African Journal of Microbiology Research Vol. 6(18), pp. 4017-4022, 16 May, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.004
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Characterization and identification of a *Bacillus* strain TS02 as bio-control agent of strawberry powdery mildew

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Accepted 7 February, 2012

Powdery mildew of strawberry is a major limitation in most fruit production areas in the world. The effects of the *Bacillus* strain TS02 on bio-controlling powdery mildew of strawberry were tested in the fields. The results indicated that the fermented liquid of TS02 above 3×10^7 CFU/ml live bacteria concentration could control the disease of strawberry powdery mildew. The best effect was 52.23% with 3×10^9 CFU/ml live bacteria. There were 48.98, 34.89 and 24.63% bio-control effects, respectively with the bacteria fermented liquid, pure live bacteria and filtrated bacteria fermented liquid on strawberry powdery mildew, which showed the corporate results of live bacteria and secretion of bacteria. The bio-control mechanism was primary searched in laboratory with the leaves *in vitro*, and there was 100% growth inhibition of TS02 on *Sphaerotheca macularis* at 5 days after inoculation of powdery mildew pathogen. TS02 might inhibit the location of pathogen on the surface of leaves. TS02 also formed a monophyletic group with the species of the *Bacillus thuringiensis* complex group in the 16SrDNA sequence analysis, and shared similarity values of 99.9% with the *B. thuringiensis* type strains in the sequence analysis. It has a 6 bp difference from the most homologous strain *B. thuringiensis* AF290545.

Key words: Strawberry, powdery mildew, bio-control, *Bacillus s*train, 16S rDNA.

INTRODUCTION

Powdery mildew of strawberry caused by *Sphaerotheca macularis*, has recently become a severe problem in most areas of strawberry fruit production. Major epidemics of powdery mildew disease reduce crop yields by causing decreased fruit set, inadequate ripening, fruit cracking and deformation. It also causes poor flavor development and reduces post-harvest storage time. Strawberry cultivars differ widely in their resistance to powdery mildew, but the most popular cultivars are quite susceptible to the disease (Maas, 1998). The production of strawberries is largely dependent on using a number of chemicals to control *S. macularis*. However, pesticide residues are becoming a major issue, resulting in legislative actions to limit and regulate pesticide nowadays. As a result, new alternatives such as bio-control agents (BCAs) are required to reduce pesticide residues on fruits. Biological agents of plant disease prevention are efficient and pollution-free for the crop production (Reuveni et al., 1994; Falk et al., 1995; Daayf et al., 1997; Yang et al., 2009).

Some isolates of *Bacillus* spp. were reported as antagonists of several pathogens in the microbial products (Selvakumar et al., 2007; Felici et al., 2008; Dhillon and Sharma, 2009). Recently, these microbial agents have been given growing awareness, acceptance and applications (Obagwu and Korsten, 2003; Szczech and Shoda, 2004; Weyens et al., 2009) on the plant disease prevention, but the products of available BCAs of strawberry powdery mildew have been limited (Pertot et al., 2008). At present, the development of novel effective strains is the focus of attention during the application of

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microbial agents. In this work, our research focused on the identification and evaluation of strain TS02 for the disease control of strawberry powdery mildew. The aim is to develop a new and effective bio-control agent, specifically for the control of strawberry powdery mildew in protective planting conditions.

MATERIALS AND METHODS

The experiment was conducted in the greenhouses of China Agricultural University Science Center, using the strawberry cultivar "Toyonoka".

Microbial preparation

TS02 strain was picked from the slant medium, inoculated in the flask containing 100 ml beef extract peptone medium, and then cultured for 6 h to make the seed culture liquid. Taking 10 flasks each filled with 500 ml beef extract peptone medium respectively, 5 ml of the seed culture liquid was inoculated in each flask respectively, cultivated for 24 h to make the bacterial fermented liquid, and enable the amount of live bacteria reach to 3×10^9 CFU/ml. Five bottles of bacterial fermented liquid were used to make sterile filtrated liquid and live bacteria by 8,000 rpm centrifugation for 5 min. After that, by adding sterile water, live bacteria liquid which contained 3×10^9 CFU/ml bacteria was produced.

Field trials

Effects of different concentration of bacterial fermented liquid on strawberry powdery mildew

Four different concentration (3×10^9, 3×10^8, 3×10^7 and 3×10^6 CFU/ml) treatments of bacterial fermented liquid were prepared. Thirty plants were used per treatment, and three leaves were used for each plant, totally ninety leaves. Each treatment was replicated three times. The treatment blocks are randomly arranged. Sterile water and Triadimefon 1500X liquid were set as control.

Effects of the bacteria fermented liquid, live bacteria liquid and filtrated bacteria fermented liquid on strawberry powdery mildew

Three treatments were set as follows: bacteria fermented liquid (3×10^9 CFU/ml), pure live bacteria liquid (3×10^9 CFU/ml) and filtrated bacteria fermented liquid. The treatments were performed following the same earlier described.

Spraying, survey and statistical analysis

The first TS02 spraying on strawberry leaves is in the early onset stage of powdery mildew in the fields; spraying was done twice during the trial period with 10 days interval. The disease index (DI) was investigated 5 and 7 days after first spraying, respectively, and 5 days after the second spraying. Three representative leaves of each plant were selected to be labeled. The DI was investigated and control effect (CE) was calculated for the treatments. The disease level of powdery mildew was defined as the percentage of diseased leaf area. Level 0: with no bacteria symptoms; level 1, 3, 5, 7 and 9: the powdery white patches of fungus area accounts for the whole leaf area were below 5%, 6~15%, 16~25%, 26~50% and exceed 50%, respectively. The disease index (DI) and control effect (CE) was calculated as follows:

$$\text{Disease Index (DI)} = \frac{\Sigma\ (\text{number of diseased leave} \times \text{Relative level})}{\text{Total number of investigated leaves} \times 9} \times 100$$

$$\text{Control Effect (CE)} = \frac{\text{Disease index of control} - \text{Disease index of treatment}}{\text{Disease index of control}} \times 100$$

All the data were processed and analyzed by software SAS8.0, the significance of different treatments was analyzed by ANOVA and DUNCAN'S procedures.

Laboratory trial

Inoculation test of powdery mildew pathogen of strawberry (S. macularis f. sp. Fragariae*) in laboratory trial*

Thirty disease-free leaves *in vitro* were used in the laboratory trials. The undersides of leaves were placed upside on filters wetted with sterile water in a Petri dish. Powdery mildew pathogens were inoculated on leaves by spraying; the treated leaves were cultivated under the condition of 20 ± 2°C, 80% relative humidity, and cool white fluorescent light with a photosynthetic photon flux density of 50 μmol m^{-2} s^{-1} for a photoperiod of 16 h/day. The index of diseases was investigated after inoculation 5, 7 and 10 days respectively.

The effects of TS02 on strawberry powdery mildew in laboratory trial

First, the thirty disease-free leaves were sprayed with bacteria fermented liquid of TS02 (3×10^9 CFU/ml). After 3 days, the TS02 treated leaves were inoculated with powdery mildew pathogens by spraying. The group with no TS02 treatment (Inoculated powdery mildew pathogen same as 1.4.1) was set as control. DI was surveyed 5, 7 and 10 days after inoculated powdery mildew pathogens respectively. The investigation and statistical analysis were performed same as that in the field trials.

Identification of 16S rDNA of *Bacillus* TS02 strain

Bacterial identification was complemented by analysis of the 16S rDNA sequence. Polymerase chain reaction (PCR) was performed using universal bacterial primer pairs of 16SrDNA (16SF5'-AGTTTGATCCTGGCTCAG-3' and 16SR5′-GGTTACCTTGTTACGACT-3'). TS02 genomic DNA was set as template for PCR amplification. PCR system was 50 μL, which included template DNA 50 ng, 10 × PCR buffer 10 μL, the four dNTP mixtures each 200 μmol/L, positive and negative primer each 20 pmol/L, and *Pyrococcus furiosus* DNA (Pfu) polymerase 1.5 U and Mg^{2+} 2 mmol/L. The PCR was programmed at: 94°C for 2.5 min, 94°C for 45 s, 52°C for 1 min, 72°C for 1.5 min; 35 cycles, 72°C extension for 5 min and finally stored at 4°C for preservation. Amplified fragments were separated by 1% agarose gel electrophoresis, and purified by DNA Gel Extraction Kit (Tiangen, China). Sequencing of the PCR product was performed by Genome Express (Sangon, China). The resulting sequences of TS02 were aligned manually with the sequences of representative *Bacillus* strains obtained from the GenBank database. All of the analyses were made using the MEGA program, Version 4.1.

Table 1. Effects of different concentration of TS02 fermented liquid on strawberry powdery mildew.

Treatments	5 days after 1st spraying		7 days after 1st spraying		5 days after 2nd spraying		Significant difference	
	DI*	CE*	DI	CE	DI	CE	0.05	0.01
3×10^9 CFU/ml	17.23(±0.32)	42.25(±0.26)	16.45(±0.27)	45.56(±0.39)	15.32(±0.23)	50.23(±0.36)	A	a
3×10^8 CFU/ml	16.25(±0.32)	40.32(±0.34)	14.25(±0.36)	44.56(±0.35)	14.56(±0.34)	49.65(±0.33)	A	a
3×10^7 CFU/ml	17.63(±0.37)	41.56(±0.21)	15.36(±0.16)	42.89(±0.26)	14.32(±0.30)	47.98(±0.28)	A	a
3×10^6 CFU/ml	16.36(±0.13)	25.69(±0.17)	20.25(±0.33)	24.56(±0.32)	21.32(±0.33)	26.89(±0.34)	B	b
Triadimefon1500X liquid	10.98(±0.41)	66.56(±0.39)	10.56(±0.32)	67.56(±0.36)	11.36(±0.35)	67.36(±0.38)	C	c
Control	27.56(±0.24)		28.89(±0.28)		35.65(±0.30)			

The data in the table are average of three repetitions, the same letters in the same column mean no significant differences among treatments (P<0.05 and P<0.01). Index of diseases ± standard error and Control effects ± standard error are given. *DI: Disease Index. *CE : Control effect.

Table 2. Effects of bacteria fermented liquid, pure live bacteria and filtrated bacteria fermented liquid on strawberry powdery mildew.

Treatments	5 days after 1st spraying		7 days after 1st spraying		5 days after 2nd spraying		Significant difference	
	DI	CE	DI	CE	DI	CE	0.05	0.01
bacteria fermented liquid	17.65(±0.45)	44.56(±0.26)	16.26(±0.28)	45.96(±0.38)	14.56(±0.32)	48.98(±0.34)	A	a
pure live bacterial liquid	19.96(±0.27)	30.21(±0.15)	18.32(±0.34)	32.56(±0.35)	14.32(±0.23)	34.89(±0.18)	B	b
filtrated bacteria fermented liquid	21.25(±0.32)	24.26(±0.43)	19.63(±0.35)	25.36(±0.28)	13.56(±0.40)	24.63(±0.38)	C	c
Control	27.36(±0.34)		29.52(±0.35)		33.32(±0.36)			

RESULTS

Effects of different concentrations of TS02 fermented liquid on strawberry powdery mildew in the field

Based on the field trials of pesticides, the spraying of TS02 was carried out during the first stage of natural incidence of powdery mildew. The results indicated that the different concentrations of TS02 fermented liquid had a degree of inhibiting effect on strawberry powdery mildew (Table 1). The control effect of each concentration was increased with the increasing of spraying times. The 3×10^6 CFU/ml treatment effect was significantly lower than other treatments at the level of 0.05 and 0.01. There were no significant differences among the concentrations of 3×10^9, 3×10^8 and 3×10^7 CFU/ml. It is obvious that the concentration of fermented liquid must reach a certain concentration (3×10^7CFU/ml) so as to achieve the control effect on strawberry powdery mildew.

Effects of bacterial fermented liquid, pure live bacteria and filtrated bacteria fermented liquid on strawberry powdery mildew in the field

The bacteria fermented liquid, pure live bacteria and filtrated bacteria fermented liquid had inhibition effects on strawberry powdery mildew compared to the control treatment (Table 2). The control effects of the three treatments increased gradually with the increase of spraying times. Five days after the second spraying, bacteria fermented liquid had the best control effect, which was up to 48.98%; and filtrated bacteria fermented liquid had the lowest control effect of 24.63%; the control effect of pure live bacteria liquid was 34.89%. There was a significant difference between the treatments at the level 0.05 and 0.01.

Effects of TS02 on the pathogen of strawberry powdery mildew in laboratory

Strawberry powdery mildew fungus almost cannot

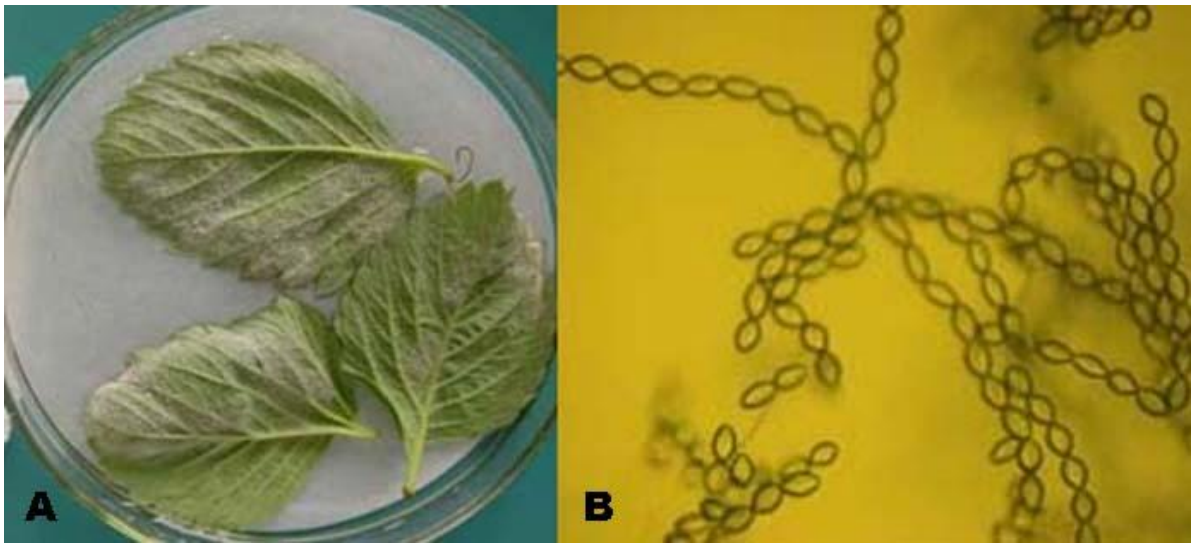

Figure 1. The infected leaves *in vitro* by powdery mildew pathogen (A), and the powdery mildew conidia (B).

Table 3. Effects of TS02 on strawberry mildew powder in laboratory.

Treatments	DI after 5 days	DI after 7 days	DI after 10 days
TS02 live bacterial liquid	0	2.52(±0.17)	9.46(±0.13)
control	100	100	100

live in artificial culture medium. Therefore, the culture of *S. macularis* f. sp. *Fragariae* needs to be operated on the i*n vitro* leaves of strawberry in laboratory trials. After 5 days of inoculation of powdery mildew pathogen on the disease-free strawberry leaves by spraying, the DI was 100% (Figure 1A). The conidia parts of the powdery mildew disease were observed under a microscope with magnification of 200X (Figure 1B). Five days after inoculation of powdery mildew pathogen, the DI was 0 with TS02 live bacteria liquid treatment, while the DI of control treatment had reached 100% (Table 3). Moreover, the DI was 2.52 and 9.46 with TS02 live bacteria liquid treatment after 7 and 10 days inoculation of powdery mildew pathogen, respectively. The results showed that pre-treated *in vitro* leaves by TS02 live bacteria liquid might reduce colonization of powdery mildew pathogen on the leaves, thereby reducing the incidence and spread of powdery mildew disease.

Identification of the antagonistic bacteria strain TS02

Genomic DNA of *Bacillus* TS02 strain was used as template. PCR amplification was performed using the 16S rDNA universal primer 16SF5'-AGTTTGATCCTGGCTCAG-3' and 16SR5'-GGTTACCTTGTTACGACTT-3'. A 1.5 kb fragment of the 16SrDNA as expected was amplified (Figure 2). After purification of the PCR products, verification and analysis of the ligation and clones, positive clone samples were sequenced. The results showed that 16S rDNA sequences of TS02 strain were 1511 bp. The phylogenetic tree (Figure 3) of 16S rDNA sequences of TS02 and related type strains was constructed. TS02 exhibited a phenotypic similarity with *Bacillus* spp.; the sequence analysis of the 16S rDNA confirmed that TS02 belonged to the *Bacillus,* and revealed that the TS06 strain share similarity values of 99.9% with the *B. thuringiensis* type strain AF290545 (Figure 3). TS02 is a new strain; it has 6 bp differences in the 16S rDNA sequence compared to the strain *B. thuringiensis* type strain *AF290545*.

DISCUSSION

For the credible application of bacterial antagonisms in strawberry fields, a good combination result of *in vitro* and *in vivo* inhibition test of antagonists is needed. For most diseases of strawberry, the culturing of fungal are easier to be established *in vitro* for the pathogenicity tests than on strawberry powdery mildew fungus, the reason being that the pathogen *S. macularis* f. sp. Fragariae of strawberry powdery mildew almost cannot live in artificial culture medium.

In this study, an innovative culture was operated on the

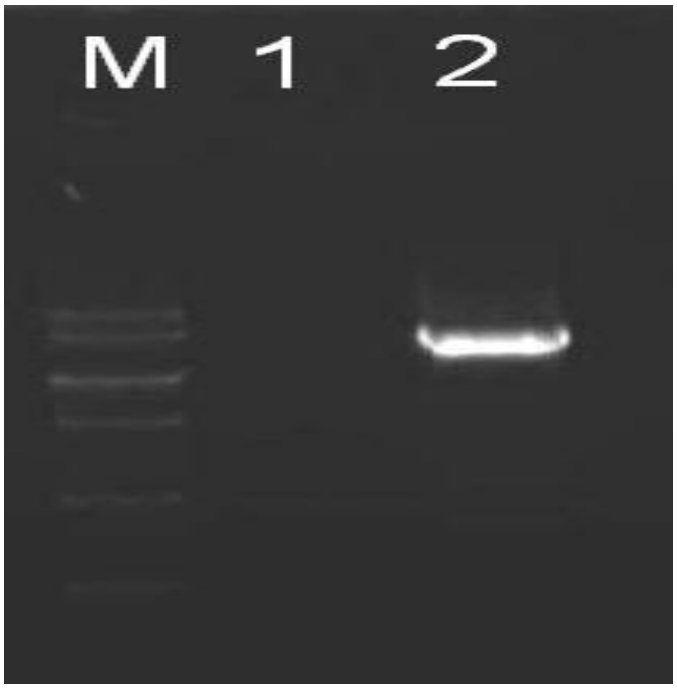

Figure 2. PCR products of 16S rDNA of strain TS02. Lane M, DNA Marker; 1, negative control; 2, PCR product of 16S rDNA.

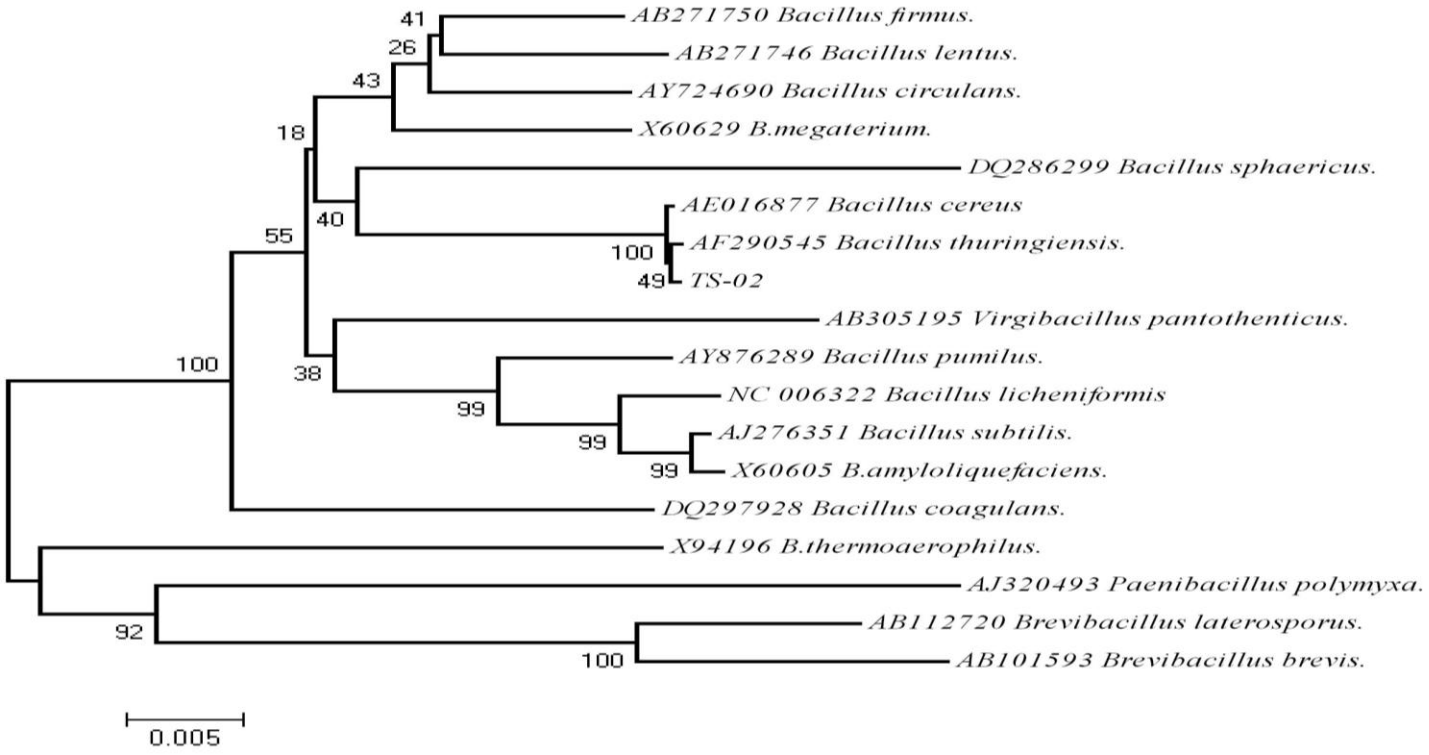

Figure 3. Phylogenetic tree based on 16S rDNA sequence homology of strain TS02.

in vitro strawberry leaves in laboratory trials. The inhibition effects of TS02 on strawberry powdery mildew were observed significantly, not only *in vitro* but also *in vivo* performance. The control effect of TS02 on

strawberry powdery mildew *in vivo* is consistent to the results of antagonism test of TS02 strain *in vitro*. The strain TS02 has antifungal efficacy against pathogens of strawberry powdery mildew, there were 48.98% bio-control effects with the bacteria fermented liquid on strawberry powdery mildew. The precise inhibition effect of TS02, *in vitro* and *in vivo*, gives TS02 the potential to be exploited as a bio-control agent for strawberry powdery mildew disease in strawberry production. Previous reports have shown the reasons for antagonism between bacteria and fungi (Mille et al., 2006). In this study, there was 100% growth inhibition of TS02 on *S. macularis* f. sp. Fragariae with the leave *in vitro* at 5 days after inoculation of powdery mildew pathogen. The actual mechanism responsible was not investigated here, but the results suggest that TS02 might inhibit the location of pathogen on the surface of leaves during the initial time.

The identification technology of bacterial antagonism needs to be developed, because the identification of *B. thuringiensis*-like organisms has become difficult and laborious, and they cannot be distinguished from each other by conventional phenotypic tests. The 16S rRNA sequencing is often used to define species (Willam et al., 1991). TS02 shares a similarity value of 99.9% with the *B. thuringiensis* type strains in the 16S rDNA sequence analysis. It has 6 bp differences from the most homologous strain *B. thuringiensis* AF290545. Although TS02 was identified as a strain of *B. thuringiensis* by the16S rRNA sequencing at last in this study, we were also puzzled with the difference of *B. thuringiensis* and *Bacillus cereus* in the phylogenetic tree at beginning. Whatever, the TS02 was so closed to the two species that further data are needed to discriminate. A previous report (Wu et al., 2006) showed that the presence of highly conserved sequences in the 16S rRNA gene was difficult to discriminate some species and subspecies of *Bacillus* group; this was also observed in our study.

Furthermore, based on previous works in our laboratory, the amplified ribosomal DNA restriction analysis (ARDRA) was also employed as an assistant method to identify TS02. The 16S rDNA PCR amplicon of TS02 was digested by six restriction enzymes (*Alu*I, *Taq*I, *Mse*I, *Bst* UI, *Hha*I and *Tsp*509I), and ARDRA results helped to make sure of the difference of TS02 from the type strains between the two species of *B. thuringiensis* and *B. cereus* (data not published). These results reaffirm that not only the biochemical, morphological or cultural characteristics of TS02 but also the combination of molecular methods were needed for a convenient system of the identification of TS02-like *Bacillus* strains.

ACKNOWLEDGEMENTS

This work was supported by the Commonwealth Science and Technology Program of Agricultural Ministry (201003064) and College PhD Subject Science and Technology Program of Education Ministry (20110008130003). We thank our NZ and USA friends, Steven Nichols and Ian Smith, for the help to review the English in this manuscript.

REFERENCES

Daayf F, Schmitt A, Belanger RR (1997). Evidence of phytoalexins in cucumber leaves infected with powdery mildew following treatment with leaf extracts of *Reynoutria sachalinensis*. Plant Physiol., 113: 719-727.

Dhillon MK, Sharma HC (2009). Effects of *Bacillus thuringiensis* δ-endotoxins Cry1Ab and Cry1Ac on the coccinellid beetle, *Cheilomenes sexmaculatus* (Coleoptera, Coccinellidae) under direct and indirect exposure conditions. Biocontrol Sci. Techn., 19: 407-420.

Falk SP, Gadoury DM, Cortesi P, Pearson RC, Seem RC (1995). Parasitism of Uncinula necator ascomata by the mycoparasite *Ampelomyces quisqualis*. Phytopatholog, 85: 794-800.

Felici C, Vettori L, Giraldi E, Forino LMC, Toffanin A, Tagliasacchi AM, Nuti M (2008). Single and co-inoculation of *Bacillus subtilis* and *Azospirillum brasilense* on Lycopersicon esculentum: Effects on plant growth and rhizosphere microbial community. Appl. Soil Ecol., 40: 260-270.

Maas J (1998). Compendium of Strawberry Diseases. APS Press, St. Paul, MN, USA.

Mille LC, Fischer H, Tranvik LJ (2006). Antagonism between bacteria and fungi: substrate competition and a possible tradeoff between fungal growth and tolerance towards bacteria. Oikos. 113: 233-242.

Obagwu J, Korsten L (2003). Integrated control of citrus green and blue molds using *Bacillus subtilis* in combination with sodium bicarbonate or hot water. Postharvest Biol. Tec., 28: 187-194.

Pertot I, Zasso R, Amsalem L, Baldessari M, Angeli G, Elad Y (2008). Integrating biocontrol agents in strawberry powdery mildew control strategies in high tunnel growing systems. Crop Prot., 27: 622-631.

Reuveni R, Agapov V, Reuveni M, Raviv M (1994). Effects of foliar sprays of phosphates on powdery mildew (*Sphaerotheca pannosa*) of roses. J. Phytopathol., 142: 331-337.

Selvakumar G, Mohan M, Sushil SN, Kundu S, Bhatt JC, Gupta HS (2007). Characterization and phylogenetic analysis of an entomopathogenic *Bacillus cereus* strain WGPSB-2 (MTCC 7182) isolated from white grub, *Anomala dimidiata* (Coleoptera: Scarabaeidae). Biocontrol Sci. Techn., 17: 525-534.

Szczech M, Shoda M (2004). Biocontrol of Rhizoctonia damping-off of tomato by B*acillus subtilis* combined with *Burkholderia cepacia*. Phytopathology, 152: 549-556.

Weyens N, van der Lelie D, Taghavi S, Newman L, Vangronsveld (2009). Exploting plant-microbe partnerships to improve biomass production and remediation. Trends Biotechnol., 27(10): 591-598.

Willam GW, Susan M, Barns (1991). 16s Ribosomal DNA amplification for phylogenetic study. J. Bacteriol., 173(2): 697-703.

Wu XY, Walker MJ, Hornitzky M, Chin J (2006). Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. J. Microbiol. Meth., 64: 107-119.

Yang JW, Kloepper JW, Ryu CM (2009). Rhizosphere bacteria help plants tolerate abiotic stress. Trends of Plant Sci., 14:1-4.