Use of chitin and chitinolytic bacteria for control of late leaf spot of groundnut (*Arachis hypogaea* L.) with

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ABSTRACT

Chitinolytic *Bacillus circulans* GRS 243 and *Serratia marcescens* GPS 5, were selected among a collection of 393 groundnut-associated bacteria. Both the strains were applied as a foliar spray for biological control of LLS. Chitin-supplemented application of *B. circulans* GRS 243 and *S. marcescens* GPS 5 resulted in improved biocontrol of LLS disease. Supplementation with 1% chitin reduced the lesion frequency by 60%, compared to the application of bacterial cells alone, in greenhouse. Chitin-supplemented application also resulted in improved and stable control of LLS in field conditions tested both in on-station and on-farm trials.

Key words: biocontrol, chitin and chitinolytic bacteria

INTRODUCTION:

Biological control of foliar diseases received less attention, owing to the poor establishment of the introduced biocontrol agents and resulting variations in disease control. Maintenance of threshold populations of the introduced biocontrol agents on the phylloplane remained the focus of biocontrol research. Nutrient supplemented application of biocontrol agents augments the rate and time of suvival of the intorudced biocontrol agent in the phylloplane.

Chitin, a linear polymer of *N*-acetyl glucosamine (NAG), is selectively degraded by the chitinolytic organisms and used as a carbon source for their growth and multiplication. Chitinases inhibit the fungal spore germination, germ tube elongation (Majula et al. 2004), and lyse hyphal tips (Ordentlich et al. 1988). Chitinolytic microorganisms, therefore, may provide a broad-spectrum fungal disease control and also an opportunity to apply alongwith chitin for better survial of the introduced agents for foliar disease control.

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; Sleesman and Leben, 1976). Hence, the introduced antagonists lose their viability within a short duration and need to be reapplied frequently. However, chitin-supplemented application improved the performance of chitinolytic biocontrol agents in the phylloplane (Kokalis Burelle et al. 1992; Yuen et al. 2001).

Late leaf spot (LLS) disease caused by *Phaeoisariopsis personata* is the major foliar disease and major yield-reducing factor of groundnut or peanut (*Arachis hypogaea* L.) in India and some other countries of Asia and America. Yield losses of >50% were common due to LLS infection (McDonald et al. 1985). The present study was an attempt to isolate and select chitinolytic bacteria for LLS control and characterization of the chitinolytic ability of the selected strain. Groundnut-associated bacteria, with strong antifungal activity against *P. personata*, were isolated from different habitats of groundnut and screened for their ability to control LLS in the greenhouse. Selected strains were field-tested and their chitinolytic activity was partially characterized.

MATERIALS AND METHODS:

Microorganisms:

Single lesion isolate of *P. personata* collected from LLS infected plants from field grown plants was maintained on detached groundnut leaves (Subrahmanyam et al. 1995). Two bacterial isolates *Bacillus circulans* GRS 243 and *Serratia marcescens* GPS 5 selected for their chitinolytic and *in vitro* antifungal activity against *P. personata*, from a collection of 393 groundnut-associated bacteria (Kishore et al. 2003). GRS 243 was initially isolated from groundnut rhizosphere and GPS 5 from groundnut phylloplane.

Greenhouse testing of chitinolytic bacteria:

Foliar application with phosphate buffer alone and colloidal chitin suspension served as controls. After 24 h, the plants were challenge inoculated with a conidial suspension of *P. personata* (2×10^4 conidia ml^{-1}), using an atomizer. Inoculated plants were incubated in alternate wet (16 h) and dry (8 h) periods of leaf wetness up to 8 days after inoculation (DAI). Temperature was maintained at 24 ± 2°C. Each treatment consisted of 24 plants in three replications and the experiments were repeated twice.

Disease severity in different treatments was compared as a) lesion frequency (LF) - number of lesions cm^{-2} leaf area at 15 DAI, and b) disease score (DS) on a 1-9 rating scale (1 = no disease, and 9 = >80% disease) at 30 DAI. LF was scored on the third leaf from the top tagged before pathogen inoculation.

Greenhouse testing of the cell free culture filtrates:

GPS 5 and GRS 243 were grown in minimal medium with 1% colloidal chitin as sole carbon source for 144 h. The fermentation conditions were 30°C and 180 rpm. The cultures were centrifuged for 10 min at 10,000 rpm and 4°C. The supernatants were filter sterilized and applied as a foliar spray 24 h before *P. personata* inoculation for control of LLS.

Development and greenhouse evaluation of peat-based formulations:

Peat-based formulations of *S. marcescens* GPS 5 and *B. circulans* GRS 243 were prepared. Initial pH of the peat was 6.1 and adjusted to 7.0 by adding $CaCO_3$. The formulations were also supplemented with 1% colloidal chitin as a carrier material. The carrier material was packed in individual high molecular and high density polyethylene bags, and sterilized by autoclaving at 121°C for 20 min. Cells of GPS 5 and GRS 243 harvested from mid-log phase cultures in LB broth were resuspended in equal volume of 10 mM phosphate buffer, pH 7.0. The cell suspension was diluted 100-fold and aseptically added to the carrier material at 50% (v/w). Moisture loss of the formulations was compensated by the addition of SDW at regular intervals. Viability of bacteria in the carrier material was determined at frequent time intervals up to 180 DAI, by dilution plating and expressed as log CFU g^{-1}. Each treatment was replicated four times and the experiment was repeated twice.
For greenhouse evaluation of LLS control the formulations were suspended in 10 mM phosphate buffer, pH 7.0 for 30 min at a concentration of 10% (w/v) and filtered through a cheese cloth. The filtrate was applied as a foliar spray at 24 h before *P. personata* inoculation with sterile peat filtrate as control and the disease control was compared with fresh cells of each bacterium.

Field evaluation of the biocontrol isolates:

In each treatment, LLS susceptible cv. TMV 2 was planted in four rows of 9 m length with an intra- and inter-row spacing of 15 and 60 cms. After every four test rows, one infector row of cv. TMV 2 was planted. Three replications of each treatment were arranged in a completely randomized block design. *P. personata* inoculation and subsequent maintenance of leaf wetness required for disease development was provided according to Subrahmanyam *et al*. (1995).
Quantification of disease severity at regular intervals of 10 days starting from 45 DAS till harvest was done based on a 1–9 rating scale (Subrahmanyam et al. 1983). After harvest, the pods were sundried and the yield was recorded in each treatment and calculated to ha.

Phylloplane survival of the bacterial isolates:

Survival and multiplication of *S. marcescens* GPS 5 and *B. circulans* GRS 243 in groundnut phylloplane was determined by using rifampicin resistance as a marker. Spontaneous mutants of these rifampicin-sensitive bacterial isolates were obtained by plating the cell suspension (~10^9 CFU ml^{-1}) on nutrient agar added with 100 μg ml^{-1} and 50 μg ml^{-1} rifampicin, respectively. Mutants observed after an incubation of 96 h at 30°C were checked for stability of the rifampicin resistance by subculturing the mutants for 20 times on agar medium with 100 μg ml^{-1} rifampicin.

In field, at 60 DAS, stable mutants of GRS 243 and GPS 5 (10^8 CFU ml^{-1}) were applied as a foliar spray. To determine the populations of rifampicin resistant GPS 5 and GRS 243 at 24 h interval, four randomly selected leaflets from different plants were excised and suspended in 50 ml of 10 mM phosphate buffer, pH 7.0 and incubated for 1 h at 180 rpm and 30°C. Serial dilutions were plated on nutrient agar with 100 µg ml^{-1} rifampicin and the populations observed after 48 h were expressed as log CFU g^{-1} leaf. The observations were based on three plates in each dilution, and the experiment was repeated with three replications.

RESULTS

Greenhouse testing of chitinolytic bacteria:

Foliar application of GPS 5 and GRS 243 reduced the LF by 20.5% and 23.7%, respectively (Table 1).

Table 1. Greenhouse evaluation of peat-based formulations of chitinolytic isolates for control of late leaf spot of groundnut.

Isolate	Formulation[a]	Lesion frequency[b]	Disease score[c]
B. circulans GRS 243	mid-log phase cells	2.81±0.31[d]	9.0±0.0
B. circulans GRS 243	peat formulation	2.93±0.27	9.0±0.0
B. circulans GRS 243	chitin-supplemented peat formulation	1.63±0.12	6.0±0.4
S. marcescens GPS 5	mid-log phase cells	2.73±0.23	8.8±0.3
S. marcescens GPS 5	peat formulation	3.71±0.18	9.0±0.0
S. marcescens GPS 5	chitin-supplemented peat formulation	1.78±0.12	6.2±0.4
Control	sterile peat	3.89±0.24	9.0±0.0
LSD ($P = 0.01$)		0.44	0.67

[a] Ninety-day-old formulations were suspended in 10 mM phosphate buffer, pH 7.0 (10% w/v). The filtrate was applied as a foliar spray 24 h before the pathogen inoculation.
[b] Lesion frequency (number of lesions cm^{-1} leaf area) was measured 15 days after inoculation.
[c] Disease score on a 1-9 rating scale was measured 30 days after inoculation.
[d] Data points are the mean of nine replications from three sets of the experiment.

Chitin-supplementation significantly improved the biocontrol activity of both the isolates.

Greenhouse testing of the cell free culture filtrates:

Cell free culture filtrates of GPS 5 and GRS 243 grown in minimal medium with colloidal chitin as a carbon source, reduced LF by 58.4% and 55.5% compared to control (Fig. 1).

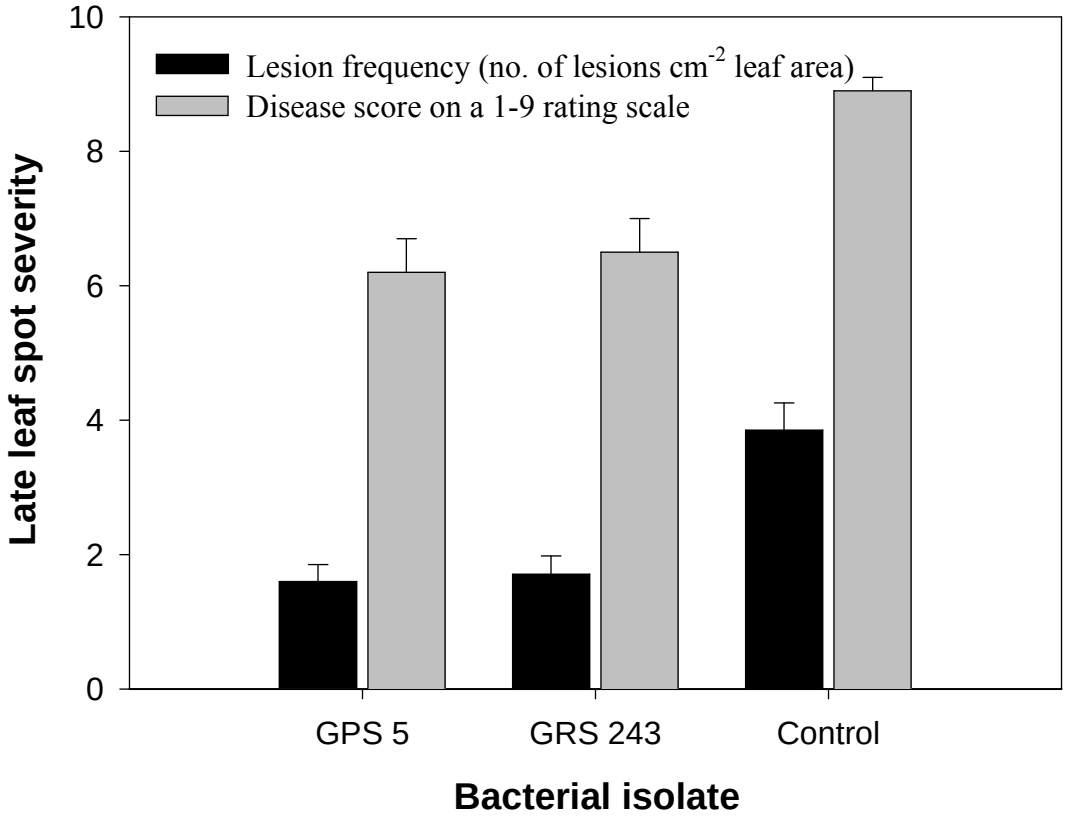

Fig. 1. Effect of cell free culture filtrates of chitinolytic *B. circulans* GRS 243 and *S. marcescens* GPS 5, on the development of late leaf spot of groundnut in greenhouse. Lesion frequency and disease score on a 1-9 rating scale were measured at 15 and 30 days after pathogen inoculation. Data points are the mean of nine replications.

Development and greenhouse evaluation of biocontrol formulations:

In peat formulation, GRS 243 had a good shelf life (log 7.4 CFU g^{-1}) up to 180 DAI, whereas GPS 5 was not detectable 120 DAI (Fig. 2).

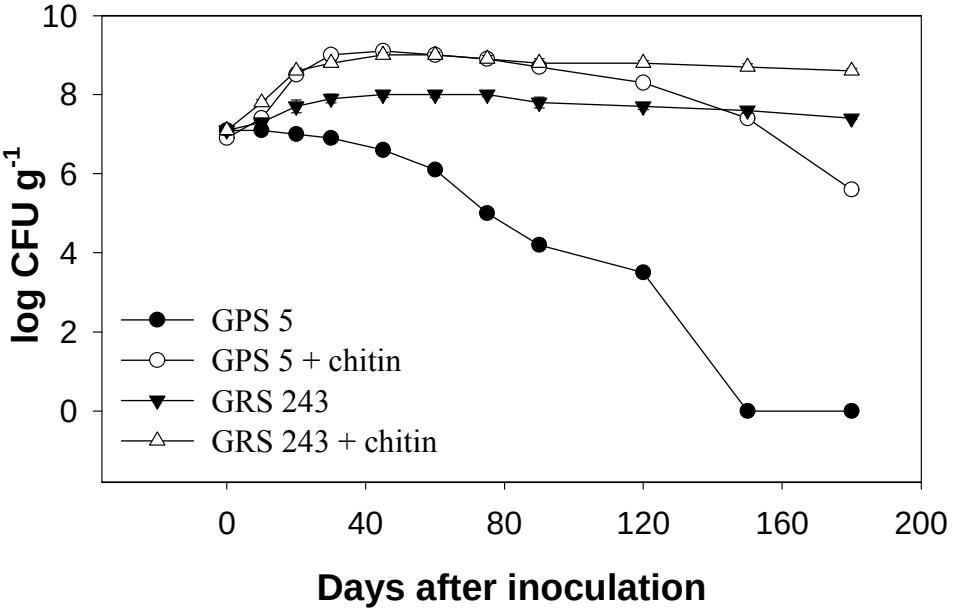

Fig. 2. Survival of chitinolytic *S. marcescens* GPS 5 and *B. circulans* GRS 243 in peat based formulations. For chitin supplementation 1% (w/v) chitin was added to neutralized peat. Data points are the mean of 12 replications in three sets of experiments.

Chitin-supplementation had a significant effect on the survival of both the isolates. The populations of GRS 243 increased from log 8.0 to log 9.0 CFU g^{-1}, and for GPS 5 from log 7.1 to log 9.1 CFU g^{-1}.

Field evaluation of the biocontrol isolates:

In both the crop seasons, LLS severity in *S. marcescens* GPS 5 and *B. circulans* GRS 243 treatments was insignificant from control (Fig. 3).

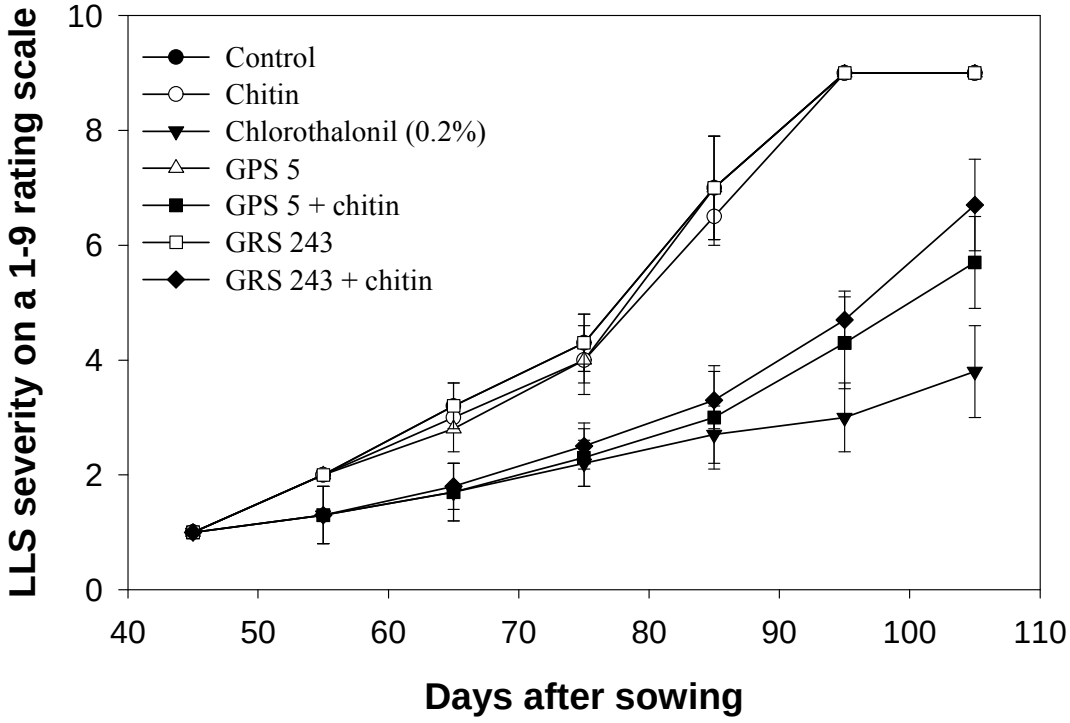

Fig. 3. Field evaluation of *S. marcescens* GPS 5 and *B. circulans* GRS 243 in supplementation with chitin (1% w/v) chitinolytic bacterial isolates for control of late leaf spot of groundnut. Disease score was measured on a 1-9 rating scale (Subrahmanyam *et al*., 1995) and the mean values of six replications from a repeated field experiment were presented.

Chitin-supplemented application of these two isolates resulted in improved control of LLS and the DS at 95 DAS was 4.3 and 4.7, compared to 9.0 in control.

Phylloplane survival of the bacterial isolates:

Chitin-supplementation enhanced the phylloplane survival of both GPS 5 and GRS 243 by >10-fold (Fig. 4).

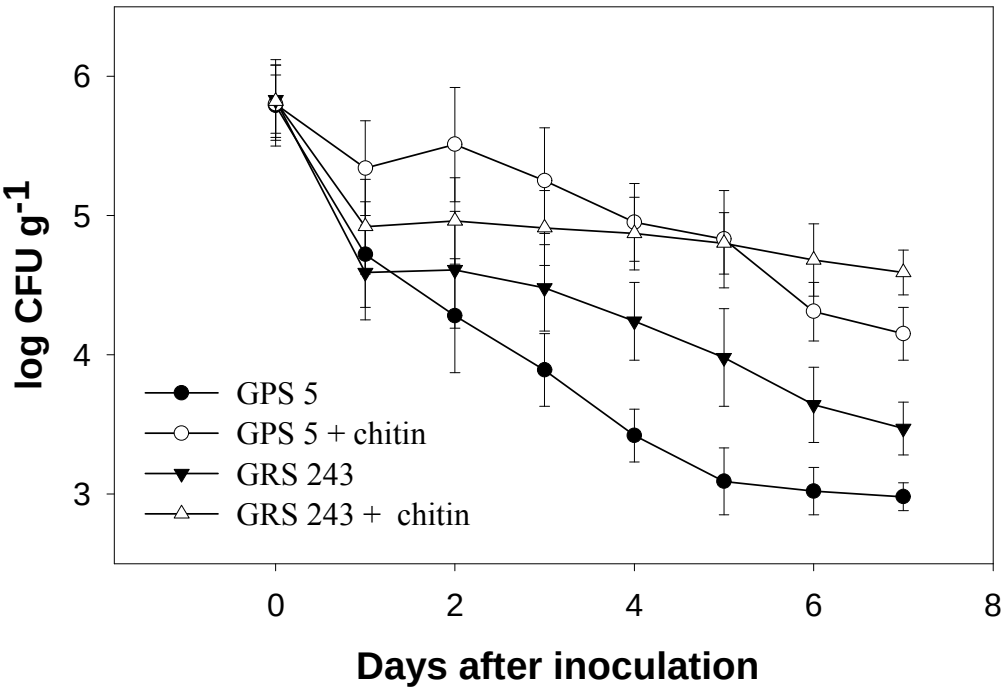

Fig. 4. Population dynamics of *B. circulans* GRS 243 and *S. marcescens* GPS 5 applied in supplementation with chitin, on the phylloplane of field-grown groundnut. Data points are the mean of six replications from a repeated experiment.

At 7 DAI, the populations of GPS 5 and GRS 243 applied in chitin supplementation were log 4.2 and log 4.6 CFU g^{-1} in comparison to log 3.0 CFU and log 3.5 CFU g^{-1}, when the bacterial cells were applied alone.

DISCUSSION

Chitin-supplemented application of *S. marcescens* GPS 5 and *B. circulans* GRS 243 resulted in improved control of LLS compared to the application of bacterial cells alone. Chitin-supplemented application of GPS 5 and GRS 243 increased their populations by >log 1.0 CFU g^{-1}. Presence of chitin improved the survival of chitinolytic *B. cereus*, which colonized and distorted the hyphae and spores of *C. arachidicola* (Kokalis Burelle et al. 1992). Improved biocontrol of LLS with an increase in the concentration of chitin, indicated a possible relation between the chitinolysis and disease control activities of both GPS 5 and GRS 243. 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. Chitin-supplementation has also been successful in enhancing the populations of chitinolytic organisms in the rhizosphere and improved control of soil-borne fungal and nematode diseases (Tian et al. 2000).

Chitin-supplementation improved the survival of *S. marcescens* GPS 5 and *B. circulans* GRS 243 in peat formulation. Chitin-supplementation enhanced the multiplication of *B. subtilis* AF 1 in peat, and the maximum cell density was >1.5 log units, compared to non-

amendment of chitin (Manjula and Podile, 2001). Chitin-supplementation improved the biocontrol efficacy of GPS 5 and GRS 243 compared to the bacteria formulated in peat alone. Thus, GPS 5 and GRS 243 pre-induced for chitinase production performed better in the phylloplane, compared to bacteria formulated in peat. Thus, chitin-supplemented peat as a carrier material facilitated an improvement in the populations of chitinolytic bacteria and also their biocontrol efficacy, and served as an ideal carrier for formulation of chitinolytic bacteria.

Field evaluation of the chitinolytic bacterial isolates, in supplementation with chitin, further confirmed the effectiveness of these treatments in LLS control. Supplementation of nutrients or other additives to the application of biocontrol agents is commonly practised to increase the survival of biocontrol agents in the phylloplane and biocontrol of foliar diseases. Chitin-supplemented application of *S. maltophilica* C3 improved the control of leaf spot of *Festuca arundonacea*, in field, compared to C3 alone (Zhang et al. 1999). Chitin-supplemented application of GPS 5 remained effective in control of LLS in the farmers' participatory on-farm evaluations. The increase in the pod yields obtained in this treatment was significant compared to the pod yields obtained with the application of chlorothalonil thrice. However, there is a need for search of cheaper sources of chitin for supplementation of both the formulations and foliar application of the chitinolytic isolates. The present study indicated the possibility of developing a suitable foliar spray for control of LLS of groundnut with chitinolytic bacteria. Chitinolytic ability of the bacteria can be exploited to develop improved formulations with increased shelf life and better survival on phylloplane.

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