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Delivery Methods for Introducing Endophytic *Bacillus* into Tomato and Their Effect on Growth Promotion and Suppression of Tomato Wilt

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Abstract: The effectiveness of three methods for delivery of four endophytic *Bacillus* into tomato root, stem and leaves tissues was studied in greenhouse conditions at Zhejiang University, Hangzhou. The delivery methods included seed treatment+soil drench, soil drench and foliar spray. The endophytic bacteria was previously isolated from tomato rhizosphere and reinoculated and recovered after treatments from tomato CV. Hezou. Seed treatment+soil drench and soil drench methods of application led to establishment and recovery of endophytic bacteria from root tissues but foliar spray no bacteria were detected from roots. All the isolates were recovered from stem by the three delivery method except B_8 was not detected by foliar spray method. From the leaves all isolates were detected by foliar spray method but only B_2 and B_7 were detected by seed treatment+soil drench method and B_2 only detected by soil drench method. The seed treatment+soil drench was the most efficient method to deliver endophytic bacteria into tomato tissues. All the isolates in the three methods of application were enhanced the growth of tomato and reduced wilt infection with varied degrees among the isolates and methods of delivery. The results demonstrated that endophytic bacteria can be recovered from tomato tissues following inoculation by different methods described, but the delivery depends on the methods used and the endophytic bacteria isolate.

Key words: *Bacillus*, deliver methods, colonization, endophytic bacteris, isolation, tomato

INTRODUCTION

Endophytic bacteria have been defined as bacteria that reside internally in the plant tissues without causing visible harm to their hosts which can be re-isolated from surface disinfected plant parts and internal plant extracts[1]. Internal colonization of plant tissues by bacteria seems to be a widespread, natural phenomenon and several vegetables have been found to harbour endophytic bacteria[2,3]. In plant tissues, bacterial endophytes may originate from seeds[4,5], vegetative material[6], soil[5] and the phylloplane[7]. Entry of these bacteria into the plant can be through sites of emergence of lateral roots, other wounds, natural openings, including stomata and lenticels and germinating radicles[1]. Studies on the population dynamics of bacterial endophytes have been carried out on the total indigenous population[2,8,9] and on the population of introduced bacterial strains in the plant tissues[2,9,10]. The indigenous endophytic population seems to be highest in the root and at the base of the stem, decreasing gradually up to the stem and into the petioles and leaves[2,8,9]. Many studies have demonstrated that certain Plant Growth-promoting Rhizobacteria (PGPR) strains, particularly those belonging to the genera *Bacillus* and *Pseudomonas*, proliferate not only on and around plant roots, but also inside root tissues of agriculturally important plant species[11,12]. Endophytic bacteria may stimulate host plant growth through any of several possible mechanisms including biological control[2], induced systemic resistance to plant pathogens[1], nitrogen fixation[13], phytohormone production and enhancement of nutrient and water uptake[14,15]. The ability to colonize plants endophytically has also been identified as an important feature of biological control agents intended for use against vascular pathogens[16]. *Bacillus* species are among the most common bacteria found to colonize plants endophytically[17,18] and it is likely that their endophytic ability could play a role in the biocontrol of vascular plant pathogens. The main objective of this study was to compare delivery methods of inoculation of endophytic bacteria in tomato, to examine the ability of the endophytic bacteria to suppress the wilt disease and enhance the growth of tomato.

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MATERIALS AND METHODS

Bacterial strain: *Bacillus* isolates (strain B_2, B_5, B_7 and B_8), which where previously identified as *Bacillus brevis, Bacillus subtilis* KL-077, *Bacillus subtilis* BS-2 and *Bacillus subtilis* BS-1, respectively were isolated from tomato rhizosphere. All these isolates were previously shown to be antagonistic to *Ralstonia solanacearum* in tests conducted under green house conditions. The strains were grown and preserved in LB agar slopes after which cultures were maintained by periodic transfer followed by storage at 4°C.

Inoculum preparation: Inoculum was prepared with a 24-h-old bacterial culture grown on LB agar. At this stage, bacterial cells were active and were found in the exponential growth phase. Twenty milliliters of sterile saline water (0.85% NaCl) were added to the bacterial culture and scraped with a glass rod. The bacterial suspension was then transferred to sterile tubes and centrifuged at 3000 g for 10 min at 4°C. Subsequently, the supernatant was discarded and the pellet was re-suspended in 20 mL sterile saline. The amount of inoculum used to inoculate the seeds was OD600=1.00 (10^8CFU mL^{-1}).

Seed disinfection: Tomato seeds were surface disinfected by immersion in 70% ethanol for 1min, transferred to 2% sodium hypochlorite for 2 min and subsequently rinsed three times in sterile distilled water. Seeds were dried in a flow cabinet for 12 h. After seed disinfection, a sterility control was performed by plating 100 seeds per lot on LB agar and incubating at 25°C for 48 h. If no microbial growth was detected on the plates, the seed samples were considered surface disinfected and used in the subsequent stages of the experiment.

Colonisation of roots, stems and leaves of tomato seedlings by endophyte *Bacillus* isolates: Four isolates, (*Brevibacillus brevis*, *Bacillus subtilis* KL-077, *Bacillus subtilis* BS-1 and *Bacillus subtilis* BS-2) were used in each of three delivery methods in this study (seed treatment+soil drench, soil drench and foliar spray). For seed treatment+soil drench, seed were submerged in the inoculum preparation for 24 h before planting and soil drench 10 days from seedlings were planted, the surrounding soil was drenched with approximately 100 mL of inoculum 10 day after transplanting. For the foliar spray method bacterial inoculum was sprayed on to surface of all leaves after transplanting. The soil drench method was carried out as described above. Controls consisted of disinfected seeds without inoculation. Seedlings were transplant at the rate of six seedling/pot and thinned to four seedlings five days after emergence. A four by three factorial design with three replications arranged in a randomized complete block design was used.

Re-isolation and quantification of *Bacillus* isolates by direct plating on LB media after treatment: Sampling was carried out 15 days after inoculation. Plants were rinsed under tap water to remove adhered soil. Individual 2 cm stem and root segments were cut from each plant with a sterile scalpel and placed for 5 min into sterile test-tubes with 10 mL of aqueous H_2O_2 solution (20% v/v) solution amended with 0.05% (v/v) Triton X-100[19]. Sterility checks to monitor the efficiency of the disinfestations procedure were used according to Shishido *et al.*[20] Surface-disinfected pieces were aseptically transferred through three washes of 30 mL of sterile water and triturated with 10 mL of 0.02 M-phosphate buffer pH 7.0 using autoclaved mortars and pestles. To assure that the sections had been completely surface disinfected 100 μL of the last wash was transferred to LB and incubated at 28°C for 48 h. If contamination was detected, the sample was discarded. Fifty microlitres of the resulting suspensions was then spread-plated on LB medium with three replications. Bacterial populations were estimated by hand-counting the colonies after incubation for 48 h at 28°C. Control plates were prepared with the inoculum suspension of the reference isolates serially diluted and plated in the same way on the same medium. Colonies similar in morphology and color to the reference isolate were picked up with a sterile needle and placed on the LB agar. The same was done with the reference isolate. Colonies that grew similarly to the colonies on the reference plates were tentatively considered to be the same as the inoculated strain. Re-isolated colonies were kept in LB gar for identification by PCR analysis.

Confirmation of colony identity by direct plating using PCR: Genomic DNA from *Bacillus* strains was prepared as described previously[21]. The 16s rDNA gene fragments were amplified by PCR using universal primer (BIOASIA, Shanghai, China), forward BSF8/20:5'-AGAGT TTGAT CCTGG CTCAG-3', reverse BSR1541/20 5'-AAGGA GGTGA TCCAG CCGCA. The polymerase chain reaction Reactions were carried out in 50 μL volumes, containing Buffer (10x) 5 μL, MgCl (25 mmol L^{-1}) 4 μL, dNTP (5 mmol L^{-1}) 2 μL, Forward primer 1 μL reverse primer 1 μL, Template DNA 1 μL, Tag enzyme (10000 u mL^{-1}) 0.5 μL, DD water 35.5 μL (PCR) temperature program was 94°C for 2 min, followed by 94°C for 1 min (denaturation), 56°C min (annealing), 72°C 2 in accounting 29 cycle (extension), 72°C 10 min, 60°C 10 min,

Following amplification, the PCR product was purified and sequenced by the same company mention above. The sequences were compared with database in gene bank.

Comparison of *Bacillus* delivery methods on its growth promotion and suppression of bacterial wilt: Seedlings were maintained under green house conditions and watered daily. Plant growth promotion was determined by measuring plant height, fresh and dry weight and number of fruits per plant. Plant height was measured from the base to the tip of the plant at 15, 30 and 45 days after transplanting. The number of fruits was counted after 50 days and fresh weight was determined by weighing the uprooted plant whereas dry weight was determined by drying the plant in an oven at 80°C for 24 h after 2 months. 30 day-old seedlings were challenge-inoculated with *Ralstonia solanacearum* by cutting their roots with a sterile scissors and then drenching the cut roots with the inoculum suspension (10^8 CFU mL^{-1}; 100 mL/pot). Inoculated plants were maintained under green house conditions and observed for disease development 11, 21 and 35 days after challenge. The rate of disease infection was calculated using the following formula: 100x (number of wilted plants per pot/total number of plants per pot). Reduction of infection was calculated using the following formula: Reduction of infection= ([disease infection of control-disease infection of treatment group]/disease infection of control)X 100%[22].

Statistical analysis: A four by three factorial design with three replications arranged in a randomized complete block design was used. All data were subjected to analyses of variance (ANOVA) by using the General Linear Model (GLM) procedure of SAS (SAS Institute, Cary and NC.USA). And the treatment means were separated by using LSD test (p=0.05) among the treatments.

RESULTS

Colonisation of roots, stems and leaves of tomato by endophytic *Bacillus* isolates: The number of isolates recovered from inoculated plants varied with the plant tissue and the delivery method used. Seed treatment+soil drench was the most efficient treatment to deliver endophytic bacteria into tomato plants. No isolate was detected with foliar spray in root tissue (Fig. 1).

With the notable exception of isolate B_8 which was not recovered from stem of tomato seedlings inoculated by the foliar spray delivery method, all the other isolates were recovered from stems of tomato seedlings inoculated by the other delivery methods (Fig. 2).

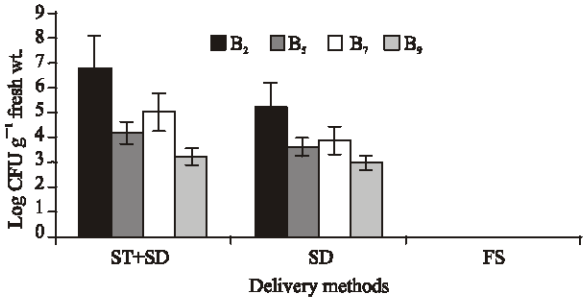

Fig. 1: Number of colonies of endophytic *Bacillus* isolates recovered from tomato roots. ST (seed treatment), SD (soil drench), FS (foliar spray). Error bars represent standard deviation

Fig. 2: Number of colonies of endophytic Bacillus isolates recovered from tomato stems. ST (seed treatment), SD (soil drench), FS (foliar spray). Error bars represent standard deviations

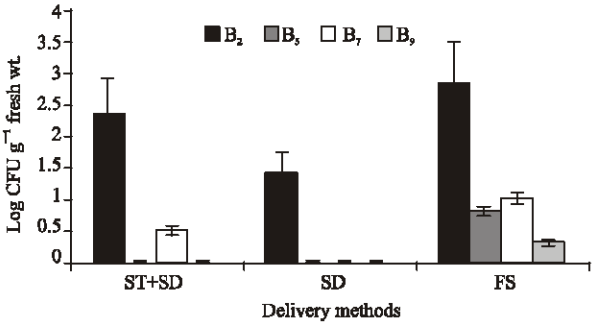

Fig. 3: Number of colonies of endophytic Bacillus isolates recovered from tomato leaves. ST (seed treatment), SD (soil drench), FS (foliar spray). Error bars represent standard deviations

All *Bacillus* isolates were detected from the leaves of tomato seedlings inoculated by the foliar spray delivery method. However only isolate B_2 and B_7 were detected from the leaves of tomato seedlings inoculated by seed treatment+soil drench method. Only isolate B_2 was detected from leaves of tomato seedlings inoculated by the soil drench method (Fig. 3).

Confirmation of colony identity: The universal primer formed many and distinct bands on the DNA of *Bacillus*

Table 1: Effect of treatment with *Bacillus* isolates on growth promotion of tomato plants under greenhouse conditions

Treatments	Height (cm) (15, 30, 45 d)[y]	Fresh wt./plant (60 d) (g)	Dry wt./plant (60 d) (g)	No. of fruits/plant (50 d)	% of reduction
Non treated control	21.77c	17.23d	1.43c	2.33c	0
Seed treatment+soil drench					
B_2	40.66a[z]	60.70a	7.00a	7.33a	71.88
B_5	36.33ab	39.73b	4.46b	5.66ab	43.75
B_7	37.99ab	39.33b	4.56b	6.33a	53.12
B_8	33.66b	34.30c	3.50b	5.00ab	34.37
Soil drench					
B_2	35.33a	43.33a	5.16a	6.66a	62.50
B_5	34.21a	31.80b	3.80b	5.33ab	34.37
B_7	33.55ab	30.76b	3.83b	5.33ab	43.75
B_8	30.55b	28.46c	2.96b	4.00b	24.99
Foliar sprayer					
B_2	32.55a	29.53a	3.70a	5.00a	53.12
B_5	28.77b	23.96bc	2.86b	4.33ab	24.99
B_7	30.66ab	26.46ab	3.20a	4.66a	37.49
B_8	29.10b	21.23c	2.16b	3.66ab	18.74

[x]Experiments were repeated two times. One representative trial is presented, [y]Values represents the average of height at 15, 30, 45 day after planting
[z] Means with the same letter within a column are not significantly different according to Turkey's HSD test at p=0.05

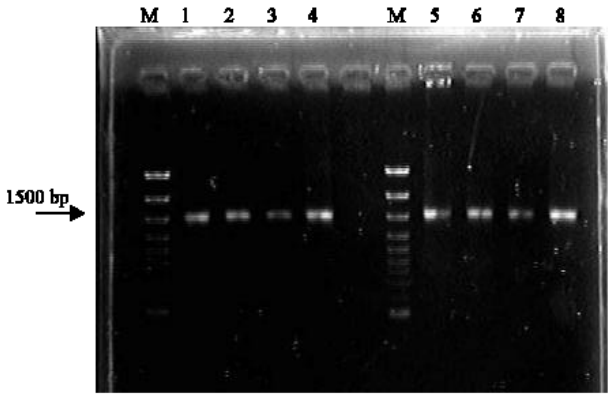

Fig. 4: Agarose 1.2% gel electrophoresis analysis of the PCR products from nucleic acids of bacillus isolates with universal primers. Lane 1-4 represents the Reference isolates, Lane 5-8 represents the reisolates of *Bacillus* M: 100 bp DNA ladder

isolates. Results showed that the re-isolated colonies and reference had the same UP-PCR banding profiles using this primer. Although other indigenous bacteria were present in the system, PCR was only performed on colonies that were similar in colour and morphology to the reference strain. These colonies were selected randomly from leaf, stem and root segments. The reference strains were identified by this method as being *Brevibacillus brevis, Bacillus subtilis (*strains KL-077, BS-2 and BS-1) with 89, 100, 99,99% sequence similarity, respectively. The re-isolated colonies obtained from different plant extracts were identified as *Brevibacillus brevis, Bacillus subtilis* strains KL-077, BS-2 and BS-1 (100% sequence similarity). These isolates has been deposited in the gene bank database under accession numbers AJ831420, AJ831421, AJ831422 and AJ831423 for isolates B_2, B_5, B_7 and B_8, respectively (Fig. 4).

Comparative of delivery methods of *Bacillus* isolates in growth promotion and suppression of bacterial wilt: In general all application methods tested increased growth promotion under greenhouse conditions. However, the degree of growth promotion varied among the isolates and methods of application. All the isolates when applied as seed treatment+soil drench enhanced the height of the plant when compared to the nontreated control (Table 1). Also the height was enhanced by the isolates with soil drench and foliar spray when compared to the nontreated control, but with seed treatment+soil drench the height was taller than with foliar spray and no significantly difference from soil drench. Fresh weight, dry weight and number of fruits were significantly enhanced by all methods of application with no significant different between isolates but significantly difference from nontreated control. Among the four isolates tested B_8 showed the lowest growth promotion effect in comparison with the other isolates and foliar spray showed less effect in growth promotion when compared with others delivery methods (Table 1).

Effects of delivery methods on disease suppression: In general all the isolates tested protected tomato plant against bacterial wilt under greenhouse conditions, but the degree of protection varied considerably with isolates and method of application. A visual assessment of treatment indicated a noticeable difference in growth promotion and disease incidence when compared to the nontreated control. The highest protection resulted from isolate B_2, which gave 71.88, 62.50 and 53.12% protection when used as seed treatment+soil drench, a soil drench and foliar spray, respectively. The lowest level of protection resulted with isolate B_8, which showed 34.37, 24.99 and 18.74% protection when applied as seed treatment+soil drench, soil drench and foliar spray, respectively. Protection against tomato wilt was more

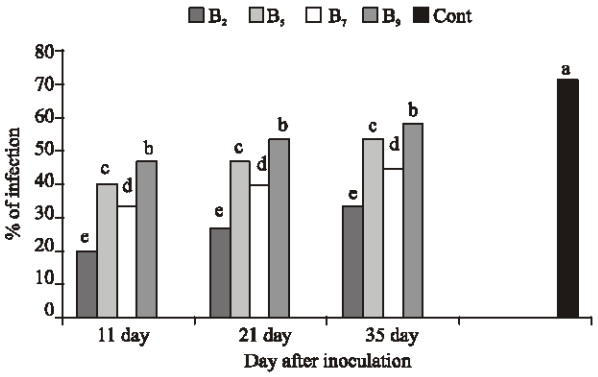

Fig. 5: Efficacy of *Bacillus* isolates delivery methods against bacterial wilt of tomato under green house conditions. (Percentage of wilt is the mean of three replicates. Means with the same letter are not significantly different at p=0.05 according to analysis of variance followed by Fisher's LSD

effective with seed treatment+soil drench rather than foliar spray. Soil drench was almost equal in protection to seed treatment+soil drench application (Fig. 5).

DISCUSSION

All the four *Bacillus* isolates, antagonistic against *Ralstonia solanacearum* were able to establish as an endophyte in tomato and were detected in root, stem and leaf section after inoculation by seed treatment, soil drench and foliar spray using direct plating method. Although, four isolates were recovered from root tissue and stem of tomato seedlings inoculated by seed treatment+soil drench and soil drench method, this finding may be explained by the role of the spermosphere as a source of bacterial endophytes. It is also evident that bacterial endophytes introduced as seed treatment colonize the internal tissues of root radicles as they emerge from the seed coat, as previously reported by Mahaffe *et al.*[23]. The lack of isolates detection in the root tissues of seedlings inoculated by the foliar spray may be explained by the inability of the bacterial cell to move through plant tissues. Once inside plant tissue, endophytic bacteria either remain localized in a specific plant tissue like the root cortex or else colonize the plant systematically by transport through the conducting elements or apoplast. The population levels of the inoculated bacterium were generally higher in the root parts and base of the stem, falling considerably in the upper plant parts[24-26]. The recovery of isolates from root and stem of tomato seedlings inoculated by soil drench delivery method, indicate that the entry into plant tissue may have occurred via germinating radicles as reported by Huang[27]. However, the main entry appears to be through wounds that naturally occur as a result of root growth, or through root hairs and epidermal conjunctions. Several authors have reported extensive colonization of the secondary root emergence zone (sites of root branches) by bacterial endophytes[23,28,29]. Besides providing entry avenues, wounds also create favorable conditions for the approaching bacteria by allowing leakage of plant exudates, which serve as a source of enrichment for the bacteria. Due to occurrence of breaks in the endodermis at these points, bacteria colonizing the cortex can cross the endodermis into vascular tissue and colonize stem tissues. Foliar spray treatment was only effective in inoculate bacterial endophytes in the leaf tissues. All endophytic bacteria significantly improve growth and development of tomato and control bacterial wilt diseases. And also resulted in significant increase in fresh and dry weight, plant height and number of fruits per plant. PCR analysis confirmed the reliability of the detection method considering used in bacterial identification. This study demonstrates that bacterial endophytes can be recovered from tomato plants following inoculation by different delivery methods. However, among the three methods of application tested foliar spray was less effective in colonization of plant roots, while the seed treatment+soil drench delivery method showed the highest level of endophytic bacteria colonization of tissues and the greatest degree of growth promotion and wilt suppression. Isolate specific attributes appear to be important in determining whether an isolate will colonize plants internally. The results suggest that any one method will not be similarly effective for all strains, but a method will have to be tested specifically for the endophytic bacterial isolate to be delivered.

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