Selection, characterization and application of fungicide-tolerant or chitinolytic bacteria for control of fungal diseases of groundnut with special reference to the late leaf spot

A thesis submitted for the degree of

Doctor of Philosophy

by

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CERTIFICATE

This is to certify that Mr. Krishna Kishore Gali has carried out the research work embodied in the present thesis entitled **"Selection, characterization and application of fungicide-tolerant or chitinolytic bacteria for control of fungal diseases of groundnut with special reference to the late leaf spot"** for the degree of **Doctor of Philosophy** under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Selection, characterization and application of fungicide-tolerant or chitinolytic bacteria for control of fungal diseases of groundnut with special reference to the late leaf spot"** 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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Krishna Kishore Gali

I dedicate this work to the memories of

my sister

Dr. Madhavi Latha

who has been a source of motivation and inspiration

Contents

List of Abbreviations

°C	degree centigrade
CAS	chrome azurol S
cfu	colony forming units
CCF	cell free culture filtrate
cm	centi meter
CMC	carboxy methyl cellulose
CMCase	carboxy methyl cellulase
conc	concentration
CWDE	cell wall degrading enzymes
DAI	days after inoculation
DAPG	2,4-diacetyl phloroglucinol
DAS	days after sowing
DDW	double distilled water
DEF	percentage defoliation
DNS	dinitro salicylate
DS	disease score
EDTA	ethylene diamine tetra acetic acid
endo-PG	endopolygalacturonase
exo-PG	exopolygalacturonase
FYM	farm yard manure
g	gram
glucanase	β-1,3 -glucanase
h	hour
ha	hectare
HAI	hours after inoculation
LB	Luria-Bertani
LF	lesion frequency

lit	liter
LLS	late leaf spot
mg	milli gram
min	minute
ml	milli liter
MM	minimal medium
NA	nutrient agar
NAG	N-acetyl glucosamine
NAGase	β-1,4-*N*-acetyl glucosaminidase
nano	meters
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia lyase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDA	potato dextrose agar
PDB	potato dextrose broth
PGPR	plant growth promoting rhizobacteria
PL	pectin lyase
PME	pectin methyl esterase
RH	relative humidity
RM	Richard's medium
SDW	sterile distilled water
SDS	sodium dodecyl sulphate
Tween 20	polyoxy ethylene sorbitan monolaurate

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Introduction

1.1. Biocontrol of plant pathogenic fungi

Fungal diseases of plants significantly contribute to the yield losses in agriculture. Fungicides were developed and used extensively to reduce the crop losses due to fungal diseases and maximize crop productivity. The need for greater sustainability in agriculture and public concern about the harmful environmental effects of fungicides necessitated the development of alternate disease control strategies. Development of fungicide tolerance in pathogen strains and non-availability of both fungicides and their appropriate application technologies to resource-poor fanners further supported the need for alternate strategies. In this context, control of fungal diseases of plants by the use of naturally occurring antagonistic microorganisms has been the focus of intense research throughout the world. This approach, popularly known as biological control of plant pathogens, was successful in a number of host-pathogen systems. Biological control is eco-friendly with a potential to emerge as an alternative to chemical control.

1.2. Biocontrol agents as PGPR

Biocontrol research received a shot in the arm when some rhizosphere bacteria termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978) were reported to control the disease and also promote the growth and yield of crop plants. Subsequently, the identified rhizobacteria have been used for control of diseases in plant parts other than roots. The beneficial effects of PGPR are due to the inhibition of pathogens or other deleterious microorganisms and/or due to the production of plant growth hormones (auxins and gibberillins) or enhanced availability of nutrients to the host plant. Most often, there is no clear separation of growth promotion and biological control brought about by PGPR. Bacterial strains selected for *in vitro* antibiosis frequently demonstrate growth promotion in absence of the target pathogen. Similarly, PGPR selected initially for growth promotion even in absence of the pathogens may demonstrate biological control activity when challenged with the pathogen (reviewed by Kloepper, 1993).

1.3. Diversity of biocontrol bacteria

Several genera including *Actinoplanes*, *Agrobacterium*, *Alcaligenes*, *Amorphosporangium*, *Arthobacter*, *Azotobacter*, *Bacillus*, *Cellulomonas*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Hafnia*, *Micromonospora*, *Pseudomonas*, *Pasteuria*, *Rhizobium* and *Bradyrhizobium*, *Serratia*, *Streptomyces* and *Xanthomonas* were identified as biocontrol agents (Weller, 1988). However, when compared to other bacteria, *Bacillus* and *Pseudomonas* spp. were widely used for disease suppression.

Bacillus spp. survive under unfavorable environmental conditions by producing endospores and are highly suitable to develop commercial formulations. *B. subtilis* A 13 isolated from lysed mycelium of *Sclerotium rolfsii* had broad-range antifungal and growth promoting capabilities and was commercialized as QUANTUM - 4000 (Kloepper ***et al.***, 1991). *B. subtilis* AF 1, a PGPR strain isolated from pigeon pea wilt non-conducive soils, reduced the incidence of crown rot in groundnut and Fusarium wilt in pigeonpea (Manjula and Podile, 2001).

Pseudomonas spp. received particular attention as biocontrol agents because of their catabolic versatility, excellent root-colonizing abilities and production of broad range antifungal metabolites such as 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin and phenazines. Control of take-all disease of wheat by *P. fluorescens* isolated from soils suppressive to *Gaeumannomyces graminis* var. *tritici*, was one of the most intensively studied biocontrol systems (Weller and Cook, 1983).

1.4. Disease control by biocontrol bacteria

Disease control by antagonistic bacteria involves antagonism and elicitation of host defense responses, that may operate either individually or collectively (Bloemberg and Lugtenberg, 2001; Haas *et al.*, 2002). Antagonism includes antibiosis, competition for space and nutrients, parasitism or lysis and degradation or inhibition of enzyme or toxin production by the pathogen. Elicitation of host defense responses induces structural alterations in the cell wall and/or biochemical or physiological changes in the host plants.

1.4.1. Antibiosis: Antibiosis is due to the production of antibiotics, siderophores and other secondary metabolites like cyanide.

The antibiotics commonly produced by different antagonistic bacteria include ammonia, butyrolactones, DAPG, kanosamine, Oligomycin A, Oomycin A, phenazine-1-carboxylic acid, pyoluterin, pyrrolnitrin, viscosinamide, xanthobaccin, and zwittermycin A (Whipps, 2001). Many of these antibiotics possess a broad-spectrum activity (Raaijmakers *et al.,* 2002). The role of antibiotics in biological control was clearly established using mutation analysis and molecular genetic tools (Thomashow and Weller, 1988; Delany *et al.*, 2001; Timms-Wilson *et al.,* 2000).

Siderophores, the low molecular weight, iron chelating compounds produced by certain microbes and plants are also involved in antibiosis and nutrient competition. Siderophores primarily help the producing organism in iron acquisition under iron-limiting conditions. Buyer *et al.* (1993), using monoclonal antibodies, confirmed the production of siderophores by PGPR in rhizosphere under iron-limiting conditions. Purified siderophores had disease-suppressive effect similar to the producer strain (Kloepper *et al.,* 1980; Neilands and Leong, 1986). A siderophore over-producing mutant of *P. putida* was more effective than the wild type in control of Fusarium wilt of tomato (Vandenburgh and Gonzalez, 1984), while a siderophore-deficient mutant of *P. aeruginosa* lost its biocontrol ability (Buysens *et al.,* 1994). In majority cases, the observed disease control was not entirely due to siderophore production, but due to a combination of this trait with other traits involved in disease control (Ongena *et al.,* 1999).

Certain antagonistic bacteria produce volatile antibiotics like cyanide that inhibit the cytochrome oxidase of many organisms. The producer strains possess an alternate cyanide-resistant cytochrome oxidase and are relatively insensitive to cyanide. Mutants of *P. fluorescens* CHAO deficient in cyanide production were less effective in control of take-all of wheat and black root rot of tobacco. Complementation of mutant strain by cloned wild type *hcn*$^{+}$ genes restored the biocontTo\ ability (Voisard, 1989).

1.4.2. Competition: Siderophores by virtue of their ability to sequester most of the available Fe^{3+} in the rhizosphere, inhibit the growth of other pathogens in the vicinity of

the root system due to lack of iron (O'Sullivan and O'Gara, 1992). Suppression of Fusarium wilt of radish by *Pseudomonas* strain WCS358 is due to siderophore-mediated competition for iron (Costa and Loper, 1994). *Pseudomonas* spp. colonize both the plant roots and hyphae of pathogenic fungi (Lagopodi *et al.*, 2002) and thus compete with other organisms in the rhizoplane.

1.4.3. Parasitism or lysis: Parasitism of pathogenic fungi facilitated by the production of hydrolytic enzymes is one of the major mechanisms involved in biological control of fungal diseases. Chitinases, among the hydrolytic enzymes, are of prime importance since chitin, a linear polymer of β-(1,4)-*N*-acetylglucosamine is a major cell wall constituent in majority of the phytopathogenic fungi. Purified chitinases of *Trichodermaharzianum* (El-Katatny *el al.*, 2001), *Gliocladium virens* (Di Pietro *el al.*. 1993). *Serratia marcescens* (Ordentlich *et al.*, 1988), *S. plymuthica* (Frankowski *el al.,* 2001) and *Streptomyces* sp. (Gomes *et al.,* 2001) were highly antifungal. **That** the chitinolysis plays an important role in biological control of plant diseases has been substantiated with increased disease control by chitin-supplemented application of chitinolytic biocontrol agents (Zhang and Yuen, 2000; Manjula and Podile, 2001), greater field efficiency of chitinase preparations in disease control (Shternshis *el al.*, 2002) and enhanced biocontrol potential of genetically engineered chitinase non-producing organisms (Limon *et al.*, 1999). Mutants of biocontrol strains, deficient in chitinase production, exhibit reduced antifungal or disease control (Zhang and Yuen, 2000). Biocontrol efficacy of non-chitinolytic bacteria was enhanced by expression of the chitinases from other organisms (Downing and Thomson, 2000). **The** other important group of hydrolytic enzymes, glucanases degrade the β-1,3-glucans in the cell walls of pathogenic fungi. Diseases caused by *Rhizoctonia solani, S. rolfsii* and *Pylhium ultimum* were effectively controlled by a p-l,3-glucanase producing strain of *P. cepacia* (Fridlender *et al.,* 1993). The synergistic action of chitinases and β-1,3-glucanases was more effective in control of pathogenic fungi than either enzyme alone (Tanaka and Watanabe, 1995).

1.4.4. Degradation or inhibition of enzymes **or toxin production by the pathogen:** Pathogenic fungi produce certain extracellular hydrolytic enzymes which degrade the polymers present in plant cell walls and facilitate the fungal infection by disintegrating the cell wall. These enzymes include pectolytic enzymes (exo- and endo- polygalacturonases. pectin lyases. pectin methyl esterases), cellulases and cutinase. A reduction in the activity of these enzymes correlates with a reduction in virulence (Beraha *et al.*, 1983). *Bacillus megaterium* B 153-2-2 inhibited cellulase. pectin lyase and pectinase of *R. solani*, by producing an extracellular endoproteinase (Bertagnolli *et* ***ed.,*** 1996).

1.4.5. Induction of systemic resistance: The induction of systemic resistance by rhizobacteria was referred to as induced systemic resistance (ISR), whereas that by other agents was termed systemic acquired resistance (SAR) (van Loon ***et*** *al.*, 1998; Pieterse *et al.*, 2001). ISR operates differently from that of SAR in that salicylic acid and pathogenesis related (PR) proteins do not play a role in ISR and is mediated by the plant hormones jasmonate and ethylene as demonstrated in *Arabidopsis thaliana* (Hoffland *et al.*, 1995; Pieterse *et ed.,* 1996, 1998 and 2001). *A. thaliana* treated with rhizobacteria when challenge inoculated with the pathogen show augmented expression of genes involved in ISR (Verhagen *et al.*, 2004), suggesting that these genes were primed to respond faster or more strongly upon pathogen attack (Verhagen ***et*** *al.*, 2004). Some rhizobacteria trigger the salicylic acid dependent SAR pathway by producing salicylic acid at the root surface (DeMcyer *et al.,* 1999).

Structural alterations of plant cell walls, increase in activity of defense-related enzymes and enhanced phytoalexin production are commonly observed in plants expressing ISR (Bargabus *et al.*, 2002; Benhamou *et al.*, 2000; van Peer *et al.*, 1991).

(a) *Structural alterations:* Deposition of structural barriers like lignin and callose, and other phenolic compounds in the plant cell walls at sites of fungal penetration delay the infection process and facilitate the host plant to build up other defense reactions to restrict the pathogen growth to outer most layers. Seed treatment of pea with *P. fluorescens* 63-28, tomato with *B. pumulis* SE 34 (Benhamou *et al.,* 1996a, 1996b) and cucumber with

S. plymuthica (Benhamou *et al.,* 2000) induced the deposition of structural barriers to protect these plants from invasion of *Fusarium oxysporum* and *P. ultimum.*

(b) *Increased activity of defense-related enzymes:* Enhanced activities of PR- proteins such as chitinases and glucanases in host plants lead to the degradation of the cell walls/germ tubes of the invading fungi (Benhamou ***et*** *al.,* 1996a; M'Piga ***et*** *al.,* 1997). Chitinases and glucanases disintegrate the cell wall of the invading fungi, thus early and increased expression of these genes results in protection against several pathogens. Induction of these enzymes and numerous other PR-proteins following **the** application of biocontrol agents has been observed in different host plants. Involvement of PR-proteins in inhibition of the pathogen infection process has also been suggested by their location in the apoplast (vanLoon, 1997). Seed bacterization of pigeonpea with *B. subtilis* AF 1 increased the activities of phenylalanine ammonia lyase (PAL) and peroxidase against infection by *F. udum* (Podile and **Laxmi,** 1998). *Bacillus mycoides* increased the activities of chitinases. induced two new isoforms of both peroxidase and glucanase and protected sugar beet against Cercospora leaf spot (Bargabus *et al.,* 2002).

(c) *Enhanced* ***phytoalexin*** *production:* Accumulation of phytoalexins, the low molecular weight antimicrobial compounds, inhibits the establishment of pathogens in the host plants. Protection of carnation against wilt by *Pseudomonas* sp. WCS 417r (van Peer *et al.,* 1991) and cucumber against *P. aphanidermatum* by *P. putida* BTP1 (Ongena *et al.,* 2000) was mediated by the accumulation of phytoalexins in the host plants.

1.4.6. Additional mechanisms of growth promotion or disease control: Production of volatile antibiotics has been recently identified as a mechanism of PGPR action. Volatiles like 2,3-butanediol and acetoin produced by *B. subtilis* and *B. amyloliquefaciens* were equally effective as the whole cells in induction of plant growth promotion. Bacterial isolates blocked in the synthesis of these two volatile compounds were devoid of growth promoting activity (Ryu *et al-,* 2003). Tha activaton of strawberry defense systems by *B. mycoides* is partly mediated by the volatile antifungal compounds (Guetsky *et al.,* 2002).

1.5. Biological control in the phylloplane

Unlike rhizosphere, in which the plant roots provide nutrients to the surrounding microorganisms and soil acts as a buffer, phylloplane does not favor the survival and rapid multiplication of microorganisms. The microorganisms in the phylloplane are continuously subjected to rapid and extreme variations in moisture and temperature, exposure to ultraviolet radiation, and limited nutrient availability (Blakeman, 1982; Leben *et al.,* 1965; Sleesman and Leben, 1976). Hence, the introduced antagonists lose their viability within a short duration and need to be reapplied frequently.

In controlled environment, foliar diseases of different crop plants were effectively managed by the foliar application of antagonistic bacteria (Rytter *et al.,* 1989; Baker *et al.,* 1983; Paulitz and Belanger, 2001), but in natural environments the bacteria failed to offer disease protection except for a few instances (Baker *et al.,* 1985). Failure of the introduced antagonists to maintain cell numbers, at high enough levels, to offer disease control was the major factor contributing to failure of the antagonists in field demonstrations. Alteration of the microclimate in favor of the survival of introduced antagonists is, to some extent, possible in greenhouse, but is impracticable in field. Use of phylloplane microorganisms for disease control can be attempted for better results since these bacteria are normally expected to survive well in the phylloplane and avoid frequent reapplication. Strategies to enhance the survival and subsequent performance of the antagonists in the phylloplane by nutrient supplementation and improvement in the inoculant delivery systems are likely to overcome the deficiencies in biocontrol of foliar diseases.

1.6. Formulations of biocontrol bacteria

An ideal formulation is expected to facilitate the delivery of living biocontrol agent in its active state, at the right place, at the right time. The formulated microbial products must be effective at the site of action and compatible with agronomic practices, easy to apply and adhere to plant parts.

Chitosan amended zeolite based formulations increased the survival and biocontrol efficacy of chitinolytic bacterial strains, *Paenibacillus* sp. and *Streptomyces* sp. (Singh *et al.*, 1999). Supplementation of chitin to peat increased the multiplication and survival of a chitinolytic strain, *B. subtilis* AF 1 and improved its biocontrol and plant growth promoting efficiency compared to fresh cells and AF 1 cells formulated in peat alone as a carrier material (Manjula and Podile, 2001). Presence of chitin improved the biocontrol efficacy of *B. cereus* and *B. subtilis* against early leaf spot of groundnut (Kokalis-Burelle *et al.*, 1992) and *Stenotrophomonas maltophilia* against rust of bean (Yuen *et al.*, 2001).

1.7. Biocontrol bacteria as components of integrated disease management

Integrated disease management (IDM) was defined as the optimization of disease control measures in an economically and ecologically sound manner, accomplished by the co-ordinated use of multiple tactics to assure stable crop production and to maintain yield losses below the economic level whilst minimizing hazards to humans, animals, plants and the environment (Jeger, 2000). Integration of biocontrol agents with moderate levels of host plant resistance or reduced doses of chemical fungicides or application of **a** combination of different biocontrol agents fits into the basic principle of IDM.

Integrated application of *T. harzianum* T39 and a fungicide, based on a weather-based disease warning system for control of *Botrytis cinerea* in non-heated greenhouse vegetables, reduced the mean number of fungicide applications without any significant differences in yield (Shtienberg and Elad, 1997). The combined use of reduced doses of fungicides and biocontrol agents offered an effective control of soil-borne diseases where chemical control alone is unaffordable (Duffy, 2000; Kondoh *et al.*, 2001). This strategy also avoids much of the risk imposed by the application of standard doses of fungicides.

Combined use of two or more biocontrol agents with identifiable differences in mechanisms can be used for improved disease protection and to overcome the narrow spectrum activity of biocontrol agents compared to synthetic fungicides. A combination of compatible PGPR strains capable of inducing systemic resistance in host plants, but with no significant antifungal activity, provided greater disease suppression than individual

PGPR strains in vegetable crops (Jetiyanon and Kloepper, 2002). Application of a mixture of three PGPR strains resulted in **intensive** plant **growth** promotion and **disease** protection against multiple pathogens in cucumber (Raupach and Kloepper. 1998) and rice (NandaKumar *et al*.,2001).

1.8. Elicitation of **systemic resistance** by biotic and abiotic elicitors

Localized treatment of plants with certain biotic or abiotic elicitors results in the local or systemic induction of disease resistance in the treated plant to subsequent pathogen attack (Hammerschmidt, 1999). The biotic inducers of systemic resistance include killed pathogens, non-pathogens, microbial pectin-degrading enzymes, plant cell wall galacturonide fragments, and fungal cell wall β-glucans, glycopeptides and fatty acids (Dong and Cohen, 2001; Durbey and Slater, 1997). Some of the abiotic agents are acibenzolar-S-methyl (benzo| 1,2,3 |thiadiazole-7-carbothioic acid-S-methyl ester, bezothiadiazole), chitosan, salicylic acid, ethylene, dichloro-isonicotinic acid and β-aminobutyric acid, heavy metals and ultraviolet light. Alterations in the phytoalexin accumulation, synthesis of hydroxy-proline rich glycoproteins and induction of PR-proteins have been identified in **the** host plants after application of these biotic and abiotic elicitors. Tomato plants sprayed with P-aminobutyric acid showed a high level accumulation of P-l,3-glucanase and chitinase, and were protected against late blight infection (Cohen *et al*., 1994). Exogenous application of salicylic acid resulted in increased activity of peroxidase and p-l,3-glucanase in the callus cultures of *Zingiber officinale* (Prachi *et al*., 2002).

1.9. Importance of fungal diseases in groundnut production

Groundnut or peanut (*Arachis hypogaea* L.) is a major legume crop in most of the tropical and subtropical areas of the world with a production area of ~3.1 X 10^7 ha and its annual production ranges up to ~3.8 X 10^7 metric tons (FAO, 1998). The crop is

vulnerable to several fungal pathogens that cause both quantitative and qualitative losses in pod and haulm yields.

1.9.1. Late leaf spot (LLS): Late leaf spot, caused by *Phaeoisariopsis personata* (Berk. & Curt.) v. Arx [=*Cercosporidium personatum* (Berk. & Curt.) Deighton] is the major foliar disease of groundnut in India and some other countries of Asia and America. Yield losses of >50% were common due to LLS infection (Mc Donald *et al*., 1985). LLS is identified by dark, circular lesions on the abaxial (lower) surface of the leaflets (Plate Fig. 1 A). Sporulation occurs on the abaxial surface and the sporulated lesions appear slightly rough (Plate Fig. 1 B). As the disease progresses, the lesion diameter increases, adjacent lesions coalesce, the leaves become chlorotic, necrotic and defoliate (Plate Fig. 1 C). Gradually, the lesions also develop on the petioles, stems and pegs (Plate Fig. 1 D and E). LLS is commonly associated with rust caused by *Puccinia arachidis* Speg. (Plate Fig. 1 F).

1.9.2. Stem rot: Stem rot caused by *Sclerotium rolfsii* Sacc. causes 20-25% yield losses in several temperate and warm regions and the yield losses can be up to 80%. Initial symptoms of stem rot are yellowing and wilting of a lateral branch or, if the main stem is attacked, of the whole plant, followed by chlorosis and drying of leaves. These symptoms are a result of the shredded stem tissue and sheaths of white mycelium can be observed at or near the soil line around the affected stems (Plate Fig. 1 G). The mycelium produces spherical, white colored sclerotia, which turn to brown as they mature (Plate Fig. 1 H). Infected pods show light tan colored lesions, become completely covered with a white mycelial mat (Plate Fig. 1 I) and eventually decay (Plate Fig. 1 J, Mehan *et al*., 1995).

1.9.3. Crown rot: *Aspergillus niger* van Tieghem causes pre-emergence rotting of groundnut seed and the infected seed fails to germinate. In young seedlings, sudden wilting followed by shredding of the collar region occurs. Infected collar regions are profusely covered with black masses of mycelium and conidia (Plate Fig. 1 K; Middleton *et al*., 1994).

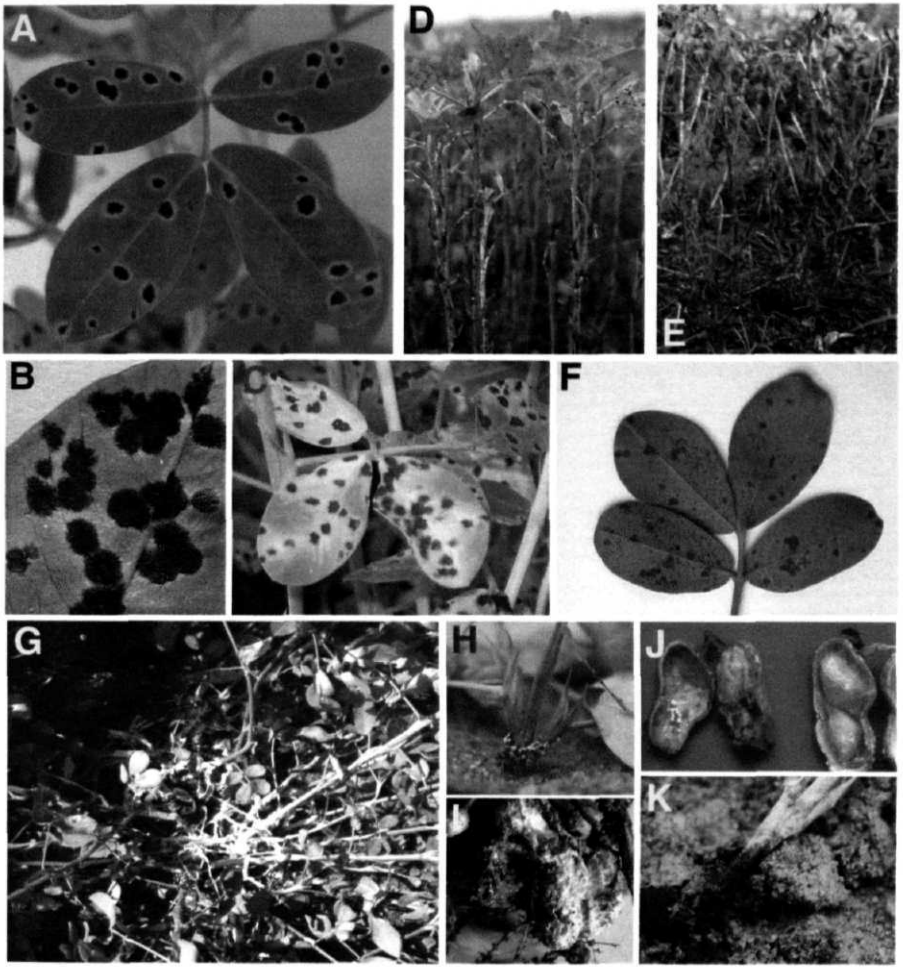

Plate Fig. 1. Symptoms of fungal diseases of groundnut - late leaf spot, stem rot and crown rot, for which biological control has been attempted in the present study.

A-F -Late leaf spot: (A) Visible lesions on the abaxial leaf surface, (B) Sporulation on the adaxial leaf surface, (C) Necrosis and defoliation of infected leaf lets, (D) Defoliation of lower leaves and development of lesions on stem portions, (E) Necrotized stems, and (F) Combined infection of late leaf spot and rust.

*G-J -**Stem rot:*** (G) Mycelium of *Sclerotium rolfsii* visible on infected stems, (H) Sclerotia formed at the base of infected stems, (I) Mycelium of *S. rolfsii* on infected pods, and (J) subsequent seed decay.

K *- Crown **rot:*** Black masses of *Aspergillus niger* at the collar region of infected plants.

1.10. Objectives of the present study

The major objective of the present study was to identify potential biocontrol bacteria for management of economically important fungal diseases of groundnut with emphasis on LLS. Efforts were made to isolate, identify, screen and field-test bacterial isolates (against LLS) individually and also in combination with the fungicides for the control of LLS, stem rot and crown rot diseases. Further, the studies were concentrated on making use of the chitinolytic potential of the bacterial isolates to develop effective formulations for control of LLS.

Materials and Methods

2.1. *In vitro* antifungal activity of bacterial isolates against major pathogens of groundnut

2.1.1. Bacterial isolates: Bacteria were isolated from different habitats - rhizosphere, phyllosphere, geocarposphere and internal tissues of root and seed of groundnut plants from farm fields of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru and farmer fields in nine districts of Andhra Pradesh, India. Ten g of rhizosphere or geocarposphere soil or leaves were suspended in 90 ml of 20 mM phosphate buffer, pH 7.0 and incubated for 1 h at 200 rpm and 30°C. Five g of seed or root surface sterilized with 70% ethanol were homogenized in a mortar and pestle using 20 ml of the same buffer. Appropriate dilutions of all the suspensions were plated on $1/4^{th}$ strength of LB agar. The inoculated plates were incubated for 72 h at 30°C and observed for bacterial growth. Isolated bacterial colonies with distinct morphologies in each treatment were subcultured and maintained as glycerol stocks at -70°C. Groundnut rhizobacterial isolates from an earlier culture collection in the lab, maintained as glycerol stocks, were also included in the present study.

2.1.2. Fungal isolates: Different fungi were isolated from diseased groundnut plants in the experimental fields of ICRISAT, Patancheru. Five soil or seed-borne fungi - *Aspergillus niger, A. flavus, Rhizoctonia bataticola, R. solani* and *Sclerotium rolfsii* were maintained on PDA at 4°C. Three foliar pathogens - *Cercospora arachidicola, Phaeoisariopsis personata* and *Puccinia arachidis* were maintained on detached leaves of groundnut cv. TMV 2 as described by Subrahmanyam *et al.* (1983) (Plate Fig. 2 A and B).

2.1.3. Antifungal activity of bacterial isolates: *In vitro* antifungal activity of bacterial isolates against the soil or seed-borne fungi was determined by dual culture method and against three foliar pathogens by their ability to inhibit the *in vitro* spore germination, since these pathogens cannot be easily cultured on nutrient media.

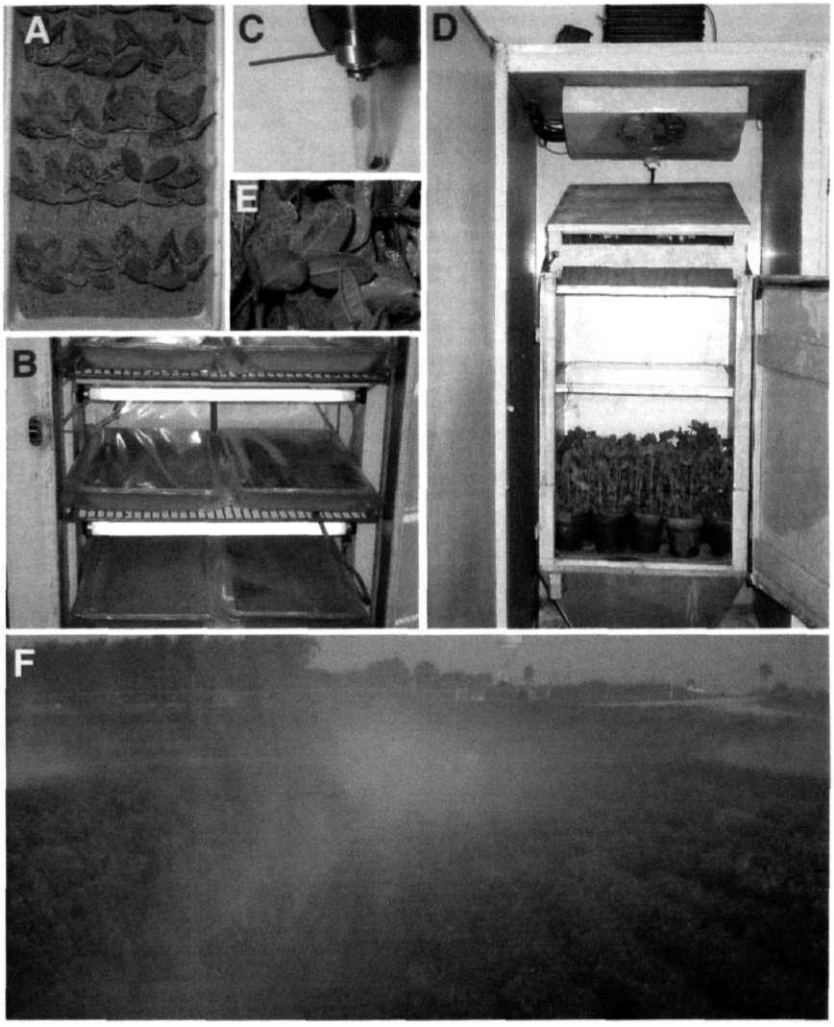

Plate Fig. 2. Multiplication and artificial inoculation of *Phaeoisariopsis personata*, pathogen of late leaf spot disease in groundnut.
(A) Maintenance of *P. personata* on infected groundnut leaves using detached leaf technique, (B) Incubation of detached leaves at optimum temperature and lighting to induce sporulation, (C) cyclone spore collector used to collect conidia from sporulated lesions, (D) Post-inoculation incubation of artificially inoculated plants in dew chamber to maintain temperature and leaf wetness, (E) Accumulation of water drop lets on plants incubated in dew chambers, and (F) sprinkler irrigation in field to maintain leaf wetness required for disease development, during dry weather.

(a) ***Dual culture method:*** Bacteria were inoculated as a line at one edge of a 9 cm diameter petri plate containing potato dextrose agar (PDA), pH 6.1 and incubated at 30°C. After 24 h, a 5 mm diameter agar disc from the edge of an actively growing fungal culture was inoculated at the center. The plates were incubated at 28°C with a 12 h photoperiod. Plates inoculated with the fungus alone served as control. The inhibition zone between the bacterial and fungal cultures was measured when the mycelium in control plates has reached both the edges of the plate. The experiment was conducted in triplicate and repeated twice.

(b) ***In vitro spore germination*** *assay:* Fresh spores were harvested from fungal cultures maintained on detached leaves using a cyclone spore collector (Fischer scientific co., USA) (Plate Fig. 2 C). The spores were suspended in 10 mM phosphate buffer, pH 7.0 containing 0.01% Tween 20 and the concentration was adjusted to 10^5 spores ml^{-1} using a haemocytometer. Bacterial cells were harvested from log phase cultures grown in LB broth by centrifugation at 5,000 rpm for 5 min, resuspended in 0.5% (w/v) dextrose and adjusted to 2 X 10^8 cfu ml^{-1}. Fifty µl each of the fungal spore and bacterial cell suspensions were mixed well on a cavity slide. Control was maintained by adding the dextrose solution to the spore suspension. The slides were placed in humid chambers prepared by lining 90 mm diameter petri plates with wet blotting paper, and incubated in dark at 24 ± 1°C. Immediately after incubation, a drop of lactophenol-cotton blue (glycerol - 40 ml, lactic acid - 20 ml, phenol - 20 g and 5 ml of 1% aqueous cotton blue) was added to each slide to prevent further spore germination and observed under a microscope. In each replication, 100 spores were observed for germination and the percentage inhibition with respect to control was calculated. Three replications were maintained in each treatment and the experiments were conducted thrice.

2.1.4. Antifungal activity of extracellular metabolites of antagonistic bacteria on test fungi:

(a) ***Inhibition of radial growth:*** The effect of extracellular metabolites of selected antagonistic bacteria on the radial growth of soil or seed-borne fungi was determined by

cellophane overlay method of Dennis and Webster (1971) and also by the addition of cell free culture filtrates (CCF) to the agar medium at lower concentrations. In cellophane overlay method, bacterial isolates were inoculated in different directions in 9 cm diameter petri plates with sterile cellophane membrane overlayed on PDA, pH 6.1 and incubated at 30°C. Petri plates inoculated with sterile distilled water (SDW) were maintained as control. After 48 h, the membrane was removed and a 7 mm diameter agar disc from the edge of an actively growing fungal culture was inoculated at the center of each petri plate and incubated at 28°C with a 12 h photoperiod. The colony diameter was measured at regular intervals of 48 h up to 8 days after inoculation (DAI).

Bacterial cells were harvested, from stationary phase cultures grown in LB broth at 180 rpm and 30°C by centrifugation at 10,000 rpm and 4°C. The culture filtrate was sterilized by passing through a 0.22 µm filter and at final concentrations of 10-50% (v/v) were added to pre-cooled agar medium before dispensing into petri plates. A 5 mm diameter agar disc from an actively growing fungal culture was inoculated at the center of each plate. The inoculated plates were incubated at 28°C with a 12 h photo period. The colony diameter in each treatment was recorded at regular intervals of 48 h up to 8 DAI. Fungi inoculated on PDA without any cell free culture filtrate served as control.

(b) ***Inhibition of fungal biomass*:** The effect of culture filtrates on the fungal dry biomass was determined by growing the fungi in potato dextrose broth (PDB) added with culture filtrates. To PDB (30 ml final volume) in 100 ml conical flasks, CCF was added at a final concentration of 10-50%. The flasks were inoculated with a 5 mm disc from an actively growing culture and incubated for 96 h in a rotary shaker at 120 rpm and 28°C. Mycelium was separated from the cultures using a Whatman No. 1 filter paper, dried in an oven at 60°C and the dry weight was recorded.

(c) ***Inhibition of spore germination:*** The effect of cell free culture filtrates on the *in vitro* spore germination of the three foliar pathogens was determined by the *in vitro* spore germination assay as mentioned in section 2.1.3. In different treatments, the final

concentration of CCF ranged from 10-50% (v/v). The experiments were conducted in four replications and repeated once.

(d) *Effect of CCF on hyphal morphology:* The effect of CCF of selected antagonistic bacteria on the mycelial growth was studied. A 4 mm disc from the edge of the fungal colony grown on PDA in presence of culture filtrate was fixed on a glass slide. The mycelium was stained with lactophenol-cotton blue and changes in hyphal morphology were observed under a photomicroscope.

(e) *Effect of CCF on production of extracellular cell wall degrading enzymes **(CWDE)***: The effects of CCF of *P. aeruginosa* GSE 18 and GSE 19 on the production of pathogenesis-related enzymes- exopolygalacturonase (exo-PG), endopolygalacturonase (endo-PG), petcin lyase (PL), pectin methyl esterase (PME) and carboxy methyl cellulase (CMCase) by *A. niger* and 5. *rolfsii*, were determined. Richard's medium (RM) was inoculated separately with each of these fungal pathogens and the carbon source was changed depending on the enzyme activity to be assayed. The carbon sources used were sodium polypectate (Sigma Chemical Co.,) for exo- and endo-PG, pectin (Sigma Chemical Co.,) for PL and PME, and carboxy methyl cellulose (CMC) to measure CMCase activity. Twenty ml of RM in 100 ml conical flasks added with CCF was inoculated with 5 mm discs of an actively growing fungal culture. The inoculated flasks were incubated for 6 days at 120 rpm and 28°C and the enzyme activities were determined at 2, 4 and 6 DAI. The culture filtrate was separated by filtration through a Whatman No. 1 filter paper followed by centrifugation for 10 min at 10,000 rpm and 4°C. The supernatant was filter sterilized and used as crude enzyme extract. The experiment was conducted by maintaining three replications in each treatment and repeated once.

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using the recorded optical densities with known amounts of galacturonic acid. One unit of enzyme activity was defined as the amount of enzyme required for the release of 1 µM of galacturonic acid min^{-1} ml^{-1} of the culture filtrate, under the assay conditions.

***Endo-PG** assay:* Endo-PG was assayed by measuring the reducing groups released by the random breakdown of polygalacturonic acid (Collmer *et al.*, 1988). The reaction mixture contained 1 ml each of 2% sodium polygalacturonic acid, 0.4 M NaCl, 0.2 M sodium acetate buffer, pH 5.2, double distilled water (DDW) and 50 µl of enzyme extract. The released reducing groups were measured by the Nelson Somogy method. One unit of enzyme activity was defined as the amount of enzyme which released 1 µM of reducing groups min^{-1} ml^{-1} of extract, under the assay conditions.

PL assay: The potassium salt of citrus fruit pectin (Sigma Chemical Co., USA) at a concentration of 1 mg ml^{-1} was used as the substrate. The increase in absorbance, due to the production of unsaturated bonds during the depolymerisation of polygalacturonic acid was measured at 235 nm (Albersheim, 1966). One unit of enzyme activity was defined as the production of 0.1 µM unsaturated product min^{-1}, which is equivalent to an increase of 0.555 in A_{235}.

***PME** assay:* The reaction mixture contained 3 ml of 1.2% pectin (pH 6.0) and 0.5 ml of enzyme extract. The pH of the reaction mixture was measured immediately after adding the enzyme extract and incubated for 60 min at 30°C. After incubation period, the pH of the reaction mixture was measured and restored to initial pH by the addition of 0.02 M NaOH (Elad *et al.*, 1994). One unit of PME activity was defined as the amount of enzyme that, under the assay conditions, required the addition of 1 µg of NaOH min^{-1} ml^{-1} of the enzyme extract to maintain the initial pH.

***CMCase** assay:* The reaction mixture contained 300 µl of 1% CMC in 50 mM acetate buffer, pH 5.2 and 200 µl of enzyme extract. Enzyme activity was measured as the amount of reducing sugar released in the reaction mixture. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 µM glucose min^{-1} ml^{-1} of culture filtrate, under the assay conditions.

2.2. Characterization and identification of bacterial isolates

All the bacterial isolates were evaluated for *in vitro* production of extracellular chitinase, glucanase, protease, auxin, siderophore, HCN and solubilization of inorganic phosphate. These tests were done in triplicate and repeated once. Selected bacterial isolates with plant growth promoting or biocontrol activity were identified at Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.2.1. Production of chitinase:

(a) *Preparation of colloidal chitin:* To 400 ml of conc. HC1, 10 g of chitin (Sigma Chemical Co., USA) was added, stirred at 4°C until the chitin dissolved and incubated at 37°C until viscosity decreased. The whole mixture was added to 4 lit of DDW and left overnight at 4°C. The precipitate was collected on a filter paper after decanting the supernatant and washed extensively with DDW to attain neutral pH and then was lyophilized and stored at -20°C (Berger and Reynolds, 1988).

(b) *Detection of chitinase production:* The bacterial isolates were characterized for production of chitinase by using minimal medium (MM) with colloidal chitin as a sole carbon source. The medium composition was yeast extract - 3.0 g, $MgSO_4.7H_2O$ - 0.3 g, KH_2PO_4 - 1.36 g, $(NH_4)_2SO_4$ - 1 g, colloidal chitin - 15 g, agar - 15 g, DDW - 1000 ml and pH adjusted to 7.0 with 1N NaOH. The bacterial isolates were inoculated as a spot and incubated at 30°C. After 120 h of incubation, the plates were observed for lysis of chitin around the bacterial colonies. The chitinolytic bacteria formed a zone of clearance around the colonies and depending on the diameter of the clearing zone, the bacteria were rated as +, ++ and +++ for production of extracellular chitinase.

2.2.2. Production of β-1,3-glucanase: Production of β-1,3-glucanase was determined by spotting the isolates on LB agar added with 0.4% (w/v) lichenan (Sigma Chemical Co., USA) (Cantwell and Mc Connell, 1983). The inoculated plates were incubated for 72 h at

30°C and enzyme production was observed by staining the plates with 1% (w/v) aqueous congo red solution. Depending on the diameter of the clear zone around the colonies, in a pink background, the isolates were rated as +, ++ and +++ for glucanase production.

2.2.3. Production of protease: Gelatin solution (8%) in water was separately autoclaved and added to autoclaved NA, pH 6.0 cooled to room temperature at a final conc. of 0.4%. The bacteria were inoculated as a spot and incubated at 30°C for 48 h. The plates were flooded with saturated $(NH_4)_2SO_4$ solution and the proteolytic activity was observed as a clearance zone. The diameters of both the colony and clearance zone were measured in mm.

2.2.4. Phosphate solubilization: Bacterial isolates were screened for their phoshpate solubilizing ability by inoculating on Pikovsky's agar containing $Ca_3(PO_4)_2$ as the insoluble-P source (Pikovskaya, 1948). Phosphate solubilization was identified as a clear zone around the colony after an incubation of 7 days at 27°C. Both the colony and zone diameters were measured in mm.

2.2.5. Production of auxins: The bacterial isolates were screened qualitatively for production of L-tryptophan derived auxins by immobilization on to a nitrocellulose membrane (Bric *et al.*, 1991). The medium consisted of tryptone - 10 g, yeast extract - 5 g, NaCl - 5 g, L-tryptophan - 0.3 g, agar - 20 g, DDW - 1000 ml and the pH was adjusted to 7.0. The bacteria were inoculated on agar plates (50 mm diameter) with toothpicks into a grid pattern and immediately covered with a nitrocellulose membrane. After 48 h, the nitrocellulose membrane was removed and placed on a filter paper saturated with Salkowski's reagent (2 ml of 0.5 M $FeCl_3$ added to 100 ml of 35% perchloric acid). The reaction was allowed to proceed until adequate color developed. All reagent incubations were conducted at room temperature. Bacteria producing auxin were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony.

2.2.6. Production of siderophores: Production of siderophores by the bacterial isolates was determined using chrome azurol S (CAS) agar medium (Schwyn and Neilands, 1987). To prepare one litre of CAS agar, 60.5 mg of CAS was dissolved in 50 ml DDW and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HC1). While stirring this solution was slowly added to 72.9 mg cetyl tri methyl ammonium bromide (CTAB) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. A mixture of 750 ml DDW, 100 ml of 10 X MM9 salts, 15 g agar, 30.24 g PIPES (Himedia Laboratories Ltd., India), and 12.0 g of a 50% (w/v) NaOH solution (to raise the pH to the pK_a of PIPES i.e., 6.8) was autoclaved separately. After cooling to 50°C, 30 ml of casamino acid solution (10% w/v) was added as carbon source. The dye solution was finally added along the glass wall with enough agitation to achieve mixing without generation of foam, and dispensed into petri plates. The casamino acid solution used was deferrated before autoclaving using 8-hydroxyquinoline as a chelating agent. Bacteria were inoculated on to these plates and incubated for 48 h at 30°C. Siderophore production by bacterial isolates was indicated by an orange halo around the colony.

2.2.7. Production of HCN: The qualitative production of HCN was determined by inoculating the bacteria on LB agar supplemented with 4.4 g lit^{-1} glycine. Inoculated petri plates were inverted and a piece of filter paper impregnated with 0.5% picric acid in 2 % Na_2CO_3 was placed in the lid (Bakker and Schippers, 1987). The petri plates were sealed with parafilm and incubated at 25°C for 96 h. A change of the filter paper color from yellow to light brown, brown, and reddish brown was recorded as weak (+), moderate (++) and strong **(+++)** cyanogenic potential, respectively. Plates without bacterial inoculation were maintained as control.

2.3. Evaluation of plant growth promoting activity of bacterial isolates

2.3.1. Seed bacterization: Seeds of groundnut cv. TMV 2 were surface sterlized by treatment with 0.02% (w/v) $HgCl_2$ for 5 min and washed thrice with SDW to remove traces of $HgCl_2$. Bacterial isolates were grown as a lawn on LB agar in 9 cm diameter petri

plates for 48 h. The cells were scrapped into 20 ml of 0.5% CMC and the surface sterilized seeds were suspended in this cell suspension for 30 min. Bacterized seeds were dried under a flow of sterile air in a laminar flow for 4-5 h and then used for sowing. The viable cell count as determined by dilution plating was 10^6-10^7 cfu $seed^{-1}$.

2.3.2. Greenhouse evaluation: For evaluation of plant growth promoting ability of bacterial isolates in groundnut, 5 bacterized seeds were planted in 15 cm diameter plastic pots filled with red alfisol and sand (2:1). Seeds treated with 0.5% CMC alone were planted simultaneously, as control. The temperature in the greenhouse was maintained at 28 ± 2°C and the pots were adequately watered daily. The emergence of seedlings was recorded 7 DAS. The plants were uprooted 20 days after sowing (DAS), and the root and shoot lengths were recorded. The plants were washed, dried in an oven at 100°C for 24 h and the dry weight of individual plant was recorded. Ten seeds were planted in each treatment and the experiment was conducted in triplicates and repeated twice.

2.3.3. Development of bacterial formulations: Selected plant growth promoting bacterial isolates viz. *Bacillus firmis* GRS 123, *B. megaterium* GPS 55 and *Pseudomonas aeruginosa* GPS 21 were formulated using peat (Biocare Technology Pvt. Ltd., Australia) as a carrier material. Initial pH of the peat was 6.1 and adjusted to 7.0 by adding $CaCO_3$. Ten g of neutralized peat was packed in individual high molecular and high density polyethylene bags, and sterilized by autoclaving at 121°C for 20 min. Bacterial cells were harvested from mid-log phase cultures grown in LB broth by centrifugation and resuspended in equal volume of 10 mM phosphate buffer, pH 7.0. The cell suspension was diluted 100-fold using the same buffer and 5 ml of the diluted cell suspensions was aseptically added to 10 g of peat in each pack with a syringe and sealed (Plate Fig. 3 C). Inoculated peat packets were mixed well and thoroughly kneaded to ensure uniform adsorption of the bacterial cells into the carrier material and incubated at 30°C. Moisture loss from the packets was compensated by adding SDW at regular intervals, based on the loss in initial weight. Variability of the formulated bacteria was determined at frequent time intervals up to 180 DAI.

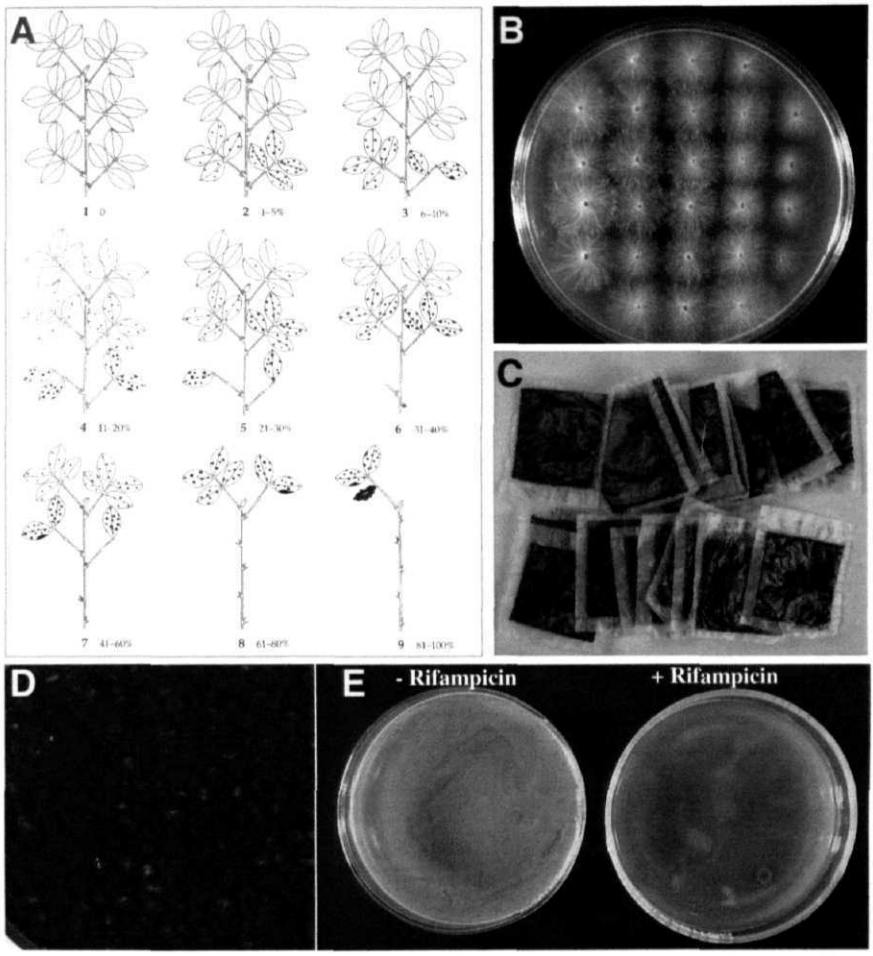


Plate Fig. 3. Representation of few materials or techniques used in the present study.
(A) A modified 9-point scale for field evaluation of late leaf spot, (B) Germinated sclerotia of *Sclerotium rolfsii* used for artificial inoculation of groundnut seedlings, (C) Formulations of bacterial isolates using peat as a carrier material, and sealed in high density polyethylene bags, (D) *Aspergillus niger* multiplied on sorghum seed and used as inoculum for soil infestation, and (E) Spontaneous mutant of *Serratia marcescens* GPS 5 resistant to rifampicin, obtained by plating the cell suspension on rifampicin added nutrient agar.

To determine the survival of bacteria in the formulations, 2 g of the carrier material was diluted with 10 mM phosphate buffer, pH 7.0 and appropriate serial dilutions were plated on nutrient or LB agar. Inoculated plates were incubated at 30°C and the number of cfu was recorded after 36 h and expressed as $\log_{10}$ cfu g^{-1} carrier material. The experiment was repeated twice with four replications in each treatment.

2.3.4. Field evaluation of bacteria and their formulations: Three bacterial isolates, *B. firmis* GRS 123, *B. megaterium* GPS 55 and *P. aeruginosa* GPS 21 that were effective in growth promotion in greenhouse, and their peat formulations were further evaluated in field. The experiments were conducted at ICRISAT, Patancheru during 2001 and 2002 rainy seasons using groundnut cv. TMV 2. Gypsum was applied at the time of pod formation, and the experimental plots received no other fertilizer. Chlorothalonil (Kavach®) was applied twice at 55 and 80 DAS to prevent the development of foliar diseases. Each treatment consisted of 4 rows of 5 m length with an intra- and inter-row spacing of 15 and 60 cm, respectively. The experiment was arranged in a completely randomized block design with triplicates in each treatment.

Seeds treated with either mid-log phase cells or 90-day-old peat formulations were used in different treatments with CMC treated seeds as control. For seed treatment with peat formulations, 100 g of surface sterilized seeds were mixed thoroughly with 5 g of the formulation using 0.5% (w/v) CMC as an adhesive. All the treated seeds were air dried in a laminar flow and used for planting. The experimental plots were irrigated and a 6 cm deep furrow was opened to facilitate sowing and covered immediately after sowing. In each treatment, two additional rows were planted for sampling at regular intervals of 20 days, from sowing till harvest. At each sampling, four randomly selected plants were uprooted to determine the root length, shoot length and dry weight. At harvest, the plants were uprooted, pods were hand picked, sun dried and the recorded dry weight of pods in each treatment was calculated to ha.

2.4. Biocontrol of stem rot disease of groundnut

Three hundred and ninety three bacterial isolates were evaluated in greenhouse for control of stem rot disease in groundnut caused by *S. rolfsii*. Bacterized seeds of groundnut cv. TMV 2 along with CMC treated seeds as control, were planted in a potting mixture consisting of red alfisol, farm yard manure (FYM) and sand (2:1:1). Five seeds were sown in 15 cm diameter pots and trimmed to four after emergence. Additionally, 2 ml of stationary phase cell suspension (~10^8 cfu ml^{-1}) was applied at the base of the emerged seedlings 5 DAS. Pathogen inoculation was done at 14 DAS as described by Ganesan and Gnanamanickam (1987) with minor modifications. Dried sclerotia were allowed to geminate on PDA at 28°C (Plate Fig. 3 B). After 36-48 h, 1 cm diameter agar disc containing germinated sclerotia along with actively growing mycelium was cut with a sterile cork borer and placed at the base of the plant, with mycelium touching the stem. Inoculated pots were covered with coarse sand up to a height of 1.5 cm and watered. Sand was kept moist and temperature was maintained at 27 ± 2°C. Stem rot severity on each plant was assessed on a 1-5 rating scale (Shokes *et al.*, 1996) 15 DAI. The experiment was repeated with three replications in each treatment.

Rating scale for stem rot assessment
(Shokes *et al.*, 1996)

Disease rating	*Description*
1	Healthy plant
2	Lesions on stem only
3	Up to 25% of the plant symptomatic (wilt, dead or drying)
4	26-50% of the plant symptomatic
5	>50% of the plant symptomatic

2.5. Biocontrol of crown rot disease of groundnut

2.5.1. Greenhouse screening: Three hundred and ninety three bacterial isolates were evaluated for their effect on the development of crown rot in greenhouse. The potting mixture consisted of red alfisol, FYM and sand (2:1:2) filled in 15 cm diameter pots. The pathogen, *A. niger* was multiplied on autoclaved sorghum seed for 8 days at 30°C, and the profusely sporulated fungus was used as inoculum (Plate Fig. 3 D). The top 6 cm potting

mixture in each pot was uniformly added with 20% (w/w) inoculum and covered with a clear polyethylene sheet for 48 h to maintain adequate and uniform moisture for establishment of the pathogen. Ten bacterized seeds of cv. TMV 2 were sown in each pot with CMC treated seeds as control. Temperature in the greenhouse was maintained at 30±2°C and the pots were regularly watered to maintain the minimal soil moisture required for germination and growth of seedlings. The incidence of crown rot in each treatment was based on pre-emergence rotting and post-emergence wilting observed at 7 DAS and 20 DAS, respectively. Each pot was considered as one replication and the experiment was repeated once with four replications.

2.5.2. Effect of *P. aeruginosa* GSE 18 on the activity of defense-related enzymes during crown rot infection: The effect of *P. aeruginosa* GSE 18 on induction of host resistance in groundnut was evaluated based on the activities of defense related enzymes in treated groundnut seedlings. Chitinase, P-l,3-glucanase, peroxidase and phenylalanine ammonia lyase (PAL) were assayed at 24 h intervals, up to 15 DAS.

(a) *Preparation of enzyme extract:* Phosphate buffer (pH 6.8) consisting of 2 mM ethylene diamine tetra acetic acid (EDTA), 1 mM ascorbic acid, 1 mM dithiothreitol and sodium meta bisulphite (950 mg lit^{-1}) was used as extraction buffer. Seed or plant material was homogenized in 25% (w/v) extraction buffer, centrifuged for 10 min at 10,000 rpm and 4°C. The supernatant was filtered through a cheese cloth, desalted by passing through a Sephadex G 25 column and used as crude extract for enzyme assays.

(b) *Estimation of total proteins:* The total protein content of the crude extract was determined according to Lowry *et al.* (1951).

(c) *Chitinase assay:* The reaction mixture consisted of 500 µl each of the enzyme extract, 10 mM sodium acetate buffer, pH 5.2 and 0.5% (w/v) colloidal chitin. The reaction mixture was incubated for 3 h at 37°C and then centrifuged for 2 min at 2000 rpm. To 500 µl of the supernatant, 20 µl of 3% (w/v) cytohelicase was added and incubated for 1 h at

37°C, and used for estimation of aminosugars (Reissig *et al.*, 1955). Fifty µl of reagent A (6.1 g of dipotassium tetraborate tetrahydrate in 100 ml of DDW) was added to the cytohelicase-treated supernatant and incubated for 3 min at 100°C. The solution was then rapidly cooled to room temperature and 1.5 ml of reagent B (10 g of para dimethyl amino benzaldehyde dissolved in a mixture of 1.5 ml of DDW, 11 ml of conc. HC1 and 87.5 ml of glacial acetic acid, and diluted 10-fold with glacial acetic acid just before use) was added. All the samples were incubated for 30 min at 37°C and absorbance was measured at 585 nm. One enzyme unit was expressed as the amount of enzyme that released 1 µM of *N*-acetyl glucosamine (NAG) from colloidal chitin in 1 h under the assay conditions.

(d) ***Estimation of fi-1,3-glucanase:*** Equal volumes (62.5 µl) of enzyme extract and laminarin (4% w/v) were mixed and incubated for 10 min at 40°C. The amount of reducing sugars released from laminarin was determined by the addition of 375 µl of DNS reagent to the reaction mixture. The solution was heated in a boiling water bath for 5 min, cooled to room temperature. To this solution 4.5 ml of DDW was added, vortexed and the absorbance was measured at 500 nm (Pan *et al.*, 1991). One unit of enzyme activity corresponded to the amount of enzyme that produced reducing sugar equivalent to 1 µM of glucose in 10 min, under the assay conditions.

(e) ***Estimation of peroxidase*****:** The reaction mixture consisted of 2 ml of 0.1 M potassium phosphate buffer, pH 7.0, 100 µl of the enzyme extract and 100 µl of 0.4 mM guaicol. To this reaction mixture, 30 µl of 2 mM H_2O_2 was added and the change in absorbance was measured at 470 nm for 2 min. One unit of enzyme was expressed as the change in absorbance by 1.0 min^{-1} (Shinishi and Noguchi, 1975).

(0 ***PAL*** *assay:* Three hundred µl of enzyme extract was incubated at 40°C with 600 µl of 0.25 M borate buffer, pH 8.8 containing 6 mM L-phenylalanine. After 2 h of incubation at 40°C, the reaction was stopped by the addition of 100 µl of 6 M HC1. The product, cinnamic acid was extracted using 1.0 ml chloroform. The chloroform layer was separated

by centrifugation and from the lower chloroform phase, 200 µl of the aliquot was separated. The chloroform was evaporated by boiling for 1-2 min. The residue was redissolved in 1.0 ml of borate buffer and the absorbance was read at 270 nm (Beaudoin-Eagan and Thorpe, 1985). The amount of cinnamic acid released in the enzyme reaction was determined by using cinnamic acid (1-10 $\mu g\, ml^{-1}$) dissolved in borate buffer, pH 8.8 as standard. One unit of PAL activity was calculated as the amount of enzyme that formed 1 mg cinnamic acid h^{-1}.

In all the above enzyme assays, controls were maintained without the addition of substrate in the reaction mixture and the activities were expressed as specific activity i.e., enzyme units per mg of total protein.

2.6. Biocontrol of LLS disease of groundnut

2.6.1. Preparation of bacterial inoculum: Bacterial isolates were grown in 50 ml of LB broth in 250 ml erlenmeyer flasks at 180 rpm and 30°C. Bacterial cells were harvested by centrifuging the log phase cultures at 5,000 rpm for 5 min and 4°C. The pelleted cells were resuspended in 10 mM phosphate buffer, pH 7.0 and the concentration was adjusted to 10^8 cfu ml^{-1} and used as a foliar spray for control of LLS.

2.6.2. Preparation of *P. personata* inoculum: Conidia of *P. personata* were harvested from infected lesions on detached groundnut leaves and suspended in SDW containing 0.01% (w/v) Tween 20. The concentration of the spore suspension was adjusted to 2 X 10^4 conidia ml^{-1} using a haemocytometer, and used as inoculum for artificial inoculation of groundnut plants.

2.6.3. Artificial inoculation in controlled environment: Groundnut cv. TMV 2, highly susceptible to LLS was used in all the experiments on biocontrol of LLS. Seedlings were raised in a potting mixture consisting of red alfisol, FYM and sand (2:1:1) in greenhouse. Five seeds were planted in 15 cm diameter plastic pots and the seedlings were thinned to four after germination. Thirty-day-old plants were used for artificial inoculation.

Inoculation was done at 1600 h by the foliar application of the inoculum evenly with an atomizer. Inoculated plants were allowed to air dry and then shifted into dew chambers (Clifford, 1973) to maintain leaf wetness (RH 100%) during night hours (Plate Fig. 2 D and E). The plants were returned to greenhouse on the morning of the following day to provide dry period. The alternate wet (16 h) and dry (8 h) period treatments were repeated up to 8 DAI (Butler *et al.*, 1994), and then the pots were retained in a greenhouse till end of the experiment. In both dew chambers and greenhouse, temperature was maintained at 24 ± 2°C. A total of eight plants were maintained in each treatment and all the experiments were conducted with three replications and repeated twice.

2.6.4. Disease scoring: Severity of LLS in different treatments was determined based on a) lesion frequency (LF) (number of lesions cm^{-2} leaf area), b) percentage defoliation (DEF) (calculated based on the number of leaflets defoliated in a quadrifoliate leaf), and c) disease score (DS) on a 1-9 rating scale (1 = no disease, and 9 = >80% disease). LF was measured 15 DAI, and DEF and DS were measured 30 DAI. In each inoculated plant, fourth quadrifoliate from the top tagged at the time of inoculation was used for measuring the **LF** and DEF.

2.6.5. Selection of effective biocontrol isolates: Fifteen bacterial isolates which inhibited the *in vitro* conidial germination of *P. personata* by >90% were evaluated for LLS control in greenhouse. In different treatments, the test isolates were applied as a foliar spray, 24 h before the pathogen inoculation. Plants sprayed with 10 mM phosphate buffer, pH 7.0 were maintained as control.

2.6.6. Integrated use of biocontrol bacteria and chlorothalonil: Effective biocontrol isolates, *P. aeruginosa* GSE 18 and GSE 19 were tested for their tolerance to chlorothalonil (Kavach®). Chlorothalonil was added at a final concentration of 62.5-2000 $\mu g\,ml^{-1}$ to 20 ml of LB medium in 100 ml conical flasks. The flasks were inoculated with 200 µl of log phase cultures and incubated at 180 rpm and 30°C. Bacterial growth, in each treatment, was determined by measuring the absorbance at 600 nm using respective blanks

at regular intervals of time. The fungicide tolerance of the two bacterial isolates was further confirmed by dilution plating on chlorothalonil amended LB agar.

Both the isolates were tolerant to chlorothalonil, and used along with reduced concentrations of chlorothalonil for LLS control. In different treatments, foliar application of GSE 18 and GSE 19, 24 h before the pathogen inoculation, was supplemented with chlorothalonil at a final concentration of 10-2000 µg ml^{-1}. The disease severity was compared to the individual application of GSE 18, GSE 19 and chlorothalonil. Plants sprayed with 10 mM phosphate buffer, pH 7.0 were maintained as control.

2.6.7. Evaluation of chitinolytic bacteria for LLS control: Two chitinolytic bacterial isolates, *S. marcescens* GPS 5 and *B. circulans* GRS 243 were evaluated as biocontrol agents of LLS. The two isolates were selected based on their chitinolytic and antifungal activity against *P. personata.* Biocontrol efficacy of these isolates was compared with that of GSE 18 and GSE 19. Foliar application of these bacteria was supplemented with colloidal chitin for improved control. The optimum concentration of colloidal chitin required for the spray was determined by varying its concentration from 0.2%-1.4% (w/v). In all the treatments, bacteria (10^8 cfu ml^{-1}) were applied 24 h before *P. personata* inoculation. Control plants were sprayed with 10 mM phosphate buffer, pH 7.0 at the same time.

2.6.8. Effect of CCF of antagonistic and chitinolytic bacteria on the severity of LLS: Cell free culture filtrates of *P. aeruginosa* GSE 18 and GSE 19 were obtained from cultures grown in LB broth for 48 h. Chitinolytic isolates GPS 5 and GRS 243 were grown in MM broth with 1% colloidal chitin as sole carbon source for 6 days and the culture filtrate was obtained by centrifugation at 10,000 rpm for 10 min and 4°C. The fermentation conditions were 30°C and 180 rpm for all the isolates. The culture filtrates were filter sterilized and applied as a foliar spray 24 h before *P. personata* inoculation. Simultaneously, control plants were sprayed with sterile LB broth and MM broth with colloidal chitin, respectively.

2.6.9. Evaluation of formulations of broad-spectrum antagonistic or chitinolytic isolates for LLS control: Peat formulations of *P. aeruginosa* GSE 18 and GSE 19 and chitin-supplemented peat formulations of *S. marcescens* GPS 5 and *B. circulans* GRS 243 were evaluated for control of LLS in greenhouse. Selected bacteria were mixed with peat and the survival of the bacteria was determined as mentioned in section 2.3.3. Simultaneously, GPS 5 and GRS 243 were also formulated in peat supplemented with 1% (w/w) powdered chitin.

Bacterial cells were harvested from the formulated peat by suspending 10 g of the formulation in 100 ml of 10 mM phosphate buffer, pH 7.0. The suspension was stirred for 30 min and filtered through a cheese cloth. The filtrate was used as a foliar spray, 24 h before the pathogen inoculation. Peat formulations of GSE 18, GSE 19, GPS 5 and GRS 243 and chitin-supplemented peat formulations of GPS 5 and GRS 243 were used in the experiment and the biocontol efficacy of the formulations was compared with that of fresh cells. Plants treated with the filtrate of sterile peat suspension were maintained as control.

2.6.10. Effect of biotic and abiotic elicitors on the development of LLS: Four biotic and abiotic elicitors - chitosan, salicylate, methyl jasmonate and heat killed conidia of *P. personata* were tested for control of LLS. Chitosan (Sigma Chemical Co., USA) was purified as described by Benhamou and Theriault (1992) and 1% (w/v) purified chitosan was dissolved in 0.5 N HC1 under continuous stirring. The pH was adjusted to 5.5-6.0 using 2 N NaOH, dialysed for 12 h against DDW and autoclaved. The stock solution was diluted to 0.1% with DDW, just before use and the final pH was adjusted to 5.6. 10 mM salicylate and methyl jasmonate were prepared in SDW. Conidia of *P. personata* suspended in DDW (5 X 10^4 conidia ml^{-1}) were killed by autoclaving at 12I°C for 20 min.

The four elicitor preparations (as above), and SDW as control, were applied as a foliar spray at 24 h, 48 h and 96 h, before the pathogen inoculation, and also as seed treatment by soaking the seeds in respective solutions for 3 h before sowing.

2.6.11. Induction of host resistance in groundnut by biocontrol bacteria: The effect of foliar application of P. *aeruginosa* GSE 18, *S. marcescens* GPS 5 and chitin-supplemented

application of GPS 5, 24 h before the pathogen inoculation, on the induction of resistance of groundnut was studied. Third or forth leaf from the top was excised from the treated plants at 24 h intervals till 14 DAI. The excised leaves were used to determine the activities of chitinase, β-1,3-glucanase, peroxidase and PAL. The preparation of enzyme extract, assay of enzyme activities was described in section 2.5.2. The experiment was conducted with three replications in each treatment and repeated twice.

2.6.12. Field evaluation of the biocontrol isolates: Two antagonistic isolates *Pseudomonas* sp. GSE 18 and GSE 19, and the chitinolytic isolates *S. marcescens* GPS 5 and *B. circulans* GRS 243, effective against LLS in greenhouse environment, were further evaluated in field. The experiments were conducted in the farm fields of 1CR1SAT, Patancheru during 2001 and 2002 rainy seasons using groundnut cv. TMV 2. Planting was done with an intra- and inter-row spacing of 15 and 60 cms, respectively. Four rows each of 5 m length were maintained in each treatment. All the treatments were arranged in a completely randomized block design with three replications. To facilitate uniform disease pressure throughout the experimental plots, an infector row of cv. TMV 2 was planted on either side of each treatment. At 35 DAS, infected crop debris collected during the previous season was evenly spread on the infector rows. Further, at around 40 DAS, all the infector rows were spray inoculated with a conidial suspension (2 X 10^4 ml^{-1}) of *P. personata* between 1800 and 1900 h. Just before inoculation, and in the subsequent 10 days, if the weather conditions are dry sprinkler irrigation was provided between 1800 and 1830 h, to maintain high humidity during night hours (Plate Fig. 2 F). With an interval of 7 days sprinkler irrigation was provided for another 10 days to favor the secondary infection cycles. The following were the foliar spray treatments at 45, 60, 75 and 90 DAS @ 500 lit ha^{-1}.

(i) GSE 18, GSE 19, GPS 5 and GRS 243 in 10 mM phosphate buffer, pH 7.0 (10^8 cfu ml^4),

(ii) GSE 18 and GSE 19 in 10 mM phosphate buffer, pH 7.0 (10^8 cfu ml^{-1}) along with chlorothalonil (0.05% w/v),

(iii) GPS 5 and GRS 243 in 10 mM phosphate buffer, pH 7.0 (10^8 cfu ml^{-1}) supplemented with 1% (w/v) colloidal chitin,

(iv) Chlorothalonil (0.05% w/v),

(v) Chlorothalonil (0.2% w/v),

(vi) 1% colloidal chitin (w/v), and

(vii) 10 mM phosphate buffer, pH 7.0.

In all the experimental plots, LLS severity was scored on a 1-9 rating scale (1 = no disease and 9 = maximum disease) (Plate Fig. 3 A; Subrahmanyam *et al.*, 1995) at regular intervals of 10 days starting from 45 DAS till harvest. At harvest, the plants were uprooted, pods were hand picked and sun dried. The dry weight of pods in each treatment was recorded separately and calculated to ha.

Modified 9-point scale for field evaluation of late leaf spot (Subrahmanyam *et al.*, 1995)

Disease score	Description	Disease severity (%)
1	No disease	0
2	Lesions present largely on lower leaves; no defoliation	1 -5
3	Lesions present largely on lower leaves, very few on middle leaves; defoliation of some leaflets evident on lower leaves	6-10
4	Lesions on lower and middle leaves but severe on lower leaves; defoliation of some leaflets evident on lower leaves	11-20
5	Lesions present on all lower and middle leaves; over 50% defoliation of lower leaves	21 -30
6	Severe lesions on lower and middle leaves; lesions present but less severe on top leaves; extensive defoliation of lower leaves; defoliation of some leaflets evident on middle leaves	31 -40
7	Lesions on all leaves but less severe on top leaves; defoliation of all lower and some middle leaves	41 -60
8	Defoliation of all lower and middle leaves; severe lesions on top leaves; some defoliation of top leaves evident	61-80
9	Almost all leaves defoliated, leaving bare stems; some leaflets may remain, but show severe leaf spots	81-100

2.7. Survival of bacterial isolates in the rhizoplane or phylloplane

Population of the introduced bacterial isolates in groundnut rhizosphere, phylloplane and internal habitats was determined by using rifampicin resistance as a marker. Rifampicin sensitivity of the wild isolates was determined by plating on nutrient or LB agar with rifampicin at concentrations of 1-100 $\mu g\ ml^{-1}$. Spontaneous mutants of different bacterial isolates with resistance to rifampicin were obtained by plating 100 |il of cell suspension (~10^9 cfu ml^{-1}) in 10 raM phosphate buffer, pH 7.0 on nutrient or LB agar added with 100 $\mu g\ ml^{-1}$ rifampicin. Inoculated plates were incubated at 30°C for 96 h and then observed for bacterial growth (Plate Fig. 3 E). Spontaneous mutants, if observed were subcultured and the stability of their antibiotic resistance was checked by subculturing for 20 times on nutrient or LB agar with 100 $\mu g\ ml^{-1}$ rifampicin. Stable mutants were applied as seed bacterization or foliar spray and their survival in the introduced habitats was determined.

(i) Plant growth promoting isolates *B. firmis* GRS 123, *B. megaterium* GPS 55 and *P. aeruginosa* GPS 21 applied as seed treatment in field,

(ii) Biocontrol isolates *P. aeruginosa* GSE 18 and GSE 19 applied as seed bacterization both in native and *A. niger*-infested potting mixture in greenhouse.

(iii) Biocontrol isolates *P. aeruginosa* GSE 18 and GSE 19 applied as a foliar spray both alone or with chlorothalonil (0.5 $mg\ ml^{-1}$) at 60 DAS in the field,

(iv) Chitinolytic isolates *S. marcescens* GPS 5 and *B. circulans* GRS 243 applied as a foliar spray with and without chitin supplementation in field at 60 DAS.

The establishment and multiplication of the introduced bacterial isolates in the rhizoplane was determined by uprooting the plants at regular intervals after sowing. A 2-3 cm root segment, along with the tightly adhering soil, was suspended in 50 ml of 10 mM phosphate buffer, pH 7.0 and incubated for 1 h in a orbital shaker at 180 rpm and 30°C. To determine the internal colonization of bacteria applied as seed treatment, 2-3 cm of root or stem portions were cut, surface sterilized with 70% ethanol and homogenized in sterile 10 mM phosphate buffer, pH 7.0. To study the establishment of introduced bacteria in the phylloplane, four leaflets were randomly excised from each plant at regular intervals of

24 h, weighed and suspended in 50 ml of 10 mM phosphate buffer, pH 7.0 by incubating for 1 h at 180 rpm and 30°C.

Serial dilutions of all the above cell suspensions were plated on nutrient or LB agar with 100 µg ml^{-1} rifampicin with three plates for each dilution. The inoculated plates were incubated at 30°C for 48 h and the recorded cfu were expressed as log_{10} cfu g^{-1}. In each replication sampling was done from two plants and the experiments consisted of three replications and repeated once.

2.8. Characterization of chitinase(s) of *S. marcescens* GPS 5

2.8.1. Chitinase assay (Boiler and Munch, 1988): The reaction mixture consisting of 0.5 ml of 1.5% colloidal chitin, 0.5 ml of cell free culture filtrate and 0.5 ml of sodium acetate buffer, pH 5.2. The reaction mixture was incubated at 37°C for .1 h, centrifuged for 2 min at 2000 rpm and 500 µl of the supernatant was used for estimation of aminosugars. The reaction was stopped by adding 50 µl of reagent A and then 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°C for 45 min. The color developed was measured at 585 nm using appropriate blanks. One unit of enzyme activity was defined as µM of NAG 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).

2.8.2. Purification of chitinase from CCF of *S. marcescens GPS 5:* *Serratia marcescens* GPS 5 was grown in minimal medium supplemented with colloidal chitin as a sole carbon source for 8 days at 30°C and 180 rpm. CCF was obtained by centrifugation at 10,000 rpm for 10 min followed by filter sterilization of the supernatant. Proteins in the culture filtrate were pelleted by adding ammonium sulphate to 100% saturation and the pellet was dissolved in minimal volume of 10 mM PBS and dialyzed against the same.

(a) ***Affinity chromatography***: About 30 g of powdered chitin was suspended in 1 lit of 1 N HC1 and allowed to swell at 4°C for 16 h. The swollen chitin was washed extensively

with deionized water to bring to neutral pH. A final wash was given with 20 mM sodium acetate buffer, pH 5.8. The washed chitin was packed in to a column (10 x 2.5 cm) at 4°C and equilibrated with 20 mM sodium acetate buffer, pH 5.8.

The extracellular proteins present in culture supernatant were loaded on to the chitin column at 10 mg protein g^{-1} matrix and the flow rate was adjusted at 15 ml h^{-1}. The flow through was re-applied on the column to ensure complete binding of chitosan or chitin binding proteins to the swollen chitin. The unbound proteins were eluted using 100 mM sodium acetate buffer, pH 5.8. The bound chitinase or chitin binding proteins were eluted in two steps using 100 mM sodium acetate buffer pH 4.8 and pH 3.6. About 130 fractions of 1 ml each were collected. Fractions with maximum protein concentration were analyzed for chitinase activity (by colorimetery) and SDS-PAGE (Laemmlli *et al.*, 1970) followed by coomassie blue staining for purity.

(b) *Gel* ***fitlration:*** Ten gram of Sephadex G 100 was suspended in 500 ml of 20 mM Tris *C* buffer pH 7.0 and allowed to swell for 24 h. The swollen gel matrix was packed in to a 75 X 1.5 cm column. Flow rate of the packed column was adjusted at 15 ml h^{-1}. The column was washed extensively with deionized water and then with 100 mM sodium acetate buffer pH 5.8. Chitinase or chitin binding proteins eluted from the chitin column were pooled, concentrated and suspended in 1 ml volume of sodium acetate buffer pH 5.8 were loaded on to the column and eluted using sodium acetate buffer pH 5.8. Standard molecular weight protein markers (blue dextran 2,000, albumin 66,000, ovalbumin 43,000, chymotrypsinogen A 25,000 and ribonuclease A 13,200) dissolved in 1.0 ml of 20 mM Tris Cl pH 7.0 were also loaded on to the column carefully. Initially 2 ml fractions were collected until all the blue dextran was eluted and the remaining proteins were eluted in 1.0 ml fractions. Fractions showing maximum protein concentration were analyzed on SDS-PAGE for their purity. Some fractions were also analyzed for their enzymatic activity using colorimetry. Elution profile of protein standards was plotted and used to determine the molecular weight of chitinase or chitin binding proteins.

(c) ***Detection of enzymatic activity after SDS-PAGE (Trudel and Asselin, 1989):*** After electrophoresis, the gel was incubated for 2 h at 37°C with reciprocal shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton x-100 purified through a mixed-bed resin deionizing column. The gel was then incubated in 150 mM sodium acetate buffer, pH 5.0 for 5 min. SDS-PAGE gel was then covered with a 7.5% (w/v) polyacrylamide overlay gel containing 0.01% (w/v) glycol chitin in 100 mM sodium acetate buffer, pH 5.0 and incubated at 37°C for 1 h in a plastic container under moist conditions. The gel was then stained with freshly prepared 0.01% (w/v) Calcofluor white M2R in 500 mM Tris-HCl, pH 8.9 and lytic zones were visualized by observing under UV transilluminator.

(d) ***N-terminal aminoacid sequencing:*** The chitinase or chitin binding proteins obtained after gel filtration chromatography were resolved in 10% denaturing polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Biorad). The protein bands were excised and used to determine N-terminal amino acid sequence with an automated aminoacid sequencer (Procise cLC machine, Applied biosystems). Amino acid sequence similarity search was performed using BLAST search from NCBI world wide web server.

2.8.3. Antifungal activity of *S. marcescens* GPS 5 β-1,4-N-acetyl glucosaminidase (NAGase): Purified NAGase of 5. *marcescens* GPS 5 was tested for *in vitro* antifungal activity against *P. personata* and *P. arachidis*. For the *in vitro* spore germination assay, 25 µl of the spore suspension (10^5 cells ml^{-1}) and equal volume of the enzyme in 10 mM phosphate buffer, pH 7.0 at a final concentrations of 10, 20, 25, 30, 35 and 40 µg ml^{-1} were mixed on a cavity slide. The slides of *P. personata* and *P. arachidis* were incubated in a humid chamber in dark at 28°C and the spore germination was observed under a light microscope after 24 and 8 h, respectively. Observations were made at 48 HAI for lysis of spores and/or germ tubes.

2.8.4. Evaluation of NAGase for control of LLS: Purified NAGase of *S. marcescens* GPS 5 was tested for control of LLS in greenhouse. The enzyme at a concentration of

35 µg ml^{-1} was used as a foliar spray, at 24 h before the pathogen inoculation, on to 30-day-old plants of groundnut cv. TMV 2. Pathogen inoculation and post-inoculation incubation conditions were the same as mentioned in section 2.6.3. Plants sprayed with 10 raM phosphate buffer, pH 7.0 were considered as control. Disease assessment in all the treatments was based on lesion frequency, percentage defoliation and disease score on **a** 1-9 rating scale (section 2.6.4). The experiment was conducted twice in triplicate treatments with four plants in each replication.

2.9. Statistical analysis

The experiments were conducted in a completely randomized block design. The data were subjected to analysis of variance (ANOVA) using Genstat 5 stastical package. The mean values in each treatment were compared using least significant differences at 5% ($P = 0.05$) or 1% ($P = 0.01$) level of significance.

Results

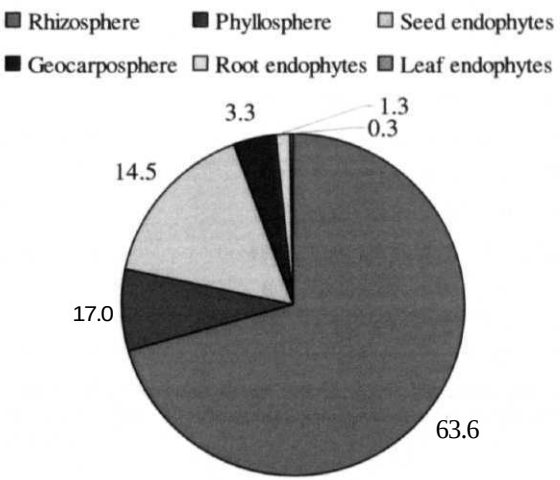
3.1. *In vitro* antifungal activity of bacterial isolates against major pathogens of groundnut

3.1.1. Collection of bacterial isolates: Bacterial isolates were collected from different habitats of groundnut plants from 55 farmers fields in Anantapur, Chittoor, Cuddapah, Kurnool, Mahaboobnagar, Nalgonda, Medak, Ranga Reddy and Guntur districts in the state of Andhra Pradesh, India. Two hundred and forty three distinct bacterial colonies were isolated based on their colony morphology. Single colonies of these isolates were subcultured and stored as glycerol stocks at -70°C to maintain their genetic purity. In addition to the 243 bacterial isolates collected, another 150 groundnut rhizobacterial isolates from an earlier culture collection in the lab were also used in the present study. The isolates represented different habitats of the groundnut plant (Fig. 1); 63.6% were from the rhizosphere, 17.0% from phyllosphere and 3.3% from geocarposphere. Endophytes of leaf, root and seeds constituted 0.3%, 1.3% and 14.5% of the total collection of isolates. The bacterial isolates were designated based on their habitat of isolation: GRS - rhizosphere, GPS - phyllosphere, GGS - geocarposphere, GSE - seed endophytes, GRE - root endophytes and GLE - leaf endophytes (G stands for groundnut).

3.1.2. Antifungal activity of bacterial isolates: The bacterial isolates were evaluated for their *in vitro* antifungal activity against eight fungal pathogens of groundnut. Antifungal activity against the necrotrophic soil-borne fungi *A. flavus*, *A. niger*, *R. bataticola*, *R. solani* and *S. rolfsii* was determined in dual culture method. Antagonism of bacterial isolates to the biotrophic foliar pathogens *C. arachidicola*, *P. personata* and *P. arachidis* was studied in an *in vitro* spore germination assay. The incubation period was dependent on the growth rate of the individual test fungus.

Of the 393 bacterial isolates evaluated, 77 had a significant antifungal activity among which 52 were antagonistic to two or more fungi and 11 were inhibitory to six or more fungi (Table 1). Among the 77 antifungal isolates the number of isolates effective against individual fungus were as follows (Fig. 2): *A. flavus* - 10 (13.0%), *A. niger* - 27 (35.1%), *C. arachidicola* - 58 (75.3%), *P. personata* - 53 (68.8%), *P. arachidis* -

Fig. 1. Categorization of bacterial isolates tested for plant growth promotion or disease control in the present study.



Two hundred and forty three bacteria] isolates were collected from different habitats of groundnut plants from 55 farmers fields in Anantapur, Chittor, Cuddapah, Kurnool, Mahaboobnagar, Nalgonda, Medak, Ranga Reddy and Guntur districts in the state of Andhra Pradesh, India. Additionally, 150 groundnut rhizobacterial isolates from an earlier culture collection in the lab were also used in the present study. The figure represents the percentage of bacterial isolates from each habitat, among the total collection.

Table 1. Antifungal activity of selected bacterial isolates against eight fungal pathogens of groundnut.

S. No.	Isolate[a]	Test fungus							
		AF[b]	AN[b]	RB[b]	RS[b]	SR[b]	PP[c]	CA[c]	PA[c]
1	*Pseudomonas aeruginosa* GPS 21	10	14.0	15.5	15.5	14.5	99.1	99.0	99.3
2	*Pseudomonas* sp. GRS 175	10.0	14.0	15.5	15.5	14.5	99.1	99.0	99.3
3	GRS 223	0	6.5	5.5	7.5	10.0	32.9	38.2	52.1
4	GRS 224	2.0	19.0	14.0	13.0	13.0	49.3	58.2	92.2
5	GRS 225	0	18.0	7.0	15.0	0	97.7	81.3	98.3
6	GSE 5	0	10.0	21.0	21.0	8.5	45.0	52.2	85.4
7	GSE 6	0	11.0	11.0	14.5	7.5	39.9	62.2	89.0
g	*P. aeruginosa* GSE 18	11.5	9.5	23.0	16.0	16.0	97.7	98.2	98.9
9	*P. aeruginosa* GSE 19	11.0	7.5	23.5	23.0	12.5	97.7	98.3	97.8
10	GSE 23	6.5	17.0	13.5	11.0	5.0	68.2	72.9	20.5
11	*P. aeruginosa* GSE 30	13.5	11.0	20.5	21.5	14.5	100.0	100.0	100.0
12	Control	0	0	0	0	0	0	0	0
	LSD (P = 0.01)	3.8	5.2	5.7	7.2	7.4	36.5	34.3	30.0

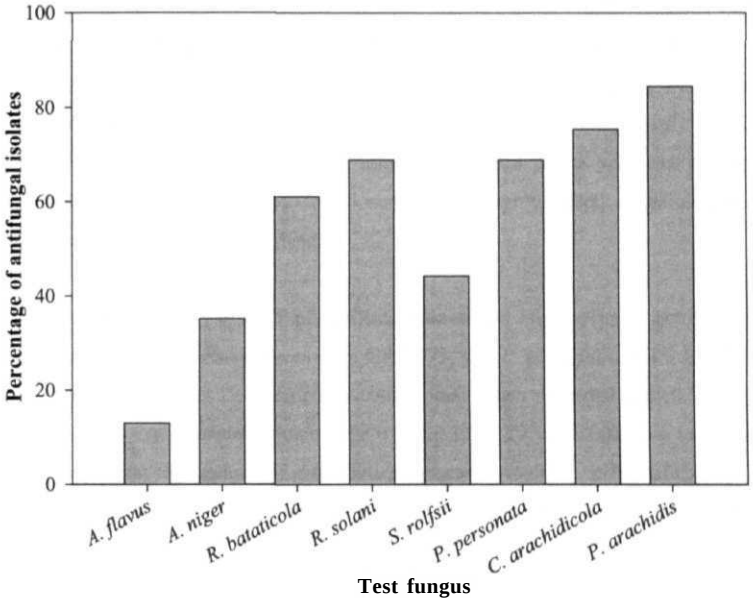
Abbreviations used: AF=*Aspergillus flavus;* AN=*A. niger,* RB=*Rhizoctonia bataticola*; RS=*R. solani;* SR=*Sclerotium rolfsii;* PP=*Phaeoisariopsis personata; CA=Cercospora arachidicola;* PA=*Puccinia arachidis.*

[a] Bacterial isolates antagonistic to six or more test fungi are shown and the values are the mean of nine replications in three repeated experiments.

[b] The mean values represent the inhibition zone measured in mm in dual culture method, where the bactena were streaked at one edge of a petri plate and fungus was inoculated at the center after 24 h. For *A. flavus* and *A. niger* the inhibition zone was measured 8 DAI and for *R. bataticola, R. solani* and *S. rolfsii* the inhibition zone was measured 3 DAI.

[c] The mean values represent the percentage inhibition of spore germination with respect to control in *in vitro* spore germination assay. Fifty µl of fungal spore suspension (10^5 spores ml^{-1}) was mixed with equal volume of bacterial cell suspension (2 x 10^8 cells ml^{-1}) on a cavity slide and incubated in dark at 24 ± 1°C. Conidia of *C. arachidicola* and *P. personata* were observed for germination 24 HA1. Urediniospores of *P. arachidis* were observed 8 HAL

Fig. 2. Activity spectrum of the identified antifungal bacterial isolates against eight fungal pathogens of groundnut.



Three hundred and ninty three bacterial isolates were evaluated for their *in vitro* antifungal activity against eight fungal pathogens of groundnut. Antifungal activity against the necrotrophic soil-borne fungi *Aspergillus flavus, A. niger, Rhizoctonia bataticola, R. solani* and *Sclerotium rolfsii* was determined in dual culture method. Antagonism of bacterial isolates to the biotrophic foliar pathogens *Cercospora arachidicola, Phaeoisariopsis personata* and *Puccinia arachidis* was studied in an *in vitro* spore germination assay. Of the 393, bacterial isolates tested, 77 were antifungal to fungal pathogens of groundnut and differed in their spectrum of activity. The percentage of antifungal isolates significantly *(P* = 0.01) effective against each fungus, were shown separately in the figure.

65 (84.4%), *R. bataticola* 47 (61.0%), *R. solani* 53 (68.8%) and 5. *rolfsii* 34 (44.2%). Five bacterial isolates, *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GRS 175, GSE 18, GSE 19 and GSE 30, had a broad-spectrum antifungal activity (Plate Fig. 4 and 5) and were highly effective against all the eight test fungi. These isolates inhibited the *in vitro* spore germination of the three foliar pathogens by 97-100% compared to control (Table 1). In addition, ten bacterial isolates GGS 1, GGS 11, GPS 15, GRS 7, GRS 70, GRS 94, GRS 100, GRS 134, GRS 225 and GSE 3 inhibited the conidial germination of *P. personata*, the pathogen of prime economic interest, by >90%. *R. bataticola, P. personata, C. arachidicola* and *P. arachidis* were more susceptible to the antagonistic activity of bacterial isolates, compared to other four test fungi, while *A. flaws* was least susceptible. Three bacterial isolates GRS 225, GSE 5 and GSE 6, highly effective against other test fungi, were ineffective against *A. flavus*.

3.1.3. Antifungal activity of CCF of antifungal bacteria: The antifungal activity of CCF of five selected bacteria, *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, was determined by testing their effect on radial growth or conidial germination and dry biomass. Since GSE 18 and GSE 19 were effective in control of crown rot and stem rot diseases of groundnut in greenhouse. The effect of CCF of these two bacterial isolates on the production of CWDE of *A. niger and S. rolfsii* was also determined.

(a) *Inhibition of radial growth:* The antifungal activity of extracellular metabolites of the selected five antagonistic isolates, *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, against the soil-borne necrotrophic fungi was determined by cellophane overlay method. The metabolites of these five isolates inhibited the radial growth of *A. flavus* by 79.5%, 79.8%, 59.9%, 59.3% and 57.0%, respectively measured 8 DAI (Table 2) and completely inhibited the growth of other four test fungi *A. niger, R. bataticola, R. solani* and *S. rolfsii* (Plate Fig. 6). Further, antifungal activity of different concentrations of cell free culture filtrates of these bacteria was tested using poisoned food technique. At the highest concentration tested i.e. 50% (v/v), culture filtrates of GPS 21,

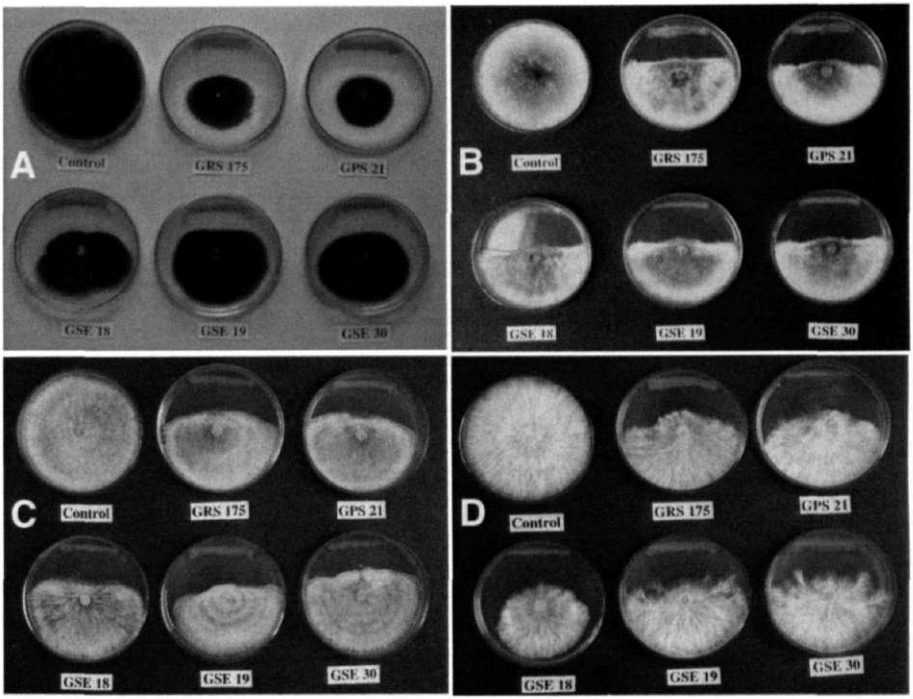


Plate Fig. 4. Showing the antifungal activity of *Pseudomonas* sp. GRS 175 and four isolates of *P. aeruginosa* - GPS 21, GSE 18, GSE 19 and GSE 30 against necrotrophic soil-borne fungal pathogens of groundnut.

(A) *Aspergillus niger,* (B) *Rhizoctonia bataticola,* (C) *R. solani,* and (D) *Sclerotium rolfsii*, as determined by dual culture method. Test fungus was inoculated at the center of each petri dish after 48 h of bacterial inoculation on PDA, and the plates were incubated at 28°C. Photographs were taken 8 DAI in case of *A. niger* and 4 DAI for the other three test fungi.

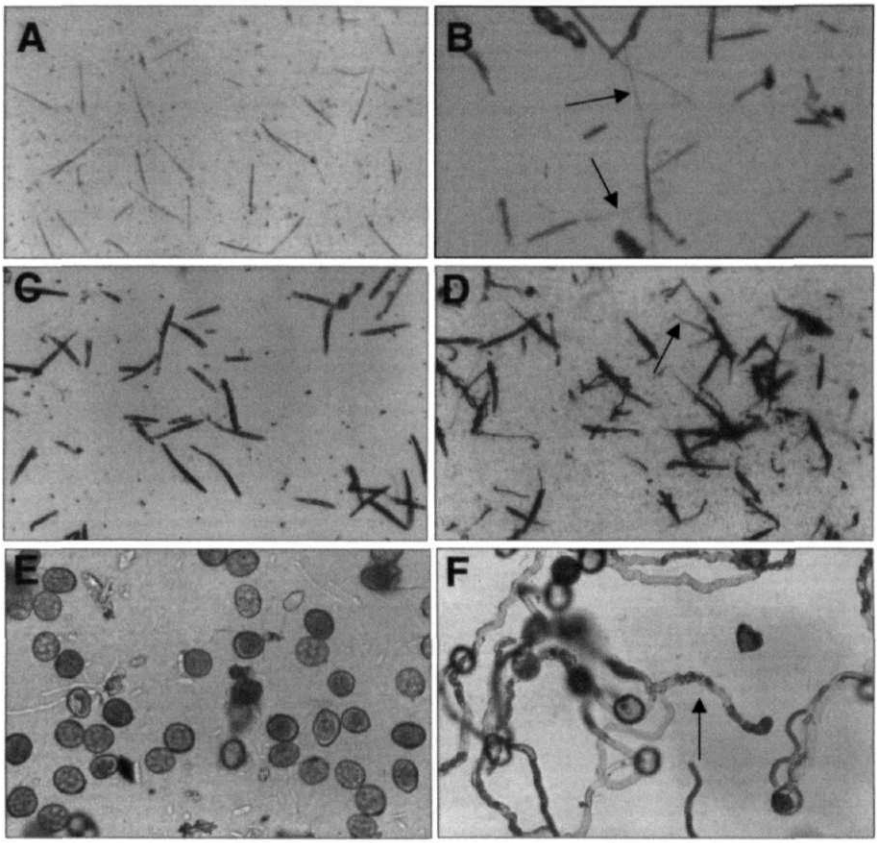


Plate Fig. 5. Showing the antifungal activity of *Pseudomonas aeruginosa* GSE 18 biotrophic foliar fungal pathogens of groundnut.
(A) *Cercospora arachidicola*, (C) *Phaeoisariopsis personata*, and (E) *Puccinia arachidis* compared to the respective controls (B), (D)and (F). Fifty µl of fungal spore suspension (10^5 spores ml^{-1}) was mixed with equal volume of bacterial cell suspension (2 x 10^8 cfu ml^{-1}) on a cavity slide and incubated in dark at 24°C. Photomicrographs of C. *arachidicola* and *P. personata* were taken 24 HAI and *P. arachidis* 8 HAI.

Table 2. Effect of the extracellular low molecular metabolites of selected antifungal isolates, *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, on the radial growth of five fungal pathogens of groundnut.

S. No.	Isolate	Colony diameter in mm				
		Aspergillus flavus	*Aspergillus niger*	*Rhizoctonia bataticola*	*Rhizoctonia solani*	*Sclerotium rolfsii*
1	GPS 21	18.0±0.63	5.0	5.0	5.0	**5.0**
2	GRS 175	17.7±1.03	5.0	5.0	5.0	5.0
3	GSE 18	35.2±1.17	5.0	5.0	5.0	5.0
4	GSE 19	35.710.82	5.0	5.0	5.0	5.0
5	GSE 30	37.711.51	5.0	5.0	5.0	5.0
6	Control	87.711.03	90.010	90.010	90.010	90.010
	LSD (P =0.01)	1.52	0	0	0	0

Bacterial isolates were inoculated on a cellophane membrane overlaid on PDA in 90 mm diameter agar plates and incubated for 48 h at 30°C. The membrane was removed and a 5 mm diameter actively growing mycelial disc was inoculated at the center and incubated at 28°C. The values represent the fungal colony diameter measured 8 DAI in each treatment and are the mean of six replicates in two sets of experiments. Colony diameter of 90 mm was maximum and 5 mm was the size of the inoculated disc.

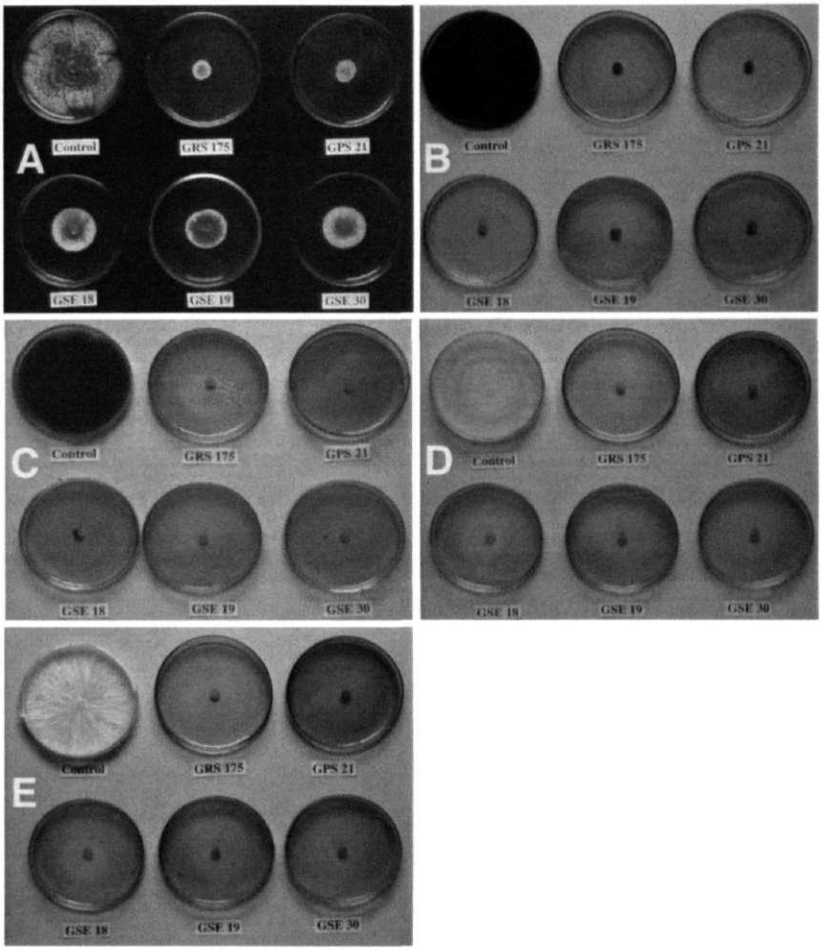


Plate Fig. 6. Effect of secondary metabolites of isolates of *Pseudomonas* sp. GRS 175 and four isolates of P. *aeruginosa* - GPS 21, GSE 18, GSE 19 and GSE 30 against necrotrophic soil-borne fungal pathogens of groundnut.
(A) *Aspergillus flavus,* (B) *A. niger,* (C) *Rhizoctonia bataticola,* (D) *R. solani,* and (E) *Sclerotium rolfsii.* Bacterial isolates were inoculated on a cellophane membrane overlaid on PDA and incubated for 48 h at 30°C. The membrane was removed and a 5 mm diameter myceliai disc was inoculated at the center and incubated at 28°C. Photographs of *A. flavus* and *A. niger* were taken 8 DAI, and the other three fungi were photographed 4 DAI.

GRS 175, GSE 18, GSE 19 and GSE 30 reduced the colony diameter of *A. flavus* by 71.0%, 71.6%, 63.9%, 63.1% and 36.0%, respectively (Table 3). **The** culture filtrates were not inhibitory to *A. flavus* at 15% (v/v). CCF of GSE 18, GSE 19 and GSE 30 completely inhibited the radial growth of *A. niger, R. bataticola, R, solani* and *S. rolfsii* at 50% (v/v). The increase in radial growth upon the decrease in the concentration of CCF varied depending on the test fungus and bacterium. Radial growth of *S. rolfsii* was completely inhibited by 15% (v/v) culture filtrates of GPS 21, GRS 175 and GSE 18.

Mycelial discs of *A. niger, R. bataticola, R. solani* and *S. rolfsii* that failed to grow in presence of CCF, when transferred to PDA, 4 DAI, fungal growth could not be revived suggesting the fungicidal nature of CCF.

(b) *Inhibition of fungal biomass:* Cell free culture filtrates of selected antifungal isolates, *Pseudomoans* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, reduced the biomass of five necrotrophic soil-borne fungi grown in PDB (Table 4). The reduction in biomass of each fungus was proportional to the concentration of CCF added to the growth medium. All test fungi except *A. flavus* failed to grow in presence of 50% (v/v) CCF in the growth medium. At a concentration of 25% (v/v), growth was observed in all the fungi, but the fungal biomass was greatly reduced compared to control. The reduction in fungal biomass in presence of 25% (v/v) CCF varied from 86.7% (GSE 18 *vv R. solani)* to 31.4% (GSE 18 *vs A. flavus),* compared to PDB control. At a concentration of 10% (v/v), in comparison to the other four bacteria, CCF of GSE 18 effectively reduced the biomass of *A. niger* (62.4%) and *R. solani* (66.1%), whereas CCF of GRS 175 effectively reduced the biomass of *S. rolfsii* (77.4%) and *R. bataticola* (49.1%).

(c) *Inhibition of spore germination:* The inhibitory effect of CCF of *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, against *C. arachidicola, P. personata* and *P. arachidis* was studied *in vitro* by spore germination assay. The culture filtrates at ≥25% (v/v), inhibited the *in vitro* conidial germination of *C. arachidicola* and *P. personata* completely, and urediniospores of *P. arachidis* by >90% (Table 5). The CCF were significantly inhibitory even at the lowest

Table 3. Effect of cell free culture filtrates of selected antifungal isolates, *Pseudomonas* sp. GRS 175, and *P. aeurginosa*GPS 21, GSE 18, GSE 19 and GSE 30, on the radial growth of five necrotrophic soil-borne fungal pathogens of groundnut.

S. No.	Isolate	CCF (% v/v)	Colony diameter (mm)				
			Aspergillus flavus	*Aspergillus niger*	*Rhizoctonia bataticola*	*Rhizoctonia solani*	*Sclerotium rolfsii*
1	GPS 21	50	25.5±1.29	12.8±2.22	5.0	5.0	5.0
2	GPS 21	25	58.3±6.80	57.0±1.83	5.0	35.012.58	5.0
3	GPS 21	15	82.5±1.29	75.313.30	29.5±2.38	42.811.71	5.0
4	GRS 175	50	25.0 ±2.16	10.010.82	5.0	40.012.16	5.0
5	GRS 175	25	50.5±2.65	54.3±1.71	5.0	41.812.50	5.0
(.	GRS 175	15	71.8±2.50	72.512.08	27.0±2.94	53.5±3.42	5.0
7	GSE 18	50	31.8±4.50	5.0	5.0	5.0	5.0
S	GSE 18	25	62.3±4.99	27.3±2.22	42.313.86	5.0	5.0
9	GSE 18	15	85.5±0.58	50.814.86	47.314.03	27.511.29	5.0
to	GSE 19	50	32.5±2.38	5.0	5.0	5.0	5.0
11	GSE 19	25	61.0±4.55	36.512.38	5.0	31.012.58	5.0
12	GSE 19	15	84.3±0.50	54.311.71	33.011.83	49.8±2.75	17.011.83
13	GSE 30	50	56.3±5.06	5.0	5.0	5.0	5.0
14	GSE 30	25	82.511.29	44.3±2.22	36.5±2.38	17.511.29	24.5±1.29
15	GSE 30	15	87.310.50	57.011.83	28.312.63	45.811.71	35.012.58
16	Control	50	88.0±0.82	89.110.50	90.010.0	90.0±0.0	90.010.0
17	Control	25	87.5±0.58	89.410.50	90.010.0	90.010.0	90.010.0
18	Control	15	87.810.50	88.911.00	90.010.0	90.010.0	90.010.0
	LSD (P = 0.01)		4.87	3.74	4.53	2.97	5.90

Cell free culture filtrate was obtained by centrifugation of stationary phase cultures grown in LB broth followed by filter sterilization of the supernatant. The filtrate was added to pre-cooled PDA just before pouring the plates. A 5 mm disc of an actively growing mycelium was inoculated at the center and incubated at 28°C. Colony diameter was recorded 8 DAI for *A. flavus* and *A. niger*, and 4 DAI for *R. bataticola*, *R. solani* and *S. rolfsii*. Colony diameter of 90 mm was maximum and 5 mm was the size of the inoculated disc. The values are the mean of eight replications from a repeated experiment

Table 4. Effect of cell free culture filtrates of five selected antifungal isolates, *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, on the growth of five groundnut pathogenic fungi in terms of inhibition of biomass dry weight.

S. No.	Isolate	CCF (% v/v)	Mycelial dry weight (mg)				
			Aspergillus flavus	***Aspergillus niger***	*Rhizoctonia bataticola*	***Rhizoctonia solani***	*Sclerotium* ***rolfsii***
1	GPS 21	25	189.8±2.2	66.015.5	95.315.4	95.8±5.4	104.018.6
2	GPS 21	15	232.5±16.7	109.317.1	140.517.1	141.8111.0	127.017.4
3	GPS 21	10	260.3±5.9	267.3122.2	157.517.3	187.316.2	147.817.5
4	GRS 175	25	168.3120.0	37.814.1	81.813.3	72.512.9	90.814.7
5	GRS 175	15	236.3121.4	80.314.3	148.812.5	92.015.0	101.514.7
6	GRS 175	10	243.318.1	234.3±14.6	150.515.5	160.818.9	101.8±6.7
7	GSE 18	25	198.8125.9	49.316.08	130.815.9	38.814.7	97.517.0
8	GSE 18	15	255.818.5	69.816.3	170.0±9.6	72.313.0	129.518.7
9	GSE 18	10	285.0119.7	103.016.1	234.016.7	98.817.3	142.5±12.0
10	GSE 19	25	186.817.9	78.3±2.2	103.816.1	43.317.1	71.517.19
11	GSE 19	15	209.317.0	81.514.0	118.5±8.4	83.814.6	79.013.4
12	GSE 19	10	251.3112.1	176.315.9	185.5115.3	128.3110.8	121.315.3
13	GSE 30	25	160.815.50	95.817.9	129.513.1	83.012.9	90.313.5
14	GSE 30	15	165.8±5.0	151.8110.7	146.012.6	129.318.8	152.516.5
15	GSE 30	10	232.314.7	252.8118.7	201.819.4	167.516.6	232.017.3
16	Control	25	289.817.8	277.0122.8	274.314.1	291.0115.3	446.517.9
17	Control	15	289.3±3.3	288.5113.7	273.517.2	279.5113.1	425.3115.0
IK	Control	10	293.319.7	273.8111.5	295.5114.6	291.8±9.5	451.0113.7
	LSD(P = 0.01)		23.81	21.12	18.32	14.04	13.64

Cell free culture filtrate was obtained by separating the bacterial cells from a stationary phase culture grown in LB broth. The supernatant was filter sterilized and added to PDB in 100 ml conical flask. The final volume of each flask was 30 ml and inoculated with a 5 mm disc of an actively growing fungal culture. After 96 h of incubation at 125 rpm and 28°C, the mycelium was harvested on to a pre-weighed Whatman filter paper, dried in an oven at 60°C and weighed. The values are the mean of eight replications in two sets of experiments.

Table 5. Effect of cell free culture filtrates of five selected antifungal isolates, *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, on the *in vitro* spore germination of three biotrophic foliar fungal pathogens of groundnut.

S. No.	Isolate	CCF (% v/v)	Percentage germination		
			Cercospora arachidicola	*Phaeoisariopsis personata*	*Puccinia arachidis*
1	GPS 21	25	O.OiO.O	0.010.0	5.5±1.7
2	GPS 21	10	23.8±2.6	29.3±3.4	39.8±1.7
3	GPS 21	5	38.0±2.2	43.016.8	53.0±3.4
4	GRS 175	25	0.0±0.0	O.OiO.O	0.010.0
5	GRS 175	10	22.5± 3.7	15.0±2.2	27.812.8
6	GRS 175	5	28.3±3.0	23.012.2	47.3±3.1
7	GSE 18	25	0.0± 0.0	0.010.0	0.010.0
8	GSE 18	10	15.512.7	18.012.2	25.3±2.2
9	GSE 18	5	23.8±2.6	28.312.8	42.0±4.2
10	GSE 19	25	0.0± 0.0	O.OiO.O	1.011.2
11	GSE 19	10	16.511.3	15.8±1.7	23.015.7
12	GSE 19	5	27.012.9	29.011.8	38.8±4.5
13	GSE 30	25	0.0± 0.0	O.OiO.O	3.0±2.2
14	GSE 30	10	26.012.9	23.0±2.2	34.3±3.6
15	GSE 30	5	49.8±6.2	44.313.9	56.514.7
16	Control	25	86.0±2.6	91.512.9	92.8±3.0
17	Control	10	82.512.7	92.013.7	90.3±3.6
18	Control	5	80.8±5.3	88.3±3.0	91.812.2
	LSD ($P = 0.01$)		4.68	5.07	5.88

Cell free culture filtrate was obtained by separating the bacterial cells from a stationary phase culture grown in LB broth. The supernatant was filter sterilized and tested for inhibition of fungal spore germination *in vitro.* Fifty µl each of CCF and spore suspension (10^5 spores ml^{-1}) were mixed on a cavity slide and incubated in a humid chamber in dark at 24°C and observed for spore germination under a photomicroscope. The values are the mean of eight replicates in two sets of experiments. Germination of *C. arachidicola* and *P. personata* conidia was measured 24 HAI and *P. arachidis* urediniospore germination was measured 8 HAI.

concentration tested i.e. 5% (v/v). At this concentration, maximum inhibition of conidial germination of *C. arachidicola* by CCF of GSE 18 (70.5%) **and** GSE 19 (66.5%), *P. personata* by GRS 175 (74.0%) and GSE 19 (67.2%) and urediniospores of P. *arachidis* was by GSE 19 (57.7%) and GSE 18 (54.2%).

(d) ***Effect of CCF on hyphal morphology:*** Microscopic observation of the five necrotrophic fungi grown on PDA in presence of CCF of five antagonistic isolates, *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30, revealed differences in the mycelial structure and arrangement compared to control. The CCF were added at a minimum concentration at which the individual fungus was able to grow. In presence of CCF, hyphal growth direction of all the test fungi was observed to be either perpendicular or backward rather than progressive (Plate Fig. 7 B and F). Increase in hyphal diameter, granulation of the cytoplasm and vacuolization of the cytoplasm (Plate Fig. 7 A and E) were commonly observed in all the fungi grown in presence of CCF of GRS 175 and GPS 21. CCF of *P. aeruginosa* GRS 175 was effective than other treatments in disruption of the fungal cytoplasmic contents. In presence of CCF of GRS 175, vacuolization of hyphae was commonly observed in different fungi (Plate Fig. 7 C and G). CCF of GRS 175 and GPS 21 induced more frequent hyphal bulging in *R. hataticola* than the other four fungi (Plate Fig. 7 D, H and I). In presence of CCF of GSE 18, GSE 19 and GSE 30, the fungal hyphae appeared similar to control except for an increase in hyphal diameter, and unbranched and coiled hyphal tips.

(e) ***Effect of CCF on production of extracellular CWDE:*** The effect of CCF of GSE 18 and GSE 19 on the production of plant CWDE by *A. niger* and *S. rolfsii* was tested. The enzymes studied were exo-PG, endo-PG, PL, PME and CMCase. Both the culture filtrates at a concentration of 25% (w/v) inhibited the enzyme production by *A. niger* (Fig. 3) and 5. *rolfsii* (Fig. 4) to higher significant levels, with GSE 18 being highly effective. Inhibition of extracellular enzyme production by *A. niger* and *S. rolfsii* in presence of culture filtrates of GSE 18 and GSE 19 varied depending on the enzyme. Culture filtrate of GSE 18 inhibited the production of PL by *S. rolfsii* and *A. niger* by 73.8% and 55.9%,

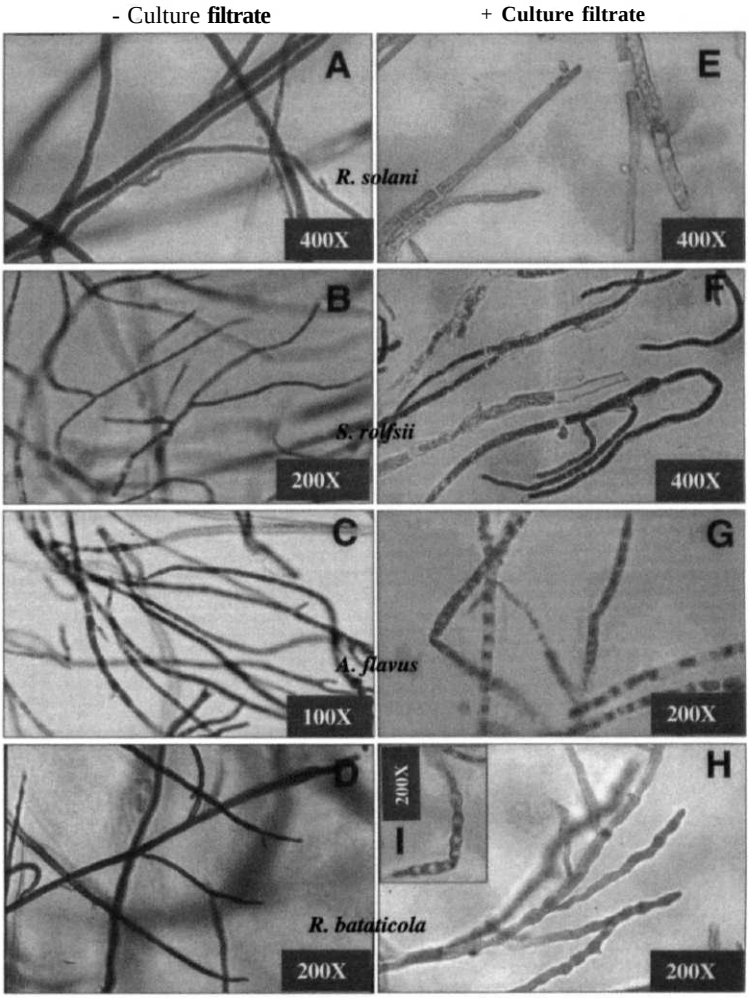
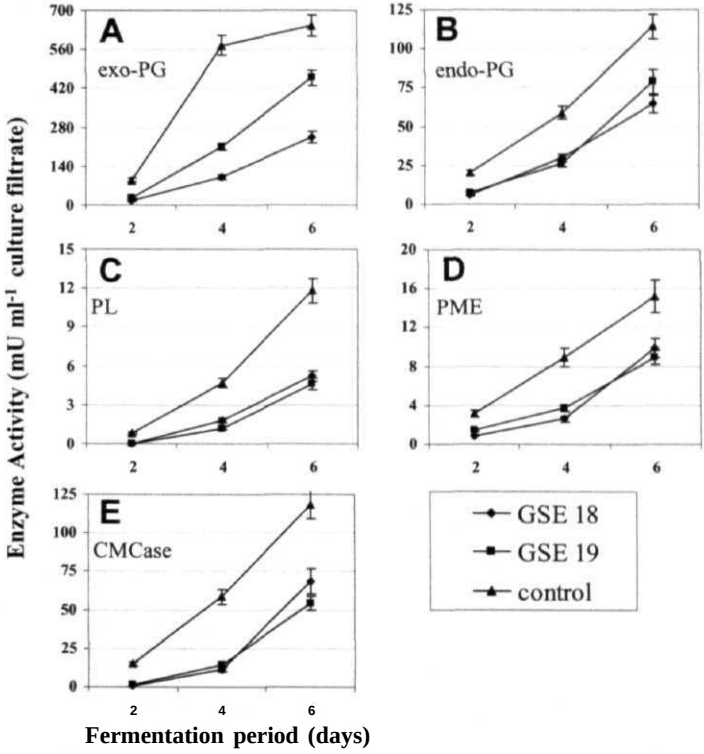


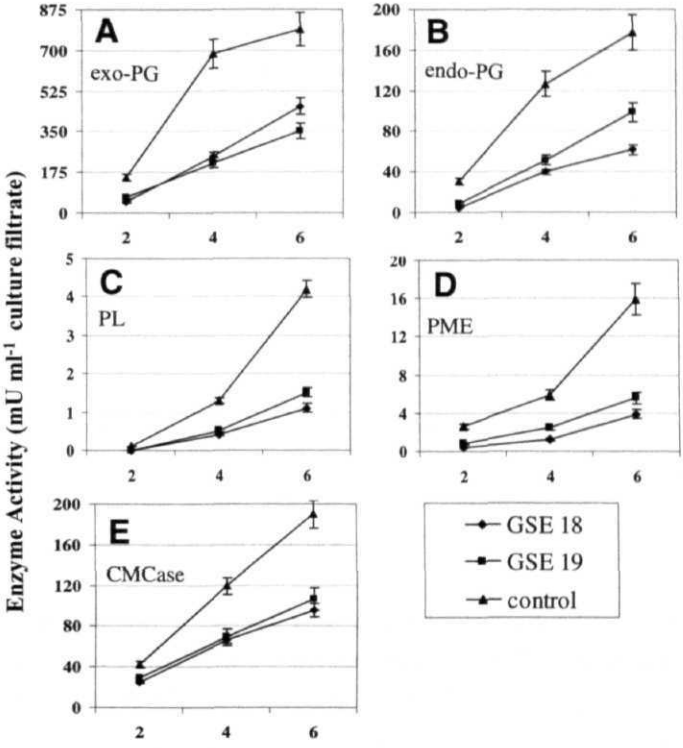
Plate Fig. 7. Effect of cell free culture filtrates of *Pseudomonas* sp. GRS 175 on the hyphal structure of soil-borne necrotrophic fungal pathogens of groundnut.
(A) *Rhizoctonia solani* (B) *Sclerotium rolfsii.* (C) *Aspergillus flavus* and (D) *Rhizoctonia bataticola,* grown on PDA alone. (E) *R. solani* in presence of 15% (v/v) culture filtrate; (F) *S. rolfsii* in presence of 10% (v/v) culture filtrate; (G) *A. flavus* in presence of 50% (v/v) culture filtrate; (H) and (I) *R. bataticola* in presence of 15% (v/v) culture filtrate.

Fig. 3. Effect of cell free culture filtrates of *Pseudomonas aeruginosa* GSE 18 and GSE 19 on the production of cell wall degrading enzymes, (A) Exopolygalacturonase (exo-PG), (B) Endopolygalacturonase (endo-PG), (C) Pectin lyase (PL), (D) Pectin methyl esterase (PME), and (E) Carboxy methyl cellulase (CMCase), by the groundnut crown rot pathogen *Aspergillus niger*.



Twenty ml of Richard's medium in 100 ml conical flask added with CCF (final concentration 25% v/v) was inoculated with four agar discs (5 mm diameter) of actively growing *A. niger*. The carbon source of the medium was changed depending on the enzyme activity to be assayed, sodium polypectate for exo- and endo-PG, pectin for PL and PME, and CMC for CMCase. The inoculated flasks were incubated at 28°C and 120 rpm, and the enzyme activities were determined using culture filtrate as crude enzyme extract.

Fig. 4. Effect of cell free culture filtrates of *Pseudomonas aeruginosa* GSE 18 and GSE 19 on the production of cell wall degrading enzymes, (A) Exopolygalacturonase (exo-PG), (B) Endopolygalacturonase (endo-PG), (C) Pectin lyase (PL), (D) Pectin methyl esterase (PME). and (E) Carboxy methyl cellulase (CMCase), by the groundnut stem rot pathogen *Sclerotium rolfsii.*



Twenty ml of Richard's medium in 100 ml conical flask added with CCF (final concentration 25% v/v) was inoculated with four agar discs (5 mm diameter) of actively growing 5. *rolfsii.* The carbon source of the medium was changed depending on the enzyme activity to be assayed, sodium polypectate for exo- and endo-PG, pectin for PL and PME, and CMC for CMCase. The inoculated flasks were incubated at 28°C and 120 rpm, and the enzyme activities were determined using culture filtrate as crude enzyme extract.

PME by 75.5% and 34.8%, exo-PG by 41.9% and 62.1%, endo-PG by 44.5% and 43.5% , and CMCase by 49.6% and 42.0%, respectively, when measured 6 DAI.

3.2. Characterization and identification of bacterial isolates

3.2.1. Characterization of bacterial isolates: The bacterial isolates used in the present study were further characterized in terms of their ability for *in vitro* production of extracellular metabolites or enzymes viz., chitinase, β-1,3-glucanase, protease, auxin, siderophore, HCN and solubilization of inorganic phosphate, that contribute to antifungal activity and/or plant growth promotion. Of the 393 bacterial isolates tested, 95 (24.2%) produced extracellular chitinase, 54 (13.7%) produced β-1,3-glucanase, 354 (90.1%) produced protease, 153 (38.9%) produced siderophores on CAS medium, 39 (9.9%) produced auxin, 10 (2.5%) produced HCN, and 50 (12.7%) solubilized tricalcium phosphate (Fig. 5 A and B).

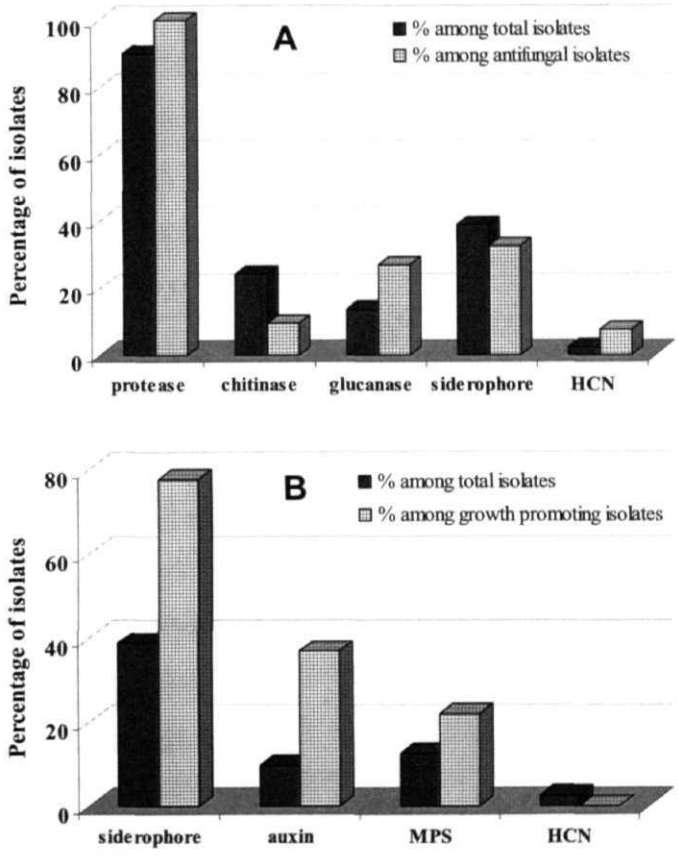
The growth promoting and antifungal bacterial isolates differed in their *in vitro* ability to produce metabolites or enzymes that contribute to these activities (Fig. 5 A and B). Among the antifungal isolates, the percentage of chitinase and siderophore-producing bacteria was less compared to their percentage among the total bacteria. Among the growth promoting isolates, the percentage of siderophore and auxin producers and mineral phosphate solubilizing bacteria was high.

3.2.2. Identification of bacterial isolates: Selected bacterial isolates were identified at Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh, India, based on morphological, physiological and biochemical tests (Table 6).

3.3. Evaluation of plant growth promoting activity of bacterial isolates

33.1. Greenhouse evaluation: Three hundred and ninety three bacterial isolates from different habitats of groundnut were applied as seed treatment and tested for their plant growth promoting ability in groundnut. Twenty seven isolates significantly (P = 0.05)

Fig. 5. *In vitro* evaluation of bacterial isolates for production of metabolites or enzymes that contribute to (A) Antifungal activity, and (B) Plant growth promotion.



MPS = mineral phosphate solubilization

Percentage of isolates positive for individual characteristic was calculated separately among the total 393 bacterial isolates and 77 antifungal isolates or 27 growth promoting isolates.

Table 6. Morphological, physiological and biochemical characteristics of selected growth promoting or broad-spectrum antifungal or chitinolytic bacterial isolates.

S. No.	Characteristic test	GPS 5	GPS 55	GRS 123	GRS 149	GRS 243	*Pse*[a]	Remarks[b]
1	Grams Reaction	-	+	+	-	+	-	
2	Shape	Rod	Rod	Rod	Rod	Rod	Rod	
3	**Size**	*	**T**	**T**	•	**M**	**S/M**	
4	Spore: *Endospore*	-	+	+	-	+	-	
5	*Position*	*	C	**C**	•	**C**	*	
6	*Sporangia bulging*	*	+	+	*	+	•	
7	Motility	+	+	+	+	+	+	GRS 175 & GSE 30: -
s	Fluoresence (UV)	-	-	-	-	-	+	
7	Growth at temp. : *4 V*	\Y	-	-	-	-	-	
	15V	+	-	-	+	-	+	
	42 V	+	+	+	+	+	+	
	55 V	-	-	-	-	-	-	
8	Growth at pH: *5.0*	•	+	±	*	-	+	
	5.7, 6.8, 8.0, 9.0 and 11.0	+	+	+	+	+	+	
9	Growth on NaCl (%): *2.5*	+	+	+	+	+	+	
	5.0	+	+	+	+	+	+	
	7.0	+	+	+	+	-	-	
	10.0	+	+	-	+	-	-	
10	Anaerobic growth	W	+	+	-	+	-	
11	Growth on Mac Conkey agar: Lac fermenter	+	-	-	+	-	+	
12	Indole test		-	-	-	-	-	
13	Methyl red test		+	+	-	+	+	GRS 175 & GSE 30: -
14	Voges Proskauer test		-	-	-	-	-	
15	Citrate utilization		+	-	-	-	+	
16	Casein hydrolysis	+	+	+	-	-	+	
17	Starch hydrolysis	-	+	+	-	+	-	
18	Urea hydrolysis	-	-	-	-	-	+	GRS 175 & GSE 30: -
19	Nitrate reduction	+	+	+	-	-	+	GRS 175 & GSE 30: *
20	Nitrite reduction	*	-	-	*	-	+	GPS 21:-

Contd...

S. No.	Characteristic test	GPS 5	GPS 55	GRS 123	GRS 149	GRS 243	*Pse*[a]	Remarks[b]
21	H_2S production	*	-	-	*	-	+	GPS 21:- GRS 175 & GSE 30: •
22	Oxidase test	+	+	+	+	+	+	
23	Catalase test	+	+	+	+	+	+	
24	Oxidation/fermentation (O/F)	-	F	-	-	F	F	GRS 175 & GSE 30: O
25	Gelatin liquefaction	·	+	+	•	+	t	GRS 175 & GSE 30: •
26	Acid production from carbohyrdates: *Arabinose*	+	-	-	+	-	-	GSE 30: +
27	***Dextrose***	+	+	+	+	+	+	
2S	***Galactose***	+	-	-	+	+	+	
29	***Lactose***		-	-	-	+	-	GRS 175 & GSE 30: *
30	***Maltose***		-	+	-	+	-	GRS 175 & GSE 30: •
31	***Mannitol***	+	±	-	+	+	-	GRS 175: +
32	*Raffinose*		-	-	+	+	-	
33	***Salicin***	+	+	+	+	+	-	
34	***Sorbitol***		-	-	-	-	-	GRS 175 & GSE 30: •
35	***Sucrose***	+	+	+	+	+	-	
36	***Trehalose***		-	+	*	t	-	GSE 18: +
37	***Xylose***	+	-	-	-	+	-	GSE 19: -

* Not determined.

[a] Characteristics of five *Pseudomonas* isolates, GRS 175, GPS 21, GSE 18, GSE 19 and GSE 30 were tested.

[b] Isolates of *Pseudomonas* sp. that differed in a particular characteristic were mentioned separately.

Abbreviations used: + = positive and - = negative for a particular character; T = Thick, S = Short and M = Moderate cell size; C = centre; W = weak; F = fermentation and O = oxidation.

Based on the above mentioned characters, identification of the test isolates was as follows:

GPS 5 = *Serratia marcescens* (Chitinase and prodigiosin production were also considered).
GPS 55 = *Bacillus megaterium*
GRS 123 = *Bacillus firmis*
GRS 149 = *Achromobacter xylosoxidans* sub sp. *denitrificans*
GRS 243 = *Bacillus circulans*
GRS 175 = *Pseudomonas* sp.
GPS 21, GSE 18, GSE 19 **and** GSE 30 = *Pseudomonas aeruginosa*

improved the plant growth. Seed treatment with these isolates resulted in an increase of root and shoot lengths, and also dry weight of groundnut seedlings observed 20 DAS. Bacterial isolate GRS 180, increased the root length to the maximum i.e. 60.2%, compared to control (Table 7). Following seed bacterization, a rhizosphere isolate *B. firmis* GRS 123 and two phylloplane isolates, *B. megaterium* GPS 55 and *P. aeruginosa* GPS 21 increased the plant biomass by 23.6%, 26.2% and 24.5%, respectively. In plants treated with GRS 123, GPS 55 and GPS 21, the root length increased by 42.8%, 42.9% and 47.2%, and shoot length increased by 35.4%, 42.9% and 47.2%, respectively than control (Table 7 and Plate Fig. 8 A). Four bacterial isolates (1.3%) were inhibitory to the plant growth as observed by significant reduction in plant biomass compared to control (data not shown).

Of the 27 plant growth promoting isolates, 21 (77.7%) produced siderophores, 10 (37%) produced auxins, and six (22.2%) solubilized tricalcium phosphate, and none produced HCN *in vitro* (Fig. 5 B). Six rhizobacterial isolates, GRS 7, 49, 96, 115, **143,** 180 and GPS 21 produced both siderophores and auxins *in vitro.* A definite correlation between auxin production and increase in root length was not observed in groundnut seedlings treated with the auxin-producing strains used in this study.

3.3.2. Development of bacterial formulations: Peat-based formulations of three selected plant growth promoting bacterial isolates *B.firmis* GRS 123, *B. megaterium* GPS 55 and *P. aeruginosa* GPS 21 were stored at 30°C. Survival and multiplication of bacterial isolates in the carrier material was monitored at frequent time intervals up to 180 DAI.

Of the three selected bacterial isolates, *B. firmis* GRS 123 and *B. megaterium* GPS 55 had a good shelf-life in peat up to 180 DAI (Fig. 6). At this time period, their mean populations were $\log_{10}$ 7.7 and 7.1 cfu g^{-1} peat. At 90 DAI and 120 DAI, the populations of these two bacteria were logio 7.9 and 7.8 cfu g'", and $\log_{10}$ 7.7 and 7.5 cfu g^{-1}, respectively.

3.3.3. Field evaluation of bacteria and their formulations: The three bacterial isolates *B. firms* GRS 123, *B. megaterium* GPS 55 and *P. aeruginosa* GPS 21, and peat-based formulations of these isolates were applied as seed treatment and tested for plant growth

Table 7. Plant growth promoting activity of bacterial isolates in greenhouse.

S. No.	Isolate	Root length (cm)	Shoot length (cm)	Dry weight (g)
1	GGS 6	18.58 (36.3)*	12.10(22.0)	2.73(16.6)
2	*Pseudomonas aeruginosa* GPS 21	19.48 (42.9)	13.03(31.4)	2.91 (24.5)
3	GPS 28	17.78(30.5)	12.18(22.8)	2.75(17.4)
4	GPS 32	18.60(36.5)	12.37 (24.7)	2.74(17.3)
5	GPS 38	16.18(18.7)	12.27(23.7)	2.70(15.5)
6	*Bacillus megaterium* GPS 55	20.07 (47.2)	14.15(42.6)	2.95 (26.2)
7	GRS 2	17.62 (29.2)	11.93 (20.3)	2.80(19.9)
8	GRS 7	15.82(16.0)	10.95 (10.4)	2.71 (15.8)
9	GRS 11	16.17(18.6)	11.65 (17.4)	2.80(19.6)
10	GRS 18	17.05(25.1)	10.95(10.4)	2.71(15.7)
11	GRS 49	17.40(27.7)	10.88(9.7)	2.68(14.6)
12	GRS 60	16.43 (20.6)	10.52(6.0)	2.69(15.2)
13	GRS 69	18.60(36.5)	12.17(22.6)	2.71 (15.9)
14	GRS 73	16.35(20.0)	12.32 (24.2)	2.68 (14.7)
15	GRS 86	15.90(16.7)	10.80(8.9)	2.74(17.0)
16	GRS 96	17.73(30.1)	12.87(29.7)	2.68(14.5)
17	GRS 115	18.88(38.5)	12.90(30.0)	2.77(18.3)
18	*B. firmis* GRS 123	19.47(42.8)	13.43(35.4)	2.89 (23.6)
19	*Serralia marcescens* GRS 128	17.45(28.0)	12.27 (23.7)	2.77(18.4)
20	GRS 143	18.75(37.6)	12.50(26.0)	2.74(17.1)
21	GRS 180	21.83(60.2)	12.23(23.3)	2.74(17.1)
22	GRS 183	16.75 (22.9)	12.02(21.1)	2.79(19.3)
23	GRS 192	18.15(32.2)	12.50(26.0)	2.75(17.5)
24	GRS 203	19.08(40.0)	11.57(16.6)	2.78(18.6)
25	GRS 241	18.68(37.1)	11.70 (17.9)	2.70(15.3)
26	*P. aeruginosa* GSE 18	16.25(19.2)	11.45 (15.4)	2.69(15.1)
27	GSE 28	20.00 (46.7)	12.02(21.1)	2.70(15.5)
28	Control	13.63(0.00)	9.92 (0.00)	2.34 **(0.00)**
	LSD (P =0.05)	1.41	1.05	0.33

*** Values in parenthesis indicate the percentage increase over control.**

Three hundred and ninety three bacterial isolates were applied as seed treatment (10^6-10^7 cfu seed^{-1}) in groundnut cv. TMV 2. Bacterized seeds were planted in a potting mixture (red alfisol and sand, 3:1) and maintained in a greenhouse at 28±2°C. At 20 DAS, the plants were observed for root length, shoot length and dry biomass. Ten seeds were planted in each replication and the experiment was repeated twice with three replications. Only the isolates that were significantly effective in increase of dry weight were listed.

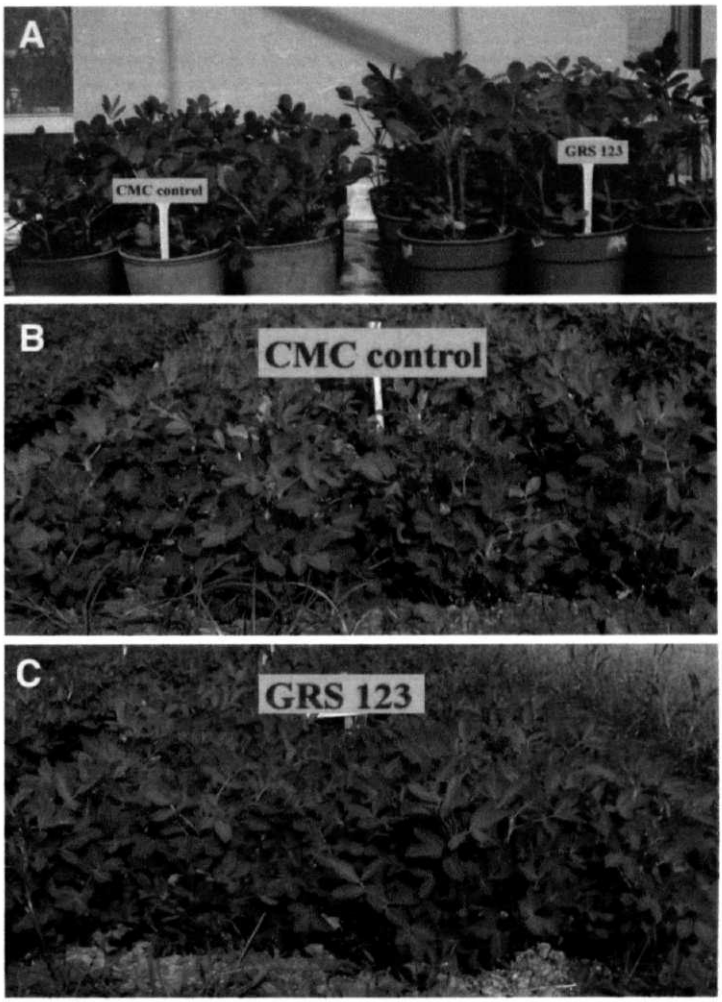
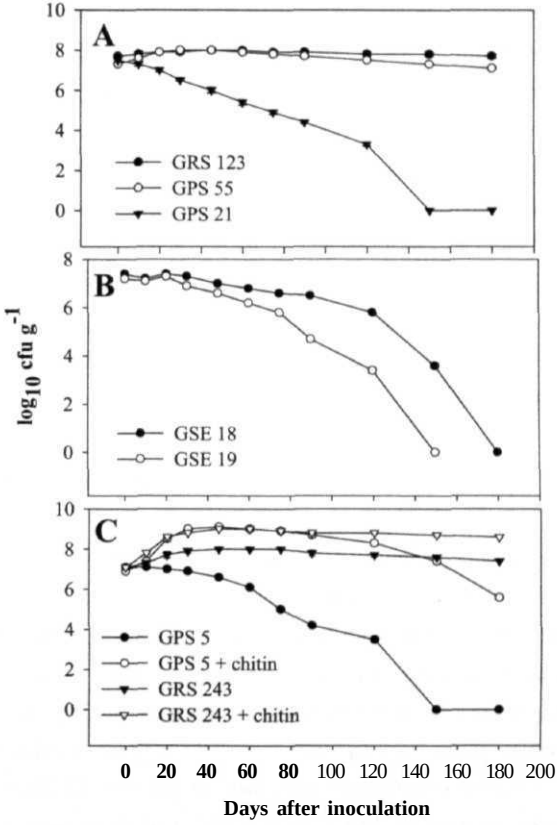


Plate Fig. 8. Greenhouse and field evaluation of *Bacillus firmis* GRS 123 applied as seed treatment (~5 x 10^6 cfu $seed^{-1}$), for plant growth promotion in groundnut. Growth of GRS 123 treated seedlings, (A) in greenhouse at 20 DAS, in comparison to CMC control; (B) and (C) in field, in comparison to CMC control, photographed 100 DAS.

Fig. 6. Survival of bacterial isolates in peat-based formulations. (A) Growth promoting *Bacillus firmis* GRS 123, *B. megaterium* GPS 55 and *Pseudomonas aeruginosa* GPS 21, (B) Biocontrol *P. aeruginosa* GSE 18 and GSE 19, and (C) Chitinolytic *Serratia marcescens* GPS 5 and *B. circulans* GRS 243.



For chitin-supplementation 1% (w/v) chitin was added to neutralized peat. The survival of all the test isolates in fonnulations was measured at regular time intervals up to 180 DAI. The values are the mean of 12 replications in three sets of experiments.

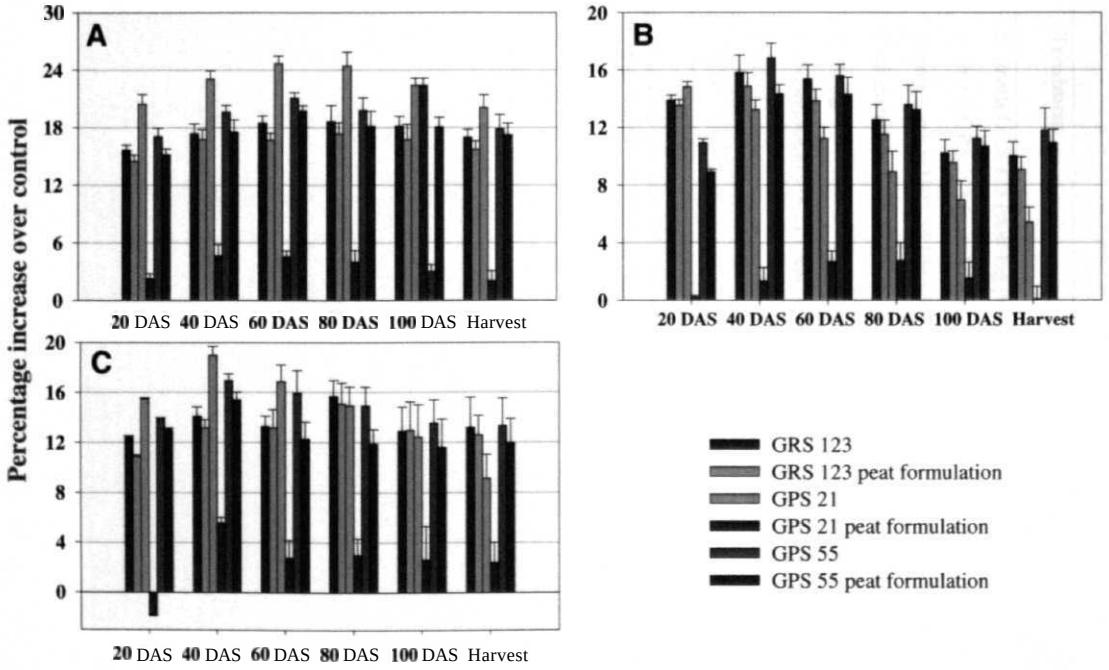
promotion in field. Seed treatment with mid-log phase cells of these isolates increased the seedling emergence, shoot and root length (Fig. 7, Plate Fig. 8 B and C), and yield of groundnut (Table 8) in field. Peat formulations of GRS 123 and GPS 55 were also equally effective as the application of mid-log phase cells of these isolates. However, peat formulation of GPS 21 was ineffective in growth promotion and the plant growth in this treatment was similar to control. Significant increase in seedling emergence, root and shoot lengths was observed in all the treatments, except for peat formulation of GPS 21, from seedling stage till harvest. Among all the treatments, maximum root length was recorded in GPS 21 treated seedlings and in this treatment the root length observed at 60 DAS was 24.6% greater than control (Fig. 7 A). Maximum increase in shoot length (16.83%) compared to control was observed in GPS 55 treated plants at 40 DAS (Fig. 7 B). Highest dry weight was recorded in GPS 21 treated plants up to 60 DAS, but at harvest GPS 55 treated plants recorded maximum dry biomass (13.3% than control) (Fig. 7 C).

In the three treatments GRS 123, GPS 21 and GPS 55 an increase of 16.4%, 15.0% and 19.0% was observed in pod yield (Table 8). Peat formulations of GRS 123 and GPS 55 also enhanced the pod yield. Peat formulation of GPS 21 did not affect the pod yield.

3.4. Biocontrol of stem rot disease of groundnut

Modification of the inoculation procedure by using germinated sclcrotia along with the actively growing mycelium as inoculum resulted in mortality of all the inoculated plants of groundnut cv. TMV 2. This inoculation method was used to evaluate 393 bacterial isolates for control of stem rot disease in greenhouse. Bacterial isolates were applied both as seed bacterization and soil amendment. Twelve bacterial isolates from different groundnut habitats viz., one from geocarposphere (GGS 12), two from phylloplane (GPS 21 and GPS 38), two from rhizosphere (GRS 175 and GRS 223), and seven seed endophytes (GSE 3, GSE 5, GSE 6, GSE 18, GSE 19, GSE 21 and GSE 30) significantly ($P = 0.01$) reduced the incidence and severity of stem rot compared to control (Table 9). Maximum reduction in the mortality of *S. rolfsii* inoculated plants was in GSE 19 treatment (58.3%) (Plate Fig. 9) followed by GSE 18 (54.1%), and

Fig. 7. Field evaluation of three plant growth promoting bacterial isolates, *Bacillus firmis* GRS 123, *Bacillus megaterium* GPS 55 and *Pseudomonas aeruginosa* GPS 21, and their peat formulations for their effects on (A) Root length, (B) Shoot length, and (C) Dry biomass of groundnut cv. TMV 2.



Mid-log phase cells and peat formulations of the three bacterial isolates were applied as seed treatment. In each treatment four plants were uprooted at regular time intervals and measured for root length, shoot length and dry weight. The values presented are the percentage increase over control and are the mean of six replications in a repeated field experiment.

Table 8. Effect of mid-log phase cells and peat formulations of *Bacillus firmis* GRS 123, *B. megaterium* GPS 55 and *Pseudomonas aeurginosa* GPS 21 applied as seed treatment on the emergence and yield of groundnut in field.

S. No.	Treatment	Emergence (%)	Yield (t ha^{-1})
1	*B. firmis* GRS 123 (mid-log phase cells)	95.3±3.1	1.32±0.10
2	*B. firmis* GRS 123 (peat formulation)	94.4±2.2	1.2910.14
3	*B. megaterium* GPS 55 (mid-log phase cells)	97.911.0	1.35±0.11
4	*B. megaterium* GPS 55 (peat formulation)	92.414.5	1.3410.15
5	*P. aeruginosa* GPS 21 (mid-log phase cells)	96.311.7	1.3010.13
6	*P. aeruginosa* GPS 21 (peat formulation)	85.711.9	1.14±0.06
7	Control	82.414.4	1.1310.10
	LSD ($P = 0.05$)	2.97	0.13

Mid-log phase cells and bacterial formulations were applied as seed treatment (10^6-10^7 log cfu $seed^{-1}$) using groundnut cv. TMV 2. Emergence was observed 15 DAS and pod yield was recorded at harvest (105-110 DAS) as tons per hectare. The values are the mean of six replications in a repeated field experiment.

Table 9. Biological control of stem rot disease of groundnut by selected bacterial isolates in greenhouse.

S.No.	**Isolate**	**Disease Score**	**Mortality** (%)
1	GGS **12**	**4.2**	81.3
2	*Pseudomonas aeruginosa* GPS 21	**4.0**	75.0
3	GPS 38	4.3	81.3
4	*Pseudomonas* sp. GRS 175	3.7	66.7
J	GRS 223	**3.9**	72.9
6	GSE 3	**4.6**	89.6
7	GSE 5	4.2	79.2
8	GSE 6	4.5	87.5
9	*P. aeruginosa* GSE 18	2.9	45.8
10	*P. aeruginosa* GSE 19	2.8	41.7
11	GSE 21	4.5	87.5
12	*P. aeruginosa* GSE 30	3.8	68.8
13	Control	5.0	100.0
	LSD ($P = 0.01$)	0.39	9.73

The bacterial isolates were applied as seed treatment (10^6-10^7 cfu $seed^{-1}$) followed by soil amendment of cell suspension (10* cells ml^{-1}) 5 DAS. Groundnut cv. TMV 2 was used in the experiments. *Sclerotium rolfsii* was inoculated 14 DAS by placing a 1 cm diameter disc containing a sclerotia germinated on PDA along with actively growing mycelium at the base of the stem and covered with coarse sand. Disease score on a 1-5 rating scale (Shokes *el a/.*, 1996) and percentage mortality were recorded 15 DAI. The experiment was conducted with three replications, repeated once and the mean values were presented.

Plate Fig. 9. Biological control of stem rot disease of groundnut using *Pseudomonas aeruginosa* GSE 19 as seed treatment (~6.8 x 10^6 cfu $seed^{-1}$) followed by soil amendment (10^8 cfu ml^{-1}) 5 DAS.

Sclerotium rolfsii was inoculated 14 DAS by placing an agar disc with freshly germinated sclerotium along with actively growing mycelium at the stem base and covered with coarse sand. The pots were watered daily to maintain the sand moisture. Photographs were taken 15 DAI.

GSE 3 was least effective (10.4%). The identified biocontrol isolates were also antifungal to *S. rolfsii* in *in vitro* dual culture assay. There was a positive correlation (correlation coefficient of 0.99) between the two mean disease rating and percentage mortality in different treatments.

3.5. **Biocontrol of crown rot disease of groundnut**

3.5.1. Greenhouse screening: Three hundred and ninety three bacterial isolates, applied as seed treatment were tested for control of crown rot disease of groundnut. Bacterized seeds were planted in *A. niger*-infested potting mixture and observed for pre-emergence rotting and post-emergence wilting. In CMC treated control, 45.8% of the seeds were infected by *A. niger* and rotted before emergence, and of the emerged seedlings 38.7% observed wilted 20 DAS (Table 10). Eleven bacterial isolates, including three from rhizosphere (GRS 86, *Achromobacterxylosoxidans* GRS 149 and *Pseudomonas* sp. GRS 175), three from phylloplane (GPS 9, *P. aeruginosa* GPS 21 and *B. megaterium* GPS 55) and five seed endophytes (GSE 6, *P. aeruginosa* GSE 18, *P. aeruginosa* GSE 19, GSE 23 and *P. aeruginosa* GSE 30) were significantly ($P = 0.01$) effective against pre-emergence *A. niger* infection. Among the 11 biocontrol isolates, four isolates GPS 55, GRS 149, GSE 6 and GSE 23, remained insignificant in control of post-emergence wilting. Two effective biocontrol isolates, *P. aeruginosa* GSE 18 and GSE 19, reduced the pre-emergence infection by 63.5% and 54.4%, and post-emergence infection by 65.6% and 59.9%, respectively compared to control. Two bacterial isolates, GRS 149 and GPS 55 though were not antagonistic to *A. niger* in dual culture tests, significantly ($P = 0.01$) reduced the pre-emergence infection by 40.8% and 31.6%.

3.5.2. Effect of *P. aeruginosa* GSE 18 on the activity of defense-related enzymes during crown rot infection: The effect of *P. aeruginosa* GSE 18, applied as seed treatment, on the activation of defense mechanisms in groundnut, in presence of *A. niger* was determined. Specific activities of chitinase, glucanase, peroxidase and PAL were significantly higher ($P = 0.01$) in GSE 18 treated seedlings compared to control, up to the

Table 10. Biological control of crown rot disease of groundnut by selected bacterial isolates in greenhouse.

S. No.	Isolate[a]	Pre-emergence rotting (%)	Post-emergence wilting (%)
1	GPS 9	25.0±7.9	24.8±7.9
2	*Pseudomonas aeruginosa* GPS 21	22.9 ±9.4	21.6±7.6
3	*Bacillus megaterium* GPS 55	31.3±6.9	30.6±10.0
4	GRS 86	25.0±7.9	22.2±13.6
5	*Achromobacterxylosoxidans* GRS 149	27.1 ±9.4	26.8±12.3
6	*Pseudomonas* sp. GRS 175	22.9±12.3	16.6±9.5
7	GSE 6	25.0±11.2	25.5±10.2
8	*P. aeruginosa* GSE 18	16.7±6.5	13.1112.4
9	*P. aeruginosa* GSE 19	20.8 ±6.5	15.9110.6
10	GSE 23	29.2 ±6.5	26.7±10.1
11	*P. aeruginosa* GSE 30	22.9 ±9.4	21.617.6
12	Control	45.8±6.5	38.3±11.3
	LSD ($P = 0.01$)	13.47	15.28

Three hundred and ninety three bacterial isolates were applied as seed treatment (10^6-10^7 cfu seed^{-1}) using groundnut cv. TMV 2. *Aspergillus niger* multiplied on autoclaved sorghum grain for 8 days was used as inoculum and mixed with the potting mixture consisting of red alfisol, farm yard manure and sand (2:1:2). Bacterized seeds were sown in the pathogen infested potting mixture and observed for pre-emergence rotting 7 DAS and post-emergence wilting 20 DAS. The experiment was conducted in four replications, with ten seeds in each replication, repeated once and mean values of best eleven treatments were presented.

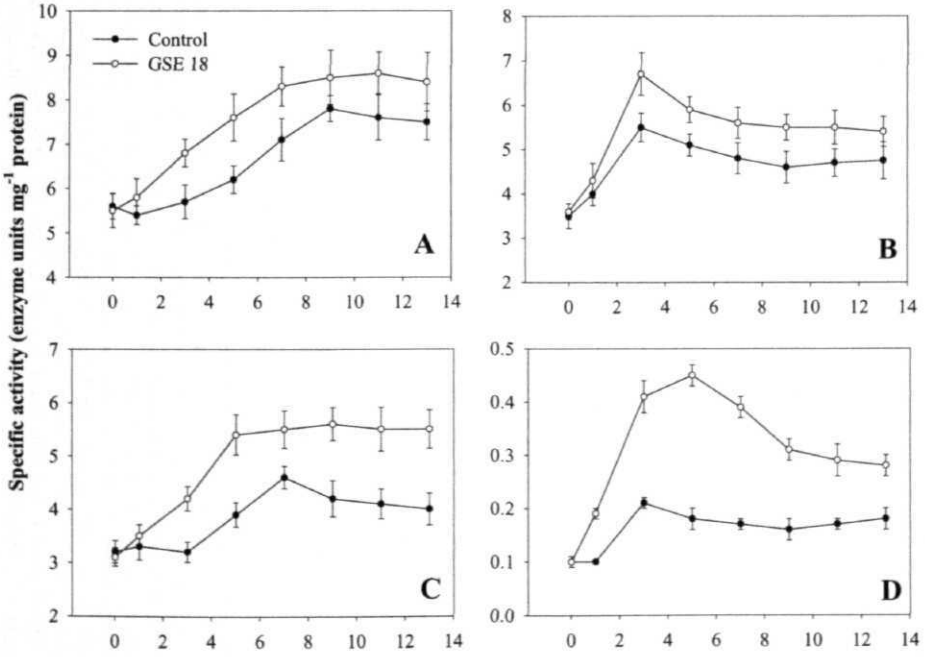
measured 13 DAS (Fig. 8). Of the four enzymes assayed, PAL activity was more prominent in GSE 18 treated seedlings followed by peroxidase.

3.6. Biocontrol of LLS disease of groundnut

3.6.1. Selection of effective biocontrol isolates: Fifteen bacterial isolates, two from geocarposphere, two from phylloplane, seven from rhizosphere and four seed endophytes, which inhibited the *in vitro* conidial germination of *P. personata* by >90% in repeated tests, were selected and evaluated for control of LLS in greenhouse. The bacteria (10^8 cfu ml^{-1}) were applied as a foliar spray 24 h before the pathogen inoculation. Frequency of LLS lesions (number of lesions cm^{-2} leaf area) was minimum in plants treated with *P. aeruginosa* GSE 18 followed by GSE 19 (Table 11 and Plate Fig. 10). In these two treatments, the decrease in lesion frequency at 15 DAI was 69.9% (1.06 lesions cm^{-2} leaf area) and 67.2% (1.15 lesions cm^{-2} leaf area) compared to control (3.51 lesions cm^{-2} leaf area). There was no defoliation of leaflets compared to 100% defoliation in control 30 DAI and the disease severity (measured on a 1-9 rating scale) was 3.9 and 4.0 in GSE 18 and GSE 19 treated plants compared to 9.0 in control.

3.6.2. Integrated use of biocontrol bacteria and chlorothalonil: *Pseudomonas aeruginosa* GSE 18 and GSE 19 effective in control of LLS were tolerant to chlorothalonil (2 mg ml^{-1}) in the growth medium (Fig. 9). Hence, a combination of either of the two isolates and reduced concentrations (compared to recommended dose of application) of chlorothalonil were tested for LLS control. The antagonists (10^8 cells ml^{-1}) were applied as a foliar spray in combination with chlorothalonil at concentrations ranging from 0.01 to 2 mg ml^{-1}. In plants treated with chlorothalonil @ $\geq$0.25 mg ml^{-1}, the development of LLS lesions decreased significantly (Table 12). Reduced concentrations of chlorothalonil @ 100 $\mu g\ ml^{-1}$ applied in combination with either of the two bacterial isolates, significantly reduced LLS severity compared to either bacterial isolate or the same concentration of chlorothalonil applied alone. A combination of GSE 18 and chlorothalonil @ 10 $\mu g\ ml^{-1}$ was effective than chlorothalonil alone @ 100 $\mu g\ ml^{-1}$. The reduction in lesion frequency

Fig. 8. Effect of a broad spectrum antifungal isolate *Pseudomonas aeruginosa* GSE 18 on the activities of defense-related enzymes, (A) Chitinase, (B) Glucanase, (C) Peroxidase, and (D) Phenylalanine ammonia lyase, in groundnut during crown rot infection.



Pseudomonas aeruginosa GSE 18 was applied as a seed treatment (~4×10^6 cfu seed^{-1}) in groundnut cv. TMV 2. Bacterized seeds were planted in potting mixture infested with *Aspergillus niger* multiplied on autoclaved sorghum grains. Seeds treated with 0.5% (w/v) CMC were treated as control. In both the experiments seeds/seedlings were uprooted at frequent intervals of time and the specific activities of each enzyme were determined.

Table 11. Biological control of late leaf spot disease of groundnut by selected bacterial isolates in greenhouse.

S.No.	Isolate	Lesion frequency	Defoliation (%)	Disease score
1	GGS 1	2.48±0.29	78.1±5.8	7.610.4
2	GGS 11	1.88±0.19	63.517.2	6.410.6
3	GPS 15	2.27±0.29	80.915.5	8.0±0.2
4	*Pseudomonas aeruginosa* GPS 21	1.9810.20	74.316.0	7.610.5
5	GRS 7	1.9710.27	75.016.4	7.410.4
6	GRS 70	2.44±0.34	85.4±6.1	8.010.2
7	GRS 94	1.7610.22	53.8±7.9	5.810.4
8	GRS 100	1.6510.34	53.4±8.7	5.610.4
9	GRS 134	1.9210.22	54.919.7	5.610.5
10	*Pseudomonas* sp. GRS 175	2.0810.23	68.1±6.7	7.3±0.6
11	GRS 225	3.2110.30	96.2±5.3	8.610.4
12	GSE 3	3.2410.24	95.815.3	8.810.3
13	*P. aeruginosa* GSE 18	1.0610.16	0.0	3.910.4
14	*P. aeruginosa* GSE 19	1.1510.16	0.0	4.010.2
15	*P. aeruginosa* GSE 30	2.3610.34	78.815.4	7.910.4
16	Control	3.5110.26	100.010.0	9.010.0
	LSD (P = 0.01)	0.37	5.44	0.45

Fifteen bacterial isolates that significantly inhibited *Phaeoisariopsis personata* conidial germination *in vitro* by >90% were tested in greenhouse for LLS control. Bacterial cells from a log phase culture were pelleted and resuspended in 10 mM phosphate buffer (pH 7.0) at a concentration of 10^8 cfu ml^{-1}. The cell suspension was applied as a foliar spray 24 h before the pathogen inoculation. Severity of LLS in all the treatments was measured based on (A) lesion frequency - number of lesions cm^{-2} leaf area, measured 15 DAI, (B) percentage defoliation - calculated based on the number of leaf lets defoliated, measured 30 DAI, and (C) Disease score based on a 1-9 rating scale (1 = no disease and 9 = maximum disease). Lesion frequency and percentage defoliation were measured on third or fourth leaf from the top tagged before the pathogen inoculation, and disease score was rated separately for each plant. The experiment was conducted thrice and the values are the mean of nine replications in three different experiments.

Plate Fig. 10. Biological control of groundnut late leaf spot in greenhouse using mid-log phase cells of (A) *Pseudomonas aeruginosa* GSE 18, and (B) *P. aeruginosa* GSE 19 (10^8 cfu ml^{-1}).

Bacterial cells were applied as a foliar spray 24 h before the pathogen inoculation. Severity of late leaf spot in different treatments was photographed 20 DAI.

Fig. 9. *In vitro* evaluation of sensitivity of *Pseudomonas aeruginosa* GSE 18 and GSE 19 to chlorothalonil (kavach®). (A) Growth of GSE 18, and (B) GSE 19 in chlorothalonil added LB broth, measured as optical density at 600 nm. (C) Growth of GSE 18 and (D) GSE 19 in LB agar added with chlorothalonil (2 mg ml^{-1}) and photographed 72 HAI.

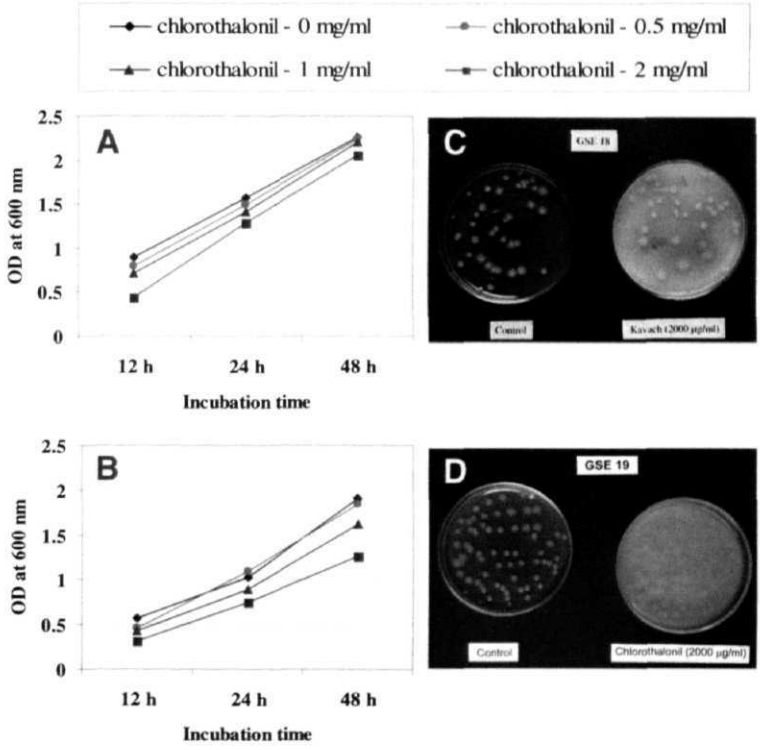


Table 12. Evaluation of *Pseudomonas aeruginosa* GSE 18 and GSE 19 in combination with reduced concentrations of chlorothalonil for control of late leaf spot of groundnut in greenhouse.

S. No.	Isolate	Chlorothalonil (mg ml^{-1})	Lesion frequency	Defoliation (%)	Disease score
1	GSE 18	0.00	1.11±0.14	10.116.5	3.910.5
2	GSE 18	0.01	0.85±0.12	9.416.5	3.7±0.3
3	GSE 18	0.05	0.29±0.06	0.010.0	3.1±0.2
4	GSE 18	0.10	0.04±0.10	0.010.0	2.0±0.0
5	GSE 18	0.25	0.01±0.05	0.0±0.0	2.010.0
6	GSE 19	0.00	1.24±0.15	11.5±7.2	4.110.4
7	GSE 19	0.01	1.1910.12	11.1±6.6	4.0±0.2
8	GSE 19	0.05	0.8110.11	7.3±6.9	3.510.4
9	GSE 19	0.10	0.1310.11	0.010.0	2.010.0
10	GSE 19	0.25	0.0810.05	0.010.0	2.010.0
11	Control	0.00	3.6010.17	100.010.0	9.010.0
12	Control	0.01	3.4310.13	100.0±0.0	9.010.0
13	Control	0.05	2.34±0.27	95.117.3	8.710.4
14	Control	0.10	0.9110.15	26.016.9	5.110.7
15	Control	0.25	0.1510.21	0.010.0	3.010.2
	LSD($P = 0.01$)		0.13	3.24	0.21

The two bacterial isolates had an *in vitro* compatibility with chlorothalonil (Kavach®). Resuspended mid log phase cells of the bacteria (10^8 cells ml^{-1}) along with chlorothalonil were applied as a foliar spray 24 h before the pathogen inoculation. Severity of LLS in all the treatments was measured based on (A) lesion frequency - number of lesions cm^{-2} leaf area, measured 15 DAI, (B) percentage defoliation - calculated based on the number of leaf lets defoliated, measured 30 DAI, and (C) Disease score based on a 1-9 rating scale (1 = no disease and 9 = maximum disease). Lesion frequency and percentage defoliation were measured on the leaves tagged before the pathogen inoculation, and disease score was rated separately for each plant. The values are the mean of nine replications in three sets of experiments.

in these two treatments was 75.2% and 74.7%, respectively. In all the treatments, the reductions in lesion frequency corresponded with that of percentage defoliation and disease score on a 1-9 rating scale.

3.6.3. Effect of chitinolytic bacteria for LLS control: Chitin-supplemented foliar application of two chitinolytic isolates, *S. marcescens* GPS 5 and *B. circulans* GRS 243 was tested for improved biocontrol of LLS in greenhouse and compared with the chitin-supplemented application of non-chitinolytic isolates *P. aeruginosa* GSE 18 and GSE 19. GPS 5 and GRS 243 were selected based on their ability to inhibit *in vitro* germination of *P. personata* conidia by >80%.

Foliar application of GPS 5 and GRS 243 reduced the lesion frequency by 20.5% and 23.7%, compared to phosphate buffer control. The reduction in lesion frequency did not affect the defoliation and the overall disease severity at 30 DAI, compared to control (Table 13). Supplementation with 1% (w/v) colloidal chitin improved the biocontrol efficacy of both the chitinolytic isolates. The reductions in lesion frequency was >65% (Plate Fig. 11).

Further, the effect of different concentrations of colloidal chitin used as a foliar amendment on the biocontrol efficacy of GPS 5 and GRS 243 was studied by varying the concentration of colloidal chitin from 0.2% to 1.4% (w/v). With an increase of colloidal chitin concentration up to 1.0% (w/v) the decrease in lesion frequency, defoliation and disease score was significant ($P = 0.01$) (Table 14).

3.6.4. Effect of CCF **of antagonistic and chitinolytic bacteria on the severity of LLS:** The CCF of P. *aeruginosa* GSE 18 and GSE 19 grown in LB broth for 48 h, and *S. marcescens* GPS 5 and *B. circulans* GRS 243 grown in MM broth with colloidal chitin as sole carbon source for 6 days were evaluated for their suppressive effects on LLS development in greenhouse. CCF of the four test isolates reduced the lesion frequency between 46.4% to 58.4% and percentage defoliation from 40.4% to 63.2%, compared to control (Table 15). Among all the treatments, CCF of GPS 5 was most effective in

Table 13. Effect of colloidal chitin supplementation on the biocontrol efficacy of chitinolytic bacterial isolates *Serratia marcescens* GPS 5 and *Bacillus circulans* GRS 243 in comparison to non-chitinolytic isolates *Pseudomonas aeruginosa* GSE 18 and GSE 19, against late leaf spot disease in groundnut.

S.No.	**Treatment**	**Lesion frequency**	Defoliation (%)	Disease score
1	*P. aeruginosa* GSE 18	1.00±0.10	0.0	3.810.4
2	*P. aeruginosa* GSE 18 + CC	0.99±0.12	0.0	3.810.3
3	*P. aeruginosa* GSE 19	1.09±0.11	0.0	3.910.4
4	*P. aeruginosa* GSE 19 + CC	1.06±0.12	0.0	3.810.3
5	*S. marcescens* GPS 5	2.68±0.27	95.8±7.3	8.610.4
6	*S. marcescens* GPS 5 + CC	1.17±0.17	0.0	4.1±0.4
7	*B. circulans* GRS 243	2.57±0.29	96.2± 5.3	8.810.3
x	*B. circulans* GRS 243 + CC	1.09±0.13	0.0	4.010.4
9	Colloidal chitin	3.31±0.31	97.217.2	9.010.0
10	Control	3.3710.30	99.3±2.0	9.010.0
	LSD (P = 0.01)	0.23	5.88	0.29

The foliar application of the two chitinolytic bacterial isolates GPS 5 and GRS 243 was supplemented with 1% (w/v) colloidal chitin (CC) and tested for control of LLS. The efficacy of chitin supplementation of chitinolytic isolates was compared with the chitin supplemented application of two non-chitinolytic isolates GSE 18 and GSE 19. Severity of LLS in all the treatments was measured based on (A) lesion frequency - number of lesions cm^{-2} leaf area, measured 15 DAI, (B) percentage defoliation - calculated based on the number of leaf lets defoliated, measured 30 DAI, and (C) Disease score based on a 1-9 rating scale (1 = no disease and 9 = maximum disease). Lesion frequency and percentage defoliation were measured on the leaves tagged before the pathogen inoculation, and disease score was rated separately for each plant. Data presented was the mean of nine replications in three sets of experiments.

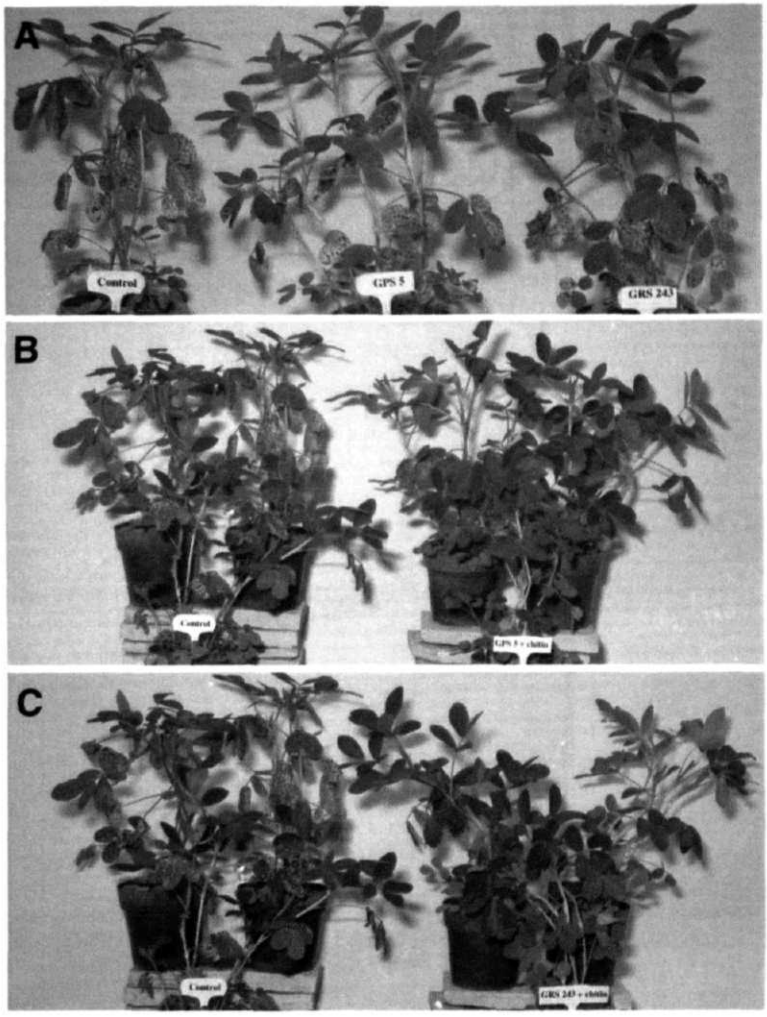


Plate Fig. 11. Effect of chitin supplementation on the biocontrol efficacy of chitinolytic *Serratia marcescens* GPS 5 and *Bacillus circulans* GRS 243.
(A) Foliar application of GRS 243 and GPS 5 (10^8 cfu ml^{-1}) resuspended in 10 mM phosphate buffer, pH 7.0; Foliar application of resuspended cells of (B) GPS 5, and (C) GRS 243 in supplementation with 1% (w/v) colloidal chitin. The photographs represent the disease severity in different treatments 30 DAI.

Table 14. Biocontrol efficacy of two chitinolytic bacterial isolates, *Serratia marcescens* GPS 5 and *Bacillus circulans* GRS 243, with different concentrations of colloidal chitin against late leaf spot disease of groundnut.

S. No.	Isolate	Colloidal chitin (% w/v)	Lesion frequency	Defoliation (%)	Disease score
1	*S. marcescens* GPS 5	0.0	2.95±0.17	87.5112.3	8.010.3
:	5. *marcescens* GPS 5	0.:	2.73±0.34	84.0±11.6	7.8±0.5
3	*S. marcescens* GPS 5	0.4	2.30±0.41	77.7113.1	7.410.5
4	*S. marcescens* GPS 5	().(	1.41±0.21	34.3±7.7	5.810.6
S	*S. marcescens* GPS 5	0.8	1.19±0.17	5.314.6	4.410.6
6	*S. marcescens* GPS 5	1.0	1.08±0.13	0.0	3.8±0.5
7	*B. circulans* GRS 243	0.0	2.86±0.39	84.717.8	8.010.5
8	*B. circulans* GRS 243	0.:	2.79±0.29	79.918.5	7.610.4
9	*B. circulans* GRS 243	0.4	2.50±0.30	70.119.7	7.210.5
10	*B. circulans* GRS 243	0.6	1.9210.15	59.0±9.9	6.610.6
11	*B. circulans* GRS 243	0.8	1.2110.14	13.514.4	5.010.5
12	*B. circulans* GRS 243	1.0	1.0910.12	0.0	4.210.6
[3	Control	0.0	3.60±0.51	100.0±0.0	9.010.0
14	Control	0.2	3.6410.40	99.711.5	9.010.0
15	Control	0.4	3.5810.37	100.010.0	9.010.0
[6	Control	0.6	3.55±0.42	99.711.5	9.010.0
17	Control	0.8	3.5110.31	99.013.2	9.010.0
18	Control	1.0	3.4510.43	99.711.5	9.0±0.0
	LSD (P =0.01)		0.33	5.86	0.33

Foliar application of the two chitinolytic isolates (10^8 cfu ml^{-1}) was supplemented with different concentrations of colloidal chitin (0.2-1.4%) and tested for control of late leaf spot disease. Severity of LLS in all the treatments was measured based on (A) lesion frequency - number of lesions cm^{-2} leaf area, measured 15 DAI, (B) percentage defoliation - calculated based on the number of leaf lets defoliated, measured 30 DAI, and (C) Disease score based on a 1-9 rating scale (1 = no disease and 9 = maximum disease). Lesion frequency and percentage defoliation were measured on the leaves tagged before the pathogen inoculation, and disease score was rated separately for each plant. The experiment was repeated thrice and the mean values of nine replications were presented in the table. The data for treatments with 1.2% and 1.4% colloidal chitin is not shown.

Table 15. Effect of cell free culture filtrates of broad-spectrum antifungal *(Pseudomonas aeruginosa* GSE 18 and GSE 19), and chitinolytic *(Bacillus circulans* GRS 243 and *Serratia marcescens* GPS 5) bacterial isolates, on the severity of late leaf spot of groundnut in greenhouse.

S.No.	Source of culture filtrate	Lesion frequency	Defoliation (%)	Disease score
1	*P. aeruginosa* GSE 18	1.98±0.48	56.6± 8.5	7.010.4
2	*P. aeruginosa* GSE 19	2.06±0.44	60.3±11.2	7.310.9
3	*B. circulans* GRS 243	1.71±0.27	39.2±7.4	6.5±0.5
4	*S. marcescens* GPS 5	1.60±0.25	34.717.2	6.210.5
5	Control	3.85±0.41	94.417.1	8.910.2
	LSD (*P* = 0.01)	0.32	7.54	0.48

Culture filtrates of *P. aeruginosa* GSE 18 and GSE 19 were obtained from stationary phase (48 h) cultures grown in LB broth. Culture filtrates of *S. marcescens* GPS 5 and *B. circulans* GRS 243 were obtained from cultures grown for 144 h in minimal medium with colloidal chitin as a sole carbon source. The culture filtrates were filter sterilized and applied as a foliar spray 24 h before *P. personata* inoculation. Severity of LLS in all the treatments was measured based on (A) lesion frequency - number of lesions cm^{-2} leaf area, measured 15 DAI, (B) percentage defoliation - calculated based on the number of leaf lets defoliated, measured 30 DAI, and (C) Disease score based on a 1-9 rating scale (1 = no disease and 9 = maximum disease), recorded 30 DAI. Lesion frequency and percentage defoliation were measured on the leaves tagged before the pathogen inoculation, and disease score was rated separately for each plant. The experiment was repeated thrice and the mean values of nine replications were presented in the table.

reducing LLS severity, where the lesion frequency and defoliation decreased by 58.4% and 63.2%, compared to control and disease score at 30 DAI was 6.2 against 8.9 in control.

3.6.5. Evaluation of formulations of broad-spectrum antagonistic or chitinolytic isolates for LLS control: *Pseudomonas aeruginosa* GSE 18 and GSE 19 did not survive in peat up to 180 days (Fig. 6 B). The initial populations of GSE 18 and GSE 19 in peat were $\log_{10}$ 7.4 and 7.2 cfu g^{-1} peat. At 90 DAI and 120 DAI, the populations of these two bacteria were logio 6.5 and 4.7 cfu g^{-1}, and $\log_{10}$ 5.8 and 2.8 cfu g', respectively.

The two chitinolytic isolates *S. marcescens* GPS 5 and *B. circulans* GRS 243 differed in their survival in peat (Fig. 6 C). GRS 243 had a good shelf life (logio 7.4 cfu g^{-1}) up to 180 DAI, whereas GPS 5 maintained a population of logic 6.1 cfu g'" at 60 DAI, and its populations were not detected after 120 DAI. Chitin-supplementation positively affected the survival of both the isolates. From 20 DAI, significant differences were observed between the mean populations of the two isolates formulated in peat and chitin-supplemented peat, respectively. Populations of GRS 243 increased from logio 8.0 cfu g^{-1} to logio 9.0 cfu g^{-1}. Similarly, there was an increase in the populations of GPS 5 from logio 7.1 cfu g^{-1} to logio 9.1 cfu g^{-1}.

Ninety-day-old formulations of different bacterial isolates were suspended in 10 mM phosphate buffer, pH 7.0 (10% w/v) and the filtrate was used as a foliar spray at 24 h before the pathogen inoculation. Chitin-supplementation of the peat formulations of 5. *marcescens* GPS 5 and *B. circulans* GRS 243 resulted in reduction of lesion frequency by 52.2% and 44.3% compared to their peat formulations and proved better than the application of mid-log phase cells of the two cultures, respectively (Table 16). Peat formulations of GSE 18 and GSE 19 reduced the lesion frequency by 72.2% and 71.4% in comparison to control. The effect of different treatments on leaf defoliation and the overall disease severity were similar to lesion frequency.

3.6.6. Effect of biotic and abiotic elicitors on the development of LLS: Four elicitors, chitosan, salicylate, methyl jasmonate and heat killed conidia of *P. personata* applied at different time intervals before the pathogen inoculation and also as seed treatment were

Table 16. Evaluation of different formulations of antifungal *(Pseudomonas aeruginosa* GSE 18 and GSE 19) and chitinolytic *(Bacillus circulans* GRS 243 and *Serratia marcescens* GPS 5) bacterial isolates for control of late leaf spot disease in groundnut.

S.No.	Isolate	Treatment	Lesion frequency	Defoliation (%)	Disease score
1	GSE 18	Mid-log phase cells	1.08±0.09	0.0±.0.0	3.810.2
2	GSE 18	Peat formulation	1.59±0.11	26.0±8.3	5.510.3
3	GSE 19	Mid-log phase cells	1.11±0.07	0.0±0.0	4.010.3
4	GSE 19	Peat formulation	3.09±0.28	95.815.6	8.6±0.3
5	GRS 243	Mid-log phase cells	2.81±0.31	100.010.0	9.010.0
6	GRS 243	Peat formulation	2.93±0.27	100.010.0	9.010.0
7	GRS 243	Chitin-supplemented peat formulation	1.63±0.12	46.6±6.3	6.0±0.4
7	GPS 5	Mid-log phase cells	2.73±0.23	98.3±3.6	8.810.3
8	GPS 5	Peat formulation	3.71±0.18	100.010.0	9.010.0
9	GPS 5	Chitin-supplemented peat formulation	1.78±0.12	53.8±7.8	6.210.4
10	Control		3.89±0.24	100.010.0	9.010.0
	LSD (P =0.01)		0.44	11.91	0.67

In all the formulation treatments, 90-day-old formulations were stirred in 10 mM phosphate buffer, pH 7.0 (10% w/v) and filtered. The filtrate was used as a foliar spray at 24 h before the pathogen inoculation. Severity of LLS in all the treatments was measured based on (A) lesion frequency - number of lesions cm^{-2} leaf area, measured 15 DAI, (B) percentage defoliation - calculated based on the number of leaf lets defoliated, measured 30 DAI, and (C) Disease score based on a 1-9 rating scale (I = no disease and 9 = maximum disease). Lesion frequency and percentage defoliation were measured on the leaves tagged before the pathogen inoculation, and disease score was rated separately for each plant. The experiment was repeated twice with three replications and the mean of nine replications were given in the table.

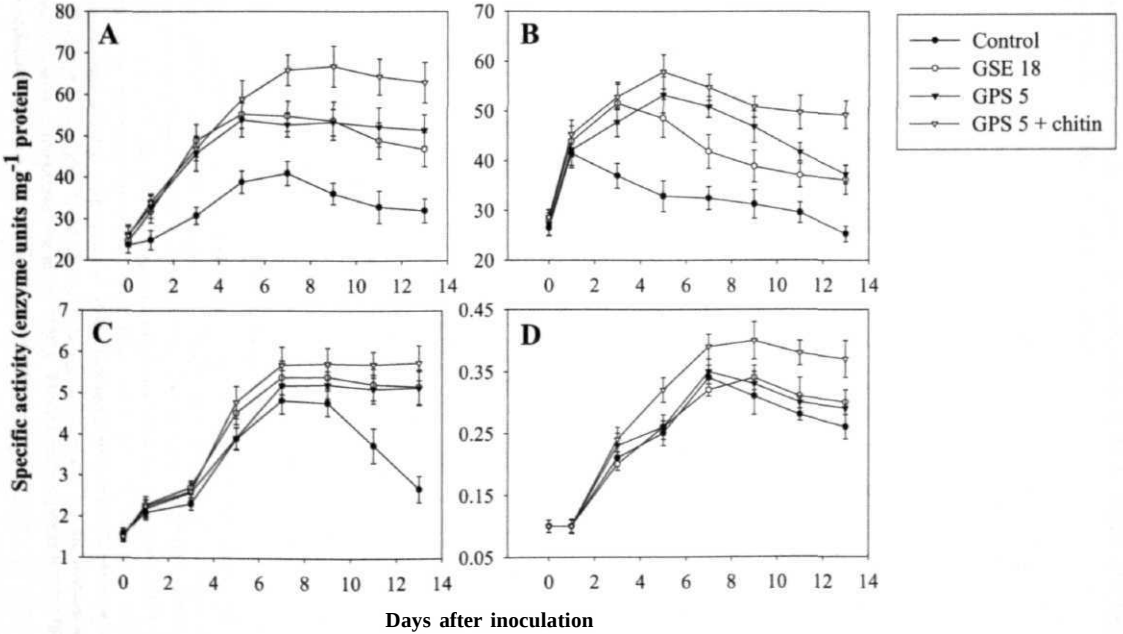
tested for their effect on LLS development in greenhouse. The severity of LLS measured as lesion frequency, defoliation and disease score on a 1-9 rating scale was not significantly ($P = 0.01$) different compared to control (data not shown).

3.6.7. **Induction** of host resistance in groundnut by selected biocontrol bacterial isolates: *Pseudomonas aeruginosa* GSE 18, and 5. *marcescens* GPS 5 along with colloidal chitin were applied as a foliar spray on 30-day-old groundnut plants (cv. TMV 2) and their effects on the defense-related enzymes of groundnut, during LLS infection were determined. The specific activity of chitinase was significantly higher ($P = 0.01$) in GPS 5 and GSE 18 treated plants compared to control, from 1 to 13 DAI (Fig. 10). Chitin-supplemented spray of GPS 5 significantly increased the chitinase activity from 7 to 13 DAI, compared to the GPS 5 spray. Chitin-supplemented spray of GPS 5 was also effective in induction of glucanase, peroxidase and PAL. The specific activities of peroxidase and PAL were almost similar in GPS 5 and GSE 18 treatments, whereas the enhanced levels of glucanase activity continued for prolonged period in GPS 5 treatment compared to GSE 18.

3.6.8. Field evaluation of the biocontrol isolates: Fungicide tolerant bacterial isolates *P. aeruginosa* GSE 18 and GSE 19 in combination with reduced concentrations of chlorothalonil, and chitinolytic isolates *S. marcescens* GPS 5 and *B. circulans* GRS 243 in supplementation with colloidal chitin were evaluated for control of LLS in field. The effect of combined application of bacteria and chlorothalonil or chitin on the severity of LLS was compared with the application of bacterial isolates alone. All the treatments were applied as a foliar spray at 45, 60, 75 and 90 DAS. The experimental field has been regularly used for groundnut foliar disease screening before this experiment. Additionally, the infector rows were inoculated with a conidial suspension of *P. personala* at 40 DAS to ensure uniform disease development.

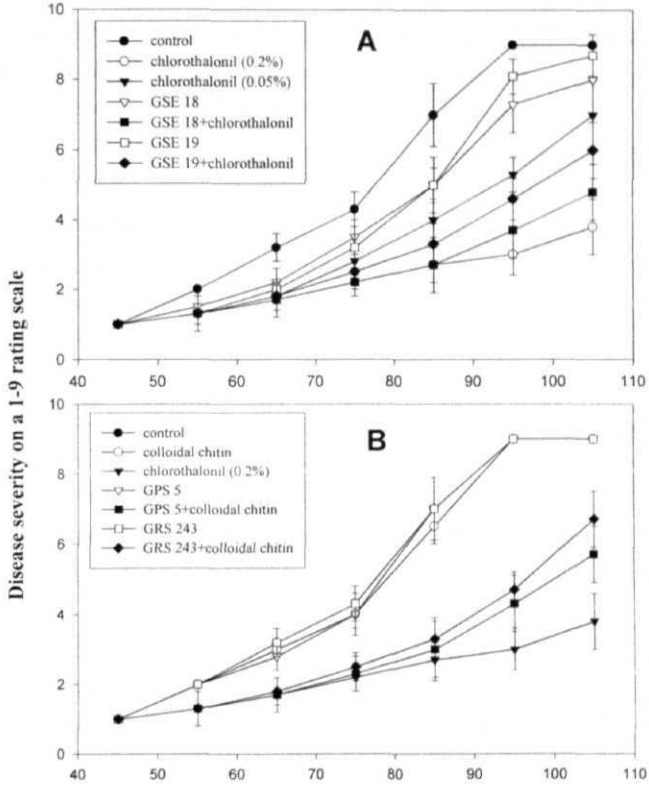
Bacterial isolates GSE 18 and GSE 19 significantly ($P = 0.01$) reduced LLS severity up to 95 and 85 DAS, respectively, and thereafter were insignificant from control (Fig. 11 A). Chlorothalonil (0.2%) was highly effective in control of LLS and the disease

Fig. 10. Effect of broad-spectrum antifungal isolate *Pseudomonas aeruginosa* GSE 18 and chitinolytic isolate *Serratia marcescens* GPS 5, applied in supplementation with 1% (w/v) colloidal chitin on the activities of defense-related enzymes, (A) Chitinase, (B) Glucanase, (C) Peroxidase, and (D) Phenylalanine ammonia lyase, in groundnut during LLS infection.



Mid-log phase cells resuspended in 10 mM phosphate buffer, pH 7.0 (10^8 cells ml^{-1}) were applied as a foliar spray on 30-day-old groundnut plants of cv. TMV 2. In control treatment, plants were sprayed with phosphate buffer alone. Plants in all the treatments were inoculated with *Phaeoisariopsis personata* (20,000 conidia ml^{-1}) after 24 h. Third or forth leaves in each treatment were harvested at regular intervals and used to determine the specific activities of different defense-related enzymes.

Fig. 11. Field evaluation of (A) Fungicide tolerant, and (B) Chitinolytic bacterial isolates for control of late leaf spot of groundnut.



Fungicide tolerant isolates *Pseudomonas aeruginosa* GSE 18 and GSE 19 in supplementation with chlorothalonil @ 0.05%, and chitinolytic isolates *Serratia marcescens* GPS 5 and *Bacillus circulans* GRS 243 in supplementation with colloidal chitin (1% w/v) (10^8 cfu ml^{-1}) were applied as a foliar spray 45, 60, 75 and 90 DAS. The infector rows planted after every 4 test rows were infested with crop debris and inoculated with conidial suspension of *Phaeoisariopsis personata*. Disease severity in each treatment was scored based on a 1-9 rating scale, where 1 • no disease and 9 = maximum disease (Subrahmanyam *et al*., 1995). Disease scores presented arc the mean of six replications from a repeated field experiment.

Note: In colloidal chitin treatment, the disease severity was similar to control.

severity was 3.8 on a 1-9 rating scale at 105 DAS. Chlorothalonil, at a reduced concentration (0.05%), was moderately effective against LLS and the disease severity was 5.3 and 7.0 at 95 and 105 DAS. The combined application of GSE 18 and chlorothalonil (0.05%) was highly effective than the individual treatments, GSE 18 or chlorothalonil (0.05%) (Plate Fig. 12). This treatment was comparable to the application of recommended dose of chlorothalonil (0.2%). Combined application of GSE 19 and chlorothalonil (0.05%) also was significantly effective than either GSE 19 or chlorothalonil (0.05%) applied alone (Fig. 11 A), but was less effective than the combined application of GSE 18 and chlorothalonil.

Chitinolytic bacterial isolates *S. marcescens* GPS 5 and *B. circulans* GRS 243 did not affect the development of LLS at any of the growth stages. Chitin-supplemcnted application of these two isolates resulted in improved control of LLS development (Fig. 11 B). Disease severity measured on a 1-9 rating scale at 95 DAS was 4.3 and 4.7 in treatments with chitin-supplemented application of GPS 5 and GRS 243, respectively.

Highest pod yield (1.21 t ha^{-1}) was recorded in chlorothalonil (0.2%) treatment (Table 17). When chlorothalonil was applied at a reduced concentration i.e. 0.05%, pod yield was 0.8 t ha^{-1} (45.5% higher than control). Bacterial isolates, applied alone, had no significant effect on the pod yield. Combined application of GSE 18 and chlorothalonil (0.05%) had a pod yield (1.05 t ha^{-1}) which was 96.3% higher than control. Combined application of GSE 19 and chlorothalonil (0.05%) resulted in 65.5% increase in pod yield (0.91 t ha^{-1}). Foliar application of GPS 5 in supplementation with colloidal chitin resulted in 75.0% increase in pod yield (0.96 t ha^{-1}) followed by 61.8% by the application of GRS 243 and chitin.

3.7. Survival of bacterial isolates in the rhizoplane or phylloplane

Three selected plant growth promoting isolates *B. firmis* GRS 123, *B. megaterium* GPS 55 and *P. aeruginosa* GPS 21 in field, and two biocontrol isolates *P. aeruginosa* GSE 18 and GSE 19 in greenhouse were tested for their colonization of groundnut rhizosphere using rifampicin resistance as a marker. The phylloplane survival of GSE 18 and GSE 19

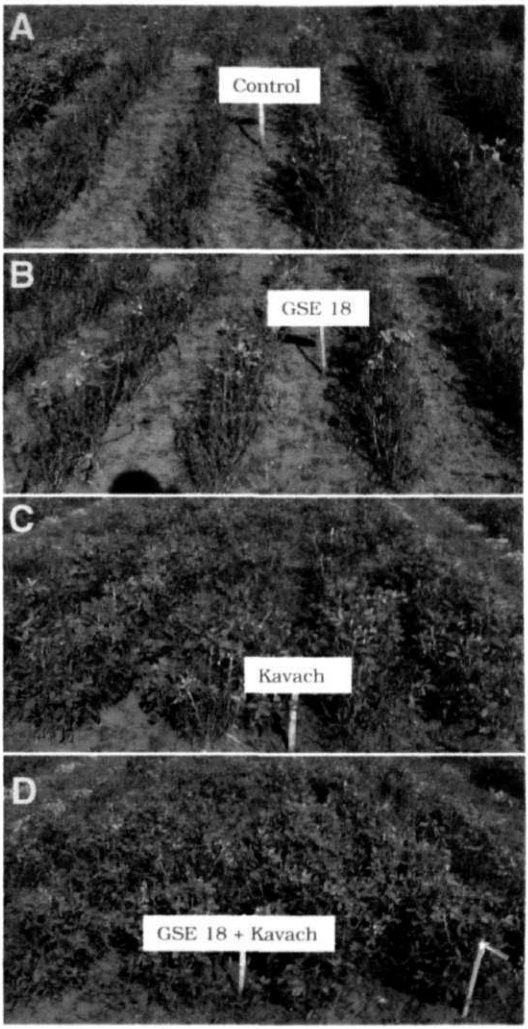


Plate Fig. 12. Field evaluation of *Pseudomonas aeruginosa* GSE 18 for control of late leaf spot disease of groundnut.
(A) Control, (B) GSE 18 (10^8 cfu ml^{-1}), (C) Chlorothalonil (kavach®) @ 0.5 g lit^{-1}, and (D) GSE 18 (10^8 cfu ml^{-1}) and chlorothalonil @ 0.5 g lit^{-1}. Mid-log phase cells were resuspended (10^* cells ml^{-1}) in phosphate buffer, pH 7.0 and used as a foliar spray 45, 60, 75 and 90 DAS. The disease severity was measured on a 1-9 rating scale at regular time intervals and photographed 105 DAS.

Table 17. Effect of fungicide tolerant (*Pseudomonas aeruginosa* GSE 18 and GSE 19) and chitinolytic *(Bacillus circulans* GRS 243 and *Serratia marcescens* GPS 5) bacterial isolates, tested for control of late leaf spot in field, on the pod yield at harvest.

S. No.	Treatment	Pod yield (t ha^{-1})
1	*P. aeruginosa GSE* 18	0.69±0.05
2	*P. aeruginosa* GSE 19	0.64±0.04
3	*S. marcescens* GPS 5	0.53±0.05
4	*B. circulans* GRS 243	0.55±0.03
5	*P. aeruginosa* GSE 18 + chlorothalonil (0.05% w/v))	1.08±0.07
6	*P. aeruginosa* GSE 19 + chlorothalonil (0.05% w/v)	0.91 ±0.08
7	5. *marcescens* GPS 5 + colloidal chitin (1% w/v)	0.96±0.06
8	*B. circulans* GRS 243 + colloidal chitin (1% w/v)	0.89±0.07
9	Colloidal chitin (1% w/v)	0.57±0.04
10	Chlorothalonil (0.05% w/v)	0.80±0.07
11	Chlorothalonil (0.2% w/v)	1.21±0.06
12	Control	0.55±0.03
	LSD (P = 0.01)	0.16

Bacterial cells from mid-log phase cultures were resuspended in phosphate buffer, pH 7.0 and applied as a foliar spray in supplementation with chlorothalonil or colloidal chitin 45, 60, 75 and 90 DAS. The infector rows after every four test rows were infested with crop debris and artificially inoculated with *Phaeoisariopsis personata* conidial suspension to ensure uniform disease development. The dry pod yields in each treatment were the mean of six replications from a repeated field experiment.

applied with and without chlorothalonil, and GPS 5 and GRS 243 applied either alone or in chitin supplementation was also tested. To determine the root colonization, the bacteria were applied as seed treatment, and for phylloplane colonization the bacteria were applied as a foliar spray.

In field, GRS 123 and GPS 55 maintained their populations (log_{10} 3.58 and 3.05 cfu g^{-1} fresh weight) in the rhizosphere up to harvest, whereas GPS 21 was not recovered in the rhizosphere 60 DAS (Fig. 12 A). Starting from 40 and 80 DAS, the isolates GPS 21 and GPS 55 were recovered in the endorhizosphere. In the native potting mixture, the two biocontrol isolates GSE 18 and GSE 19 had higher populations of log 4.58 and 4.26 cfu g^{-1} up to the measured 28 DAS, in the groundnut rhizosphere (Fig. 12 B). The populations of these bacteria in *A. niger*-infested potting mixture were lower than in the native potting mixture, in the same environment.

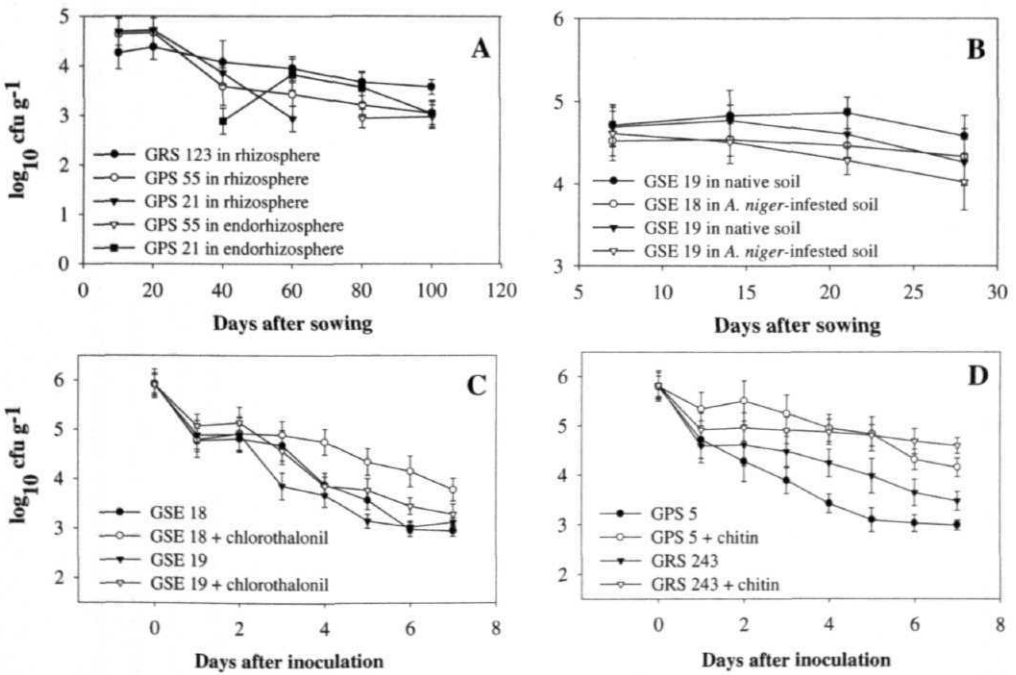
The populations of bacteria, GSE 18 and GSE 19 applied as a foliar spray (10^8 cfu ml^{-1}) decreased from ~log 5.9 to -3.0 cfu g^{-1} at 7 DAI (Fig. 12 C). Foliar application of chlorothalonil increased the populations of these two bacteria on the phylloplane and the increase in the populations of GSE 18 was significant. Similarly, supplementation of chitin enhanced the phylloplane survival of GPS 5 and GRS 243 by > 10-fold (Fig. 12 D).

3.8. **Characterization of chitinase(s) of** 5. *marcescens* GPS 5

3.8.1. Purification of chitinase from CCF **of** *S. marcescens* **GPS 5:** Cell free culture filtrate of GPS 5 grown in minimal medium with colloidal chitin as sole carbon source was used as the enzyme source. Chitinase was purified after ammonium sulphate precipitation, followed by affinity chromatography using acid swollen chitin as a matrix and gel filtration using Sephadex G 100, and SDS-PAGE.

(a) ***Affinity chromatography:*** Proteins eluted from chitin affinity column were monitored by noting the absorbance at 280 run, and the fractions with maximum protein concentration were analyzed on SDS-PAGE. Protein fractions eluted with 100 mM sodium acetate buffer, pH 4.8, contained a prominent 21 kDa protein. Elution with 100 mM sodium

Fig. 12. Population dynamics of the introduced (A) Growth promoting isolates in the rhizoplane and root system in field, (B) Antifungal isolates in the rhizoplane in native and *A. niger* infested potting mixtures, (C) Antifungal isolates in the phylloplane when applied alone and in supplementation with chlorothalonil in field, and (D) Chitinolytic isolates in the phylloplane when applied alone and in chitin-supplementation in field.



GRS 123 = *Bacillus firmś;* GPS 55 = *B. megaterium*; GPS 21, GSE 18 and GSE 19 = *Pseudomonas aeruginosa*; GPS 5 = *Serratia marcescens.* and GRS 243 = *Bacillus circulans.*

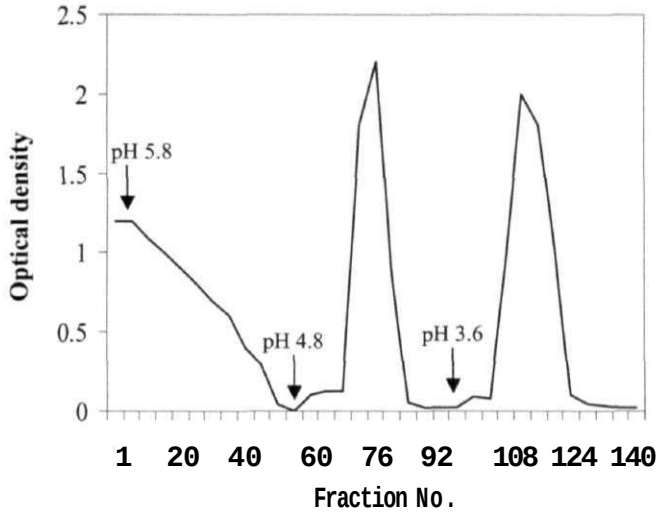
acetate buffer, pH 3.6 resulted in a single peak consisting of a 55 kDa protein (Fig. 13). This fraction had 102.3 units of chitinase activity and contained 26.2% of the total activity (Table 18). In both the peaks the proteins were co-eluted with trace amounts of contaminating protein (Plate Fig. 13 A). Hence, the fractions were pooled and further separated on a Sephadex G 100 column.

(b) *Gel filtration **chromatography**:* The protein eluted near 43 kDa range on Sephadex G 100 column (Fig. 14) resolved as 21 kDa dimeric protein on 10% denaturing polyacrylamide gel. The second protein eluted using 100 mM sodium acetate buffer, pH 3.6 resolved as a single band of 55 kDa both on native and denaturing polyacrylamide gels (Plate Fig. 13 A). When analyzed for enzymatic activity, the 21 kDa protein did not show chitinase activity, where as the 55 kDa protein showed high NAGase activity. This protein fraction had a specific activity of 120.1 units and retained 22.8% of the total enzymatic activity (Table 18).

(c) *Detection of eznymatic activity after **SDS-PAGE**:* The protein fractions eluted from gel filtration column were checked for their purity using SDS-PAGE. Individual bands of separated proteins were further tested for their enzymatic activity using glycol chitin as a substrate and Calcofluor white staining. Separated band of 55 kDa protein showed a clear zone of lysis of glycol chitin observed under UV illumination (Plate Fig. 13 B), whereas the 21 kDa band has no such lytic activity.

(c) ***N-terminal** aminoacid sequence:* The N-terminal amino acid sequence of 21 kDa protein was determined by automated aminoacid sequence. The sequence of the first 14 aminoacids obtained was A H G Y V E X P A S R A X Q. This N-terminal sequence corresponds to the reported aminoacid sequence of 21 kDa chitin binding protein of *S. marcescens* from amino acids 27 to 40, and also chitin binding proteins from other microorganisms (Table 19).

Fig. 13. Elution profile of dialysed culture filtrate of *Serratia marcescens* GPS 5 passed through acid swollen chitin matrix.



Serratia marcescens GPS 5 was grown in minimal medium with colloidal chitin as a sole carbon source for 8 days and the proteins in the culture filtrate were pelleted by adding ammonium sulphate to 100% saturation. The pellet was dissolved in minimal volume of 10 mM PBS and dialyzed against the same. The dialyzed sample was loaded on to the chitin column at 10 mg protein g^{-1} matrix and the flow rate was adjusted at 15 ml h^{-1}. The flow through was re-applied on the column to ensure complete binding of chitosan or chitin binding proteins to the swollen chitin. The unbound proteins were eluted using 100 mM sodium acetate buffer, pH 5.8. The bound chitinase or chitin binding proteins were eluted in two steps using 100 mM sodium acetate buffer pH 4.8 and pH 3.6.

Table 18. Purification of a 55 kDa β-1,4-N-acetyl glucosaminidase from the culture filtrate of *Serratia marcescens* GPS 5.

Purification step	Total protein (mg)	Enzyme units	Specific activity	Purification fold	Yield (%)
Culture filtrate	455	4732	10.4	1.0	100.0
Affinity column	12.1	1238	102.3	9.8	26.2
Sephadex G 100	9.0	1081	120.1	11.5	22.8

*One unit of enzyme activity was defined as the micromoles of N-acetyl glucosamine released per ml per hour at 37°C and pH 5.2. Specific activity is the enzyme units per mg protein.

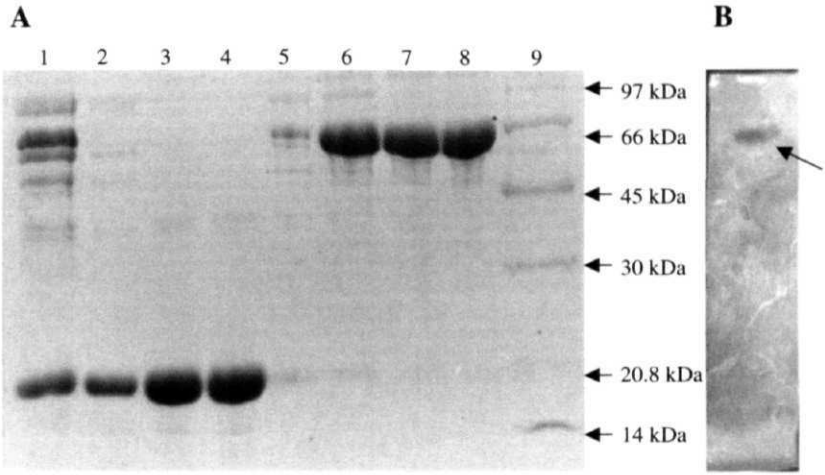


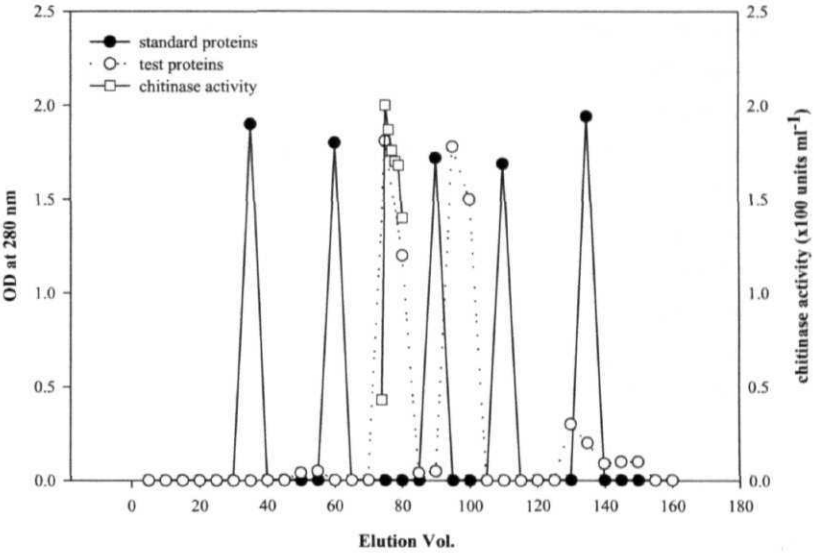
Plate Fig. 13. (A) Sodium dodecyl sulfate (SDS)-PAGE. and (B) Activity staining of a purified N-acetyl glucosaminidase from the culture filtrate of *Serralia marcescens* GPS 5.

(A) lane 1: Crude culture filtrate; lane 2: eluent of affinity column with sodium acetate buffer, pH 3.6; lane 3 and lane 4: fractions of gel filtration eluent with chitin binding protein; lane 5: eluent of affinity column with sodium with buffer pH 3.4; lane 6 to 8: fractions of gel filtration eluent with N-acetyl glucosaminidase; lane 9: molecular weight marker.

(B) Activity staining of 55 kDa NAGase of 5. *marcescens* GPS 5 after native PAGE using glycol chitin as a substrate and Calcoflour white as brightener.

Proteins in the crude culture filtrate of *Serratia marcescens* GPS 5 grown on minimal medium with colloidal chitin as sole carbon source, were concentrated by ammonium sulphate precipitation. Concentrated proteins were separated on affinity column using acid swollen chitin as matrix. The chitin binding proteins were further concentrated by gel filtration using Sephadex G 100 as matrix.

Fig. 14. Elution profile of chitin binding proteins of *Serratia marcescens* GPS 5 eluted from affinity column passed through sephadex G 100.



Serratia marcescens GPS 5 was grown in minimal medium with colloidal chitin as a sole carbon source for 8 days. Proteins precipitated at 100% ammonium sulphate saturation were separated by affinity chromatography using acid swollen chitin as a matrix. Chitin binding proteins were eluted in two steps using 100 raM sodium acetate buffer pH 4.8 and pH 3.6. The proteins were further purified by gel filtration using sephadex G 100 as a matrix.

Table 19. Comparison of N-terminal aminoacid sequence of 21 kDa chitin binding protein (CBP) of *Serratiamarcescens* GPS 5 with related proteins of different microorganisms.

S. No.	Organism	Protein	Sequence
1	*Serratia marcescens* GPS 5	**CBP**	**AHGYVEXPASRAXQ**
I	*Serratia marcescens*	**CBP** 21 **precursor**	**27 AHGYVESPASRAYQ 40**
2	*Bacillus cereus* ATCC 14579	**CBP**	**34** AHGYVESPASR **44**
3	*Bacillus anthracis* str. Ames	**CBP**	**34 AHGYVESPASR 44**
4	*Bacillus halodurans*	**CBP**	**33 AHGY1ENPSSRA 44**
5	*Enterococcus faecalis* V583	**CBP**	**28 AHGYVASPGSRA 39**
6	*Yersinia enterocolitica*	**ChiY protein**	21 **AHGYIENPPSR** 31
7	*Oceanobacillus iheyensis* HTE831	**CBP**	**33** AHGYIEEPQSR **43**
9	*Bacillus cereus* ATCC 14579	**CBP**	**40** AHGFVEKPGSR **50**
10	*Bacillus amyloliquefaciens*	**CBP**	**27** AHGYIKEPVSRA **38**
11	*Lactococcus lactis* subsp. *lactis*	**CBP**	**28** AHGYVQSPPAR **38**
12	*Shewanella oneidensis* MR-1	**CBP**	**43** AHGYVVSPESR **53**

***Amino acid sequences matching with GPS 5 N-terminal sequence were highlighted.**

3.8.2. Antifungal activity of *S. marcescens* GPS 5 NAGase: Chitinase at concentrations of 10 to 40 $\mu g\ ml^{-1}$ was tested for antifungal activity against the conidia of *P. personata* and urediniospores of *P. arachidis* determined by *in vitro* spore germination assay. Chitinase at a concentration of 10 $\mu g\ ml^{-1}$ was significantly ($P = 0.01$) inhibitory to the *in vitro* germination of both the fungi and the inhibitory action increased with an increase in enzyme concentration (Table 20). At concentrations of 35 and 25 (ig ml^{-1}, *P. personata* conidia and germ tubes of *P. arachidis* (Plate Fig. 14 A-E) lysed. At the highest concentration tested i.e. 40 $\mu g\ ml^{-1}$, spore germination of both the fungi was inhibited by 94.4% and 98.5%, respectively.

3.8.3. Evaluation of NAGase **for** control **of** LLS: Foliar spray of chitinase at 35 $\mu g\ ml^{-1}$, 24 h before the pathogen inoculation, reduced LLS severity. The lesion frequency measured in chitinase treatment (1.42) decreased by 59.7% in comparison to control (3.53; Plate Fig. 14 F). At 30 DAI, the disease severity in chitinase treated plants was 4.6 on a I-9 rating scale against 9.0 in control plants.

Table 20. *In vitro* inhibition of spore germination of *Phaeoisariopsis personata* and *Puccinia arachidis* by β-1,4-*N*-acetyl glucosaminidase of *Serratia marcescens* GPS 5.

S.No.	NAGase (μg ml^{-1})	Percentage germination inhibition	
		P. personata	*P. arachidis*
1	**10**	60.3±3.2	72.813.8
2	20	89.7±2.2	95.311.4
3	25	93.9±1.1	97.111.0
4	30	92.8±1.0	98.3±0.7
5	35	94.6±1.0	98.010.5
6	**40**	94.411.3	98.510.4

To 25 μl of the spore suspension (10^5 cells ml^{-1}) on a cavity slide, equal volume of the enzyme suspended in 10 mM phosphate buffer, pH 7.0 was added at a final concentration of 10 to 40 fig ml^{-1}. The slides were incubated in a humid chamber in dark at 28°C. Germination of *P. personata* and *P. arachidis* spores was observed under a photomicroscope after 24 and 8 h, respectively.

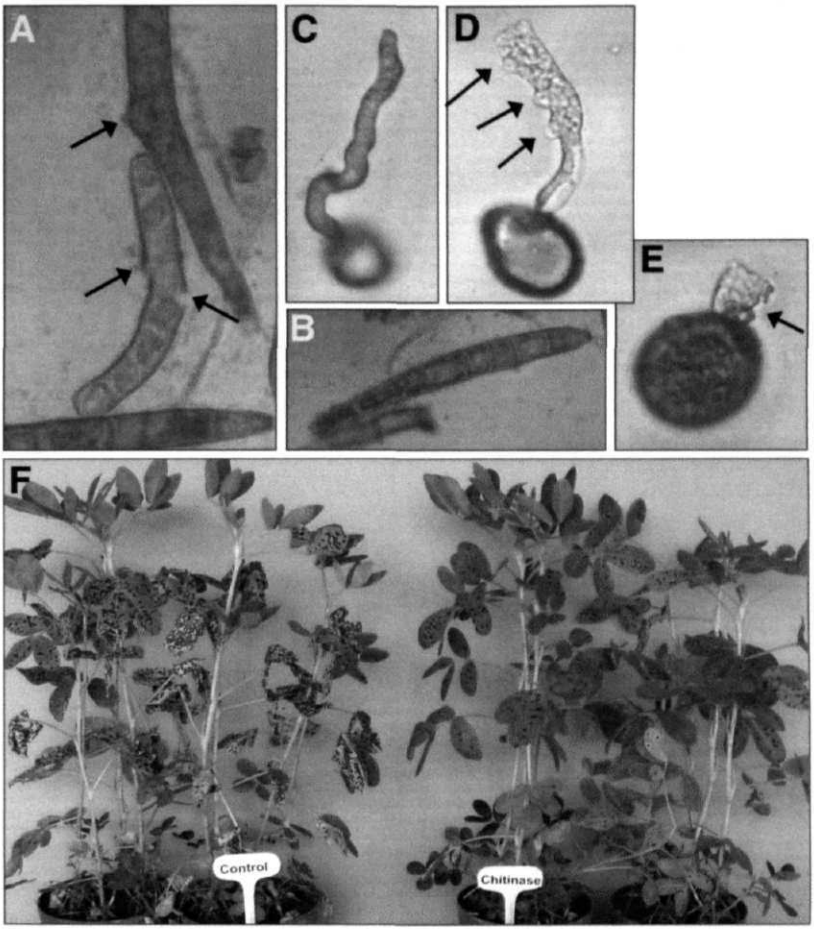


Plate Fig. 14. Effect of β-1,4-*N*-acetyl glucosaminidase of Serratia *marcescens* GPS 5 on the conidia of *Phaeoisariopsis personata* and urediniospores of *Puccinia arachidis in vitro*, and development of late leaf spot of groundnut *in vivo*.

(A) Lysed conidia of *P. personata* in comparison to (B) Control; (D) and (E) Lysis of *P. arachidis* germ tubes in comparison to (C) Control; (F) Reduced lesion frequency by the foliar application of chitinase 24 h before *P. personata* inoculation.

For *in vitro* studies, the conidia or urediniospores were mixed with chitinase of *S. marcescens* GPS 5 on a cavity slide and incubated in dark at 28°C. Lysis of conidia or urediniospores and germ tubes was recorded 24 HAI. For *in vivo* testing, the enzyme (35 μg ml^{-1}) was used as a foliar spray 24 h before the pathogen inoculation.

Discussion

Modem green revolution aimed at increased food grain production was largely dependent on chemical fertilizers, pesticides and fertilizer responsive crop varieties. Steady increase in the global use of chemicals in different agricultural systems led to yield stagnations, decrease in the fertilizer responsiveness of crops and development of pesticide tolerant pathogens. In this context, biological control appeared as a low-cost and eco-friendly strategy for disease management.

Biological control of foliar diseases has been less successful because of the inconsistent performance of introduced agents on the aerial plant parts. Modification of application systems, nutrient supplementation or other additives are likely to enhance the performance of biocontrol agents. In the present study, biocontrol of LLS of groundnut was adopted as a model system for application of groundnut-associated bacteria as biocontrol agents in the phylloplane. Compatibility of the biocontrol agents with chlorothalonil and/or their chitinolytic ability was utilized to achieve improved disease control, particularly in the IDM practices. Simultaneously, the bacteria were also tested for other beneficial effects like plant growth promotion and control of soil-borne diseases.

4.1. Selection and characterization of antifungal bacterial isolates

4.1.1. Broad-spectrum antifungal activity of bacterial isolates: Bacterial isolates were collected from diverse habitats of groundnut in different geographical locations and evaluated for their beneficial effects in groundnut production, either by growth promotion or disease control. The bacterial isolates were tested for broad-spectrum antifungal activity against eight major fungal pathogens that cause yield losses in the semi-arid tropics (SAT). Of the 393 isolates tested, individual isolates from different habitats differed in their antifungal activity. Isolates with antifungal activities were distributed in different habitats of groundnut, but their frequency was high among the endophytes. Fourteen isolates had a broad-spectrum activity and inhibited the growth/germination of six or more test fungi. Variation in the spectrum of antifungal activity of microorganisms or their metabolites is not uncommon (Leifert *et al.*, 1995).

Pseudomonas sp. GRS 175 and four isolates of *P. aeruginosa*, GPS 21, GSE 18, GSE 19 and GSE 30 were highly inhibitory to all the test fungi. The antifungal activity of these isolates was comparable with several of the antagonists documented in the literature. *Pseudomonas* spp. quite often emerged as antifungal isolates in several screening programs. Among a collection of 849 bacterial isolates, six isolates with broad-spectrum antifungal activity were identified as *P. aeruginosa* (Viji *et al.*, 2003). Pseudomonads arc widely distributed in diverse agricultural ecosystems (Garbeva *et al.,* 2004) as they are good colonizers of plant root systems due to their competitive advantage. In the present study, the five antagonistic isolates of *Pseudomonas* spp. were cither seed endophytes or phylloplane inhabitants, from different locations, but had a similar antifungal activity. Keel *et al.* (1996) observed that the occurrence of phenotypically and genotypically similar groups of DAPG producing *Pseudomonas* strains did not correlate with the geographic origin of the isolates.

Pseudomonas spp. produces broad-spectrum and diffusible antibiotics. Phenazines, DAPG, pyoluteorin, pyrrolnitrin, lipopeptides and HCN have been well characterized for their distribution, production, antifungal activity and *in vivo* disease control (Haas and Keel, 2003). Thomashow and Weller (1988) first reported disease suppression by an antibiotic and demonstrated the *in vivo* fungicidal activity of phenazine-l-carboxylic acid produced by *P. fluorescens* 2-79. Lee *et al.* (2003) isolated aerugine [4-hydroxymethyl-2-(2-hydroxyphenyl)-2-thiazoline] from the culture filtrates of *P. fluorescens* MM-B16. Treatment with aerugine exhibited a significantly high protective activity against development of *Phytophthora* disease on pepper and anthracnose on cucumber.

4.1.2. Antifungal activity of CCF: In the present study, the antifungal activity of CCF of selected *Pseudomonas* spp. was demonstrated initially by cellophane overlay method and subsequently by the addition of diluted CCF in the growth medium. In cellophane overlay method or in presence of diluted CCF, the test fungi except for *A. flavus*, failed to grow or germinate. Mycelial discs that didn't grow in presence of extracellular metabolites of *Pseudomonas* spp. were not viable when transferred to fresh medium. This fungicidal activity of the extracellular metabolites provides additional advantage in reducing the

pathogen populations. Bano and Musarrat (2003) reported the antifungal, plant growth promotion and biocontrol activities of secondary metabolites *of P. aeruginosa* NJ-15. The production of secondary metabolites in *Pseudomonas* spp. is highly regulated and is very much dependent on the environmental conditions, hence the antifungal activity of CCF may vary according to the growth conditions (Haas and Keel, 2003).

4.1.3. Changes in hyphal morphology: Macroscopic observation of the test fungi in agar cultures added with diluted CCF showed reduced and compact colonies, in contrast to normal spread in control. Microscopic observations revealed the degradation of cytoplasmic contents, vacuolization and bulging of hyphae. The extent **of** hyphal deformations varied depending on the antagonist and pathogen. Deformation of hyphae in presence of antagonistic bacteria or their culture filtrates is a common phenomenon in many of the antagonistic-pathogen interactions. In the presence of an antagonistic *Bacillus* spp. or its culture filtrate hyphae of *Colletotrichum capsici* and **C.** *gloeosporioides* were observed to be thickened, vacuolated and swell at their tips (Meon, 1994). In presence of CCF of *B. brevis*, hyphal tips of *F. udum* showed a characteristic swelling. Additionally, cells were found to be bulbous and swollen with shrunken, granulated cytoplasm compared to hyaline cytoplasm, and rectangular cells seen in control mycelia (Bapat and Shah, 2000). CCF of *B. subtilis* AF 1 showed a concentration dependent growth inhibition of *F, udum* and induced the formation of bulbous structures in the hyphae (Podile and Laxmi, 1998).

4.1.4. Effect of CCF on production of extracellular CWDE: Inhibitory effect of CCF or active metabolites of antagonistic microorganisms on the production of CWDE by the pathogenic fungi was thought to be one of the mechanisms involved in the action of biocontrol agents. *T. harzianum* inhibited the production of cutin esterase, exo-PG, endo-PG, PL, PME and CMCase by *R. solani*. A calcium dependent endoproteinase produced by *B. megaterium* inactivated the cellulase, pectinase and pectin lyase produced by *R. solani* (Bertagnoili *et al.*, 1996). *T. harzianum* T39 and NCIM1185 inhibited the production of cutin esterase, polygalacturonases, PME and PL by *Botrytis cinerea* on bean

leaf surface (Kapat *et al.*, 1998). Extracellular proteases of these two fungi were associated in inhibition of *B. cinerea* enzyme production (Elad and Kapat. 1999).

In the present study, in addition to inhibition of radial growth and conidial germination, CCF of GSE 18 and GSE 19 also inhibited the production of CWDE by *S. rolfsii* and *A. niger*. The enzymes including pectinases and cellulases degrade the plant cell wall polymers ahead of penetration by necrotrophs facilitating the infection process. Inhibition of production of CWDE possibly contributed for effective control of stem rot and crown rot infection by GSE 18 and GSE 19.

4.1.5. Characterization of extracellular enzyme production: **The** lytic action of chitinase and P-1.3-glucanase (laminarinase) could be the major antifungal mechanism or supplement the antagonistic action of other antifungal metabolites produced by the same bacterial isolate. Extracellular chitinase and β-1,3-glucanase produced by *P. stutzeri* YPL-1 caused lysis of *F. solani* mycelia and germ tubes (Lim *et ed.*, 1991). A penetration hole was formed on the hyphae resulting in the leakage of protoplasm in the region of interaction with the antagonist. The antifungal activity of *B. subtilis* AF 1 against *A. niger* in dual cultures was due to the lysis of fungal mycelium by the extracellular chitinase (Podile and Prakash, 1996). Similarly, β-1,3-glucanase produced by a non-chitinolytic isolate of *P. cepacia* caused damage to *R. solani* hyphae (Fridlender *et al.*, 1993). A strong correlation was observed between the chitinolytic potential of different bacterial isolates and *in vitro* lysis of *Gaeumannomyces graminis* mycelium (Renwick *et ed.*, 1991). A definite relation between the production of *in vitro* chitinase and antifungal activity was not observed (Frandberg and Schnwer, 1998). In the present study, since chitinase and P-1,3-glucanase are inducible enzymes under catabolite repression, correlation between *in vitro* chitinase production and antifungal activity was not seen.

4.2. Evaluation of plant growth promoting activity of **bacterial isolates**

4.2.1. Greenhouse evaluation: Groundnut-associated bacteria isolated from various habitats (rhizosphere, phylloplane, geocarposphere and seed endophytes) promoted

groundnut growth in greenhouse. Bacterial isolates. *B. firmis* GRS 123 from the rhizosphere, and *B. megaterium* GPS 55 and *P. aeruginosa* GPS 21 from the phylloplane of groundnut were effective than other test isolates. Positive effects of PGPR, as seed treatment, in maize (Jacoud *et al.*, 1999), wheat (Creus *et al.*, 1996), rice (Mehnaz *et <;/.,* 2001) and several other crops (Podile and Dube, 1988; Kloepper *et al.*, 1991) have been reported.

Production of auxins, cytokinins (Garica de Salamone *et al.*, 2001), gibberellins (Gutierrez-Manero *et al.*,2001) and siderophores, and solubilization of mineral phosphates by PGPR help to promote the plant growth. Bacterial production of plant hormones influences the root morphogenesis by overproduction of root hairs and lateral roots, and subsequent increase in ion uptake. The percentage of isolates that produce auxins, siderophores and solubilize mineral phosphate was high among the best growth promoting isolates compared to the 393 test isolates. However, there was no clear relation between the ***in*** *vitro* production of auxins by the growth promoting isolates in presence of L-tryptophan and their effect on the root growth in greenhouse. Auxin producing PGPR isolates and their culture filtrates increased the surface area of aerial plant parts, shoot length, number of leaves and total nitrogen content (Manero *et al.*, 1996). A positive correlation was observed between L-tryptophan-derived auxin production by PGPR ***in*** *vitro* and grain yield, number of pods and branches per plant of *B. juncea* (Asghar *et al.*, 2002). Suppression of root growth by auxin-producing PGPR isolates was also reported (Barazani and Friedman, 1999). The differences in the performance of auxin producing PGPR could be due to their greater dependency on the availability and composition of root exudates for production of auxins. Moreover, the optimal concentration of auxin required for plant growth promotion is extremely narrow (Xie *et al.*, 1996) and doses of auxin above the threshold levels are deleterious for root growth. This optimal concentration is in turn dependent on the sensitivity of the plant and also cultivar.

4.2.2. Field evaluation of PGPR and their formulations: In field, the three bacterial isolates GRS 123, GPS 55 and GPS 21 promoted seedling emergence, root length, shoot length, dry weight and pod yield. *Pseudomonas* sp. were earlier observed to effectively

colonize the groundnut rhizosphere and enhance root length, shoot length, biomass, nitrogen and phosphorous uptake, and yield in field (Pal *et al.*. 2000). *Pseudomonas* sp. GRC2 increased the seed germination, early seedling growth, fresh nodule weight and grain yield (Gupta *et al.*, 2002). *P. cepacia* and *P. putida* as seed inoculants increased the number of tillers, and grain yield of winter wheat by 11% in field (DeFreitas and Germida, 1992). Inoculation with PGPR increased the dry weight of rapeseed seedlings in addition to increase in emergence (Kloepper *et al*., 1986). Seed treatment of hybrid spruce seedlings with *Bacillus* and *Pseudomonas* sp. resulted in the rhizosphere endophytic colonization of these isolates and significantly enhanced the dry weight up to 57% in field (Chanway *et al.*, 2000). *P. putida.* a plant growth promoting isolate, increased the yield of potato (Burr *et al.*. 1978) and chickpea (Trapero-Casas *et al*., 1990). The performance of PGPR is greatly influenced by soil type and environment (Difluza and Gisela. 2003) which determines their root colonization.

Bacillus firmis GRS 123 and *B. megaterium* GPS 55 formulated in peat maintained almost the same populations up to 180 days of storage at room temperature, whereas the populations *of P. aeruginosa* GPS 21 declined gradually and were $<\log_{10}$ 4.0 cfu g'" peat after 90 days of storage. In an earlier study, *B. subtilis* B6 formulated in peat had a good shelf life at room temperature, with a drop of only log 0.1 cfu g^{-1} peat even after storage for 2 years (Georgakopoulos *et al.*. 2002). In the same study, *Pseudomonas* sp. also maintained higher populations in peat with a decline of only log 1-2 cfu g^{-1} peat after 2 years. Our results indicate that the formulation of *P. aeruginosa* GPS 21 in peat is not beneficial for practical use. *Pseudomonas* spp. usually have a short shelf-life compared to spore-forming bacilli. Storage *of Pseudomonas* spp. up to 8 months, without a decrease in populations and effectiveness, has been reported in powder formulations (Vidhyasekaran and Muthamilan, 1995). In the present study, the peat formulations of GRS 123 and GPS 55 were equally effective as the seed treatment in plant growth promotion in field. The peat formulation of GPS 21 was not effective in growth promotion.

The results of the present study indicate that bacterial isolates from other habitats like phylloplane and internal tissues were also effective, like rhizobacteria, in plant growth promotion when applied on to the seeds. Phylloplane isolates normally survive in low

moisture and other adverse conditions, thus may effectively colonize the nurtient-rich rhizosphere. Earlier, rhizobacteria were isolated, screened and selected as PGPR. It may be possible to identify better plant growth promoting bacteria from parts of the plants other than roots.

4.2.3. Root colonization of PGPR: Root colonization by **PGPR** is critical in biological control and plant growth promotion (Kloepper and Beauchamp, 1992). Root colonization is highly variable as it is **affected** by a variety of chemical, physical and biological factors (Weller, 1988). Determining the dynamics of root **colonization** by the introduced PGPR is essential for their effective use. Our results showed that in the groundnut rhizosphere, the populations of *Bacillus* spp. was more stable compared **to *P.*** *aeruginosa*, which was not recovered after 60 DAS. When applied as a seed treatment, populations of the introduced **PGPR** in the rhizosphere increased rapidly and then declined as the **groundnut** roots grew. In several earlier studies, **the PGPR** population in the rhizosphere grew exponentially during **the** initial plant growth and then declined (Hebbar ***et*** *al*., 1992; Kluepfel, **1993).**

4.3. Biocontrol of stem rot disease of groundnut

The method used for *S, rolfsii* inoculation resulted in complete mortality in non-treated control. Seed treatment and soil amendment with /'. ***fluorescens*** **(Ganesan** and Gnanamanickam, 1987), *T. harzianum* and ***(!.*** *virens* (SreenivasaPrasad and ManibhushanRao, 1993) effectively protected the groundnut seedlings from stem rot infection. *Pseudomonas* spp. applied as seed treatment were also effective in control **of** *S. rolfsii* in other crops. Seed treatment with *P. fluorescens* controlled *S. rolfsii* infection in betelvine (Singh ***et*** *at.,* 2003) and chickpea (Sarma ***et*** *al*., 2002), and elicited phenolic compounds, gallic, ferulic, chlorogenic and cinnamic acids in chickpea. We found that **of** the 393 isolates tested, 12 bacterial isolates that were antagonistic to *S. rolfsii in vitro* showed a significant control of stem rot. Seed endophytes, /'. ***aeruginosa*** **GSE** 18 and GSE 19 were highly effective in reducing the stem rot infection compared to other isolates. *P.* ***aeruginosa*** is known to produce broad-spectrum antifungal metabolites and

siderophores that contribute to *in vivo* disease control. The biocontrol action of *P. aeruginosa* PNA1 against *Pythium myriotylum* on Cocoyam, Fusarium wilt of chickpea and Pythium damping-off of tomato was due to the production of phenazines, as supported by the loss of biocontrol activity of phenazine-deficient mutants (Anjaiah *el al.*. 1998; Tambong and Hofte, 2001). The involvement of siderophores. pyochelin and pyoverdin in suppression of Pythium damping-off of tomato by *P. aeruginosa* 7NSK2 was established by the use of siderophore-deficient mutant strains and further complementation of pyochelin and pyoverdin production (Buysens *et al.,* 1996). Culture filtrates of *P. aeruginosa* GSE 18 and GSE 19 inhibited the production of CWDE by *S. rolfsii,* which could probably be one of the mechanisms of their action.

Combined use of the two or more biocontrol agents can be attempted for improved control of *S. rolfsii* on groundnut. Combined seed treatment with *P. **fluorescens*** and *P. aeruginosa* protected chickpea seedlings from *S. rolfsii* infection and also induced phenolic acids in the host plant (Singh *et al.*. 2003a). These results were futher substantiated by the increased inhibition of root-infecting fungi of tomato by a combinational use of *P. fluorescens, P. aeruginosa* and *Bradyrhizobium japonicum* (Siddiqui and Shaukat, 2002a), or *P. aeruginosa* and *Pochonia chlamydosporia* (Siddiqui and Shaukat, 2003).

4.4. Biocontrol of crown rot disease of groundnut

Observations on the incidence of *A. niger* infection in each treatment was based on pre-emergence rotting and post-emergence wilting of young seedlings.

4.4.1. Greenhouse screening: Eleven among the 393 bacterial isolates were effective in reducing *A. niger* infection, both pre- and post-emergence, with *P. aeruginosa* GSE 18 and GSE 19 being the best. Earlier attempts for control of crown rot were focused on the application of an antifungal and chitinolytic bacterium, *B. sublilis* AF 1. In dual cultures, AF 1 lysed *A. niger* mycelium (Podile and Prakash, 1996). Different isolates of *P. aeruginosa* were effective in control of other soil-borne diseases like Fusarium wilt of chickpea and pigeonpea, when screened in pathogen-infested soils (Anjaiah *et al.*, 2003).

Seed treatment with *Pseudomonas* sp. GRC_2 reduced charcoal rot disease **of** groundnut in *R. bataticola*-infestedsoils by 99% (Gupta *et al.*, 2002). We observed that two bacterial isolates *Achromobacter xylosidans* GRS 149 and GSE 6 that were non-antagonistic to *A. niger* reduced the pre-emergence rotting. Since these two isolates were potent siderophore producers, inhibition *of A. niger* in the rhizosphere by iron limitation also may contribute to the observed disease control. Suppression of Fusarium wilt of carnation by *P. putida* WCS 358 was by siderophore-mediated competition for iron (Duijff *et al.*, 1994).

4.4.2. Effect of *P. aeruginosa* GSE 18 on the activity of defense-related enzymes during crown rot infection: Seed treatment with ***P.*** *aeruginosa* GSE 18 enhanced **the** activities of chitinase. β-1,3-glucanase, peroxidase and PAL in treated seedlings. Augmented defense responses in the host plant are effective, non-specific and durable. *B. subtilis* AF 1 applied as seed treatment increased the lipooxygenase activity (Sailaja *et al.*, 1998). altered the phytoalexin metabolism (Sailaja and Podile, 1998) for the host benefit. *P. aeruginosa* is a known inducer of systemic resistance in bean and tomato **plants. Root-dip** treatment or soil drench with *P. aeruginosa* induced systemic resistance in tomato and protected against *R. solani* (Siddiqui and Shaukat, 2002b). When tested in split-root assay, the bacterium also protected tomato roots against *Meloidogyne javanica* infection, by salicylic acid independent induction of systemic resistance (Siddiqui and Shaukat, 2004). *P. aeruginosa* 7NSK2 indcued systemic resistance in bean by production of salicylic acid (DeMeyer and Hofte, 1997), as observed by increased PAL activity in roots and leaves of treated seedlings (DeMeyer *et* al., 1999). In subsequent studies, pyochelin and pyocyanin, rather than salicylic acid, were identified as determinants for induced resistance in tomato (Audenaert *et al.*, 2002).

4.5. Biocontrol of **LLS** disease of groundnut

4.5.1. Selection of effective biocontrol isolates: Bacterial isolates that were highly inhibitory to *P. personata* in *in vitro* experiments were further evaluated in greenhouse for LLS control. Two broad-spectrum antifungal isolates, *P. aeruginosa* GSE 18 and GSE 19

applied as a prophylactic spray were inhibitory to the development of LLS. *Pseudomonas* spp. applied as a foliar spray were also effective in control of several foliar diseases. Foliar application of broad-spectrum antifungal isolates of *P. aeruginosa* prior to pathogen inoculation, protected perennial ryegrass turf from gray leaf spot infection caused by *Pyricularia grisea* (Vizi *et al.*, 2003). Seed treatment and foliar application with talc-based powder formulation of *P. fluorescens* pf1 effectively reduced the **severity of** leaf spot and rust, and increased the pod yield in greenhouse and field tests (Meena *et al.*. 2002). In contrast, *P. fluorescens* of Mathivanan *et al.* (2000) was ineffective in LLS control. *Bacillus* sp. also proved effective in control of leaf spot diseases. *Bacillus* sp. BacB was effective in control of sugar beet leaf spot caused by *Cercospora* ***beticola*** (Collins and Jacobsen, 2003). Foliar application **of** BacB as vegetative cells instead **of** spores, at 1-5 days before inoculation, significantly increased the disease control.

4.5.2. Integrated use of biocontrol bacteria and chlorothalonil: The two isolates *P. aeruginosa* **GSE** 18 and GSE 19 were tolerant to **chlorothalonil, the fungicide** commonly used for control of both LLS and rust. Foliar application **of** these isolates in combination with reduced dosage **of** chlorothalonil was effective than either **of** the components used alone. It is possible that the application **of** chlorothalonil might have reduced the pathogen populations below a threshold level, wherein the biocontrol agents are more effective. Also, the application **of** chlorothalonil will reduce or eliminate the competition from native microflora for the successful establishment of the introduced biocontrol agents. Such strategies may help to achieve the consistency of biocontrol in different environments. Soil application of chlorothalonil followed by a short incubation period, resulted in community changes including both enhancement and inhibition of a variety of dominant organisms (Sigler and Turco. 2002). Fungicide resistance is frequently observed in *Pseudomonas* spp. An antagonistic isolate *P. fluorescens* EPS 288 was tolerant to benomyl, imazalil and folpet fungicides. The combination **of** EPS288 and 25% of the standard dose of fungicide (imazalil + folpet) was as effective as the standard fungicide alone, in control of *Penicillium expansum* on pear fruits (Frances *et al.*, 2002). Similar results were also obtained by using a combination of reduced dosage of the

fungicides and *Rhodotorula glutinis* for control of *B. cinerea* on geranium seedlings (Buck, 2004). This treatment effectively controlled the fungicide tolerant pathogen strains. Flutolanil has no effect on the ***in*** *vitro* growth and iturin A production of *B subtil is* RB14-C. Integration of RB14-C and flutolanil reduced the fungicide requirement to $1/4^{th}$ for effective control of damping off in tomato plants (Kondoh ***et*** *al*., 2001).

4.5.3. Evaluation of chitinolytic bacteria for LLS control: The foliar application of the two chitinolytic isolates *S. marcescens* GPS 5 and *B. circulans* **GRS** 243 in supplementation with colloidal chitin improved their efficacy against **LLS.** Production of chitinase contributes to increased inhibition/lysis of pathogen on the leaf surface. **Presence** of chitin on the leaf surface increased the biocontrol effectiveness of chitinolytic bacterial isolates against early leaf spot of groundnut and other foliar diseases (Kokalis-Burelle *el al.,* 1992). Presence of chitin resulted in better phylloplane **survival of** a **chitinolytic** isolate *B cereus* compared to non-amended leaves of groundnut. In chitin- amended leaves, hyphae and spores of C. *arachidicola,* were colonized and distorted by *B cereus.* Chitin supplementation of ***Stenotophomonas maltophilica*** **C3** improved the control of bean rust in greenhouse (Yuen *et al.,* 2001). compared to cells suspended in buffer alone. Inducible production of chitinases by the introduced chitinolytic bacteria on the phylloplane. in presence of chitin. leads to germination inhibition and lysis of the conidia of *P. personata.* The disease severity gradually decreased with an increasing concentration of colloidal chitin, indicating a strong relation between the chitinolysis and disease control activities of the test chitinolytic isolates. Chitin amendment has been successful in enhancing the populations of chitinolytic organisms resulting in improvement of the biocontrol systems for management of soil-borne fungal diseases and nematode diseases (Tian *et al.,* 2000).

4.5.4. Evaluation of formulations of broad-spectrum antagonistic or chitinolytic isolates for LLS control: Peat formulations of *P. aeruginosa* GSE 18 was moderately effective in control of LLS in greenhouse. Foliar application of a talc-based formulation of *P. fluorescens* pf1 effectively controlled LLS and rust in field (Meena *et al.,* 2002). In the

present study, the reduced effectiveness of formulations of GSE 18 and GSE 19 compared to the mid-log phase cells could be mostly due to the reduced cell density in 90-day-old formulations.

Chitin-supplementation improved the survival of *S. marcescens* GPS 5 and *B. circulans* GRS 243 in peat formulation by improving the nutrient status of the formulation. In this treatment, also there was an improvement in the biocontrol efficacy compared to the bacteria formulated in peat alone. Chitin-supplementation of the formulations induces chitinase production by the chitinolytic bacterium. Thus, GPS 5 pre-induced for chitinase production performed better in the phylloplane, compared **to** CiPS 5 formulated in peat. Earlier observations on improved biocontrol efficacy of peat formulation of a chitinolytic bacterium by chitin-supplementation (Manjula and Podile, 2001), further support the usefulness of chitin amendment for chitinolytic bacterial formulations for foliar disease control.

4.5.5. Effect of biotic and abiotic elicitors on the development of LLS: Elicitation of disease resistance in host plants by different biotic and abiotic elicitors imparts a broad-spectrum of resistance against fungal pathogens (Oostendorp *el al.*, 2001) and is an economical and feasible method of disease control. Exogenous application of salicylic acid (Naylor *et al.*, 1998), methyl jasmonate (Walters *et al.*, 2002) and **chitosan** (Sathiyabama and Balasubramanian, 1998) induced systemic resistance against fungal, bacterial and viral pathogens in different crops. Foliar application of salicylic acid was observed to induce the accumulation of a 25 kDa thaumatin-like protein and a 30 kDa P-l,3-glucanase in groundnut (Meena *et al.*, 2001). In the present study, these three elicitors, in addition to heat-killed conidia *of P. personala* applied as a foliar spray were not effective in protection of groundnut against **LLS** infection. The responsiveness of the groundnut to different elicitors could be genotype-specific.

4.5.6. Induction of host resistance in groundnut by biocontrol bacteria: The bacterial isolates *P. aeruginosa* GSE 18 and GPS 5 enhanced the activities of chitinase and glucanase in groundnut leaves. *P. fluorescens* pf1 as a foliar spray increased the activities

of PAL, phenolic compounds, chitinase and glucanase in groundnut plants, and protected from LLS and rust infection (Meena *el al.*. 2000). Chitin-supplemented application of GPS 5 further enhanced the enzyme activities compared to GPS 5 alone. This could be due to the released chito-oligosaccharides, which function as elicitors of host defense responses. Chitin fragments. N-acetyl chitooligosaccharides induced various defense-related cellular responses in suspension-cultured rice cells. These responses include transient membrane depolarization **(Kikuyama *et al.*,** 1997), ion flux (Kuchitsu *et at.*, 1997), reactive oxygen generation (Kuchitsu *et al.*. 1995). expression of PR genes (He *et al.*, 1998; Takai *el al.*, 2001) and biosynthesis of phytoalexins (Yamada *et al.*, 1993). Hence, the application of chitinolytic bacteria or chitinases can act both by direct antagonism and elicitation of host defense responses, which synergistically enhance the disease control.

4.5.7. Field evaluation of the biocontrol isolates: Field evaluation of the fungicide tolerant and chitinolytic bacterial isolates for LLS control further confirmed the effectiveness of these treatments in disease control. In agreement with greenhouse experiments, integrated application of *P. aeruginosa* GSE 18 along with reduced concentration of chlorothalonil was highly effective than cither of the components applied alone. Similar synergistic action of fungicides and fungicide tolerant biocontrol isolates has been reported to be beneficial in control of Rhizoctonia aerial blight and root rot of rosemary (Conway *et al.*. 1997).

Chitin-supplemented application of GPS 5 was effective than that of GRS 243 in control of LLS in field. Presence of chitin on the leaf surface increased the populations of GPS 5 on the phylloplane. Chitin supplementation of *S. maltophilica* C3 improved the control of leaf spot of *Festuca arundonacea*, both in growth chamber and field, compared to application of biocontrol agent alone (Zhang and Yuen, 1999). Chitin supplemented application of GPS 5 in the present study was further validated in field experiments. In the field experiment, both GPS 5 and GSE 18 showed improved control of LLS upon supplementation with chitin and chlorothalonil, respectively. **These** two isolates can be further tested and recommended for large-scale use by the farmers.

4.6. Characterization of chitinase(s) of *S. marcescens* GPS 5

Chitinases catalyse the conversion of chitin (polymer of P-1.4-N-acetyl glucosamine) to its monomeric and oligomeric components. Chitinases are widely distributed in different organisms including bacteria, fungi, insects, crustaceans and plants (Patil *et al.*, 2000). *S. marcescens* GPS 5 exhibited strong chitinolytic activity, as determined by degradation of colloidal chitin **both** in solid and liquid media. *S. marcescens* is known to possess a highly active chitinolytic system consisting of at least four enzymes and a chitin-binding protein (CBP) viz. *Chi*A (57-58 kDa), *Chi*B (52-45 kDa). *Chi*C1 (48-52 kDa). *Chi*C2 (35-36 kDa) and CBP 21 (21-22 kDa) (Brurberg *el ed.*, 2000).

4.6.1. Purification of chitinase from CCF of *S. marcescens* **GPS 5:** One of the major objectives is to utilize the chitinolytic potential of antagonistic bacteria for biocontrol of LLS. In this direction, we made an attempt to characterize extracellular chitinases of *S. marcescens* GPS 5, by evaluating the antifungal and disease control activities of purified chitinase of GPS 5.

One-step purification of chitinase using chitin affinity chromatography was earlier described by Roberts and Cabib (1982). In the present study, two chitin binding proteins were eluted from the affinity column using elution buffer of different pI I values. Different types of chitinolytic enzymes having affinity towards chitin would bind to the matrix, and a pure enzyme may not be obtained in a single step. Fuchs *et al.* (1986) reported that individual chitinolytic enzymes from *S. marcescens* were not completely separated by several known purification techniques. Gel filtration resulted in complete fractionation of the eluents of affinity column. SDS-PAGE separation of the two chitin binding proteins followed by activity staining revealed the enzymatic action of 55 kDa protein, while the 21 kDa protein has no detectable enzymatic activity. The enzyme recovery after gel filtration was 22.9%. Roberts and Cabin (1982) reported to recover 27.5% of the initial chitins

activity. NAGase was purified from *Bipolaris sorlkiniana*, with a purity of 70-fold and the yield was 41% (Geimba *et al.*, 1998).

The molecular mass of the chitin binding protein estimated by gel filtration was ~43 kDa. Analysis of the enzymes by SDS-PAGE indicated a single protein band of ~21 kDa. suggesting that the native protein was a dimer. The molecular weight of NAGase evaluated by gel filtration was 55 kDa. and SDS-PAGH analysis revealed the monomeric nature of this enzyme. This molecular weight matches with the reported NAGases of *S. marcescens*. According to a recent nomenclature chitinases that hydrolyse chitobiose or release NAG monomer from chitin were termed as β-N-acetylglucosaminidases (Harman *et al*., 1993).

4.6.2. **N-terminal** aminoacid sequence of chitin **binding** protein: The **determined N-**terminal aminoacid sequence of the 21 kDa chitin binding protein of **GPS** 5 matches with reported sequence of similar proteins of *S. marcescens*, starting al aminoacid 27. A chitinase of 22 kDa molecular weight has been reported from *S. marcescens* and the first 27 aminoacids had characteristic features of a signal sequence, i.e. a positively charged **aminoterminal** segment followed by a hydrophobic sequence (Gal *et al*.. 1997).

4.6.3. **Antifungal** activity of *S. marcescens* GPS 5 NAGase: In several *in vitro* studies chitinases inhibited the fungal growth and/or spore germination. The purified 55 kDa NAGase of *S. marcescens* GPS 5 inhibited the *in vitro* conidial germination of *P. personata*, in a concentration dependent manner. This protein also inhibited the urediniospore germination of *P. arachidis* also lysed the germ tubes. Anti fungal activity of chitinases and the resultant lysis of germ tubes has been frequently reported. Purified chitinase of *F. chlamydosporum* inhibited the urediniospore germination of *P. arachidis* in a concentration dependent manner. The enzyme also lysed the walls of urediniospores (Mathivanan *et al.*, 1998). Degradation of hyphae of *S. rolfsii* in presence of chitinase of *S. marcescens* was observed. and 63% of the cells of the hyphal tips lysed (Ordentlich *et al.*, 1988).

4.6.4. Evaluation of NAGase for control of LLS: Chitinase-based preparations had varying levels of disease control in greenhouse and field. Foliar application of chitinase had a significant reduction in lesion frequency confirming chitinolysis as a major mechanism of action of GPS 5 in control of LLS. The crude culture filtrate of ***S.*** *marcescens* grown in minimal medium **with** colloidal chitin as a sole carbon source also had significant suppressive effects on the development of LLS. These observations support earlier report that a 4-fold decrease in the severity of midge blight and spur blight **of** raspberry was obtained by the foliar application of a chitinase preparation (Shternshis *el al.*, 2002). Chitinase E (family 19; class IV) from a yam *(Dioscorea opposila)* protected strawberry berries and leaves from powdery mildew infection, and the disease protection lasted for 2 weeks. Scanning electron microscopic observations indicated **the** degradation of the hyphae and conidia of *Sphaerolheca humuli* even in presence **of** 0.3 μM chitinase E. The enzyme did not cause undesired **effects** in **the** strawberry plants (Karasuda *el al.*, 2003). The results of these studies form a strong base for further evaluation of chitinases as eco-friendly fungicides. Foliar application of chitinases may be advantageous in terms of **public** acceptance.

Summary and Conclusion

5.1. **Background and objective:** Biocontrol agents effective in control of soil-borne fungal diseases were identified and used in agriculture. Biocontrol of foliar diseases met with limited success due to the poor survival of introduced biocontrol agents on the phylloplane. Attempts have been made for improved biocontrol of foliar diseases by nutrient supplemented application **of** the biocontrol agents. Integrated use **of** biocontrol agents along with the existing disease management technologies is desirable **to** achieve a stable biocontrol of foliar diseases. A combination **of** fungicide-tolerant biocontrol isolates and reduced concentration of fungicides was effective in control **of** plant diseases. Chitin is a major cell wall constituent in majority of the plant pathogenic fungi. Hence, chitinolytic biocontrol agents are expected to have a broad-spectrum antifungal and biocontrol activities. The main objective of this study is to select bacterial isolates effective in groundnut growth promotion and control of fungal diseases of groundnut, with an emphasis on late leaf spot (LLS) disease caused by *Phaeoisariopsis personata*. Fungicide tolerance and chitinolytic potential of selected biocontrol agents was utili/cd to improve biocontrol of groundnut LLS by bacterial isolates.

5.2. Collection of **bacterial** isolates: Three hundred and ninety three **groundnut-associated** bacterial isolates were used, of which 243 were collected from 55 farmer's fields in nine districts of Andhra Pradesh, during the present study. The bacterial isolates represented a wide range of habitats of the groundnut plant. The isolates were numbered using a suffix based on their source of isolation: GRS - rhizosphere, GPS - phyllosphere, GGS - geocarposphere, GSE - seed endophytes, GRE - root endophytes and GLE - leaf endophytes (G stands for groundnut).

5.3. **Plant** growth promoting activity of bacterial isolates: In greenhouse, 27 of the 393 bacterial isolates significantly ($P = 0.05$) increased the early plant growth and biomass. Three selected isolates, *Bacillus firmis* GRS 123, *B. megaterium* GPS 55 and *Pseudomonas aeruginosa* GPS 21 increased the root length, shoot length and biomass in a repeated field experiment. An increase of 15-20% in yield was observed following seed treatment with either of the three isolates.

There was a positive correlation between the *in vitro* siderophore production and growth promoting ability of the test isolates. No correlation was observed between auxin production and plant growth promoting activity of the isolates. The results **of greenhouse** and field experiments indicate that bacterial isolates from habitats like phylloplane and internal tissues were also effective, like rhizobacteria. in plant growth promotion when applied as seed treatment. It may be possible to get better plant growth promoting bacteria from parts of the plants other than roots.

5.4. Broad-spectrum antifungal activity of bacterial **isolates: Five** isolates *Pseudomonas* sp. GRS 175, and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30 were highly inhibitory to the eight groundnut pathogenic fungi - *Aspergillus flavus, A. niger, Cercospora arachidicola, Phaeoisariopsis personata, Puccinia arachidis, Rhizoctonia bataticola, R. solani* and *Sclertoium rolfsii.* Additionally, cell free culture filtrates **(CCF)** of these bacteria completely inhibited the mycelial growth or spore germination of the test fungi, except *for A. flavus.* The CCF were antifungal up to 10% (v/v) dilution. Growth of the test fungi in presence of culture filtrates resulted in cytoplasmic disruption, increased vacuolization, hyphal bulging and altered hyphal orientation.

5.5. **Biocontrol** of soil-borne diseases (stem rot and **crown** rot) of groundnut: The inoculation method used for *S. rolfsii* resulted in complete mortality of the control plants. Twelve bacterial isolates, which were antagonistic to *S. rolfsii* in vitro, reduced the stem rot incidence significantly *(P* = 0.01) compared to CMC treated control. *P. aeruginosa* GSE 18 and GSE 19 reduced the plant mortality by >50%. Eleven bacterial isolates, among which *P. aeruginosa* GSE 18 and GSE 19 were the most effective, significantly reduced the incidence of groundnut crown rot in greenhouse. *P. aeruginosa* GSE 18 had a considerable shelf life in peat (logic 6.5 cfu g^{-1}) up to 90 DAI when stored at 30°C and retained its biocontrol activity. CCF of *P. aeruginosa* GSE 18 and GSE 19 inhibited the production of cell wall degrading enzymes, pectin lyase, pectin methyl esterase, exo- and endo-polygalacturonases and carboxy methyl cellulase by *A. niger* and *S. rolfsii.* The inhibition of plant cell wall degrading enzymes production might be one of the

mechanisms of action of the two biocontrol isolates. *P. aeruginosa* GSE 18 applied as a seed treatment enhanced the activities of chitinase. β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase (PAL) in groundnut seedlings against crown rot infection augmenting the host resistance.

5.6. Biocontrol of LLS disease **of groundnut:** Fifteen bacterial isolates that inhibited the *in vitro* conidial germination *of P. personata* by >90% significantly reduced the severity of LLS in greenhouse. *P. aeruginosa* GSE 18 and GSE 19 reduced the lesion **frequeoc)** (number of lesions cm^{-2} leaf area) by >65% compared to control and were tolerant to **chlorothalonil** @ 2 mg ml^{-1} **(the** recommended dosage for field application). In greenhouse, chlorothalonil @ ≥250 μg ml^{-1} completely inhibited the development of leaf spot lesions, whereas similar reductions in lesion development were obtained by a combination of GSE 18 and 100 μg ml^{-1} chlorothalonil. In field, GSE 18 or **GSE** 19 combined with chlorothalonil @ 0.5 mg ml^{-1} and applied at 45. 60, 75 and 90 DAS resulted in an improved disease control. Combined application of **GSE** 18 and reduced chlorothalonil application increased the pod yield by ~95% compared to 120% by the recommended dose of chlorthalonil.

Chitin-supplemented application of the two antifungal chitinolytic isolates *Serratia marcescens* GPS 5 and *Bacillus circulans* GRS 243 reduced the lesion frequency by >60% in greenhouse. This treatment remained effective in field. Chitin-supplemented application of the chitinolytic strains significantly enhanced the survival of these isolates on the leaf surface as determined by rifampicin resistance as a marker. Also, chitin-supplemented peat formulations of GPS 5 and GRS 243 were effective in reducing the LLS lesion frequency up to 40% compared to the bacteria formulated in peat alone. *P. aeruginosa* GSE 18, *S. marcescens* GPS 5 and chitin-supplemented application of GPS 5 enhanced the activities of chitinase. glucanase, peroxidase and PAL in treated groundnut plants, the latter being the most effective.

5.7. **Characterization of chitinolytic enzymes of** *S. marcescens* GPS **5:** Chitin-binding

proteins were purified from the CCF of GPS 5 grown with colloidal chitin as a sole carbon source following affinity chromatography and gel filtration. Two chitin binding proteins of 21 kDa and 55 kDa molecular weights were detected in **SDS-PAGE** following gel filtration. Activity staining detected the chitinolytic action of the 55 **kDa** protein. The 21 kDa protein has no chitinase activity. N-terminal aminoacid sequence of a 21 kDa chitin binding protein. A H G Y V E X P A S R A X Qwas similar to the reported sequence of chitin binding protein of *S. marcescens.* The 55 kDa β-1,4-*N*-acetyl glucosaminidase lysed the conidia and urediniospores of *P. personata* and *P. arachidis.* This enzyme as a foliar spray reduced the lesion frequency of LLS up to 50% in greenhouse.

5.8. Major Findings of **the** present study:

- Bacterial isolates with broad-spectrum antifungal activity or **groundnut** growth promoting activity were identified among a collection of 393 groundnut-associated bacteria (Table 21).
- Bacterial isolates effective against stem rot. crown rot and LLS diseases of groundnut were identified (Table 21).
- A combination of fungicide tolerant biocontrol isolate and reduced concentrations of chlorothalonil. or chitinolytic bacteria and chitin improved LLS control both in greenhouse and field.
- The purified chitinase also protected groundnut against LLS.

5.9. Significance of the findings in the global context: The selection and characterization of broad-spectrum antifungal bacterial isolates inhibitory to the major fungal pathogens of groundnut, and simultaneous evaluation as biocontrol agents helped to identify useful strains for application in groundnut production systems. Earlier attempts for biocontrol of LLS mainly focused on the use of mycoparasites, which were successful in field only to a limited extent. In the present study, exploitation of fungicide tolerance and chitinolytic potential of the bacterial isolates provided an improved biocontrol of LLS, and such strategies are crucial in production systems where the application of fungicides alone are losing acceptability for sustained agriculture. Chitinase as a foliar application

provided protection against LLS and is worthwhile for further **evaluation as a new** generation fungicide **with** broad-spectrum **activity.**

5.10. Future Research:

- Fungicide-tolerant and chitinolytic biocontrol isolates to be evaluated in more fields at different locations for their commercial release.
- A combination of host-plant resistance and the identified biological **control** technologies can be evaluated for effective management of LLS.
- Chitinase based formulations can be developed **for** simultaneous control **of** LLS and rust.

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