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Selection of bioinoculants for tomato growth enhancement and pathogen resistance

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Abstract

Bioinoculants were selected based on their ability to produce chitinase solubilize phosphate and demonstrate antifungal activity both on agar medium and in a quantitative assay in broth medium. It was demonstrated that *Serratia marcescens* and *Bacillus licheniformis* exhibited these attributes in significantly ($P<0.1$) greater amounts than other strains tested. The effects of *S. marcescens***,** *B. licheniformis* and a mixed culture of *S. marcescens* and *B. licheniformis* on seed germination of tomato (*Lycopersicon esculentum* Mill. cv. Seeda) were evaluated in *in vitro* culture. *S. marcescens* and the mixed culture showed a significantly ($P<0.01$) higher percentage of seed germination, and greater height and weight of seedlings than those recorded when using *B. licheniformis* or the control (no bioinoculant). *S. marcescens* and the mixed culture were also studied for their effects on growth and pathogen resistance of tomato in pot culture under field conditions. The results demonstrated that both of these bioinoculants significantly ($P>0.01$) increased fresh and dry weights and the height of tomato when compared with the control. Upon infestation of tomatoes with the pathogenic fungus *Fusarium solani*, both bioinoculants significantly ($P<0.1$) increased the resistance of tomato to the pathogen. It was clear that bioinoculants could enhance growth and pathogen resistance of tomato under field conditions as well as under *in vitro* culture.

Keywords: plant growth promoting rhizobacteria (PGPR), *Serratia marcescens***,** *Bacillus licheniformis.*, chitinase, phosphate solubilization, antifungal activity

1. Introduction

In agricultural production, famers conventionally use agrochemicals in many instances for increasing crop yield and controlling pathogens. Consequently, some of these chemicals may persist in the environment. A range of bioinoculants, microorganisms in the rhizosphere including biofertilizers, biocontrol agents and organic decomposers have, therefore, been introduced in an attempt to achieve safer disease control measures in agriculture. The use of bioinoculants is as an alternative to synthetic fungicides can assist farmers to save money and to reduce the buildup of undesirable chemicals [1, 2]. Plant growth promoting rhizobacteria (PGPR) are known to enhance plant growth by colonization of soil surrounding the root zone and improving nutrient cycling, effecting biological control of plant pathogens and enhancing crop growth. PGPRs can promote plant growth through the production of plant growth regulators and other compounds (such as indoleacetic acid (IAA), gibberellic acid, cytokinin, ethylene, ammonia (NH_3), hydrogen cyanide (HCN), and siderophore) and can exhibit antifungal activity [3, 4]. PGPRs are characterized by their ability to colonize the root zone, survive and multiply in the rhizosphere [5]. They consist of beneficial microorganisms such as symbiotic or free-living N_2-fixing organisms, mycorrhizal fungi, phosphate solubilizers and a large group of bacteria including species of *Enterobacter, Pseudomonas, Azospirillum, Azotobacter, Serratia, Klebsiella, Alcaligenes, Arthrobacter, and Bacillus* [3]. George *et al*. [6] have shown that *Serratia marcescens* KiSII isolated from the rhizosphere of coconut palms exhibited phosphate solubilization, ammonification, production of many useful bioactive compounds such as indole acetic acid, chitinase enzyme, siderophore and antibiotics. These traits indicate that *S. marcescens* would be a good candidate for development as a novel bioinoculant.

Many studies have reported that the use of individual bioinoculants as well as in combination could enhance plant growth. Gupta *et al.* [7] have reported a significant increase in growth of the legume *Cajanus cajan* was observed when treated with the mixture of three bioinoculants (*Bacillus megaterium, Pseudomonas fluorescens and Trichoderma harzianum*). Additionally, the use of these microorganisms in combinations as bioinoculants showed no negative impacts on the indigenous microbial community [8].

However, bioinoculants used to colonize the areas surrounding roots could have undesirable interactions with plants under field conditions. Various environmental factors including climate, weather conditions, soil characteristics and the activity of indigenous microflora and other microorganisms can affect the activity of bioinoculants [3]. Thus, it is necessary to select and develop suitable and compatible strains of microorganisms for effective bioinoculant use under field conditions. This study was aimed at selecting appropriate bioinoculants and investigating their effects on seed germination of tomato (*Lycopersicon esculentum* Mill.cv.Seeda) under *in vitro* conditions. Growth and pathogen resistance of tomato were also evaluated using bioinoculants with or without the infestation of the fungal pathogen *Fusarium solani* in pot culture of tomato under field conditions were used for evaluating the growth and pathogen resistance.

2. Materials and Methods

2.1 Bacteria selection and culture conditions

Bacteria strains with the ability to produce chitinase and to solubilize phosphate, according to previous reports [8, 9], were used in this study. These included *Bacillus subtilis* TISTR 001, *B. coagulans* TISTR 352, *B. thuringiensis* TISTR 126, *B. megaterium* TISTR 003, *B. licheniformis* TISTR1010 and *S. marcescens* TISTR1354. The pathogenic fungus used in this study was *F. solani* TISTR 3436. All microorganisms were obtained from the Thailand Institute of Scientific and Technological Research (TISTR). The bacterial strains were stored at -20□C in nutrient broth (NB) containing 20% glycerol. Bacterial inoculum was prepared by growing the bacteria on nutrient agar (NA) for 24 h at 37□C. Bacterial cells were suspended in sterile water to give a final concentration of 10^9cfu/mL using the viable plate count method and an optical density measurement at 600 nm.

2.2 Selection for bacteria as bioinoculants

The selection of chitinase producing bacteria comprised two steps. Each step was performed with three replicates. The first step was performed by point inoculating the bacteria on NA containing 1.5% colloidal chitin (w/v). Colloidal chitin was prepared from crab shell chitin (Sigma, UK) using the method of Shanmugaiah *et al.* [9]. Bacterial strains showing a clear zone against the creamy background after incubation at 37□C for 5 days were selected as chitinase producing strains and chosen for the next step. In the second step, 5mL bacterial cells of 10^9 cfu/mL in sterile water were added into the final NB (containing 1.5% colloidal chitin (w/v)) volume of 100 mL and incubated at 200 rpm, 37□C for 24 h. Crude enzyme was harvested by centrifugation at 4066 g for 15 min at 4°C. The supernatant was assayed for chitinase activity by a colourimetric method described by Imoto and Yagishita [10].

The bacteria with chitinase activity in both steps of selection were then evaluated for antifungal activity *in vitro* by determining the inhibition of *F. solani* growth on potato dextrose agar (PDA) media. Three chitinolytic bacterial strains were streaked on the right side of the plate while the agar disk (0.6 cm diameter) of *F. solani* was placed on the left side. The plates were incubated at 28□C for five days and the diameter of mycelium was then measured. The experiments were repeated three times. The bioinoculants were subsequently confirmed for their effectiveness by their ability to inhibit fungal growth in liquid medium (PDB). Two disks of agar from the margin of active growth of the fungal colonies on the plates were co-cultured with 5% bacterial inoculum or sterile distilled water (control) in PDB (50 mL) on a rotary shaker at 28□C and 180 rpm for 5 days. The mycelium of the fungi was collected by centrifugation at 5000 rpm for 10 min and then washed with hot distilled water three times to remove the bacterial cells and the medium substrate before measuring the dry weight of the mycelium. The inhibition of fungal growth was calculated using the following formula [11]:

$$\% \text{ inhibition} = 1 - (\text{fungal growth} / \text{control growth}) \times 100 \quad (1)$$

fungal growth = dry weight of fungal mycelium co-cultured with bacteria
control growth = dry weight of fungal mycelium without bacteria

The selection of phosphate solubilizing bacteria comprised two steps. Each step was performed with three replicates. For the first step, *B. licheniformis* and *S. marcescens* were streaked onto modified Pikovskaya (MPVK) solid medium [12]. The bacteria were incubated at 37□C for 5 days. The ability of bacteria to dissolve tricalcium phosphate was estimated by the appearance of a clear zone around the bacterial colonies. In the second step,

quantitative analysis of phosphate solubilizing ability was carried out by inoculating 1% bacterial inoculum (sterile distilled water used for the negative control) in MPVK broth (150 mL) containing tricalcium phosphate, incubating at 30□C, 200 rpm. The cultures were harvested by centrifugation at 8604 g for 30 min. The supernatant was assayed for phosphate solubilizing ability using the colorimetric method of Apha *et al* [13].

2.3 Effect of bioinoculants on seed germination

The effect of bacteria on seed germination was evaluated using three inocula: the suspensions (prepared by previous protocol) of *B. licheniformis, S. marcescens* individually and the mixed culture of both *B. licheniformis* + *S. marcescens*(1:1 v/v). Each inoculum test was carried out with four replicates of 100 seeds. Seeds of tomato (*L. esculentum* Mill.cv. Seeda) were washed with mild detergent and surface-sterilized by soaking in 70% ethanol for 1 min and in 0.95% sodium hypochlorite containing a wetting agent (Tween 20) for 15 min. After three washes in sterile water, these seeds were inoculated with bacteria by immersion in 20 mL either the bacterial inoculum (10^9cfu/mL) or in sterile distilled water (uninoculated) for 1 h. A germination assay was performed according to the International Seed Testing Association (14). Inoculated and uninoculated tomato seeds were placed on filter papers in sterile Petri dishes (9.0 cm diameter) and moistened with 10 mL of ½ MS medium [15]. The tomato seeds were cultured under light conditions (illumination of 2000 lux from cool-white fluorescent lamp) for 16 h photoperiod at 25□ 2□C. After 5, 10 and 15 days the number of germinating seeds was recorded and expressed as germination percentage. At day 15, the seedlings were measured for height, wet weight and dry weight (drying at 65□C for 3 days).

2.4 Effect of bioinoculants on growth and pathogen resistance of tomato

Sandy loam was collected from Mahasarakham province and autoclaved at 121□C and 15 psi for 30 min. Plastic pots (20 cm diam.) were sterilized with 20% sodium hypochlorite solution and filled with 1.5 kg of autoclaved soil. The experiment was arranged as a completely randomized design with four replications. Six treatments were imposed using the following inoculations: sterile distilled water (control), *S. marcescens* and the mixed culture of *B. licheniformis* + *S. marcescen*s (1:1 v/v), with or without infestation by the pathogenic fungi (*F. solani*) prepared by the procedure of Kamil *et al.* [16] with a slight modification by using millet (*Sorghum bicolor*) instead of barley grains. Sterile tomato seeds were inoculated by immersion in either sterile distilled water or bacterial inoculum for 1 h and then six seeds were placed into each pot at the same depth (approximately 1 cm below the soil surface). Seedlings were thinned to two per pot after 7 days. Two weeks after transplanting, 10 mL of inoculum (10^9 cfu/mL) were added to the inoculation treatment by pipette directly and plants were infected with inoculum of *F. solani* (10 g of millet per pot) around the seedlings. Tap water was supplied daily with the water holding capacity adjusted to 60 %. The height of tomato shoots were measured at 7, 14, 21, 28 and 35 days after planting. The plants were harvested on day 35 after planting and fresh weight and dry weight were measured.

2.5 Statistical analysis

The study was arranged as a completely randomized design. The data were analyzed by analysis of variance (ANOVA) and comparisons between treatments were carried out using a least significant difference (LSD) test. All statistical analyses were performed using Statistix version 8.0 (NH Analytical Software, USA.).

3. Results and Discussion

3.1 Selection for bacteria as bioinoculants

An important property of bioinoculants is the ability to promote plant growth and biological control activity. One of the biological control mechanisms is the production of chitinase that can degrade chitin in cell walls of higher fungi. In this study, suitable bacteria for bioinoculant production were selected using the characteristics of chitinase production, inhibition of fungal mycelium growth and phosphate solubilization. Among the six bacterial strains tested on NA medium containing colloidal chitin, only three strains *B. subtilis*, *B. licheniformis* and *S. marcescens* produced a clear zone on the culture, indicating chitinase activity. From three strains of chitinolytic bacteria, *S. marcescens* showed the highest activity of chitinase enzyme (31.67 U/mL) followed by *B. licheniformis* (25.67 U/mL) and *B. subtilis* (19.73 U/mL) (Figure 1).

The antifungal activity of bacteria was evaluated by the ability to inhibit *F. solani* growth on PDA. All bacteria inhibited the growth of *F. solani* when compared with the control. Among the three species, *B. subtilis* clearly showed less inhibitory ability of *F. solani* than *B. licheniformis* or *S. marcescens* (Figure 2). Co-cultures of *F. solani* and the bacteria in liquid medium were tested to confirm the antifungal ability of these bacteria (assessed as inhibition of fungal growth). In this study, *S. marcescens* had the highest inhibition (80.05%) followed by *B. licheniformis* (65.74%) (Figure 1). From measurement of the ability to produce chitinase enzyme and of the

inhibition of fungal growth, *S. marcescens* and *B. licheniformis* were found to be similar in their effectiveness (P<0.01) (Figure 1). *S. marcescens* is known to be efficient in producing chitinase, which is from an endochitinase named chitinase A or Chi A [17]. In addition, various reports have demonstrated that *S. marcescens* is an important bacterium that exhibits resistance to various pathogens such as *Rhizoctonia solani* and *Fusarium oxysporum*, [18, 19]. Our results are in accordance with previous studies that have shown the effectiveness of *S. marcescens*. *S. marcescens* may exhibit antifungal activity via the production of antimicrobial secondary metabolites, such as the red pigment prodiogiosin, the antibiotic carbapenem, salicyclic acid and siderophores [6, 20]. For chitinase production by *Bacillus* sp., only *B. licheniformis* showed a high activity of the enzyme. This is in agreement with the previous work of Kami *et al*. [16].

Three strains of bacteria were selected for examination of phosphate solubilizing ability. The results showed that *S. marcescen*s and *B. licheniformis* were both able to produce clear zones on MPVK agar plates. Further, when assayed by culturing in MPVK broth, both bacteria revealed high phosphate solubilizing ability (Figure 1). Phosphorus (P) is an important plant growth limiting nutrient but there are a number of organic and inorganic forms of P in soil [21]. The low availability of P to plants is often caused by P being in an insoluble form. Ahemad and Kibret [22] demonstrated that phosphate solubilizing bacteria (PSB) in the rhizosphere can be considered as being a biofertilizer for the supply P. Bacterial genera reported as being the most significant for phosphate solubilization have been reported in strains belonging to *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Rhizobacterium*, and *Serratia* [23, 24, 25]. Previous reports are in agreement with the result of the present study. *S.marcescens* and *B. licheniformis* were able to solubilize phosphorus and can, therefore, be considered to be bioinoculants.

3.2 Effect of bioinoculants on seed germination

The effects of different bioinoculants on the germination of tomato seeds is shown in Table 1 and Figure 3. Seed germination of samples treated with bioinoculants, *S. marcescen*s (83.7%), *B. licheniformis* (83.0%) and the mixed culture of *S. marcescens* + *B. licheniformis* (86.3%) was higher than that of the control (78.7%) at 10 days after sowing. A significantly increased germination percentage was found in the inoculated seeds with the mixed culture of *S. marcescens* + *B. licheniformis* at 15 days. Seedlings treated with bioinoculants had higher fresh weight compared with seeds with no bioinoculation. The highest seedling dry weight was found in the inoculated seeds with the mixed culture of *S. marcescens* + *B. licheniformis*, followed by using *B. licheniformis* and *S. marcescens* individally. These results with bioinoculants were significantly higher than that of the control. Similarly seed treatment with bioinoculants increased seedling height over the control. Among the bioinoculants, *B. licheniformis* had a much lower seedling height than the mixture of *S. marcescens* + *B. licheniformis* and *S. marcescens* alone. The mixture of *S. marcescens* + *B. licheniformis* or *S. marcescens* alone were, therefore, effecive bioinoculants of tomato growth using these measures. Recently, successful applications of bioinoculants for promoting plant growth have been reported [1, 2, 3, 5, 8]. The functional activity of PGPRs as biofertilizers, phytostimulators, rhizoremediators, biopesticides and antifungal metabolites have been investigated by Ahemad and Kibret [22] and their results were consistent with the observations from this study. Among the bioinoculants, the mixed culture of *S. marcescens* + *B. licheniformis* showed that the significantly highest of seed germination, fresh and dry weights of tomato seedlings. The statistical analysis showed that fresh weight of control and inoculants were not significantly different at P<0.01. Moreover, height of control and inoculant *B. licheniformis* were not significantly different at P<0.05. Such bacteria have been shown to enhance seed germination through production of phytostimulators such as IAA [22]. The results showed that inoculation with *B. licheniformis* increased growth of tomato *in vitro* similarly to that under field conditions. Previous reports demonstrated that *B. licheniformis* promoted plant growth through ability to produce gibberellin and to enhance phosphate solubilization [26, 27]. Inoculation with *S. marcescens* and *B. licheniformis* yielded similar results for both seed germination and growth.

Table 1 The effects of different bioinoculants on the percentage of seed germination and seedling growth of tomato at 15 days after sowing.

Treatment	Height (cm)	Weight (g)		Germination (%)
		fresh	dry	
Control	5.96 ± 0.41^{C}	0.398 ± 0.02^{B}	0.355 ± 0.00^{C}	84.33 ± 4.51^{A}
S. marcescens	6.52 ± 0.07^{AB}	0.410 ± 0.00^{B}	0.370 ± 0.00^{BC}	82.33 ± 3.21^{A}
B. licheniformis	6.12 ± 0.23^{BC}	0.417 ± 0.01^{B}	0.379 ± 0.01^{B}	84.33 ± 2.08^{A}
S. marcescens + *B. licheniformis*	6.69 ± 0.16^{A}	0.444 ± 0.01^{A}	0.404 ± 0.01^{A}	91.00 ± 1.00^{B}
	*	**	**	*
LSD (P<0.01)	-	0.0237	0.018	-
LSD (P<0.05)	0.47	-	-	5.65

Each value represents mean ± SD of three replication per treatment. Values are means of three replicates. Different letters show significant difference at P< 0.01 (**) or P<0.05 (*) and ns, not significant.

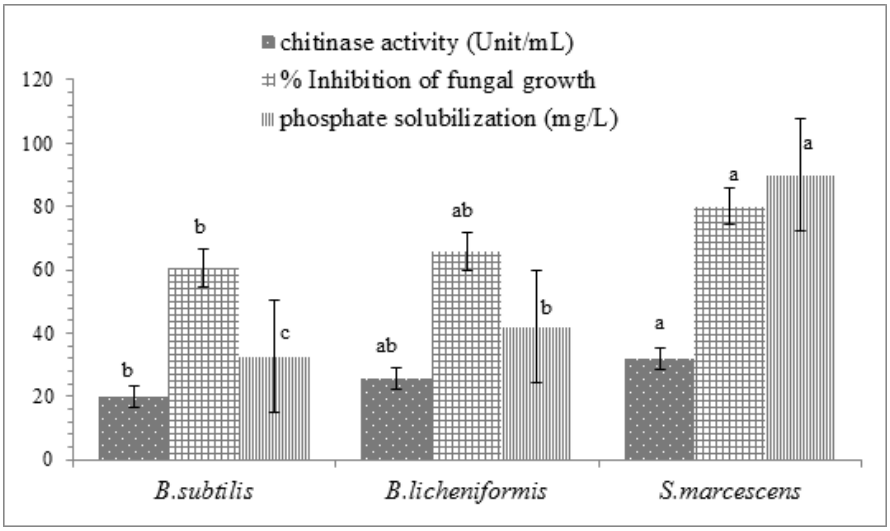

Figure 1 The determination of chitinase production, phosphate solubilization and fungal growth inhibition of three species of selected bacteria in liquid medium. Values are the means of three replicates. Control values were 0 in all instances.

Figure 2 The antifungal assay using point inoculation of *F. solani* on the left side and the antagonistic bacteria streaked on the right side of PDA plates.

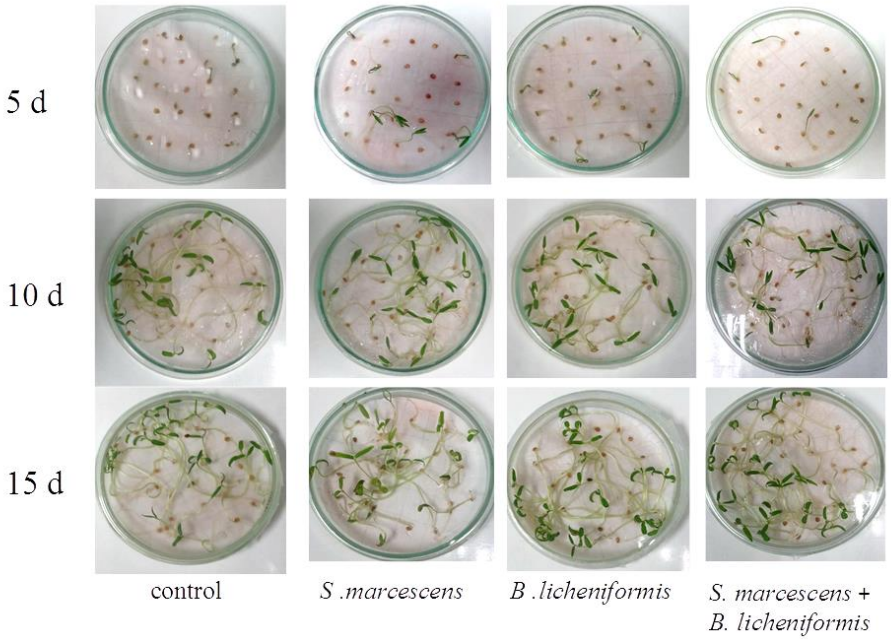

Figure 3 The effect of different bioinoculants on seed germination of tomato cultured under light conditions at 5, 10 and 15 days after sowing.

3.3 Effect of bioinoculants on growth and pathogen resistance of tomato

The effect of different bioinoculants on tomato growth studied using pot culture under field conditions is presented in Table 2. Growth of tomato was determined by measuring the height of tomato shoots weekly. The results showed no significant difference in shoot height in all treatments 14 days after transplanting but increased growth was evident after 3 weeks. Inoculation of *S. marcescens* and *B. licheniformis* produced significantly higher

growth than no inoculation (control). The effect of bioinoculants on pathogen resistance of tomato was evaluated by infecting plants treated with or without the inoculation of bioinoculants with the fungal pathogen *F. solani*. Treatments with bioinoculants increased the resistance of tomato to the pathogen. The greatest height was observed with *S. marcescens* (13.8 cm) followed by treatment with the mixture of *S. marcescens + B. licheniformis* (11.9 cm). Significant differences in tomato height were observed in the treated plants compared to the tomato infected with *F. solani* only at 4 weeks. Similarly, fresh and dry weights, from treatments with bioinoculants were significantly greater than from infected plants. The results showed dry weights of tomato between the bioinoculant *S. marcescens* and the mixture of *S. marcescens + B. licheniformis* was significant difference at P<0.01. Overall, inoculation with individual bacteria or with a combination of bacteria enhanced both growth and pathogen resistance. The results showed that *S. marcescens* and the mixture of *S. marcescens + B. licheniformis* could be potential in plant growth promoting bacterium as it promotes growth and pathogen resistance of tomato plants by phosphate solubilization, the activity of enzyme chitinase. This result was in accordance with Ahemad and Kibret [22] who suggested that 1-aminocyclopropane-1-carboxylate (ACC) deaminase produced by bacteria, such as *Bacillus* and *Serratia* can inhibit growth of phytopathogenic microorganisms**.**

Table 2 The effect of different bioinoculants on the growth and pathogen resistance of tomato grown in pot culture under field conditions.

Treatment	Weight (g)		Height of tomato shoot (cm.)				
	fresh	dry	1 wk	2 wk	3 wk	4 wk	5 wk
Control (no bioinoculant, no infection)	4.72±0.38^{C}	1.44±0.02^{E}	2.83±0.14	4.69±0.08	7.40±0.10^{BC}	11.50±0.61^{B}	14.66±2.84
S.marcescens	12.04±3.01^{A}	5.29±0.13^{B}	2.82±0.20	5.41±0.52	7.60±0.10^{BC}	14.66±0.57^{A}	18.00±2.00
S.marcescens + B.licheniformis	12.93±2.47^{A}	6.29±0.38^{A}	2.60±0.10	5.05±0.51	9.26±0.74^{A}	14.36±0.51^{A}	18.66±2.47
Control +*F. solani*	4.14±1.53^{C}	1.67±0.14^{E}	2.88±0.13	4.30±0.62	6.83±0.85^{C}	9.33±1.15^{C}	13.73±3.11
S.marcescens+ F.solani	9.23±1.92^{AB}	2.23±0.24^{D}	2.60±0.36	4.87±0.33	8.40±0.26^{AB}	13.76±0.25^{A}	15.93±3.06
S.marcescens +B.licheniformis + F.solani	9.49±0.93^{AB}	4.24±0.36^{C}	2.70±0.10	4.78±0.64	7.96±0.21^{BC}	11.96±0.75^{B}	16.50±0.66
	**	**	ns	ns	**	**	ns
LSD (P<0.01)	4.79	0.62	-	-	1.20	1.73	-

Each value represents mean ± SD of three replication per treatment. Values are mean of three replicates. Different letters show significant difference at P< 0.01 (**) and ns, not significant.

4. Conclusions

In summary, *B. licheniformis* and *S. marcescens* separately and in combination showed the potential to be used as bioinoculants to enhance tomato growth and increase pathogen resistance. In addition, use of microorganisms as biofertilizers and for biological control provides option for introducing sustainable production method in agriculture.

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