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Endophytic bacterial communities in tomato plants with differential resistance to *Ralstonia solanacearum*

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The endophytic bacterial communities in tomato varieties having differing resistance (or susceptibility) to *Ralstonia solanacearum* were investigated using both cultivation dependent and independent approaches. Both approaches revealed the differences between resistant (Xiahong-1) and susceptible (Baoshi-5) cultivars in terms of diversity and abundance of endophytic bacteria. The amount of the endogenous bacteria in Xiahong-1 at different growth stages was significantly higher than that in Baoshi-5. Furthermore, there were more culturable and antagonistic endophytic bacteria in Xiahong-1 than that in Baoshi-5. Seven endophytic bacterial genetic groups were identified in Xiahong-1 by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) and 16S rDNA sequence, and they were highly similar to *Sphingomonas yanoikuya*, *Pseudomonas pseudoalcaligenes, Serratia marcescens, Bacillus megaterium, Paenibacillus polymyxa, B. pumilus* and *B. cereus.* Four groups were identified in Baoshi-1 which were highly similar to *S. yanoikuyae, Pseudomonas fluorescens, Arthrobacter globiformis* and *Paenibacillus polymyxa*. In addition, antagonistic endophytes were identified by 16S rRNA gene analysis, and tested for their abilities to protect tomato plants from infection with *R. solanacearum.* The relationships between plant resistance and endophytic bacteria diversity are discussed.

Key words: Tomato, *Ralstonia solanacearum,* resistance, endophytic bacteria, diversity, biological control, 16S rRNA gene.

INTRODUCTION

Tomato bacterial wilt disease is an important tomato disease that is widely distributed in tropical, subtropical and temperate regions. The disease is caused by *Ralstonia solanacearum* (synonym *Pseudomonas solanacearum* E.F. Smith) (Yabuuchi et al., 1995), and has been recorded to infect more than 200 species representing over 50 families of plants (Hayward, 1991; Salanoubat et al., 2002). In China, the disease normally occurs at the flower and young fruit stages of tomato plant development, and there has been no effective control method to date (Liu and Zeng, 1999). *R. solanacearum* can live in the soil for a long time in non-hosts (Grey and Steck, 2001) which results in ineffectiveness for the control of bacterial wilt disease by crop rotation. The use of resistant varieties is thought to be the most effective way of controlling tomato bacterial wilt, however the development of resistant varieties takes a long time and their use is also limited by strain resistance specificity. Therefore, developing effective biological control agents is very important for the control of tomato bacterial wilt.

A large number of plant endophytic bacteria reside in plants which establish harmonious and close relationships with their hosts resulting from co-evolutionary processes. Endophytes offer a wide range of benefits to plants (Sturz et al., 2000) such as promoting growth (Barka et al., 2002; Kang et al., 2007), reducing disease severity (Coombs et al., 2004; Kloepper et al., 2004; Senthilkumar et al., 2007), inducing plant defense mechanisms

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(Coombs et al., 2004; Kloepper et al., 2004; Senthilkumar et al., 2007), inducing plant defense mechanisms (Bargabus et al., 2002; Mishra et al., 2006; Bakker et al., 2007), producing anti-herbivory products (Scott, 2001; Sullivan et al., 2007), biologically fixing nitrogen (Martı′nez et al., 2003; Jha and Kumar, 2007) and increasing plant mineral uptake (Malinowski et al., 2000). The investigation of endophytes in the biological control of diseases were reported on annual, biennial and perennial crops (Lodewyckx et al., 2002; Bargabus et al., 2004; Kloepper et al., 2004). There have been some reports on the biological control of tomato bacterial wilt (Li et al., 2003; Hu et al., 2006; Zhao et al., 2006; Nguyen and Ranamukhaarachchi, 2010; Almoneafy et al., 2012), but applications in the field have been limited by inconsistent disease control effects. Similarly, there have been some reports on the relationship between plant resistance and endophytic bacteria diversity (Sturz et al., 1999; Araujo et al., 2002; Reiter et al., 2002), however the roles of endophytic bacterial communities in resistant/susceptible tomato plants to *R. solanacearum* have yet to be investigated. In the present study, the endophytic bacterial communities of tomato plants resistant, or susceptible, to *R. solanacearum* were characterized by means of cultivation as well as culture-independent methods. The antagonistic endophytic bacteria from tomato and their control effects to *R. solanacearum* were also investigated.

MATERIALS AND METHODS

Experimental materials

Tomato varieties Xiahong-1 and Baoshi-5 with high resistance, and high susceptibility, to *R. solanacearum,* respectively were obtained from Horticultural Development Corporation in South China Agricultural University. The virulent strain *R. solanacearum* Tm89 isolated from tomato plant was provided by Bacteria Laboratory of South China Agricultural University.

Isolation of endophytic bacteria in tomato plant

Tomato seeds were surface-sterilized and sown with 5 seeds per flowerpot with soil and grown in a greenhouse. Six tomato plants from different growth periods were taken from 3 flowerpots for the isolation of endophytic bacteria. Ten grams of tomato roots and stems were each collected from 4 tomato growth periods: the germination stage (2 ~ 3 true leaves), the seedling stage (9 ~ 10 true leaves), the flowering period (the first panicle to flowering) and fruit period (the first white cluster fruit). The tomato roots and stems were sterilized by treating with 70% alcohol for 30 s, 5% sodium hypochlorite for 15 min, and 3-fold rinsing with sterile water. Aliquots of rinse water were cultured to examine for bacterial growth. The tomato roots and stems were then cut into pieces and ground using a mortar and pestle in 90 mL of sterile water. A 10-fold dilution series were made with sterile water and planted into MPN plates. Isolation and MPN counting for bacteria were done as described by Zhao and He (2004). The endophytic bacteria from tomato plant stems at the flowering period were also isolated and incubated at 30°C for 4 days on NA plates. Different single colonies were isolated and stored at 4°C.

Screening of antagonistic endophytic bacteria to *R. solanacearum*

One hundred milliliter of NA media was liquefied and cooled to 45°C. After adding 200 µL of *R. solanacearum* Tm89 bacterial suspensions with a concentration of 3×10^9 CFU/mL, the NA media was then poured into plates. Endophytic bacteria isolates were cultured for 24 h and then inoculated on to the surface of the NA medium plate with Tm89 bacterial suspension. Plates were incubated at 30°C for 48 h and the size of inhibition zones was measured.

Antagonistic endophytic bacteria against tomato bacterial wilt in the pot experiments

Two antagonistic endophytic bacteria strains isolated from Xiahong-1, X-3 and X-6, were cultured in liquid medium and used for tomato bacterial wilt control. Five Baoshi-5 tomato seedlings at 5-leaf growth stage were planted in a pot with 2.5 kg of soil containing *R. solanacearum*. In each pot, 300 mL of endophytic bacterial suspensions with a concentration of 1×10^8 CFU/mL were added into soil when the tomato seedlings were planted and at 10 days after the seedlings were planted. Thirty tomato plants were used for X-3 and X-6 endophytic bacteria stocks. The tomato plants were also treated with pure water as a control. The treated plants were monitored for disease development over a 25 day period after treatment and disease was rated using the following scale: 0, no wilting; 1, 1-25% wilt symptom; 2, 26-50% wilt symptom; 3, 51-75% wilt symptom; 4, 76-100% wilt symptom or dead (Park et al., 2007). The disease severity and biocontrol efficacy were calculated accordingly.

Identification of antagonistic endophytic bacteria by amplifying and sequencing the 16S rDNA

The total bacterial DNA from the X-3 and X-6 strains was extracted as per Araújo et al. (2002). Briefly, bacterial 16S rDNA was amplified by PCR with bacterial 16S rRNA universal primers 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-TACCTTGTTACGACTT-3' (Teng et al., 2006). The primers were synthesized by Shanghai Yingjun Biological Technology Co. Ltd. Twenty five microliter PCR reactions included 10x buffer 2.5 µL, dNTPs (2.5 mmol/L) 2 µL, primers 27F (5 µmol/L) 1 µL, primer 1492R (5 µmol/L) 1 µL, TaqDNA polymerase (5u/µL) 0.2 µL, ddH_2O 17.3 µL, and template DNA 1.0 µL. The reaction program was at 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 1.5 min and a final extension at 72°C for 10 min. PCR products were detected by 1.2% agarose gel electrophoresis and were sequenced by Shanghai Yingjun Biological Technology Co. Ltd. The sequencing results were analyzed with BLAST in Gene Bank.

Molecular diversity of endophytic bacteria in tomato plants

Ten grams of roots and stems of tomato plants at flowering period were collected and sterilized by treating with 70% alcohol for 30 s and then with 5% sodium hypochlorite for 15 min. The sterilized tissues were rinsed 5-6 times with sterilized water. The extraction of tomato total DNA was done according to the procedure described by Sambrook and Russell (2001).

Endophytic bacterial 16S rDNA PCR amplification and cloning

Bacterial 16S rDNA from tomato plant was amplified by PCR with bacterial 16S rRNA universal primers described above and tomato plant total DNA as template. *R. solanacearum* Tm89 was used as a positive control and double-distilled water as a negative control. The

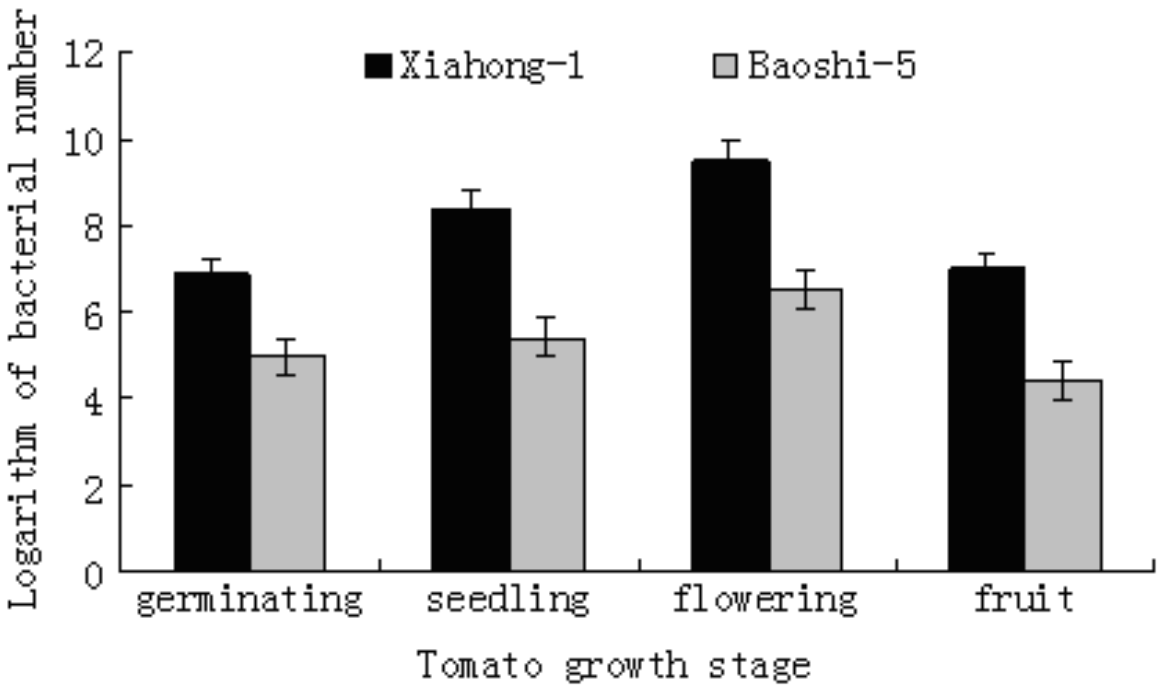

Figure 1. The number of bacteria in Xiahong-1 and Baoshi-5 isolated from roots and stems at germination, seedling, flowering and fruit growth stages, as monitored with MPN counting. Each value and error bar represents the mean and standard deviation, respectively, of 3 replicates.

PCR products were purified with a General UNIQ-10 column DNA purification kit (Shanghai Biological Engineering Technology Services Limited). The purified PCR products were ligated with pMD18-T vector and transformed into *E. coli* DH5α. Transformants were screened on LB plates containing 50 mg/L ampicillin.

At least 500 single white transformants were chosen randomly and their plasmid DNA were extracted by a conventional alkaline lysis method. The resultant plasmid DNA were amplified with pMD18-T universal primers RV-M: 5'-GAGCGGATAATTTCACACAGG-3 'and M13-47: 5'-CGC CAGGGTTTTCCCAGTCACGA-3' (Teng et al., 2006). The PCR reaction and program were the same as described above. Amplification products were detected with 1.2% agarose gel electrophoresis.

16S rDNA - RFLP analysis of endophytic bacteria

Confirmed PCR products were digested with the restriction enzymes *Hha* I and *Rsa* I. The digestion mixture had 10 µL PCR products, 2 µL 10 × NEB buffer, 0.5 µL *Hha* I (5U), 0.5 µL *Rsa* I (5U) and 7 µL sterile water, and was incubated at 37°C overnight. The results of digestion were detected with 2% agarose gel electrophoresis and analyzed with gel analysis software. By using NTSYS software package, the clusters for the results were obtained using an unweighted pair-group method with arithmetic averages (UPGMA) and the similarity and phylogenetic tree were generated automatically.

Sequencing and analysis of endophytic bacterial 16S rDNA

16S rDNA PCR original products in each section of 16S rDNA-RFLP were sequenced by Shanghai Chun-Ying Biological Technology Co. Ltd.

RESULTS

Differences in the population of endophytic bacteria in resistant and susceptible tomato plants

The colonization profiles of endophytic bacteria in both resistant and susceptible tomato plants were monitored with MPN counting at different growth stages. The amount of the endophytic bacteria in the roots and stems of tomato plants of the resistant variety, Xiahong-1, at germination, seedling, flowering and fruit growth stage were 7.5×10^6 cfu/g, 2.19×10^8 cfu/g, 3.16×10^9 cfu/g and 9.55×10^6 cfu/g, respectively; while there were 8.92×10^4 cfu/g, 2.512×10^5 cfu/g, 3.02×10^6 cfu/g and 2.512×10^4 cfu/g, respectively in the susceptible variety, Baoshi-5 (Figure 1). This result indicated that the population of bacteria was higher in the flower stage than those of other growth stages in both the resistant and susceptible tomato plants. Furthermore, the populations of bacteria in resistant tomato plants at different growth stages were significantly higher than those in susceptible plants.

The endophytic bacteria from tomato plant stems at the flowering period were isolated and incubated at 30°C for 4 days on NA plates. Based on the characteristics of bacterial colonies, 41 and 37 endophytic bacterial isolates could be obtained from Xiahong-1 and Baoshi-5, respectively indicating that the species of endophytic bacteria in the disease-resistant cultivar was more than that in the disease-susceptible cultivar.

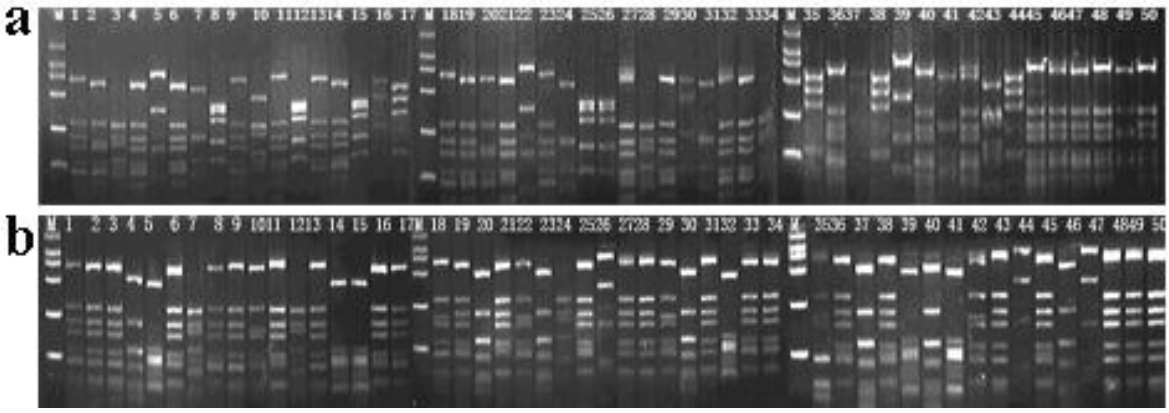

Figure 2. 16S rDNA-RFLP analysis of endophytic bacteria from Xiahong-1 **(a)** and Baoshi-5 **(b)**. M: DL-2000 Marker; Lanes 1-50: plasmid DNA clones of 16S rDNA of endophytic bacteria.

Molecular diversity and population analysis for endophytic bacterial 16Sr DNA from tomato plants

Total tomato DNA at the flowering stage from both Xiahong-1 and Baoshi-5 were extracted, amplified, and ligated with pMD18-T to obtain 16S rDNA libraries of tomato endophytic bacteria. Fifty positive 16S rDNA clones were randomly selected from both the resistant and susceptible tomato plant clone libraries to be used to amplify the 16S rDNA by PCR with 16S rRNA universal primers. The PCR products were then digested with *Rsa* I and *Hha* I and analyzed (Figure 2).

The clustering for 16S rDNA-positive clones of endophytic bacteria indicated that Xiahong-1 could be clustered into two groups, group A and B. When the similarity was 0.9, group A could be divided into 6 subgroups, while group B was divided into 3 subgroups (Figure 3a). Endophytic bacteria in Baoshi-5 were also clustered into group X and Y, which could be divided into 4 and 2 subgroups, respectively (Figure 3b). This indicated that endophytic bacteria in the 2 tomato varieties were different, and that the population diversity of endophytic bacteria in the disease-resistant variety was richer than that in the susceptible variety.

To identify the bacterial species from each RFLP subgroup, one clone from each was selected to sequence the 16S rDNA PCR product and the sequences were subjected to BLAST analyses in the NCBI database. The results (Table 1) showed that the RFLP subgroups A3, A4, A5, A6, B1, B2 and B3 from Xiahong-1 were identified to be similar to *Sphingomonas yanoikuyae, Pseudomonas pseudoalcaligenes, Serratia marcescens, Bacillus megaterium, Paenibacillus polymyxa, Bacillus pumilus* and *Bacillus cereus*, respectively. The RFLP subgroups X3, X4, Y1 and Y2 from Baoshi-5 were similar to *Sphingomonas yanoikuyae, Pseudomonas fluorescens, Arthrobacter globiformis* and *Paenibacillus polymyxa*, respectively. Not surprisingly, the sequences of the RFLP subgroups A1 and A2 from Xiahong-1, and X1 and X2 from Baoshi-1, were found to have 100% identities with tomato chloroplast DNA (*Lycopersicon esculentum* cultivar IPA-6 chloroplast AM087200), as disturbances from chloroplast 16S rDNA have been a problem in the characterization of bacteria in plants by using 16S rDNA sequences (Chelius and Triplett, 2001; Sun et al., 2008).

Isolation and identification of antagonistic endophytic bacteria in tomato plants to *R. solanacearum*

To investigate the differences of endophytic bacteria from the resistant and susceptible varieties including whether the disease-resistant variety has more antagonistic endophytic bacteria to *R. solanacearum*, the endophytic bacteria were tested in plate inhibition experiments. Of the 41 endophytic bacterial isolates obtained by culture method from Xiahong-1, 6 isolates were found to have antagonistic abilities against *R. solanacearum* Tm89 by plate inhibition tests. Of the 37 endophytic bacterial isolates from Baoshi-5, 3 isolates had antagonistic abilities against *R. solanacearum* Tm89. This indicated that the number of species of endophytic bacteria and antagonistic bacteria in the disease-resistant cultivar were greater than that in the disease-susceptible cultivar.

Two antagonistic isolates, X-3 and X-6, isolated from Xiahong-1 in the plate inhibition screening experiments above were tested in greenhouse for their effectiveness in controlling tomato bacterial wilt disease. At 25 days after the application of endophytic bacteria X-3 and X-6 in tomato soil, the control effect to *R. solanacearum* were 84.5, and 50.0%, respectively (Table 2). This demonstrated that the 2 antagonistic endophytic bacterial isolates had control effects on the tomato bacterial wilt disease and suggested that the existence of these endophytic bacteria in disease-resistant varieties might play a good role for the plant to be resistant to the infection of *R. solanacearum*.

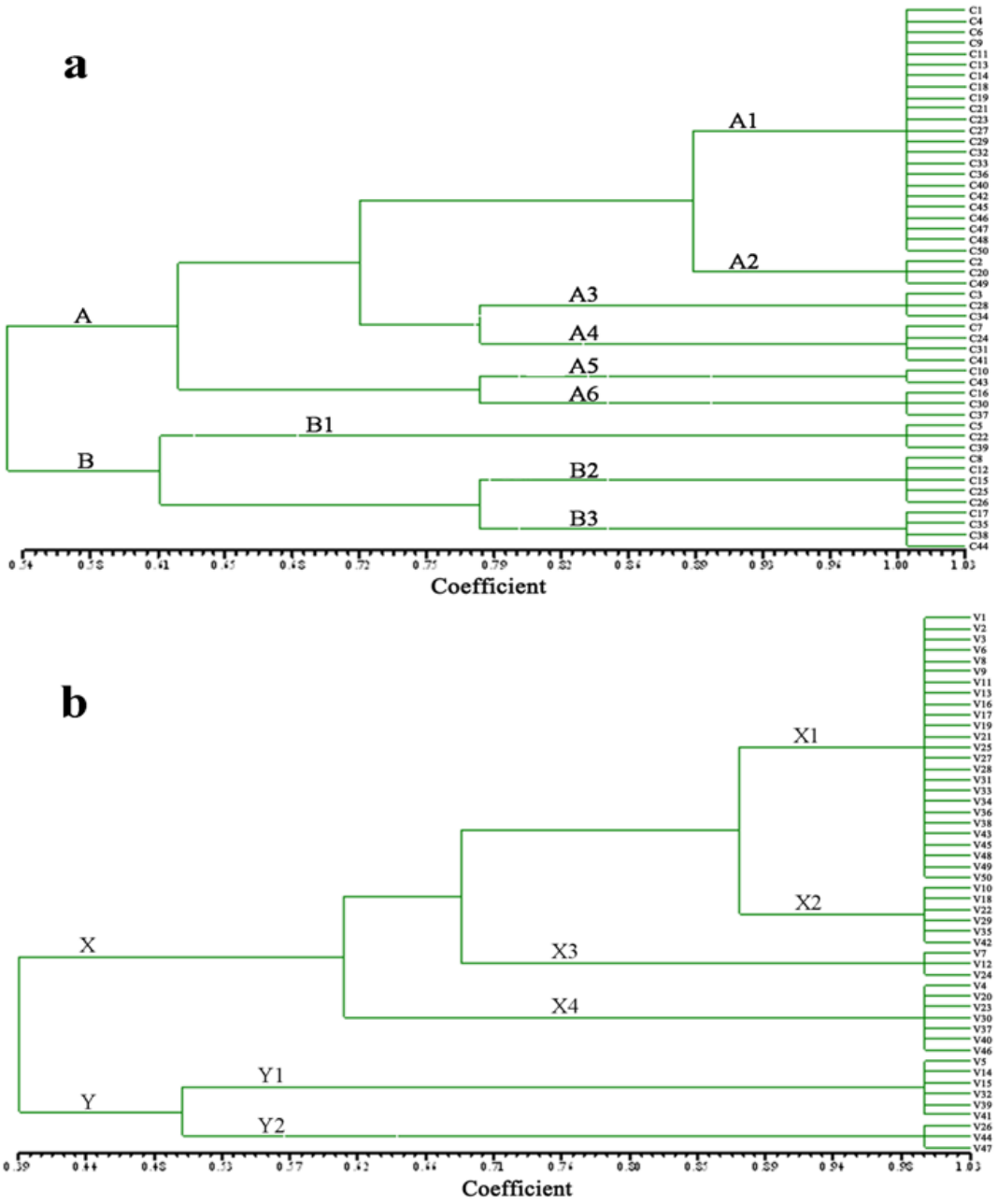

Figure 3. RFLP groups of 16S rDNA of 50 endophytic bacterial clones in Xiahong-1**(a)** and Baoshi **(b)**. By using NTSYS software package, the clusters for the results of Figure 2 (a) and 2(b) were determined in an unweighted pair-group method with arithmetic averages (UPGMA), and the similarity and phylogenetic tree were generated automatically.

To identify which bacterial species of antagonistic endophytes X-3 and X-6 are, the 16S rDNA from the two isolates were amplified using 16S rDNA primers, and sequenced. The results show that 16S rDNA of X-3 and X-6 had 100% identities with *B. megaterium* (EU627686) and *B. cereus* (AY129651), respectively. Both *Bacillus* species had been isolated from arable soils and were shown to have disease control effects in tomato crops (Nishijima et al., 2005).

DISCUSSION

The present results showed that the population of endophytes in resistant tomato cultivar at different growth stages was significantly higher than that in susceptible cultivar by a traditional MPN counting method. More endophytic bacteria were also obtained by cultivation-independent methods from resistant tomato plants (Xiahong-1) than that from susceptible plants (Baoshi-5).

Table 1. The BLAST results for sequences for endophytic bacterial 16S rDNA in Xiahong-1 and Baoshi-5.

RFLP subgroup	Clone Number	Description	Gene Bank Accession Number	Nucleotide identities (%)
A1	C1	*Lycopersicon esculentum* cultivar IPA-6 chloroplast	AM087200	100
		Solanum tuberosum cultivar Desiree chloroplast	DQ386163	100
A2	C2	*Lycopersicon esculentum* cultivar IPA-6 chloroplast	AM087200	100
		Solanum tuberosum cultivar Desiree chloroplast	DQ386163	100
A3	C3	*Sphingomonas yanoikuyae*	U37525	100
A4	C7	*Pseudomonas pseudoalcaligenes*	AB109887	100
A5	C10	*Serratia marcescens*	EF415649	99
A6	C16	*Bacillus megaterium*	AB271751	100
B1	C5	*Paenibacillus polymyxa*	AY302439	95
B2	C8	*Bacillus pumilus*	EF197942	100
B3	C17	*Bacillus cereus*	DQ207729	99
X1	V1	*Lycopersicon esculentum* cultivar IPA-6 chloroplast	AM087200	100
		Solanum tuberosum cultivar Desiree chloroplast	DQ386163	100
X2	V10	*Lycopersicon esculentum* cultivar IPA-6 chloroplast	AM087200	100
		Solanum tuberosum cultivar Desiree chloroplast	DQ386163	100
X3	V7	*Sphingomonas yanoikuyae*	U37525	100
X4	V4	*Pseudomonas fluorescens*	AB266613	99
Y1	V5	*Arthrobacter globiformis*	AB089741	99
Y2	V26	*Paenibacillus polymyxa*	AY302439	95

One clone from each RFLP subgroup in Figures 3 and 4 were selected to sequence 16S rDNA PCR products, and the sequences were subjected to BLAST analysis with the NCBI database.

Table 2. Biological control of *Ralstonia solanacearum* with antagonistic endophytic bacteria in pot experiments.

Antagonistic endophytic bacteria	Disease incidence (%)	Index of disease	Control effect (%)
X-6	50.0	24.2±0.6b	50.0
X-3	20.0	7.5±0.5c	84.5
CK	73.3	48.3±0.3a	

Values are given as the mean or standard deviation of three replicates. Mean values for Index of disease that share the same letter are not significantly different ($p > 0.05$).

In addition, the number of endophyte species with the abilities of antagonistic to *R. solanacearum* in resistant tomato plants was more than that in susceptible plants. These results indicated that there was a positive relationship between populations of endophytic bacteria and tomato resistance to bacterial wilt disease. Similarly, there have been numerous reports on the relationship between endophytic bacterial populations and plant resistance. By studying the population dynamics of endophytic bacteria from different resistant cotton varieties, it has been found that the number of endophytic bacteria in a cotton cultivar resistant to *Fusarium* wilt disease is significantly higher than that in a susceptible cultivar (Wang et al., 1997). The populations of endophytic bacteria in tobacco plants, at various planting times and in various tissues, showed certain differences in tobacco varieties with different resistance (Ma et al., 2004). The diversity of endophytic bacteria between healthy tobacco seedlings and *R. solanacearum*–inffected tobacco seedlings are different (Bottomly et al., 2004). Further study with RFLP analysis of bacterial 16S rDNA proved that populations of endophytic bacteria in tobacco resistant varieties are different from susceptible varieties. In comparing the populations of endophytic bacteria in healthy citrus plants with those in healthy citrus plants (no significant symptoms or Citrus variegated chlorosis (CVC)), Araujo et al. (2002) found that the endophytic bacteria *Curtobacterium flaccumfaciens* has a higher density only in asymptomatic citrus plants, and hence suggested that *C. flaccumfaciens* may play a key role in citrus resistance to CVC. It has been proposed that potato endophytic bacterial communities with biological functions determine potato resistance to soft rot bacterial disease (Sturz et al., 1999). In addition, there is a significant correlation between endophytic bacterial communities that colonized potato and the presence or absence of pathogenic bacterium *Erwinia carotovora*

(Reiter et al., 2002).

Further works will be needed to determine what mechanisms drive tomato cultivars resistant to pathogens contain higher number of endophytes than in susceptible cultivars. It will also be interesting to investigate the role of antagonistic endophytic bacteria in tomato plant resistance to *R. solanacearum*.

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