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Full Length Article

Organic Management of Tomato Fusarium wilt using a Native *Bacillus subtilis* Strain and Compost Combination in Saudi Arabia

Abdur Rashid Khan[1], Mahmoud H. El_Komy[1,2*], Yasser E. Ibrahim[1,2], Younis K. Hamad[1,3], Younes Y. Molan[1] and Amgad A. Saleh[1,4]

[1]*Department of Plant Protection, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia*
[2]*Plant Pathology Institute, Agriculture Research Center (ARC), Giza, Egypt*
[3]*Plant Pathology Department, Faculty of Agriculture, Alexandria University, Alexandria, Egypt*
[4]*Agriculture Research Center, Agricultural Genetic Engineering Research Institute, Giza, Egypt*
[*]For correspondence: malkomy@ksu.edu.sa
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Abstract

One of the most destructive tomato diseases is Fusarium wilt, caused by *Fusarium oxysporum* f. spp. *lycopersici* (FOL), which leads to substantial losses in tomato production in Saudi Arabia. Under organic farming conditions, disease management based on chemical treatment is restricted. Therefore, environmentally friendly alternative control strategies are needed. In this study, we investigated the effectiveness of the use of a native rhizobacteria integrated with organic compost to manage FOL. Twenty rhizobacterial strains were isolated from the rhizosphere of commercial tomato fields. Molecular identification of the 16S rRNA gene showed that the strains belonged to five genera, namely: *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Enterobacter*, and *Achromobacter*. Screening of antagonistic activity using dual cultures and culture-filtrate assays identified *Bacillus subtilis* strain KSU-110 as having the highest activity against FOL. Treating tomato plants with this antagonistic strain either alone or in combination with compost resulted in the reduction of the wilt disease under both greenhouse and field conditions. The combination of KSU-110 with organic compost significantly ($P < 0.05$) reduced the FOL population in the tomato rhizosphere and improved plant growth. Moreover, systemic resistance was enhanced in tomato plants through the induction of defense-related enzymes, including peroxidase and polyphenol oxidase. Taken together, the results of this study suggest that the combination of the native rhizosphere strain KSU-110 and organic compost has the potential to control tomato wilt under organic farming conditions in Saudi Arabia. © 2020 Friends Science Publishers

Keywords: Organic management; Fusarium wilt; Tomato; Plant growth-promoting rhizobacteria; Compost

Introduction

The government of the Kingdom of Saudi Arabia is taking considerable steps toward the support of more environmental-friendly farming systems. One such system is organic farming, which the Saudi government is actively encouraging the private sector to adopt for the production of safe and healthy foods (Hartmann *et al.* 2012). The total area of organically farmed land is approximately 35,000 ha, and future increases are expected (Hartmann *et al.* 2012). Tomato (*Lycopersicon esculentum* Mill.) is one of the most important organic crops in Saudi Arabia, with an annual production of approximately 306,000 tons (FAO 2017). However, Fusarium wilt, a devastating disease caused by *Fusarium oxysporum* f. spp. *lycopersici* (FOL), leads to extensive losses in tomato yield under both greenhouse and open field conditions (El_Komy *et al*. 2016). Under optimal infection conditions, yield losses can reach 90% (Hibar *et al*. 2006). Controlling tomato wilt disease using standard chemical methods is challenging; furthermore, this mode of control is restricted in organic farming (Finckh *et al*. 2015). Biological control with antagonistic microorganisms offers a promising and alternative strategy to manage tomato wilt disease without the deleterious environmental effects of chemical treatments (El_Komy *et al*. 2016). Inoculation with certain strains of plant growth-promoting rhizobacteria (PGPR), such as *Bacillus subtilis*, can protect plants from damaging soil-borne pathogens and promote plant growth via different mechanisms, including competition for nutrients and space, production of antifungal volatile organic compounds, antibiotics and fungal cell wall-degrading enzymes, and enhancement of plant resistance to pathogens (Kloepper *et al.* 2004; Bouizgarne 2013; Jangir *et al*. 2018).

Despite the proven effectiveness of certain rhizobacterial inoculants for the control of plant diseases and improvement of productivity, their successful application in commercial agricultural has been hampered by multiple constraints. Indeed, the efficiency of rhizobacteria is influenced by environmental conditions

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(Shirinbayan *et al*. 2019). Saudi Arabia is one of the world's arid regions. The cultivated soils in such regions are characterized by harsh environmental conditions, including lack of soil moisture and poor fertility represented in the lower contents of organic matter, as well as the higher contents of calcium carbonate and salinity (Hussain *et al*. 2010). However, such ecosystems have a diversity of microbes that are adapted to harsh environmental conditions (Soussi *et al*. 2016). Thus, identification of native rhizobacterial strains that are naturally adapted to harsh arid ecosystems may further lead to development of effective and sustainable cropping systems (Shirinbayan *et al*. 2019).

Root colonization by rhizobacteria and their persistence in the rhizosphere are major challenges in the implementation of biocontrol strategies (Abdallah *et al*. 2018). Poor root colonization and inadequate antagonistic metabolite production can account for some of the variations in the activity of antagonistic inoculants during the crop growing season (Bouizgarne 2013; Abdallah *et al*. 2018). Amendment of soil with organic substrates in combination with biocontrol strains can increase both the survival of rhizobacterial strains and their colonization of the soil near plant roots (Gava and Pinto 2016). Moreover, certain types of compost can naturally suppress disease. Therefore, the use of compost as a substrate for biocontrol applications would offer additional advantages (Termorshuizen *et al*. 2006; Gava and Pinto 2016).

Biological control may be an effective strategy to protect tomato plants against FOL (Jangir *et al*. 2018); however, novel microbial control agents that are native to specific arid conditions need to be identified. In fact, the application of exotic biocontrol agents might disrupt the local ecosystem and have detrimental ecological effects on the native rhizospheric microbial populations (Figueroa-López *et al*. 2016). In addition, exotic biocontrol agents might not remain active under all soil environments and in all agricultural ecosystems (Schmidt *et al*. 2004). Hence, this study aimed to select a native rhizobacterial strain against FOL *in vitro* and evaluate its effectiveness, either alone or in combination with organic compost, in reducing tomato wilt disease and inducing plant defense responses *in vivo*. This study was designed to develop an approach using a native rhizobacterial strain to control FOL and improve organic farming practices in arid regions.

Materials and Methods

Fungal pathogen

In this study, a pathogenic strain of *F. oxysporum* f. spp. *lycopersici* (FOL-30) was obtained from the collection of the Department of Plant Protection, College of Food and Agricultural Sciences, King Saud University. The pathogen was isolated from roots of tomato plants showing typical symptoms of Fusarium wilt. Koch's postulates were performed to confirm the pathogenicity. The pathogen was further identified morphologically and microscopically according to the criteria of Leslie and Summerell (2006), as well as molecularly by sequencing translation elongation factor 1 α (*tef1α*) and ITS-rRNA genes (Saleh *et al*. 2017). The fungal culture was revived on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 28°C after incubation for 7 days and maintained on PDA by subculturing at regular intervals.

Isolation and identification of bacterial antagonists

Rhizobacteria were isolated from the rhizosphere of healthy tomato plants grown in the Al-Kharj region of Saudi Arabia through serial dilution plating on nutrient agar media (NA; Difco Laboratories). Colonies with different characteristics were selected and grown separately. The isolated bacteria were initially identified on the basis of their morphological and physiological characteristics (Bergey *et al*. 1974), as well as by using the Biolog system (Biolog Inc., Hayward, C.A.). Molecular identification of rhizobacterial cultures was performed by isolating genomic DNA using a standard protocol (Sambrook *et al*. 1989). The 16S rRNA gene was amplified using PCR and the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5'-TACGGYTACCTTGTTA CGACTT-3') universal primers (Heuer *et al*. 1997). The PCR conditions were as follows: 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 55°C and 1.5 min at 72°C, with a final extension of 10 min at 72°C. The amplified products were sequenced by the Advanced Genetic Technologies Center (AGTC), College of Agricultural Sciences of the University of Kentucky (http://www.uky.edu/Centers/AGTC/). These DNA sequences were identified by comparison with nucleotide sequences deposited in GenBank using Bioedit software (Hall 1999; http://www.mbio.ncsu.edu/Bioedit/bioedit.html).

The *In vitro* antagonistic activity

Dual culture assay: The bacterial strains were screened for antifungal activity against FOL-30 by using the dual culture assay of Müller *et al.* (2018), with some modifications. Briefly, two straight lines, 5 cm long and 2 cm from the edge of a PDA plate, were streaked using a loop full of cells from a freshly growing bacterial culture (24 h old). The plates were incubated at 28°C for 48 h before fungal inoculation. A 4 mm diameter mycelial disc from a 7-day-old fungal culture was placed in the central position between the two lines, 1.5 cm from the streaks. Plates without bacterial antagonists served as controls. Five replicates were prepared for each treatment. Plates were incubated at 28°C and observed daily for 7 days. The percentage of fungal growth inhibition was determined by $[(R_1 - R_2)/R_1] \times 100$, where R_1 and R_2 are the radii of the pathogen colonies in the control and dual-culture plates, respectively. Signs of stress in pathogen hyphae because of the antagonistic effects of rhizobacterial strains were evaluated microscopically.

Bacterial culture filtrate assay: Rhizobacterial strains were grown in NA at 28°C for 48 h. A single colony of bacterial cultures was incubated in 100 mL of nutrient broth with continuous shaking for 72 h at 28°C. The cultures were centrifuged at 5000 × *g* for 10 min at 4°C and the supernatant was vacuum-filtered through a 0.22 μm sterile membrane (Millipore, Bedford, MA; Li *et al*. 2008). The resulting culture filtrate was added at the concentration of 15% (v/v) to molten PDA media containing the appropriate amount of agar to ensure the plates gelled properly. Plates containing the medium mixed with sterile water only were used as controls. The plates were inoculated in the center with a 0.5 cm plug from the leading edge of a 5-day-old PDA culture of FOL-30. Five replicates were prepared for each treatment, and the plates were incubated at 28°C and observed daily for 7 days. Mycelial growth was measured, and the percentage of growth inhibition was calculated as described above (Jangir *et al*. 2018).

Greenhouse experiments

The results of antagonistic experiments suggested that *Bacillus subtilis* KSU-110 was the most promising strain and it was selected for further testing of its biocontrol potential, both individually and/or in combination with organic compost, against Fusarium wilt disease under greenhouse conditions.

Plant growth conditions

Seeds of Farah tomatoes, a common FOL-susceptible greenhouse cultivar in Saudi Arabia, were surface-sterilized by immersion in 1% sodium hypochlorite for 30 s and then washed thrice with sterile distilled water. The seeds were pre-germinated for 3 days in Petri dishes containing sterile distilled water at 28°C. Germinated seeds were then sown in 15 cm pots containing an autoclaved mixture of potting soil, peat moss, and perlite (2:1:1, v/v/v). Plants were grown in a growth chamber with 70% relative humidity and a photoperiod of 12 h light at 26°C and 12 h dark at 20°C. Plants were fertilized weekly with a 20-20-20 (N-P-K) soluble fertilizer (1 g/L) and the seedlings were irrigated with tap water as needed. Subsequent experiments were performed when four leaves had completely expanded (6 weeks old).

Microbial inoculum preparation

Fungal inocula were prepared by culturing FOL-30 on PDA plates for 2 weeks at 28°C in the dark. Fungal colonies were subsequently scraped from PDA plates using a sterile glass rod to dislodge spores into sterile distilled water. Fungal hyphae and residue were removed by filtering the suspension through four layers of gauze. Spores were counted using a hemocytometer, and the conidial suspension was adjusted to 1×10^7 conidia per milliliter. For antagonistic bacterial inocula, a suspension of the KSU-110 strain was obtained from overnight cultures incubated on NA at 28°C. Bacterial cells were scraped off the agar plate into 10 m*M* magnesium sulfate buffer, centrifuged at 3000 × *g* for 10 min, and resuspended in sterile distilled water. The bacterial cell concentration was adjusted to $1 \times l0^7$ colony forming units (cfus) per milliliter by measuring the OD_{660} (optical density at 660 nm) spectrophotometrically (Youssef *et al*. 2016).

Compost material

The sterilized organic compost (Al-Reef Organic Fertilizers Factory, Co., Kingdom of Saudi Arabia, Riyadh) used in this study consisted of cow manure and vegetable material, with the former being the major component and the latter representing only 20% of the amendment. The compost had the following major physicochemical characteristics: pH 6.3, 1% nitrogen, 91.8% organic matter, 78.3% carbon and a 37.3 C: N ratio. It is commercially available in Saudi Arabia.

Control of fusarium wilt in tomatoes using the combination of the KSU-110 strain and compost

A pot experiment was conducted in the greenhouse of the Plant Protection Department, College of Food and Agricultural Sciences, King Saud University. Pots were arranged in a completely randomized design with eight treatments: (T1) non-infested soil (healthy control), (T2) non-infested soil amended with KSU-110, (T3) non-infested soil with compost, (T4) non-infested soil with KSU-110 and compost, (T5) soil infested with FOL-30, (T6) soil infested with FOL-30 amended with compost, (T7) soil infested with FOL-30 and amended with KSU-110, and (T8) soil infested with FOL-30 and amended with KSU-110 and compost. Each treatment consisted of 30 replicates, with one plant per replicate (pot).

Plastic pots (15 cm in diameter) were filled with a sterilized mixture of sand, clay and soil (1:1:1, v/v). The compost treatments were incorporated into the potting mixture at a rate of 25% (v/v). The conidial suspensions of the wilt pathogen were incorporated into each pot to ensure a final concentration of 1×10^3 conidia per gram of soil to promote disease development. Pots inoculated with an equal volume of distilled water served as controls. One week after soil infestation with FOL-30 inocula, tomato seedling roots were dipped into a bacterial suspension (1×10^7 cfu mL^{-1}) for 30 min and then transferred to pots, with one tomato seedling per pot. The seedlings were placed in a greenhouse maintained at 28°C with 50–70% relative humidity and a 12:12 h light-dark photoperiod. At 45 days after transplanting (DAT), 15 plants from each treatment were arbitrarily selected for biomass analysis and disease scoring. The experiment was repeated twice.

Incidence and severity of fusarium wilt disease

Plants were examined for the incidence and severity of Fusarium wilt after 45 DAT. Disease incidence (DI) was calculated as DI = (number of diseased plants/total number of plants) × 100. Disease severity (DS) was evaluated using two different disease rating scales (1–5); one was based on foliar symptoms as proposed by (Hibar *et al.* 2006) and the other was based on vascular browning (Horinouchi *et al.* 2008). Disease scores were converted to DS using the following formula: DS = [(*A* × 1) + (*B* × 2) + (*C* × 3) + (*D* × 4) + (*E* × 5)]/(total number of plants) × 100, where, *A*, *B*, *C*, *D*, and *E* are the number of plants corresponding to 1, 2, 3, 4, and 5 disease rating scores, respectively. Control efficiency (%CE) for each treatment was calculated using the estimates of DI and DS in control and treated plants (D_1 and D_2, respectively) using the following formula: CE = $[(D_1 - D_2)/D_1] \times 100$.

The synergistic interaction of KSU-110 + compost in controlling wilt disease was estimated according to Abbott's formulae (Levy *et al*. 1986). The expected control efficiency (P_{exp12}) for the combined application was calculated using the formula: $P_{exp12} = (P_1 + P_2) - (P_1 \times P_2)/100$, where P_1 and P_2 are the P data observed for the single application of KSU-110 and compost, respectively. The observed protection efficiency (P_{obs}) was estimated from the P data for the combined application. The synergism factor (SF) was estimated using the formula: SF = (P_{obs}/P_{exp12}). As a decision rule, SF > 1 indicated the interaction was synergistic, SF = 1 indicated the interaction was additive, and SF < 1 indicated the interaction was antagonistic (Levy *et al*. 1986).

Plant growth measurements

At the end of the experiment, length (cm) and dry weight (g; oven dried at 80°C for 72 h) of the root and shoot systems of each plant were measured separately. The improvement efficiency (%IM) was calculated using the following formula: [(*C* - *T*)/*C*] × 100; where, *C* and *T* are the growth parameters of control and treated plants, respectively. The synergistic interaction of KSU-110 + compost in promoting plant growth was estimated, as described above.

Assay of defense enzymes

Root samples of three plants from each treatment were collected at 15, 30, and 45 DAT (three replicates per time interval for each treatment). The roots were washed, briefly dried, snap frozen in liquid nitrogen, stored at −80°C, and maintained separately for biochemical analysis representing three biological replications. Plant tissues were ground into a fine powder under liquid nitrogen using a mortar and pestle. The fine powder was suspended in 100 m*M* sodium phosphate buffer (pH 7.0) at 4°C (1 mL g^{-1} leaf tissue). The solution was centrifuged at 10,000 × *g* for 20 min. The supernatant was collected and used as a crude enzyme extract to assay peroxidase (POD) spectrophotometrically by measuring the oxidation of pyrogallol in the presence of H_2O_2 as OD_{425} and polyphenol oxidase (PPO) as OD_{575} (Tuzun *et al*. 1989).

Monitoring FOL-30 and KSU-110 in the tomato rhizosphere

KSU-110 and FOL-30 populations in the soil were monitored by collecting rhizosphere samples at 15, 30, and 45 DAT. A 1 g rhizosphere soil sample (four replicates per time interval for each treatment) was suspended in 9 mL of sterilized/ distilled water and vortexed at the maximum speed for 5 min. Next, a 1:200 soil dilution was spread onto plates containing Komada's selective medium for FOL-30 (Komada, 1975) and NA medium for KSU-110. After incubation at 28°C for 48 h, the number of cfus of FOL-30 and KSU-110 per gram of rhizosphere soil was recorded. Re-isolated bacteria showing morphological similarities to KSU-110 were identified using the Biolog system to confirm the association of the applied strain with the rhizosphere samples. The pathogen reduction efficiency (%RE) was calculated using the following formula: [(*C* - *T*)/*C*] × 100; where, *C* and *T* are the FOL populations in infected control and treated rhizosphere soils, respectively. The synergistic interaction of KSU-110 + compost in the reduction of the FOL population was estimated at intervals, as described above.

Field experiments

Experiments were conducted in a field naturally infested with FOL at the Experimental Farm of the College of Food and Agriculture Sciences, King Saud University. This field was naturally highly infested with the wilt pathogen during the previous season. The experiment was arranged in a completely randomized block design with four treatments (T5, T6, T7 and T8) and was replicated six times, with 15 plants per replicate. For the experimental treatments, tomato plants were treated with KSU-110 and compost as described above. At the end of the experiment (45 DAT), ten plants were arbitrarily selected from each replicate and used to evaluate the % DI and % DS. Furthermore, the dry weight and length of the root and shoot systems for each plant were recorded at 45 DAT as described above. The synergistic interactions of KSU-110 + compost in promoting plant growth and controlling wilt disease were estimated, as described above.

Statistical analysis

All experiments were repeated twice. The analyses did not indicate any significant differences between the two repeats of the treatments; hence, the results from the duplicate tests were combined for the final analysis. All the data are presented as mean values (average of two experiments). All analysis of variance tests were conducted using SAS

Version 9.1 software (SAS Institute Inc 2003). The data for disease measurements were analyzed following an arcsine transformation. The population data were transformed with a square-root [sqrt (x + 0.5)] transformation before analysis to obtain homogeneity of variances (Gomez and Gomez 1984). The least significant difference at $P < 0.05$ was applied to detect differences between treatments (Gomez and Gomez 1984).

Results

Isolation and identification of fungal pathogens and bacterial antagonists

The results of culture morphology and microscopic examination revealed that the FOL-30 strain was *F. oxysporum*. In addition, the *tef1α* and rDNA-ITS sequences confirmed the identity of the FOL-30 strain. The *tef1α* and rDNA-ITS sequences of FOL-30 were deposited in the GenBank database under the accession numbers MN514860 and MN508482, respectively.

Twenty rhizobacterial strains were isolated from the rhizosphere of commercial tomato fields. The 16S rRNA gene-based analysis showed that 13 rhizobacterial strains belonged to the genus *Bacillus* (Table 1). Eleven were assigned to a species, whereas two were identified only to the genus level. The remaining strains belonged to the genera *Pseudomonas*, *Stenotrophomonas*, *Enterobacter*, and *Achromobacter* (Table 1).

In vitro antagonistic activity

All the strains were significantly antagonistic to FOL-30 ($P < 0.05$) and inhibited its growth (Fig. 1). However, these antagonistic responses varied by strain (Fig. 1). In the dual culture plate assay, the highest level of antagonistic activity against FOL-30 was observed for *B. subtilis* strain KSU-110 (68%; Fig. 1–2). This strain induced distortions and deformations in FOL-30 mycelia, including increased branching, hyphal swelling, and cytoplasm collapse. Moreover, the KSU-110 culture filtrate had the greatest inhibitory activity toward pathogen growth (46%) among all the rhizobacterial culture filtrates (Fig. 1).

Incidence and severity of fusarium wilt disease

The highest mean DI and DS values ($P < 0.05$) were detected in tomato plants grown in the presence of FOL alone (Table 2). Application of KSU-110 and compost, either separately or in combination, significantly ($P < 0.05$) reduced disease development compared to that of infected control plants (Table 2). Indeed, compared to the infected control, the combined rhizobacteria + organic compost treatment resulted in the largest reduction in DI (71%), DS based on foliar symptoms (63%), and stem discoloration (69%; Table 2). However, the control efficiency for the tomato plants treated with KSU-110 was higher than that of plants treated with organic compost (Table 2). According to Abbott's formulae, the interaction of KSU-110 with compost was synergistic (SF > 1) in the reduction of tomato wilt disease (Table 2).

Table 1: List of rhizobacterial strains identified on a molecular basis and their GenBank accession numbers

Strain code	Strain identified	GenBank Accession No.
KSU-18	*Pseudomonas* spp.	MN208459
KSU-67	*Pseudomonas* spp.	MN208458
KSU-25	*Pseudomonas aeruginosa*	MN208460
KSU-87	*Stenotrophomonas* spp.	MN208462
KSU-E3	*Stenotrophomonas* spp.	MN208463
KSU-B3	*Enterobacter* spp.	MN208461
KSU-191	*Achromobacter spanius*	MN208464
KSU-B17	*Bacillus* spp.	MN208475
KSU-XR	*Bacillus* spp.	KY123A56
KSU-06	*B. cereus*	MN208465
KSU-103	*B. megaterium*	MN208466
KSU-A7	*B. pumilus*	MN208469
KSU-12	*B. safensis*	MN208467
KSU-31	*B. amyloliquefaciens*	MN208468
KSU-2.2	*B. pumilus*	MN208474
KSU-Y1	*B. subtilis*	MN208472
KSU-50	*B. subtilis*	MN208470
KSU-110	*B. subtilis*	MN208473
KSU-B2	*B. subtilis*	MN208476
KSU-43	*B. subtilis*	MN208471

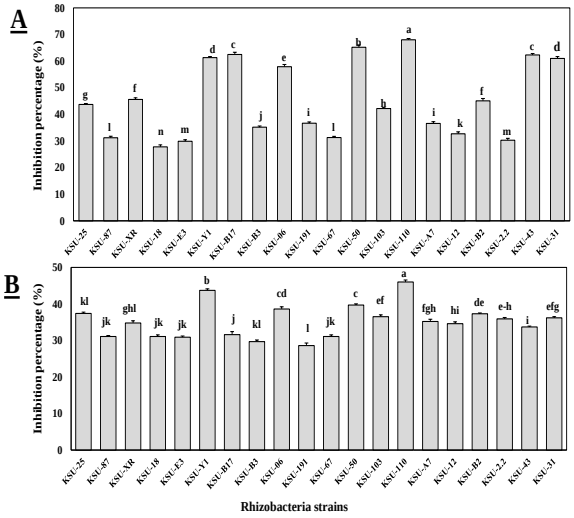

Fig. 1: Percentage mean inhibition of FOL-30 growth by the rhizobacterial strains obtained using a dual culture plate assay (**A**) and bacterial culture filtrate (**B**) after six days of inoculation at 28 ± 1°C. Each bar represents the average of two experiments with five replicates per treatment per experiment. Percentage inhibition data were analyzed after arcsine transformation. Bars with the same letter are not significantly different at $P < 0.05$ according to the LSD test

Plant growth measurements

Plant growth parameters were significantly ($P < 0.05$)

Table 2: The effects of *Bacillus subtilis* strain KSU-110 and organic compost applied alone or in combination on tomato wilt incidence, foliar symptoms, and discoloration severity caused by the Fusarium wilt pathogen under greenhouse conditions

Treatments	Wilt incidence (%)[a]	(%CE)[b]	Foliar symptoms (%)[a]	(%CE)[b]	Discoloration (%)[a]	(%CE)[b]
FOL (infected control)	92.4 a	-	50.0 a	-	48.2 a	-
FOL + compost	75.8 b	17.9	38.0 b	24.0	29.5 b	38.8
FOL+ KSU-110	56.1 c	39.3	24.8 c	50.4	26.7 b	44.6
FOL+ compost + KSU-110	26.4 d	71.4	18.3 c	63.4	15.2 c	68.5
Synergism factor [c]	1.43		1.02		1.05	

[a] Each value represents the average of two experiments with 15 replicates for each treatment per experiment

[b] The control efficiency (%CE) was calculated according to the following formula: $[(D_1 - D_2)/D_1] \times 100$; where, D_1 and D_2 are the disease measurements of the control and treated plants, respectively

[c] The synergism factor (SF) was estimated using the following formula: $SF = (P_{obs}/P_{exp})$; where, P_{obs} and P_{exp} are the observed and expected protection efficiency achieved by the combined application, respectively

- Values in each column followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test

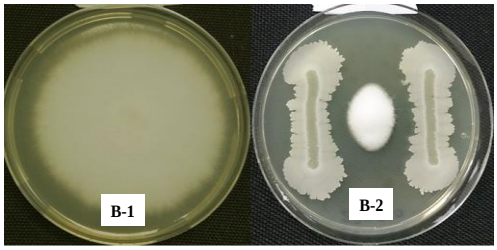

Fig. 2: Dual culture assay of *B. subtilis* strain KSU-110 and FOL-30 on PDA after 6 days of incubation at 28 ± 1°C (B). The control B-1 plate was inoculated only with FOL-30. Plate B-2 shows the antagonistic action of KSU-110 against FOL-30

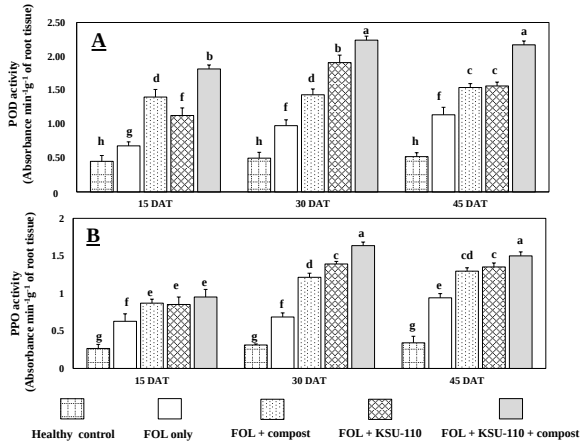

Fig. 3: Changes in peroxidase (POD) (**A**) and polyphenol oxidase (PPO) (**B**) activities in root tissues of FOL-infected tomato plants treated with *Bacillus subtilis* strain KSU-110 and organic compost, applied either singly or in combination at 15, 30, and 45 days after transplanting. Each bar represents the average of two experiments with three replicates per treatment per experiment. Bars with the same letter are not significantly different at $P < 0.05$ according to the LSD test. Error bars represent the standard deviations of the mean

reduced in tomato plants infected with FOL-30 compared with those of non-infected controls (Table 3). In FOL-30-infested soil, tomato plants treated with the antagonistic bacteria along with organic compost showed the greatest increase in root and shoot length (68 and 58%, respectively), as well as root and shoot dry weight (50 and 66%, respectively) compared to those of the infected control plants (Table 3). However, treatment with KSU-110 or compost alone also significantly ($P < 0.05$) improved the growth of pathogen-infected plants (Table 3). Regardless of the presence or absence of FOL-30, the combined effect of KSU-110 + organic was synergistic (SF > 1) in most plant growth measurements (Table 3).

Activity of defense enzymes

Infection of tomato plants with the wilt pathogen resulted in significant increases in POD and PPO enzyme activities (Fig. 3). The activities of POD (1.5- to 2.2-fold increases in absorbance per minute per gram of root tissue) and PPO (2.4- to 2.8-fold increases in absorbance per min per gram of root tissue) significantly ($P < 0.05$) increased in infected plants (Fig. 3). The activities of both enzymes were greater in KSU-110- and/or compost-treated tomato plants than in the non-treated, infected plants (Fig. 3). Treating tomato plants with KSU-110 + compost resulted in significantly ($P < 0.05$) higher POD (1.3- to 1.45-fold increases in absorbance) and PPO (1.1- to 1.26-fold increases in absorbance) activities than those in plants treated with FOL-30 only (Fig. 3).

Monitoring of FOL-30 and KSU-110 in the tomato rhizosphere

KSU-110 efficiently colonized the tomato rhizosphere and persisted at high levels for up to 45 DAT (range from 9.7×10^4 to 12.3×10^4 cfu g^{-1} of rhizosphere soil at 45 DAT; Table 4). However, the highest level of KSU-110 colonization occurred in the composted soil (Table 4). The pathogen population increased over time in the FOL-30-only infested (control) soil (from 2.2×10^5 to 5.0×10^5 cfu g^{-1} of rhizosphere soil; Table 4). At 45 DAT, the FOL-30 populations were significantly reduced ($P < 0.05$) by 94–99.6% in the tomato rhizosphere treated with KSU-110 and/or organic compost compared to that in soil treated only with FOL-30 (Table 4). The FOL-30 population decreased in the soil of plants treated with KSU-110, the population of which increased markedly over time (Table 4). In terms of FOL reduction efficiency, the mean synergy factor calculated over study intervals was 0.98 (Table 4). This

Table 5: The effects of *Bacillus subtilis* strain KSU-110 and organic compost applied alone or in combination on tomato wilt incidence, foliar symptoms, and discoloration severity caused by the Fusarium wilt pathogen under field conditions

Treatments	Wilt incidence (%)[a]	(%CE)[b]	Foliar symptoms (%)[a]	(%CE)[b]	Discoloration (%)[a]	(%CE)[b]
FOL (infected control)	66.0 a	-	32.6 a	-	25.5 a	-
FOL + compost	50.5 b	23.4	20.0 b	38.7	19.3 b	24.3
FOL+ KSU-110	39.6 c	40.0	19.0 c	42.0	16.4 c	36.0
FOL+ compost + KSU-110	26.7 c	59.5	10.7 d	67.2	11.3 d	55.7
Synergism factor[c]	1.1		1.05		1.1	

[a] Each value represents the average of two experiments with 15 replicates for each treatment per experiment
[b] The control efficiency (%CE) was calculated according to the following formula: $[(D_1 – D_2)/D_1] \times 100$; where, D_1 and D_2 are the disease measurements of the control and treated plants, respectively
[c] The synergism factor (SF) was estimated using the following formula: $SF = (P_{obs} / P_{exp})$; where, P_{obs} and P_{exp} are the observed and expected protection efficiency achieved by the combined application, respectively
- Values in each column followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test

Table 6: The effects of *Bacillus subtilis* strain KSU-110 and organic compost applied alone or in combination on the length and dry weights of both root and shoot systems of tomato plants infected with the Fusarium wilt pathogen under field conditions

Treatments	Length (cm)[a]				Dry weight (g)[a]			
	Root	(%IM)[b]	Shoot	(%IM)[b]	Root	(%IM)[b]	Shoot	(%IM)[b]
FOL (infected control)	10.7 c	-	37.8 c	-	3.7 b	-	12.5 d	-
FOL + compost	14.5 ab	35.4	50.8 b	34.4	5.9 b	60.5	19.9 b	37.3
FOL+ KSU-110	13.4 b	24.9	48.5 b	28.2	4.8ab	29.7	16.2 c	29.6
FOL+ compost + KSU-110	15.5 a	44.2	58.5 a	54.7	6.1 a	65.7	23.0 a	83.6
Synergism factor[c]	0.86		1.04		0.92		1.49	

[a] Each value represents the average of two experiments with 15 replicates for each treatment per experiment
[b] The calculation of improvement efficiency (%IM) was according to the following formula: $[(C - T)/C] \times 100$, where C and T are the growth parameters of control and treated plants, respectively
[c] The synergism factor (SF) was estimated using the following formula: $SF = (IM_{obs} / IM_{exp})$; where, IM_{obs} and IM_{exp} are the observed and expected improvement efficiency achieved by the combined application, respectively
- Values in each column followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test

value did not significantly differ according to the one-tailed t test ($P > 0.05$).

Field experiments

The greatest protection against wilt disease was noted in tomato plants treated with a combination of KSU-110 and organic compost, for which the disease incidence, foliar symptom severity, and internal stem discoloration were reduced by 60, 67 and 56%, respectively (Table 5). The same application led to significant increases ($P < 0.05$) in root and shoot length (44 and 55%, respectively), as well as root and shoot dry weight (66 and 84%, respectively), compared to those of the non-treated control (Table 6). Application of either KSU-110 or compost alone was next in degree of effectiveness, significantly suppressing wilt disease and improving plant growth ($P < 0.05$; Tables 5 and 6). In general, the combined KSU-110 + organic compost treatment resulted in synergistic effects (SF > 1) in the control of wilt disease and promotion of tomato growth (Table 5 and 6).

Discussion

Selecting native PGPR strains that are well adapted to Saudi ecosystems is a requisite step towards improving the efficacy of managing tomato wilt disease under organic farming conditions. In the present study, we identified 20 rhizobacterial strains from the tomato rhizosphere and screened them for their antagonistic potential against FOL-30 *in vitro*. All rhizobacterial strains had significant antifungal activity against the wilt pathogen and inhibited its growth. *B. subtilis* strain KSU-110 had the highest antagonistic activity against FOL-30. This strain also induced distortions and deformations in the mycelia of the pathogen, including increased hyphal branching, swelling, and cytoplasm collapse. The antagonistic responses observed in the *in vitro* tests suggested that the selected KSU-110 strain could release antifungal substances that restricted pathogen growth. Direct antagonism of pathogenic fungi because of antibiosis (*e.g*., antibiotics, lytic enzymes, and volatile organic compounds) is one of the biocontrol mechanisms used by the soil *Bacillus* strains (Ahemad and Kibret 2014; Grobelak *et al*. 2015). This could provide a potential basis for selecting antagonistic strains for biological control under field conditions (Bubici *et al*. 2019).

The addition of organic compost to the soil provided an environment-friendly method of managing soil-borne diseases, including FOL (Dukare *et al*. 2011; Hadar and Papadopoulou 2012; Bahramisharif *et al*. 2013; Gava and Pinto 2016). In the present study, we evaluated the hypothesis that the integrated combination of KSU-110 with compost could enhance the biological control efficacy against FOL because of additive or synergistic interactions. Our results showed that the application of KSU-110 and organic compost applied alone or in combination, significantly ($P < 0.05$) reduced disease development under greenhouse and field conditions. This suggested that the suppressive effects of KSU-110 detected in the *in vitro* antagonistic assays were linked to the management of

Table 3: The effects of *Bacillus subtilis* strain KSU-110 and organic compost applied alone or in combination on the length and dry weights of both root and shoot systems in tomato plants regardless of the presence or absence of the Fusarium wilt pathogen under greenhouse conditions

Treatments	Length (cm)[a]				Dry weight (g)[a]			
	Root	(%IM)[b]	Shoot	(%IM)[b]	Root	(%IM)[b]	Shoot	(%IM)[b]
Healthy control	7.1 bc	-	22.6 c	-	1.30 bc	-	3.90 cd	-
Compost	8.7 a	22.5	28.2 b	24.8	1.60 a	23.0	5.10 b	30.8
KSU-110	8.0 a	12.7	26.4 b	16.8	1.40 ab	07.7	4.70 bc	20.5
KSU-110 + compost	9.1 a	28.2	31.4 a	38.9	1.70 a	30.8	6.11 a	56.7
Synergism factor[c]	0.89		1.05		1.1		1.26	
FOL (infected control)	3.7 e	-	10.3 f	-	0.68 e	-	1.93 g	-
FOL + compost	5.0 d	**35.1**	13.6 e	**32.0**	0.97 d	**42.6**	2.78 ef	**44.0**
FOL+ KSU-110	4.9 d	**32.4**	13.0 e	**26.2**	0.88 de	**29.4**	2.20 fg	**14.0**
FOL+ compost + KSU-110	6.2 c	**67.6**	16.3 d	**58.3**	1.02 cd	**50.0**	3.20 cd	**65.8**
Synergism factor[c]	**1.21**		**1.17**		**0.85**		**1.27**	

[a] Each value represents the average of two experiments with 15 replicates for each treatment per experiment

[b] The improvement efficiency (%IM) was calculated using the following formula: $[(C - T)/C] \times 100$; where, *C* and *T* are the growth parameters of control and treated plants, respectively. Values in normal font are the effects of different bio-organic treatments on plant growth parameters compared with those in the healthy control (no FOL stress). **Values in bold font** are the effects of different bio-organic treatments on plant growth parameters compared with those in the infected control (under FOL stress)

[c] The synergism factor (SF) was estimated using the following formula: $SF = (IM_{obs} / IM_{exp})$; where, IM_{obs} and IM_{exp} are the observed and expected improvement efficiency achieved by the combined application, respectively

- Values in each column followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test

Table 4: Rhizosphere soil populations of *Bacillus subtilis* strain KSU-110 and Fusarium wilt pathogen strain FOL-30 (cfu g^{-1} of rhizosphere soil) sampled in the vicinity of tomato plants at 15, 30, 45 days after transplantation (DAT) under greenhouse conditions

Treatments	Population of KSU-110[a]			Population of FOL-30[a]					
	15 DAT	30 DAT	45 DAT	15 DAT	(%RE)[b]	30 DAT	(%RE)[b]	45 DAT	(%RE)[b]
KSU-110	6.0×10^4 e	7.7×10^4 d	9.8×10^4 c	-		-		-	
KSU-110 + compost	7.7×10^4 d	10.0×10^4 c	12.3×10^4 a	-		-		-	
FOL	-	-	-	2.2×10^5 C	-	3.3×10^5 B	-	5.1×10^5 A	-
FOL + compost	-	-	-	3.1×10^4 D	85.9	3.5×10^4 D	89.4	3.2×10^4 D	93.7
FOL + KSU-110	4.5×10^4 f	6.5×10^4 e	9.7×10^4 c	3.0×10^4 D	86.4	3.2×10^4 D	90.3	3.4×10^4 D	93.3
FOL + compost + KSU-110	6.2×10^4 e	9.6×10^4 c	11.4×10^4 b	1.8×10^4 E	91.8	2.3×10^3 F	99.3	2.0×10^3 F	99.6
Synergism factor[c]				0.98					

[a] Each value represents the average of two experiments with four replicates for each time point per experiment

[b] The pathogen reduction efficiency (%RE) was calculated using the following formula: $[(C - T)/C] \times 100$; where, *C* and *T* are the FOL populations in infected control and treated rhizosphere soils, respectively

[c] The mean synergism factor (SF) was estimated using the following formula: $SF = (R_{obs} / R_{exp})$; where, R_{obs} and R_{exp} are the observed and expected FOL reduction efficiency achieved by the combined application at 15, 30, and 45 DAT

- Values followed by the same lowercase or uppercase letters are not significantly different at $P < 0.05$ according to the LSD test

Fusarium wilt disease in tomatoes *in vivo*. Interestingly, mixing KSU-110 with organic compost caused the highest reduction in DI and DS compared to that of the individual applications even under field conditions. Moreover, the observed control efficiency in tomato plants treated with KSU-100 + compost was higher than that expected (SF > 1), indicating a synergistic effect. This suggests that the application of KSU-110 + compost represents a promising option for organic growers of tomatoes. The synergistic disease suppression elicited by this combination could result from nutrients in the compost that enhance rhizobacterial competitive ability, or from the presence of specific agents that evoke an antibiosis effect or induce resistance against pathogen infection (Abbasi *et al*. 2002; Huang *et al*. 2011). Moreover, volatiles released during manure decomposition, such as sulfur-containing compounds, organic acids, and ammonia, may increase disease suppression (Coventry *et al*. 2006).

In addition to suppressing wilt disease, the application of KSU-110 and compost, either separately or in combination, had a positive growth-promoting effect on tomato seedlings under both greenhouse and field conditions. Application of KSU-110 and organic compost led to the highest ($P < 0.05$) increase in tomato growth parameters, irrespective of the presence or absence of the wilt pathogen. These effects may be attributed to the ability of *Bacillus* strains to promote plant growth and health by enhancing nutrient uptake from the soil by plant roots, as well as the synthesis of plant hormones (Grobelak *et al*. 2015). Moreover, the addition of organic compost to the soil probably improves the nutritional status of the plants and contributes to soil health through increased soil microbial activity (Abbasi *et al*. 2002). Together, these factors could explain the remarkably synergetic effects of the combined treatment on the growth parameters of tomato plants. In fact, improved growth enhances the resistance of plants to the detrimental effects of infection-related stress while promoting plant health and decreasing harvest losses (Ahemad and Kibret 2014; Grobelak *et al*. 2015).

Our results showed that POD and PPO activities were higher in tomato plants treated with different bioorganic compounds than that in the non-treated infected plants. Furthermore, their activities were remarkably higher in plants treated with KSU-110 + organic compost than in

those receiving either treatment alone. In fact, POD is a key enzyme that participates in lignin biosynthesis. It also catalyzes reactive oxygen species generated in plant tissues caused by pathogen attack (Caverzan *et al*. 2012). Furthermore, PPO is another plant defense enzyme responsible for the oxidation of phenolic compounds into anti-microbial quinones in plant tissues attacked by plant pathogens, thereby inducing disease resistance (Arora and Bajaj 1985). Therefore, integrated application of KSU-110 + compost had likely induced defense responses of POD and PPO that might have increased tomato plant tolerance against FOL stress. These results are consistent with those of Krause *et al*. (2003) and Kloepper *et al*. (2004) who revealed that the application of *Bacillus* strains and compost amendments induced systemic disease resistance in affected plants.

Determining the population density of both biocontrol agents and pathogens in the plant rhizosphere is important in predicting the success of biological control (Leandro *et al*. 2007). Notably, KSU-110 could efficiently colonize the tomato rhizosphere and persist at a high level in the treated soils. In fact, extensive colonization of the plant rhizosphere by inoculant rhizobacteria was essential for its biocontrol and growth-promoting activities (Bouizgarne 2013; Abdallah *et al*. 2018). We showed that plants with reduced wilt disease incidence had an increased KSU-110 population and decreased FOL-30 population. Importantly, mixing KSU-110 + organic compost resulted in additive activity in the reduction of the FOL-30 population compared with that of their separate application. Moreover, the KSU-110 could be was attributed to the additional nutrients supplied by the amended compost. This suggests that competition for nutrients and space may be the key mechanism for biocontrol of the pathogen by KSU-110. The additional organic substrates enhanced the competitive action of KSU-110 and its survival in the tomato rhizosphere. Taken together, these findings may explain why the combined treatment was more effective in decreasing wilt disease than the application of KSU-110 alone. The results are consistent with those of previous studies showing that compost amendments positively enhanced microbial biomass and activity in the plant rhizosphere and resulted in a deleterious competitive environment for pathogens (Hadar and Papadopoulou 2012; Bahramisharif *et al*. 2013).

Conclusion

Our findings highlight the advantages of deploying native antagonistic bacteria for crop health and management in arid regions. The application of KSU-110 decreased the FOL-30 population in the tomato rhizosphere and hence could be a potential biological control agent against FOL. Furthermore, the combination of KSU-110 and compost greatly enhanced disease suppression and enhanced tomato growth under both greenhouse and field conditions. Further evaluations are needed to verify the efficacy of combining KSU-110 with compost against diverse soil-borne pathogens under a wider range of field conditions, before commencing any large-scale application.

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