African Journal of Biotechnology Vol. 11(28), pp. 7193-7201, 5 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2963
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Characterization and evaluation of *Bacillus* isolates for their potential plant growth and biocontrol activities against tomato bacterial wilt

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Accepted 3 February, 2012

About 200 *Bacillus* isolates were isolated from tomato and potato rhizosphere and examined for their antagonistic activities against *Ralstonia solanacearum* T-91, the causal agent of tomato bacterial wilt (TBW), *in vitro* and *in vivo*. Four strains, AM1, D16, D29 and H8, have shown high potential of antagonistic activity against the pathogen in laboratory and greenhouse experiments. In greenhouse, 81.1 to 89.0% reduction of disease incidence of TBW was recorded in treated tomato plants with 4 isolates, which also significantly ($p > 0.05$) increased plant height by 22.7 to 43.7% and dry weight by 47.93 to 91.55% compared with non-treated control. 16SrRNA gene sequence, the biochemical and physiological tests and fatty acid methyl esters analysis assigned strains AM1 and D29 as *Bacillus amyloliquefaciens*, while strains D16 and H8 as *Bacillus subtilis* and *B. methylotrophicus,* respectively. In addition, the 4 strains showed ability to inhibit growth of the three soil-borne fungi, produce indole-3-acetic acid, siderophores and also with exception of strain D16, the other 3 strains were capable of solubilizing phosphate. Therefore, these results suggest that out of 200 isolates, *Bacillus* stains AM1, D16, D29 and H8 support good antagonistic activity and could be applied as biocontrol agents against TBW under greenhouse conditions beside their potential to promote tomato plants growth.

Key words: Tomato, *Ralstonia solanacearum*, *Bacillus* spp, biological control, plant growth promotion activities.

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most popular vegetables in the world. It is the second most important vegetable crop next to potato. The world production was about 141,400,629 tons fresh fruit produced on 4,980,424 hectares in 2009 (http://faostat.fao.org/). Tomato crop has suffered from several biotic and abiotic stresses during its growing season. Among those stresses, bacterial wilt of tomato caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995) is one of the most devastating and wide-spread diseases of crops worldwide (Poussier et al., 1999). *R. solanacearum* infects more than 200 species in 50 families (Hayward, 1991), including tomato, potato, eggplant, pepper, tobacco, banana, chilli and peanut (French and Sequeira, 1970). This aforementioned reason therefore makes this soil-borne pathogen difficult to control. Many methods like soil solarization, field sanitation, crop rotation and use of bactericides have been applied to control the disease, but with only limited success (Ciampi-Panno et al., 1989). Resistance cultivars have been used as an important component of integrated disease management. However, it is generally agreed that breeding for resistance is not completely effective, producing only limited gains and often lacking stability or durability (Boucher et al., 1992; Hayward,

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1991). Furthermore, the high variability of strains of *R. solanacearum* combined with the influence of environmental factors on host–pathogen interactions often restricts the expression of resistance to specific regions (Hayward, 1991).

Many studies report that biological control is a promising alternative strategy to be used in the integrated control of various bacterial plant diseases (Weller, 1988). Biological control strategies may either help development of alternative management measures or be integrated with other practices for effective disease management at the field level. Plant growth-promoting rhizobacteria (PGPR), a group of root associated bacteria, intimately interact with the plant roots and consequently influence plant health and soil fertility. They offer an excellent combination of traits useful in disease control and plant growth promotion. This group can produce bioactive substances to promote plant growth and/or protect them against pathogens (Harish et al., 2009). Some PGPR may influence plant growth by synthesizing plant hormones or facilitating uptake of nutrients from the soil through different direct mechanisms such as atmospheric nitrogen (N) fixation, solubilization of phosphate, and synthesis of siderophores for iron sequestration, thus making nutrients more available to plants (Glick et al., 2007). Among PGPR cluster, *Bacillus* is one of the most potential genera due to their spore forming ability, thereby increasing the adaptation of *Bacillus* strains to commercial formulation and field application (Liu and Sinclair, 1993). Some species of *Bacillus* suppress plant pathogens and insect pests by producing antibiotic metabolites, while others stimulate plant host defenses prior to pathogen infection (van Loon, 2007), which indirectly contributes to increase crop production.

Published reports on endophytic colonization and biofilm formation by *Bacillus* and *Paenibacillus* spp. have suggested that the endophytic colonization and biofilm formation improve the bacterium's ability to act as a biocontrol agent against plant pathogens (Davey and O'toole, 2000; Hallmann et al., 1997; Timmusk et al., 2005). So applying cultivated plants and soil with these microorganisms will be important in preventing the environmental pollution that results from pesticides and chemical fertilizers. Hence, this study aimed to isolate and characterize *Bacillus* spp. from cultivated soil by solanaceous vegetables, screen them against *R. solanacearum* under laboratory conditions, evaluate their plant growth promotion activities *in vitro* and finally screen their efficacy against *R. solanacearum* under greenhouse conditions.

MATERIALS AND METHODS

Isolation of potential antagonistic isolates

A total of 200 isolates have been isolated from ten rhizosphere soil samples of healthy tomato and potatoes collected from three locations in Yemen (Amran, Sana'a and Thamar areas), and one sample was collected from Hangzhou, China. Each sample was placed in plastic bag during sample collection in the field and 100 g were taken from each sample for isolation and maintained in suitable plastic cans at 4°C until use. Serial dilution method was applied to isolate rhizobacteria from soil, where 5 g of soil sample were added to 50 ml of sterile distilled water then shaken in rotary shaker at 160 rpm until homogenization of soil suspension. The suspensions were plated in Luria-Bertani agar (LBA) medium incubated at 28°C for 48 h. The appeared colonies of each representative isolate were picked and streaked in new LBA plates and incubated at 28°C for 48 h. Typical single colony for every acquired isolates was sub-cultured in nutrient agar slant for further study. For long term preservation, obtained isolates were stored in Eppendorf tubes containing LB with 20% glycerol at -20ºC.

Plant pathogen and culture conditions

R. solanacearum strain T-91 belonging to race 1 biovar 3, which is a standard virulent strain on tomato (Li et al., 2010), was provided by the Institute of Biotechnology, Zhejiang University, China. It was grown on yeast extract peptone glucose agar (YPGA) medium (Xue et al., 2009).

Preliminary screening for antagonistic activity *in vitro*

This test was performed to screen antagonistic activity of obtained isolates from soil against *R. solanacearum* according to the method described by Li et al. (2008b). Cultures of *R. solanacearum* was grown overnight in nutrient broth; 0.5 ml of the liquid culture was mixed with 15 ml lukewarm molted nutrient agar in sterile plates and allowed to solidify. Afterwards, each tested isolate was spotted on the surface of agar plates and incubated them at 28°C for 72 h. Consequently, those bacteria that displayed positive inhibition activity were considered as antagonistic isolates and selected for further investigations.

Laboratory *in vitro* assay for studying antagonism

Antagonistic activity of selected isolates from preliminary test was evaluated against 3 plant pathogenic fungi (*Fusarium graminearum, Pythium aphanidermatum* and *Rhizoctonia solani*) by applying two techniques; dual culture method described by Ganesan and Gnanamanickam (1987) and double layer agar method given by Pawar and Puranik (2008). To check the antagonism ability against *R. solanacearum,* YPGA medium was seeded by adding 10 ml of *Ralstonia* suspension (overnight liquid culture of *Ralstonia* centrifuged and then the cell pellets were re-suspend with sterilized saline solution (0.85% NaCl) to final concentration~10^8 CFU/ml) and 5 ml of 2,3,5-triphenyl tetrazolium chloride (TZC) to 1 L of melted YPGA and pouring into sterilized plates. Thereafter, each test antagonist was transferred to surface of agar using sterile toothpicks and incubated at 28°C. Plates were checked after 72 h and clear haloes without pinkish coloration around the tested isolates indicating inhibition of *Ralstonia* growth were measured (Adesina et al., 2007). All experiments were done under completely randomized design (CRD) with three replicates.

Evaluation of antagonistic isolates in greenhouse

To determine whether the potential selected antagonistic isolates can suppress tomato bacterial wilt (TBW) and enhance plant growth under greenhouse conditions or not, this experiment was carried out in greenhouse of the Agriculture and Biotechnology College during the period 25th May to 5th August, 2010. Ten selected

isolates which offer inhibition diameter (15 to 20 mm) during *in vitro* test were used for this investigation. Seeds of Tomato Cv. "Hezuo" (susceptible to infection by *R. solanacearum*) were surface-sterilized by immersing in 2% sodium hypochlorite solution for 2 min, and then washed thoroughly three times by sterilized water. Pots of 18 cm diameter filled by sterilized potting mixture (1 soil: 1 peat moss), were used in study. Cultivated pots were maintained under greenhouse conditions with range of temperature of 25 to 30°C and relative humidity between 70 to 90%. Two control treatments were considered; control one (C1) was treated only with pathogen and control two (C2) was not treated either with pathogen or tested isolates. Pots were arranged in completely randomized block design with four replicates for each treatment and 16 plants for each replicates.

Application by antagonistic strains

Tomato plants were applied with tested antagonist twice; the first was before cultivation, when seeds were treated by tested isolates through overnight soaking in bacterial suspension adjusted to 10^8 CFU/ml and seeds that were immersed with sterilized saline served as control. Then bacterized seeds were transferred to dry sterilized filter paper and allowed to dry in laminar flow for 8 h. Before planting the microbiolized seeds into the pots, they were pre-germinated on moistened filter paper with sterilized distilled water for 5 days. Each pot was sowed with the 4 pre-germinated seeds. The second application with tested antagonist was carried out before one week of inoculation with pathogen by soil drenching. About 50 ml of antagonist suspension (1.0×10^8 CFU ml^{-1}) were poured in each pot and pots with 50 ml of saline served as control.

Inoculation of *R. solanacearum*

Ralstonia was cultured in yeast extract peptone glucose (YPG) broth medium and incubated at 30°C in rotary shaker with 160 rpm for 18 h and centrifuged at 10000 rpm for 10 min and afterward cell pellets were harvested and diluted to obtain the final concentration of 1.0×10^9 $CFUml^{-1}$. Thereafter, tomato plants in all treatments except C2 were inoculated with the pathogen at the third to fourth leaf stage by punching each plant with sterilized needle at the base of stem above upper secondary root, subsequently 80 ml of the suspension were poured in every pot over wounded area. Pots in C2 treatment were treated by pouring 80 ml of sterilized saline solution in each of them. After inoculation, all pots were covered by polyethylene bags for 24 h to maintain high humidity (Algam et al., 2010).

Disease assessment

Tomato plants were monitored for development of wilt symptoms. Disease index data were recorded according to the scale ranged from 0 to 4 (Park et al., 2007). Based on disease index collected data, two parameters; disease incidence and biocontrol efficacy of antagonistic isolates were estimated as follows (Xue et al., 2009):

$$\text{Disease incidence} = \frac{\text{Disease index} \times \text{number of diseased plants in this index}}{\text{Total number of plants investigated} \times \text{the highest disease index}} \times 100\%$$

$$\text{Biocontrol efficacy} = \frac{\text{Disease incidence of control - Disease incidence of antagonist-treated group}}{\text{Disease incidence of control}} \times 100\%$$

Plant growth parameters assessment

After one month from second application by antagonistic isolates, the effect of tested antagonist on plant growth was measured in term of plant height. Thereafter at the end of experiment, above ground parts of plants were cut into small pieces and shoot fresh weight was recorded, then those parts were dried at 60ºC for three days and dry weight of shoot pieces was recorded. Moreover growth promotion efficacy was calculated to clarify the relative effect of tested antagonist on plant biomass compared with C1 or C2 treatments according to following formula:

$$GPE\ (\%) = \frac{G_T - G_C}{G_C} \times 100$$

Where, GPE refers to growth promotion efficacy, G_T refers to growth parameter in antagonist-treated group and G_C refers to growth parameter in control group.

Detection of plant growth promotion activities for antagonistic isolates

Indole acetic acid production

Selected isolates were investigated for their ability to produce indole acetic acid (IAA). Each isolate was grown in LB media supplemented with (40 µg/ml) L-tryptophan and incubated in shaker with 30°C and 160 rpm for 48 h. Next, bacterial culture was centrifuged at 10000 rpm for 15 min, and 1 ml of culture filtrate was mixed with 1 ml of Salkowski's reagent (1.5 ml of $FeCl_3.6H_2O$ 0.5 M solution, in 80 ml of 60% H_2SO_4) and the mixture incubated at room temperature for 30 min; presence of pink color indicates that isolate can produce indole acetic acid (IAA). Meanwhile, IAA concentration for each tested strain was quantified colorimetrically in 550 nm by spectrophotometer comparing with IAA standard curve (Gordon and Weber, 1951).

Phosphate solubilization

Capacity of selected isolates to solubilize phosphate in form of calcium phosphate was checked qualitatively by using glucose yeast extract agar (GYA) medium containing per 1 L distilled water; 10 g glucose, 2 g yeast extract and 15 g agar. In addition, two other solutions were prepared separately; first 5 g K_2HPO_4 was dissolved in 50 ml distilled water and second 10 g $CaCl_2$ in 100 ml distilled water. These two solutions were added to 1 L GYA just before pouring medium to plates (Beneduzi et al., 2008b). Each tested isolate was grown in GY broth for 24 h, and then 10 µL of bacterial culture were dropped in each plate and incubated for 7 days at 28ºC. Isolates which showed clear halos around their colonies were considered as phosphate solubilizers.

Siderophores production

Evaluation for Siderophores production of selected isolates was carried in LBA medium supplemented with chrome azurol S (CAS) complex (Schwyn and Neilands, 1987). Each isolate was grown in LB medium for 24 h, then one drop of culture was spotted in CAS plates and incubated in 28°C for 3 days; presence of orange halos around the colonies indicated that bacterial isolate were able to produce siderophores.

Statistical analysis

Obtained data were subjected to analysis of variance (ANOVA) test

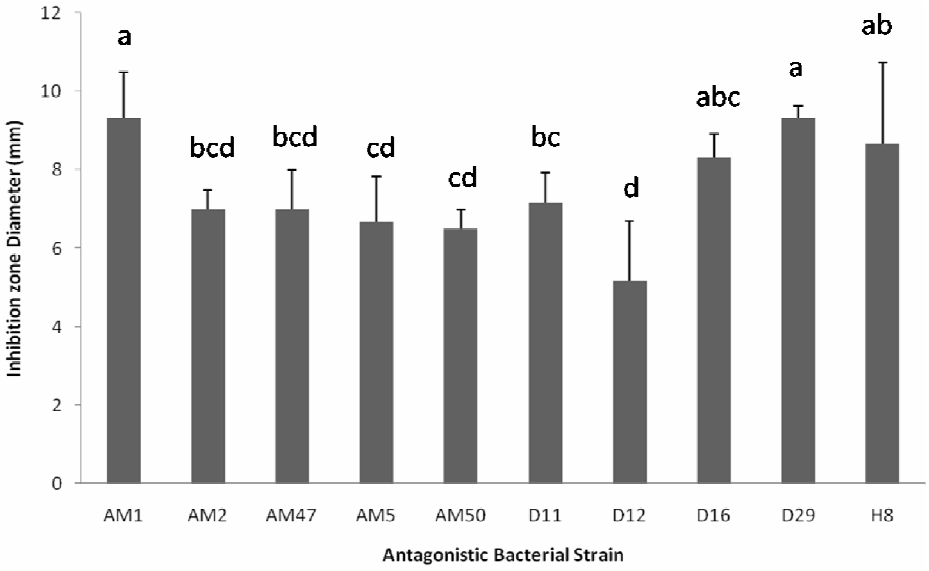

Figure 1. The inhibition effect expressed by inhibition area diameter (mm) of ten antagonistic bacterial strains on *R. solanacearum* growth on tetrazolium chloride (TZC) medium. Columns with the same letters are not significantly different based on LSD test ($p < 0.05$), Error bars in each column represent the standard error within same treatment.

by using SAS software (SAS Institute, Cary, NC). General linear model (GLM) procedure was used to check the significant differences among main treatments. Individual comparisons between mean values were performed by using the least significant differences (LSD) test (P = 0.05). Correlation analysis was performed by CORR procedure.

Identification of selected bacterial strains

Sequence analysis for 16S rRNA gene

Total DNA from each bacterial strain was extracted using TIANamp Bacteria DNA Kit (Tiangen Biotech (Beijing) co., Ltd.). Thereafter, the 16S rRNA gene was amplified using universal primers; 16sP0 (5´-GAA GAG TTT GAT CCT GGC TCA G - 3´) and 16sP6 (5´-CTA CGG CTA CCT TGT TTAC GA - 3´). The amplified products were purified using DNA gel extraction kit. Purified products were submitted to Shanghai Sangon, China, for sequencing. Obtained sequenced data of 16S rRNA were compared with attainable sequences in GENBANK by using BLAST sequence search to determine the phylogenetic affiliation; consequently identification of bacterial isolates was performed according to the similarity of 16SrRNA genes. Furthermore, a phylogenetic tree was created based on partially sequence of 16S rRNA and closely related sequences using MEGA5 (version 5.03) (Kumar et al., 2004). Sequences of four isolates were submitted to GenBank to get accession number for isolates H8, D16, AM1 and D29 which were assigned as JN411089, JN411090, JN411091 and JN411092, respectively.

Fatty acid methyl esters (FAMEs) analysis

Respective isolates were grown in TSB agar plates at 28°C for 24 h, and then each isolate was subjected to saponification, methylation and purification processes of FAMEs. Purified esters were analyzed according to the recommendations of the commercial identification system, Microbial identification System (MIDI). FAMEs profiles were compared with the MIDI identification database TSBA50, version 5.00 (MIDI Inc., Newark, DE, USA).

Biochemical and physiological characterizations

The four selected isolates were characterized based on their biochemical and physiological reactions according to Bergey's Manual of Systematic Bacteriology (Vos et al., 2009) to confirm the results of 16S rRNA and FAME analysis. Four isolates were checked for gram reaction, motility, spore formation, colony color, cell shape, anaerobic growth, growth in different pH values, growth in different NaCl concentrations, hydrolysis of starch and gelatin, catalase reaction, nitrate reduction, citrate utilization and utilization of some carbon sources including sucrose, L-rhamnose, arginine, glycerol, lactic acid, inositol, D-rhafinos and D-Sorbitol.

RESULTS

In vitro screening of bacterial antagonistic activity

Among 200 rhizobacteria isolates screened during preliminary test, ten isolates AM1, AM2, AM5, AM50, AM47, D11, D12, D16, D29 and H8 presented inhibition activity against *R. solanacearum*. Therefore, these 10 bacterial isolates were considered as bacteria with antagonistic characteristics. Among them, four isolates Am1, D16, D29 and H8 gave the highest means of inhibition diameter of 9.33, 8.33, 9.33 and 8.66 mm, respectively, while the other isolates offer a wide range of antagonistic activity from 5 to 7 mm in laboratory *in vitro* test (Figure 1).

Table 1. Reduction of tomato bacterial wilt incidence and biocontrol efficacy of ten antagonistic bacterial isolates against *Ralstonia solanacearum.*

Treatment	Disease incidence (%)	Biocontrol efficacy (%)
AM1+ R.s	9.40 ± 7.65^{c}	88.98 ± 9.69^{a}
AM2 + R.s	81.11 ± 16.59^{a}	9.66 ± 19.79^{c}
AM47 + R.s	83.37 ± 14.80^{a}	8.10 ± 7.02^{c}
AM5 + R.s	88.43 ± 10.05^{a}	1.39 ± 14.63^{c}
AM50 + R.s	82.09 ± 18.81^{a}	9.83 ± 12.38^{c}
D11 + R.s	79.86 ± 17.49^{a}	12.15 ± 10.67^{c}
D12 + R.s	52.26 ± 12.36^{b}	41.21 ± 18.47^{b}
D16 + R.s	16.48 ± 7.03^{b}	81.14 ± 9.77^{a}
D29 + R.s	14.12 ± 10.12^{b}	83.68 ± 11.88^{a}
H8 + R.s	12.96 ± 3.21^{b}	85.35 ± 4.98^{a}
R. s.	78.89 ± 19.66^{a}	-

Data are presented as mean value ± standard error of four replicates, and each replicate contains four plants. Values with same letters within each column indicate no significant difference according LSD test at $p < 0.05$. R.s = *Ralstonia solanacearum*.

Greenhouse study

Antagonistic bacterial strains showed significant differences for their abilities to suppress TBW. Isolate AM1 showed the lowest value of disease incidence as well as the highest value of biocontrol efficacy against *R. solanacearum* of 9.40 and 88.98%, respectively. While isolate Am5 exhibited the highest disease incidence and lowest value of biocontrol efficacy with 88.43% and 1.39% respectively. Isolates D16, D29 and H8 sustained significantly lower disease incidence and biocontrol efficacy compared with control. In contrast, the rest isolates failed to suppress bacterial wilt in greenhouse despite their offering antagonistic activity against *Ralstonia* in the *in vitro* assay (Table 1).

Moreover, noteworthy significant differences among treatments regarding plant height and biomass were noted. Plants treated with isolates D16 and D29 presented the highest values of plant fresh weight by 34.38 and 35.34 g, respectively, with high significant differences, compared with pathogen infected control and plants treated by isolates AM2, AM5, AM50, AM47 and D11. For dry weight, isolates D16, D29 and H8 significantly increase the plant dry weight (g) compared with other treatments with 5.71, 5.42 and 5.48 g respectively. Likewise high GPE (%) increase was recorded with plants treated with isolates D16, D29 and H8 by 56.43, 60.78 and 40.01% and 91.55, 81.77 and 84% for fresh and dry weight, respectively. However, treatments by other isolates did not show high increasing of biomass compared with control except treatment with isolate AM1 which was 47.93% higher than control (Table 2).

In addition, in terms of plant height, significant differences were also noted with treatments of D16 and D29 which significantly increased the plant height with 61.33 and 61.28 cm, respectively with GPE (%) increasing by 43.67 and 43.57%, respectively compared with control (Table 2). According to their high antagonistic activity against *R. solanacearum* (*in vitro* and *in vivo* assays) and their high performance regarding to enhance plant growth. The four isolates (AM1, D16, D29 and H8) were selected for further investigations.

Plant growth promotion traits and inhibition activity against fungi

Plant growth promotion traits for selected strains and their activity against three plant pathogenic fungi presented in Table 3 showed that all four strains gave significance levels of IAA ranging from 44.25 µg/ml by strain AM1 to 59.56 µg/ml for strain H8, with significant difference between four strains. The four strains have ability to produce siderophores as evidenced formation of orange halo around the colony, although isolate H8 showed bigger halo than others. For phosphate solubilization, all the strains were able to solubilize phosphate by giving a clear halo around bacterial colony in GYA medium except isolate D16, where its colony did not surround by any clear area in GYA medium. All strains presented inhibition activity against all of three fungi *F. graminearum*, *P. aphanidermatum* and *R. solani* in dual culture test. However, isolate D29 displayed the highest inhibition percentage against *F. graminearum* which was significantly higher than other isolates, while strains D16 and D29 gave also high inhibition percentage 95.19 and 87% against *P. aphanidermatum* in double layer test. Moreover, all four strains totally suppressed the growth of *R. solani* in double layer test.

Bacterial strains identification

The results of fatty acid analysis for isolates Am1, D29,

Table 2. Effect of application of antagonistic bacterial isolates and *Ralstonia solanacearum* on tomato plants fresh, dry weight and plant height.

Treatment	Plant fresh weight (g)		Plant dry weight (g)		Plant height (cm)	
	Mean	GPE (%)	Mean	GPE (%)	Mean	GPE (%)
AM1+ R.s	26.24 ± 3.13^{bcd}	19.40	4.41 ± 0.37^{b}	47.93	52.36 ±2.88^{bcd}	22.65
AM2 + R.s	13.80 ± 6.14^{f}	-	3.02 ± 0.61^{d}	1.42	46.53 ± 1.79^{ef}	8.99
AM47+ R.s	16.18 ± 5.28^{ef}	-	3.32 ± 0.25^{cd}	11.30	53.14 ± 2.17^{bcd}	24.48
AM5 + R.s	10.50 ± 0.95^{f}	-	2.42 ± 0.48^{d}	-	48.28 ± 2.11^{de}	13.09
AM50 + R.s	17.14 ± 6.72^{ef}	-	3.31 ± 0.30^{cd}	11.11	46.28 ± 4.02^{f}	8.40
D11 + R.s	23.47 ± 2.82^{cde}	6.76	3.09 ± 0.51^{cd}	3.84	53.47 ± 5.80^{bc}	25.26
D12 + R.s	27.33 ± 1.51^{abcd}	24.33	4.03 ± 0.34^{bc}	35.35	50.19 ± 2.27^{dce}	17.58
D16 + R.s	34.38 ± 5.38^{a}	56.43	5.71 ± 0.86^{a}	91.55	61.33 ± 0.55^{a}	43.67
D29 + R.s	35.34 ± 6.91^{a}	60.78	5.42 ± 0.77^{a}	81.77	61.28 ± 1.63^{a}	43.54
H8 + R.s	30.77 ± 3.25^{abc}	40.01	5.48 ± 0.69^{a}	84.00	56.44 ± 1.45^{ab}	32.22
R. s.	21.98 ± 6.15^{de}	-	2.98 ± 0.86^{d}	-	42.69 ± 5.01^{f}	-
Saline solution	31.83 ± 1.87^{ab}	-	4.99 ± 0.22^{ab}	-	48.89 ± 2.27^{cde}	-

Data are presented as a mean value ± standard error of four replicates, and each replicate contains four plants. Values with same letters within each column indicate no significant difference according LSD test ($p < 0.05$). R.s = *Raistonia solanacearum*.

D16 and H8, showed that low similarity percentage 0.27, 0.28 and 0.28 with *Bacillus lentus* for isolates Am1, D29 and D16, respectively and 0.32 similarity percentage with *Paenibacillus lentimorbus* for isolate H8. Although these results showed closest matches to the above mentioned bacillus species, their low similarity values were non considerable and inadequate to identify bacterial strains to species level. However, acquired FAMEs profiles could be suggestible to identify those strains within Bacillus genus. The obtained sequences (1200 to 1500 bp) of the 4 isolates (AM1, D16, D29 and H8) revealed 100, 99, 100 and 100% homology with 16S rRNA sequence of *Bacillus amyloliquefaciens* (accession number JN582030), *B. subtilis* (accession number EF472266), *B. amyloliquefaciens* (accession number JF460733) and *B. methylotrophicus* (accession number HQ662588) in GenBank, respectively.

For biochemical and physiological characteristic tests, data in Table 4 revealed that the 4 selected isolates are gram positive, rod shaped, motile, aerobic growth and able to form spores. All strains gave positive reaction for catalase test and starch hydrolysis. Moreover, they were positive for utilization of lactic acid and D-sorbitol. The results of biochemical and physiological tests were therefore guidable to discriminate four isolates from each other.

DISCUSSION

Bacterial species which inhabit in rhizosphere area have an impressive effect to protect the plant roots from soil-borne pathogens as well as to improve plant growth (Glick et al., 2007; Harish et al., 2009; Singh et al., 2011). In this study, rhizobacterial isolates belonging to genus *Bacillus* were isolated from tomato and potato fields with the objective of obtaining the efficient strains representing high disease control performance against TBW, and also to offer several plant growth promoting activities contributing positively to improve health and growth of plant. *In vitro* laboratory test, all of 10 isolates represent remarkable antagonistic activity against *R. solanacearum*. The four isolates (*B amyloliquefaciens* AM1, *B. subtilis* D16, *B amyloliquefaciens* D29 and *B. methylotrophicus* H8) represented the highest inhibition effect against pathogen.

These results are similar with previous studies reporting the antagonistic activity of *B. subtilis* (Leifert et al., 1995; Lemessa and Zeller, 2007; Pinchuk et al., 2002) and *B. amyloliquefaciens* (Li et al., 2008a; Yoshida et al., 2001) against plant pathogenic bacteria and fungi. In this study, YPGA medium was considered for *in vitro* screening assay because this medium was reported as suitable for *R. solanacearum* growth, especially when YPGA was amended with TZC which makes the medium more selective to *R. solanacearum* (Xue et al., 2009). Moreover, tested antagonistic isolates demonstrated an abundant growth in this medium and this is in agreement with other studies which emphasize that the type of culture medium strongly affects antagonistic activity by mediating the production of substances responsible for inhibition (Montesinos et al., 1996). Meanwhile, Nguyen and Ranamukhaarachchi (2010) reported that the best antagonistic activity is obtained with culture medium containing 2.5% sucrose and 2% peptone (w/v) with 28°C and initial pH value of 7. The mechanism of antagonistic effect in the plate may be due to antibiosis or production of siderophores or both of them (Adesina et al., 2007; Lemessa and Zeller, 2007).

Despite a large proportion of potential biocontrol agent,

Table 3. IAA and siderophores production and phosphor solubilization ability of the four bacilli selected strains.

Strain	IAA production[a] (µg/ml)	Siderophore production[b]	Phosphor solubilization[c]	Inhibition activity against fungi (%)					
				Fusarium graminearum		*Pythium aphanidermatum*		*Rhizoctonia solani*	
				Dual culture[d]	Double layer[a]	Dual culture	Double layer[a]	Dual culture	Double layer
AM1	44.25 ± 3.52[c]	+	+	+	68.22[b]	+	55.00[b]	+	100
D16	53.21 ± 2.30[b]	+	-	+	70.89[b]	+	95.19[a]	+	100
D29	51.54 ± 1.72[b]	+	+	+	83.11[a]	+	87.04[a]	+	100
H8	59.56 ± 4.60[a]	+	+	+	66.82[b]	+	63.52[b]	+	100

([a]): values followed by same letter means no significant difference based on LSD test ($p < 0.05$); ([b]): +: able to produce Siderophore; ([c]): + : able to solubilize Phosphor, - : not able to solubilize; ([d]): + : able to inhibit fungal growth.

strains present considerable inhibition activity against targeted pathogen *in vitro*, and a few of them kept this ability while applying them in greenhouse or in field (Lugtenberg and Kamilova, 2009). Several soil-borne plant pathogens co-exist in the same field, while potential biocontrol agents was selected based on their ability to control a certain targeted pathogen (Xue et al., 2009). Therefore, selected isolates from *in vitro* and *in vivo* test were further checked for their ability to suppress the growth of some soil-borne plant pathogenic fungi to increase their chance by offering sufficient protection to the host plant against wide spectrum of soil-borne pathogens. In greenhouse study, application of tomato plants by strain *B. amyloliquefaciens* AM1, *B. subtilis* D16, *B. amyloliquefaciens* D29 and *B. methylotrophicus* H8 gave the best suppression of disease incidence of TBW and this suppression was significantly higher than other studied isolates. Furthermore, plants treated with the four strains supported high values of plant height and biomass (fresh and dry weight) compared with the control treatment. The *in vivo* inhibitory activity of *B. amyloliquefaciens* against *R. solanacearum* in this study is in line with that of Hu et al. (2010) who reported that antibacterial activity of suspension of *B. amyloliquefaciens* against Capsicum bacterial wilt caused by *R. solanacearum* in greenhouse and field. Furthermore, Jetiyanon (2007) has found that a mixture of *B. amyloliquefaciens* strain IN937a and *B. pumilus* strain IN937b induced the production of defense related enzymes against *R. solanacearum* and other plant pathogens. Regarding *B. methylotrophicus* H8, this strain maintains stable antagonistic activity against *R. solanacearum* in the *in vitro* and in planta tests, and according to Madhaiyan et al. (2010), this species is a novel species in genus of Bacillus.

So far our study is the first report that emphasizes the antagonistic effect of *B. methylotrophicus* (isolate H8) against TBW. Four *Bacillus* strains, AM1, D16, D29 and H8 have demonstrated a high ability to produce IAA, with their production ranging from 44.25 µg/ml of AM1 to 59.56 µg/ml of H8. In general, poor correlation (0.423, 0.473) ($p < 0.05$) was observed between the amount of IAA with plant height and fresh weight. However, relatively strong correlation value (0.801) was noted between IAA amount and dry weight of plants; this could explain to some extent why the plant growth parameters in case of isolate AM1 had low values compared to the other three isolates, inspite of its high antagonistic activity *in vitro* and *in vivo*. Likewise, Idris et al. (2007) reported that inactivation of gene responsible for IAA biosynthesis in *B. amyloliquefaciens* FZB42 cause reduction in IAA concentration and consequently resulting in low plant growth promotion activity. Regarding phosphate solubilization, all the 4 strains except D16 showed capability to solubilize inorganic phosphate. Bacillus species have been documented for their capacity to increase availability of phosphorus in soil; for example *B. amyloliquefaciens* can increase the availability of phosphorus from insoluble myoinositol hexaphosphate or phytate by producing an extracellular phytase which can catalyze sequential hydrolysis of phytate to less-phosphorylated myoinositol derivatives and inorganic phosphate (Jorquera et al., 2008).

In addition, obtained results showed that the four strains were able to produce siderophores *in vitro*. There are many reports indicating that bacilli can produce siderophores (Beneduzi et al., 2008a; Yadav et al., 2011), although a mechanism of biocontrol activity of Bacillus spp. mostly refers to their ability to produce a wide spectrum of antimicrobial compounds as well as elicitation of induced systemic resistance of plants (Haas and Defago, 2005). However, Yu et al. (2011) found that *B. subtilis* CAS15 which can produce siderophores was able to suppress *Fusarium* wilt

Table 4. Differential biochemical characteristic of four Bacilli isolates AM1, D29, D16 and H8 with three other related Bacilli species.

Biochemical characteristic	1	2	3	4	5	6	7
Pigmentation	Opaque	Opaque	Opaque	Creamy white	Opaque	Opaque	Creamy white
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Spore formation	+	+	+	+	+	+	+
Anaerobic growth	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Gram reaction	+	+	+	+	+	+	+
pH 4	-	-	-	+	-	-	+
pH 5	-	-	-	+	-	-	+
pH 6	+	+	+	+	+	+	+
Growth in 1%NaCl	+	+	+	+	+	+	+
Growth in 4%NaCl	+	+	+	+	+	+	+
Growth in 8%NaCl	+	+	+	+	+	+	-
Nitrate reduction	-	-	+	-	+	-	-
Tri-sodium citrate	-	-	+	+	+	-	+
Catalase	+	+	+	+	+	+	+
Gelatin hydrolysis	-	-	-	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
L-Rhamnose	-	-	-	+	-	-	+
Arginine	+	+	+	-	+	+	-
Inositol	+	+	-	-	+	-	-
Glycerol	-	+	-	+	+	+	+
Lactic acid	+	+	+	+	+	+	+
Sucrose	+	+	+	-	+	+	-
D-Sorbitol	+	+	+	+	+	+	+
D-rhafinos	+	+	-	+	-	+	+

1: D29; 2:AM1, 3:D16 and 4:H8, 5: *Bacillus subtilis,* 6: *B. amyloliquefaciens,* 7: *B. methylotrophicus* , +: positive reaction or growth, -: negative reaction or no growth.

incidence in greenhouse, and this suppression was negatively affected after iron supplementation. Furthermore, ability of tested four isolate to inhibit the growth of the three soil born fungi means these isolates have several mechanisms to suppress plant pathogenic microorganisms, and have a high chance to keep their activity for protection of host plants in greenhouse and field.

Finally, we can conclude that the four Bacilli strains AM1, D29, D16 and H8 have proved to be potential biocontrol agents against TBW, in addition to their capability to improve tomato growth under greenhouse conditions. Further studies are, however, needed to investigate the mode of action of these strains in terms of inducing systemic resistance and enhancing their antibiosis activity against plant pathogens, as well as confirming the antagonistic ability of these strains in field trials at different locations.

ACKNOWLEDGMENT

This project was supported by the Special Fund for Agro-scientific Research in t he Public Interest (201003029, 201003066), National Natural Science Foundation of China (30871655) and Zhejiang Provincial Agricultural Departments of China (2008C22072, SN200811).

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