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Full Length Research Paper

Inhibitory activity of *Paenibacillus macerans* and *Paenibacillus polymyxa* against *Ralstonia solanacearum*

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The inhibitory activities of seven *Paenibacillus polymyxa* strains and nine *Paenibacillus macerans* strains against *Ralstonia solanacearum* strains were examined. Result from this study indicated that the growth of all *R. solanacearum* strains except strain E406 were inhibited by *P. macerans* MB02-992 and *P. polymyxa* MB02-1007, while the other fourteen *Paenibacillus* strains had no *in vitro* inhibitory effect against *R. solanacearum* strains. In addition, suspensions of the two antagonistic bacteria showed antibacterial activities against *R. solanacearum* under different treatments and reduced the disease incidence and severity of tomato bacterial wilt. Overall, this study clearly demonstrates that antagonistic substances may play an important role in biocontrol of the two antagonistic bacteria. However, antimicrobial activities of *P. macerans* and *P. polymyxa* depend on the *Paenibacillus* strains and the target pathogen. This is the first report about the antibacterial activities of *Paenibacillus* strains against *R. solanacearum* strains isolated from different host plants.

Key words: *Paenibacillus*, antagonistism, biovar, *Ralstonia solanacearum*, tomato wilt.

INTRODUCTION

Ralstonia solanacearum is an important soilborne bacterial phytopathogen with a worldwide distribution and a large host range of more than 200 species in 50 families (Kleman, 1954; Aliye et al., 2008). In China, its hosts include economically important crops such as tomato, potato, tobacco, banana, eggplant, pepper, peanut, ginger and mulberry (Guo et al., 2004). Strains of *R. solanacearum* have been grouped into five races according to hosts primarily affected and five biovars according to the use of selected biochemical properties (Hayward, 1991). Although the complete genome sequence of *R. solanacearum* strain GMI1000 has been recently determined and annotated (Salanoubat et al., 2002), control of this disease is still a problem.

Paenibacillus macerans and *Paenibacillus polymyxa* have previously been isolated from the rhizosphere of various plants including tomato (von der Weid et al., 2000; Ryu et al., 2005; Li et al., 2008a). Furthermore, they have been suggested to be involved in plant growth promotion (Jeon et al., 2003; Son et al., 2009) and suppression of pathogens (Akhtar and Siddiqui, 2007; Li et al., 2007; Saber et al., 2009; Timmusk et al., 2009). In particular, a number of researches showed that bacteria from the genus *Paenibacillus* (formerly *Bacillus*) were able to produce a variety of antibiotics effective against a range of plant pathogenic fungi (Raza et al., 2008; He et al., 2009; Chen and Chen, 2010). However, it is not fully clear whether the *Paenibacillus* strains could inhibit the

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Table 1. *In vitro* antibacterial activities of *P. macerans* MB02-992 and *P. polymyxa* MB02-1007 against *R. solanacearum* strains from different host plants.

R. solanacearum			Diameter of inhibition zone (mm)	
Strain number	**Host plant**	**Biovar**	**MB02-992**	**MB02-1007**
G77	Ginger	I	16.9 ± 1.8	15.4 ± 1.0
P75	Pepper	II	12.6 ± 1.1	7.4 ± 0.7
T91	Tomato	III	17.9 ± 1.4	16.9 ± 1.5
E406	Eggplant	IV	0.0 ± 0.0	0.0 ± 0.0
E69	Eggplant	New*	10.9 ± 2.8	15.5 ± 1.3
T1130	Tomato	I	14.9 ± 1.4	11.9 ± 1.4
M5	Mulberry	V	16.1 ± 1.6	12.7 ± 0.9
M7	Mulberry	V	15.1 ± 0.4	18.2 ± 0.7

The data were shown as means ± standard error from a representative experiment repeated twice with similar results. Each value represents the average of six replicates. * The biovar is different from all known biovars of *R. solanacearum*.

growth of the bacterial pathogen in particular *R. solanacearum* through production of antagonistic substances.

The purpose of this study was to examine the antibacterial activities of the *Paenibacillus* strains against *R. solanacearum* strains isolated from different host plants and to evaluate biocontrol potential of selected *Paenibacillus* strains against bacterial wilt caused by *R. solanacearum* in tomato plants.

MATERIALS AND METHODS

Bacterial strains

Sixteen strains of *Paenibacillus* were obtained from previous study (Mansfeld-Giese et al., 2002) which included 7 strains of *Paenibacillus polymyxa* and 9 strains of *Paenibacillus macerans*. In addition, eight strains of *R. solanacearum* isolated from different host plants were used in this study, and this represent all currently known biovars (Table 1). All bacterial strains involved in this study were deposited in the culture collection of the Institute of Biotechnology, Zhejiang University, China.

In vitro antagonistic activity

Antibacterial activities of *Paenibacillus* strains

The antibacterial activities of sixteen *Paenibacillus* strains against *R. solanacearum* were performed as described by Li et al. (2008b). *R. solanacearum* was grown overnight in nutrient broth at 28°C and 0.5 ml of suspension was added to 15 ml of melted nutrient agar (NA) in Petri dish and allowed to solidify. Then, the 2-day-culture of the *Paenibacillus* strains was transferred onto the surface of the solidified bacterial lawn with 6 replications. The plates were incubated at 28°C and the inhibition zone (if any) was measured after 2 days. Only those strains that produced a clear inhibition zone were accessed as antagonistic bacteria.

Antibacterial activities of bacterial suspension

The antagonistic bacteria were inoculated into nutrient broth and incubated at 160 rpm shaking at 28°C for 36 h. The cell-free supernatant was obtained by centrifugation at 5000 *g* (Beckman J2-21 M/E centrifuge, USA) for 15 min and filtered on a 0.45 m millipore filter. In addition, the cell-free supernatant was further treated with heat sterilization at 121°C for 20 min or Proteinase K (0.2 mg/ml) at 37°C for 1 h, respectively. The antibacterial activities of antagonistic bacterial suspensions under different treatments were detected by the method of stainless steel cylinders (Li et al., 2008b).

Antibacterial activity of protein crude extracts

The cultures of antagonistic bacteria grown in nutrient broth were centrifuged at 10,000 *g* for 10 min. The crude protein was precipitated from the supernatant (200 ml) with ammonium sulfate at 30 - 70% saturation and dissolved into 1.5 ml sterile water. The antibacterial activities of the crude protein extract against *R. solanacearum* strain T91 were evaluated as described earlier. In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the crude protein extracts with strong antibacterial activities was performed by the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue solution to observe protein bands.

Greenhouse evaluation of antagonistic bacteria

Experimental design

The biocontrol effects of two antagonistic bacterial suspensions under different treatments were evaluated by inhibition of the bacterial wilt of tomato. The experiment had a fully randomized 4 × 2 factorial design with bacterial suspensions and *Paenibacillus* species as the main factors. Four different levels of bacterial suspensions were used: 1, none; 2, bacterial suspension; 3, bacterial filtrate; and 4, the crude protein extract; and two different levels of *Paenibacillus* species: 1, inoculation with *P. macerans* and 2, inoculation with *P. polymyxa* were employed. Then, all treatments were inoculated with *R. solanacearum* strain T91. In addition, the treatment without bacterial suspension and without *R. solanacearum* was used as the control. The experiment was conducted twice with completely randomized design. Treatments were replicated eight times with one plant per replication.

Growth of plants

A potting medium that constitutes a mixture (2:1) of sand: commercial potting substrate (Multi-element nutritional soil; Zhenjiang Lvdao Horticulture Company, China) was filled in a sterilized plastic tray. Tomato seeds (c.v Zhefen-202) were surface sterilized with 2% sodium hypochlorite for 2 min (Guo et al., 2004), washed thoroughly with sterilized water and planted in a plastic tray with 12 cm diameter and 10 cm height. The plants were maintained in a greenhouse at 24 – 28°C and 75 – 90% relative humidity in 12 h light and 12 h dark conditions. The seedlings were watered with sterile water when necessary.

Bacterial application and pathogen inoculation

The antagonistic bacteria were grown for 24 h in nutrient broth at 28°C on a rotary shaker (200 rpm) and harvested by centrifugation for 5 min at 3000 *g* at 20°C. Following two washes in a phosphate buffer solution, the bacteria were re-suspended in 1/10 strength nutrient broth before they were applied to the seeds. At sowing, each tomato seed was applied with 1 ml of bacterial suspension (approximately 3×10^9 cells/ml), bacterial filtrate, the crude protein extract and sterile water, respectively. The number of colony forming units (CFU) was counted after plating on NA plates.

The 6-week-old tomato seedlings were inoculated with *R. solanacearum* strain T91 by cutting their roots with a sterile scissors and then drenching the cut roots with 5 ml of pathogen suspension (10^8 CFU/ml). The pathogen was prepared by culturing in CPG broth (Kleman, 1954) for 48 h at 28°C and 150 rpm on rotary shaker. Cultures were centrifuged at 10 000 *g* for 10 min at 10°C. Bacterial pellets were suspended in distilled water and adjusted to 10^8 CFU/ml.

Plant weight and growth promotion assessment

Two-weeks after inoculation of *R. solanacearum*, the tomato seedlings including the stems, leaves and roots were harvested from the pots and fresh weights were recorded. For dry weight measurements, plants were dried in an oven at 60°C for 3 days before the weights were evaluated. Dry and fresh weights were used for data analysis.

Disease assessment

Disease incidence was examined according to the number of diseased plants. Determination of disease severity (P) was made by the method of Schihata (1974): $P = (0A + 1B + 2C + 3D + 4E + 5F) / T$, where 0, 1, 2, 3, 4 and 5 refers to none, 1 - 20%, 20 - 40%, 40 - 60%, 60 - 80% and 80% of the foliage wilted, respectively; and A-F is the corresponding number of leaves of plant in that infection category. T is the total number of the plant.

Population dynamics of *R. solanacearum*

To assess the effect of antagonistic bacteria on the population density of *R. solanacearum* in rhizosphere soil, 10 g of pathogen–antagonist infested soil samples were taken from each treatment pot at the time of harvest. The soil was mixed thoroughly, and then 1 g was added to sterile distilled water (1:9, w/v) and shaken for 30 min on a rotary shaker, serial dilutions were made and 0.1 ml aliquots were spread on the surface of a semi-selective TZC medium (Kleman, 1954). After incubating plates at 28°C for 3 days, colonies of *R. solanacearum* were counted and CFU were calculated per gram (dry weight) of potting medium. The experiment was conducted twice and eight replicates were prepared for each sample.

Statistical analysis

The software STATGRAPHICS Plus, version 4.0 (Copyright Manugistics Inc., Rockville, Md., USA) was used to perform the statistical analysis. Levels of significance ($P < 0.5$) of main treatments and their interactions were calculated by analysis of variance after testing for normality and variance homogeneity.

RESULT AND DISCUSSION

In vitro antagonistic effect of *Paenibacillus*

Both *P. macerans* MB02-992 and *P. polymyxa* MB02-1007 showed strong inhibitory activities against seven strains of *R. solanacearum*, which were isolated from ginger, tomato, eggplant and mulberry, respectively, while the *in vitro* growth of strain E406 was unaffected by the two antagonistic bacteria (Table 1). In contrast, Li et al. (2007) found that the two *Paenibacillus* strains had no *in vitro* inhibitory effect against all the tested fungi. However, these results are consistent with the observation of He et al. (2007), who found that *P. polymyxa* OSY-DF exhibited strong antibacterial activity but had no activity against fungi. The other fourteen *Paenibacillus* strains have no *in vitro* inhibitory effect against *R. solanacearum*.

Antibacterial activities of bacterial suspension

Suspensions of the two antagonistic bacteria inhibited the growth of seven strains of *R. solanacearum* (Tables 2 and 3). However, the antibacterial activities of *P. macerans* MB02-992 against strain M7, M5 and G77 and *P. polymyxa* MB02-1007 against strain G77, T91 and P75 was reduced by filter sterilization when compared to the corresponding control (Tables 2 and 3). The antibacterial activities of *P. macerans* MB02- 992 against strain M7, M5 and E69 and P75 and *P. polymyxa* MB02-1007 against strain M7, M5, G77, E69 and P75 was reduced by filter sterilization together with Proteinase K treatment when compared to the corresponding control (Tables 2 and 3). The antibacterial activities of the two antagonistic bacteria against 7 strains of *R. solanacearum* were reduced by filter sterilization together with heat sterilization when compared to the corresponding control (Tables 2 and 3). The antibacterial activities of the two antagonistic bacteria against the other strains of *R. solanacearum* were unaffected by these treatments when compared to the corresponding control (Tables 2 and 3).

In this study, suspensions of the two antagonistic bacteria showed strong antibacterial activities against *R. solanacearum* strains under different treatments, which is consistent with the result of Aliye et al. (2008), who found that *P. macerans* BS-DFS and PF9 have strong *in vitro*

Table 2. *In vitro* antagonistic activity of suspensions of *P. macerans* MB02-992 against *R. solanacearum* strains under different environments.

Treatments of bacterial suspension	Diameter of inhibition zone (mm)						
	M7	M5	G77	E69	T1130	T91	P75
Bacterial suspension (untreated)	14.3c	16.9c	14.4c	14.4c	14.4bc	14.3bc	14.7c
Filter sterilization	11.8b	12.1b	12.9b	15.0c	15.2c	13.6b	15.0c
Filter sterilization + proteinase K	12.0b	11.8b	13.9c	11.9b	13.5b	15.0c	13.6b
Filter sterilization + heat sterilization	9.3a	8.8a	8.8a	9.8a	12.0a	9.3a	9.4a

The data were obtained from a representative experiment repeated twice with similar results. Each value represents the average of six replicates. Means in a column followed by the same letter are not significantly different ($P < 0.05$).

Table 3. *In vitro* antagonistic activity of suspensions of *P. polymyxa* MB02-1007 against *R. solanacearum* strains under different environments.

Treatments of bacterial suspension	Diameter of inhibition zone (mm)						
	M7	M5	G77	E69	T1130	T91	P75
Bacterial suspension (untreated)	12.1c	12.3c	15.0d	14.5c	15.8b	12.9c	13.7d
Filter sterilization	11.7bc	12.0bc	14.9c	14.1c	15.6b	12.0b	12.0b
Filter sterilization + Proteinase K	11.5b	11.4b	14.4b	12.4b	15.2b	13.2c	12.9c
Filter sterilization + heat sterilization	9.1a	10.0a	9.0a	9.6a	11.3a	8.7a	9.2a

The data were obtained from a representative experiment repeated twice with similar results. Each value represents the average of six replicates. Means in a column followed by the same letter are not significantly different ($P < 0.05$).

inhibitory activity against *R. solanacearum* from potato plants. In addition, He et al. (2007) found that the cell-free culture supernatant of *P. polymyxa* OSY- DF exhibited a broad spectrum of antimicrobial activity against gram-negative and positive bacteria, which may be attributed to the fact that *P. polymyxa* is able to produce two types of peptide antibiotics, one type which is only active against bacteria and the other against fungi, gram positive bacteria and actinomycetes (Awais et al., 2008; Raza et al., 2008). However, in the present study, the antibacterial activities of cell-free culture supernatants against all strains of *R. solanacearum* were significantly reduced by heat sterilization, which indicated that the active substance of the two antagonistic bacteria is sensitive to high temperature.

Antibacterial activity of protein crude extracts

The crude extract precipitated with ammonium sulfate at 50% saturation exhibited the strongest antibacterial activity. The inhibition zone diameter of the crude proteins from *P. polymyxa* MB02-1007 against *R. solanacearum* strain T91 were 12.8 mm, while the inhibition zone diameter of crude proteins from *P. macerans* MB02-992 were 14.4 mm. SDS-PAGE showed that the protein profiles of the two antagonistic bacteria were similar. In fact, Raza et al. (2008) found that *P. polymyxa* strains produce many hydrolytic enzymes, potentially making them valuable antagonists to control plant pathogens.

In vivo antagonistic activities of *Paenibacillus*

Disease incidence and disease severity

Inoculation of tomato with *R. solanacearum* resulted in 100% disease incidence (Figures 1a and 2a) and the highest disease severity score of 5.00 (Figures 1b and 2b). However, the disease incidence was significantly reduced by the two antagonistic bacteria when tomato seeds were treated with bacterial suspensions, but was unaffected by bacterial filtrate and the crude protein extract when compared to the pathogen control (Figures 1a and 2a). The disease severity was significantly reduced by suspensions or filtrate of *P. macerans* MB02-992, while unaffected by the crude protein extract when compared to pathogen control (Fiure. 1b). However, the disease severity was reduced in the suspension, filtrate and crude protein extract of *P. polymyxa* MB02-1007 when compared to the pathogen control (Figure 2b). No symptoms were observed when plants were inoculated with sterile water.

Plant fresh weight and dry weight

Inoculation of tomato with *R. solanacearum* alone resulted in the reduction of root and shoot fresh and dry weight as well as the root/shoot dry weight ratio, while the root/shoot fresh weight ratio was unaffected when compared to the control (Tables 4 and 5). However, root

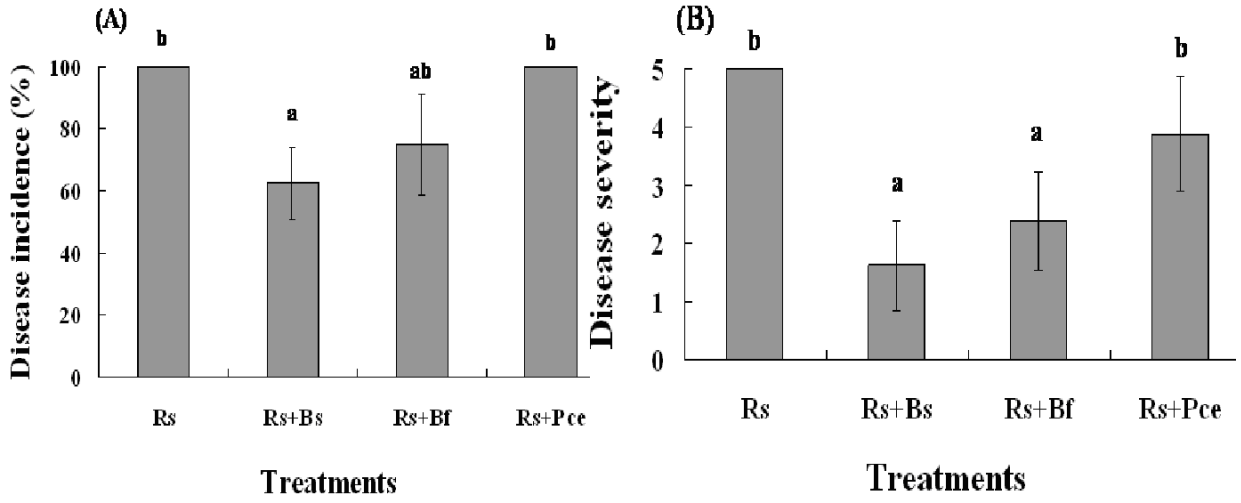

Figure 1. Biocontrol effect of *R. solanacearum* wilt in tomato plants by *P. macerans* MB02-992 under different treatments. (a) Disease incidence; (b) Disease severity. The data were obtained from a representative experiment repeated twice with similar results. Error bars represent standard errors and columns with the same letters are not significantly different (n = 8). Rs: *R. solanacearum*; Bs: Bacterial suspension; Bf: Bacterial filtrate; Pce: Protein crude extract.

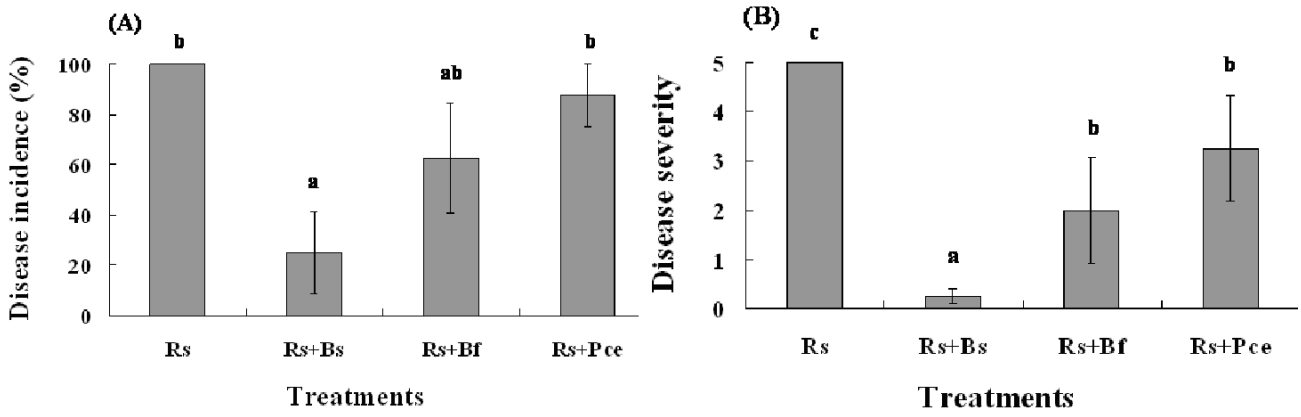

Figure 2. Biocontrol effect of *R. solanacearum* wilt in tomato plants by *P. polymyxa* MB02-1007 under different treatments. (a) Disease incidence; (b) Disease severity. The data were obtained from a representative experiment repeated twice with similar results. Error bars represent standard errors and columns with the same letters are not significantly different (n = 8). Rs: *R. solanacearum*; Bs: Bacterial suspension; Bf: Bacterial filtrate; Pce: Protein crude extract.

Table 4. Effect of *P. macerans* MB02-992 on biomass of tomato plants inoculated with *R. solanacearum*.

Treatments	Fresh weight (g)			Dry weight (g)		
	Root	Shoot	R/S	Root	Shoot	R/S
None	0.23b	2.02b	0.12ab	0.02b	0.18b	0.14c
Rs control	0.12a	1.07a	0.11a	0.01a	0.11a	0.09a
Rs + Bs	0.22b	1.49a	0.15b	0.02b	0.15ab	0.12bc
Rs + Bf	0.13a	1.27a	0.11a	0.01a	0.13a	0.10ab
Rs + Pce	0.12a	1.18a	0.10a	0.01a	0.13a	0.09a

The data were obtained from a representative experiment repeated twice with similar results. Each value represents the average of eight replicates with each replicate containing one plant. Means in a column followed by the same letter are not significantly different ($P < 0.05$). Rs: *R. solanacearum*; Bs: Bacterial suspension; Bf: Bacterial filtrate; Pce: Protein crude extract; R/S: Root/Shoot.

Table 5. Effect of *P. polymyxa* MB02-1007 on biomass of tomato plants inoculated with *R. solanacearum*.

Treatments	Fresh weight (g)			Dry weight (g)		
	Root	Shoot	R/S	Root	Shoot	R/S
None	0.23b	2.02b	0.12bc	0.02c	0.18b	0.14d
Rs control	0.12a	1.07a	0.11ab	0.01a	0.12a	0.09ab
Rs + Bs	0.20b	1.40a	0.14c	0.02b	0.14a	0.12cd
Rs + Bf	0.18b	1.40a	0.13bc	0.02b	0.15ab	0.11bc
Rs + Pce	0.10a	1.11a	0.08a	0.01a	0.12a	0.07a

The data were obtained from a representative experiment repeated twice with similar results. Each value represents the average of eight replicates with each replicate containing one plant. Means in a column followed by the same letter are not significantly different (P < 0.05). Rs: *R. solanacearum*; Bs: Bacterial suspension; Bf: Bacterial filtrate; Pce: Protein crude extract; R/S: Root/Shoot.

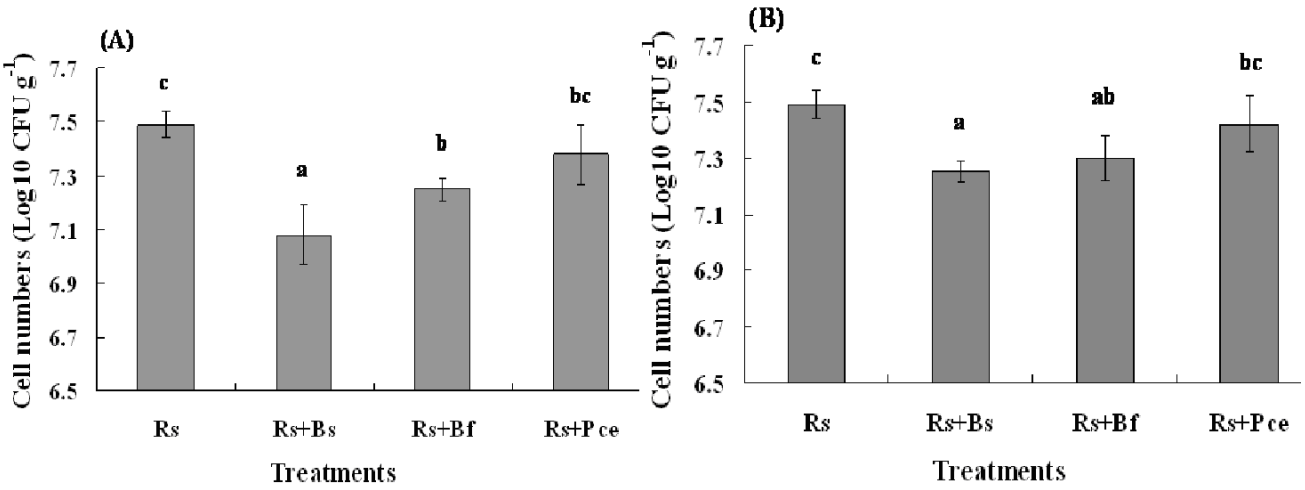

Figure 3. Effect of antagonistic bacteria on *R. solanacearum* population densities in rhizosphere soil of tomato plants. (a) *P. macerans* MB02-992; (b) *P. polymyxa* MB02-1007. The data were obtained from a representative experiment repeated twice with similar results. Error bars represent standard errors and columns with the same letters are not significantly different (n = 8). Rs: *R. solanacearum*; Bs: Bacterial suspension; Bf: Bacterial filtrate; Pce: Protein crude extract.

fresh and dry weight as well as the root/shoot fresh and dry weight ratio were increased while shoot fresh and dry weight was unaffected when compared to pathogen control after tomato seeds were treated with suspension of the 2 antagonistic bacteria (Tables 4 and 5). A suspension of *P. macerans* MB02- 992 reduced the root fresh and dry weight as well as the root/shoot fresh weight ratio, while the shoot fresh and dry weight as well as the root/shoot dry weight ratio was unaffected by filter sterilization (Table 4). However, effect of *P. polymyxa* MB02-1007 on root and shoot fresh and dry weights as well as the root/shoot fresh and dry weight ratio was unaffected by filter sterilization (Table 5) . In addition, the root and shoot fresh and dry weights as well as the root/shoot fresh and dry weight ratio were unaffected by the crude protein extract from the two antagonistic bacteria when compared to the pathogen control (Tables 4 and 5), which indicated that the crude protein extract had no effect on disease control and plant growth promotion. In general, this data is consistent with the result of disease incidence and severity.

Population dynamics of *R. solanacearum*

The cell numbers of *R. solanacearum* were 7.49 Log10 CFU/g in rhizosphere soil when tomato seeds were inoculated with the pathogen alone (Figures 3a and b). However, *R. solanacearum* cell numbers were significantly reduced after tomato seeds were treated with either suspensions or filtrates of the two antagonistic bacteria, but were unaffected by the crude protein extract when compared to the pathogen control (Figures 3a and b). The data is consistent with the results of disease incidence and severity as well as plant biomass, suggesting that tomato seeds should be treated with a suspension or filtrate of one of the two antagonistic bacteria, but not with the crude protein extract.

This is the first report of *in vitro* antibacterial activities of *P. polymyxa* and *P. macerans* against *R. solanacearum* strains. The results clearly indicate that *P. macerans* MB02-992 and *P. polymyxa* MB02-1007 had strong *in vitro* antagonistic activity against *R. solanacearum* and protected tomato from *Ralstonia* infection in growth chamber conditions when tomato seeds were treated with suspensions or filtrates of either of the two antagonistic bacteria. Therefore, production of antagonistic substances may play an important role in inhibition of *Ralstonia* wilt of tomato by these bacteria.

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