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Chitin-supplemented Foliar Application of *Serratia marcescens* GPS 5 Improves Control of Late Leaf Spot Disease of Groundnut by Activating Defence-related Enzymes

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Abstract

Chitinolytic *Serratia marcescens* GPS 5 and non-chitinolytic *Pseudomonas aeruginosa* GSE 18, with and without supplementation of chitin, were tested for their ability to activate defence-related enzymes in groundnut leaves. Thirty-day-old groundnut (cv. TMV 2) plants pretreated with GPS 5 and GSE 18 (with and without supplementation of 1% colloidal chitin) were challenge inoculated after 24 h with *Phaeoisariopsis personata*, the causal agent of late leaf spot (LLS) disease of groundnut. GPS 5 and GSE 18, applied as a prophylactic spray, reduced the lesion frequency by 23% and 67%, respectively, compared with control. Chitin supplementation had no effect on the control of LLS by GSE 18, unlike GPS 5, which upon chitin supplementation reduced the lesion frequency by 64%, compared with chitin alone. In a time course study the activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase were determined for the different treatments. There was an enhanced activity of the four defence-related enzymes with all the bacterial treatments when compared with phosphate buffer and colloidal chitin-treated controls. In correlation to disease severity in bacterial treatments, chitin-supplemented GSE 18 was similar to GSE 18, whereas chitin-supplemented GPS 5 was much more effective than GPS 5, in activation of the defence-related enzymes. The high levels of enzyme activities following chitin-supplemented GPS 5 application continued up to the measured 13 days after pathogen inoculation.

Introduction

Phylloplane biocontrol is a complex phenomenon due to the poor establishment of the introduced biocontrol agents (Andrews, 1992). Nutrient and spatial competition, antagonism and activation of host defence responses are involved in the activity of biocontrol agents in the phylloplane. Extracellular chitinolytic enzymes produced by the biocontrol agents lyse chitin, one of the major constituents of fungal cell walls, hence chitinolysis has a significant role in management of phytopathogenic fungi. Chitin supplementation of *Bacillus cereus* and *B. subtilis* enhances the control of foliar diseases such as early leaf spot of groundnut (Kokalis-Burelle et al., 1992) and bean rust (Yuen et al., 2001). The role of chitinolytic organisms in disease control has been further substantiated by the use of partially purified chitinases as disease protectants (Karasuda et al., 2003; Manjula et al., 2004).

Different biocontrol agents, avirulent pathogens and abiotic elicitors activate an array of inducible defence reactions in plants, including the synthesis of lignin and phytoalexins, pathogenesis-related (PR) proteins and hydroxyproline-rich glycoproteins. Among the PR-proteins, β-1,3-glucanases (PR-2) and chitinases (PR-3) possess *in vitro* (Mauch et al., 1988) and *in vivo* (Zhu et al., 1994) antifungal activity. Peroxidase and phenylalanine ammonia lyase are key components in induction of local and systemic disease resistance (Kombrink and Somssich, 1995). Peroxidase is involved in cross-linking of extensin molecules to form lignin (Brisson et al., 1994), which strengthens the plant cell wall against fungal invasion. Phenylalanine ammonia lyase is associated with biosynthesis of phytoalexins, phenols, lignins and salicylic acid (Mauch-Mani and Slusarenko, 1996). Rapid activation of these enzymes results in protection of plants against fungal pathogens (Podile and Laxmi, 1998).

Change in the host defence activities after application of biocontrol strains against soil-borne (Podile and Laxmi, 1998; Sailaja et al., 1998), and foliar fungal diseases (Meena et al., 2000) have been reported. Detailed investigations on the factors that can accelerate and/or increase such activities to the benefit of the host plant are needed for efficient use of biocontrol strains for foliar disease control. Chitin oligomers and chitosan in

the phylloplane, enhanced groundnut resistance to rust pathogen (Sathiyabama and Balasubramanian, 1998). We made an attempt to see whether the application of a chitinolytic strain along with chitin can alter the host defence response of groundnut against late leaf spot (LLS; caused by *Phaeoisariopsis personata*) disease, an economically important disease of groundnut. Here, we report the effect of selected chitinolytic and non-chitinolytic biocontrol agents on the induction of defence-related enzymes, with and without chitin supplementation, in groundnut.

Materials and Methods

Two bacterial isolates, non-chitinolytic *Pseudomonas aeruginosa* GSE 18 (associated with groundnut seed), and chitinolytic *Serratia marcescens* GPS 5 (groundnut phylloplane isolate), were used in the present study. These two isolates have been selected for their *in vitro* antifungal activity against *P. personata* and control of LLS in the greenhouse (Kishore et al., 2003; Pande et al., 2003).

The effect of foliar application of mid-log phase cells (10^8 cfu/ml) of *P. aeruginosa* GSE 18 and *S. marcescens* GPS 5 suspended in 10 mM phosphate buffer, pH 7.0, with and without supplementation of 1% (w/v) colloidal chitin (Berger and Reynolds, 1988), on LLS severity and the activity of defence-related enzymes was quantified. Thirty-day-old plants of groundnut cv. TMV 2 (highly susceptible to LLS), grown in greenhouse at 28 ± 3°C and 12-h photoperiod were used in the experiments. Bacteria were applied to the foliage of 30-day-old plants using a hand-operated atomizer, and the plants were challenge inoculated 24 h later by foliar spray with *P. personata* conidial suspension in water (2×10^4/ml). Following pathogen inoculation, the plants were incubated in alternate wet (16 h) and dry (8 h) periods of leaf wetness up to 8 days after inoculation (DAI), by alternate incubation in dew chambers (Clifford, 1973) and greenhouse at 24 ± 2°C. In all the inoculated plants, the fourth leaf from the top was tagged before inoculation for measurement of lesion frequency (number of lesions/cm^2 leaf area) at 15 DAI. Each treatment consisted of 24 plants in three replications and the experiments were repeated twice.

Young leaves from four plants in each treatment were randomly excised at 1, 3, 5, 7, 9, 11 and 13 days after pathogen inoculation, to determine the activities of defence-related enzymes. Specific activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase were quantified in each treatment. The experiment was in three replications and repeated twice.

Two grams of leaf tissue was homogenized in 8 ml of extraction buffer (phosphate buffer, pH 6.8 added with 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ascorbic acid, 1 mM dithiothreitol and 950 mg sodium *m*-bisulphate/l) and centrifuged for 10 min at 14 500 g (4°C). The supernatant was filtered through cheesecloth, desalted by passing through a Sephadex G-25 column and used as crude enzyme extract.

For the chitinase assay, 500 μl of each of the enzyme extract, 10 mM sodium acetate buffer, pH 5.2, and 0.5% (w/v) colloidal chitin, were mixed and incubated for 3 h at 37°C. The reaction mixture was centrifuged for 2 min at 2000 rpm, and 20 μl of 3% (w/v) cytohelicase was added to 500 μl of the supernatant. The solution was incubated for 1 h at 37°C and used for estimation of aminosugars (Reissig et al., 1955). 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.

For quantification of β-1,3-glucanase activity, 62.5 μl each of enzyme extract and laminarin (4% w/v), and 200 μl of sodium acetate buffer, pH 5.2, were mixed and incubated for 10 min at 40°C. The amount of reducing groups released from laminarin was determined by using dinitro salicylic acid reagent with glucose as standard (Pan et al., 1991). One unit of enzyme activity was defined as the amount of enzyme that produced reducing groups equivalent to 1 μM of glucose in 10 min, under the assay conditions.

In the peroxidase assay, 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 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 (Shinishi and Noguchi, 1975).

The phenylalanine ammonia lyase assay reaction consisted of 300 μl of enzyme extract, incubated at 40°C with 600 μl of 0.25 M borate buffer, pH 8.8 containing 6 mM L-phenylalanine. The mixture was incubated for 2 h at 40°C and the product cinnamic acid was quantified following chloroform extraction (Beaudoin-Eagan and Thorpe, 1985). One unit of enzyme activity was calculated as the amount of enzyme that formed 1 mg cinnamic acid/h.

Each sample of protein extract was tested in duplicates and the enzyme activities determined were expressed as specific activity, i.e. enzyme units/mg of total protein, determined according to Lowry et al. (1951).

Data from different experiments were subjected to ANOVA using GENSTAT 5 statistical package. Mean values of different treatments were compared using least-significant difference (LSD) at 1% ($P = 0.01$) level of significance.

Results

In the greenhouse, *P. aeruginosa* GSE 18 and *S. marcescens* GPS 5, both applied as a prophylactic spray, significantly ($P = 0.01$) reduced the lesion frequency of LLS by 67% and 23%, respectively compared with phosphate buffer-treated controls, measured 15 DAI. The lesion frequency of LLS after foliar application of colloidal chitin was similar to water sprayed controls. Chitin-supplemented GSE 18-treated plants had the same lesion frequency of LLS as

GSE 18-treated plants. In contrast, chitin-supplemented application of *S. marcescens* GPS 5 reduced the lesion frequency by 64% compared with chitin-treated controls and by 67% compared with phosphate buffer-treated controls (Fig. 1).

There was a steady increase in chitinase activity up to 7 DAI in control and chitin-treated plants. In GSE 18 (with and without chitin supplementation) and GPS 5 application, the enzyme activity continued to increase up to 5 DAI, and was significantly ($P = 0.01$)

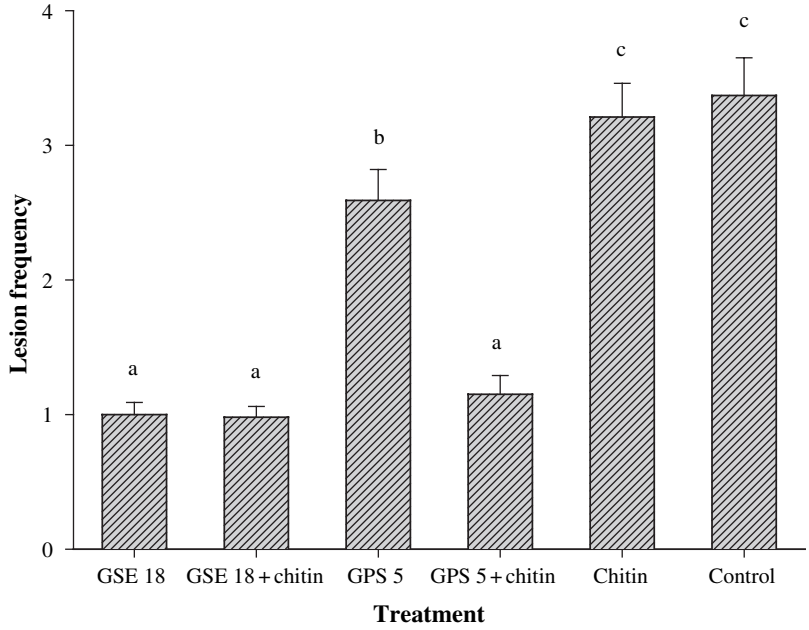

Fig. 1 Greenhouse evaluation of chitin-supplemented application of *Pseudomonas aeruginosa* GSE 18 and *Serratia marcescens* GPS 5 for control of late leaf spot disease of groundnut. Mid-log phase cells of the bacteria (10^8 cfu/ml), with or without supplementation of 1% colloidal chitin were applied as a foliar spray 24 h before *Phaeoisariopsis personata* inoculation. Disease severity was measured as lesion frequency (number of lesions/cm^2 leaf area) 15 days after inoculation. Data points are the mean of nine replications in three sets of the experiment. Individual bars denoted with the same letter are insignificant ($P = 0.01$) from each other

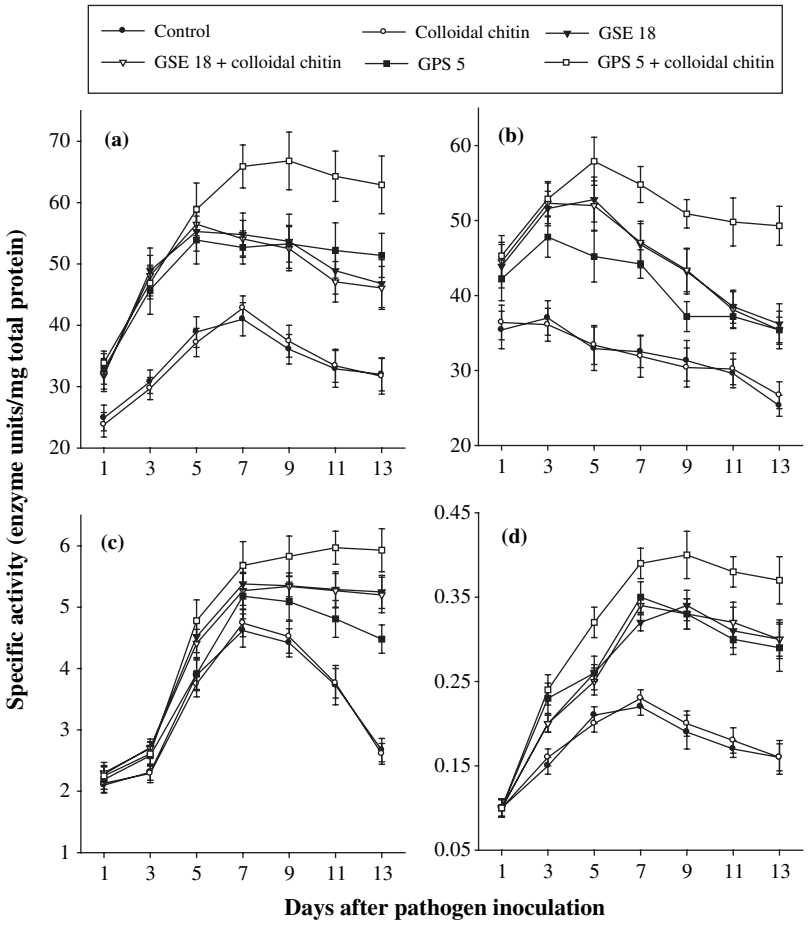

Fig. 2 Changes in the activities of defence-related enzymes of groundnut upon application of *Pseudomonas aeruginosa* GSE 18 and *Serratia marcescens* GPS 5: (a) chitinase, (b) β-1,3-glucanase, (c) peroxidase and (d) phenylalanine ammonia lyase. Mid-log phase cells of the bacteria (108 cfu/ml), with or without supplementation of 1% colloidal chitin were applied as a foliar spray 24 h before *Phaeoisariopsis personata* inoculation. Data points are the mean of nine replications in three sets of the experiment

higher than colloidal chitin and phosphate buffer-treated controls from 3 DAI. In chitin-supplemented application of GPS 5 the enzyme activity increased up to 9 DAI and was significantly higher than all other treatments from 7 DAI (Fig. 2a).

β-1,3-Glucanase activity of groundnut in GSE 18, and chitin-supplemented GPS 5 and GSE 18 treatments was significantly ($P = 0.01$) higher than chitin and phosphate buffer-treated controls from 24 h after pathogen inoculation. Maximum activity of β-1,3-glucanase was observed at 5 DAI in chitin-supplemented application of GPS 5 and this high activity was maintained up to 13 DAI (Fig. 2b).

In all the treatments, except for chitin-supplemented application of GPS 5, peroxidase activity increased up to 7 DAI and thereafter decreased in control, chitin and GPS 5 treatments, but remained the same in GSE 18 treatments. In chitin-supplemented GPS 5 treatment, peroxidase activity increased up to 11 DAI. GSE 18 (with and without chitin supplementation) and GPS 5 were equally effective in induction of the enzyme. Chitin-supplemented GPS 5 was significantly ($P = 0.01$) more effective than all other treatments, at 13 DAI (Fig. 2c).

Phenylalanine ammonia lyase activity increased up to 7 DAI in phosphate buffer and colloidal chitin-treated controls, 9 DAI in GSE 18 and chitin-supplemented GPS 5 treatments, and 7 DAI in GPS 5 and chitin-supplemented GSE 18 treatments. Starting from 3 DAI, the PAL activity in all the bacterial treatments was significantly ($P = 0.01$) higher than phosphate buffer and chitin-treated controls, with chitin-supplemented application of GPS 5 being the most effective (Fig. 2d).

Discussion

Pseudomonas aeruginosa GSE 18 applied as a foliar spray both reduced the lesion frequency of LLS and activated the defence responses of groundnut, as observed by a significant increase in chitinase, glucanase, peroxidase and phenylalanine ammonia lyase activities. These four defence-related enzymes are known to protect the plants from fungal attack either by direct inhibition of the pathogen or indirect activation of host defence responses including cell wall thickening (Zhu et al., 1994; Kombrink and Somssich, 1995). The observations of the present study are in agreement with reports on induction of systemic resistance in groundnut by foliar application of biocontrol agents/elicitors. *Pseudomonas fluorescens* pf1 induced the activities of phenylalanine ammonia lyase, phenolic compounds, chitinase and glucanase in groundnut (Meena et al., 2000). Induction of chitinase and β-1,3-glucanase activities was also observed in chitosan-treated groundnut plants after inoculation with *Puccinia arachidis* (Sathiyabama and Balasubramanian, 1998). Interestingly, a difference in the biocontrol potential of *S. marcescens* GPS 5 and *P. aeruginosa* GSE 18 was observed, although the bacteria did not differ much in activation of groundnut defence responses. This could be due to the potent antifungal activity and siderophore production of GSE 18 (Kishore et al., 2005) possibly coupled with its better colonization of the phylloplane compared with GPS 5. GSE 18 being broad spectrum antifungal (Kishore et al., 2005) and tolerant to chlorothalonil (Pande et al., 2003), a recommended fungicide for LLS control, can be a suitable biocontrol agent for control of LLS.

Improved control of LLS by chitin-supplemented application of *S. marcescens* GPS 5 is related to the enhanced and prolonged activities of defence-related enzymes of groundnut. Induction of chitinase production of *S. marcescens* GPS 5 by colloidal chitin also might have contributed for improved disease control by direct lysis of *P. personata* (unpublished results). Increase in enzyme activities of groundnut over a long period of time upon treatment with chitin-supplemented GPS 5 could be due to the prolonged survival of GPS 5 in the phylloplane in presence of chitin. Under field conditions, GPS 5 in presence of chitin had a better phylloplane survival, which was higher by 1.5 log cfu/g leaf compared with the absence of chitin, even at 10 DAI (Kishore et al., 2003). Elicitation by chitin breakdown products might also have contributed for the enhanced activities of defence-related enzymes in groundnut leaves. Although activation of groundnut defence responses by chitin oligomers has never been reported, they are known to elicit defence responses in other crops. *N*-acetyl chito-oligosaccharides induced various defence-related cellular responses including expression of PR genes (Takai et al., 2001) and biosynthesis of phytoalexins (Yamada et al., 1993), in suspension-cultured rice cells. Cell wall fragments of pathogenic fungi resulting from the action of chitinases induced PR-proteins in plants (Ryan and Farmer, 1991). Our earlier observations suggest the advantage(s) of chitin-supplemented application of chitinolytic biocontrol agents for improved biocontrol through activation of host plant resistance.

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