell free culture filtrate, fermentation conditions were optimised. Details are presented in Table 2.2.

Cell numbers in every individual experiment were determined by plating the serially diluted culture on NA plates supplemented with 100 |ig ml^{-1} ampicillin.

Fermentation period: *B subtilis* AF 1 was grown in MM containing 0.5% chitin and the cell free culture filtrate was assayed for NAGase activity over a period of 10 days (Fig 3.1a). Up to six days the glucosaminidase activity increased steadily and decreased there after. The cell density also reached a maximum by sixth day. *B. subtilis* Al" 1 produced NAGase during the active growth period on colloidal CC.

B. subtilis AF 1 was grown in MM containing 0.5% laminarin and the cell free culture filtrate was assayed for β-1,3-glucanase activity over a period of 96 h. Up to 48 h, the glucanase activity increased steadily and decreased there after (Fig 3.2a). Cell numbers increased over a period of 72 h. *B. subtilis* AF 1 produced β-1,3-glucanase during the active growth period on laminarin.

Substrate for induction: *B. subtilis* AF 1 secreted NAGase in to the medium in presence of colloidal chitin, *A. niger* cell walls, N-acetyl glucosamine (NAG), N. N`-diacetyl chitobiose and chitosan. CC was the most suitable substrate to induce production of NAGase by *B. subtilis* AF 1 (Fig 3.1b) followed by

Fig. 3.1 Extracellular NAGase production by *B subtilis* AF 1: *B. subtilis* AF 1 was grown in MM containing 0.5% *CC* as sole source of carbon at 30°C and 180 rpm for eight days. The activity of NAGase was assayed using the cell free culture filtrate (b-i, 6 days grown AF 1 culture filtrate was used). The cell numbers were determined in terms of log CFU in the fermentation medium. NAGase activity (—●— or □) and cell numbers in terms of log CFU (—■— or ▨) were presented a) over a period of 10 days; b) different inducing substrates; c) different concentrations of CC; d) pH of medium; e) yeast extract; f) Tween 20; g) EDTA: h) PMSF and i) different trace metals.

The values represent averages of three independent determinations and the vertical bars represent standard deviation.

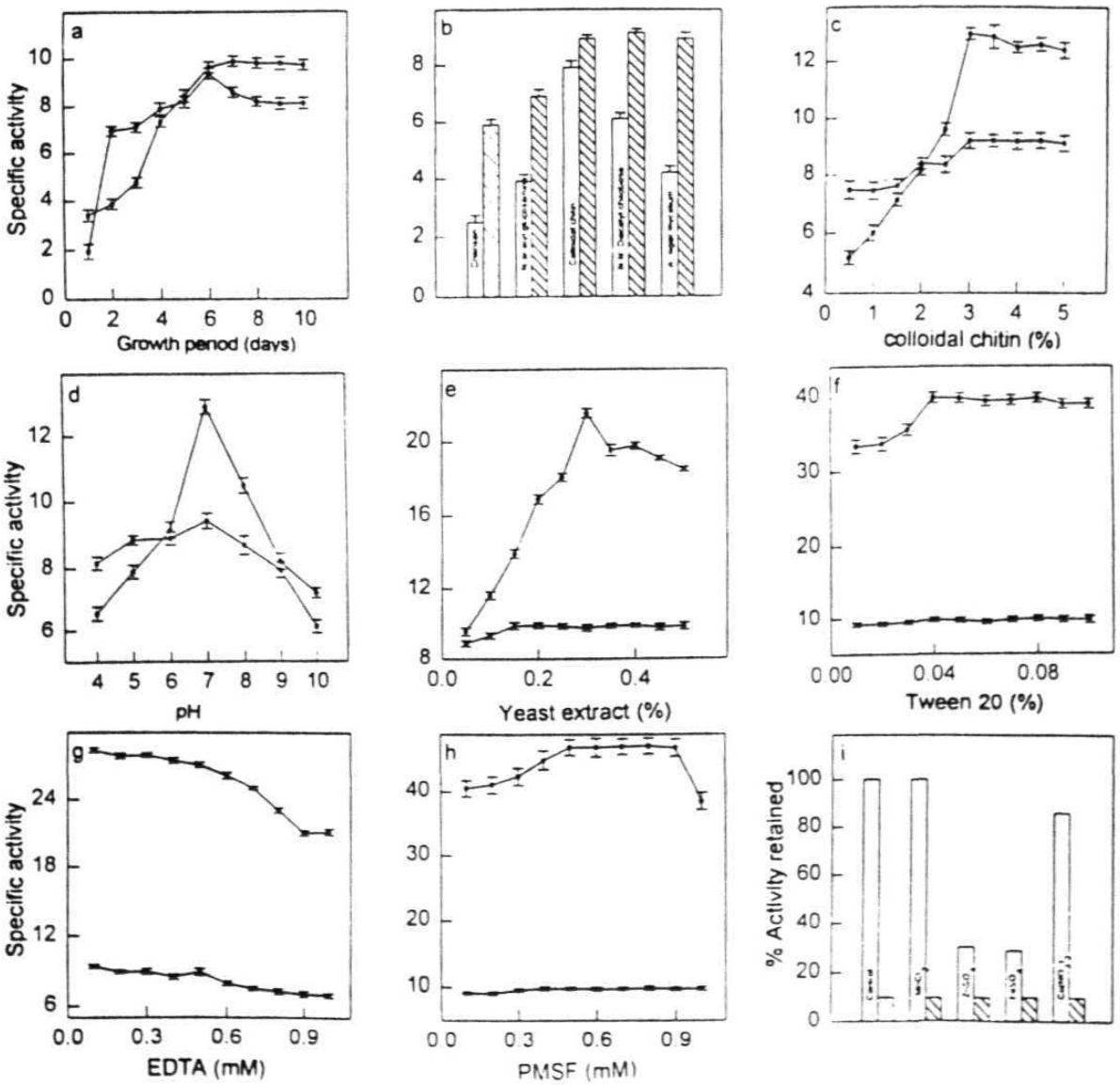

a
Specific activity
Growth period (days)
b
c
colloidal chitin (%)
d
Specific activity
pH
e
Yeast extract (%)
f
Tween 20 (%)
g
Specific activity
EDTA (mM)
h
PMSF (mM)
i
% Activity retained

Fig. 3.1

Fig. 3.2 Extracellular β-1,3-glucanase production by *B. subtilis* AF 1: *B. subtilis* AF 1 was grown in MM containing 0.5% laminarin as sole source of carbon at 30°C and 180 rpm for four days. The activity of β-1,3-glucanase was assayed using the cell free culture filtrate (b-f, 2 days grown AF 1 culture filtrate was used). The cell numbers were determined in terms of log CFU in the fermentation medium. β-1,3-glucanase activity (—●— or ▭) and cell density in terms of log CFU (•—■—-or ▨) were presented a) over a period of 3 days; b) different concentrations of laminarin, c) pH of medium; d) yeast extract; e) EDTA and f) different trace metals.

The values represent averages of three independent determinations and the vertical bars represent standard deviation.

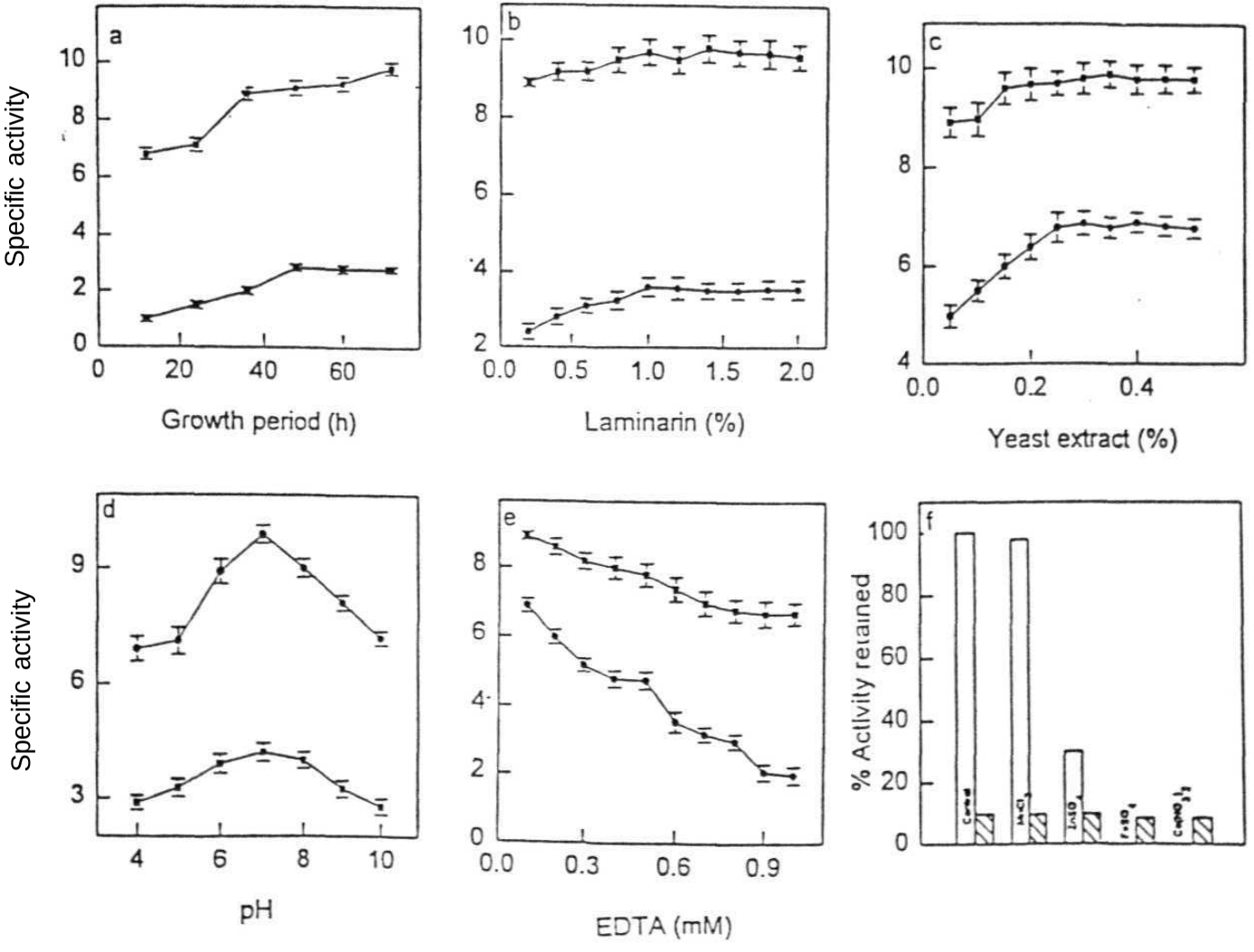

a
b
c
d
e
f
Specific activity
Specific activity
Growth period (h)
Laminarin (%)
Yeast extract (%)
pH
EDTA (mM)
% Activity retained

Fig. 3.2

chitobiose, *A. niger* mycelium, NAG and chitosan. The cell numbers reached a maximum in media containing chitobiose, colloidal chitin and *A. niger* mycelium. With chitosan as substrate both the cell density and the enzyme activity were low. The enzyme levels were maximum when 3.0% colloidal chitin was used as inducing substrate (Fig 3.1c).

The optimum concentration of inducing substrate for p-l,3-glucanase was 1.0%, when a range from 0.2 to 2.0% laminarin was used in the fermentation medium (Fig 3.2b). Further increase in laminarin concentration did not affect the enzyme production in to the medium. There was a marginal increase in cell numbers when the laminarin concentration was increased from 0.2 to 1.0%.

pH of the medium: *B subtilis* AF 1 was grown in MM in presence of 3.0% CC for six days at 30°C. The pH of the medium was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, seperately. At neutral pH NAGase production was high (Fig 3.1d) and in both alkaline and acidic pH the enzyme levels declined. Therefore, 7.0 was noted as the ideal pH for production of NAGase.

B. subtilis AF 1 was grown in MM in presence of 1.0% laminarin for two days at 30°C at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, seperately. At pH 7.0 and 8.0, β-1.3-glucanase production was high. The levels of enzyme were less in both alkaline and acidic pH (Fig 3.2c). A similar trend was observed in cell numbers for both the enzymes (Fig 3.1d & 3.2c).

Concentration of yeast extract (YE): To test the effect of yeast extract (YE) on production of glucosaminidase, *B. subtilis* AF 1 was grown in MM supplemented with different concentrations of YE (0.05% to 0.5%) for six days at 30°C at pH 7.0. AF 1 produced maximum NAGase in medium supplemented with 0.3% YE (Fig 3.1e). Further increase in YE concentration negatively influenced the enzyme production. Up to 0.15% YE the cell numbers also increased and further increase up to 0.5% did not affect the growth (Fig 3.1e).

Similarly *B. subtilis* AF 1 was grown in medium supplemented with different concentrations of YE (0.05% to 0.5%) and the cell free filtrate was assayed for P-l,3-glucanase. The enzyme production was maximum in presence of 0.3% YE in the growth medium and further increase in YE concentration did not result in increase in enzyme levels (Fig 3.2d). The cell numbers increased

up to 0.15% YE and no further increase up to 0.5%.

Concentration of Tween 20: Surfactants like Tween 20 facilitate release of enzymes into surrounding medium. *B. subtilis* AF 1 was grown in MM supplemented with different concentrations of Tween 20 (0.01% to 0.1%) with all other optimum conditions described above. Addition of Tween 20 to the growth medium resulted in increase in NAGase levels at 0.04% concentration (Fig 3.1f). Further increase (>0.04%) did not affect the enzyme production. Tween 20 had no effect on cell growth at all concentrations used (Fig 3.1f).

Addition of Tween 20 to the growth medium did not alter (\$-1.3-glucanase levels.

EDTA & **PMSF:** *B subtilis* AF 1 was grown in MM supplemented with EDTA or PMSF from 0.1 mM to 1.0 mM, for six days at 30°C. In presence of EDTA both glucosaminidase levels and cell numbers decreased in the medium (Fig 3.1g) while at 0.5 mM PMSF there was about 9% increase in enzyme levels with no significant difference in cell numbers (Fig 3.1h).

β-1.3-glucanase production by *B. subtilis* AF 1 was not affected by the addition of PMSF. In presence of EDTA there was decrease in enzyme levels with a corresponding decrease in cell numbers (Fig. 3.2e).

Trace metals: Different trace metals were included (1 mM) in the fermentation medium and *B, subtilis* AF 1 was grown under optimum conditions. Zn^{+} and Fe^{2+} caused 70% and 68% decrease, respectively, in production of NAGase where as *Co* ' caused 14% decrease in activity. There is no difference in cell numbers in presence of all the trace metals (Fig 3.1i).

Fe^{2+} and Co^{2+} completely inhibited β-1,3-glucanase production while Zn^{+2} inhibited 70% of the enzyme activity (Fig 3.2f). There was no difference in cell numbers except in presence of Fe^{2+} and Co^{2+} where the log CFU decreased by 0.9 units.

With the optimization of the above parameters a 5-fold increase in NAGase and a 3-fold increase in β-1,3-glucanase production was achieved.

Partial purification of NAGase: NAGase was partially purified by affinity chromatography on a chitin column.

The eluted fractions with 0.35 to 0.50 absorbance at A_{280} (fraction no. 45 to 85) contained NAGase with high specific activity (Fig. 3.3 and Table 3.1).

Fig. 3.3 Partial purification of NAGase from culture filtrate of *B. subtilis* AF 1: *B. subtilis* AF 1 was grown in MM pH 7.0 for six days at 30^{0}C at 180 rpm. The cell free culture filtrate was lyophilized and applied to a chitin column at one unit activity per 200 mg of matrix and eluted with 100 mM sodium phosphate buffer pH 7.0 at 37 C. Fractions with 0.35 to 0.5 absorbance at 280 nm were analysed for NAGase activity.

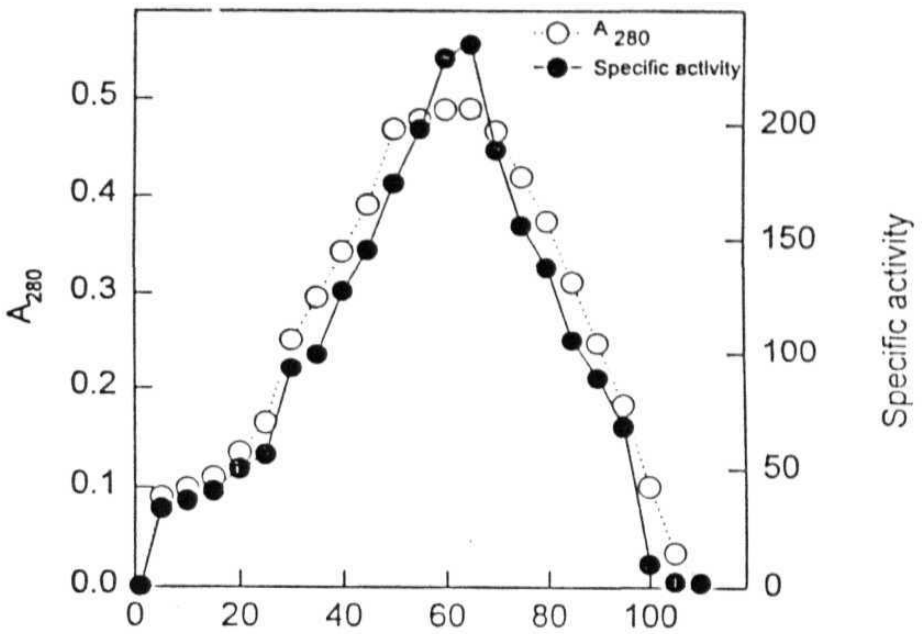


Fig.3.3

Table 3.1 Partial purification of p-l,4-N-acetyl glucosaminidase from culture filtrate of B *subtilis* AF 1.

Enzyme step	Total protein (mg)	Enzyme activity µ mole/ml/h	Specific activity	Purification fold	% Yield
Crude	156	6271	40.2	1.0	100%
Fractions 40-80	10.96	2779	253.55	6.30	44.31
Dialysed fractions 40-80	8.93	2041	228.55	5.68	32.54

B. subtilis AF 1 was grown in Minimal medium pH 7.0 for 6 days nt 30°C, 180 rpm and the cell free culture filtrate was used for purification (for details sec legend for the Fig 3.3).

Fractions with high specific activity, resolved on native and SDS-PAGE gels, showed four distinct bands corresponding to 67. 40. 37. and 32 kDa molecular mass. When stained for activity, a single band corresponding to the top most 67 kDa protein showed NAGase activity and the remaining three proteins showed no enzyme activity (Plate Fig. 2).

***In vitro* inhibitiion of *A. niger* by culture filtrate proteins and partially purified NAGase** : Growth of *A. niger* was studied in microtitre plates in presence of cell free culture filtrate, partially purified NAGase of AF 1, casein and peptone separately in an Enzyme Linked Immunosorbant Assay (EL1SA) reader at 405 nm.

In potato dextrose broth. *A niger* showed a steady increase in growth up to 72 h (Table 3.2). Crude culture filtrate proteins of AF 1 at 1.0 mg ml^{-1} retarded the growth of the fungus up to 48 h. Growth in presence of partially purified NAGase at 1.0 µg ml^{-1} was almost identical which remained static up to 48 h. Growth in presence of AF 1 culture filtrate proteins and partially purified NAGase started late and was always less when compared with the control. The growth of the fungus was significantly high in potato dextrose broth with casein or peptone at 1.0 mg ml" indicating the absence of negative effect of external protein supplementation. Sporulation started early in microtitre plates containing casein or peptone.

b) Effect of B. subtilis AF 1 on hydrolytic enzymes of A. niger/ F. udum:

Inhibition of the activity of pathogenecity-related enzymes of pathogens including cutinolytic, pectinolytic and cellulolytic enzymes is one of the mechanisms by which the biocontrol agent limits diseases caused by fungal pathogens of plants. By altering the enzyme levels of fungal pathogens it is possible to suppress the disease pressure. To study the effect of *B. subtilis* AF 1 on the production of plant cell wall hydrolytic enzymes by *A. niger* and *F. udum*, both the cultures were inoculated with live and autoclaved cells of AF 1 separately, and the enzymes were assayed in the cell free culture filtrates.

Exopolygalacturonase (Exo-PG): With 1% polygalacturonic acid as carbon source in RM (pH 5.6) both *A. niger* and *F. udum* produced copious amounts of exo-PG (Fig 3.4a and 3.4b), with a maximum production of 654 mU ml^{-1} by *A. niger* and 962 mU ml^{-1} by *F. udum.* AF 1 alone produced negligible amounts

Plate Fig 2 Polyacrylamide gel electrophoresis of partially purified NAGase: A) Polyacrylamide gel electrophoretic pattern of crude culture filtrate proteins and partially purified NAGase. Lane 1: Molecular weight standards, Lane 2: crude culture filtrate proteins, Lane 3: partially purified NAGase from affinity column. B) Activity staining of partially purified NAGase and crude culture filtrate proteins. Lane 1: 200 μg of crude protein, Lane 2: 20 ug of partially purified NAGase.

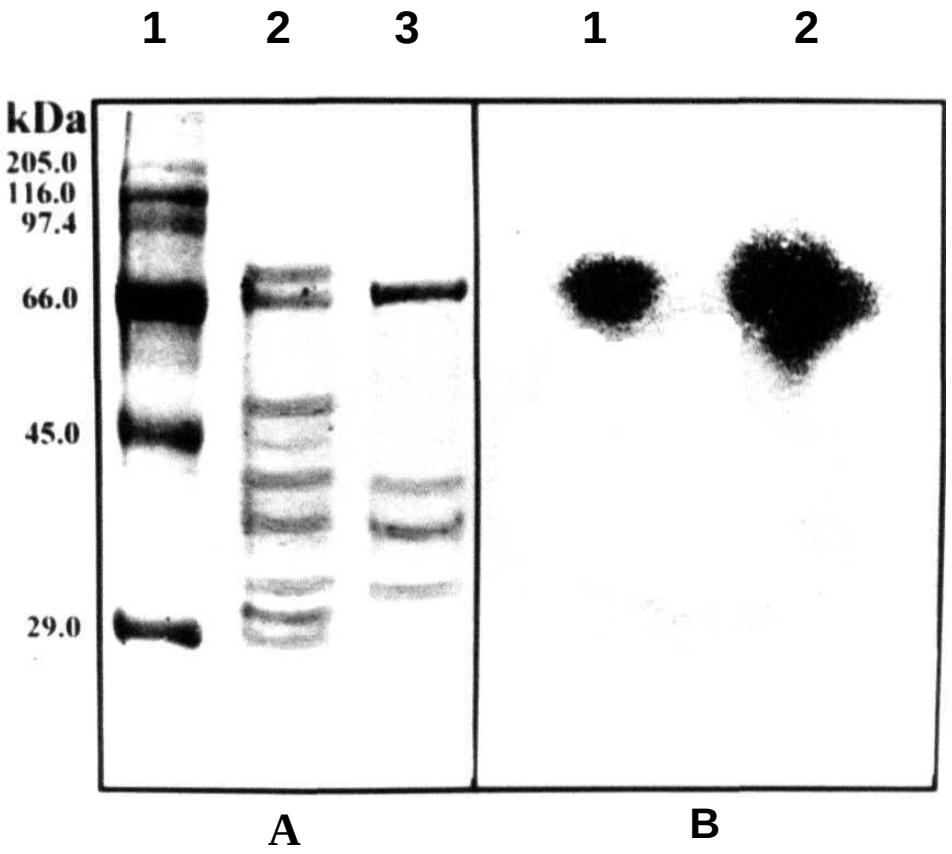


Plate Fig 2

Table 3.2 The effect of crude culture filtrate proteins and 1,4-β-N-acetyl glucosaminidase of *B. subtilis* AF 1 in comparison with casein and peptone on the growth of *A. niger*.

Incubation period (h)	Control (PDB alone)	Crude culture filtrate proteins 1mg/ml	p-1,4-N-acetyl glucosaminidasc 1µg/ml	Casein 1mg/ml	Peptone 1mg/ml
0	0.441	1.46	1.120	1.46	1.40
24	0.761	1.43	1.090	1.71	1.73
48	1.142	1.51	1.047	2.39	2.46
72	2.690	1.87	1.540	>3.00	>3.00

25 µl of *A. niger* spores(10^7/ml) was inoculated in to 175 µl of Potato Dextrose Broth containing 1.0 mg/ml crude culture filtrate proteins or 1.0 µg/ml partially purified 1,4-β-N-acetyl glucosaminidase in microtitre plates and the growth of the fungus was monitored using ELISA reader at 405 nm. PDB (175 µl) with 25 µl of *A. niger* spore inoculum served as control

Fig. 3.4 Production of exo-PG by *A. niger* and *F. udum* in presence of *B. subtilis* AF 1: *A. niger* /*F. udum* was grown for 24 h in RM containing 1% sodium polypectate and AF 1 in MM with 0.1% sodium polypectate as sole source of carbon. *A. niger*/*F. udum* cultures were co-inoculated with 6 h grown live/autoclaved cells of AF 1 and were allowed to grow for further 144 h.

a, c, e, g and i: AF 1 alone (—●—). *A. niger* alone (—■—), *A. niger* culture co-inoculated with live AF 1 cells (—▲—) and autoclaved cells of AF 1 (—▼—).

b, d. f, h and j: AF 1 alone (—●—). *F. udum* alone (—■—), *F. udum* culture co-inoculated with live AF 1 cells (—▲—) and autoclaved cells of AF 1 (—▼—).

a & b, c & d, e & f, g & h, i & h present the profiles for exo-PG, endo-PG, PL, PME and CMCase in *A. niger* and *F. udum*, repectively.

The values are averages of three independent determinations and the vertical bars represent standard deviation

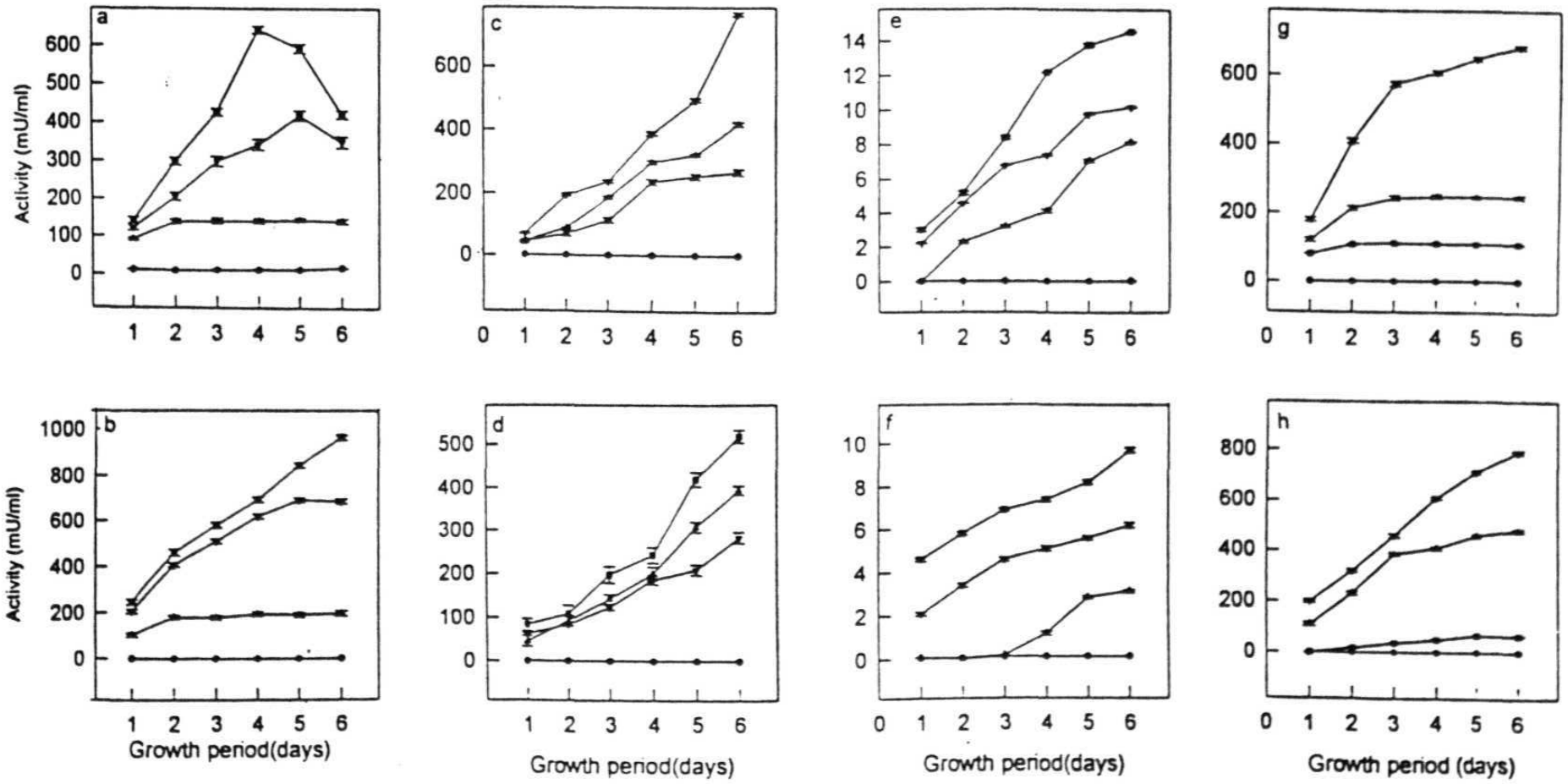
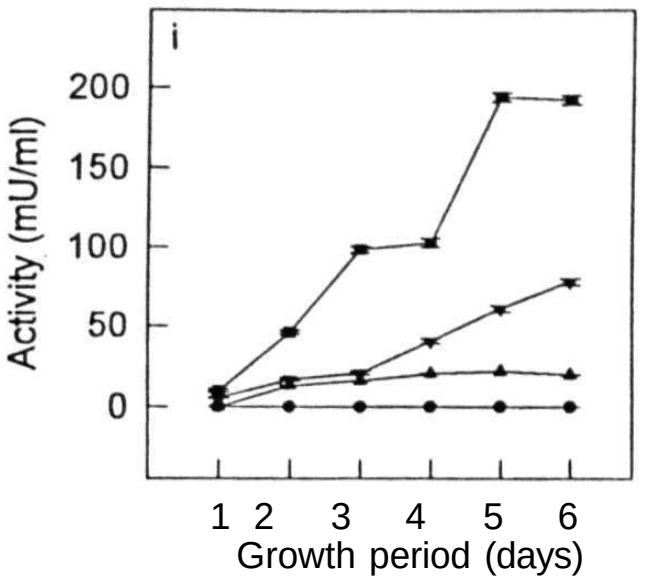

a
b
c
d
e
f
g
h
Activity (mU/ml)
Activity (mU/ml)
Growth period(days)
Growth period(days)
Growth period(days)
Growth period (days)

Fig. 3.4

Cont'd.


i
200
150
100
50
0
Activity (mU/ml)
1 2 3 4 5 6
Growth period (days)

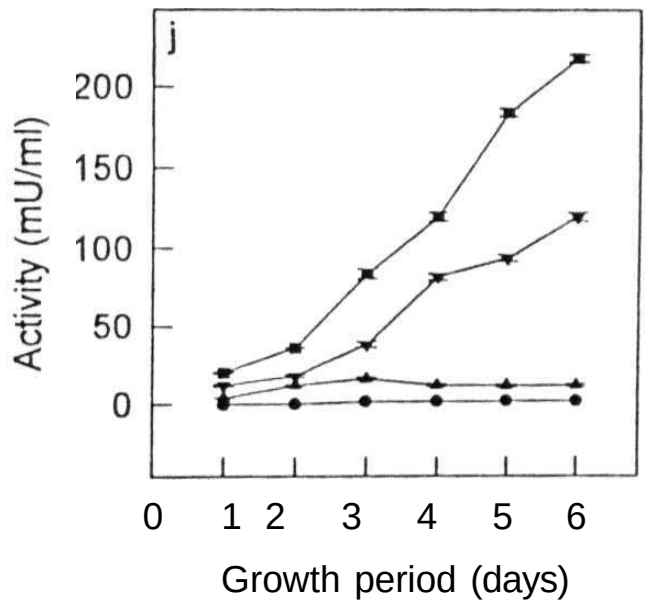

j
200
150
100
50
0
Activity (mU/ml)
0 1 2 3 4 5 6
Growth period (days)

Fig. 3.4

of exo-PG (< 10 ml') through out the growth period compared with fungal pathogens. In presence of live AF 1 cells in fermentation medium exo-PG production by both the pathogens decreased significantly. A five-fold decrease in exo-PG activity of *A. niger* and *F. udum* was recorded as compared to the production of the enzyme when pathogens were grown without *B. subtilis* AF 1. The enzyme levels were intermediary in pathogen cultures inoculated with autoclaved cells of AF 1. The reduction in exo-PG levels in fermentation media added with live cells of AF 1 was 80% for *A. niger* (on 4th day) and 79% for *F, udum* (on 6th day), respectively (Fig 3.4a & 4b). In fermentation media added with autoclaved cells of AF 1 maximum production of exo-PG was observed on 5th day in both *A. niger* and *F. udum* The decrease in enzyme production in presence of autoclaved cells was significantly less (39% and 37%, respectively) than the media inoculated with live AF 1 cells.

Endo-polygalacturonase (endo-PG): By 6th day endo-PG production reached maximum in the cultures of both the pathogens and dual cultures (Fig 3.4c & 3.4d). AF 1 alone produced negligible amounts of endo-PG compared with fungal pathogens (<10 mU through out the growth period). In dual cultures of pathogen with live cells of AF 1, > 50% decrease in endo-PG production occurred. In medium added with autoclaved cells of AF 1. there was a decrease in endo-PG activity by 45% in *A. niger* and 26% in *F. udum* cultures, respectively.

Pectate lyase (PL): Both *A. niger* and *F. udum* produced PL into growth medium from 1st day. The levels of PL in *A. niger* and *F. udum* culture filtrates was maximum on 6th day (14.2 and 9.8 mU ml^{-1}) (Fig 3.4e & 3.4f). In media inoculated with live cells of AF 1, PL activity decreased by 48% in *A. niger* and 75% in *F. udum* cultures. In *A. niger* and *F. udum* inoculated with autoclaved cells of AF 1, the PL production decreased by 28% and 39%, respectively on 6th day.

Pectin methyl esterase (PME): PME of *A. niger* and *F. udum* culture filtrates was also maximum on 6th day (740 mU ml^{-1} in *A. niger,* 790 mU ml^{-1} in *F. udum)* (Fig 3.4g & 3.4h). PME production decreased (> 80%) when live cells of AF I were inoculated in to medium (by 6th day). However, in fermentation media added with autoclaved cells of AF 1, the decrease was 58% in *A. niger*

and 38% in *F. udum* cultures (by 6th day).

Carboxy methyl cellulalse (CMCase): Both the fungal pathogens produced CMCase with CMC as carbon source in the fermentation medium. AF 1 also produced trace amounts of CMCase in to growth medium (Fig. 3.4i & 3.4j). The level of CMCase produced by the pathogens increased with fermentation time and reached maximum on 6th day (192 mU ml^{-1} in *A. niger* and 248 mU ml^{-1} in *F. udum).* When live cells of AF 1 were included in fermentation medium > 90% decrease of CMCase production by *A niger* and *F. udum* was recorded on 6th day. The decrease in enzyme production when autoclaved cells of AF 1 were added to the fermentation medium was 64% and 48% on 6th day for *A. niger* and *F. udum,* respectively.

II. Formulation of 8. *subtilis* AF 1 and application of formulation products:

As an initial step towards commercialization of this beneficial plant growth promoting biocontrol rhizobacterium, attempts were made to develop formulations using calcium alginate, peat with and without chitin related supplements and spent compost. Survival of AF 1 was monitored in all the formulations at 4 and 30 C. Depending on the survival of AF 1 cells, different formulations were tested for their plant growth promoting and disease control activities.

a) *Formulation of* ***B.*** *subtilis AF* ***1:***

Aqueous suspensions of PGPR fail to remain at high viable populations after application on to seed unless various adhesive and preservative polymers are added. Formulations of these microbes in a suitable carrier material with other additives extend the shelf life facilitating easy application on to seeds. AF 1 was formulated using calcium alginate, peat and mushroom spent compost as carrier materials. Survival of AF 1 in chitin/A *niger* mycelium-supplemented peat and mushroom spent compost were compared with conventional peat formulation. Performance of these chitin-supplemented formulations with conventional formulations was also compared with respect to their ability to promote growth and control diseases in plants.

Alginate formulation: Mid log. late log and sporulating phase grown cells of *B. subtilis* AF 1 were immobilized in 2% calcium alginate beads. The beads were stored at 4°C (hydrated state) and 30°C (dehydrated state).

Solubility of beads: The release of AF 1 cells from the above beads was determined in 0.2 M potassium phosphate buffer pH 7.0 and 0.2 M citrate phosphate buffer pH 7.0.

Both potassium phosphate and citrate phosphate buffers pH 7.0 (0.2 M) solubilized the hydrated and dehydrated alginate beads within 20 and 40 min, respectively (Fig 3.5a). Therefore, 0.2 M potassium phosphate buffer pH 7.0 was used for solubilization of alginate beads in subsequent experiments.

Survival of AF 1 after dissolution of alginate beads: Survival of AF 1 after dissolution of alginate beads containing mid log. late log and sporulating phase of cells in 0.2 M potassium phosphate buffer pH 7.0 was assessed for 12 h (Fig 3.5b). Up to 6 h the number of cells in alginate beads prepared from mid log phase grown cells remained same as (marginal decrease of 0.4 log CFU) that of zero time and there after gradually declined. There was a decrease of 2.6 log CFU compared to the initial log CFU. In alginate beads prepared from AF 1 cells grown to sporulating phase. there was negligible decrease in cell numbers compared to the initial cell numbers even after 12 h. In alginate beads prepared from late log phase grown cells also, the cell numbers declined. The decline was slower to that of mid log phase grown cells and rapid when compared with sporulating phase grown cells.

Shelf life of **alginate-based** formulation : Survival of AF 1 was monitored for six months by solubilizing single alginate bead in one ml of buffer and plating the serially diluted aliquots on NA supplemented with 100 fig ml^{-1} ampicillin. Shelf life of alginate-based formulation of AF 1 prepared from mid log, late log and sporulating phase cells was assessed by dissolving the alginate beads in 0.2 M potassium phosphate buffer pH 7.0 and enumerating the number of cells per bead after every 10 days over a period of six months (Fig 3.5c). The effect of storage temperature on survival of AF 1 cells was determined by storing the dehydrated beads at 30°C and hydrated beads at 4°C.

Fig 3.5a Rate of release of AF 1 from alginate beads: Single alginate bead was destabilized in one ml of the buffer with gentle vortexing and after every five min the aliquots were plated on NA supplemented with 100 $\mu g\ ml^{-1}$ ampicillin. Rate of release of AF 1 cells was presented in hydrated alginate beads in 0.2 M potassium phosphate buffer pH 7.0 (—•—), in 0.2 M citrate phosphate buffer pH 7.0 (—•—); dehydrated alginate beads in 0.2 M potassium phosphate buffer (—▲—) and 0.2 M citrate phosphate buffer (—▼—).

Fig. 3.5b Survival of *B. subtilis* AF 1 cells after dissolution of alginate beads in 0.2 M KH_2PO_4 buffer pH 7.0: Single alginate bead was allowed to destabilize in one ml. of 0.2 M KH_2PO_4 buffer pH 7.0 and serially diluted samples were plated NA-ampicillin after 1 to 12 h at 1 h intervals to check the viability of the bacterium in the buffer used for dissolution of alginate beads. The log CFU were recorded for alginate beads containing immobilized mid log (—■—), late log (—●—) and sporulating phase AF 1 cells (—▲—).

Fig. 3.5c Shelf life of alginate-based formulation: Alginate beads prepared from mid log late log and sporulating phase grown AF 1 cells and stored at 4°C and 30^0C were destabilized in 0.2 M KH_2PO_4 buffer. Aliquots were plated on NA-ampicillin plates after serial dilutions. Log CFU of AF 1 in formulations stored at different temperatures were determined over a period of 180 days. The log CFU were recorded for alginate beads containing immobilized mid log phase AF 1 cells stored at 4^0C (—▼—) & 30^0C (—■—); late log phase AF 1 cells stored at 4^0C (—●—) & 30^0C (—▲—): sporulating phase AF 1 cells stored at 4^0C (—◆—) & 30^0C (—●—).

The values are means of triplicate determinations. Standard deviations from the mean values are represented as error bars.

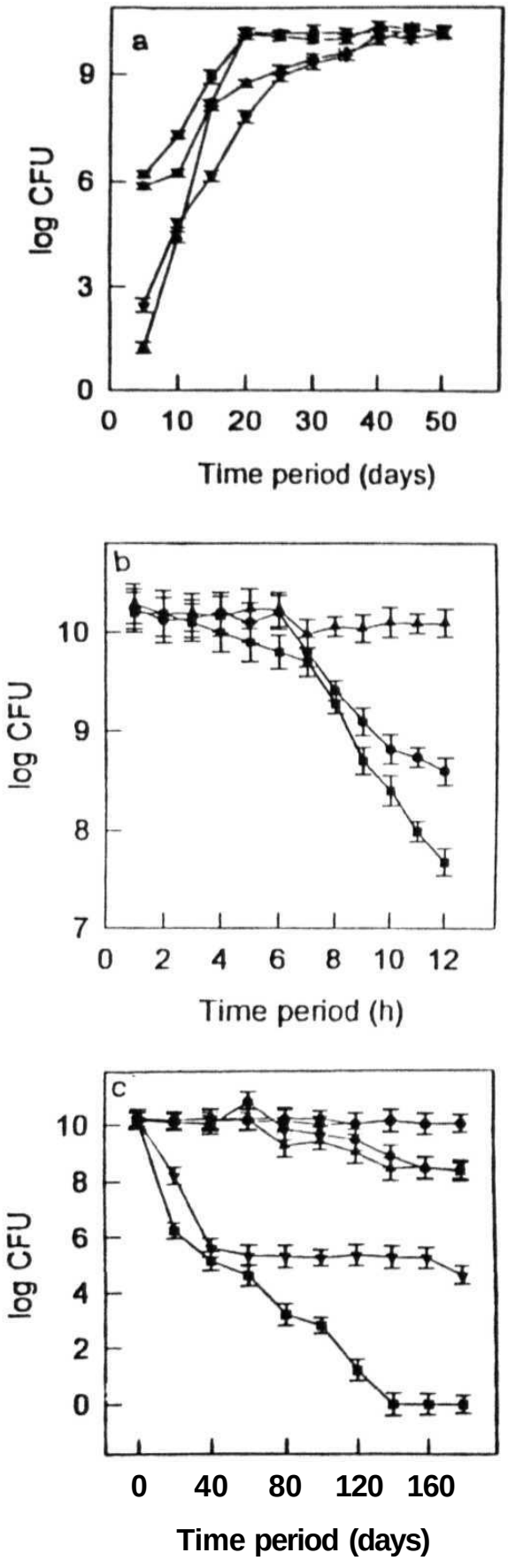

a
log CFU
0
3
6
9
0 10 20 30 40 50
Time period (days)
b
log CFU
7
8
9
10
0 2 4 6 8 10 12
Time period (h)
c
log CFU
0
2
4
6
8
10
0 40 80 120 160
Time period (days)

Fig. 3.5

The number of AF 1 cells decreased from 10.14 log CFU to 5.24 log CFU at the end of 6 months in hydrated beads stored at 4°C prepared from mid log phase grown AF 1 cells. The cell numbers in alginate beads stored at 30°C prepared from mid log phase grown AF 1 cells rapidly decfeased when compared to the 4°C stored beads. There was a decrease of 38% of the cells within the first 20 days of storage which further decreased to 78% after 90 days. In the alginate beads prepared from late log phase grown AF 1 cells stored at 4°C and 30°C, the decrease in cell numbers was slow where the log CFU decreased from 10.2 to 8.4 at the end of 180 days. In alginate beads prepared from AF 1 cells grown to sporulating phase, the recovery of bacterial cells was highest and was same in case of both 4°C and 30°C stored products. Viability of cells was maximum only in sporulating phase cells immobilized in alginate irrespective of the storage temperature. The viability in mid log and late log phase cells immobilized in alginate was better in 4°C stored product.

These results indicate that alginate formulation is not suitable to support the multiplication of cells but can retain the cells in viable form, where the number of viable cells depends on the growth phase and storage temperature. From the late log and sporulating phase cells of AF 1 immobilized in alginate. > 90% cells were recoverable even after storage at 30°C after 80 days and 180 days, respectively.

Shelf life of peat-based formulation: Shelf life of peat-based formulation prepared from mid log. late log and sporulating phase cultures, stored at 4°C and 30°C was assessed. One gram of peat sample was suspended in saline and AF 1 cells were enumerated on plates containing NA supplemented with 100 $\mu g\ ml^{-1}$ ampicillin.

The log CFU of AF 1 cells decreased from 5.1 to 4.1 per gram peat formulation prepared from mid log, late log and sporulating phase grown AF 1 cultures stored at 4°C by the end of 6 months (Fig 3.6a). In peat inoculated with mid log phase culture of AF 1, and stored at 30°C there was a gradual increase in log CFU from 5.1 to 9.1 per gram product up to 100 days after which it decreased slightly to 8.7 by 180 days. In the late log phase culture

Fig 3.6a Shelf life of peat-based formulation: Peat-based formulations were prepared by inoculating mid log, late log and sporulating phase cultures of AF 1 in to presterilized peat and stored at 4^0C and 30^0C, separately. The log CFU of AF 1 cells in 1.0 g of peat was determined by vortexing the sample in saline and plating the aliquots on NA-ampicillin after serial dilution with saline. Log CFU of AF 1 were presented in formulations inoculated with mid log phase AF 1 cells and stored at 4^0C (—◆—) & 30^0C (—■—); late log phase AF 1 cells stored at 4^0C (—●—) & 30^0C (—▼—); sporulating phase AF 1 cells stored at 4^0C (—▲—) & 30^0C (—●—).

Fig 3.6b Shelf life of peat-based formulation prepared from diluted liquid cultures: 100-fold diluted mid log, late log and sporulating phase AF 1 culture with H_2O and PDB separately was inoculated in to presterilized peat and stored at 30^0C. The log CFU of AF 1 cells in 1.0 g of peat was determined as described above and presented in peat sample inoculated with mid log phase AF 1 cells diluted with H_2O (—▲—) & PDB (—■—); late log phase AF 1 cells diluted with H_2O (—◆—) & PDB (—▼—); sporulating phase AF 1 cells diluted with H_2O (—●—) & PDB (—●—).

Fig 3.6c Shelf life of peat-based formulation supplemented with 0.5% chitin/ *A. niger* mycelium: Mid log, late log and sporulating phase AF 1 culture was inoculated in to presterilized peat supplemented with 0.5% chitin/*A. niger* mycelium and stored at 30^0C. The log CFU of AF 1 cells in 1.0 g of peat was determined as described above and presented in peat+chitin inoculated with mid log phase AF 1 cells (—●—), late log phase AF 1 cells (—■—) and sporulating phase AF 1 cells (—▲—); peat+ *A. niger* mycelium inoculated with mid log phase AF 1 cells (—◆—), late log phase AF 1 cells (—▼—) and sporulating phase AF 1 cells (—●—).

The values are the means of triplicate determinations. Standard deviations from the mean values are represented as error bars.

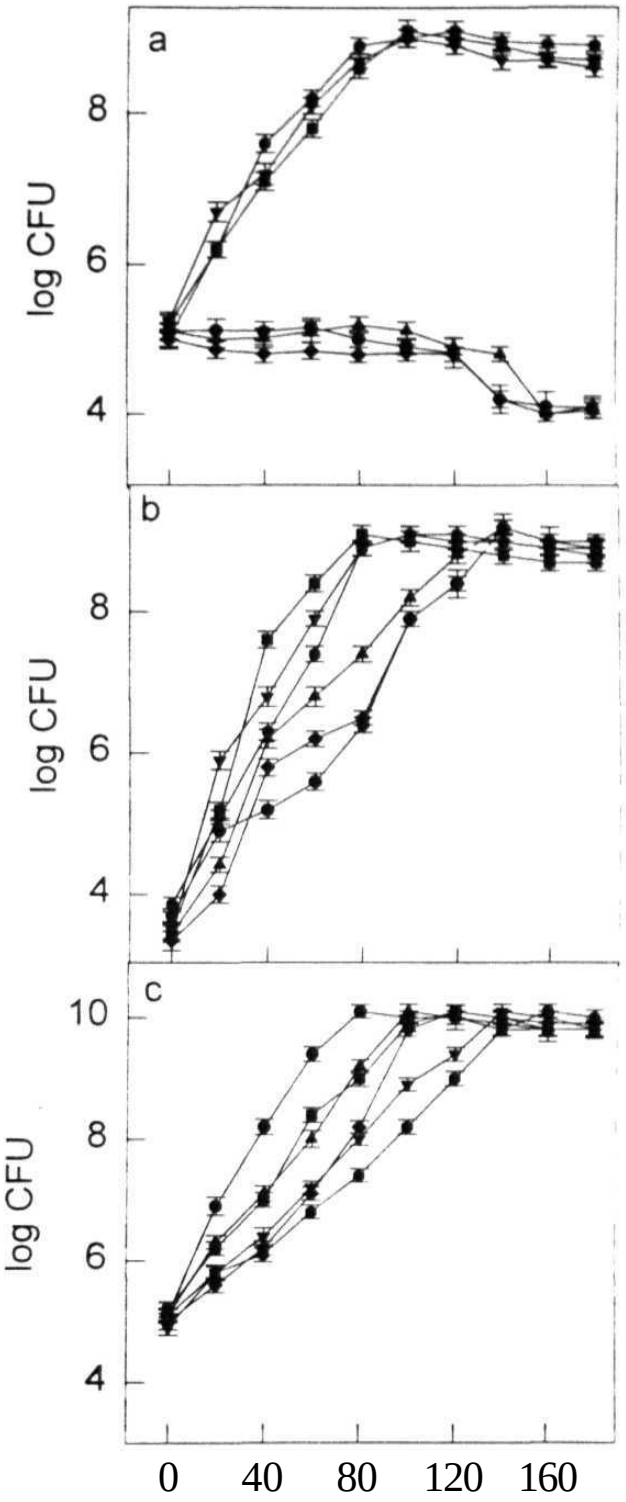

a
b
c
log CFU
log CFU
log CFU
4
6
8
10
0
40
80
120
160
Time period (days)

Fig 3.6

inoculated peat, stored at 30°C, AF 1 populations increased up to 100 days and later decreased by 0.4 log CFU over the remaining 80 days. Peat inoculated with sporulating phase AF 1, stored at 30°C, the cell density increased up to 120 days and later decreased by 0.2 log CFU over the remaining 60 days. The increase in AF 1 populations during storage at 30°C indicates multiplication of the bacterium in peat, which was not observed in the 4°C stored product.

Peat-based formulation with diluted liquid cultures of *B. subtilis* AF 1 : Diluted cultures of AF 1 were used to prepare peat inoculants, to assess the difference in maximum cell numbers attainable in these preparations, separately.

The multiplication in 100-fold diluted cultures (DW and **PDB,** separately) of AF 1 inoculated in to peat did not differ from that of undiluted cultures except for the time required to reach maximum log CFU (Fig. 3.6b). With undiluted cultures. stored at 30°C, the mid log phase, late log phase and sporulating phase inoculants reached maximum cell numbers (from 5.1 log CFU at zero time of formulation product preparation to 9.2 log CFU) by 100 and 120days, respectively. The maximum cell numbers (from 3.2 log CFU at zero time of formulation product preparation to 9.2 log CFU) reached by 130, 140, 160 days in the distilled water diluted mid log phase, late log phase and sporulating phase cultures of AI 1 inoculated in to peat While in potato dextrose broth diluted cultures. the time taken to reach maximum cell density (9.1 log CFU. initial log CFU at zero time of formulation product preparation being 3.1) was 80, 90 and 100 days. respectively.

Chitin/A *niger* mycelium supplemented peat formulation: Peat supplemented with 0.5% chitin and *A. niger* mycelium separately, was used as a carrier material. Chitin or mycelial supplements serve as carbon source for chitinolytic AF 1. Survival of AF 1 was assessed as described earlier for peat samples.

The amendment of peat with chitin/mycelium increased the cell multiplication. In chitin-supplemented peat stored at 30°C, with mid log, late log and sporulating phase formulations, cell density reached to a maximum of 10.1 log CFU by 80, 100 and 120 days, respectively (Fig. 3.6c). In peat supplemented with mycelium (0.5%) the multiplication of cells was slow compared to chitin-supplemented peat. The maximum cell density of 10.1 log

CFU was attained only after 120, 140 and 160 days with mid log. late log and sporulating phase cultures of AF 1 inoculated in to 0.5% mycelium-supplemented **peat.**

Mushroom spent compost formulation: Spent compost is similar to peat in containing organic matter and in addition has chitin-rich mycelial mass of *Agaricus bisporus* for better survival of chitinolytic microorganisms. Spent compost from *A bisporus* cultivation was used as a carrier material and survival of AF 1 was monitored for six months at 4°C and 30°C (Fig 3.7). The number of AF 1 cells decreased by 0.4 log CFU in spent compost inoculated with mid log, late log and sporulating phase cultures stored at 4°C. In the 30°C stored spent compost, inoculated with mid log phase culture of AF 1, there was a gradual increase of log CFU from 5.2 to 10.2 up to 100 days which decreased to 9.9 by 180 days. The increase in AF 1 populations during storage at 30°C indicates multiplication of cells, similar to the peat-based formulations. Both the log phase and sporulating phase cultures inoculated in to spent compost, stored at 4°C, did not show increase in AF 1 cell density. The late log phase culture inoculated spent compost stored at 30°C has shown an increase of AF 1 populations up to 80 days and shown a decrease of 0.4 log CFU over the remaining 100 days. In the sporulating phase AF 1 inoculated spent compost, stored at 30°C, the increase in cell density occurred up to 80 days and a marginal decrease of 0.3 log CFU over the remaining 100 days.

b) Application of formulation products:

To check the efficacy of the developed formulations, plant growth promoting activity was tested in both glass house and field experiments. The biocontrol activity was studied in glass house experiments. Root colonizing ability of AF1 in different formulation product treatments was studied in both growth promotion and disease control experiments. In all the experiments formulation products prepared from sporulating phase of AF 1 cells and stored at 30^0C were used.

Pigeon pea growth promoting activity of formulation products in glass house: Plant growth promoting activity of formulation products at zero time and after storage for three months and six months was studied in pigeon pea (ICPL 85010) after seed bacterization. The cell density after seed bacterization

Fig. 3.7 Mid log, late log and sporulating phase AF 1 culture was inoculated in to presterilized spent compost. The log CFU of AF 1 cells in 1.0 g of spent compost were determined by vortexing the sample in saline and plating the aliquots on NA-ampicillin. Log CFU of AF 1 were presented in spent compost inoculated with mid log phase AF 1 cells stored at 4^0C (—◆—) & 30^0C (—■—); late log phase grown AF 1 cells stored at 4^0C (—●—) & 30^0C (—●—); and sporulating phase AF 1 cells stored at 4^0C (—▲—) & 30^0C (—▼—).

The values are the means of triplicate determinations. Standard deviations from the mean values are represented as error bars.

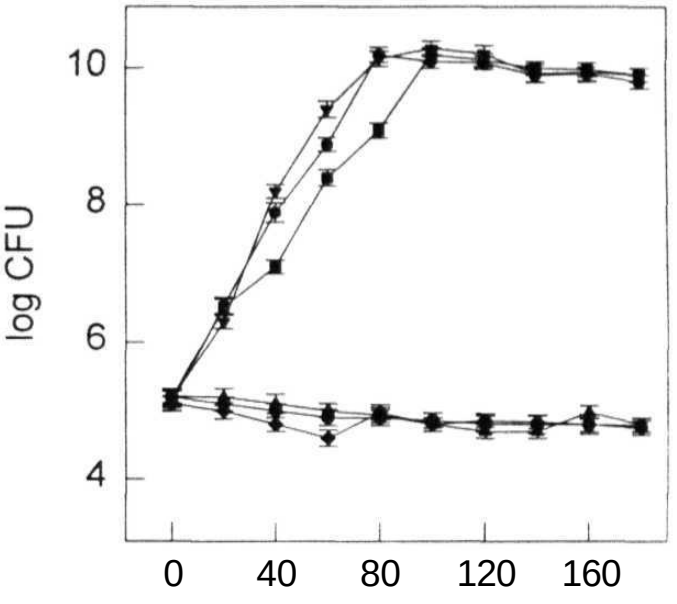


Fig. 3.7

was between 6.2 to 6.6 log CFU per seed. The percent increase in seedling emergence/plant length/dry weight over control in different treatments was compared in freshly prepared (zero time), three months and six months stored formulation products.

Seedling emergence:

Emergence promoting activity of formulation products and freshJy grown AF 1 culture was compared in terms of the percent emergence on 7 day of sowing. The increase in emergence over control in different formulation product treatments varied between 16% to 33% where the higher limit was equal to that of fresh culture bacterized seeds (Fig 3.8a). The increase in emergence over control with freshly grown AF 1 culture, alginate. peat, peat supplemented with chitin/ *A. niger* mycelium and spent compost with zero time formulation products was 24%, 24%. 20%, 30%, 22% and 26%, respectively. When three months stored formulation products were used the increase in emergence over control was 23%, 22%, 19%, 28%, 20% and 24% and in six months stored formulation product was 24% 20%, 16%, 28%. 17% and 24%, in the same order.

Plant length:

Growth promoting activity of formulation products was assayed in terms of increase in plant length and dr\ weight after 3<> days of sowing. Increase in plant length over control in different formulation product treatments varied between 6.2% to 13.0% (Fig 3.8b). The increase in plant length over control in freshly grown AF 1 culture. alginate. peat. peat supplemented with chitin/ *A niger* mycelium and spent compost at zero time of formulation product preparation was 12%, 10%, 9%, 9%, 8.2%, and 10%, respectively. The increase in plant length over control in 3 months stored formulation product treated seeds was 12%. 9.2%, 8%, 8%, 7%. and 9% and with six months stored formulation products the increase was 13%. 7%, 6%, 8%, 7% and 8% in the same order.

Plant dry weight:

The increase in dry weight over control in freshly grown AF 1 culture, alginate, peat, peat supplemented with chitin /. *A. niger* mycelium and spent compost at zero time of formulation preparation was 28%, 26%, 18%. 34%, 19% and 21%. respectively (Fig 3.8c). When three months stored formulation products were used the increase in dry weight over control was 28%, 24%, 16%, 32%,

Fig. 3.8 Plant growth promoting activity of AF 1 formulations in glass house: Pigeon pea (ICPL 85010) seeds were treated with freshly grown AF 1 and different formulations at zero time of their preparation and after storage for three and six months, sown in native soil. Percent increase in a) emergence b) plant length and c) dry weight was recorded in treatments with freshly grown AF 1 (), alginate (), peat (), peat+chitin (), peat+*A. niger* mycelium () and spent compost ().

All values are the means of triplicate determinations.

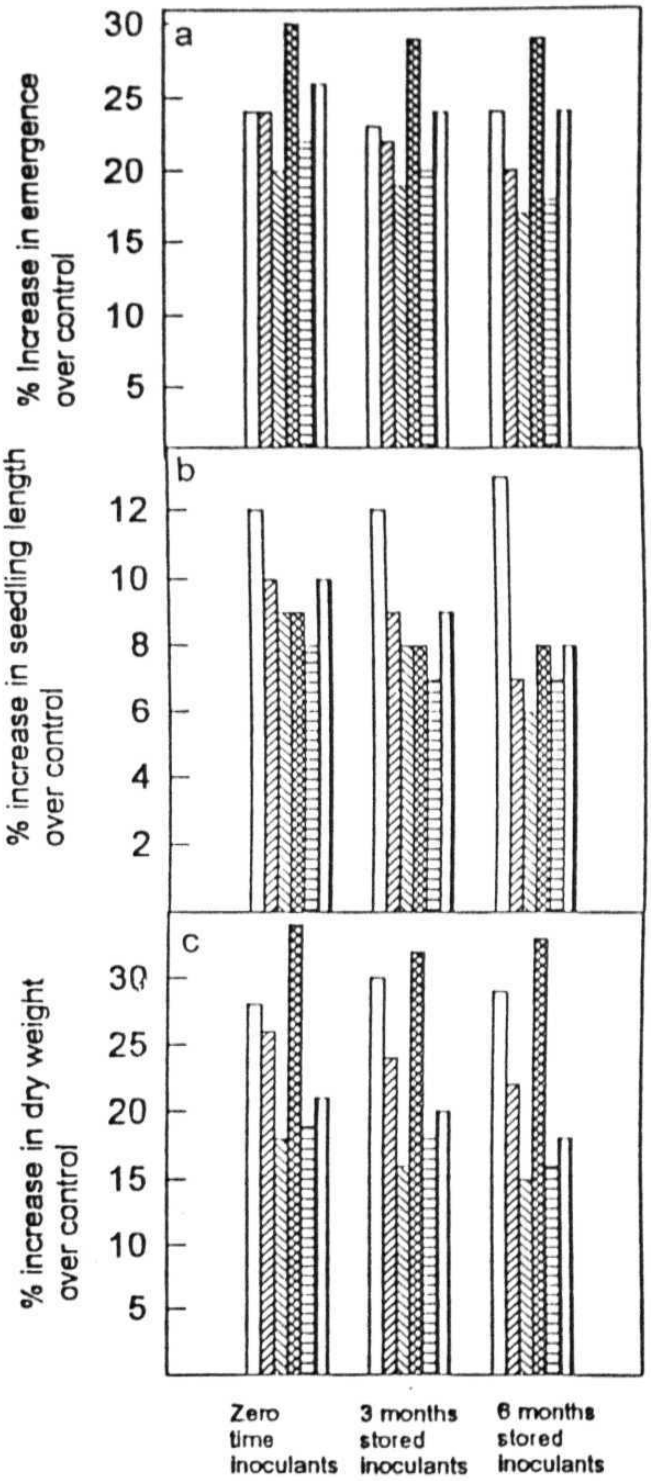

a
% Increase in emergence over control
30
25
20
15
10
5
b
% increase in seedling length over control
12
10
8
6
4
2
c
% increase in dry weight over control
30
25
20
15
10
5
Zero time inoculants
3 months stored inoculants
6 months stored inoculants

Fig. 3.8

18% and 19% in the same order. The increase in dry weight over control in six months stored formulation product treated seeds was 28%, 22%, 14%, 33%, 16%. and 17% in the same order. The increase in dry weight of formulation product treated plants varied between 14% to 34% where the higher limit was comparable with that of fresh culture bacterization.

Pigeon pea growth promoting activity of formulations in two different cropping seasons in the field:

Since the storage time of formulations had no effect on their performance compared with freshly grown *B subtilis* AF1 cells, four months stored formulation products were used for pigeon pea seed treatments, and the emergence and growth promoting activities were recorded in two different cropping seasons (Kharif and Rabi) in field experiments.

Seedling emergence:

The increase in emergence over control in freshly grown AF 1 culture, alginate, peat, peat supplemented with chitin/ *A niger* mycelium and spent compost treatments during Kharif season was 22%, 19%. 18%, 28%, 20%. and 23% (Fig 3.9a). In the following Rabi season the increase in emergence was 19%, 18%, 17%, 29%, 8%, and 22%, respectively (Fig 3.9a).

Plant length:

The increase in plant length over control in freshly grown AF 1 culture, alginate, peat, peat supplemented with chitin/ *A nigcr* mycelium and spent compost in Kharif season was 12%. 11%, 8%, 9%. 7%. and 8% (Fig 3.9b). Increase in plant length over control in case of AF 1 culture, alginate. peat, peat supplemented with chitin/ *A. niger* mycelium and spent compost in Rabi season was 13%, 10%, 8%. 8%, 6%, and 9%, respectively (Fig 3.9b).

Plant dry weight:

Increase in plant dry weight over control in AF 1 culture, alginate. peat, peat supplemented with chitin/ *A. niger* mycelium and spent compost treatments sown in Kharif and Rabi seasons was 29%, 24%, 16%, 32%, 16%, 20% and 30%, 25%, 15, 33%, 17%, 21%, respectively (Fig 3.9c and Plate Fig 3).

The increase in emergence over control in different formulation product treatments in glass house experiments varied between 33% to 16% and in field experiments it was 28% to 18% (Kharif season) and 29% to 8% (Rabi season). Increase in plant length over control in glass house experiments varied between

Fig.3.9 Plant growth promoting activity of AF 1 formulations in the field: Pigeon pea (ICPL 85010) seeds were treated with freshly grown AF 1 and different formulations stored for four months after their preparation. Treated seeds were sown in native soil in Kharif and Rabi seasons. Percent increase in a) emergence; b) plant length; c) plant dry weight was recorded in treatments with freshly grown AF 1 ( ), alginate ( ), peat ( ), peat+chitin ( ), peat+*A. niger* mycelium ( ) and spent compost ( ).

The values are the means of triplicate determinations.

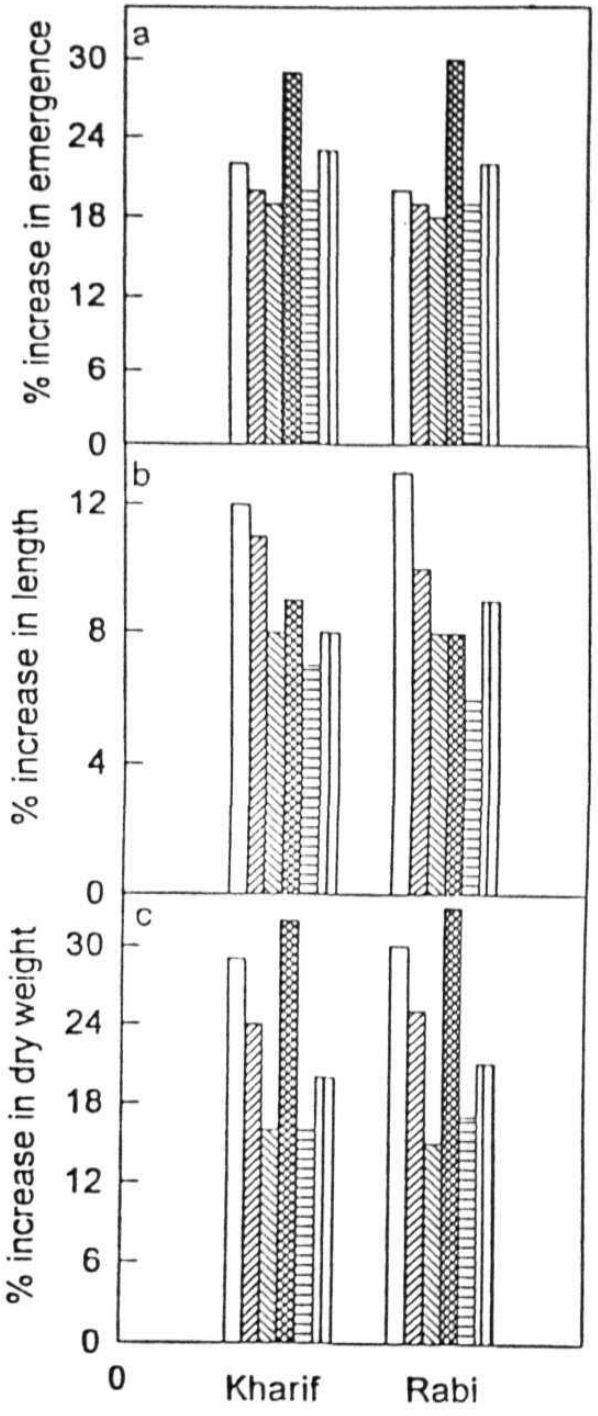

a
% increase in emergence
30
24
18
12
6
0
b
% increase in length
12
8
4
0
c
% increase in dry weight
30
24
18
12
6
0
0
Kharif
Rabi

Fig. 3.9

Plate Fig 3 Plant growth promoting activity of AF 1 formulations in the field: Pigeon pea (1CPL 85010) seeds were treated with freshly grown AF 1 and different formulations stored for four months. Formulation product treated seeds were sown in native soil in field plots. Growth of treated plants was compared with that of control (CMC treated) plants. Different treatments include

A) Comparison of CMC and AF 1 treated plants

B) AF 1 culture

C) Peat

D) Peat+*A. niger* mycelium

E) CMC control

F) Alginate

G) Peat+chitin

H) Spent compost

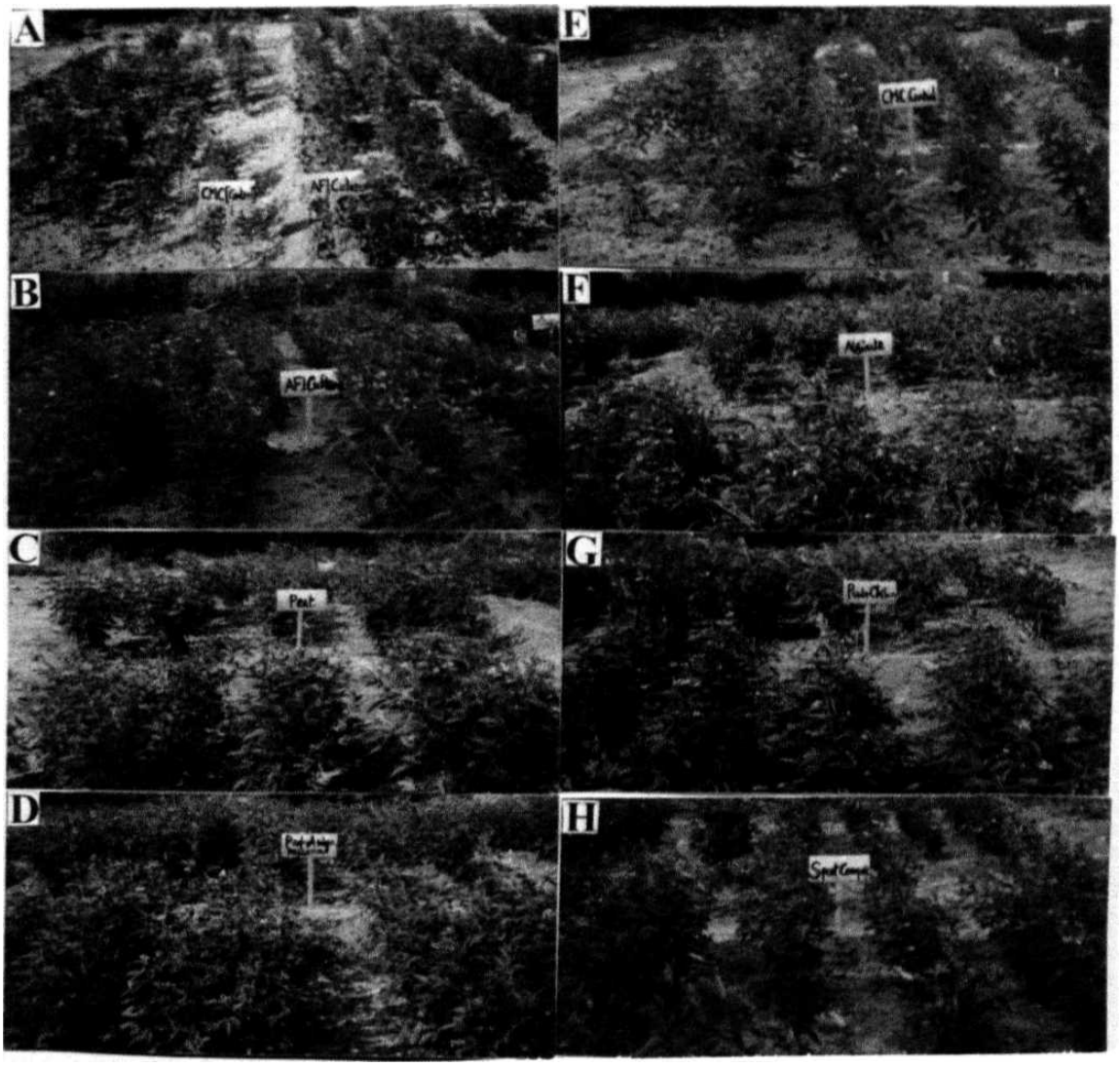


Plate Fig 3

13% to 6% and in field experiments it was 12% to 7% (Kharif season) and 13% to 6% (Rabi season). The increase in dry weight of formulation product treated plants varied between 34% to 14% in glass house experiments and in field experiments it was between 32% to 16% (Kharif) and 33% to 15% (Rabi).

Biocontrol activity of formulations in the glass house:

Biocontrol activity of AF 1 formulations on pigeon pea wilt and groundnut crown rot was compared with the fungicide treatments in glass house. Experiments were conducted with wilt susceptible and resistant genotypes of pigeon pea. crown rot susceptible and resistant genotypes of groundnut. The seedling emergence, plant length and dry weight of plants grown in pathogen- infested and native soil were recorded in different treatments.

Control of pigeon pea wilt:

Wilt incidence in two pigeon pea genotypes ICP 2376 (wilt-susceptible) and ICP 8863 (wilt-resistant) was studied using alginate, peat-supplemented chitin, spent compost formulations and freshly grown Al' 1 culture as seed treatments.

Disease control in wilt susceptible genotype (ICP 2376):

The wilt incidence in control plants was found to be 68% when a wilt susceptible genotype of pigeon pea (ICP 2376) was used. In presence of freshly grown AF 1 culture, and formulations in alginate. peat-supplemented with chitin and spent compost treatments the disease control was 52%, 50%, 68% and 54%. respectively, while it was 90% in thiram treatment (Fig. 3.10a). The disease control in different formulation treatments varied between 50% to 68%. Peat-supplemented with chitin offered a highest of 68% protection when compared to other formulations (Plate Fig. 4).

Disease control in wilt resistant genotype (ICP 8863):

Wilt incidence in a wilt resistant genotype of pigeon pea (ICP 8863) was studied using the same formulation products. Thiram conferred 98% disease control while disease control in AF 1 culture, alginate, peat-supplemented chitin and spent compost formulation treatments was 48%, 42%, 58% and 54%, respectively (Fig 3.10b and Plate Fig 4).

Fig 3.10 Disease control and plant growth promoting activities of different formulations on wilt susceptible (ICP 2376) and resistant (ICP 8863) pigeon pea genotypes in presence/absence of *F. udum*: Seeds were treated with freshly grown AF 1 and different formulations stored for four months after preparation. Treated seeds were sown in native and *F. udum*-infested soil separately. Disease control in a) susceptible (ICP 2376) and b) resistant (ICP 8863) genonpes.

Plant growth promoting activity of formulations (c - h) in terms of percent increase in emergence (c & d), plant length (e & f) and dry weight (g & h) in thiram ( ), freshly grown AF 1 cells ( ), alginate ( ), peat+chitin ( ) and spent compost ( ) treatments.

The values are means of triplicate determinations.

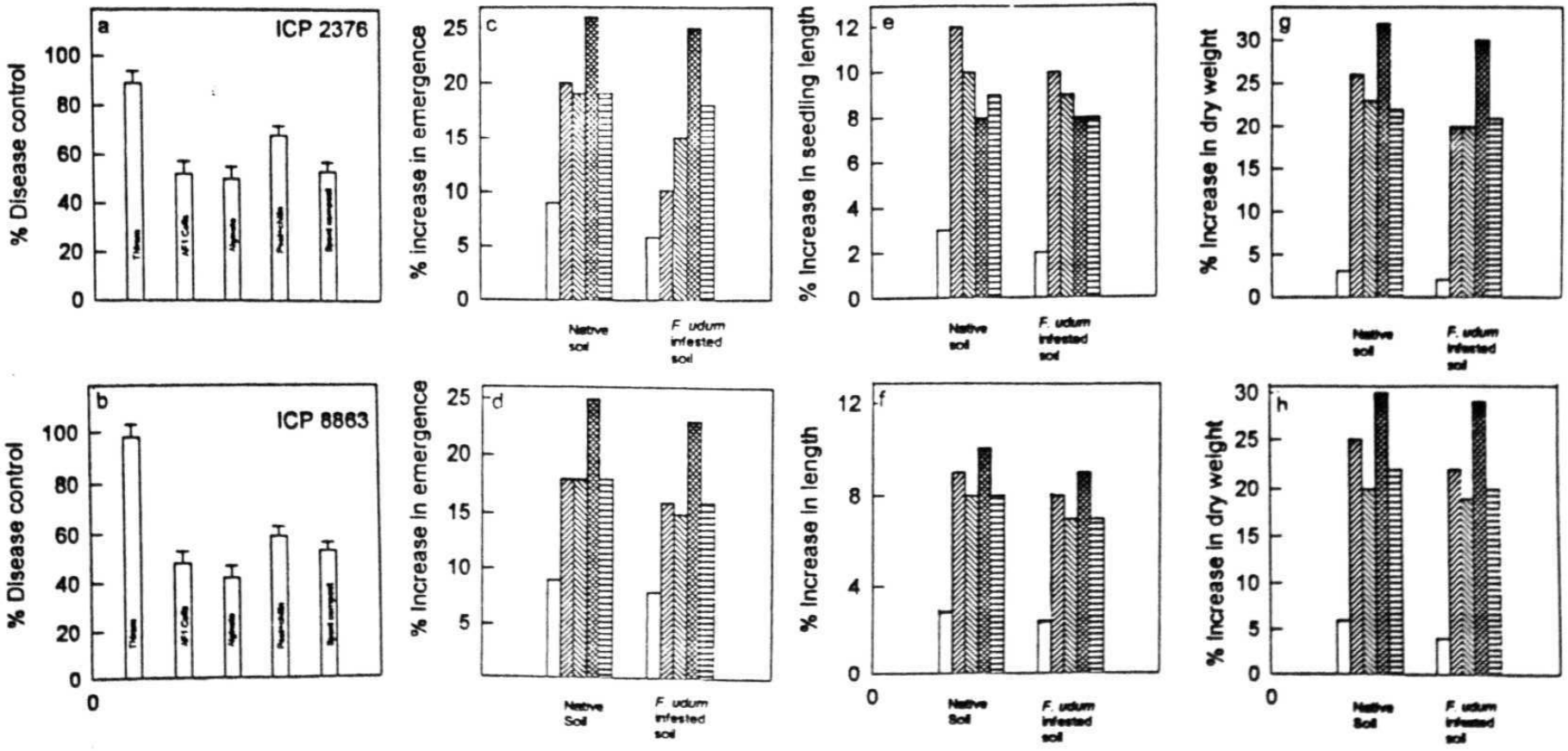

a
ICP 2376
% Disease control
b
ICP 8863
% Disease control
c
% increase in emergence
Native soil
F. udum infested soil
d
% Increase in emergence
Native Soil
F. udum infested soil
e
% Increase in seedling length
Native soil
F. udum infested soil
f
% Increase in length
Native Soil
F. udum infested soil
g
% Increase in dry weight
Native soil
F. udum infested soil
h
% Increase in dry weight
Native Soil
F. udum infested soil

Fig. 3.10

Plate Fig 4 Biological control of Fusarial wilt in pigeon pea: Disease incidence was compared in different treatments in wilt susceptible genotype (Left panel, ICP 2376) and in resistant genotype (Right panel, ICP S863).

Different treatments include: A) *F. udum* Control

B) Thiram

C) Peat+Chitin

D) AF 1 cells

E) Alginate

F) Spent Compost

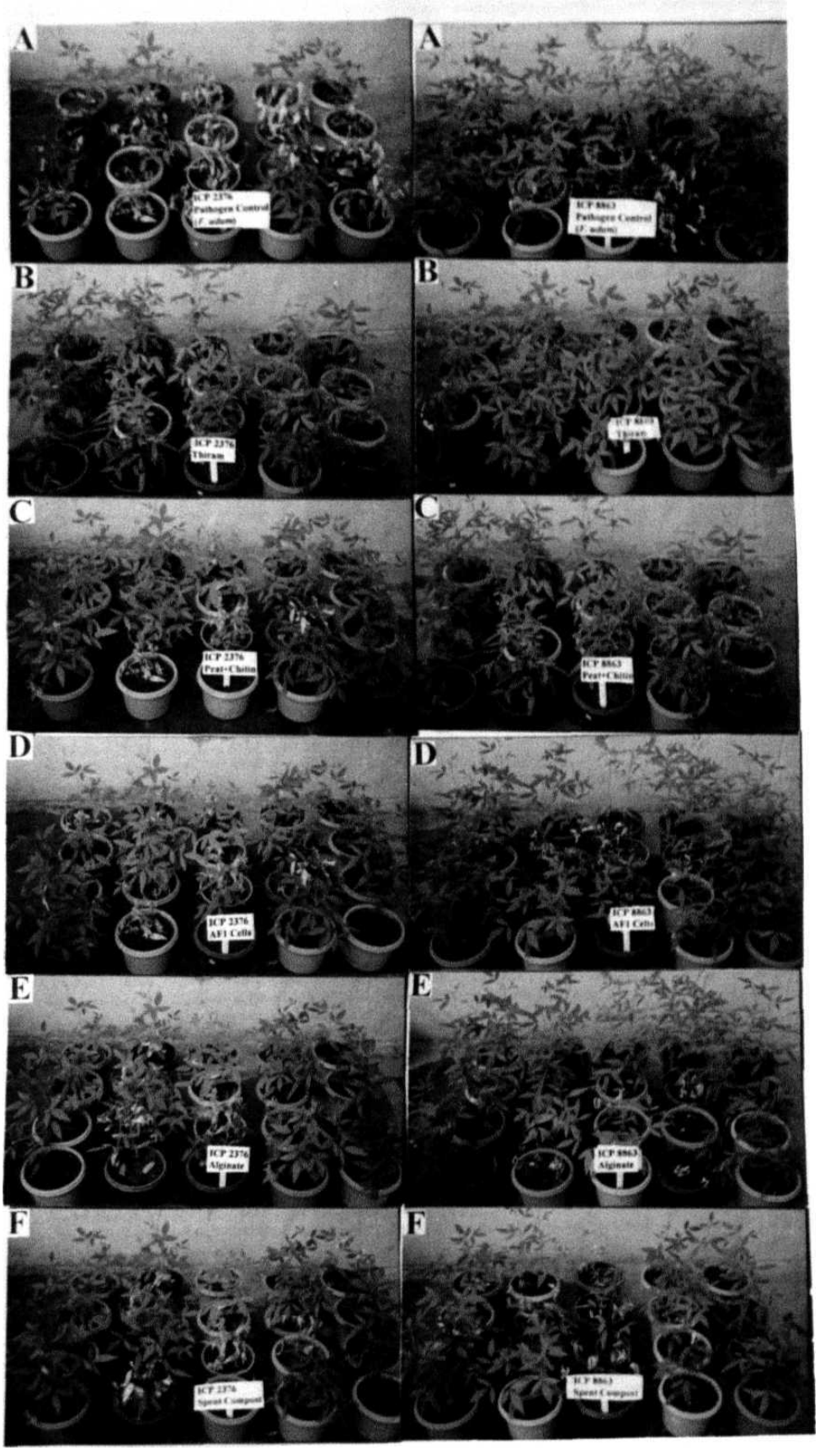


Plate Fig 4

Growth promotion in native and pathogen-infested soils:

Increase in seedling emergence. plant length and plant dry weight were recorded in formulation product treated seedlings grown either in pathogen infested or in native soils. Wilt resistant and susceptible genotypes were used and efficiency of AF 1 formulations was compared with thiram treatment.

Growth promotion in susceptible genotype (1CP 2376):

Seedling emergence: The increase in emergence over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin. spent compost and thiram treatments in pathogen infested soil was 7%, 15%, 25%, 17% and 10%, and in the native soil it was 20%, 18%, 26%. 18% and 8%, respectively (Fig 3.10c).

Plant length: The increase in plant length over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 10%, 9%. 8%, 8% and 2%, respectively. The increase in plant length over control in native soil was 12%, 10%, 8%, 9% and 3%, in the same order (Fig 3.1 Oe).

Plant dry weight: The increase in plant dry weight over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin. spent compost and thiram treatments in pathogen infested soil was 20%, 20%, 28%, 21% and 2% and in the native soil it was 26%. 23%. 32%. 22% and 3%. respectively (Fig 3.10g and Plate Fig 5).

Growth promotion in resistant genotype (ICP 8863):

Seedling emergence: In wilt-resistant genotype of pigeon pea the increase in emergence in AF 1 culture. alginate. peat supplemented with chitin. spent compost and thiram treatments in pathogen infested soil was 16%, 15%. 23%. 16% and 7%, respectively. In native soil the increase in emergence was 17%. 17%, 25%, 17% and 8% in the same order (Fig 3.10d).

Plant length: The increase in plant length over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 8%, 7%, 9%. 7% and 2%, respectively. The increase in plant length over control in native soil was 9%, 8%, 10%, 8% and 3%, in the same order (Fig 3.1 Of).

Plant dry weight: The increase in plant dry weight over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 22%, 18%, 28%, 20% and 4% and in

the native soil it was 25%, 20%, 30%, 22% and 6%, respectively (Fig 3.1 Oh and Plate Fig 5).

Thiram treatment resulted in maximum disease protection associated with increase in emergence but no significant increase in plant length or dry weight. Chitin- supplemented peat formulation showed better disease protection associated with increased emergence and dry weight but no significant increase in plant length, compared to other formulation products.

In presence of freshly grown AF 1 culture, and formulations in alginate, peat-supplemented with chitin and spent compost treatments the disease control in wilt susceptible genotype (ICP 2376) was 52%, 50%, 68% and 54%, respectively, while it was 90% in thiram treatment. In wilt resistant genotype (ICP 8863) thiram conferred 98% disease control while disease control in AF 1 culture, alginate, peat-supplemented chitin and spent compost formulation treatments was 48%, 42%, 58% and 54%, respectively. The increase in seedling emergence, plant length and dry weight in susceptible genotype varied between 25% to 10%. 10% to 8% and 28% to 20%, respectively. In the resistant genotype increase in emergence, plant length and dry weight varied between 23% to 15%, 9% to 7% and 28% to 18%, repectively. In thiram treatment the increase in seedling emergence, plant length and dry weight in susceptible genotype was 7%, 2%, and 2%, while in resistant genotype it was 7%, 2%, and 3%, respectively.

Control of crown rot in groundnut: Disease incidence in crown rot susceptible (TMV *2)* and resistant (FDRS 10) genotypes was studied using different formulation products in comparison with bavistin seed treatment.

Disease control in crown rot susceptible genotype (TMV 2) :

The disease control in susceptible genotype (TMV 2) was 49%, 48%, 68% and 59% with freshly grown AF 1 cells, alginate, peat+chitin and spent compost treatments, respectively (Fig.3.11a and Plate Fig 6). Bavistin treatment conferred 90° o disease control in TMV 2.

Disease control in crown rot resistant genotype (FDRS 10) :

In a resistant genotype (FDRS 10) the disease control was 42%, 38%, 59% and 52% with freshly grown AF 1 cells, alginate, peat+chitin and spent compost treatments, respectively (Fig. 3.11b). Bavistin treatment conferred 98% disease

Plate Fig. 5 Plant growth promoting activity of different formulations: Plant growth was compared in plants grown in native (left side pot) and *F. udum*-infested (right side pot) soil. Left panel presents treatments in susceptible (ICP 2376) pigeon pea genotype and the right panel in resistant (ICP 8863) genotype.

Different treatments include: A) Control & *F. udum*

B) AF 1 cells

C) Peat+Chitin

D) Thiram Control

E) Alginate

F) Spent Compost

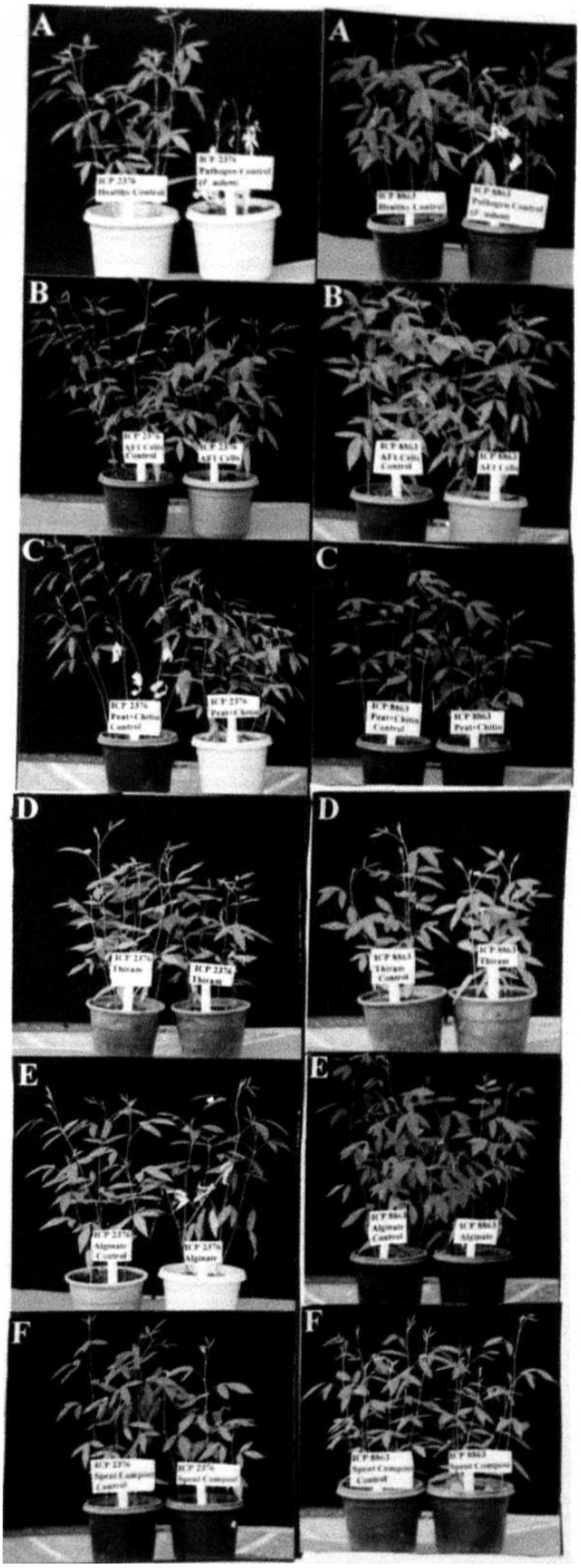


Plate Fig 5

Fig. 3.11 Disease control and plant growth promoting activities of different formulations on crown rot susceptible (TMV 2) and resistant (FDRS 10) groundnut genotypes in presence/absence of *A. niger:* Seeds were treated with freshly grown AF 1 and different formulations stored for four months after preparation. Treated seeds were sown in native and *A. niger*-infested soil separately. Disease control in a) susceptible (TMV 2) and b) resistant (FDRS 10) genotypes.

Plant growth promoting activity of formulations (c - h) in terms of percent increase in emergence (c & d), plant length (e & f) and dry weight (g & h) in bavistin ( ), freshly grown AF 1 cells ( ), alginate ( ), peat+chitin ( ) and spent compost ( ) treatments.

The values are means of triplicate determinations.

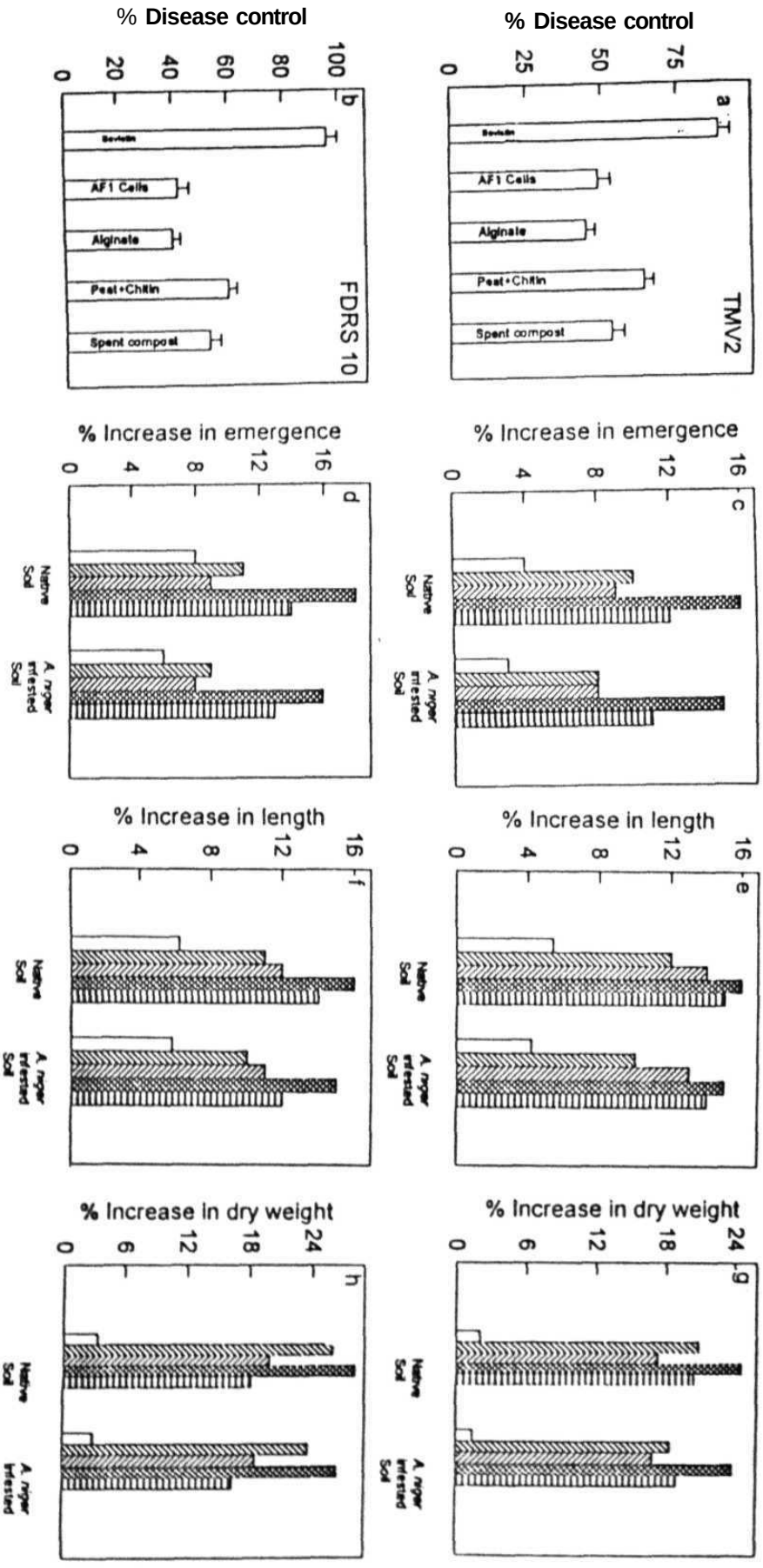

% Disease control
a
TMV2
AF1 Cells
Alginate
Peat+Chitin
Spent compost
% Disease control
b
FDRS 10
% Increase in emergence
c
% Increase in emergence
d
% Increase in length
e
% Increase in length
f
% Increase in dry weight
g
% Increase in dry weight
h
Native Soil
A. niger infested Soil

Fig. 3.11

control in FDRS 10. Peat-supplemented with chitin showed better performance followed by spent compost, fresh culture and alginate formulations.

Growth promotion in native and pathogen infested soil:

Increase in emergence, seedling length and dry weight of formulation product treated seedlings was recorded in both the susceptible and resistant genotypes in native and pathogen infested soil. The performance of formulations was compared with that of bavistin treatments.

Growth promotion in susceptible genotype (TMV 2):

Seedling emergence: The increase in emergence over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin, spent compost and bavistin treatments in pathogen infested soil was 8%, 8%, 15%, 11% and 3%, and in the native soil it was 10%, 9%, 16%, 12% and 4%, respectively (Fig 3.11c).

Plant length: The increase in plant length over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 10%. 14%, 15%, 14% and 4%, respectively. The increase in plant length over control in native soil was 12%, 14%, 16%, 15% and 6%, in the same order (Fig 3.11e).

Plant dry weight: The increase in plant dry weight over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 19%. 17%, 23%, 19% and 1% and in the native soil it was 21%, 17%, 24%, 20% and 2%, respectively (Fig 3.11g and Plate Fig 6).

Growth promotion in resistant genotype (FDRS 10):

Seedling emergence: The increase in emergence in AF 1 culture, alginate, peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 9%, 8%, 15%, 13% and 6%, respectively. In native soil the increase in emergence was 11%, 9%, 19%, 15% and 8% in the same order (Fig **3.11d).**

Plant length: The increase in plant length over control in freshly grown AF 1 culture, alginate, peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 10%, 11%, 15%, 12% and 6%, respectively. The increase in plant length over control in native soil was 10%, 12%, 16%, 14% and 6%, in the same order (Fig 3.11f).

Plate Fig. 6 Plant growth promoting activity of different formulations: Plant growth was compared in plants grown in native (left side pot) and *A. niger*- in tested (right side pot) soil. Left panel presents treatments in susceptible (TMV 2) pigeon pea genotype and the right panel in resistant (FDRS 10) genotype.

Different treatments include: A) Control & *A. niger*

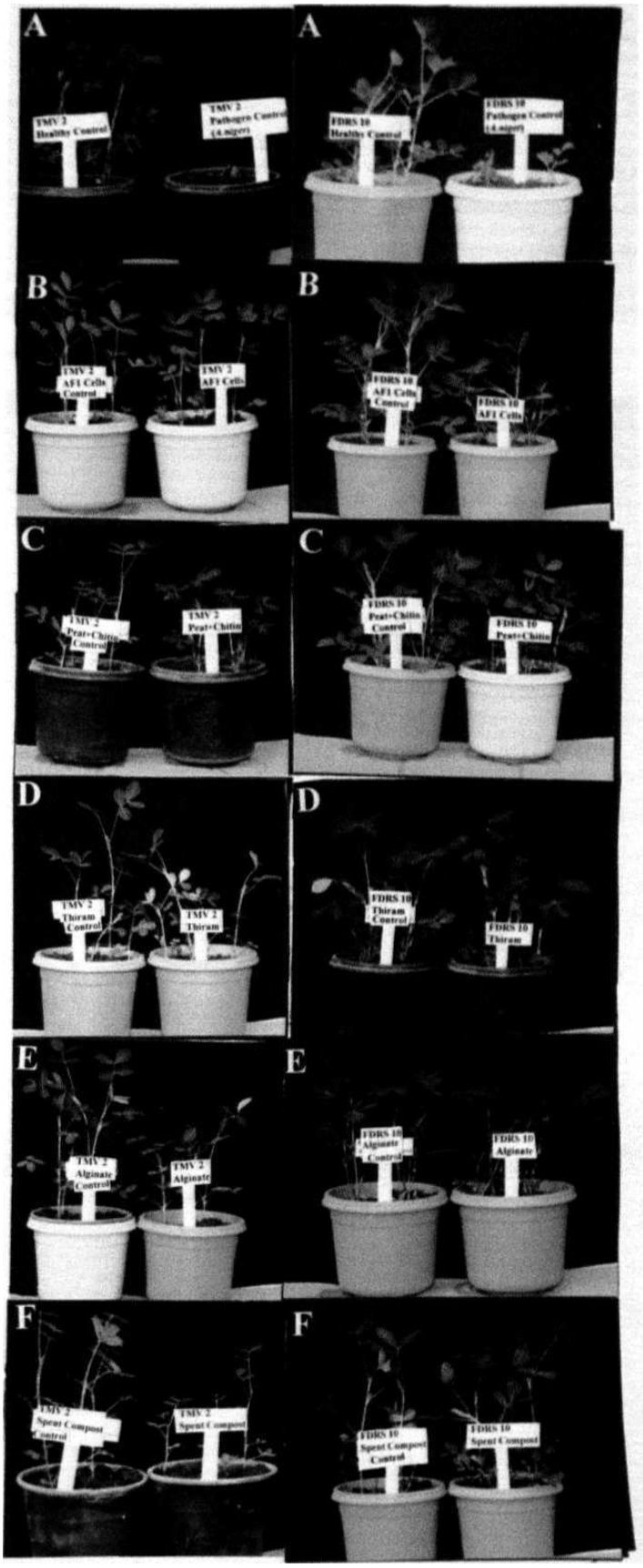
B) AF 1 cells

C) Peat+Chitin

D) Bavistin Control

E) Alginate

F) Spent Compost


A
TMV 2
Healthy Control
TMV 2
Pathogen Control
(A.niger)
FDRS 10
Healthy Control
FDRS 10
Pathogen Control
(A.niger)
B
TMV 2
AF1 Cells
Control
TMV 2
AF1 Cells
FDRS 10
AF1 Cells
Control
FDRS 10
AF1 Cells
C
TMV 2
Peat+Chitin
Control
TMV 2
Peat+Chitin
FDRS 10
Peat+Chitin
Control
FDRS 10
Peat+Chitin
D
TMV 2
Thiram
Control
TMV 2
Thiram
FDRS 10
Thiram
Control
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E
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Alginate
F
TMV 2
Spent Compost
Control
TMV 2
Spent Compost
FDRS 10
Spent Compost
Control
FDRS 10
Spent Compost

Plate Fig 6

Plant dry weight: The increase in plant dry weight over control in freshly grown AF 1 culture, alginate. peat supplemented with chitin, spent compost and thiram treatments in pathogen infested soil was 23%, 18%, 26%, 16% and 2% and in the native soil it was 26%, 20%, 28%, 18% and 3%, respectively (Fig 3.1 1h and Plate Fig 6).

In freshly grown AF 1 culture, and formulations in alginate, peat-supplemented with chitin and spent compost treatments the disease control in crown rot susceptible genotype (TMV 2) was 49%, 48%, 68% and 59%, respectively, while it was 90% in bavistin treatment. In crown rot resistant genotype (FDRS 10) bavistin conferred 98% disease control while disease control in AF 1 culture, alginate, peat-supplemented chitin and spent compost formulation treatments was 42%, 38%. 59% and 52%, respectively. The increase in seedling emergence, plant length and dry weight in susceptible genotype varied between 15% to 8%. 15% to 10% and 23% to 17%, respectively. In the resistant genotype increase in emergence, plant length and dry weight varied between 15% to 8%. 15% to 10% and 26% to 16%, repectively. In thiram treatment the increase in seedling emergence, plant length and dry weight in susceptible genotype was 3%, 4%, and 1%, while in resistant genotype it was 6%. 6%. and 2°o. respectively.

Vigour in disease resistant and susceptible genotypes of pigeon pea and groundnut:

Seedlings treated with formulated products were always healthy and vigorous compared to the untreated seedlings. In bavistin treatment disease protection was maximum with no significant increase in seedling biomass. Bavistin promoted seedling emergence comparable with that of formulation products treatments.

AF 1 and its formulation products were able to promote emergence, increase in biomass of plants grown even underpathogen pressure. The vigour of plants treated with AF 1 was less in pathogen infested soil in the beginning, they have picked up gradually. There was increase in seedling emergence and biomass similar to the plants grown in native soil (Plate Fig 5 and 6). Thiram and bavistin offered better disease protection than the bacterial treatments.

Survival of *B. subtilis* AF 1 on the rhizoplane of inoculated plants in glass **house and field** experiments: The amount of carrier material and volume of

Table 3.3. Mean populations of AF 1 (log CFU per cm rhizoplane) recovered from roots of pigeon pea (ICPL 8S010) seedlings treated with a) zero time b) three months and c) six months stored formulation products.

S.No	Time period (days)	AF 1			Alginate			Peat			Peat+chitin			Peat+mycelium			Spent compost		
		a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1.	0	6.6	6.6	6.6	6.5	6.4	6.5	6.3	6.3	6.3	6.5	6.4	6.4	6.3	6.1	6.3	6.2	6.1	6.3
2.	7	3.2	3.2	3.2	3.1	3.1	3.1	2.9	2.9	2.9	3.1	3.0	3.0	2.8	2.8	2.8	2.9	2.9	2.9
3.	14	4.3	4.1	4.2	4.1	4.1	4.2	4.0	4.0	4.0	4.4	4.3	4.3	4.0	4.0	4.0	4.1	4.0	4.1
4.	21	4.3	4.2	4.3	4.1	4.0	4.1	4.1	4.1	4.1	4.3	4.2	4.2	4.0	4.1	4.0	4.2	4.2	4.2
5.	28	4.0	4.0	4.0	4.0	4.0	4.0	3.8	3.8	3.8	4.2	4.2	4.2	3.8	3.9	3.9	4.0	4.0	4.0

Plants from difTerent treatments were uprooted at specified intervals. One cm root segments were macerated in 0.2 M potassium phosphate buffer pH 7.0. The resulting aliquots were serially diluted and plated on MM overlaid with 0.1% CC and 100 jig/ml ampicillin.

All values are average of triplicate determinations.

Table 3.4 Mean populations of AF flog CFU per cm rhizoplane) recovered from pigeon pea (ICPL 85010) seedlings in a) Kharif and b) Rabi seasons.

S.No	Time period (days)	AF 1		Alginate		Peat		Peat+chitin		Peat+mycelium		Spent compost	
		a	b	a	b	a	b	a	b	a	b	a	b
1.	0	6.5	6.6	6.4	6.4	6.3	6.3	6.5	6.5	6.4	6.3	6.3	6.4
2.	20	2.9	3.2	3.8	3.1	3.4	2.9	3.6	3.0	3.5	2.8	3.6	2.9
3.	40	4.3	4.2	4.1	4.2	4.1	4.0	4.3	4.3	3.9	4.0	4.0	4.1
4.	60	3.9	4.0	3.8	4.0	3.7	3.9	4.0	4.2	3.9	3.9	3.8	4.1
5.	80	3.8	3.9	3.6	3.8	3.6	3.8	4.0	4.0	3.5	3.5	3.6	3.9
6.	100	3.7	3.7	3.6	3.6	3.5	3.8	3.9	4.0	3.4	3.6	3.5	3.5

Plants from different treatments were uprooted at specified intervals. One cm root segments were macerated in 0.2 M potassium phosphate buffer pH 7.0. The resulting aliquots were serially diluted and plated on MM overlaid with 0.1% CC and 100 µg/ml ampicillin.

All values are average of triplicate determinations.

Table 3.5 Mean populations of AF 1 (log CFU per cm rhizoplane) recovered from pigeon pea seedlings a) ICP 2376 b) ICP 8863 treated with formulation products and sown in x) native and y) *F. udum*-infested soil.

a) ICP 2376

S.No	Time period (days)	AF 1		Alginate		Peat+chitin		Spent compost	
		x	y	x	y	x	y	*	y
1.	0	6.6	6.6	6.4	6.3	6.3	6.1	6.1	6.1
2.	7	3.1	3.1	2.9	2.9	3.1	3.0	2.5	2.4
3.	14	4.0	3.9	4.0	3.9	4.2	4.2	3.9	3.9
4.	21	3.9	3.9	3.8	3.8	4.1	4.1	4.0	4.0
5.	28	4.0	4.0	3.7	3.9	4.0	4.2	3.8	3.9

b) ICP 8863

S.No	Time period (days)	AF 1		Alginate		Peat+chitin		Spent compost	
		x	y	x	y	x	y	*	y
1.	0	6.5	6.6	6.5	6.3	6.5	6.4	6.1	6.0
2.	7	3.1	3.1	3.0	3.0	3.2	3.0	2.9	3.2
3.	14	4.0	4.0	3.8	3.9	4.4	4.2	3.8	3.9
4.	21	3.9	3.9	3.9	3.9	4.1	4.1	3.8	4.0
5.	28	3.8	3.8	3.7	3.7	4.1	4.1	3.9	3.9

Plants from different treatments were uprooted at specified intervals. One cm root segments were macerated in 0.2 M potassium phosphate buffer pH 7.0. The resulting aliquots were serially diluted and plated on MM overlaid with 0.1% CC and 100 fig/ml ampicillin.

All values are average of triplicate determinations.

Table 3.6 Mean populations of AF1 (log CFU per cm rhizoplane) recovered **from** groundnut seedlings of crown rot susceptible and resistant genotypes (a) TMV 2 **and** b) FDRS 10) treated with formulation products and sown in x) native and y) *A niger*-infested soil.

a) **TMV** 2

S.No	Time period (days)	AF 1		Alginate		Peat + chitin		Spent compost	
		x	y	x	y	x	y	x	y
1.	0	6.6	6.5	6.5	6.5	6.3	6.3	6.1	6.1
2.	7	3.2	3.1	3.2	3.0	3.1	2.8	2.6	2.5
3.	14	4.0	4.0	4.0	3.9	4.2	4.2	3.9	3.9
4.	21	3.9	3.9	3.9	3.9	4.1	4.1	3.9	4.0
5.	28	3.8	4.0	3.8	4.0	4.0	4.2	3.8	3.9

b) FDRS 10

S.No	Time period (days)	AF 1		Alginate		Peat + chitin		Spent compost	
		x	y	x	y	x	y	x	y
1.	0	6.5	6.4	6.2	6.2	6.4	6.1	6.1	6.2
2.	7	3.2	3.2	3.1	3.1	3.1	3.0	2.9	2.9
3.	14	4.2	4.1	4.1	4.1	4.3	4.3	4.0	4.1
4.	21	4.0	4.2	4.0	4.1	4.2	4.2	4.0	4.0
5.	28	4.0	4.0	4.0	4.0	4.2	4.2	3.9	3.9

Plants from different treatments were uprooted at specified intervals One cm root segments were macerated in 0.2 M potassium phosphate buffer pH 7.0. The resulting aliquots were serially diluted and plated on MM overlaid with 0.1% CC and 100 fig/ml ampicillin.

All values are average of triplicate determinations.

Discussion

Biocontrol bacteria inhibit fungal pathogens b> competing for the nutrients, synthesizing antifungal antibiotics and fungal cell wall hydrolytic enzymes. Chitin and β-1,3 glucans are essential structural cell wall components of fungal pathogens of vascular plants. These components could be used as target sites by chitinolytic enzymes of biocontrol agents. The role of microbial Chitinases and P-1,3 glucanases has, therefore, became one of the most important areas of research for plant disease control. The first part of the work deals with production of fungal cell wall degrading enzymes by *B. subtilis* AF 1 and inhibition of plant cell wall degrading enzymes of fungal pathogens in presence of AF 1. Second part includes preparation of formulations of AF 1 and their use in pigeon pea plant growth promotion and disease control in groundnut and pigeon pea.

I. Antagonism of biocontrol bacteria towards fungal pathogens:

a) Production of fungal cell wall degrading enzymes by biocontrol bacteria:

Mitchell and Alexander (1962) demonstrated that cell wall lytic bacteria added to soil lyse the fungal mycelium through their lytic activity. *Aeromonas caviae*, a chitinolytic soil isolate was shown to control *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *vasinfectum* in cotton. *A. caviae* partially lysed the live mycelium of all the three fungal pathogens, where their mycelium served as sole source of carbon in liquid medium (Inbar and Chet, 1991).

In the genus *Bacillus*, chitin degrading activity has been found in type strains of seven species *viz.*, *B. brevis, B laterosporus, B. lentus, B licheniformis, B. megaterium, B. thiaminolyticus*, and *B thuringiensis* and in some other strains of *Bacillus* species (Kuroshima *et al.,* 1996). *B. cereus* produces a chitobiosidase and was antagonistic to root rot pathogen *Rhizoctonia solani* (Pleban *et al.*. 1997) in cotton seedlings. *B. circulans* I AM1165 produces three major extracellular β-1,3-glucanases of which the 42 kDa enzyme lysed the fungal cell walls most effectively (Hamamura *et al.*, 1995). Similarly *B. circulans* WL-12. when grown in liquid medium with yeast cell walls or yeast glucan produced five β-glucanases (Rombouts and Phaff. 1976). Selective enzymolysis of cell walls of *Pyricularia oryzae* by single and combined actions of p-1,3- and 1,6- glucanases and chitinase produced by *B. circulans* WL-12

was reported by Tanaka and Watanabe (1995). Enzyme system from a Streptomycete active in lysing *Aspergillus oryzae* and *Fusarium solani* hyphal walls contains chitinase and several β-1,3-glucanase components. The extracellular P-l,3-glucanase and chitinase were shown to be instrumental in the process of dissolution of hyphal walls. *B. subtilis* AF 1 inoculated in to media containing *A. niger* suppressed >90% fungal growth in terms of dry weight. In dual cultures the fungal growth was not accompanied by formation of spores and the mycelial preparation of *A. niger* as principal carbon source supported the growth of *B. subtilis* AF 1 as much as chitin (Podile and Prakash, 1996).

B. subtilis AF 1 produced NAGase in presence of 0.5% colloidal chitin as carbon source. Different culture conditions were optimized for increased production of NAGase by *B subtilis* AF 1 to study its antifungal activity. Increase in the concentration of chitin and yeast extract in the growth medium of *Serratia marcescens*, *Enterobacter liquefaciens* and *Aspergillus fumigatus* resulted in increase in the levels of chitinase (Monreal and Reese, 1969). Supplementation of the fermentation medium of *B subtilis* AF 1 with yeast extract (0.3%), PMSF (0.5 mM), Tween 20 (0.04%) improved the yield of NAGase by five times. Yeast extract helped in better proliferation of bacterial cells in the medium. PMSF might have inhibited the serine proteases while Tween 20 facilitated better release of enzyme in to external medium. In the medium NAGase activity decreased in presence of Zn^{+2} and Fe^{+2} up to 70% and in presence of Co^{+2} up to 18%.

Chitinase and chitin-binding proteins:

Bacillus circulans WL-12, isolated as a yeast cell wall-lytic bacterium produces six distinct Chitinases in to growth medium. of which the chitinase Al was found to be responsible for the chitin degradation. N- terminal amino acid sequence analysis revealed that the four Chitinases A1. A2, B1, B2 were not distinct but A2 and B2 were derived from A1 and B1 by proteolytic removal of polypeptide fraction from C-terminal end. Chitinases C and D were distinct from others (Watanabe *et al.,* 1990). *B. licheniformis* B-6839 produced five Chitinases in to culture medium with molecular masses of 66, 62, 53, 49, and 42 kDa (Trachuk *et al.,* 1996). In addition to four different Chitinases, a 21-kDa protein with chitin-binding activity was found in the culture supernatant of

Serratia marcescens 2170. This protein bound to chitin affinity column with no detectable chitinase activity (Suzuki *et al*., 1998). Similar result was obtained when an attempt was made to purify chitinase(s) of *B. subtilis* AF 1. Of the four proteins eluted from chitin column, only the 67 kDa protein was able to hydrolyze chitin. The remaining three proteins could be chitin binding in nature.

The inhibitory effect of NAGase on *A. niger* growth was studied using microtitre plate assay. Results suggested the possible involvement of extracellular NAGase of AF 1 in the growth inhibition of *A niger*.

b) Effect of biocontrol agents on plant cell wall hydrolytic enzymes of fungal pathogens:

B. subtilis AF 1 inhibited the growth of *A. niger* under *in vitro* conditions with a corresponding decrease in crown rot incidence in groundnut (Podile and Prakash, 1996) while *F. udum* culture inoculated with AF 1 formed chlamydospore-like structures and vacuolated portions in mycelium suggesting the possibility of having a mechanism to tolerate mycolytic activity and grow slowly with regular conidiation (Harish *et al.* 1998). In spite of its resistance to the mycolytic activity of AF 1, *F. udum* failed to cause wilt in pigeon pea probably due to induced host plant resistance by AF 1 (Podile and Laxmi, 1998). To further understand the reasons responsible for pigeon pea wilt control, the effect of *B subtilis* AF 1 on production of plant cell wall degrading enzymes by *F. udum* was studied. The same experiment was repeated in *A. niger* also to sec the inhibition of plant cell wall degrading enzymes.

B. subtilis AF 1 inhibited the production of plant cell wall hydrolytic enzymes like exo-PG. endo-PG, PL. PML and CMCase by *A niger* and *F. udum.* The inhibition of pathogen-produced plant cell wall hydrolytic enzymes was > 50% when live AF 1 cells were inoculated, and about 20% when autoclaved cells were used. These results suggested that *B. subtilis* AF 1 possibly affects disease development in plants by inhibiting the plant cell wall degrading enzymes of fungal pathogens.

Zimand *et al.* (1995) reported that the enzymatic activity of polygalacturonase, pectin methyl esterase and pectate lyase was less in presence of the antagonist *Trichoderma harzianum* T-39 with a corresponding decrease in

Botrytis cinerea infection in bean leaves. A biocontrol strain of *T. harzianum* T-39 was able to inhibit germination of conidia, reduce the level of hydrolytic enzymes produced by *B. cinerea* with a corresponding decrease in disease incidence in bean leaves compared with a normal strain (*T. harzianum* NCIM 1185) (Kapat *et al.,* 1998). The PGPR strains *of P. fluorescens* strain B6/2 and B6/4 produced some unidentified compounds which significantly reduced phytotoxicity of culture filtrates of *Fusarium culmorum*, *F. oxysporum* and *F. solani.* More over these compounds inhibited the activity of crude cellulase. pectinase and xylanase extracted from culture filtrates of the above pathogens (Pietr, 1990). Bertangnolli *et al.* (1996) attributed the inactivation of extracellular enzymes produced by *Rhizoctonia solani* by an endo-proteinase produced by the biocontrol bacterium *Bacillus megaterium* B153-2-2.

II. Biocontrol bacteria: Formulation and application

Broadbent *et al.* (1977) used different species of Bacilli for infesting ten plant genera and observed a varied response of increased germination and top weight, or both , to no effect. Bacterization of plants with rhizobacteria led to enhanced growth and yields (Suslow and Shcroth, 1982). Bacterized and non-bacterized groundnut plants were inoculated with the root rot pathogen *Sclerotium rolfsii* and significant increase in plant growth as seen in bacterized plants (Ganesan and Gnanamanickam, 1987). Savithry and Gnanamanickam (1987) used *P. fluorescens* as seed inoculant and observed significant increase in plant height, number of pods per plant and fresh weight of pods in groundnut.

a) *Formulation of biocontrol bacteria:*

Production and use of formulations of biocontrol PGPR is desirable since aqueous suspensions of these microbes tend to perish soon after application on to seeds. Disease control by AF 1 in distantly related pathogen systems like wilt (Podile and Laxmi, 1998) and crown rot (Sailaja *et al.*, 1998) indicated the non-specific antagonistic action of *B subtilis* AF 1 against a wide spectrum of fungal pathogens. Thus the preparation and use of formulations of AF 1 for plant growth promotion and disease control, was an attempt towards commercialization.

The nature and suitability of carrier material is an important aspect for formulating a microorganism. Different carrier materials like peat (Roughley

and Vincent, 1967). filter mud (Philpotts. 1976). lignite (Kandaswamy and Prasad. 1971). coal (Crawford and Berrybill, 1983). coal bentonite mixture (Deschodt and Strijdom,1976), cellulose (Pugashetti *et al.*, 1971), compost of coir dust and soil (John, 1966) and charcoal, manure. compost, powdered coconut shells, ground teak leaves and combinations of these substrates (Tilak and Subba rao, 1978) were effectively used for formulating microbes.

All the above carrier materials were used for formulating *Rhizobium* spp. and use of perlite, rock wool, wood fibres from pinus tree bark for production of *Pseudomonas* formulations was established (Digat and Lemaire, 1991; Digat, 1988). Chao and Alexander (1984) used mineral soils as carrier material for production of inoculants of *R meliloti* and *R. phaseoli.* Vermiculite supplemented with nutrients served as a direct fermentation medium for *R japonicum* and the matrix supported viability of bacterial cell numbers between 10 to 10 per gram final product e\en after storage at room temperature for one year (Weiss *et al.,* 1987) *Pseudomonas fluorescens* pf 7-14 formulated in methyl cellulose : talc was found to be viable for 10 months where the number of cells decreased from 10 to 10 at the end of 7 months and it further decreased to 10^2 at the end of 10 months (Krishnamurthy & Gnanamanickam.1998).

Alginate formulation: Alginatc immobilization is most frequently used method for entrapping microbes for different industrial / microbiological purposes. This technique employed for production of inoculants entrapping different PGPR (Bashan. 1986) has advantages in maintaining water content around the cells and minimizing the loss in viability due to dehydration. Alginate pellets formulated with *Trichodcrma* and *Gliocladium* propagules showed better viability at 5^0C than at 25^0C. Despite the reduction in propagule viability in stored pellets numbers of CFU formed after adding these pellets to soil were comparable with those formed from freshly prepared pellets (Lewis and Papavizas, 1985). *Talaromyces flavus, Gliocladium virens, Penicillium oxalicum, Trichoderma viride* and *Pseudomonas cepacia* were formulated in alginate-pyrax clay, and the survival was monitored for 12 weeks (Fravel *et al.* 1985). The initial populations ranged from 10 to 10 propagules per ml alginate suspension where the populations declined during the test period by 10 to 100 fold after 4 weeks. Use of polymer entrapped *Rhizobium* for legumes

was proposed by Jung *et al.* (1982). Recently Bashan and Gonzalez (1999) advocated the use of dry form of alginate inoculant for *Azospirillum brasilens* and *Pseudomonas fluorescens*. Modifications in alginate bead preparation using a specially designed machine for production of beads of micro-alginate consisting of a central core containing bacterial inoculum and an envelope made up of calcium alginate was reported by Digat. (1993).

The results in this study indicated that the alginate formulation retains cells in viable form and is not suitable to support multiplication of cells. The recovery of cells depended on the growth phase of the cells used for immobilization and the temperature at which the product was stored. The recovery of mid log phase grown cells, immobilized in alginate and stored at 30^0C was least. More than 90% of the cells were recoverable from the late log and sporulating phase cells of AF 1 immobilized in alginate even after storage at 30°C.

Peat-based formulation: In the three growth phases of AF 1 cultures inoculated in to peat and stored 30°C, cell numbers increased slowly and steadily. The maximum log CFU reached per gram product was 9.2. where the initial log CFU was 5.1. The conventional means to produce the rhizobacterial inoculants involves inoculating neutralized peat with bacterial suspension of 10^7 cells per gram of peat, which reaches a final population density of approximately 10 to 10 bacteria per gram of product (Roughley and Vincent, 1967; Tilak and Subba Rao, 1978).

Peat formulation with diluted liquid cultures of *B. subtilis* AF I: The maximum cell numbers reached with diluted and undiluted cultures was similar but the time required by AF 1 to reach the maximum cell density being different. The results indicated that inoculation of peat with diluted cultures affects the multiplication rate of the introduced strain but not the survival. The significance of using diluted cultures lies in the fact that *B. subtilis* AF 1 would multiply and attain high numbers in pre-sterilized peat on introduction. After reaching maximum numbers, the AF 1 cells reflect characteristics similar to those of strains in peat inoculants prepared from undiluted cultures. The use of water as a suitable diluent has an interesting implication of bringing out the intrinsic properties of peat, indicating that the peat samples used for inoculant

production are naturally self-sufficient in the nutrients required for multiplication and long term survival of AF 1 in the inoculants.

The suitability of water or un-inoculated liquid medium as a diluent may have economic implications for inoculant production systems with pre-sterilized peat, in that the diluent can be used for diluting smaller volumes of fully grown cultures of bacteria before incorporation in to autoclaved peat. Furthermore, diluting liquid cultures significantly reduces the size and capacity of fermenters required for growing bacteria. The use of diluted cultures and pre-sterilized peat may be a cheaper option for small to medium scale inoculant production units compared with the use of undiluted cultures.

These results compare well with the previous studies of Somasegaran and Halliday (1982) and Somasegaran (1985) with *Rhizobium* spp. where undiluted and 1,000-fold diluted fully grown cultures of *R. phaseoli* TAL 182 and *R. japonicum* TAL 102 showed no differences in the survival or shelf life of the inoculants prepared in peat.

Chitin / *A. niger* mycelium-supplemented peat formulation: Supplementation of peat with chitin or fungal mycelium significantly increased (by 1.0 log CFU) the highest cell numbers per gram of final product compared with peat-based formulation. Peat supplemented with chitin (0.5%) and inoculated with mid log, late log and sporulating phase cultures (stored at 30°C), reached maximum cell density (10.1 log CFU) with in 80. 100 and 120 days, respectively. Peat supplemented with mycelium (0.5%) showed slow rate of multiplication of cells compared to chitin-supplemented peat and the maximum cell density of (10.1 log CFU) was reached after 120, 140 and 160 days in mid log, late log and sporulating phase cultures of AF 1. The ability to degrade chitin resulted in better multiplication of *B. subtilis* AF1 in carrier materials supplemented with chitin or chitin-related substances. Mushroom spent compost based formulation: Recent reports on use of water extracts of composts in inducing systemic resistance against fungal pathogens of plants (Weltzien, 1992) and role of composts in selective proliferation of PGPR (Alvarez *et al.,* 1995) have led to the idea of using mushroom spent compost as a carrier material for formulating *B. subtilis* AF 1.

Over a period of six months the cell numbers decreased by 0.40 to 0.70

log CFU in spent compost inoculated with mid log. late log and sporulating phase cultures of AF 1 stored at 4°. In 30°C stored spent compost inoculated with mid log. late log and sporulating cultures of AF1, there was a gradual increase in cell numbers up to 100, 80 and 80 days after which it decreased by 0.24, 0.39 and 0.29 log CFU, respectively over the remaining time period. The increase in AF 1 populations during storage at 30°C indicates multiplication of the bacterium, which is a similar phenomenon observed in peat inoculants. The highest cell numbers attained per gram of the final formulation product was same as that of peat-supplemented with chitin/ *A. niger* mycelium. The result suggests suitability of naturally occurring agro-wastes like mushroom spent compost rich in mycelial residues as carrier materials for formulating chitinolytic biocontrol bacteria.

Physiological state of the microbe vs. Multiplication in carrier material:

The differences in multiplication/survival of AF 1 in different formulations depends on nature of carrier material and physiological state of *B. subtilis* AF 1.

Carrier materials with or without adjuvant generally extend the shelf life of the formulation. Microbes may remain viable in formulations either in a dormant or a metabolically active state. *B. subtilis* AF 1 was formulated both in physiologically active and dormant state i.e. AF 1 was grown till mid log, late log and sporulating phase and used for preparation of formulations.

The mid log phase culture containing metabolically active cells of AF 1 immobilized in calcium alginate, when stored at 4°C, showed a decrease of 4.90 log CFU over a period of 6 months. The same product stored at 30°C showed no recovery of cells after 140 days. Sporulating phase of AF 1 cells immobilized in calcium alginate had shown highest recovery of cells with intermediary values in case of late log phase AF 1 cells. In alginate beads stored at 30°C, the mid log phase cells showed least recovery due to complete dehydration. Recovery of cells from alginate beads immobilized with late log phase cells is intermediate to that of mid log phase and sporulating phase cells. Sporulating phase culture of AF 1 used in alginate immobilization stored at 4°C and 30°C showed negligible decrease in cell numbers. In beads prepared with

sporulating cells of AF 1 the cell numbers remained constant and showed no further decrease beyond 9.72 log CFU even after 6 months of storage.

The mid log. late log and sporulating phase cells of *B. subtilis* AF 1 inoculated in to peat and stored at 4°C showed a decrease of 1.1 to 0.9 log CFU, respectively. At 30°C stored products all the three phases of cells showed increase in AF 1 numbers over a period of six months, indicating slow multiplication. Similar trend was observed in case of diluted cultures inoculated into peat, peat supplemented with 0.5% chitin/*A niger* mycelium and mushroom spent compost formulations stored at 30°C. In all the cases the carrier material served as a multiplication medium at 30°C.

Survival of *B. subtilis* AF 1 was good in alginate beads and multiplication was good in peat and spent compost. AF 1 inoculated in to spent compost from *Agaricus bisporus* cultivation showed better multiplication of cells. Mushroom spent compost used in formulations serves the dual purpose of waste utilization and as organic fertilizer apart from serving as a carrier medium for the bacterial inoculum. Peat supplemented with chitin / *A. niger* mycelium and spent compost containing *A. bisporus* mycelium used for inoculating AF 1 has other implications apart from serving as a nutritive source for chitinolytic bacteria. Recent studies on use of chitin chitosan in farming have established the yield increases along with impro\ement of beneficial micro flora of plants and induction of various self defense compounds like phyloalexins. PR-proteins, proteinase inhibitors and lignins (Hirano, 1997).

b) Application of formulation products:

Alginate formulation containing sporulating phase AF 1 cells, peat with and without supplements and spent compost formulations stored for four months at 30°C (most of them contain sporulating phase of AF 1 cells in them) were used as seed treatments and the plant growth promoting and biocontrol activities were assessed in pigeon pea and groundnut, respectively. Seed treatment with sporulating phase of AF 1 cells has an advantage over metabolically active phase of AF 1. The metabolically active Bacilli coated on to seeds may become inactive by the time the seed germinates which is not the case with sporulating phase of cells. Sporulating Bacilli coated on to seeds may have the opportunity to be the first colonizers of host plant roots. *Bacillus* spp. are

highly competitive colonizers of ecological niche because they are relatively acid tolerant where seed surface pH is not as important as for the gram negative bacteria. *Bacillus* spp. were not significantly affected by seed treatment fungicides also.

Plant growth promoting activity:

Plant growth promoting activity in terms of seedling emergence, plant length and dry weight of freshly grown AF 1, alginate. peat, peat-supplemented chitin/*A. niger* mycelium and spent compost formulations was determined using pigeon pea (ICPL 85010), both in glass house and field experiments. Seedling emergence, plant length and dry weight with different formulations in the glass house and the field experiments in two different cropping seasons (Kharif and Rabi) were not significantly different. Formulation products promoted pigeon pea growth in glass house conditions and field conditions with equal efficiency.

Plant growth promoting activity of different PGPR strains differ when assessed under controlled glass house conditions and in field conditions. The reason being sensitiveness of the PGPR strains to varied environmental conditions. But, in the present study the growth promoting activity of formulation products were not significantly different in glass house and field trials indicating the consistency in performance of the introduced PGPR strain in the given formulation product.

Biocontrol activity:

Biocontrol activity of freshly grown AF 1, alginate. peat supplemented chitin and spent compost formulations in controlling pigeon pea wilt and groundnut crown rot in both susceptible and resistant genotypes was assessed in glass house conditions.

Pigeon pea wilt: The disease control in different formulation treatments varied between 50% (alginate) to 68% (chitin- supplemented peat) in wilt susceptible variety of pigeon pea (ICP 2376). Peat supplemented with chitin offered 68% disease protection which was highest when compared to other treatments..

Similarly disease control in a wilt resistant genotype of pigeon pea (ICP 8863) varied between 42% (alginate) to 58% (chitin- supplemented peat). In presence of freshly grown AF 1 culture, and formulations in alginate, peat-supplemented with chitin and spent compost treatments the disease control in

wilt susceptible genotype was marginally higher than the control in resistant genotype. The disease control was maximum in thiram followed by chitin-supplemented peat and spent compost formulations in both the genotypes

Formulation products promoted emergence, increase in biomass of plants grown even under disease conditions. Increase in seedling emergence, plant length and dry weight in susceptible and resistant genotypes was similar. In thiram treatment, increase in seedling emergence was observed with no increase in plant length and dry weight.

Fusarial wilts are difficult to control with chemicals due to the systemic nature of the disease. Breeding for resistance also has limited success due to the complexity of the disease. Biological control can supplement the existing practices. Suppression of fusarial wilts with the use of non-pathogenic strains of *Fusarium* spp. and strains of *Pseudomonas fluorescens* (Alabouvette *el al.,* 1998) and *B subtilis* AF 1 (Podile and Laxmi, 1998) has been reported. *P fluorescens* WCS 374 has been applied commercially ("BioCoat") on radish seeds to suppress fusarial wilt. The production of BioCoat was abandoned due to high production costs coupled with less production efficiency (Alabouvette *et al.*, 1998). Seed treatments with *Gliocladium virens* strains G-4 and G-6 and with *Bacillus subtilis* strains GB03 and GB07 reduced the colonization of tap roots and secondary roots of cotton seedlings by *Fusarium* spp. (Zhang *el al.*. 1996). Rhizosphere bacteria have shown biological control *of Fusarium* spp. in carnation (Sneh. 1981). Reports on control of Fusarial wilts by *Pseudomonas* spp., have attributed the competition for iron/siderophore production and induction of host plant resistance as mechanisms for control of the disease (Van Peer *et al.*. 1991). Soil amendment with chitin along with chitin-degrading microorganisms was reported in control of wilts in tomato (Toyoda *et al.*, 1996), but soil amendment requires large amounts of chitin. Podile and Laxmi (1998) reported pigeon pea wilt control with *B. subtilis* AF 1 and in the present study, formulations of AF 1 in peat supplemented with chitin formulation has shown promising result in controlling wilt in pigeon pea.

Crown rot in groundnut: Groundnut seeds bacterized with *B. subtilis* AF 1 showed a reduced incidence of crown rot in *A. niger* infested soil, suggesting a possible role of *B. subtilis* AF 1 in biological control of *A. niger*. Based on this result, crown rot incidence in two groundnut genotypes TMV 2 and FDRS

10 was studied using the formulation products as seed treatments. The percent disease control in susceptible variety (TMV 2) varied from 48% (alginate) to 68% (peat+chitin) and in case of resistant variety (FDRS 10) it was found to be 38% to 59%. Bavistin treatment offered 98% disease control in FDRS 10 variety where as in TMV 2 it was 90%. In this case also peat supplemented with chitin has shown better performance followed by spent compost, fresh culture and alginate seed bacterization. Therfore, it is a better option to amend the carrier material with chitin or chitin related substances for better plant protection, especially in case of soil borne diseases. In freshly grown AF 1 culture and different formulations the disease control in susceptible genotype (TMV 2) was marginally higher compared to the disease control in resistant genotype (FDRS 10). The disease control was maximum with bavistin in both the genotypes followed by chitin-supplemented peat and spent compost formulations. Increase in seedling emergence, plant length and dry weight in susceptible and resistant genotypes was similar. In bavistin treatment disease protection was maximum and it promoted emergence comparable with that of formulation product treatments. Disease control in chitin-supplemented formulation and spent compost was always better compared with other formulations indicating the efficacy of chitin-supplemented formulations in disease control.

When chitin-supplemented formulations were used for seed bacterization, the degradation products of chitin in carrier material impart certain beneficial effects to plants (Hirano. 1997) like induction of PR- proteins and other defense related enzymes and improve the beneficial microflora of plants along with suppressing deleterious fungal pathogens. formulation product treated seedlings were always more healthy and vigorous comparable with that of seedlings grown in native soil. Bavistin treatment offered maximum disease protection but there was no increase in biomass of seedlings with this treatment.

Therefore, it can be concluded that formulations of *B. subtilis* AF 1 have the potential to control soil born diseases of ground nut and pigeon pea simultaneously enhancing the growth. Both resistant and susceptible genotypes of groundnut and pigeon pea responded positively to seed bacterization. The emergence, seedling length and dry weight varied in treatments with

formulation products of AF 1 in pigeon pea and groundnut in diseased conditions

Survival of *B. subtilis* AF 1 in the rhizoplane of inoculated plants:

Information regarding the fate of a bacterium introduced in to soil is essential before its use as an inoculant on large scale. This information is useful in understanding the ecology of the bacterium. its rhizosphere competency and potential as a plant growth promoting /biological control agent.

B. subtilis AF 1 when used for groundnut seed bacterization, decreased fungal and bacterial numbers and increased the actinomycete population in the rhizosphere. Over 28 days, AF 1 declined by 0.9 log CFU in sterilized soil and by 2.6 log CFU in native soil. From the seedlings raised with groundnut seeds bacterized with AF 1 at 6.6 log CFU/seed, 4.1 and 4.7 log CFU of AF 1 were recovered from the rhizosphere and total underground parts, respectively (Podile. 1994). The survival of AF 1 in different formulations treated seedlings was assessed in groundnut and pigeon pea. The colonizing ability of AF 1 was determined for formulations stored for different time periods by enumerating the number of AF 1 cells in terms of log CFU present on the rhizoplane over a period of 28 days in glass house and over a period of 90 days in field experiments in Kharif and Rabi seasons. In all the treatments the cell numbers declined to 48% to 45% after 1 week of planting. After 2 weeks the cell numbers increased to 67% to 63%, of initial population which was stable during the entire growth period of the plant in glass house experiments. A similar trend was observed in Kharif and Rabi sown crops also. Survival of AF 1 on the rhizoplane of formulation treated plants was similar in susceptible and resistant genotypes, both in native and pathogen-infested soils.

Summary and Conclusions

Plant growth promoting rhizobacteria (PGPR) are a subset of rhizosphere microorganisms which cause increased plant growth and some times associated with biological control of plant pathogens, nutrient cycling and seedling establishment. Successful inoculation of agricultural crops with biocontrol PGPR includes the delivery of sufficient inoculum to the target, economical production of large quantities of microorganisms, extended shelf life, and the development of a convenient formulation. For microbial inoculants, the type of chosen formulation and deliver)' is a key point for the efficacy of the inoculum. Application of biopolymers like chitin and chitosan to farming soils resulted in plant growth promotion associated with disease control. Addition of composts to soil increased the incidence of PGPR in the rhizosphere and decreased the disease incidence. Agricultural wastes were used for delivery of biocontrol agents like *T. harzianum*, *G virens* and *P. fluorescens*.

Earlier work from this lab established *B subtilis* AF 1 as a potential antagonist of plant pathogenic fungi, and also promotes emergence and nodulation in pigeon pea. Seed bacterization with AF 1 enhanced the levels of defense related enzymes in groundnut and pigeon pea. AF 1 produces diffusible antifungal antibiotic and also causes extensive mycolysis. In the present work an attempt has been made to characterize mycolytic activity of AF 1 and this property was used to develop improved formulations for plant growth and disease control. The production of plant cell wall hydrolytic enzymes by two fungal pathogens in presence of *B. subtilis* AF 1 was also studied to assess the effect of biocontrol strain on pathogenecity of fungi. Formulations of AF 1 in alginate. peat and spent compost were tested for plant growth promoting and biocontrol activities in pigeon pea and groundnut. The ability of AF 1 to produce chitin-degrading enzyme(s) was used to improve the formulation with chitin supplements.

B. subtils AF 1 produced NAGase and β-1,3-glucanase in to culture medium in presence of chitin and laminarin, respectively. Fermentation conditions were optimized for increased production of NAGase and β-1,3-glucanase to study the antifungal activity. Supplementation of the growth medium with colloidal chitin (3.0%), yeast extract (0.3%), PMSF (0.5 mM), Tween 20 (0.04%) and pH adjusted to 7.0 resulted in a five-fold increased

production of NAGase. Similarly supplementation of fermentation medium with laminarin (2.0%), yeast extract (0.3%) and pH adjusted to 7.0 resulted in a three-fold increased production of P-1.3-glucanase. NAGase was partially purified as a 67 kDa protein from cell free culture filtrate after chitin-affinity chromatography. Three other chitin-binding proteins of molecular mass 40, 37 and 32 kDa with no detectable enzyme activity were also eluted from the chitin column. Similar chitin-binding proteins were reported in *Serratia marcescens*. *In vitro* inhibition of *A. niger* by crude culture filtrate proteins and partially purified NAGase was studied in micro-titre plates using ELISA reader. Inhibition of with *A niger* with 1.0 mg ml^{-1} crude culture filtrate proteins and 1.0 jig ml^{-1} partially purified glucosaminidase was identical. The inhibition of *A. niger* growth could be attributed to mycolytic activity of NAGase produced by the bacterium.

The effect of *B subtilis* AF 1 on production of plant cell wall hydrolytic enzymes (pathogenecity-related enzymes) like exo-PG. endo-PG, PME, PL and CMCase by *A. niger* and *F. udum* was studied in dual liquid cultures. Inhibition of hydrolytic enzymes was > 50% in dual cultures inoculated with live AF 1 cells and the inhibition was > 20% when autoclaved cells were used for inoculation. AF 1 may possibly affect disease development in plants by interfering with the plant cell wall hydrolytic enzyme activities of both *A. niger* and *F. udum*.

Formulation of PGPR in a suitable carrier material with other additives extends shelf life facilitating easy application on to seeds and is desirable since aqueous suspensions of PGPR tend to perish soon after application on to seeds. AF 1 grown to mid log, late log and sporulating phase was formulated in alginate, peat, peat supplemented with chitin/*A. niger* mycelium and spent compost. All these formulations were stored separately at 4^0C and 30^0C.

In alginate formulation there was no increase in cell numbers compared with that of the initial cell numbers in products stored at 4^0C and 30^0C. In mid log and late log phase cells immobilized in alginate, stored at 30°C, resulted in decrease in viability compared to sporulating phase cells. Viability of cells was maximum in sporulating phase cells immobilized in alginate irrespective of the storage temperature.

In peat formulation prepared from three different growth phases, the cells multiplied in 30°C stored products. In 4°C stored products the cell numbers decreased by 1.0 log CFU by the end of 180 days. The increase in AF 1 populations during storage at 30°C indicates multiplication of the bacterium in peat. When diluted cultures of AF 1 were used to prepare peat formulations, multiplication in 100-fold diluted cultures (with DW and PDB, separately) of AF 1 inoculated in to peat did not differ from that of undiluted cultures except for the time required to reach maximum log CFU. The use of diluted liquid cultures decreases the dependency on high capacity fermenters to produce cultures with high cell numbers for production of PGPR formulations.

In peat supplemented with chitin/ *A. niger* mycelium the maximum log CFU reached per gram final product was greater by 1.0 log CFU compared to peat alone. In spent compost formulation there was increase in AF 1 populations during storage at 30°C similar to the peat-based formulations. The maximum log CFU attained per gram of spent compost was of same magnitude as peat supplemented with chitin/*A. niger* mycelium. These results suggest that supplementation of conventional carrier materials like peat with chitin or its related substances improves the survival of the select PGPR strains with chitinolytic ability. Use of naturally occurring chitin rich agro-waste substrates like mushroom spent compost for production of formulations is desirable since the mushroom spent compost provides mycelial residues for proliferation of chitinolytic bacteria. serves as a fertilizer to the plant apart from delivering beneficial PGPR strains.

Plant growth promoting activity in terms of seedling emergence. plant length and dry weight of freshly grown AF 1. alginate, peat, peat-supplemented chitin/ *A. niger* mycelium and spent compost formulations was determined using pigeon pea (ICPL 85010). both in glass house and field experiments. Seedling emergence. plant length and dry weight with different formulations in the glass house and the field experiments in two different cropping seasons (Kharif and Rabi) were not significantly different. Formulation products promoted pigeon pea growth in glass house conditions and field conditions with equal efficiency.

Biocontrol activity of freshly grown AF 1, alginate, peat supplemented chitin and spent compost formulations in controlling groundnut crown rot and

pigeon pea will was assessed in glass house conditions. Plant growth promotion with the formulations in diseased plants was also observed. In presence of freshly grown AF 1 culture, and formulations in alginate, peat-supplemented with chitin and spent compost treatments the disease control in wilt susceptible genotype (1CP 2376) was marginally higher than the control in resistant genotype (ICP 8863). The disease control was maximum in thiram followed by chitin-supplemented peat and spent compost formulations in both the genotypes. Increase in seedling emergence, plant length and dn weight in susceptible and resistant genotypes was similar. In thiram treatment increase in seedling emergence was observed with no increase in plant length and dry weight.

In freshly grown AF 1 culture, and formulations in alginate, peat-supplemented with chitin and spent compost treatments the disease control in crown rot susceptible genotype (TMV 2) was marginally higher compared to the disease control in resistant genotype (Fl)RS 10) The disease control was maximum with bavistin in both the genotypes followed by chitin-supplemented peat and spent compost formulations. Increase in seedling emergence, plant length and dry weight in susceptible and resistant genotypes was similar. Seedlings treated with formulated product were always healthy and vigorous compared to the untreated seedlings. In bavistin treatment disease protection was maximum and it promoted emergence comparable with that of formulation product treatments. Disease control in chitin-supplemented formulation and spent compost was always better compared with other formulations indicating the efficacy of chitin-supplemented formulations in disease control.

The colonizing ability of AF 1 was determined for formulations stored for different time periods by enumerating the number of AF 1 cells in terms of log CFU present on the rhizoplane over a period of 28 days in glass house and over a period of 90 days in field experiments in Kharif and Rabi seasons. In all the treatments the cell numbers declined to 48% to 45% after 1 week of planting. After 2 weeks the cell numbers increased to 67% to 63%, of initial population which was stable during the entire growth period of the plant. A similar kind of trend was observed in Kharif and Rabi sown crops also. The survival pattern was similar in different formulation treatments, in susceptible and resistant genotypes in both native and pathogen-infested soils.

Conclusions drawn from the present study:

1. *B. subtilis* AF 1 produces β-1,4-N-acetyl glucosaminidase and β-1,3-glucanase. Secretion of these enzymes could be increased by supplementing the medium with yeast extract, Tween 20 and PMSF.
2. A 67 kDa NAGase was partially purified by affinity chromatography. Three other chitin binding proteins were also separated.
3. Extracellular proteins and partially purified NAGase of *B. subtilis* AF 1 have shown inhibitory effect on growth of *A. niger*.
4. *B. subtilis* AF 1 inhibits the plant cell wall hydrolytic enzymes of both *A. niger* and *F. udum* in dual cultures.
5. Chitin-amended or spent compost-based formulations of chitinolytic *B. subtilis* AF 1 improved the performance of formulation in plant growth promotion and disease control.

Significance of the findings in global context:

The chitin-supplemented formulations of *B. subtilis* AF 1 for seed treatments have practical advantage in providing disease control and plant growth promotion in pigeon pea and groundnut. The strategy of incorporating chitin supplements in the carrier materials has added advantage for proliferation of other beneficial chitinolytic microorganisms in the rhizosphere and improving overall plant health apart from helping in better multiplication of the target organism in the formulation. This forms the first report on the successful application of chitin-supplemented formulations of *Bacillus* spp. for seed bacterization.

Future projections:

With few more trials in field conditions and scale up studies, formulations of *B. subtilis* AF 1 can be delivered to farmers for field use. Chitinase gene(s) of *B. subtilis* AF 1 can be cloned in to *E. coli* to develop novel biocontrol formulations for foliar applications as well. The application of *B. subtilis* AF 1 along with other disease control strategies will augment the benefits to the plant in integrated disease management systems.

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