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ORIGINAL PAPER

Biological control of *Phytophthora* blight in red pepper (*Capsicum annuum* L.) using *Bacillus subtilis*

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Abstract *Phytophthora* blight is one of the most important devastating diseases of red pepper plants. Forty-one bacterial isolates were obtained from rhizosphere soil and subsequently tested for antagonistic activity under in vitro and in vivo conditions. Among the 41 isolates tested, 12 exhibited a maximum antagonistic activity in dual culture assay. These 12 isolates were further screened for disease suppression on red pepper plants in both natural and greenhouse conditions. All the antagonists showed varying levels of antagonism, whereas the isolates R33 and R13 exhibited the maximum (86.8 and 71%) ability to reduce the disease severity in in vivo conditions. Based on the 16S rDNA sequencing, the most effective isolates were identified as *Bacillus subtilis*. In addition, the isolates were also screened for siderophores, hydrogen cyanide and hydrolytic enzymes. Further, the isolates increased the root and shoot length of the red pepper, which is an added advantage of the isolates while performing the desired function.

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Introduction

Red pepper (*Capsicum annuum* L.) is an important tropical and subtropical crop in Korea and worldwide. The most important devastating diseases of the red pepper plant are *Phytophthora* blight, incited by the oomycete pathogen *Phytophthora capsici.* The disease has been spreading at an alarming rate and is responsible for significant loss to growers. Crop productivity is reduced because of *Phytophthora* blight infection, which has been estimated to account for 30–80% of the total global annual red pepper production.

Phytophthora capsici can attack all parts of the red pepper plant at any stage of growth. The symptoms include root and crown rot, aerial blight in leaves, stems and fruits. The causative oospores disperse very rapidly by splashing rain as well as flowing irrigation and surface waters (Mao et al. 1998). Crop rotation and application of chemicals have been the most widely used culture practice to control this soil-borne disease. Even though chemical treatments showed promising results in controlling the disease, phytotoxicity and chemical residues may pose a serious threat to the environment (Rajkumar et al. 2005).

In recent years, biological control has emerged as an important alternative method in managing soil-borne plant diseases. Several rhizobacteria have been used extensively as biological agents to control many soil-borne plant pathogens (Amico et al. 2005; Rajkumar et al. 2005). The mechanism by which rhizobacteria exert their beneficial effect on plants includes the production of siderophores, hydrogen cyanide (HCN), antibiotics, lytic enzymes,

competition and by inducing systemic resistance (Pieterse et al. 2001; Tag et al. 2003). Rhizosphere organisms also exhibit antagonistic activity against *P. capsici*. Several authors have reported the use of *Trichoderma* species as a biological control agent (BCA) against *P. capsici* infection in black pepper, bell pepper and coorg mandarin (Anandaraj and Sarma 1995; Sawanth et al. 1995). *Pseudomonas* species isolated from the rhizosphere have been successfully used for the biological control of *P. capsici* infection in red pepper and other crops (Tag et al. 2003; Rajkumar et al. 2005). However, only limited attempts have been made to control *P. capsici* infection in red pepper using *Bacillus* species as a biological agent. Hence, the present study is aimed to isolate and characterize *Bacillus* species for biological control and also for promote plant growth activity.

Materials and methods

Soil sample collection and isolation of *Bacillus* species

In August 2006, samples were collected from the rhizosphere of healthy red pepper plants in three different locations in a *Phytophthora*-infected field. Pepper roots were uprooted and 100 g of rhizosphere soil was carefully transferred into sterile plastic bags covered with ice and transported to the laboratory and processed within 18–24 h. *Bacillus* species were isolated from the rhizosphere soil samples according to Narita et al. (2004). Virulent strains of *P. capsici* were isolated from the infected pepper plant and maintained on either Potato Dextrose Agar (PDA) or Corn Meal Agar (CMA).

Dual culture assay

All the bacterial isolates were screened for antagonism against *P. capsici* on PDA plates using the dual culture technique (Yoshida et al. 2001). The isolates were also screened for antagonistic activity against other fungal plant pathogens like *Phytophthora citrophthora*, *Phytophthora citricola*, *Colletotrichum gloeosporioides* and *Colletotrichum coccodes*.

Identification of the efficient isolates based on 16S rDNA homology

To extract the DNA, the cells were harvested from 10 ml of overnight culture and the pellets were lysed in 1 ml lysis buffer (25% sucrose, 20 mM EDTA, 50 mM Tris–HCl and 5 mg/ml of lysozyme). Chromosomal DNA was extracted according to standard procedure of Maniatis et al. (1989).

The 16S rDNA was amplified using PCR with the universal primers 27f and 1492r (Reysenbach et al. 1992). The PCR was done in a thermocycler (MJ Research) using a thermal cyclic condition at 94°C (5 min) followed by 35 cycles at 94°C (1 min), 49°C (2 min) and 72°C (2 min) with a final extension temperature at 72°C for 7 min. The PCR products were cleaned using the PCR purification kit (Qiagen, USA). The amplicons were sequenced in both forward and reverse direction by using an automated sequencer ABI PRISM (Model 3700). The sequences were compared using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) for identification of the isolates.

Detached leaf assay and biocontrol activity in the greenhouse

Based on the dual culture assay, 12 isolates were selected for the detached leaf assay and in vivo studies. Red pepper leaves (5 cm length) were collected from 1-month-old plant and leaf surface were sterilized with 1% sodium hypochlorite solution for 30 s and rinsed several times in sterile water. All the antagonistic isolates (10^8 cells/ml) were sprayed individually on to the detached leaves and placed in the petri plates that were lined with moistened filter paper. A spore suspension of 10^5 spores/ml was prepared and sprayed on to the leaves after spraying the antagonistic isolates. Later, the leaves were incubated in a growth chamber at 20°C with 12 h light/dark for 10 days. Disease severity was assessed using a scale of 0–4: 0, no symptoms; 1, 1–12%; 2, 13–25%; 3, 26–50%; 4, 51–100% of leaves covered with brown lesions. Leaves without antagonistic bacteria served as a control in this experiment. Each treatment consisted of three detached leaves in three replications (Chiou and Wu 2001).

For in vivo studies, red pepper seeds surface were sterilized and inoculated with the antagonistic isolates (10^8 cells/ml) by soaking them for 1 h. Seeds soaked in sterile water were used as a control. The inoculated and non-inoculated seeds were planted in a pot 12 cm in diameter with the following soil mix (Soil:Peat:Perlite = 1:1:1) and incubated in a greenhouse at 22°C with light–dark cycles (14 h light, 8 h dark) for 40 days. Seedlings at four leaf stages were inoculated with *P. capsici* by adding 2 ml of zoospore suspension (10^5 spores/ml). The percentage of disease incidence and severity were recorded on day 40 after sowing. Disease severity was assessed using a 0–5 scale; 0, no visible disease symptoms; 1, leaves slightly wilted with brownish lesions beginning to appear on stems; 2, 30–50% of the entire plant diseased; 3,

51–70% of the entire plant diseased; 4, 71–90% of the plant diseased; 5, plant dead.

Siderophores, HCN, indoleacetic acid (IAA) production and 1-aminocyclopropane-1-carboxylic acid deaminase (ACC) assay

Siderophore secretion was detected by the "universal" method of Schwyn and Neilands (1987). In brief, 0.5 ml of blue Chrome azurol S (CAS) solution was added to 0.5 ml of filtered supernatant of the isolates. A reference solution was prepared using the uninoculated medium. Positive reactions were estimated by changes in colour of the assay reagent from blue to orange. The assay was considered to be negative when no change in blue colour was observed within 3 h. HCN production was determined according to the method of Lee et al. (2001). The isolates were grown on tryptic soy agar (TSA) medium supplemented with 4.4 g/l of glycine, placing filter paper strips soaked in picric acid solution (2.5 g of picric acid, 12.5 g of Na_2CO_3 in 1 l of distilled water) in the lid of each Petri dish. Dishes were sealed with the Parafilm and incubated at 28°C for 3 days. The change in the filter paper colour from yellow to brown indicated HCN production by the isolates.

The production of IAA was determined according to the method of Gordon and Weber (1951). In brief, the isolates were cultured for 2 days in Dworkin and Foster (DF) minimal medium supplemented with 0.5 mg/ml of tryptophan. After incubation, 1 ml of cell suspension was transferred into the tube and mixed vigorously with 2 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled water, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$) and allowed to stand at room temperature for 20 min. Development of pink colour in the tubes indicated IAA production. The ability of the isolates to use ACC as nitrogen source is a consequence of enzymatic activity of ACC deaminase. The isolates were cultured in DF minimal medium supplemented with 3 mM ACC instead of $(NH_4)_2SO_4$ as nitrogen source. Following inoculation, the tubes were incubated at 37°C on rotatory shaker at 180 rev/min for 48 h. After incubation, the development of turbidity in the tubes when compared with control tube was considered as a positive result. The ACC was added to the DF minimal medium after autoclaving and cooling to 45–50°C from the filter-sterilized stock solution (Amico et al. 2005).

Chitinolytic and proteolytic activity of the isolates were determined according to Tahtamouni et al. (2006). The log phase culture of isolates were inoculated individually in colloidal chitin agar and milk agar plates, later the plates were incubated at 30 ± 1°C for 6–10 days and observed for the clear zone around the colonies. The phosphate solubilizing potential of the isolates was determined according to Pikovskaya (1948). The development of a clear zone at the inoculation site on the culture plates was considered to be an index of phosphate solubilization.

Seedling vigour

Plant growth-promoting potential of the isolates was carried out according to the standard roll towel method (ISTA 1966). Germination percentage of the seeds was recorded and vigour index was calculated using the following formula. Vigour Index = Percentage of germination × Seedling length (Shoot and Root length) (Baki and Anderson 1973).

Results

Forty-one morphologically different colonies were isolated from the three different rhizosphere soil samples. Among the 41 bacterial isolates tested for dual culture assay, 12 were found to be good antagonists to *P. capsici* on PDA. The results of the dual culture assay for these 12 isolates are given in the Table 1. Inhibition was clearly observed by limiting or complete inhibition of the mycelial growth around the bacterial colonies. Among these 12, isolates R15, R22, R30, R32 and R33 exhibited a maximum inhibition of *P. capsici* growth with inhibition zones of 11.5, 11, 11.5, 12 and 12 mm respectively.

The isolates also exhibited good antagonistic activity to *P. citrophthora*, *P. citricola* and *C. coccodes*. Whereas, few isolates (R15, R19, R23, R30 and R36) exhibits moderate antagonistic activity against *C. gloeosporioides* (Table 1).

In the detached leaf assay, significant differences were observed between the treated and untreated leaves (Table 2). Among the 12 isolates, R19, R32 and R33 showed a maximum suppression of *P. capsici* infection in the detached leaves, followed by R22, R27, R36, R15, R41, R23 and R13, respectively. In contrast, isolates R14 and R30 exhibited very limited biological control activity when compared with the other isolates. The disease's severity scale range was 1.0–2.6 after 10 days of incubation in the growth chamber.

In vivo efficiency of the antagonistic isolates to control *Phytophthora* infection in pepper plants was evaluated under greenhouse conditions and the results are given in the Table 2. In control plants, initially symptoms appeared on the stems and then extended (enlarged) rapidly within 31 days and spread to aerial part of the plants, accompanied by wilt of the entire plants, leaf defoliation and damping off. The plants treated with antagonistic isolates

Table 1 Inhibition of *Phytophthora* species and *Colletotrichum* species mycelial growth on potato dextrose agar by *Bacillus* species

Isolates	Degrees of inhibition				
	P. capsici	*P. citrophthora*	*P. citricola*	*C. coccodes*	*C. glyoeosporioides*
R13	++	+++	+++	+++	+++
R14	++	+++	+++	+++	+++
R15	+++	+++	+++	+++	++
R19	++	+++	+++	+++	++
R22	+++	+++	+++	+++	+++
R23	++	+++	+++	+++	++
R27	++	+++	+++	+++	+++
R30	+++	+++	+++	+++	++
R32	+++	+++	+++	+++	+++
R33	+++	+++	+++	+++	+++
R36	++	+++	+++	+++	++
R41	++	+++	+++	+++	+++

+ Represents 1–5 mm wide zone, ++ represents 6–10 mm wide zone, +++ represents more than 10 mm wide zone

Results are average of three replications

exhibited a significant difference in the disease severity and incidence. Among the isolates, strain R33 and R13 exhibited high reduction in disease incidence and severity, followed by R41, R15, R19 and R36, respectively. The isolates R14, R22, R23, R27, R30 and R32 showed a limited biocontrol activity under in vivo conditions.

The isolates R33 and R13 exhibited positivity towards siderophores, HCN, IAA, phosphatase and ACC-deaminase activity. Presence of clear zones around the colonies in colloidal chitin agar and milk agar indicated the chitinolytic and proteolytic activity of the isolates. In addition, the isolates also increased both root and shoot length of the plants (Table 3). The control seeds showed poor performance in germination as well as in length of the root and shoots.

Table 2 Effect of antagonistic *Bacillus* on controlling *Phytophthora* disease development in detached leaves assay and in plants grown in greenhouse condition

S.No.	Isolate	Degrees of inhibition	
		Detached leaves assay	In vivo studies
1	R13	1.83 ± 0.7	1.1 ± 0.4
2	R14	2.00 ± 1.0	2.5 ± 0.5
3	R15	1.50 ± 0.5	1.5 ± 0.1
4	R19	1.00 ± 0.8	1.5 ± 0.2
5	R22	1.33 ± 0.8	2.1 ± 0.1
6	R23	1.66 ± 0.8	2.6 ± 0.3
7	R27	1.40 ± 0.8	2.1 ± 0.1
8	R30	2.66 ± 0.5	2.0 ± 0.2
9	R32	1.16 ± 0.4	2.0 ± 0.1
10	R33	1.16 ± 0.4	0.5 ± 0.1
11	R36	1.33 ± 0.5	1.5 ± 0.2
12	R41	1.66 ± 0.5	1.3 ± 0.5
13	*P. capsici*	3.66 ± 0.5	3.8 ± 0.7

In detached leaf assay disease severity was assessed based on 0–4 scale: 0, no symptoms; 1, 1–12%; 2, 13–25%; 3, 26–50%; 4, 51–100% of leaf were covered with brown lesions. In in vivo studies disease severity was assessed based on 0–5 scale: 0, no visible disease symptoms; 1, leaves slightly wilted with brownish lesions beginning to appear on stems; 2, 30–50% of the entire plant diseased; 3, 51–70% of the entire plant diseased; 4, 71–90% of the plant diseased; 5, plant dead

Results are an average of three replications ± standard error

Polymerase chain reaction amplification of the targeted 16S rDNA resulted in the predicted 1.55 kbp amplicons in R33 and R13 isolates. The PCR amplified products were sequenced and compared with the 16S rDNA sequences in NCBI database. Based on the partial sequences comparison by BLAST, the isolates were identified as *Bacillus subtilis*. The nucleotide sequences were deposited in GenBank (accession number EF634482, EF648001).

Discussion

Rhizosphere bacteria are considered as an important functional group of beneficial bacteria used for promoting plant growth as well as for biological control of soil-borne plant pathogens. Hence, we focused our attention to screen the potential *Bacillus* species for their biological control against *P. capsici.* Two different in vitro and in vivo assays were employed for screening potential antagonists against the soil-borne pathogen. First, the traditional in

Table 3 Effect of *Bacillus subtilis* on seedling vigour

Isolate	Vigour index	Percent increase over control
R13	630	54.4
R33	631.4	54.7
Control	408.1	

Results are an average of three replications

vitro dual culture assay on PDA was taken as preliminary screening criteria for selecting the antagonist. The area of the inhibition zone was taken as a measure of antagonistic potential of the isolates.

The zone of inhibition by the isolated *Bacillus* species varied between 0.7 and 12 mm. Among the 41 isolates, 12 were found to be highly inhibitory of *P. capsici* growth, whereas others showed only limited activity or no activity (Table 1). This suggests that the mode of action exerted or the type of antifungal metabolites produced by the isolates may vary from each other (Williams and Asher 1996). Moreover, in the inhibition zone of the 12 isolates there was no physical contact between isolates and the pathogen suggesting that the isolated *Bacillus* species could produce certain antifungal metabolites which inhibit the growth of the mycelia (Montealegre et al. 2003). Furthermore, a highly nutrient rich PDA was used for the dual culture assay, so competition might be excluded as a mode of action for the isolates.

Secondly, an assay was performed on the basis of interaction of pathogen, antagonist and host plant, which resembled the field condition. In dual culture assay there was no involvement of the host plant, whereas in real conditions the host plant plays an important role in supporting the introduced antagonist (Anith et al. 2003). Hence a screening system involving the pathogen, antagonist and host plant were expected to give a more realistic picture than dual culture assay. Since *P. capsici* is capable of infecting all part of the red pepper plant, leaves were selected as the host part for screening the biocontrol activity of the 12 isolates. Throughout the assay period high humidity and darkness was maintained in the growth chamber, which was very conducive for *P. capsici* to grow and infect the plant. Despite environmental conditions highly favourable to *P. capsici*, few of the antagonistic isolates (R19, R32 and R33) exhibited a maximum reduction in disease severity (Table 2). However, diseases severity in some treatments (R14 and R30) were limited, this might have been due to either the antagonistic bacteria failing to suppress the growth of the pathogen during the initial stage of disease development or the isolates failing to survive in the leaves on limited period of time. These results concur with those of other researchers (Chiou and Wu 2001; Rajkumar et al. 2005) who reported that some of the antagonistic *Pseudomonas* species exhibited a limited biological control activity in detached leaf assay.

All promising isolates from the dual culture and detached leaf assay were further evaluated under greenhouse conditions. The isolates showed varying levels of antagonism, among them R33 exhibited a maximum activity in controlling the diseases incidence and severity. Though the isolates R22 and R32 showed a good antagonistic activity in the dual culture and detached leaf assay, they failed to suppress the disease severity under in vivo conditions. This could be due to the inability of the isolates to control the growth of pathogen in the early stages of disease development (Yoshida et al. 2001). Interestingly the isolates R13, R19, R36 and R41 exhibited less inhibition in dual culture assay, and were more efficient in controlling the disease severity under in vivo conditions. Similar results were also reported by Jubina and Girija (1998). Their studies on biocontrol of *P. capsici* in black pepper plants revealed that one of the *Bacillus* isolates (B13) showing poor inhibition in dual culture exhibited maximum disease suppression in the in vivo studies. The present results suggest that the preliminary screening can be used to some extent, for selecting the potential antagonist against soil-borne infection by *P. capsici.*

Bacillus species isolated from rhizosphere soil were reported to be effective in controlling variety of soil-borne plant pathogens (Williams and Asher 1996). Strains of *B. subtilis* isolated from the rhizosphere region also exhibit antagonistic activity against several plant pathogens. Compant et al. (2005) reported that *B. subtilis* produced some volatile compounds that activated an ISR pathway in *Arabidopsis*. The same *B. subtilis* isolates have also been reported as efficient BCAs for *F. oxysporum* and *Aspergillus niger* (Basha and Ulganathan 2002). Similarly, in the current study, the isolates (R33 and R13) exhibiting maximum (86.8%) suppression of *Phytophthora* lesions in red pepper plants were identified as *B. subtilis.*

Recent studies have demonstrated that exposure of phytopathogenic fungi to certain fungal hydrolysing enzymes such as chitinases, proteases, cellulases or glucanases can degrade most fungal cell walls (Wang et al. 2002). However, pseudofungi such as *Phytophthora* did not appear to be affected by chitinases or cellulases, probably because the cell walls of *Phytophthora* do not contain chitin. Interestingly, the isolate secretes a complex of hydrolytic enzymes, which inhibit the growth of several important soil-borne fungal plant pathogens including *Colletotrichum* species.

Numerous studies have been reported that most of the rhizosphere organisms have plant growth-promoting potential; these strains are collectively called as plant growth-promoting rhizobacteria designated as PGPR (Kloepper et al. 1980; Asghar et al. 2004). As shown in Table 3 the antagonistic bacteria were able to increase the vigour index of the red pepper. The increased growth responses of plants caused by antagonistic isolates were due to the synthesis of phytohormones, especially IAA, siderophores and ACC deaminase that would have triggered the activities of specific enzymes (Glick et al. 1998). Phosphate-solubilizing potential of the isolates also increased the root proliferation, thereby enhancing the uptake of minerals (Gupta et al. 2002). These results also

corroborate earlier studies on IAA production, siderophore synthesis, ACC deaminase and phosphate solubilization of *Bacillus* species and its role in plant growth promotion (Asghar et al. 2004; Rajkumar et al. 2005).

Root colonization was found to be another important factor in biological control of soil-borne plant pathogens (Bowen and Rovira 1999). Cook (1993) reported that bacteria isolated from the rhizosphere of a specific crop showed a better control of diseases than organisms isolated from other crops. Therefore, in the present study, the antagonists were isolated from the rhizosphere of the red pepper and the increased vigour (Table 3) index indicates the ability of the isolates to colonize the roots of the red pepper plants.

To conclude, based on the dual culture assay, detached leaf assay and greenhouse studies, two isolates (R33, and R13) were found to exert antagonistic activity against *P. capsici*. However, tests based on greenhouse studies do not always correlate with the biological control efficacy under natural conditions. Hence, biological control activities of these promising isolates (R33 and R13) were presently evaluated in natural conditions, prior to developing integrated control strategies including bacteria *Bacillus* species as a BCA to minimize the impact of *Phytophthora* blight in red pepper.

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