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Field suppression of Fusarium wilt and microbial population Shifts in tomato rhizosphere following soil treatment with two selected endophytic bacteria

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Abstract

Two endophytic bacteria, *Bacillus subtilis* SV41 (KR818071) and *B. amyloliquefaciens* subsp. *plantarum* SV65 (KR818073), were assessed under field conditions for their capacity to control tomato Fusarium wilt in tomato and their effects on soil microbial activity. Six months after planting, Fusarium wilt severity, estimated through the vascular browning extent in tomato stems, was significantly reduced by 82.3 and 88.2% compared to control following bacterial treatments. The frequency of *F.oxysporum* re-isolation from roots, collars and stems was also significantly lowered in treated plants compared to controls. These effects were associated with a significant improvement, by 10.6 to 16.3%over control, in plant height and root fresh weight and an increase in fruit production by 8.4-12.5%. As for microbial activity, *F. oxysporum* population in the rhizosphere of tomato plants treated with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 was reduced by 87.5-91.7%compared to the initial soil (sampled before planting) and by 88.4-92.3% relative to the rhizospheric soil of untreated plants (control soil). A significant enhancement in the total culturable bacterial community was also noted in the rhizosphere of tomato plants treated with both strains compared to initial and control soils where a significant enrichment in *Pseudomonas* and actinobacteria community was recorded.

Keywords: Endophytic bacteria, Fusarium wilt, growth-promoting, microbial community, soil, tomato.

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Introduction

Plants are naturally associated with microbes in various ways (Vijayabharathi et al., 2016). To face biotic and abiotic stresses, plants interact with different members of soil biodiversity and especially microbial community. These relations involve positive and negative feedbacks between soil microbial agents, plants, and their chemical environment (Meena and Meena, 2017). Agriculturally important microorganisms can affect the efficiency of nutrient availability to grown plants and soil biodiversity, and can also regulate the interactions between plants and pathogenic microflora (Zeilinager et al., 2016). Interactions among plants and microbes can influence soil physiochemical, biochemical and microbiological properties (Dubey et al., 2016). In fact, beneficial microbial communities or microbial inoculants can offer various positive services for plants and soils including plant growth-promotion, nutrient efficiency, bioremediation, and suppression of bio-aggressors. A profound understanding of the environmental factors influencing the viability and performance of these microbial inoculants is essential of their large-scale use in sustainable agriculture production systems (Meena and Meena, 2017).

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Due to the increased need to reduce use of chemical fertilizers and pesticides, for sustainable agriculture and environment protection, there was a growing interest in searching for beneficial microorganisms (Figueiredo et al., 2016). Among the explored microorganisms, bacteria occurring in the rhizosphere soil, rhizoplane, and/or the internal plant tissues were widely used (Hallmann et al., 1997). Their beneficial effects include, among others, efficient systems for uptake and catabolism of organic compounds present in root exudates (Barraquio et al., 1997). Several bacteria may also help to derive maximum benefits from root exudates through their capacity to attach to root surface (rhizoplane) (Compant et al., 2005). Endophytic bacteria are able to grow inside plant tissues and to protect them from biotic and biotic stresses (Sharma and Nowak, 1998). They can stimulate plant growth via direct and/or indirect mechanisms. Directly, they act by providing essential nutrients, producing phytohormones and growth regulators, or by regulating phytohormone levels. Indirect plant growth promotion (PGP) may be achieved by suppressing pathogens and/or inactivating pollutants responsible for plant growth inhibition (Vijayabharathi et al., 2016).

Beneficial agents may adversely affect population, density, dynamics and metabolic activities of soilborne pathogens through the production of hydrolytic enzymes and antimicrobial secondary metabolites and/or through the competition for nutrients (Swarupa et al., 2016). Several microbial agents such as *Trichoderma* and *Bacillus* are able to suppress fungal inoculum in soils by producing antibiotics (Larkin and Tavantzis, 2013; Bernard et al., 2014).

The soil microbial community is thought to be responsible for biological processes that are necessary for maintaining a healthy soil and suppressing plant diseases (Mazzola, 2004). The biocontrol of soilborne diseases can be achieved by manipulating the rhizosphere microflora in favor of beneficial microorganisms acting directly against soil pathogens (Yang et al., 2001). There is a considerable interest in monitoring changes in activity and composition of soil microbial communities following the application of microbial inoculants. In fact, beneficial bacteria or fungi such as *Trichoderma*, *Hypoxylon*, *Tritirachium*, *Paenibacillus*, *Bacillus*, *Haliangium* and *Streptomyces* were more abundant in biologically treated soils as compared to control whereas *Fusarium* inoculum was markedly decreased (Qiu et al., 2012).

Two endophytic bacteria *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 originally recovered from *Datura metel* and *Solanum nigrum*, respectively, were tested in this study under field conditions. They were previously (pot experiment) selected based on their ability to suppress Fusarium wilt and to promote tomato growth using their whole cells or their cell-free culture filtrates.

This study aimed to check, under field conditions, the ability of *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 to enhance growth and production of tomato plants growing in soils naturally infested with *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and to suppress wilt severity. Their subsequent effects on pathogen inoculum and soil microbial community were also investigated.

Material and Methods

Plant material

Tomato cv. Sahel was used in this study. This cultivar is known to be resistant to FOL races 1 and 2 and susceptible to race 3 (Syngenta, 2015). Seedlings were grown under greenhouse conditions (16 h photoperiod, 60-70% relative humidity and 20-30°C air temperature) in alveolus plates (3×3 cm) filled with sterilized peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg) . They were watered regularly until reaching the two-true-leaf growth stage. Seedlings with approximately similar heights were used in all trials.

Bacterial material

Two endophytic bacterial strains namely *Bacillus subtilis* SV41 (Accession number KR818071) and *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 (KR818073), originally recovered from the internal stem tissues of *Datura metel* and *Solanum nigrum*, respectively, were used in the current investigation.

Isolation procedure, characterization and identification using 16S rDNA sequencing genes were previously described in Aydi Ben Abdallah et al. (2015). These two strains were previously selected based on their higher efficiency, when used as cell suspensions or cell-free culture filtrates, in suppressing Fusarium wilt disease on tomato plants inoculated with FOL. Indeed, both *Bacillus* strains were harbor chitinase gene in their genome and *B. amyloliquefaciens* subsp. *plantarum* SV65 was able to produce the lipopetide antibiotic, *fengycin D*, which was detected in its genome. Also, the plant defense genes such as *PR1*, acidic *PR3* and lipoxygenase (*LOXD*) were expressed in tomato plants inoculated or not with FOL and treated with *B. amyloliquefaciens subsp. plantarum* SV65 (Aydi Ben Abdallah et al., 2017). Furthermore, the plant growth-promoting traits such as the production of indole-3-acetic acid, siderophores and organic acids in root exudates and the phosphate solubilization ability were recorded on both selected strains (unpublished data). Their plant growth-promoting properties and antagonististic mechanisms are detailed in Table 1.

Table 1. Plant growth-promoting (PGP) traits, antagonistic and inducing systemic resistance (ISR) properties of Bacillus subtilis SV41 and Bacillus amyloliquefaciens subsp. plantarum SV65 recovered from Datura metel and Solanum nigrum stems, respectively.

Strain	PGP traits (Unpublished data)					Antifungal and ISR properties (Aydi Ben Abdallah et al., 2017)						
	IAAa	Phosb	Ox, Mal acidsc	Sdd	Pece	Chitf	Protg	FenDh	SAi	LOXDj	CHI3-R3k	PR1l
B. subtilis SV41	+	-	+	+	+	+	+	-	+	-	-	-
B. amyloliquefaciens subsp. *plantarum* SV65	+	+	+	+	+	+	+	+	+	+	+	+

[a] IAA: Indole-3-acetic acid production after 48 h of incubation at 28 ± 2°C in Luria-Broth medium; +: Production of IAA.
[b] Phosphatase activity: Tested on Pikovskaya agar medium and incubated at 28 ± 2°C for 7 days; +: Presence of clear zone, -: Absence of clear zone.
[c] Organic acids (oxalic and malic acids) production detected in root exudates which are collected after 14 days from tomato plants cultivated in Hoagland solution (50 %).
[d] Siderophore production: Tested on Chrome Azurol Sulphonate (CAS) agar medium and incubated at 28 ± 2 °C for 5 days; +: Presence of zone of siderophore activity (yellow color).
[e] Pectinolytic activity:Tested on pectin-agar (0.5% w v-1) medium and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone. The Polygalacturonic acid was measured at 540 nm.
[f] Chitinase activity:Tested on chitin-agar (0.5 % w v-1) medium and incubated at 28 ± 2°C for 72 h; +: Presence of clear zone; Detection of ChiA gene by PCR using 5'-TTCAYGTTCAACACTACAA-3 ' and 5'-CATTAAGGCCGCGGARTG-3' primers for B. subtilis SV41 and 5'-GATATCGACTGGGAGTTCCC-3 'and 5'-CATAGAAGTCGTAGGTCATC-3' primers for B. amyloliquefaciens subsp. plantarum SV65.
[g] Protease activity: Tested on skim milk agar (3% v v-1) medium and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone.
[h] Detection of fengycin D (FenD) gene by PCR using 5'-TTTGGCAGCAGGAGAAGTTT-3' and 5'-GCTGTCCGTTCTGCTTTTTC-3'primers.
[i] Salicylic acid production after 48 h incubation at 28 ± 2°C in succinate medium; +: Production of salicylic acid.
[j] Relative expression of lipoxygenase (LOXD) gene in uninoculated tomato plants with F. oxysporum f. sp. lycopersici (FOL) using 5'-CCTGAAATCTATGGCCCTCA-3' and 5'-ATGGGCTTAAGTGTGCCAAC-3' primers for quantitative RT-PCR.
[k] Relative expression ofacidic chitinase (CHI3-PR3) gene in uninoculated tomato plants with FOL using 5'-TGCAGGAACATTCACTGGAG-3' and 5'-TAACGTTGTGGCATGATGGT-3' primers for quantitative RT-PCR.
[l] Relative expression ofPR1 gene into inoculated tomato plants with FOL using 5'-TCTTGTGAGGCCCAAAATTC-3' and 5'-ATAGTCTGGCCTCTCGGACA-3'primers for quantitative RT-PCR.

Before being used in the different bioassays, bacterial cultures were initiated from stock maintained at -20°C in Nutrient Broth (NB) supplemented with 40% glycerol and grown for 48 h at 25°C on Nutrient Agar (NA) medium.

Test of the effect of *Bacillus* spp. strains on tomato growth and Fusarium wilt severity

Bacterial treatments were prepared from cultures previously grown in NB medium for 3 days at 28 ± 2°C and under continuous stirring at 150 rpm. They were firstly applied to tomato cv. Sahel seedlings by drenching the substrate of each alveolus (3 cm in diameter) with 5 ml of a bacterial cell suspension (10^8 cells ml^{-1}). Treated and untreated tomato seedlings were transplanted into rows with a distance of 33 cm between seedlings. Planting was carried out on December 2015 under greenhouse installed in the experimental station of Teboulba, Monastir, Tunisia (N35°38'38,256'', E10°56'48,458''). Planting soil was known to be historically infected with FOL.

A second bacterial treatment was applied at planting, one week after the first treatment, by drenching the rhizospheric soil of each seedling with 100 ml of a cell suspension (10^8 cells ml^{-1}). Three treatments were tested: (i) Untreated control seedlings, (ii) seedlings treated with *B. subtilis* SV41, and (iii) seedlings treated with *B. amyloliquefaciens* subsp. *plantarum* SV65.

Two replicates of twenty seedlings each were used for each individual treatment. Tomato seedlings were grown for about six months at 24-25°C with 16/8 h photoperiod and 70% air relative humidity. They were subjected to agricultural practices commonly adopted by farmers in the region and irrigated and fertilized as needed.

Disease severity, growth and production parameters

Six months after planting, the parameters noted were the vascular browning extent (from the collar), plant height, roots fresh weight, and fruits fresh weight per plant. The frequency of colonization of roots, collars and stems of tomato plants by pathogen was determined after isolation of ten fragments per organ on PDA medium amended with 300mgl^{-1} of streptomycin sulphate. For the latest parameter and for each organ, three replicates of one plate each were used for every individual treatment. Cultures were incubated at 25°C for 4 days.

The percentage of colonization of plant tissues by various microorganisms was calculated using the formula of Moretti et al. (2008). The percentage of re-isolation from stems is the average of five counts recorded at different stem levels i.e. (0 to 5 cm), (5 to 10 cm), (10 to 15 cm), (15 to 20 cm) and (20 to 25 cm). The averages vascular browning extent, plant height, roots fresh weight were estimated from thirty plants. The average weight of fruits produced per plant was determined by calculating the total weight of fruits harvested between April and June 2016 and dividing by the total number of plants (40 plants).

Initial soil sampling and processing

Composite soil samples from each individual plot were collected twice i.e. just before planting and after the last harvest. Uprooted soil samples collected after the last harvest were removed from: (i) the rhizophere of untreated control plants, (ii) the rhizosphere of plants treated with *B. subtilis* SV41 and (iii) the rhizosphere of plants challenged with *B. amyloliquefaciens* subsp. *plantarum* SV65. At each sampling date, twenty soil cores (7cm in diameter × 15 cm in depth) collected from each treatment were combined to make one composite soil per individual treatment. Two replicates were considered for each soil sampling. Once brought to laboratory, soil samples were passed through a 2-mm sieve to remove rocks and large organic debris. They were stored in plastic bags at 10°C and processed within 1 to 4 weeks after sampling (Larkin and Honeycutt, 2006). For further assays, two subsamples were processed from each composite soil sample.

Determination of *Fusarium oxysporum* population

To confirm the presence of *F. oxysporum* in the soil and to evaluate the effect of tested bacterial treatments on the soil infectious potential, 5 g of each soil sample were placed in an Erlenmeyer flask containing 100 ml of sterile distilled water (SDW) and subjected to continuous stirring at 150 rpm for 60 min. The supernatant was filtered through a double layer of muslin followed by a series of dilutions using SDW (Daami-Remadi et al., 2009). For each sample, 200 µl of 10^{-2} dilution were plated onto selective peptone-pentachloro-nitrobenzen (PCNB) agar medium specific for *Fusarium* spp. isolation (Nash and Snyder, 1962). Plates were stirred gently by hand for homogeneous distribution of the soil extract with the culture medium. Three replicates of one Petri plate each were used for each soil sample. After incubation at 25°C for 12 days, *F. oxysporum* colonies growing on PCNB agar medium were morphologically and microscopically identified and the total number of colonies was counted.

Determination of soil properties

Soil samples were air-dried before use. Soil extracts were prepared by suspending soil in distilled water in 1:10 soil/dH_2O ratio. They were filtered through Whatman paper No 1 and analyzed for determination of their pH and conductivity using a glass electrode and conductivity meter, respectively. Water content was checked by removing 5 g of soil and weighting it before (wet weight) and after oven drying at 105°C for 24h (Larkin et al., 2006). Organic carbon was determined by drying 10 g of soil at 105°C overnight and then at 900°C for 2 h. The percentage of organic matter was calculated using the following formula:

Organic matter = (dry matter – mineralized matter) × 100 / dry matter and the percentage of organic carbon was estimated using the following formula: %Organic carbon = %Organic matter / 1.72 (Kettler et al., 2001).

Estimation of soil microbial community

General populations of culturable soil microorganisms were determined by soil dilution plating on various agar media according to Larkin and Honeycutt (2006) with some modifications. For each subsample taken from each composite soil, 10 g were added to 90 ml of sterile 0.2% water agar, vigorously stirred for 30 min, serially diluted and a-100 µl sample wasplated on 10% Tryptic Soy Agar (TSA) for total bacterial counts, selective King's B medium (KB) amended with 75 mg l^{-1} of penicillin and 75 mg l^{-1} of cyclohexamide for *Pseudomonas fluorescens* counts, Yeast Malt Agar (ISP medium No. 2) amended with 75 mg l^{-1} of nalidixic acid and 100mg l^{-1} of cyclohexamide for actinomycete counts,and Potato Dextrose Agar (PDA) amended with 300 mg $l^{-1}$of streptomycine sulphate for total fungal counts.Four replicates of one plate each were used for each soil subsample.

Bacterial and actinomycete plates were incubated at 28°C for 2 and 14 days, respectively, and fungal plates were maintained at 25°C for 7 days before counting growing colonies. Colonies of *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. were identified based on their macro- and micro-morphological traits under light microscope and counted separately.

Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0. Data were analyzed according to a completely randomized design. Means were separated using Duncan Multiple Range tests to identify significant pair-wise differences at $P \leq 0.05$.

Results

Effects of tested bacterial treatments on Fusarium wilt severity and tomato growth

Six months after planting, a significant decrease (at $P \leq 0.05$) in Fusarium wilt severity was achieved following tested bacterial treatments as compared to control,. Indeed, the vascular browning extent was reduced by 82.3 and 88.2% by *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 treatments, respectively, compared to the untreated control (Figure 1).

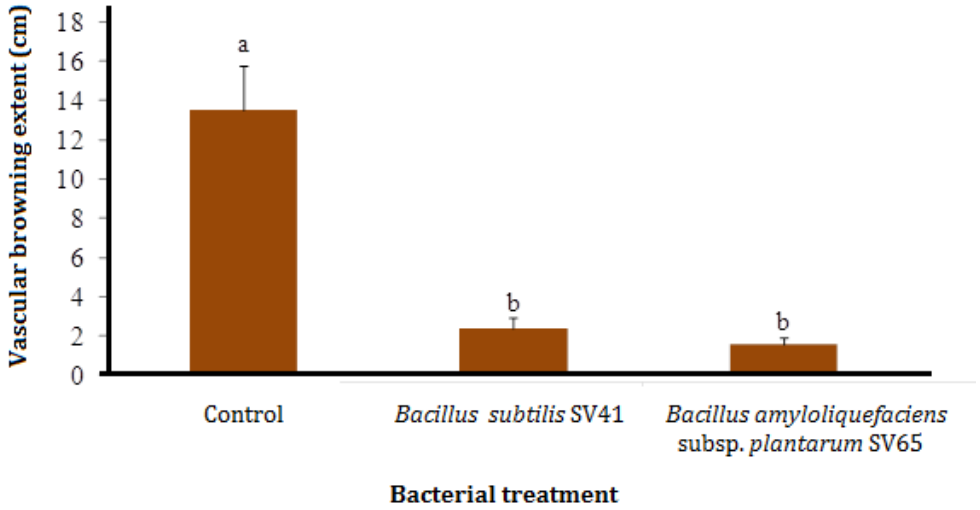

Figure 1. Effect of Bacillus subtilis SV41 and Bacillus amyloliquefaciens subsp. plantarum SV65 on Fusarium wilt severity on tomato plants cv. Sahel grown under greenhouse for six months.
Results are presented as mean ± SE (n=30, P≤ 0.05). Bars sharing the same letter are not significantly different according to Duncan Multiple Range test at P≤ 0.05.

The percentage of re-isolation of *F. oxysporum* from roots, collars and stems of plants treated with *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. subtilis* SV41 was limited to 2.6-46.6% and 10-53.3%, respectively, compared to the untreated control where the re-isolation frequency of *F. oxysporum* varied from 63.3 to 80% (Figure 2).

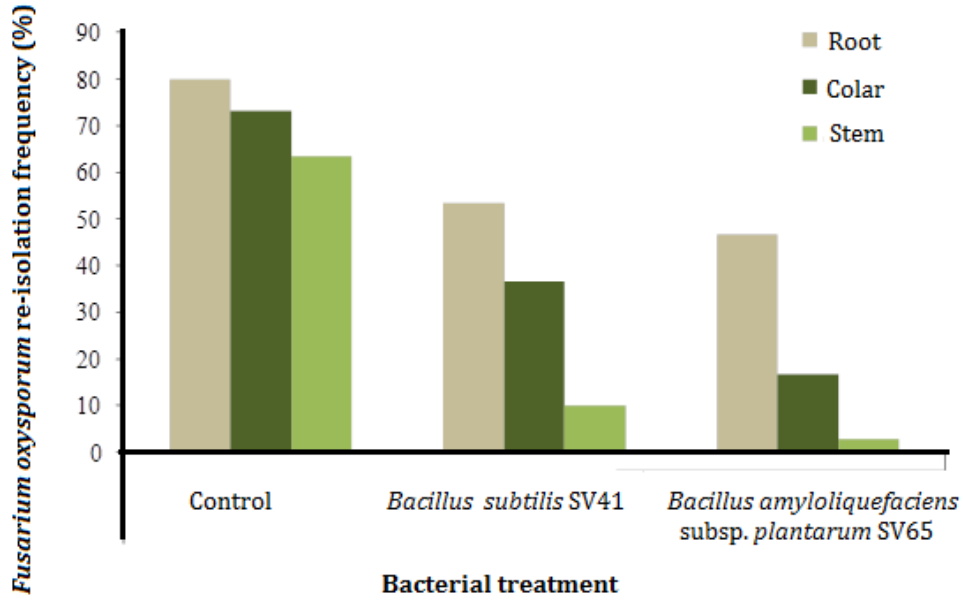

Figure 2. Effect of Bacillus subtilis SV41 and Bacillus amyloliquefaciens subsp. plantarum SV65 on Fusariumoxysporum re-isolation frequency from roots, collars and stems of tomato plants cv. Sahel grown under greenhouse for six months.

The values presented are the percentages (%) of re-isolation of F. oxysporum on Potato Dextrose Agar (PDA) medium after incubation at 25°C for 4 days. Ten fragments per organ were cultured on plates containing PDA medium supplemented with streptomycin sulfate (300 mg l-1). Each individual treatment was repeated three times (3 × 10 fragments). The percentage of re-isolation from stems is the average of five counts recorded at different stem levels i.e. (0 to 5 cm), (5 to 10 cm), (10 to 15 cm), (15 to 20 cm) and (20 to 25 cm).

As given in Table 2, growth and production parameters of tomato plants cv. Sahel (plant height, root fresh weight and fruit production per plant), noted six months after planting, varied significantly (at $P \leq 0.05$) depending on tested bacterial treatments.

Concerning plant height, a significant improvement of this parameter, of about 10.6% over control, was achieved using *B. amyloliquefaciens* subsp. *plantarum* SV65 based treatment (Table 2). The root fresh weight was significantly increased by 15.6 and 16.3% versus control in tomato plants treated with *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. subtilis* SV41, respectively. As shown in Table 2, *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. subtilis* SV41 significantly improved the fruit production by 8.4 and 12.5% compared to the untreated control where the greatest enhancement, of about 12.5% over control, was recorded in *B. subtilis* SV41 treated plants.

Table 2. Effect of two *Bacillus* spp. strains on the growth of tomato plants cv. Sahel grown under greenhouse[x] for six months.

Bacterial treatment	Plant height (cm)[a]	Root fresh weight (g)[b]	Fruit weight (g)[c]
Control	321.7 b ± 68	51.5 b ± 1.86	1622 c ± 0.73
B. subtilis SV41	326.5 b ± 67	61.5 a ± 3.15	1854 a ± 0.83
B. amyloliquefaciens subsp. *plantarum* SV65	359.17 a ± 51	61.03 a ± 12	1770 b ± 0.8

For each column, the values followed by the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$; ± SE: Standard error.

[a,b] Results are noted at harvest. The values presented are the averages obtained from 30 plants for each treatment. The broken plants were eliminated from the statistical analysis.

[c] Fruit weight was expressed per plant. For each treatment, this parameter was determined by calculating the sum of the weights of all fruits harvested between April and June 2016 divided by the total number of plants (40 plants).

[x] The greenhouse is located in the experimental station at Teboulba (N35 ° 38'38.256 '', E10 ° 56'48.458 '', Monastir), The Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia.

Effects of tested bacterial treatments on *Fusarium oxysporum* population

ANOVA analysis performed for the number of *F. oxysporum* colonies revealed a significant variation (at $P \leq 0.05$) in this parameter depending on sampled soils. As given in Figure3, *F. oxysporum* colonies were reduced by 87.5 and 91.7% in soils sampled from the rhizosphere of tomato plants cv. Sahel treated with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65, respectively, as compared to those sampled before planting (initial soil state). *F. oxysporum* population decreased by 88.4 and 92.3% in tomato rhizospheric soils, compared to controlsoils, following their treatment with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65, respectively.

Effect of tested bacterial treatments on soil properties

All collected soil samples were sandy loam. The application of bacterial treatments did not induce significant changes in soil pH which ranged between 7.3 and 7.7. However, the conductivity decreased by 26.7 and 42.1%in soils treated with *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. subtilis* SV41 compared to control and by 23.7 and 39.7% relative to the initial state, respectively (Table 3).

Table 3. Properties of sampled soils [a]

Soil sample	Sampling date	Texture	pH	EC (dS/m)	Water content (%)	Organic matter (%)	Organic carbon (%)
Initial state	December 2015	Sandy loam	7.3	0.228	15	3.16	1.83
Control	June 2016	Sandy loam	7.7	0.237	15.5	2.74	1.59
SV41-treated	June 2016	Sandy loam	7.6	0.137	15.5	3.38	1.96
SV65-treated	June 2016	Sandy loam	7.4	0.174	13.5	3.66	2.13

[a] Soil sampled from the greenhouse located in the experimental station at Teboulba (N35 ° 38'38.256 '', E10 ° 56'48.458 '', Monastir), The Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia.

Initial state: Soil sampled one week before planting. Control: Soil sampled from the rhizosphere of untreated tomato plants six months after planting.SV41: Soil sampled from the rhizosphere of tomato plants treated with *Bacillus subtilis* SV41. SV65: Soil sampled from the rhizosphere of tomato plants treated with *B. amyloliquefaciens* subsp. *plantarum* SV65.

The percentage of water content varied from 13.5 to 15.5% in all soil samples. A slight increment of about 23.4 and 33.7%i n the percentage of organic matter was noted in the soil removed around roots of tomato plants treated with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 compared to control soil sampled on June 2016 and of about 6.9 and 15.8%relative to the initial soil state sampled on December 2015, respectively. Additionally, the percentage of organic carbon varied from 1.96 to 2.13% in soil treated separately with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 compared to 1.59 and 1.83% estimated on control and initial soil samples, respectively (Table 3).

Effects of tested bacterial treatments on soil microbial community

Analysis of variance revealed that the number of bacterial colonies growing from plated soil samples, after 2 days of incubation in TSA medium, varied significantly (at $P \leq 0.05$) depending on tested treatments. Soil samples treated with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 showed significant increase in their bacterial populations of about 78.57 10^6 and 58.12 10^6 CFU g^{-1} soil, respectively, as compared to the initial soil state (before planting) estimated at 16.75 10^6CFUg^{-1}. A significant enhancement, of about 49.9%, in the total culturable bacterial community was recorded in the tomato rhizospheric soil treated with *B. subtilis* SV41 compared to control (Figure 4A).

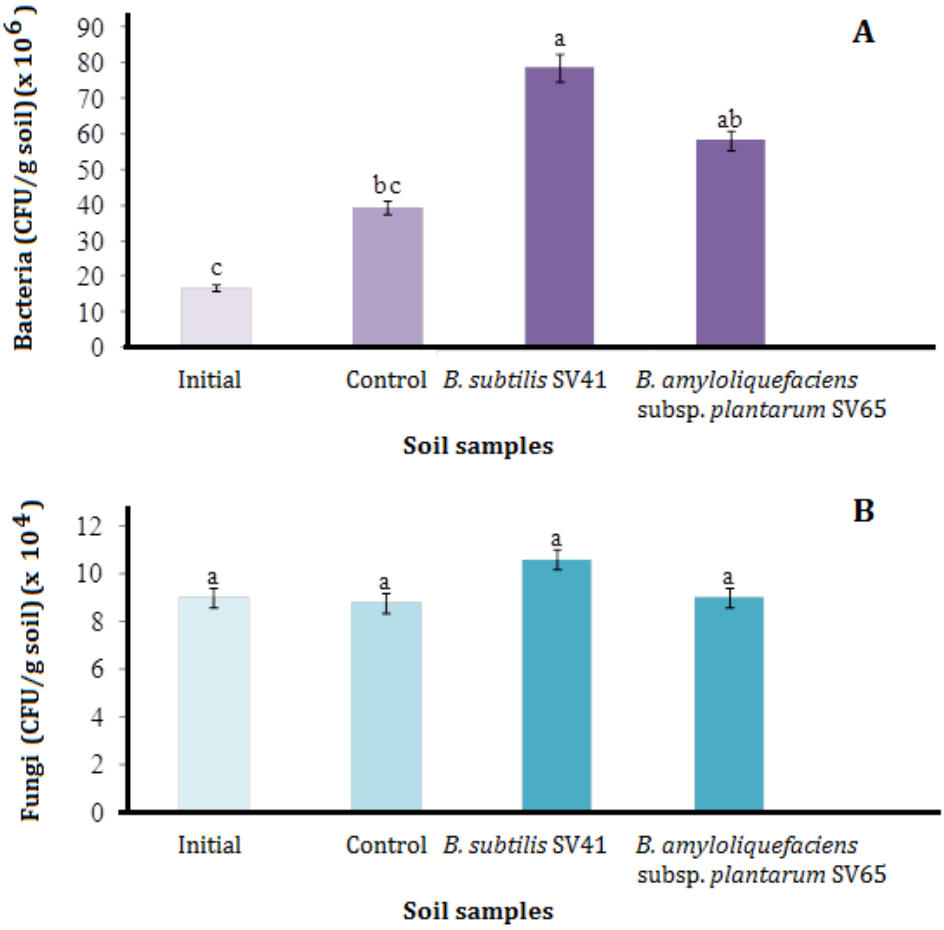

Figure 4.Effect of bacterial treatment on soil microbial population sampled from the rhizosphere of tomato plants grown under greenhouse[x] for six months.

Results are presented as mean ± SE (n = 8, $P \leq 0.05$). Bars sharing the same letter are not significantly different according to Duncan Multiple Range test at $p \leq 0.05$. Dilution was made from a concentration of 10% (w v^{-1}).Initial: Soil sampled at pre-plant (before planting). Control: Soil sampled from the rhizosphere of untreated tomato plants at the end of the trial (six months after planting). [x] The greenhouse is located in the experimental station at Teboulba (N35 ° 38'38.256 '', E10 ° 56'48.458 '', Monastir), The Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia.

As for the bacterial biodiversity, the relative abundance of *Pseudomonas* spp. community was found to be relatively important in the rhizosphere of tomato plants treated with the two bacterial strains and in the untreated ones as compared to *Pseudomonas* population determined in the initial soil state (Figure 5). Indeed, both bacterial treatments significantly enriched *Pseudomonas* community in treated soils by 57.5-66% compared to the control soils and by 92.8-94.2% versus the initial soil state. Furthermore, *P. fluorescens* colonies were three times higher in the *B. amyloliquefaciens* subsp. *plantarum* SV65-treated soil than in control soil and 11.2 times more abundant than in theinitial state. Soil treated with *B. subtilis* SV41 showed significant increase in *P. fluorescens* population which was nine times higher than that ofthe initial soil (Table 4). As shown Figure5, in each soil sample, *Actinomycetes* population was lesser when compared to the remaining bacterial groups. Indeed, Actinobacteria population significantly increased by 52 and 56.2%, compared to the initial state, in soils treated with both bacterial strains. Furthermore, *B. subtilis* SV41-treated soil showed significant increment in the total of culturable Actinomycetes of about 44.9% compared to control (Table 4).

Table 4. Soil populations of selected subgroups of microorganisms isolated from the rhizosphere of tomato cv. Sahel plants treated separately with *Bacillus subtilis* SV41 and *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 compared to the untreated and to the pre-plant soil samples, as determined by soil dilution plating on selective media.

Soil sample	Microorganism group (CFU g^{-1} soil)[x]					
	Actinomycetes ($\times 10^3$)	*Pseudomonas* ($\times 10^5$)	*P.fluorescens* ($\times 10^5$)	*Aspergillus* spp. ($\times 10^3$)	*Penicillium* spp. ($\times 10^3$)	*Fusarium* spp. ($\times 10^3$)
Initial state	15.00 c	15.00 b	5.00 c	13.75 b	0 b	25.00 a
Control	18.88 bc	88.75 b	18.75 bc	16.66 ab	0 b	26.66 a
SV41-treated	34.28 a	261.25 a	45.00 ab	27.50 a	6.25 a	7.50 b
SV65-treated	31.25 ab	208.75 a	56.25 a	21.25 ab	1.25 ab	2.50 b

[x] Soil samples were collected in December 2015 (before planting) and June 2016 (harvest) from the greenhouse located in the experimental station at Teboulba (N35 ° 38'38.256 '', E10 ° 56'48.458 '', Monastir), The Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia.

Values following with the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$. Dilution was made from a concentration of 10% (w v^{-1}). Initial: Soil sampled at pre-plant (before planting). Control: Soil sampled from the rhizosphere of untreated tomato plants at the end of the trial (six months after planting).B1: Soil sampled from the rhizosphere of tomato treated with *Bacillus subtilis* SV41. B2: Soil sampled from the rhizosphere of tomato treated with *B. amyloliquefaciens* subsp. *plantarum* SV65.

As for fungal biodiversity, there was a slight increase in the total culturable fungi, growing after 7 days of incubation in PDA medium, following bacterial treatments compared to control and initial soil samples (Figure 4B). As given in Table 3, *Aspergillus* spp. and *Penicillium* spp. colonies were more abundant in the rhizosphere of *B. subtilis* SV41-treated plants compared to the initial (before tomato planting) and control soil samples. However, a significant decrease by 71.9 and 90.6% compared to control soil and by 70 and 90% relative to the initial state, was noted in *Fusarium* spp. populations in soils treated with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65, respectively (Table 4).

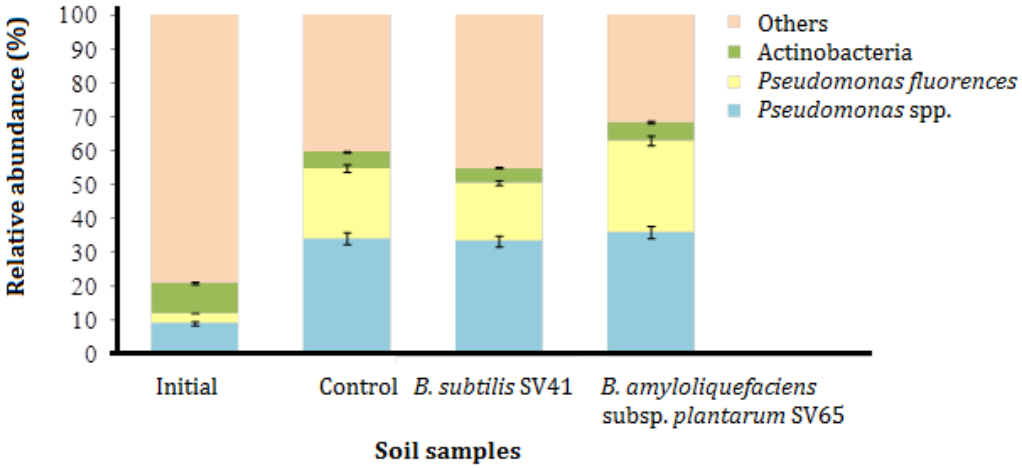

Figure 5. The bacterial community structure under bacterial soil treatment per g soil.
Results are presented as mean ± SE (n = 8, $P \leq 0.05$). The relative abundance was estimated per the total bacteria counted in each sampled soil. Dilution was made from a concentration of 10% (w v^{-1}). Initial: Soil sampled at pre-plant (before planting). Control: Soil sampled from the rhizosphere of untreated tomato plants at the end of the trial (six months after planting).

Discussion

A better understanding of what makes a plant-microbe interaction detrimental or beneficial to plants would provide an important insight into the efficient handling of microbes for agriculture production (Swarupa et al., 2016). In this study, two endophytic bacteria, *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65, were assessed for their ability to suppress Fusarium wilt disease symptoms, to enhance tomato growth and production and to reduce pathogen inoculums in soil. The effect of both bacteria on soil microbial populations was also estimated.

The exploration of endophytic bacteria as a potentially interesting and environmentally friendly alternative for the management of tomato Fusarium wilt disease has been shown to be effective in inhibiting systemic progression of the causative agent (Ramyabharathi and Raguchander, 2014). In this study, *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 successfully reduced Fusarium wilt severity by 82-88% and limited the pathogen colonization of tomato stems compared to control. Numerous plant pathogenic *F. oxysporum* isolates were successfully controlled by *Bacillus* sp. recovered from chickpea plants (Landa et al., 1997). Disease suppressive effects were also expressed by two unidentified endophytic bacteria, recovered

from healthy wild and cultivated young oilseed rape plants, which reduced Fusarium wilt severity by 75% on tomato plants (Nejad and Johnson, 2000). Kalai-Grami et al. (2014) found that endophytic *B. mojavensis* recovered from citrus plants reduced disease severity in maize plants inoculated with *Fusarium verticillioides*. In addition, most *Bacillus* species were able to inhibit the mycelial growth of *F. oxysporumin vitro* (Idris et al., 2007; Aydi Ben Abdallah et al., 2015).

The plant growth-promoting ability was induced by the beneficial microbial agents directly by the acquisition of essential nutriments and the production of phytohormones and/or indirectly by inhibiting pathogen growth and inducing plant systemic resistance (Santoyo et al., 2016). Results from the current study clearly demonstrated that application of *B. subtilis* SV41 and *B. velezenis* SV65 in the soil reduced the population of *F. oxysporum* and enhanced the number of beneficial microbial agents leading to a significant decrease in Fusarium wilt severity and to a significant promotion of plant growth and production. This result is in agreement, in part, with previous findings related to disease-suppressive effects displayed by an endophytic *B. subtilis* EPC016, isolated from cotton plants, which decreased Fusarium wilt incidence by 68.4% and increased tomato growth and yield compared to control (Ramyabharathi and Raguchander, 2014). *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 were previously tested with other *Bacillus* species in a pot experiment where authors indicated that Fusarium wilt severity suppression was significantly correlated to the decreaseof pathogen colonization of vascular tissues leading consequently to plant growth promotion (Aydi Ben Abdallah et al., 2017). Indeed, *B. subtilis* SV41 was found the highest salicylic acid producing agent that acts as an elicitor for the induction of systemic resistance against pathogen attack. Furthermore, Aydi Ben Abdallah et al. (2017) found that *B. amyloliquefaciens* subsp. *plantarum* SV65induced expression of the lipooxygenase gene (*LOXD*) and especially acidic PR-1 and PR-3 genes in treated plants and those uninoculated or inoculated with FOL. Therefore, the two *Bacillus* spp., tested in our study, have shown able to induce systemic resistance in treated tomato as reported in Aydi Ben Abdallah et al. (2017) leading indirectly to growth promotion. There are a large number of common mechanisms that plant growth-promoting bacteria (PGPB) use to indirectly promote plant growth which include production of antibiotics and cell wall-degrading enzymes, decrease in ethylene levels, induction of systemic resistance, reduction of iron available to pathogens, and synthesis of pathogen-inhibiting volatile compounds (Glick, 2015). In addition, PGPB directly promote plant growth through the acquisition of nutriments from environment including nitrogen, phosphorous and iron or through the production and/or regulation of various plant hormones including auxin, cytokinin or ethylene (Santoyo et al., 2016). These two *Bacillus* strains tested in the current investigation are shown able to produce the indole 3-acetic acid (IAA) and only *B. amyloliquefaciens* subsp. *plantarum* SV65 was able to solubilize the phosphate (unpublished data). Aydi Ben Abdallah et al. (2016) also demonstrated that *Bacillus* sp. SV101 and *B. tequilensis* SV104 were IAA-producing agents and only *Bacillus* sp. SV101 was a phosphate-solubilizing strain.

Many researchers have attempted the development of biocontrol agents for suppression of various soilborne pathogens (Domenech et al., 2005). In this study, pre-planting soil treatment with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp*. plantarum* SV65 reduced *Fusarium* (and especially *F. oxysporum*) inoculum in treated soils as compared to control. These results are in agreement with other findings (Zhang et al., 2008; Ling et al., 2010). In fact, Qiu et al. (2012) found that following treatment with abio-organic fertilizer (composed of organic fertilizer combined with *B.subtilis* SQR-9, *Paenibacillus polymyxa* SQR-21 and *Trichoderma harzianum* SQR-T037), *Fusarium* population was reduced to one fourth as compared the organic fertilizer applied without microbial supply. Indeed, among the *Fusarium* group, *F.oxysporum* population in soils unsubjected to microbial amendment were almost ten times more abundant than in the soil treated with the bio-organic fertilizer. In this study, the abundance of fungi showed different trends. A higher frequency of *Aspergillus* and *Penicillium* colonies was detected in the rhizosphere of tomato plants treated with *B. subtilis* SV41 as compared to the initial state (before planting) and to control soil. *Aspergillus* and *Penicillium* have been reported to be antagonistic to various *Fusarium* species (Dong and Cohen, 2002; Brzezinska and Jankiewicz, 2012; Mejdoub-Trabelsi et al., 2017). In fact, Sreevidya and Gopalakrishnan (2016) demonstrated that *P. citrinum* VFI-51 is able to produce siderophores, indole 3-acetic acid, hydrocyanic acid, lipase, protease, β-1,3 glucanase, and volatile compounds and also capable to compete with other pathogenic microorganisms such as *Fusarium* species (Aydi Ben Abdallah et al., 2015; Mejdoub-Trabelsi et al., 2017).

As for their effects on bacterial community structure, the number of beneficial bacteria determined in bacterized soils was three times higher than in the initial soil state (before planting). Moreover, *B. subtilis* SV41-treated soil showed enrichment of the total bacteria populations as compared to control. These

findings are in agreement with those of Qiu et al. (2012). In the current study, the soil DNA was not extracted and pyrosequenced. The colonies number of Actinomycetes and *Pseudomonas* wereestimatedbased on their macro-morphological traits on selective media. The number of *Pseudomonas* spp. was significantly higher in treated soils than in control ones (initial state and untreated control). The application of *B. amyloliquefaciens* subsp. *plantarum* SV65 in soil enhanced the population of *P. fluorescens* as compared to the initial state and to control soil. *Pseudomonas* is also known by its ability to suppress numerous plant pathogens including *F.oxysporum* (Patel et al., 2012; Munif et al., 2012; Dalal and Kulkarni, 2013). Bibi et al. (201) and Aydi Ben Abdallah et al. (2016) showed the ability of *Pseudomonas* spp. to produce hydrolytic enzymes such as chitinase, protease, pectinase and β-1,3 glucansewhich are involved in the antifungal activity against*F.oxysporum*. Also, in the current study, Actinobacteria population was significantly increased in soils treated with both bacterial strains compared to the initial soil state. In fact, *B. subtilis* SV41-treated soil showed significant enrichment in *Actinomycetes* population which was estimated to be two times higher than to that of the untreated soil. Qiu et al. (2012) found that *Streptomyces*, an Actinobacteria, accounted for 3.2% of the total bacterial sequences on soils treated with various microbial inoculants such as *Bacillus*, *Paenibacillus* and *Trichoderma*. Its abundance is approximately three times higher than in the untreated soil. This genus can produce tubercidin, phosphlactomycin and candicidin (Hwang et al., 1994) and was found to be an effective biological control agent (Etebarian et al., 2003; Nourozian et al., 2006; Shekhar et al., 2006). Actinomycetes collected from 30 rhizospheric soils of *Catharanthus roseus* and *Withania somnifera* in different locations in Ludhiana, India, are promising biocontrol agents for *Alternaria alternata*, *Fusarium oxysporum*, *Helminthosporium oryzae*, *Macrophomina phaseolina*, *Penicillium* sp., *Rhizoctoniasolani* and *Sclerotium rolfsii* control (Kamara and Gangwar, 2015).

Fusarium wilt suppression maybe, in part, attributed to the action of microbial agents associated to treated tomato plants. In this study, soil bacterial treatment increased significantly the populations of *Pseudomonas* and *Actinomycetes* in tomato rhizosphere compared to untreated soils. Patel et al. (2012) identified an endophytic *Pseudomonas aeruginosa* HR7, recovered from cultivated tomato, as an effective agent for *F. oxysporum* biocontrol. Dalal and Kulkarni (2013) also recorded a significant inhibition of *F.oxysporum* mycelial growth using endophytic *Pseudomonas* sp. originally recovered from soybean. In Aydi Ben Abdallah et al. (2016) study, *P. brenneri* S85, shown able to inhibit FOL *in vitro* and *in vivo* growth, was capable to produce chitinase, protease and pectinase. Endophytic *Pseudomonas* species i.e *P. brenneri*, *P. koreensis*, *P. viridiflava* and *P. syringae* are also commonly known as producers of antibiotics such as ecomycins and pseudomycins (Christina et al., 2013) and/or hydrolytic enzymes (Bibi et al., 2012). Indeed, two actinomycetes species i.e*Micromonospora* sp. and *M. globosa* were successfully explored as biocontrol agents against tomato wilt and Pigeons-peas wilts caused by *F. oxysporum* f. sp. *lycopersici* and *F. udum*, respectively (Smith, 1957; Ypadhyay and Rai, 1987). Various antifungal antibiotics were released in soils by different actinomycetes (Trejo-Estrada et al., 1998; El-Tarabily and Sivasithamparam, 2006). In fact, this bacterial group produced high levels of chitinases and β-1,3-glucanses which caused extensive hyphal plasmolysis, cell-wall lysis and significantly reduced the level of disease incidence under controlled greenhouse conditions (El-Tarabily et al., 2000). Furthermore, the plant growth-promoting potential noted in treated plants may be due to the presence of beneficial microorganisms in the rihzosphere of treated plants such as *Pseudomonas* and actinobacteria. Several previous study showed that phosphate solubilization potential and IAA production ability by *Pseudomonas*are involved in plant growth promotion (Ngamau et al., 2012; Patel et al., 2012; Dalal and Kulkarni, 2013; Aydi Ben Abdallah et al., 2016). Additionally, the selected actinomycetes, identified as *Streptomyces* using 16S rDNA analysis, have good plant growth-promotion and biocontrol potentials on chickpea under *in vitro* and *in vivo* conditions and were found able to produce siderophores, cellulases, lipapses, chitinases, proteases, β-1,3-glucanses, hydrocyanic acid and IAA (Sreevidya et al., 2016).

In conclusion, soil bacterial treatment using *B. subtilis* SV41 (KR818071) and *B. amyloliquefaciens* subsp. *plantarum* SV65 (KR818073) was considered to be an effective approach to suppress Fusarium wilt of tomato through the direct inhibition of the causal agent and the suppression of its inoculum in soil and also through the enhancement of the soil biofertility via its enrichment with various beneficial microbial agents.

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