Role of chitinase and β-1,3-glucanase activities produced by a fluorescent pseudomonad and in vitro inhibition of *Phytophthora capsici* and *Rhizoctonia solani*

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Abstract: A study was conducted to investigate the possibility of involvement of chitinase and β-1,3-glucanase of an antagonistic fluorescent *Pseudomonas* in growth suppression of phytopathogenic fungi, *Phytophthora capsici* and *Rhizoctonia solani*. Fluorescent *Pseudomonas* isolates GRC_3 and GRC_4 were screened for their antifungal potential against phytopathogenic fungi by using dual culture technique both on solid and liquid media. The percent inhibition was calculated. Various parameters were monitored for optimization of enzyme activities by fluorescent *Pseudomonas* GRC_3. The involvement of chitinases, β-1,3-glucanases, and antifungal metabolites of nonenzymatic nature was correlated with the inhibition of *P. capsici* and *R. solani*. The results provide evidence for antibiosis as a mechanism for antagonism. The study also confirms that multiple mechanisms are involved in suppressing phytopathogens as evidenced by the involvement of chitinase and β-1,3-glucanase in inhibition of *R. solani* but not *P. capsici* by isolate GRC_3.

Key words: fluorescent *Pseudomonas*, phytopathogenic fungi, biocontrol, antagonism, biofungicide, chitinase, β-1,3-glucanase.

Résumé : Nous avons réalisé cette étude afin d'examiner la possibilité que la chitinase et la β-1,3-glucanase d'un *Pseudomonas* antagoniste fluorescent soient impliquées dans l'inhibition de la croissance de champignons phytopathogènes, le *Phytophthora capsici* et le *Rhizoctonia solani*. Les isolats fluorescents GRC_3 et GRC_4 issus de *Pseudomonas* ont été criblés relativement à leur potentiel antifongique envers les champignons phytopathogènes grâce à une technique de double culture en milieux liquide et solide. L'inhibition a été calculée (%). Différents paramètres d'optimization de l'activité des enzymes de *Pseudomonas* fluorescent GRC_3 ont été examinés. L'implication de la chitinase et de la β-1,3-glucanase dans l'inhibition de la croissance de *P. capsici* et *R. solani* a été évaluée. L'inhibition de la croissance de *P. capsici* était corrélée avec la production de métabolites antifongiques de nature non-enzymatique alors que l'inhibition de la croissance de *R. solani* était corrélée avec la présence de chitinase et de β-1,3-glucanase. Ces résultats démontrent que l'antibiose est responsable de l'antagonisme. Cette étude confirme également que de multiples mécanismes sont impliqués dans la suppression de la croissance de phytopathogènes tel que démontré par l'implication de la chitinase et de la β-1,3-glucanase dans l'inhibition de la croissance de *R. solani* par l'isolat GRC_3, ce qui n'est pas le cas pour *P. capsici*.

Mots clés : Pseudomonas fluorescent, champignons phytopathogènes, contrôle biologique, antagonisme, fongicide biologique, chitinase, β-1,3-glucanase.

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Introduction

Fluorescent pseudomonads are easily distinguishable from other rhizospheric bacteria owing to their inherent ability to produce water-soluble, yellow–green fluorescent pigments (Stanier et al. 1966). Considerable attention has been paid to fluorescent pseudomonads that are antagonistic, resulting in the exclusion of fungal pathogens in the rhizosphere. Many biocontrol strains of fluorescent pseudomonads produce extracellular secondary metabolites that inhibit the growth of fungal pathogens and account for part of the disease-suppressive activity. They produce antibiotics, phytohormones, and siderophores, among other compounds, which contribute to their beneficial role in host-plant growth (Gupta et al. 2001; Haas and Defago 2005). Microorganisms capable of lysing other organisms are widespread in natural ecosystems, but the rigid cell wall protects fungal hyphae and is considered the main barrier. Chitin and β-glucans are the principal components of fungal cell walls (Peberdy 1990; Ruiz-Dueñas and Martinez 1996). Species of *Pseudomonas* have been shown to excrete chitinases and β-1,3-glucanases to digest the hyphae of various fungi to provide a carbon source, and the products

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of digestion are used as energy sources (Gooday 1990; Lim and Kim 1995; Lee et al. 1997; Garbeva et al. 2004). The enzymatic digestion of the fungal pathogens by chitinases and β-1,3-glucanases could present an effective method for the biological control of fungal pathogens.

Many fluorescent pseudomonads, of which several have been modified genetically, have been reported to control the fungi causing plant diseases (Bainton et al. 2004). In the present study a natural isolate of fluorescent *Pseudomonas* strain GRC_3, possessing a wide range of plant-growth-promoting active substances, chitinases, and β-1,3-glucanases, was evaluated for suppression of the phytopathogens *Phytophthora capsici* and *Rhizoctonia solani*. These enzymes may play a major role in the suppression of phytopathogens.

Materials and methods

Fluorescent pseudomonads GRC_3 and GRC_4 were taken from the corresponding author's collection and were grown on tryptic soy agar medium (TSM), King's medium B, and Luria–Bertani (LB) medium at 28 °C. These fluorescent pseudomonads were isolated from the rhizosphere of potato plants and identified as members of genus *Pseudomonas* according to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994). The plant pathogenic fungi *P. capsici* and *R. solani* were obtained from the National Institute of Agricultural Science and Technology, Suwon, South Korea. The fungi were grown on potato dextrose agar (PDA) medium and potato dextrose broth (PDB). The plates were incubated and cultures in PDB were placed on a rotary shaker, both at 28 °C for 4 days.

Screening for antifungal and plant-growth-promoting characters of bacterial isolates

Siderophore activity was determined qualitatively using chrome azurol agar medium (CAS) and the $FeCl_3$ method (Schwyn and Neilands 1987). Production of hydrocyanic acid (HCN) was determined as previously described (Kremer and Souissi 2001), as was indole acetic acid (IAA) production (Bric et al. 1991). 1-Aminocyclopropane-1-carboxylate deaminase (ACC) activity was determined as described by Penrose et al. (2001) and phosphate solubilization ability was measured as described by Dubey and Maheshwari (2002).

Screening for antifungal activity

A dual culture test was performed to study antagonism between *Pseudomonas* isolates and fungal pathogens (Gupta et al. 2001). Bacterial culture (50 μL) was spotted in the center of a 90 mm diameter TSM plate and a 7 mm agar block obtained from 4-day-old cultures of *P. capsici* and *R. solani* were placed at 2 cm juxtaposed to the bacterial culture. Antibiosis of the test strain against the fungal pathogens was recorded for up to 5 days at 28 °C in terms of the diameter of the zone of inhibition. In each TSM plate, one bacterial strain was inoculated against one fungal pathogen. Five replicates of each set were taken. The mycelia of *P. capsici* and *R. solani* were periodically recovered for microscopic examinations as described by Gupta et al. (2001).

In vitro fungal inhibition assay

Pseudomonas isolates were grown on 100 mL of LB broth for 2 days at 28 °C. After centrifugation at 18 785*g* (12 000 r/min) for 30 min at 4 °C, the cells were separated and culture supernatant was filtered using a 0.45 μm membrane filter. Two millilitres of filtrate was mixed in 18 mL of molten PDA medium on which both fungal pathogens were grown separately. The antagonism against *P. capsici* and *R. solani* was tested as previously described (Han and Kim 1999). PDA media without bacterial supernatant acted as control. In another experiment, bacterial culture supernatant (100 mL) was dialysed against phosphate buffer (pH 6.0) at 4 °C for 12 h using a Spectro/Por cellulose dialysis membrane (Spectrum Laboratories, Rancho Dominguez, California, USA) as a source of lytic enzyme preparations (Cruz et al. 1992). Another 100 mL of culture supernatant was incubated with *Bacillus subtilis* protease (Sigma-Aldrich, St. Louis, Missouri, USA) at 37 °C for 24 h. In a third experiment, 100 mL of supernatant was heated at 100 °C for 1 h in a water bath. Later, fungal discs (7 mm) were cut from pregrown (4 day old) cultures of both *P. capsici* and *R. solani* and incubated separately with the protease-treated and heat-treated supernatants at 28 °C for 6 days to determine the inhibition ratio (fungal growth in treated samples and controls) and mycelial biomass (Lee and Kim 2000). In total, five replicates were taken for each set.

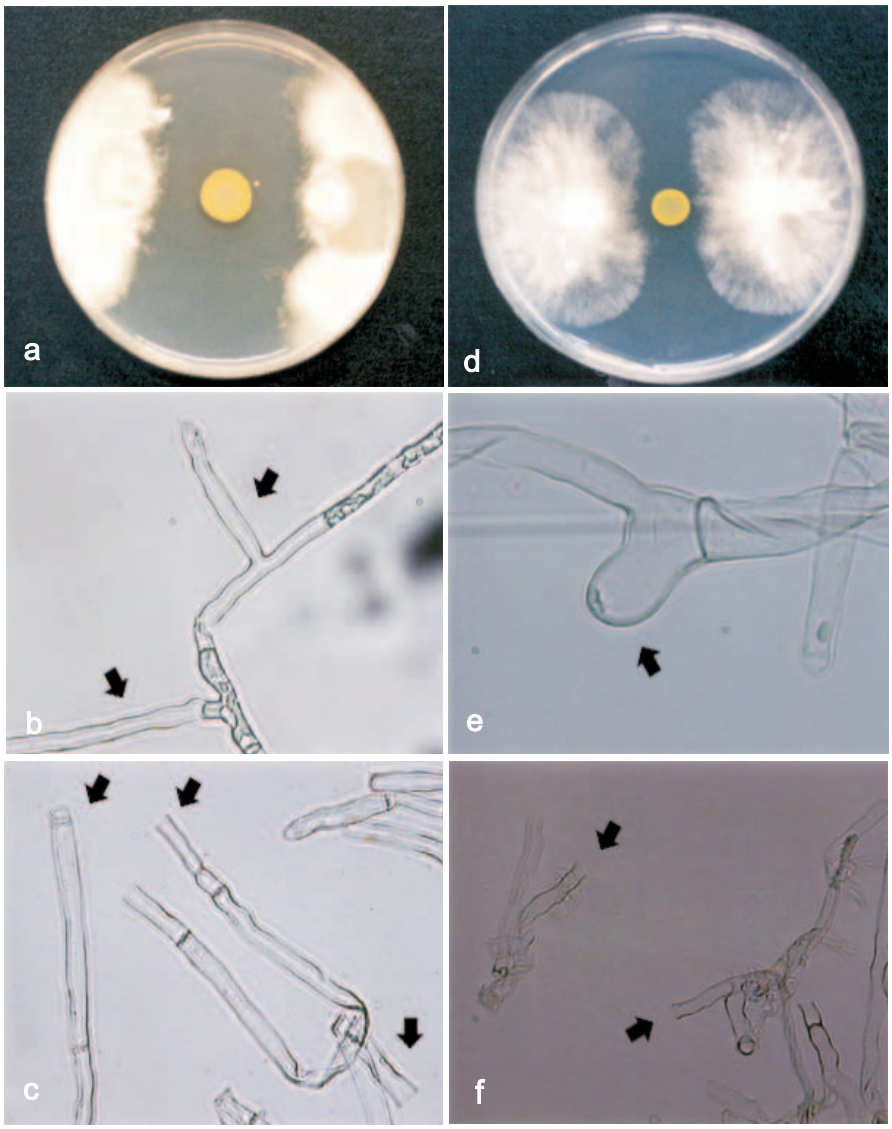
Effect of time course on chitinase and β-1,3-glucanase activities

Chitin (Sigma-Aldrich) and laminarin (Sigma-Aldrich) were used as substrates. Colloidal chitin was prepared from crab shells, which were powdered and digested overnight with concentrated hydrochloric acid at 4 °C. Digested chitin was washed repeatedly with distilled water to adjust the pH to 7.0. The chitin suspension was centrifuged and the pellet collected, dried, and washed (Berger and Reynolds 1958). For the preparations of crude chitinase and β-1,3-glucanase, bacterial isolates were grown in 100 mL of liquid LB medium at 28 °C for 1–7 days. The broth was centrifuged at 18 785*g* (12 000 r/min) supplied 20 min at 4 °C, and the supernatant was treated with ammonium sulphate up to saturation (100%). After standing overnight at 4 °C, the precipitate was dissolved in deionized water and then dialyzed against phosphate buffer (pH 6.0) using Visking dialysis tubing (Medicell International, London, UK). The dialyzed solutions were used as the source of enzyme (Lim and Kim 1990). The experiment was repeated five times.

Enzyme assays

Chitinase activity was determined by measuring the release of *N*-acetyl-D-glucosamine (NAGA) from colloidal chitin by the dinitrosalicylic acid method (Miller 1959). The assay mixture containing 0.5 mL of enzyme sample and 0.25 mL of 0.2% colloidal chitin in a 1 mol/L concentration of sodium acetate buffer (pH 5.5) was incubated at 37 °C for 1 h. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of NAGA/min. β-1,3-Glucanase enzyme was assayed according to Miller (1959). The reaction mixture was prepared by adding 0.15 mL of crude enzyme preparation and 0.25 mL of 0.1% (*m*/*v*) lami-

Fig. 1. Dual culture showing colony interactions. (*a*) *Pseudomonas* GRC_3 vs. *Phytophthora capsici*. (*b*) Degeneration of cell cytoplasm (*P. capsici*). (*c*) Lysis of hyphal fragments of *P. capsici*. (*d*) *Pseudomonas* GRC_3 vs. *Rhizoctonia solani*. (*e*) Intercalary hyphal swelling of *R. solani*. (*f*) Lysis of *R. solani* hypha.



narin in 0.25 mL of a 1 mol/L concentration of sodium acetate buffer (pH 5.5). The reaction was carried out at 37 °C for 1 h. One unit of β-1,3-glucanase was defined as the amount of the enzyme releasing 1 μmol of glucose/min under these conditions (Miller 1959).

Results

Pseudomonas isolates selected for the study were Gram-negative, motile, rod-shaped, aerobic bacteria, which produced fluorescent pigment, siderophore, HCN and IAA, solubilized phosphate, and showed ACC deaminase activity. Only isolate GRC_3 showed chitinase and β-1,3-glucanase activities. Isolate GRC_3 showed strong antagonism in dual culture against *P. capsici* and *R. solani* (Figs. 1*a* and 1*b*) (Table 1), whereas isolate GRC_4 (the negative control) could only antagonise *P. capsici.* However, inhibition of *P. capsici* by isolate GRC_4 was markedly less than that by GRC_3.

Table 1. Antifungal activity of fluorescent *Pseudomonas* GRC_3 and GRC_4 against phytopathogens after 5 days of incubation.

	Inhibition ratio* (%)	
Phytopathogen	GRC_3	GRC_4
Phytophthora capsici	49.8±0.47	12.1±0.25
Rhizoctonia solani	28.5±0.33	2.3±0.12

Note: Results are the means of five replicates ± standard deviation.

*Inhibition ratio (%) = [1 – mycelial growth of treatment (mm)/mycelial growth of control (mm)] × 100.

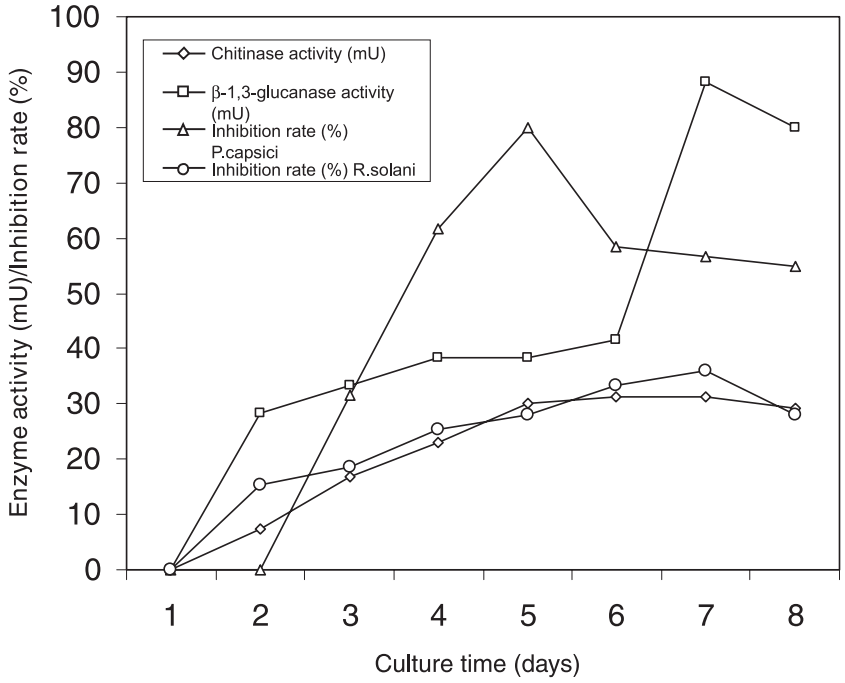
An increase in incubation time (in dual culture of GRC_3 and fungi) corresponded to an increase in the zone of inhibition up to 4 days in the case of *P. capsici* and 6 days in

Table 2. Percent inhibition of *Phytophthora capsici* and *Rhizoctonia solani* owing to fluorescent *Pseudomonas* GRC_3 after 6 days of incubation.

		Culture supernatant of *Pseudomonas* GRC_3		
Phytopathogen	Antagonism in dual culture	Dialysed	Protease treated	Heat treated
Phytophthora capsici	83.0±0.72a	23.6±0.27d	64.7±0.61b	50.8±0.48c
Rhizoctonia solani	36.0±0.31a	32.4±0.30a	4.9±0.19b	4.1±0.18b

Note: Means in each row followed by same letter are not significantly different (Duncan's multiple range test). Results are the means of five replicates ± standard deviation.

Fig. 2. Time course of changes in chitinase activity, β-1,3-glucanase activity, and antifungal activity during the culture of *Pseudomonas* sp. GRC_3 in Luria–Bertani medium at 28 °C.



R. solani. On further incubation, the fungal mycelia growing towards the interacting zone stopped and gradually lost vigour due to degeneration. Hyphae from the vicinity of the inhibition zone showed complete degeneration and failed to grow on fresh TSM plate. Alterations in structural features such as degeneration and lysis of mycelia in *P. capsici* and intercalary swelling of mycelial fragments and their lysis in *R. solani* (Figs. 1*b*, 1*c*, 1*e*, and 1*f*) led to morphological deformities finally resulting in inhibition (Figs. 1*a* and 1*d*).

The protease-treated and heat-treated culture supernatants did not suppress *R. solani* growth. However, dialyzed culture supernatant of GRC_3 exhibited significant inhibition similar to that of nontreated supernatant, suggesting involvement of enzymes. In the case of *P. capsici*, the protease-treated bacterial culture supernatants exhibited maximum inhibition, which was followed by heat-treated supernatant indicating significant involvement of nonproteinacious, heat labile substances, such as antibiotics, with less involvement of chitinases and β-1,3-glucanases (Table 2). Protease- and heat-treated supernatants of isolate GRC_4 also showed inhibition of *P. capsici.* When *Pseudomonas* GRC_3 was grown alone at different temperatures and pHs in King's medium B and LB medium, maximum chitinase and β-1,3-glucanase activities were observed in LB medium at 37 °C and pH 8.0. The glucanase activity was significantly higher than chitinase activity at different temperatures and pH. However, the level of the chitinase and β-1,3-glucanase activities increased with time and peaked at 6 days of incubation, but maximum inhibition of *P. capsici* and *R. solani* observed on day 4 and day 6, respectively, indicated the major involvement of these enzymes in the inhibition of *R. solani* (Fig. 2). Although *Pseudomonas* GRC_3 produced both the enzymes maximally at 6 days, they did not increase the inhibition rate of *P. capsici*, further confirming our earlier findings.

Discussion

Presently, *Pseudomonas* GRC_3 strongly inhibited the growth of the fungal phytopathogens *R. solani* and *P. capsici* as was evident by the clear zones of inhibition. Structural abnormalities in mycelia (from the zone of inhibition) of both the fungal pathogens by *Pseudomonas* GRC_3 were

mediated by antibiosis. Several workers have reported production of antifungal compounds by fluorescent pseudomonads that are responsible for cell wall lysis, suppression of fungi, and morphological deformities (Leah et al. 1991; Upadhyay and Jayaswal 1992). These metabolites include antibiotics, HCN, iron-chelators, and cell-wall-degrading enzymes (Gupta et al. 2001; Cherinin and Chet 2002).

The cell wall of *R. solani* is rich in β-1,3-glucans and chitin (Bartnicki-Garcia 1968; Benhamou et al. 1993), supporting the concept that the production of β-1,3-glucanases by a co-cultured organism might be sufficient to inhibit the ability of this fungus to grow normally. The results also show that the inhibition of *R. solani* peaks when the synthesis of glucanase and chitinase is at maximum by the pseudomonad. Because the cell wall of *Phytophthora* lack chitin, chitinases alone would be unlikely to affect growth (Griffin 1985). Most bacterial chitinases cannot inhibit growth of fungi completely (Robbins et al. 1988) and susceptibility of fungi to inhibition by chitinolytic bacteria is either due to bacterial antibiotic production (De Boer et al. 1998) or due to the combined action of antibiotics and lytic enzymes (Belanger et al. 2002). During the present study, we found the variable susceptibility of *P. capsici* and *R. solani* to inhibition by *Pseudomonas* GRC_3 to be correlated with antifungal metabolite production of both an enzymatic and nonenzymatic nature as observed with other chitinolytic bacteria (Fridlender et al. 1993). This was further confirmed by the results that isolate GRC_4, which was negative for enzymes chitinases and glucanases but positive for certain secondary metabolites like siderophore and HCN, inhibited the growth of *P. capsici* to a lesser extent than did isolate GRC_3.

The broad range of antifungal activity of fluorescent *Pseudomonas* GRC_3 demonstrates that multiple mechanisms of action may involve more than one antifungal metabolite. Similar findings were reported in the case of *Serratia plymuthica* against *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Kamensky et al. 2003). The present results provide evidence for antibiosis as a mechanism for antagonism because the fluorescent *Pseudomonas* GRC_3 causes significant inhibition of both the pathogenic fungi *P. capsici* and *R. solani*. This bacterial strain demonstrates potential for further testing as an antifungal agent in field trials.

Biocontrol as an economically viable addition to crop pest control is attracting major research interest, as the environmental, economic, and functional capabilities of traditional chemical fungicides and fumigants are currently being questioned (Bainton et al. 2004). However, the problem with biocontrol agents is their inconsistency. Determination of exact mechanisms of action of biocontrol agents can assist in furthering the use of ecofriendly biofungicides in a replicated manner in the field.

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