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ORIGINAL ARTICLE

Chitin-supplemented foliar application of chitinolytic *Bacillus cereus* reduces severity of Botrytis gray mold disease in chickpea under controlled conditions

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Abstract

Aim: To identify and evaluate chitinolytic bacteria for control of Botrytis gray mold (BGM), a devastating disease in chickpea.

Methods and Results: Two antifungal bacterial isolates, chitinolytic *Bacillus cereus* CRS 7 and nonchitinolytic *Pseudomonas fluorescens* CRS 31, from the rhizosphere of chickpea, were applied as a prophylactic foliar spray and evaluated for control of BGM. In a controlled environment, the two isolates reduced the severity of BGM on the susceptible cv. JG 62 to 6·0 and 5·6, respectively, compared with 9·0 in the control, measured on a 1–9 rating scale. Supplementation of the foliar application of CRS 7 with 0·5% and 1·0% colloidal chitin reduced BGM severity to 4·4 and 4·1 respectively, while chitin-supplemented application of CRS 31 was similar to CRS 31 applied alone. Partially purified 47-kDa chitinase from the cell-free culture filtrate of CRS 7 at 20 and 40 μg protein ml^{-1} (enzyme activity 3·1 units ml^{-1}) inhibited the germination and lysed the conidia of *Botrytis cinerea*, and as a prophylactic foliar spray reduced BGM severity to 5·4 and 4·8, respectively.

Conclusion: Chitin supplementation improved the biocontrol of the foliar disease BGM by chitinolytic bacterium. Disease control with partially purified chitinase of CRS 7 supported the major role of chitinolysis in improved control of BGM.

Significance and Impact of the Study: Enhanced control of BGM by chitin-supplemented application of CRS 7 is of significant in view of the frequent inconsistency in biocontrol of foliar diseases. This study supports further attempts on chitinolysis-based biocontrol methods for foliar disease biocontrol.

Introduction

Chitin, an insoluble linear β-1,4-linked homopolymer of *N*-acetylglucosamine (NAG), is an important cell wall constituent in the majority of the phytopathogenic fungi (Bartnicki-Garica 1969). Chitinases (EC 3·2·1·14) selectively degrade chitin by hydrolysis of the β-1,4-glycosidic bonds that link NAG residues of chitin and form the basis for antifungal activity of a wide range of micro-organisms. Chitinase overproducing strains of bacteria obtained either by mutation (Limon *et al.* 1999) or recombinant DNA technology (Chen *et al.* 2004), were observed to exhibit improved antifungal activity. The role of chitinolysis in inhibition of fungal growth has been precisely identified in several studies. Inhibition of fungal growth and/or lysis of actively growing germ tubes and hyphal tips by purified microbial chitinases frequently has been reported (Manjula *et al.* 2004; Kishore *et al.* 2005c).

Several attempts have been made to utilize chitinase-producing micro-organisms as fungal disease protectants,

because they are expected to inhibit the growth of phytopathogenic fungi on plant surfaces. Interest in the utilization of chitinolytic micro-organisms in management of phytopathogenic fungi has grown as chitin supplementation was observed to increase the attainable levels of disease control. Chitin supplemented application of *Bacillus cereus* Frankland and Frankland, *Serratia marcescens* Bizio and *Stenotrophomonas maltophilia* (Hugh) Palleroni and Bradbury effectively controlled leaf spot diseases caused by *Cercospora arachidicola* Hori and *Phaeoisariopsis personata* (Berk. & Curt.) v. Arx in groundnut (*Arachis hypogaea* L.) (Kokalis-Burelle *et al.* 1992; Kishore *et al.* 2005c) and rust [*Uromyces appendiculatus* (Pers. ex Pers.) Unger] of bean (*Phaseolus vulgaris* L.) (Yuen *et al.* 2001), compared with the application of bacterial cells alone. In these studies, the presence of chitin increased the populations of introduced chitinolytic bacteria on the phylloplane. This observation is valuable for the control of foliar diseases, as biocontrol can be inconsistent because of the poor establishment of the introduced biocontrol agents on the phylloplane owing to fluctuations in temperature, moisture and nutrient availability and exposure to UV radiation (Beattie and Lindow 1999).

Botrytis gray mold (BGM), caused by *Botrytis cinerea* Pers. ex. Fr., is a devastating foliar disease of chickpea (*Cicer arietinum* L.) and can result in complete yield loss (Davidson *et al.* 2004). The disease is widespread because of the broad host range of *Bot. cinerea* and has been reported from almost all the chickpea growing areas of the world. Deployment of host plant resistance has a limited potential in BGM management, as high levels of resistance have not been identified in cultivated germplasm and because of the variable nature of *Bot. cinerea* populations (Davidson *et al.* 2004). Fungicidal control of BGM is expensive and development of fungicide tolerance has been reported frequently in *Bot. cinerea* (Leroux 2004). Thus, biological control was selected as the focus for economical and eco-friendly management of BGM. Earlier attempts for biocontrol of BGM were limited to the foliar application of *Trichoderma* spp., and seed treatment with *Trichoderma* spp. and *Gliocladium* spp. to reduce seed-borne inoculum (Davidson *et al.* 2004). Therefore, the present study was an attempt to test selected antifungal and chitinolytic bacterial strains from a collection of chickpea rhizobacteria for use in a foliar spray with and without chitin supplementation to control BGM. Further, the chitinolytic ability of *B. cereus* CRS 7 was characterized and partially purified chitinase was evaluated for its antifungal activity against *Bot. cinerea* and in the control of BGM, to determine the role of chitinolysis in control of BGM.

Materials and methods

Micro-organisms

Fifteen rhizosphere soil samples from chickpea plants in 10 different fields at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India were used for bacterial isolation. Five grams of the soil was added to 45 ml of 20 mmol l^{-1} of phosphate buffer, pH 7·0 and incubated in a shake culture for 1 h at 200 rev min^{-1} at 30°C. Serial dilutions of the suspension were plated on one-fourth strength Luria-Bertani (LB) agar in three replicates and incubated for 72 h at 30°C and 12-h photoperiod. In each sample, well isolated, individual colonies with distinct morphologies were selected from the appropriate dilution treatment. The selected bacterial isolates were subcultured on LB agar to check their purity and preserved as glycerol stocks at −70°C. A total of 63 bacterial isolates were obtained and designated as CRS (chickpea rhizosphere) and numbered from 1 to 63. Two selected bacterial isolates CRS 7 and CRS 31 were identified as *B. cereus* and *Pseudomonas fluorescens* Migula at Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology (Chandigarh, India).

A highly virulent strain of *Bot. cinerea* was isolated from BGM infected chickpea plants in Kurukshetra, India. A Single-spore isolate of the fungus was maintained on potato dextrose agar at 4°C.

Selection of antifungal and chitinolytic bacteria

In vitro antifungal activity

In vitro antifungal activity of the 63 rhizobacterial isolates against *Bot. cinerea* was determined by dual cutlure assay as described earlier (Kishore *et al.* 2005a). The inhibition zone between the bacterial and fungal colonies was measured 6 days after inoculation. There were three repetitions per experiment and the test was conducted three times.

Chitinolytic activity

The 63 chickpea rhizobacterial isolates were tested for degradation of chitin in minimal medium with colloidal chitin as the sole carbon source (Cantwell and Mc Connell 1983). Actively growing bacteria were spot inoculated on agar medium containing minimal medium with 1·5% colloidal chitin (Berger and Reynolds 1988) and incubated for 96 h at 30°C. Chitinolytic bacteria formed a clear zone around the colony by degradation of colloidal chitin and the diameter of lytic zone was measured in millimetre. There were three repetitions per experiment and the test was conducted three times.

Greenhouse evaluation of bacterial isolates for control of BGM

Bacillus cereus strain CRS 7, selected for its *in vitro* antifungal activity and chitinolytic ability, and nonchitinolytic *P. fluorescens* strain CRS 31, selected for its antifungal activity, were tested for the control of BGM under controlled environment conditions. The bacteria were applied as a prophylactic foliar spray, with and without supplementation of colloidal chitin, 24 h before *Bot. cinerea* inoculation. For foliar application, mid-log phase cultures of CRS 7 and CRS 31 grown in LB broth for 16 h at 30°C and 180 rev min^{-1} were pelleted by centrifugation at 3600 ***g*** for 5 min at 4°C, and suspended in 10 mmol l^{-1} phosphate buffer, pH 7·0 at a concentration of 10^8 CFU ml^{-1}.

Seedlings of chickpea cv. JG 62, highly susceptible to BGM, were raised in 12-cm-diameter plastic pots filled with vertisol clay and sand (3 : 1) in greenhouse at 25 ± 2°C and ~12-h photoperiod. Twelve-day-old seedlings were sprayed with *B. cereus* CRS 7 and *P. fluorescens* CRS 31 cell suspensions. In separate treatments, the cell suspensions were supplemented with 0·5% and 1·0% (w/v) colloidal chitin. Foliar application with the phosphate buffer, and 0·5% and 1% (w/v) colloidal chitin alone served as two different controls.

A 7-mm-diameter disc of an actively growing *Bot. cinerea* culture was inoculated into 50 ml of potato dextrose broth in 250-ml conical flasks. Inoculated flasks were incubated at 25°C for 7 days. The mycelial mat from each flask was homogenized in 100 ml of sterile distilled water using a waring blendor and filtered through cheesecloth. The concentration of conidia in the filtrate was adjusted to 3×10^5 ml^{-1} and used as inoculum. The seedlings were challenge inoculated with *Bot. cinerea*, 24 h after the bacterial treatment, using a hand-operated atomizer. Inoculated plants were partially air-dried for 30 min to minimize run-off of the inoculum and shifted to a growth room maintained at 20 ± 1°C and 100% relative humidity (RH). Disease severity was recorded on a 1–9 rating scale 15 days after inoculation (DAI). Details of the rating scale are as follows (Anonymous 1999): 1 = no infection on any part of the plant; 2 = minute water-soaked lesions on emerging tender leaves; 3 = minute water-soaked lesions on 1–5% of the surface of emerging and upper most leaves; 4 = water-soaked lesions prominent on 6–10% of the upper most tender leaves and tender shoots; 5 = water-soaked lesions, soft-rotting and sporulation of 11–25% of tender leaves and shoots; 6 = 26–40% of top leaves and shoots affected, and minute lesions on the mid-stem; 7 = soft-rotting and fungal growth on 41–55% of the leaves and branches, and prominent lesions on mid-stem; 8 = 56–70% of the leaves, branches and stems affected; 9 = extensive soft-rotting, fungal growth on more than 70% of the leaves, branches and stems. Each treatment consisted of 24 plants in three replications, and the experiment was conducted three times.

Partial characterization of chitinase(s) of *Bacillus cereus* CRS 7

Chitinase assay

Chitinase activity in cell-free culture filtrates and fractions of affinity chromatography was determined colorimetrically using colloidal chitin as the assay substrate as described by Boller and Mauch (1988). One unit of chitinase activity was defined as micromoles of NAG released per millilitre per hour under the assay conditions. The total protein in each sample was determined as described by Bradford (1976) and the specific activity, i.e. enzyme units per milligram of protein was calculated.

Partial purification of chitinase

Five hundred microlitres of an actively growing culture of *B. cereus* CRS 7 in LB broth was inoculated into 50 ml of a minimal medium (yeast extract −0·3 g, $MgSO_4.7H_2O$ − 0·3 g, KH_2PO_4−1·36 g, $(NH_4)_2SO_4$− 1·0 g, distilled water −1000 ml, pH adjusted to 7·0) with 0·5% (w/v) colloidal chitin as a sole carbon source and grown for 6 days at 30°C and 180 rev min^{-1}. Five hundred millilitres of the culture from different flasks was pooled and centrifuged at 14 500 ***g*** for 10 min to pellet the bacterial cells. The supernatant was filter-sterilized to obtain a cell-free culture filtrate (CCF). Proteins in the CCF were pelleted by adding $(NH_4)_2SO_4$ to 100% saturation. The pellet was dissolved in a minimal volume of ×1 phosphate-buffered saline (PBS) and dialysed against the same.

Chitinase/chitin binding protein(s), present in the dialysed fraction, were partially purified by affinity chromatography using acid swollen chitin as a matrix (Kishore *et al.* 2005c). Thirty grams of chitin swollen in 1 mol of HCl was extensively washed with distilled water followed by 20 mmol l^{-1} of sodium acetate buffer, pH 5·8, then packed into a column (10 × 2·5 cm) at 4°C and equilibrated with the same buffer.

Affinity chromatography

The dialysed protein sample from the CCF was loaded onto the affinity column at 20 mg protein g^{-1} matrix, and the flow rate was adjusted to 15 ml h^{-1}. The unbound proteins were eluted using 100 mmol l^{-1} of sodium acetate buffer, pH 5·8. The bound proteins were eluted using 100 mmol l^{-1} sodium acetate buffer, pH 3·6. One-millilitre fractions with maximum protein concentration, as determined by measuring the absorbance at 280 nm, were analysed for chitinase activity by colorimetry as mentioned

earlier and purity by sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). Enzyme activity of SDS-PAGE separated proteins was further confirmed using glycol chitin as a substrate, followed by calcofluor white M2R staining (Trudel and Asselin 1989).

Antifungal activity of *Bacillus cereus* CRS 7 chitinase

The 47-kDa partially purified chitinase of *B. cereus* CRS 7 was tested for inhibition of *in vitro* germination of conidia of *Bot. cinerea*. Thirty microlitres each of the *Bot. cinerea* conidial suspension (10^5 conidia ml^{-1}) and partially purified chitinase (final concentrations of 20 and 40 $\mu g\ ml^{-1}$) in 10 mmol l^{-1} of phosphate buffer, pH 7·0 were mixed thoroughly on a cavity slide. A mixture of conidial suspension and phosphate buffer was treated as control. The slides were incubated in a humid chamber in darkness at 25°C. The conidia were observed for germination and lysis under a light microscope after 8 h. One hundred conidia were observed in each of the three replications and the experiment was conducted three times.

Greenhouse evaluation of chitinase for control of BGM

Partially purified 47-kDa chitinase (20 $\mu g\ ml^{-1}$) of *B. cereus* CRS 7 and CCF of CRS 7 grown in minimal medium with chitin as the sole carbon source were applied as a foliar spray, 24 h before *Bot. cinerea* inoculation, to test for control of BGM in controlled environment as described earlier. In two other treatments, chitin-supplemented CRS 7 and CCF of CRS 7 were also applied as a foliar spray for comparison. Seedlings sprayed with 10 mmol l^{-1} of phosphate buffer pH 7·0, 24 h before *Bot. cinerea* inoculation, served as the control. The experiment was conducted with eight plants per treatment in each of the three replications, and repeated two times.

Data analysis

Data from individual experiments were subjected to analysis of variance (ANOVA) using the Genstat 5 statistical package (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). The mean values in each treatment were compared using least significant differences at 1% ($P = 0·01$) level of significance.

Results

Selection of antifungal and chitinolytic bacteria

Of the 63 bacterial isolates evaluated, 12 isolates inhibited the growth of *Bot. cinerea* in a dual culture assay. *Pseudomonas fluorescens* CRS 31 had the most potent antifungal activity among the test isolates, and produced an inhibition zone of 21 mm (Table 1). Eight of the 63 bacterial strains were observed to lyse colloidal chitin when grown in minimal medium. Four bacterial isolates CRS 7, CRS 16, CRS 27 and CRS 44 had both antifungal and chitinolytic abilities.

Greenhouse evaluation of bacterial isolates for control of BGM

The two test bacterial isolates *B. cereus* CRS 7 and *P. fluorescens* CRS 31 that were applied as a prophylactic spray significantly ($P = 0·01$) reduced the severity of BGM under controlled environment conditions (Fig. 1). The severity of BGM in CRS 7 and CRS 31 treatments was 6·0 and 5·6, respectively on a 1–9 rating scale, compared with 9·0 for the control. Supplementation of the chitinolytic isolate *Bacillus circulans* CRS 7 with colloidal chitin increased the inhibitory effect of this isolate on BGM, and disease severity was 4·4 and 4·1 when the colloidal chitin concentrations were 0·5% and 1% (w/v), respectively. BGM severity in plants treated with chitin-

Table 1 Evaluation of chickpea rhizobacterial isolates *in vitro* for antifungal activity against *Botrytis cinerea* and chitinolysis ($n = 9$)

Bacterial isolate*	Inhibition zone against *Bot. cinerea* (mm)†	Diameter of zone of chitinolysis‡
CRS 3	8·3 ± 0·7	0·0 ± 0·0
CRS 7	11·1 ± 1·4	6·9 ± 1·3
CRS 12	14·9 ± 0·6	0·0 ± 0·0
CRS 16	5·0 ± 0·7	4·0 ± 0·7
CRS 21	0·0 ± 0·0	9·2 ± 1·3
CRS 24	5·9 ± 0·6	0·0 ± 0·0
CRS 27	10·3 ± 0·9	3·0 ± 0·9
CRS 31	20·9 ± 1·3	0·0 ± 0·0
CRS 36	0·0 ± 0·0	8·0 ± 1·0
CRS 38	4·2 ± 1·1	0·0 ± 0·0
CRS 40	5·8 ± 0·8	0·0 ± 0·0
CRS 44	5·1 ± 0·8	4·2 ± 0·7
CRS 48	9·0 ± 1·2	0·0 ± 0·0
CRS 50	0·0 ± 0·0	4·1 ± 0·8
CRS 52	14·0 ± 1·9	0·0 ± 0·0
CRS 61	0·0 ± 0·0	5·3 ± 0·7

*Sixty-three bacterial isolates were evaluated for antifungal activity and chitinolysis, and isolates that possessed either activity were reported.

†The zone of inhibition between the bacterial and fungal cultures in a dual culture assay measured 6 days after inoculation.

‡Actively growing bacteria were spot inoculated on minimal medium with 1·5% colloidal chitin as sole carbon source. Inoculated plates were incubated at 30°C for 96 h, and the diameter of chitinolytic zone around the bacterial colony was measured.

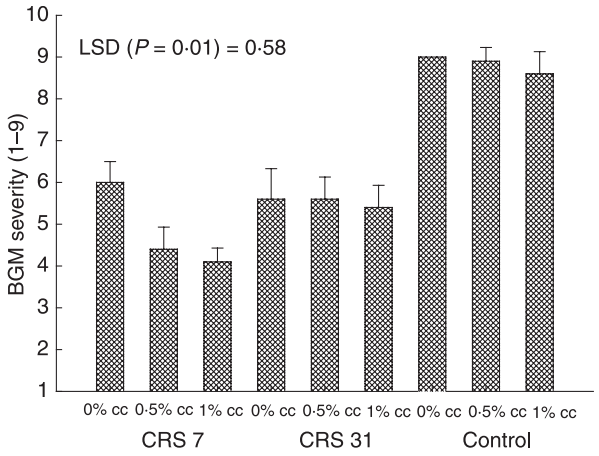

Figure 1 Effect of two antifungal bacterial strains, chitinolytic *Bacillus cereus* CRS 7 and nonchitinolytic *Pseudomonas fluorescens* CRS 31, applied with and without colloidal chitin (cc) supplementation on the severity of Botrytis gray mold (BGM) of chickpea in a controlled environment. The treatments were applied 24 h before the pathogen inoculation. Disease severity was measured on a 1–9 rating scale 15 days after pathogen inoculation. The standard error of mean of each value is represented as a vertical bar ($n = 9$).

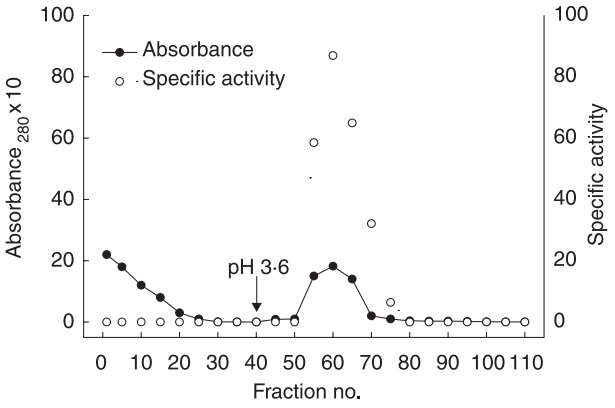

Figure 2 Elution profile of dialysed cell-free culture filtrate of *Bacillus cereus* CRS 7 in affinity column with processed chitin as matrix. One-millilitre fractions from the affinity column were analysed for protein content by measuring the absorbance at 280 nm and chitinase activity by colorimetric assay. One unit of chitinase activity was defined as micromoles of *N*-acetylglucosamine released per millilitre per hour under the assay conditions. Enzyme activity was expressed as specific activity, i.e. enzyme units per milligram of protein.

supplemented nonchitinolytic isolate of *P. fluorescens* CRS 31 was similar to that for CRS 31 alone. Disease severity in plants treated with chitin alone did not differ from that of phosphate buffer-treated control.

Partial characterization of chitinase(s) of *Bacillus cereus* CRS 7

Crude protein extract from CCF of *B. cereus* CRS 7 fractionated by affinity chromatography, using acid-swollen chitin as the matrix, resulted in a single protein peak (Fig. 2) that contained a prominent 47-kDa protein (Fig. 3a). The dialysed eluent fractions containing the 47-kDa protein had a chitinase-specific activity of 75·2 units with a purification fold of 9·2 times, and the enzyme recovery was 30·8% of the initial activity (Table 2). The chitinase activity of the prominent single band was further confirmed by lysis of glycol chitin under UV illumination after staining with calcoflour white (Fig. 3b).

Antifungal activity of *Bacillus cereus* CRS 7 chitinase

Partially purified chitinase of *B. cereus* CRS 7 was inhibitory to the germination of *Bot. cinerea* conidia. At both the concentrations tested, i.e. 20 and 40 μg ml^{-1} (enzyme activity of 1·5 and 3·1 units ml^{-1}), chitinase inhibited the conidial germination by 87% and 94%, respectively, and lysis of conidia and germ tubes was observed.

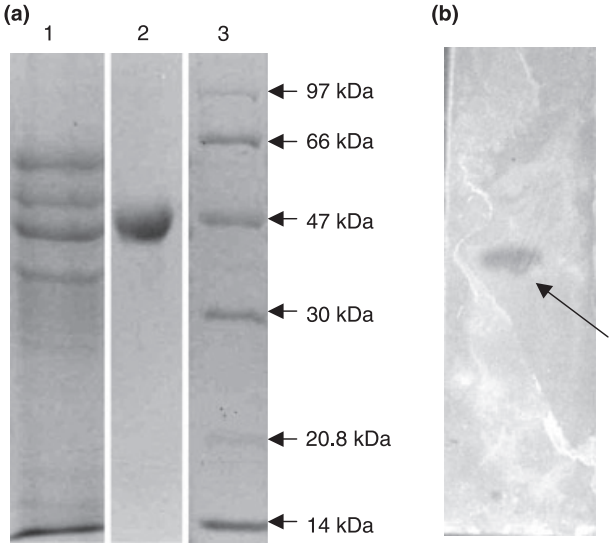

Figure 3 Analysis of the chitin-binding proteins from the culture filtrate of *Bacillus cereus* CRS 7: (a) Sodiumdodecylsulfate-polyacrylamidegel electrophoresis of eluents of affinity column, and (b) activity staining of 47-kDa chitinase of CRS 7. (a) lane 1, cell-free culture filtrate; lane 2, eluent of affinity column; lane 3, molecular weight marker.

Greenhouse evaluation of chitinase for control of BGM

Prophylactic foliar application of partially purified chitinase at both the concentrations, i.e. 20 and 40 μg ml^{-1} (1·5 and 3·1 enzyme units ml^{-1}) had a significant ($P < 0·001$) effect on the severity of BGM (Fig. 4). In

Table 2 Purification profile of a 47-kDa chitinase from the culture filtrate of *Bacillus cereus* CRS 7

Purification step	Total protein (mg)	Enzyme units*	Specific activity†	Purification fold	Yield (%)
Crude culture filtrate	568	4658	8·2	1·0	100·0
Fractions from affinity column	21·2	1622	76·5	9·3	34·8
Dialysed fractions	19·1	1436	75·2	9·2	30·8

*One unit of enzyme activity is the amount of enzyme that released 1 μmol of *N*-acetylglucosamine per millilitre per hour under the experimental conditions.
†Specific activity represents the enzyme units per milligram of protein.

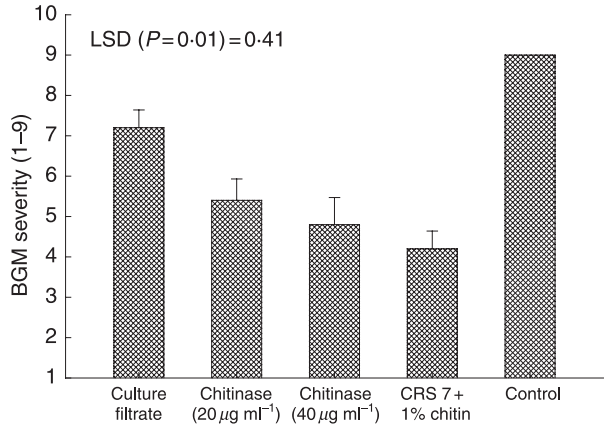

Figure 4 Controlled environment evaluation of partially purified 47-kDa chitinase of *Bacillus cereus* CRS 7 for control of Botrytis gray mold. In all the treatments, disease severity was measured on a 1–9 rating scale 15 days after pathogen inoculation. The vertical bars of each data point represent the standard error of the mean.

these two treatments, BGM severity was 5·4 and 4·8 compared with 9·0 in the control. Chitinase at a concentration of 40 μg ml^{-1} was comparable with chitin-supplemented application of CRS 7 in reducing the severity of BGM, while CCF of CRS 7 was less effective than chitin-supplemented CRS 7.

Discussion

The present study was an attempt to utilize the chitinolytic potential of antifungal bacteria to improve control of BGM on chickpea. Chitin-supplemented application of *B. cereus* CRS 7 enhanced the control of BGM compared with the application of bacterial cells alone, whereas foliar application of chitin alone had no effect on disease severity. Enhanced soil-borne and foliar diseases control by chitin-supplemented application of chitinolytic strains is known in several host–pathogen systems. Soil amendment with chitin increased the chitinolytic microbial populations and significantly reduced the incidence of fungal diseases in celery (*Apium graveolens* L. var. duice D.C.) (Bell *et al.* 1998). Chitin supplementation supported the survival of *B. cereus*, *B. circulans* Jordan and *S. marcescens* in the groundnut phylloplane and resulted in better control of early and late leaf spot diseases (Kokalis-Burelle *et al.* 1992; Kishore *et al.* 2005c). Chitin-supplemented application of *Sten. maltophilia* C3 improved the control of rust of bean caused by *U. appendiculatus* and leaf spot of tall fescue (*Festuca arundonacea* Schreb.) caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker, under field conditions (Zhang and Yuen 1999; Yuen *et al.* 2001). In all of these studies, improved disease control is associated with an increase in the populations of the introduced biocontrol agents in presence of chitin. The role of chitin supplementation in BGM control by *B. cereus* CRS 7 is further supported from the observation that the severity of BGM following chitin-supplemented application of nonchitinolytic *P. fluorescens* CRS 31 was similar to CRS 31 alone.

In a recent study, Kishore *et al.* (2005b) reported the rapid and enhanced activation of defense-related enzymes in groundnut leaves by chitin-supplemented foliar application of *S. marcescens* GPS 5, compared with GPS 5 alone. There is further need to search the mechanism in chickpea to determine if it includes the activation of defense-related enzymes that leads to broad-spectrum disease control.

Only a limited number of biocontrol agents for the control of BGM in chickpea have been identified (Davidson *et al.* 2004). In this context, identification of *B. cereus* for control of BGM and improvement in its efficacy by chitin supplementation could be of considerable significance in chickpea production. *Bacillus cereus* is known to produce antibiotics (Milner *et al.* 1995), in addition to chitinases, and is an effective biocontrol agent of foliar fungal diseases of tomato (Silva *et al.* 2004). Identification of inexpensive sources of chitin that are as effective as colloidal chitin for supplementation of foliar application with *B. cereus* is needed for sustainability of this application method. Chang *et al.* (2003) used the processed wastes of shrimp and crab shell powder as a carbon source to induce chitinase production in *B. cereus*.

Chitin supplementation is expected to induce production of extracellular chitinase(s) by *B. cereus* CRS 7 that degrades the cell wall content of the pathogen and inhibits

its growth. Induction of chitinase production in chitinolytic biocontrol bacteria is known to improve the control of fungal pathogens. Isolates of *B. circulans*, *Bacillus subtilis* (Ehrenberg) Cohn and *S. marcescens* that have been pre-induced for chitinase production by multiplication in chitin-supplemented peat formulations showed enhanced disease control activities in the rhizosphere and phylloplane of groundnut (Manjula and Podile 2001; Kishore *et al.* 2005c), compared with actively growing cells in LB broth or formulated in peat. Therefore, to evaluate the role of chitinolysis in improved control of BGM by chitin-supplemented *B. cereus* CRS 7, extracellular chitinase of CRS 7 was purified and used as prophylactic spray for disease control.

A single chitin-binding protein of 47 kDa with chitinase activity was obtained from the culture filtrate of CRS 7 grown in medium with crude chitin as the sole carbon source. *Bacillus cereus* is known to produce four different chitinases of 35, 47, 58 and 64 kDa in the culture supernatants, when grown in a medium containing colloidal chitin (Mabuchi *et al.* 2000). SDS-PAGE analysis revealed the monomeric nature of the partially purified 47-kDa chitinase. This enzyme inhibited the *in vitro* conidial germination of *Bot. cinerea* and also lysed the conidia and germtubes. Potent antifungal and lytic activities of chitinases against a range of phytopathogenic fungi are known. Examples of this phenomenon include lysis of conidia of *Puccinia arachidis* (Manjula *et al.* 2004), groundnut rust pathogen, and *Phae. personata* (Kishore *et al.* 2005c), groundnut leaf spot pathogen, by purified bacterial chitinases. The partially purified chitinase fraction, with no other detectable proteins on polyacrylamide gel, produced a reduction of BGM severity similar to chitin-supplemented CRS 7. This result indicates that chitinolysis has a major role in BGM control. In similar studies, purified chitinases of *Streptomyces* sp., yam (*Dioscorea opposite* Dhunb.) and *B. subtilis* AF 1 as a foliar spray reduced the severities of spur blight [*Didymella applanata* (Niessl) Sacc.] of raspberry (*Rubus idaeus* L.) (Shternshis *et al.* 2002), powdery mildew [*Sphaerotheca humuli* (de Candolle) Burrill] of strawberry (*Fragaria ananassa* Duch., Toyonoka) (Karasuda *et al.* 2003) and rust of groundnut (Manjula *et al.* 2004), respectively.

Mabuchi *et al.* (2000) reported that the 47-kDa chitinase of *B. cereus* had an optimum temperature of >50°C. Thermal stability of this enzyme is an advantage in developing chitinase-based formulations suitable for long-term storage and use in natural environments. Additionally, chitinase-based formulations are expected to reduce the difficulties associated with deployment of live micro-organisms for disease control and also offer broad-spectrum disease control.

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