

Effects of chitin and salicylic acid on biological control activity of *Pseudomonas* spp. against damping off of pepper

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Received 1 February 2007; received in revised form 28 November 2007; accepted 29 November 2007

Abstract

Fluorescent pseudomonads (SE21 and RD41) and resistance inducers (chitin and salicylic acid) were examined for plant growth promotion and biological control of damping off of pepper caused by *Rhizoctonia solani*. The antagonists SE21 and RD41 isolated from the rhizosphere of pepper were found to be effective in inhibiting the mycelial growth of *R. solani* in a dual culture assay and increasing the seedling vigour in a roll towel assay. Both antagonists were further characterized for biocontrol and plant growth promoting features. The addition of inducers (chitin alone) increased the antagonist's population in the culture medium. In a further study, seed treatment with antagonists showed an increase in plant growth and controlled the damping off under *in vivo* conditions. Amendment with inducers alone showed a moderate degree of plant protection against *R. solani*. However, the reduction in disease was more pronounced when inducers were applied with antagonists. Amendment with chitin alone enhanced biocontrol efficiency of both SE21 and RD41. However, amendment with SA alone or in conjunction with chitin showed a moderate effect on biocontrol efficiency of the antagonists. These results show that the biocontrol efficiency of antagonists SE21 and RD41 may be stimulated by chitin resulting in a significant increase in their population density and antagonistic effect against *R. solani*.

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Keywords: Biocontrol; Chitin; Damping off; Pepper; Salicylic acid; Siderophores

1. Introduction

Red pepper is considered as an important tropical and subtropical crop on the basis of its high consumption, nutritional and cash value to farmers and consumers in Korea and worldwide. Although, it is cultivated in several parts of Korea, its productivity is very low due to fungal infections. Pre and post emergence damping off is one of the main diseases caused by *Rhizoctonia solani* in pepper. Damping off usually cannot be prevented by crop rotation or by the development of resistant crop varieties. In spite of promising results obtained by some chemical treatments in controlling damping off, phytotoxicity and chemical residue are major problems leading to environmental pollution and human health hazards. Thus, alternative control measures for the control of damping off should be developed. Biological control is proposed to be an

effective and non-hazardous strategy to reduce crop damage caused by plant pathogens. In recent years the fluorescent pseudomonads have been extensively used for plant growth promotion and disease control. Several mechanisms have been suggested for disease control by fluorescent pseudomonads involving production of siderophores, HCN, ammonia, antibiotics, volatile compounds etc. or by competing with pathogens for nutrients or colonization space (Thomashow and Weller, 1996). In addition, fluorescent pseudomonads can trigger a plant-mediated resistance mechanism called induced systemic resistance (ISR; Pieterse et al., 2001).

Biological control of soil borne pathogens is often attributed to improved nutrition that boosts host defenses or to direct inhibition of pathogen growth and activity. Amendment with certain abiotic factors (inducers) appears to stimulate the disease resistance by indirectly stimulating indigenous populations of microorganism that are beneficial to plant growth and antagonistic to pathogens. For example chitin amendment of soil has been found to stimulate the growth of chitinolytic microorganisms (De Boer et al., 1999;

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Manjula and Podile, 2001), increase the biocontrol activity and stimulate the expression of plant defense proteins (Roby et al., 1987). All these effects may culminate in enhancing plant protection. Similarly, salicylic acid (SA) amendment was tested in combination with biocontrol agents. Saikia et al. (2003) tested the efficiency of *P. fluorescence* with or without SA amendment in chickpea against *Fusarium* wilt infection. The application of *P. fluorescence* (pf4-92) with SA recorded highest protection of chickpea seedlings against wilting.

Thus, the aim of this study is to (i) isolate and screen fluorescent pseudomonads capable of inhibiting the growth of *R. solani* and promoting the growth of red pepper, (ii) characterize isolates for auxiliary activities including production of biocontrol and plant growth promoting metabolites and (iii) determine the biocontrol efficiency of fluorescent pseudomonads, alone and in combination with inducer (chitin and SA) amendment against damping off of pepper.

2. Materials and methods

2.1. Isolation of fluorescent pseudomonads

Fluorescent pseudomonads were isolated from rhizosphere of pepper grown in an agriculture field at Jinan, South Korea, as detailed by Rajkumar et al. (2005). Fluorescence of the colonies under UV light was taken as the primary criterion for selection of the isolates. A virulent strain of *R. solani* was isolated from an infected pepper plant and maintained on either potato dextrose agar (PDA) or corn meal agar (CMA).

2.2. Screening of fluorescent pseudomonads

In order to isolate the plant growth promoting antagonists, all the fluorescent pseudomonads were screened for their ability to inhibit the growth of *R. solani* on PDA and CMA plates using the dual culture technique (Yoshida et al., 2001). A mycelial plug of *R. solani* (1 cm diameter) was placed at the center of the agar medium in a 90 mm Petri plates. Four spots were made on the edge of the plate with an actively growing suspension of the bacterial isolate after 48 h of fungal inoculation. Plates were incubated for four to seven days at 24 °C and the inhibition of fungal growth was assessed by measuring the diameter of the inhibition zone (mm). Further, the plant growth promoting efficiency of antagonists was assessed in a roll towel assay based on seedling vigour index. The pepper seeds were surface sterilized in 1% sodium hypochlorite for 30 s and rinsed several times with sterile water. The seeds were inoculated by soaking in a bacterial suspension containing 10^8 cells/ml for 1 h then placed in wet blotters and incubated in a growth chamber for 20 days. The vigour index was calculated by using the formula as described by Abdul Baki and Anderson (1973). Vigour index = (Mean root length + Mean shoot length) × germination (%).

2.3. Characterization of biocontrol features

The method to detect siderophore production was based on that described by Schwyn and Neilands (1987). Cultures of the

isolates were raised in M9 minimal medium at 30 °C for 48 h at 150 rpm. These were centrifuged at $10,000 \times g$ for 10 min, and 50 μ l of supernatant were added to 0.95 ml of chrome azural S (CAS) solution. After 30 min, the absorbance of the solution was measured at 630 nm. Production of HCN was observed according to the method of Meena et al. (2001). β -1,3-glucanase activity was determined by measuring the production of reducing sugars from laminarin. The standard assay contained 0.25 ml of enzyme solution, 0.3 ml of 0.1 M phosphate buffer (pH 5.5) and 0.5 ml of laminarin (0.2%). The reaction mixture was incubated for 2 h at 40 °C. Total reducing sugars were assayed by a colorimetric method and expressed as 1 nM of glucose released per minute per mg of protein. Chitinase activity was determined by measuring the release of reducing sugar by the method of Nelson (1944). The reaction mixture contained 0.25 ml of enzyme solution, 0.3 ml of 1 M sodium acetate buffer (pH 5.3) and 0.5 ml of colloidal chitin (0.1%). The reaction mixture was incubated for 4 h at 50 °C. One unit of chitinase was determined as 1 nM of GlcNAc released per minute per mg of protein.

2.4. Characterization of plant growth promoting features

IAA production by antagonists was determined according to the method of Gordon and Weber (1951). Cultures of the isolates were raised in tryptic soya broth amended with L-tryptophan at 30 °C for 48 h at 150 rpm. Cells were removed by centrifugation at 4000 rpm and the supernatant was assayed for IAA production. The phosphate solubilizing activity of the isolates was analyzed in NBRIP medium (Nautiyal, 1999) amended with tricalcium phosphate. The isolates were grown at 30 °C for 5 days at 150 rpm. The solubilized phosphate in the culture supernatant was quantified as detailed by Fiske and Subbarow (1925).

2.5. Effect of inducers on the growth of antagonists

The effect of inducers on the growth of antagonists was carried out in King's medium B (KMB). 20 ml of KMB broth containing 0.5% chitin (Berger and Reynolds, 1988), 0.2 mM SA or 0.5% chitin + 0.2 mM SA was inoculated with 10 μ l of starter culture. Cultures were incubated at 27 °C with shaking at 150 rpm. After 24 h the number of colonies was counted by serial dilution plate technique.

2.6. Effect of inducers on biocontrol efficiency of antagonists

The effects of chitin and SA on biocontrol efficiency of antagonists against damping off of pepper were evaluated under greenhouse conditions. Pepper seeds were surface sterilized and inoculated by soaking them for 1 h in a bacterial suspension containing 10^8 cells/ml. Seeds soaked in sterile water were used as control. The inoculated and non-inoculated seeds were planted in 12 cm diameter pots with soil mix (soil:peat:perlite; 1:1:1) and incubated in a greenhouse at 22 °C. Inoculation of *R. solani* was prepared by following this method: 10 agar discs (1 cm diameter) from an actively growing fungal culture were mixed with 100 g of sterile barley seeds and incubated for 10 days at 24 °C. Seedlings

Table 1
Efficiency of fluorescent pseudomonads on inhibition of growth of *R. solani* and vigour index of red pepper

Isolate	Zone of inhibition (mm)		Vigour index
	PDA	CMA	
SE21	7.5±0.62	8.0±0.81	785.0±18.0
RD41	9.5±0.78	9.3±0.57	824.6±17.7
Blank	–	–	630.0±18.0

Results are an average of three replicates.

± Standard deviation.

at four-leaf stage were planted in soil mixed with ten infected barley seeds. Aqueous solutions of inducers such as 0.5% chitin, 0.2 mM SA or 0.5% chitin+0.2 mM SA were prepared in deionized water and added to each pot (20 ml) before sowing red pepper seeds (12 seeds/pot). The percentage disease incidence and disease severity were recorded on the 50th day after sowing. Disease severity was assessed using a 0–5 scale: 0, no symptoms; 1, <10% diseased roots; 2, 11 to 30% diseased roots; 3, 31 to 60% diseased roots; 4, 61 to 90% diseased roots; 5, plant dead. Plant growth was measured by randomly selecting five plants from each pot. The population densities of antagonists on the root were determined by the dilution plate method. 1 g of roots were macerated and shaken with 20 ml of sterile 50 mM potassium phosphate buffer. The resulting suspensions were diluted, and plated onto KMB agar amended with 100 µg/ml ampicillin. CFU per g of root were scored after incubation at 30 °C for 48 h.

3. Results and discussion

3.1. Isolation and screening of fluorescent pseudomonads

Thirty-five isolates from the rhizosphere of pepper could be classified as fluorescent pseudomonads. The strains were fluorescent on KMB agar, gram-negative, oxidase-positive and catalase-positive. In order to isolate the plant growth promoting antagonists, all the fluorescent pseudomonads were screened using dual culture and roll towel assays. Among the 35 isolates, twelve isolates showed an inhibitory effect on the growth of *R. solani* in a dual culture assay (data not shown). Among these 12 antagonists, SE21 and RD41 exhibited maximum growth inhibition of *R. solani* (Table 1). The production of clear inhibition zones in a dual culture assay is due to the production of either antibiotics, siderophores, HCN (Thomashow and Weller, 1996) or hydrolytic enzymes, i.e. chitinases and β -1,3-glucanases (Fridlender et al., 1993) as mechanisms for biological control.

Further, in a roll towel assay, antagonists SE21 and RD41 showed an increase in the vigour index of red pepper. However, maximum increase in vigour index was observed in RD41 compared to SE21. From this screening, antagonists SE21 and RD41 were selected for further studies. Taxonomic characterization of isolates SE21 and RD41 by Gram staining, biochemical tests and tests for utilization of sugars revealed that these isolates belong to the fluorescent group of *Pseudomonas* species.

The importance of fluorescent pseudomonads in antagonistic potential and their ability to promote plant growth make them the preferred choice for biocontrol studies. In addition to biocontrol potential, the fluorescent pseudomonads could exert their beneficial effects on host plant growth by several possible mechanisms. The mechanisms include: production of phytohormones, which can enhance the growth of plants and solubilization of phosphate. Hence, both biocontrol and plant growth promoting features of the antagonists SE21 and RD41 have further been investigated in detail.

3.2. Biocontrol features

Antagonists SE21 and RD41 showed the production of siderophores in M9 minimal medium (Table 2). Maximum production was observed by RD41 compared with SE21. The siderophores have a high affinity for ferric iron, which will form a ferric-siderophore complex and make it unavailable to other organisms, but the producing organisms can utilize these complex via a specific receptor in their outer membrane (Buyer and Leong, 1986). Due to iron starvation, the growth of pathogenic fungi in the rhizosphere will be restricted. Characterization of antagonists for HCN production revealed its biocontrol potential. The maximum production of HCN was recorded in RD41. Production of fungal cell wall degrading enzymes such as β -1,3-glucanase and chitinase was analyzed because this is an important mechanism for fungal inhibition (Fridlender et al., 1993). The antagonist SE21 recorded a higher rate of β -1,3-glucanase and chitinase activity than the antagonist RD41.

3.3. Plant growth promoting features

Antagonists SE21 and RD41 utilized tryptophan as a precursor for their growth and IAA production. The maximum production of IAA was observed in SE21 compared with RD41. Similarly, both antagonists utilized tricalcium phosphate as the sole phosphate. The antagonist RD41 exhibited a higher rate of phosphate solubilization than the antagonist SE21. The IAA

Table 2
Biocontrol and plant growth promoting features of fluorescent pseudomonads SE21 and RD41

Isolate	Biocontrol features				Plant growth promoting features	
	Siderophore production (OD at 630 nm)	HCN production (OD at 625 nm)	β -1,3-glucanase activity (nM/min/mg of protein)	Chitinase activity (nM/min/mg of protein)	IAA production (µg/ml)	P solubilization (µg/ml)
SE21	0.576±0.12	0.022±0.003	475.8±4.01	137.5±2.29	64.7±2.09	14.0±1.26
RD41	2.487±0.38	0.033±0.002	347.2±5.01	33.1±1.25	14.0±1.20	21.4±2.50

Results are an average of three replicates.

± Standard deviation.

production and phosphate solubilization evidently suggests the plant growth promoting ability of the antagonists SE21 and RD41 (Table 2).

3.4. Effect of inducers on the growth of antagonists

Amendment with inducers showed an increase in the number of colonies of antagonists (Fig. 1). When compared with the control, amendment with SA alone had no significant effect on the number of colonies of both antagonists. This may be due to the various susceptibilities of antagonists to SA. Along with SA, amendment with chitin increased the colonies of SE21 to 132.4×10^8 colonies, and it was a 16% increase over the amendment with SA alone. Similarly there was a 6% increase in the number of colonies of RD41 over the amendment with SA alone. The amendment with chitin alone showed a maximum increase in the number of colonies of both antagonists. Similar trends were also observed with *P. fluorescence* and *Bacillus subtilis* (Bharathi et al., 2004). The maximum growth was observed in SE21 compared with RD41. The enhancement of SE21 with chitin alone represented an increase of 56% compared with the control, an increase of 52% compared with the amendment of SA alone and an increase of 31% compared with amendment with SA with chitin.

3.5. Effect of inducers on biocontrol efficiency of antagonists

The effect of inducers on the biocontrol efficiency of antagonists against damping off of pepper was evaluated under greenhouse condition. Inoculation with antagonists showed an increase in the growth of pepper (Table 3). This investigation confirms earlier research where the plant growth promoting effects of fluorescent pseudomonads in different crops were clearly demonstrated (Gutterson, 1990). It revealed a significant increase in plant growth due to the treatment with SE21 and RD41, compared to the control. It is likely that the IAA producing and phosphate solubilizing isolates might have helped plant root proliferation and enhanced uptake of soil minerals.

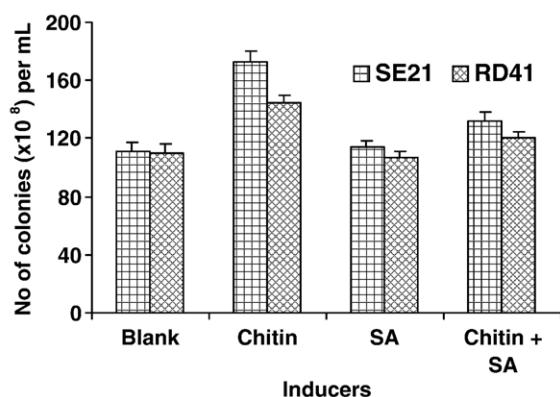


Fig. 1. Effect of inducers on growth of fluorescent pseudomonads SE21 and RD41. (Chitin — 0.5%; SA — 0.2 mM). Results are an average of three replicates. Bars indicate standard deviation.

Table 3

Effect of inducers and fluorescent pseudomonads on the growth of red pepper

Treatment	Plant height (cm)	
	Without <i>R. solani</i>	With <i>R. solani</i>
Control	6.57±0.50	5.60±0.46
Control+chitin	6.83±0.33	6.60±0.48
Control+SA	5.39±0.40	5.34±0.45
Control+chitin+SA	6.24±0.21	6.07±0.18
SE21	8.39±0.47	7.50±0.25
SE21+chitin	8.61±0.35	7.86±0.33
SE21+SA	7.13±0.18	6.94±0.45
SE21+chitin+SA	7.44±0.25	6.94±0.39
RD41	8.46±0.16	7.59±0.26
RD41+chitin	8.22±0.29	7.74±0.20
RD41+SA	7.38±0.30	7.18±0.17
RD41+chitin+SA	7.07±0.34	6.96±0.45

Results are an average of three replicates.

± Standard deviation.

The amendment with chitin alone (without antagonists) moderately increased the plant growth. However, SA with or without chitin amendment had no effect on plant growth. Amendment with inducers with antagonists showed a marked increase in plant growth as compared with inducers used alone. Inoculation of *R. solani* reduced the growth of pepper seedlings and this effect was suppressed by the treatment of antagonistic isolates. Among the treatments, chitin alone with SE21 showed a maximum increase in the growth of red pepper.

Amendment with inducers alone exhibited a varied degree of protection to pepper against *R. solani* damping off (Table 4). Disease incidence and disease severity were at a maximum in *R. solani* treated control plants, whereas significant reduction in disease was recorded when inducers were applied alone. In general, a 10–26% reduction of disease severity was recorded. Of the treatments, amendment with chitin showed maximum

Table 4

Effect of inducers and fluorescent pseudomonads on biocontrol of damping off of pepper

Treatment	Disease incidence (%)	Disease severity	Antagonists colonization (No of colonies ($\times 10^6$) g^{-1} root)
Control	71±3.8	2.01±0.14	–
Control+chitin	56±3.8	1.54±0.09	–
Control+SA	64±3.8	1.82±0.09	–
Control+chitin+SA	60±6.6	1.49±0.13	–
SE21	40±6.6	1.03±0.07	6.46±0.25
SE21+chitin	31±4.2	0.71±0.07	9.23±0.60
SE21+SA	36±3.8	1.05±0.05	6.73±0.47
SE21+chitin+SA	31±4.2	0.96±0.14	7.06±0.68
RD41	42±3.8	1.17±0.09	6.36±0.35
RD41+chitin	36±3.8	0.88±0.08	8.43±0.45
RD41+SA	40±6.6	1.11±0.10	6.53±0.35
RD41+chitin+SA	38±3.8	0.89±0.10	6.66±0.56

Disease severity was assessed based on a 0–5 scale from 0 — no symptoms to 5 — plant dead.

Results are an average of three replicates.

± Standard deviation.

protection of red pepper against damping off. This result was consistent with the observation of Benhamou et al. (1998), who found that chitosan has the capacity to induce resistance to *Fusarium oxysporum* in susceptible tomato plants when applied as a root dressing, foliar spray, and seed dressing. Likewise, the induction of resistance to *Colletotrichum lagenarium* in cucumber (Mills and Wood, 1984) and to *Erysiphe graminis* f. sp. *tritici* in barley (Walters et al., 1993) has also been demonstrated by exogenous application of SA. However, in the present study, amendment with SA alone showed minimum effect on the control of damping off of pepper. This result was concordant with the observation of Quintanilla (1995), who found that SA had little activity against infection of potato with *Phytophthora infestans*.

Greater reduction in disease occurred when inducers were applied in combination with antagonists. For instance, a 45–65% reduction of disease severity was observed when the antagonists were used in combination with inducers. Among the treatments, chitin alone with antagonist SE21 showed a maximum reduction in disease incidence (57%) and disease severity (65%). This result indicates that chitin might stimulate the growth of antagonists and/or the plants which might also facilitate plant protection. However, in SA with or without chitin treatment, both antagonists showed moderate activity on the reduction in disease severity. This may be due to the various susceptibilities of antagonists and/or plants to SA. Other studies are needed to elucidate the biochemical responses of rhizosphere bacteria and plants on varying levels of SA.

Amendment with chitin alone increased the population density and biocontrol efficiency of SE21. Earlier research indicated that the biocontrol potential of antagonists against various pathogens is correlated with their population density in the rhizosphere or roots. Raaijmakers et al. (1995) reported that the level of suppression of *Fusarium* wilt of radish was significantly related to the rhizosphere population density of the bacterial strain WCS358. In the present observations, amendment with SA alone did not increase the population density and biocontrol efficiency of the antagonists. Further, amendment with SA with chitin showed a slight increase in biocontrol efficiency of both antagonists. However, this level of disease suppression was lower than that provided by chitin alone with antagonists but was higher than that provided by SA plus chitin without antagonists. The observations indicate that there was a significant relationship between the disease reduction and the population density of added antagonists. The mechanism through which an increase in population density of antagonists resulted in an increase in the level of biological control is uncertain. It has been suggested that the amendment with chitin and/or inoculation of biocontrol agents may induce host defense responses in plants (Benhamou et al., 1998; Pieterse et al., 2001). In the present study, the antagonist SE21 with chitin amendment consistently suppressed the damping off of pepper. This may be due to the production of high β -1,3-glucanase and chitinase by the antagonist SE21 (Table 2).

The results obtained indicate that the amendment with chitin might increase plant protection by favoring the fast growth of antagonists and therefore stimulate the production of related metabolites, which may help antagonistic activity and/or induce

defense capacity of the plants. Further studies on molecular characterization of antagonists and the role of antagonists with chitin in induction of systematic resistance are under progress.

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