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Evaluation of the strains of *Acinetobacter* and *Enterobacter* as potential biocontrol agents against Ralstonia wilt of tomato

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ABSTRACT

Bacterial wilt (*Ralstonia solanacearum*) of tomato, *Lycopersicon esculentum*, causes a considerable amount of damage to tomato in Southern China. Biological control is one of the more promising approaches to reduce the disease incidence and yield losses caused by this disease. Based on antagonistic activity against *R. solanacearum* and three soil-borne fungal pathogens as well as biocontrol efficacy in the greenhouse, two bacterial strains Xa6 (*Acinetobacter* sp.) and Xy3 (*Enterobacter* sp.) were selected out of fourteen candidates as potential biocontrol agents. In order to find a suitable antagonist inoculation method, we compared the methods of root-dipping with soil-drenching in the aspects including rhizocompetence, biocontrol efficacy, and effect of promoting plant growth under greenhouse conditions. The drenching treatment resulted in a higher biocontrol efficacy and plant-yield increase, and this method was also easier to operate in the field on a large scale. Field trials were conducted for further evaluation of these two antagonistic strains. In both greenhouse and field experiments, the strain Xy3 had a better control effect against bacterial wilt than Xa6 did, while Xa6 caused higher biomass or yield increases. As recorded on the 75th day after treatment in two field experiments, biocontrol efficacy of Xy3 was about 65% in both field trials, and the yield increases caused by Xa6 were 32.4 and 40.7%, respectively, in the two trials. This is the first report of an *Acinetobacter* sp. strain used as a BCA against Ralstonia wilt of tomato.

1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995) is one of the most devastating plant diseases worldwide. *R. solanacearum* affects a wide range of plants in more than 50 families (Hayward, 1995). In China, its hosts include economically important crops such as potato (*Solanum tuberossum* L.), tomato (*Lycopersicon esculentum* Miller), tobacco (*Nicotiana tabacum* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.), peanut (*Arachis hypogaea* L.) and ginger (*Zingiber officinale* Roscoe). This disease has caused great losses in agriculture and horticulture (Li et al., 2004; Liu et al., 2005).

Ralstonia wilt control approaches, including field sanitation, crop rotation, and application of resistant varieties, have proven limited success (Ciampi-Panno et al., 1989). Although using resistant cultivars is an important part of the integrated disease management, breeding for disease resistance is a long-term task that is both expensive and difficult. In addition, disease resistance of a cultivar is usually not stable and/or durable (Hayward, 1991; Boucher et al., 1992). In specific regions, influence of environmental factors on host–pathogen interactions often restricts the expression of disease resistance (Hayward, 1991). Applying chemical pesticides is generally considered as the most effective and fastest strategy for plant disease management, however, no effective chemical product is available for Ralstonia wilt. Although streptomycin is regarded as a suitable bactericide to control the disease, Chinese farmers are reluctant to use it because large dosages are required for the expected effectiveness, probably due to the bactericide resistance that developed during its repeated and abusive use for last several decades in this country. Therefore, more efforts need to be devoted to biological control with living microbes.

Several living microbial products have been commercialized as biological control agents (BCAs). Some of these products are a wettable powder of *Bacillus subtilis* (Cohn) Y1336, a water suspension of *Pseudomonas fluorescens* (Migula), and a mixture of wettable powder and granule of *Paenibacillus polymyxa* (Ash, Priest and Collins) (Sun et al., 2004). However, living microbial BCAs have not

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been widely accepted as an alternative to antibiotics by farmers since they are often regarded as not quite effective, and there is no application method suitable for various BCAs and crop cultivation systems.

In our previous studies, 549 bacterial strains isolated from the forest soil were screened for their *in vitro* antagonistic activities against *R. solanacearum* and other biocontrol-related characteristics (unpublished). Based on these results, we selected fourteen strains for the study reported here. In this study, we employed a new strategy to select adaptable and stable potential BCAs from the fourteen strains based on their biocontrol efficacy, biomass and yield increasing potential in the greenhouse and field. We also compared two antagonists of highest biocontrol potential of the fourteen strains with two inoculation methods (soil-drenching and root-dipping) for biocontrol efficacy and yield increases.

2. Materials and methods

2.1. Bacterial strains and culture conditions

R. solanacearum strains Tm15 (Guo et al., 2004) and HN538 (Heuer et al., 2007) were isolated from the diseased tomato plants. *Ralstonia* strains were grown 2–3 days on YPGA medium (yeast extract 5 g, bacto-peptone 5 g, glucose 10 g, and agar 15 g per l). All fourteen antagonistic bacterial strains were cultured on Luria Bertani (LB) medium (Miller, 1992). In the greenhouse experiments, the antagonistic strains with rifampicin-resistance were selected on LB medium amended with rifampicin (50 mg/L).

2.2. In vitro assay for antagonistic activity

The antagonistic activities of the fourteen selected strains against four fungal pathogens (*Pythium ultimum* Trow, *Rhizoctonia solani* Kühn, *Fusarium oxysporum* Schlecht, and *Verticillium dahliae* Kleb) were tested with a dual-culture assay according to Berg et al. (2001). The strains' antagonism to *R. solanacearum* strains Tm15 and HN538 was tested as follows: the stock solution of 2, 3, 5-triphenyl tetrazolium chloride (TZC) was added into 1 l of molten YPGA medium ($\leqslant$50 °C), reaching 0.005% as the final concentration in the medium; subsequently, 10 mL of the *R. solanacearum* suspension at OD_{600} of 2.0 (about 2.0×10^8 CFU/mL) was added into medium and poured into Petri Dishes. Each of the four tested antagonistic strains was streaked onto the plates containing *R. solanacearum*. These plates were incubated at 28 °C for 2–4 days before the width of the clear halo surrounding the bacterial streak was measured. All experiments were replicated three times.

2.3. Evaluation of selected antagonistic strains in greenhouse

Cells of antagonistic strains were washed twice in sterile saline (0.85% NaCl) and then resuspended in sterile saline. The cell suspension was adjusted to an optical density of OD_{600} = 1.00 (approximately equal to 1.0×10^9 cells/mL).

Tomato seedlings (cv. Shanghai 903) at the age of 30 days were treated with antagonistic strains in one of the following two methods. In the drenching method, 20 mL suspension of antagonistic strains was poured into each pot 3 days before transplanting. In the root-dipping method, the seedling roots were soaked in suspensions of antagonistic strains for about 15 min before the seedlings were transplanted into pots. Plants treated with sterile saline served as controls for both treatments. The pots were placed in a greenhouse maintained at 28 °C with relative humidity of 30%, and a 12 h/12 h photoperiod.

In each of the greenhouse experiments, there were 24 plants in each replication and three replications for each treatment. All experiments were repeated three times.

2.3.1. Biocontrol Efficacy of selected antagonists against Ralstonia wilt

In preliminary experiments, two days after root-dipping inoculation with the antagonists Xy3, Xa6, NJ07, or NJ218, 20 mL of *R. solanacearum* strain HN538 suspension with about 1.0×10^7 CFU/mL was drenched into each pot. Thirty days after treatment with antagonists (28 days after pathogen inoculation), the disease index was recorded based on a scale of 0–4 as described by Kempe and Sequeira (1983). Due to their higher biocontrol efficacy against Ralstonia wilt in the preliminary experiments, strains Xa6 and Xy3 were selected for further evaluation in a separate experiment. In this experiment, tomato plants were treated with either Xa6 or Xy3 using either of the two inoculation methods.

Disease incidence and biocontrol efficiency were calculated as follows:

Disease incidence = [$\sum$ (The number of diseased plants in this index $\times$ Disease index)/(Total number of plants investigated $\times$ The highest disease index)] $\times$ 100%.

Biocontrol efficacy = [(Disease incidence of control − Disease incidence of antagonist-treated group)/Disease incidence of control] $\times$ 100%.

2.3.2. Colonization capacity of strains Xa6 and Xy3

Colonization capacity of both antagonists was tested in another separate greenhouse experiment. Ten days after transplanting upon treatment with either Xa6 or Xy3 using either of the two inoculation methods, three rhizosphere samples from each treatment were carefully collected from the experimental pots. Each sample consisted of the whole root system with tightly adhering soil of three individual plants. To harvest bacterial cells from the rhizosphere soil and the rhizoplane, one gram of the root samples was soaked in 9 mL of sterile saline with shaking at 200 rpm for 30 min. To determine the CFU counts of the antagonists, serial dilutions of the cell suspension were made and plated on LB medium supplemented with chloramphenicol (10 mg/L) and rifampicin (50 mg/L). Cycloheximide (100 mg/L) was added to all plates, including the control group, to prevent fungal growth. Three plates were used for each dilution. The plates were incubated at 28 °C for 2 days before the number of colonies was counted.

2.3.3. Plant growth promotion by antagonistic strains Xa6 and Xy3

One month after treatment with the antagonistic strains with either of the two methods, fresh weight of the whole plant was measured, and biomass increase was calculated with the following formula:

Biomass increase = [(Average fresh weights of plants treated with antagonist − Average fresh weights of control plants)/Average fresh weights of control plants] $\times$ 100%.

2.4. Field experiments

Field trials were conducted in Longyan (117°35′ E, and 24°52′ N), Fujian, China, and Huaian (119°05′ E, and 33°30′ N), Jiangsu, China, in 2006. The two locations selected for field test of Xa6 and Xy3 are about 1000 Km apart in distance with very different climatic conditions. In Longyan, it is warmer and more humid with the mean annual temperature being between 18 and 20 °C and the average yearly rainfall being between 1450 and 2200 mm; while in Huaian, it is dryer and colder with the mean annual temperature being around 14 °C and the average yearly rainfall being only about 940 mm.

The plots in all experiments were arranged randomly. Each plot was 32 m^2 (5.0 m $\times$ 6.4 m) with 150 tomato seedlings. There were

four treatments including 2 controls, and each treatment had 4 replications. In each plot of treatment 1 and 2, 250 mL of antagonistic bacterial preparation of either Xa6 or Xy3 at the concentration of 1.0×10^9 CFU/mL was diluted at 1:100 with water and drenched evenly into the soil. In Control 2, the same amount of water was used instead of antagonistic bacterial suspensions. In Control 1, streptomycin (200 ppm; each tomato plant received approximately 300 mL) was applied once every 10 days for a total of three times according to the instructions by the manufacturer (North China Pharmaceutical Company Aino Co., Ltd.) when the Ralstonia wilt symptoms appeared. The soil was raked and covered with a plastic film immediately after application. Three days later, 40 day old tomato plants of CVs. Xia-Hong No.1 and Mao-Feng 802 were transplanted in the plots in Longyan and Huaian, respectively.

In all experimental plots, the standard agronomic practices were applied to culture the tomato plants without use of any other pesticides. On the 75th day after inoculation with the antagonistic bacteria, disease index and tomato yield were recorded. Biocontrol efficacy was calculated as described in 2.3.1. The yield increase was calculated in the following way:

Yield increase = [(Average yield of tomato treated with antagonist − Average yield of tomato in control group)/Average yield of tomato in control plants] × 100.

2.5. Identification of biocontrol agents

Genomic DNA of both Xa6 and Xy3 was extracted using the Mini BEST Bacterial Genomic DNA Extraction Kit (TaKaRa Biotechnology Co., Ltd.). The partial nucleotide sequence of the 16S rRNA gene was amplified using PCR with the following primers: U8-27 (F): 5′-AGAGTT TGATC (AC) TGGCTCAG-3′, and L1494-1514 (R): 5′-CTACGG (AG) TACCTTGTTACGAC-3′. Amplification was performed with a Peltier Thermal Cycler PTC-200 (Bio-Rad, Watertown, MA, USA) using an initial denaturation step at 94 °C for 5 min, and subsequently 35 cycles of denaturing at 94 °C for 1 min, annealing at 56 °C for 2 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The PCR

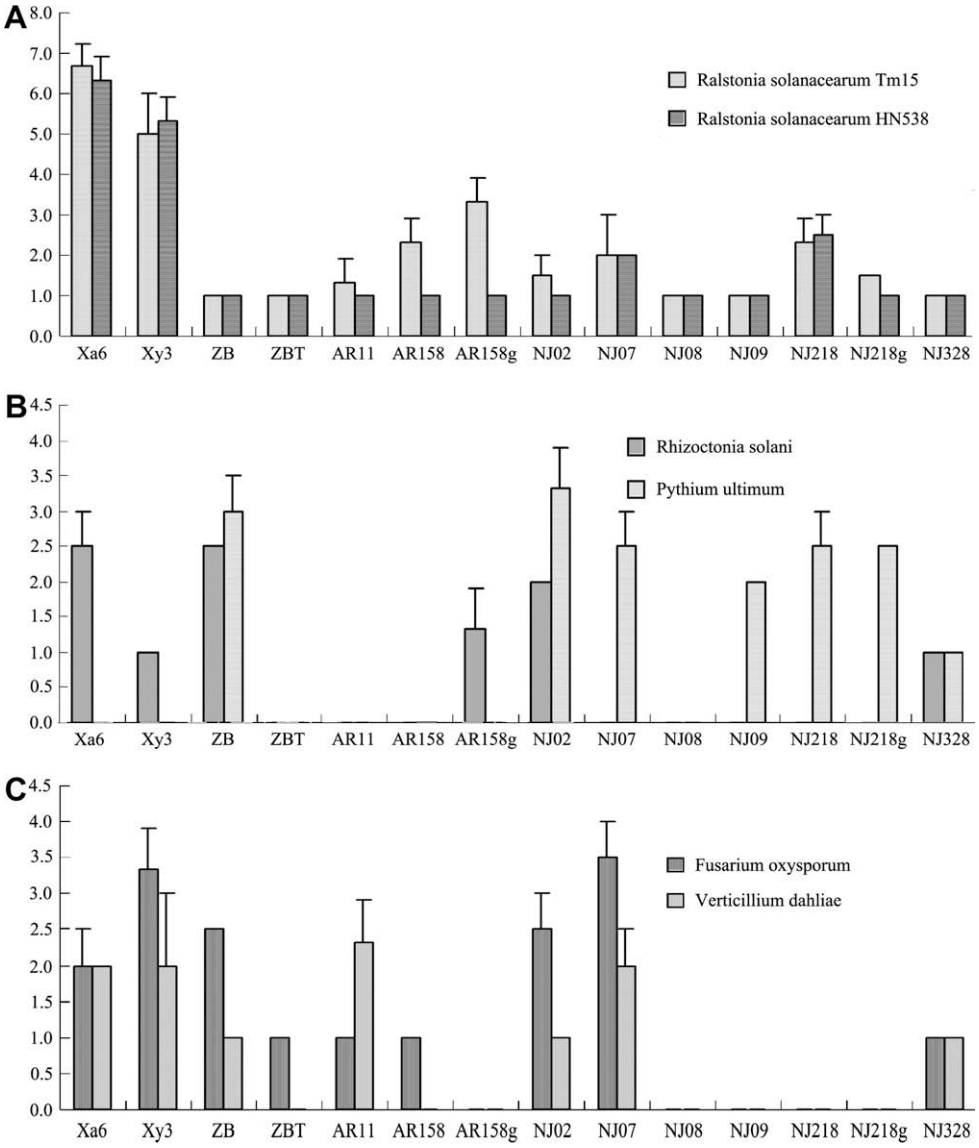

Fig. 1. The width (mm) of the inhibition zones of the fourteen bacterial strains against 2 *Ralstonia* strains (A) and four fungal pathogens on media (B and C). The inhibition zones were clear halo surrounding the antagonistic bacteria. The antagonistic bacteria were streaked on YPGA media containing *R. solanacearum* and 2, 3, 5-triphenyl tetrazolium chloride (TZC) and WA media with pathogen on them. The error bars above the bars indicate standard deviations of the means.

Table 1
Efficacy of four antagonistic bacterial strains for biological control of *Ralstonia solanacearum* on tomato after inoculation by root-dipping in greenhouse experiments.

Treatments[a]	Disease incidence (%)	Biocontrol efficacy (%)	ANOVA results of biocontrol efficacy
Xy3	36.4 ± 3.48[b]d[c]	55.2 ± 3.20a[a]	$F(3,8) = 66.789$
Xa6	42.8 ± 2.60c	47.4 ± 2.60b	
NJ07	47.7 ± 2.99c	41.3 ± 2.55c	$P < 0.0001$
NJ218	62.9 ± 3.83b	22.7 ± 3.32d	
Control	81.3 ± 2.60a	—	

[a] Plant roots were dipped into antagonist suspensions of Xa6, Xy3, NJ07 and NJ218 at a concentration of 1.0×0^9 CFU/mL while control plantlets were dipped into sterile 0.85% NaCl for the same length of time. A suspension of *R. solanacearum* (20 mL, 1.0×10^7 CFU/mL) was watered into all treatments.
[b] Standard deviation of the means.
[c] Means followed by the same letter within a column are not significantly different as determined by the LSD test ($P = 0.05$). The data were expressed as the average of three replications and three repetitions.

products were sequenced, and the 16S rRNA gene sequences were subjected to blast search in NCBI Nucleotide Sequence Database.

2.6. Data analysis

Analysis of variance for biocontrol efficiency, biomass of tomato plant, yield of tomato fruit and the population of antagonists was performed using the SAS general linear model (GLM) procedure (SAS Institute, Version 6, Cary, NC). Mean comparisons were conducted using ANOVA test and a least significant difference (LSD) test ($P = 0.05$). Standard deviations, ANOVA results and LSD results were recorded.

3. Results

3.1. In vitro antagonistic activity of fourteen antagonists

Fourteen antagonist bacterial strains showed antagonistic activity against both strains (Tm15 and HN538) of the bacterial pathogen, *R. solanacearum*, (with over 1 cm of inhibition zone width), and at least one of the four fungal pathogens of tomato except for NJ08 (Fig. 1). Strains Xa6 and Xy3 had much stronger inhibition to both *R. solanacearum* strains than other antagonists. In addition, these antagonists inhibited the three fungal pathogens including *R. solani*, *F. oxysporum* and *V. dahliae*. Among the other 12 antagonists, strains ZB, NJ02, and NJ328 had broader inhibition spectra, and NJ07 and NJ218 had a strong inhibition to *Ralstonia* strains while NJ07 also had strong inhibition against three fungal pathogens.

Table 2
Efficacy of Strains Xa6 and Xy3 for biological control of *Ralstonia solanacearum* in replicated greenhouse experiments.

Treatments[a]		Disease incidence (%)	Biocontrol efficacy (%)	ANOVA results of biocontrol efficacy
Drenching	Xa6	40.1 ± 1.29[b]bc[c]	50.2 ± 0.62c	$F(3,8) = 22.841$
	Xy3	32.7 ± 2.70d	59.5 ± 1.99a	
	Control 1	80.6 ± 2.82a	—	
Root-dipping	Xa6	42.6 ± 2.44b	47.5 ± 2.17c	$P = 0.0003$
	Xy3	36.5 ± 2.54cd	55.0 ± 2.41b	
	Control 2	81.0 ± 1.35a	—	

[a] Plants were inoculated with antagonist suspensions of Xa6 and Xy3 at a concentration of 1.0×10^9 CFU/mL while control plantlets were treated with sterile 0.85% NaCl for the same length of time. A suspension of *R. solanacearum* (20 mL, 1.0×10^7 CFU/mL) was watered into all treatments.
[b] Standard deviation of the means.
[c] Means followed by the same letter within a column are not significantly different as determined by the LSD test ($P = 0.05$). The data were expressed as the average of three replications and three repetitions.

Table 3
Comparison of the root-colonization abilities of two antagonistic strains after inoculation with two methods in greenhouse experiments.

Treatments[a]		CFU/g rhizosphere sample	ANOVA results of colonization population
Drenching	Xy3	$(3.43 \pm 0.43^{b}) \times 105b^{c}$	$F(3,8) = 76.629$
	Xa6	$(6.05 \pm 0.60) \times 10^5$a	
	Control 1	$<10^2$	
Root-dipping	Xa6	$(1.17 \pm 0.12) \times 10^5$d	$P < 0.0001$
	Xy3	$(2.23 \pm 0.38) \times 10^5$c	
	Control 2	$<10^2$	

[a] Plant roots were treated with antagonist suspensions of Xa6 and Xy3 at a concentration of 1.0×10^9 CFU/mL while control plantlets were dipped into sterile 0.85% NaCl for the same length of time.
[b] Standard deviation of the means.
[c] Means followed by the same letter within a column are not significantly different as determined by the LSD test ($P = 0.05$). The data were expressed as the average of three replications and three repetitions.

Thus, these 4 strains (Xa6, Xy3, NJ07, and NJ218) were selected for the greenhouse experiments to test their biocontrol efficiency in pots.

3.2. Antagonistic activity in greenhouse

3.2.1. Biocontrol efficiency of selected strains against Ralstonia wilt

With root-dipping inoculation method, biocontrol efficiency of Xy3 reached 55.2% which was the best of the four selected bacterial strains (Table 1). Xa6 showed the second best biocontrol efficiency of 47.4%. We chose these two strains for further experiments due to their best performance in biocontrol efficiency.

In another separate experiment comparing the effects of application methods on biocontrol efficiency of antagonists, the biocontrol efficacy of Xy3 was also significantly greater than that of Xa6 no matter what kind of inoculation methods was used (Table 2). The biocontrol efficacy of Xy3 against Ralstonia wilt by soil-drenching was significantly higher ($P = 0.05$) than that with the root-dipping method.

3.2.2. Colonization capacity of strains Xa6 and Xy3

The results of experiments determining the colonization capacity of tomato roots by Xa6 and Xy3 applied with different inoculation methods are shown in Table 3. With drenching method, Xa6 had 6.05×10^5 CFU/g rhizosphere sample, which was about two times that of Xy3 (3.43×10^5 CFU/g rhizosphere sample). The difference between colonization capacities of roots by two antagonists is significant. The rhizocompetence of Xa6 and Xy3 with the drenching method was 5 and 1.5 times higher than that with

Table 4
Biomass increases of tomato plants inoculated with strains Xa6 and Xy3 and grown in the greenhouse.

Treatments[a]		Fresh weight (g)	Biomass increase (%)	ANOVA results of biomass increase
Drenching	Xa6	29.0 ± 2.00[b]c[c]	25.6 ± 2.38a	$F(3,8) = 3.007$
	Xy3	28.4 ± 1.88c	23.0 ± 3.63ab	
	Control 1	23.1 ± 1.28a	—	
Root-dipping	Xa6	27.2 ± 1.84bc	18.6 ± 2.45bc	$P = 0.0947$
	Xy3	26.5 ± 0.87c	16.0 ± 2.21c	
	Control 2	22.9 ± 1.44a	—	

[a] Plants were inoculated with antagonist suspensions of Xa6 and Xy3 at a concentration of 1.0×10^9 CFU/mL while control plantlets were treated with sterile 0.85% NaCl for the same length of time.
[b] Standard deviation of the means.
[c] Means followed by the same letter within a column are not significantly different as determined by the LSD test ($P = 0.05$). The data were expressed as the average of three replications and three repetitions.

Table 5
The efficacy of treatments containing Xa6 and Xy3 for the control of tomato bacterial wilt and on increasing the yields in field tests conducted at Huaian, Jiangsu, China.

Treatments [a]	Disease incidence (%)	ANOVA results of disease incidence	Biocontrol efficacy (%)	Yield of each plot (kg)	ANOVA results of yield	Yield increase (%)
Xa6	20.8 ± 4.13[b]c[c]	$F(3,12) = 82.123$	58.4	131 ± 5.72c	$F(3,12) = 41.362$	40.7
Xy3	16.7 ± 2.63c		66.6	120 ± 6.73b		28.9
Control 1	29.2 ± 3.22b	$P < 0.0001$	41.6	118.3 ± 3.52b	$P < 0.0001$	21.3
Control 2	50.0 ± 2.91a		—	93.1 ± 2.98a		—

[a] Plants were treated with antagonist suspensions of Xa6 and Xy3 at a concentration of 1.0×10^9 CFU/mL while control 1 was treated with streptomycin, and control 2 was treated with water at the same time.
[b] Standard deviation of the means.
[c] Means followed by the same letter within a column are not significantly different as determined by the LSD test ($P = 0.05$). The data were expressed as the average of four replications.

root-dipping, respectively. These results indicated that inoculation by the root-dipping method caused significantly less colonization of the antagonistic strains.

3.2.3. Plant growth promotion by strains Xa6 and Xy3

With soil-drenching or root-dipping inoculation methods, biomass of tomato plants treated with Xa6 increased by 25.6% and 18.6%, respectively, while that with Xy3 increased by 23.0% and 16.0%, respectively (Table 4). The results of this experiment indicated that tomato plants significantly grew better when the antagonistic bacterial suspension was applied with soil-drenching method than that with root-dipping before transplanting.

Obviously, the antagonist application methods also affected plant growth promotion by Xa6 and Xy3 under greenhouse conditions. Based on these results, we chose soil-drenching as the application method to test the antagonists in field experiments described below.

3.3. Biocontrol efficacy of Xa6 and Xy3 in Field trials

In field trials in Huaian, the biocontrol efficacy on the 75th day after the application of Xa6 and Xy3 were 58.4 and 66.6%, respectively, while the control efficacy with streptomycin was 41.6%. The disease incidences of plants after treatment with the potential biocontrol agents were significantly lower than that of control 2 (infected with pathogen only) and control 1 (treated with streptomycin) at $P < 0.05$. The average yield increase with the inoculation of Xa6 (40.7%) was significantly higher than that of Xy3 (28.9%) (Table 5).

In field trials in Longyan, the biocontrol efficacy of Xy3 (64.8%) was also greater than that of Xa6 and streptomycin (57.2 and 56.0%, respectively) (Table 6). However, the tomato yield increase of the Xy3 treatment was only 22.9%, and that of Xa6 treatment was 32.4% (Table 6). In this field experiment, significant differences were only found between control 2 (infected with pathogen only) and other treatments.

3.4. Classification of antagonists

Sequences of 1488 bp of nearly whole 16S rRNA gene revealed that strain Xa6 belongs to *Acinetobacter* sp. (accession number: EU887288). The sequence of 1207 bp of 16S rRNA gene of Xy3 showed 100% similarity to that of *Enterobacter* sp. strain sa (accession number: EF030721).

4. Discussion

Biological control by using plant-associated microorganisms (Bargabus et al., 2003; Tjamos et al., 2005) is an efficient approach for disease management, and is regarded as friendly to the environment. The first step leading to successful biological control of plant diseases is to screen potential effective BCAs. A common screening strategy was used to select potential BCAs according to their antagonistic activity against pathogen on plates (Ahmed Idris et al., 2007). Although a large proportion of bacterial strains show antagonistic activity to target pathogens on plates, only about 1% of these control diseases in greenhouse tests, and even fewer could provide good biocontrol efficacy in field tests (Yang et al., 2008).

A major factor reducing inhibitory effects in the field may be that several distinct pathogens co-exist in the same field, while a potential antagonist is only capable of inhibiting a specific pathogen under greenhouse conditions and incapable of controlling the other diseases in the field. Therefore, when choosing potential BCAs against bacterial wilt of tomato, we included not only two strains of *R. solanacearum* but also four other fungal pathogens of tomato as the targets of the BCAs. Using this strategy, we identified strains Xa6 and Xy3 as potential BCAs because of their significant antagonism to the two *Ralstonia* strains and three of the four fungal pathogens. The results of the greenhouse and field experiments suggest that this BCA selection strategy for controlling tomato Ralstonia wilt was effective.

The inconsistent biocontrol efficacy of BCAs was presumably also caused by their uneven rhizocompetence. Several studies have showed that rhizocompetence is a crucial factor determining the

Table 6
The efficacy of treatments containing strains Xa6 and Xy3 treatments for the control of tomato bacterial wilt and for increasing yields in field tests conducted at Longyan, Fujian, China.

Treatments[a]	Disease incidence (%)	ANOVA results of disease incidence	Biocontrol efficacy (%)	Yield (kg/plot)	ANOVA results of yield	Yield increase (%)
Xa6	11.47 ± 8.96[b]ab[c]	$F(3,12) = 2.166$	57.2	131.09 ± 17.99b	$F(3,12) = 1.563$	32.4
Xy3	9.43 ± 8.18b		64.8	121.65 ± 22.18b		22.9
Control 1	11.81 ± 9.30ab	$P = 0.1451$	56.0	130.48 ± 29.92b	$P = 0.2494$	31.8
Control 2	26.82 ± 18.36a		—	98.99 ± 24.37a		—

[a] Plants were treated with antagonist suspensions of Xa6 and Xy3 at a concentration of 1.0×10^9 CFU/mL while control 1 was treated with streptomycin, and control 2 was treated with water at the same time.
[b] Standard deviation of the means.
[c] Means followed by the same letter within a column are not significantly different as determined by the LSD test ($P = 0.05$). The data were expressed as the average of four replications.

success of BCAs (Dashti et al., 2000; Kamilova et al., 2005). Thus, another key step in our strategy for screening potential BCAs was to assess the colonization capacity of strains Xa6 and Xy3. We found that both Xa6 and Xy3 demonstrated a significant level of rhizocompetence ($\geqslant 10^5$ CFU/g of a rhizosphere sample) on the 10th day after the treatment.

A suitable field application method is needed once a good potential BCA is identified through this screening procedure. Thus, we employed two application methods to evaluate the rhizocompetence, biocontrol efficicacy and plant growth promotion. The results indicated that the specificity of an application method had a significant impact on the colonization of a BCA onto the roots of tomato plants. Götz et al. (2006) found that antagonistic bacteria *Pseudomonas putida* (Migula) PRD16 and *Enterobacter cowanii* (Inoue et al.) PRF116 colonized tomato roots more effectively following root-dipping than with seed inoculation. In our study, soil-drenching, a simpler inoculation method, was compared to the root-dipping method. Our results showed that soil-drenching led to a much higher levels of colonization than root-dipping.

Dipping the seedling roots into a bacterial suspension might slightly or even severely injure the root system. These injuries may give both of the pathogen and BCA an opportunity for colonization, and the serious injury may inhibit plant growth; which might explain why both biomass increase and biocontrol efficacy in the plants inoculated by root-dipping were lower than the counterparts inoculated with the soil-drenching method (Tables 4 and 5). Therefore, we concluded that a suitable application method for potential BCAs resulted in good rhizocompetence which contributed to good biocontrol efficiency and plant growth promotion.

Moreover, both potential BCAs (Xa6 and Xy3) showed higher biocontrol efficacy in the field trials than those in the greenhouse experiments despite of the fact that we investigated the disease incidence on the 75th day after the treatment in the field and on the 30th day after treatment in the greenhouse. Similarly, Jiang et al. (2006) found the effectiveness of BF mixture (a mixture of *Bacillus* strains BB11 and FH17) against Phytophthora blight on pepper reached 70% in the field but only about 60% in the greenhouse, and Larena et al. (2003) observed that the mixture of the conidia and production substrate (a mixture of peat) of *Penicillium oxalicum* (Currie and Thom) reduced Fusarium wilt of tomato by 72% in the field (naturally infested soils), while the reduction was only 28% under greenhouse conditions (artificial inoculation). The uneven biocontrol efficacy of the same BCA shown in the two conditions might result from more fertilizer in the field than in potting soil in the greenhouse, and the nutrients from the fertilizer could enhance the multiplication and colonization of a BCA in the plant rhizosphere. In addition, the two strains, Xa6 and Xy3, showed similar high biocontrol efficacy in both fields in two locations with different climates. This indicated that performance of the two strains in biocontrol efficiency is similar under various environmental conditions.

So far, the potential BCAs against Ralstonia wilt include avirulent mutants of *R. solanacearum* (Trigalet and Trigalet-Demery, 1990); genetically engineered antagonistic bacteria (Kang et al., 1995); arbuscular mycorrhizal (AM) fungus *Glomus versiforme* (Zhu et al., 2004); and some naturally antagonistic rhizobacteria, such as *Bacillus* spp. (Silveira et al., 1995), *Pseudomonas* spp. (Lemessa and Zeller, 2007), and *Streptomyces* spp. (El albyad et al., 1996) etc. The present study is the first report of using an *Acinetobacter* sp. (Xa6) as a potential BCA for Ralstonia wilt of tomato though applying an *Acinetobacter* strain to control the plant fungal pathogen - *Botrytis cinerea* Pers has been documented (Trotel-Aziz et al., 2008).

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References

Ahmed Idris, H., Labuschagne, N., Korsten, L., 2007. Screening rhizobacteria for biological control of Fusarium root and crown rot of sorghum in Ethiopia. Biological control 40 (1), 97–106.

Bargabus, R.L., Zidack, N.K., Sherwood, J.E., Jacobsen, B.J., 2003. Oxidative burst elicited by *Bacillus mycoides* isolate Bac J, a biological control agent, occurs independently of hypersensitive cell death in sugar beet. Molecular Plant-Microbe Interactions 16, 1145–1153.

Berg, G., Fritze, A., Roskot, N., Smalla, K., 2001. Evaluation of potential biocontrol *rhizobacteria* from different host plants of *Verticillium dahliae* Kleb. Journal of Applied Microbiology 91, 963–971.

Boucher, C.A., Gough, C.L., Arlat, M., 1992. Molecular genetics of pathogenicity determinants of *Pseudomonas solanacearum* with special emphases on hrp genes. Annual Review of Phytopathology 30, 443–461.

Ciampi-Panno, L., Fernandez, C., Bustamante, P., Andrade, N., Ojeda, S., Conteras, A., 1989. Biological control of bacterial wilt of potatoes caused by *Pseudomonas solanacearum*. American Potato Journal 66, 315–332.

Dashti, N., Prithiviraj, B., Hynes, R.K., Smith, D.L., 2000. Root and rhizosphere colonization of Soybean *Glycine max* (L) Merr by plant growth promoting rhizobacteria at low root zone temperatures and under short season conditions. Journal of Agronomy and Crop Science 185, 15–22.

El Albyad, M.S., el Sayed, M.A., el Shanshoury, A.R., 1996. Effect of culture conditions on the antimicrobial activities of UV-mutants of *Streptomyces corchorusii* and *S. Spiroverticillatus* against bean and banana wilt pathogens. Microbiology Research 151, 201–211.

Götz, M., Gomes, N.C.M., Dratwinski, A., Costa, R., Berg, G., Peixoto, R., Mendonc, L., Hagler, H., Smalla, K., 2006. Survival of *gfp*-tagged antagonistic bacteria in the rhizosphere of tomato plants and their effects on the indigenous bacterial community. FEMS Microbiological Ecology 56, 207–218.

Guo, J.H., Qi, H.Y., Guo, Y.H., Ge, H., Gong, L.Y., Zhang, L.X., Sun, P.H., 2004. Biocontrol of tomato wilt by growth-promoting rhizobacteria. Biological Control 29, 66–72.

Hayward, A.C., 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual Review of Phytopathology 29, 65–87.

Hayward, A.C., 1995. *Pseudomonas solanacearum*. In: Singh, U.S., Singh, R.P., Kohmoto, K. (Eds.), Pathogenesis and Host Specificity in Plant Disease: Histopathological, Biochemical, Genetic and Molecular Bases, vol. 1. Elsevier, Tarrytown, pp. 139–151.

Heuer, H., Yin, Y.N., Xue, Q.Y., Smalla, K., Guo, J.H., 2007. Repeat Domain Diversity of *avrBs3*-Like Genes in *Ralstonia solanacearum* Strains and Association with Host Preferences in the Field. Applied and Environmental Microbiology 73 (13), 4379–4384.

Jiang, Z.Q., Guo, Y.H., Li, S.M., Qi, H.Y., Guo, J.H., 2006. Evaluation of biocontrol efficiency of different *Bacillus* preparations and field application methods against phytophthora blight of bell pepper. Biological control 36, 216–223.

Kamilova, F., Validov, S., Azarova, T., Mulders, I., Lugtenberg, B., 2005. Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environmental Microbiology 7 (11), 1809–1817.

Kang, Y., Mao, G., Lu, C., He, L., 1995. Biological control of bacterial wilt of tomato by extracellular protein defective mutant of *Pseudomonas solanacearum*. Acta Phytopathology Sinica 22, 287–288.

Kempe, J., Sequeira, L., 1983. Biological control of bacterial wilt of potatoes: Attempts to induce resistance by treating tubes with bacteria. Plant Disease 67 (5), 499–501.

Larena, I., Sabuquillo, P., Melgarejo, P., De Cal, A., 2003. Biocontrol of Fusarium and Verticillium wilt of tomato by *Penicillium oxalicum* under greenhouse and field conditions. Journal of Phytopathology 151, 507–512.

Lemessa, F., Zeller, W., 2007. Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. Biological Control 42 (3), 336–344.

Li, G.C., Jin, L.P., Xie, K.Y., Qu, D.Y., 2004. Advances in research on bacterial wilt of ginger in China. Chinese Potato 18, 350–354.

Liu, M., Zhang, M., Ji, J.C., Yin, F.Q., Zhang, Y., Tu, Y., Ye, Y.J., 2005. Advances in research on bacterial wilt of ginger in China. Chinese Agricultural Science Bulletin 21, 337–341.

Miller, J.H., 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Silveira, E.B., Da, R., Mariano de, L.R., Michereff, S.J., 1995. Antagonism of *Bacillus* spp. Against *Pseudomonas solanacearum* and effect on tomato seedling growth. Fitopathologia Brasileira 20, 605–612.

Sun, S., Wei, A.M., Wu, H.X., Wang, J., 2004. Advances in research on chemical and biological control of plant bacterial wilt. Jiangxi Plant Protection 27 (4), 157–162.

Tjamos, S.E., Flemetakis, E., Paplomatas, E.J., Katinakis, P., 2005. Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. Molecular Plant-Microbe Interactions 18, 555–561.

Trigalet, A., Trigalet-Demery, D., 1990. Use of avirulent mutants of *Pseudomonas solanacearu*m for the biological control of bacterial wilt of tomato plants. Physiological and Molecular Plant Pathology 36, 27–38.

Trotel-Aziz, P., Couderchet, M., Biagianti, S., Aziz, A., 2008. Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas* spp. Mediating grapevine resistance against *Botrytis cinerea*. Environmental and Experimental Botany 64 (1), 21–32.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., Nishiuchi, Y., 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. Nov.: proposal for *Ralstonia pickettii*, *Ralstonia solanacearum* and *Ralstonia eutropha*. Microbiology and Immunology 39, 897–904.

Yang, J.H., Liu, H.X., Zhu, G.M., Xu, L.P., Pan, Y.L., Guo, J.H., 2008. Diversity analysis of antagonists from rice associated bacteria and their application in biocontrol of rice diseases. Journal of Applied Microbiology 104 (1), 91–104.

Zhu, H.H., Yao, Q., Li, H.H., Yang, S.Z., 2004. Inhibition of *Ralstonia solanacearum* by AM fungus *Glomus versiforme* and their effect on phenols in root. Journal of microbiology 31 (1), 1–5.