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Isolation of Endophytic Bacteria from *Withania somnifera* and Assessment of their Ability to Suppress Fusarium Wilt Disease in Tomato and to Promote Plant Growth

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Abstract

Four nonpathogenic and putative endophytic bacterial isolates, recovered from *Withania somnifera* fruits (S7, S8 and S9) and stems (S15), were evaluated for their *in vivo* and *in vitro* antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (FOL), and their plant-growth promoting ability. Tomato plants challenged and/or not with FOL and treated using these bacterial isolates exhibited a significant increment in their growth parameters (plant height, aerial part fresh weight, maximum root length, and root fresh weight). The strong suppressive effect against Fusarium wilt was achieved using two isolates (namely S15 and S8) leading to 92-96% lower disease severity compared to pathogen-inoculated and untreated control. Both isolates were characterized and only the isolate S8 was identified as *Alcaligenes faecalis* subsp. *faecalis* str. S8 (KR818077) using 16S rDNA gene sequencing. The unidentified bacterial isolate S15 had improved germination of bacterized tomato seeds relative to the untreated ones. Tested using streak and sealed plates methods, diffusible and volatile compounds from S15 and S8 isolates inhibited FOL by 10.7-16.8% and 53.8-20.7%, respectively. Moreover, an inhibition zone (8.5-8.25 mm) was formed around FOL colonies using the disc diffusion method. *Alcaligenes faecalis* subsp. *faecalis* str. S8 and the unidentified bacterium str. S15 were shown able to produce chitinolytic, proteolytic and pectinolytic enzymes and hydrogen cyanide. Production of indole-3-acetic acid and phosphate solubilizing ability were also investigated for elucidation of their plant growth-promoting traits.

Keywords: Antifungal activity; Endophytic bacteria; *Fusarium oxysporum* f. sp. *lycopersici*; Tomato growth; *Withania somnifera*; Wilt severity

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops worldwide. However, this culture is very susceptible to many diseases caused by fungi, bacteria and viruses. Among soilborne diseases, Fusarium wilt incited by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hans (FOL) led to important crop losses in open field and under greenhouse crops [1]. The main disease symptoms are half-leaf yellowing, browning of vascular tissues, plant wilting, stunting and ultimately death [2].

The long survival of pathogen resting structures (chlamydospores) in the soil, even in absence of host plants, limits the suppressive effect of crop rotation. Furthermore, due to the endophytic pathogen progress within vascular tissues, chemical control fails to successfully control disease and risks of development of fungicide resistance are frequent. Moreover, genetic control of tomato Fusarium wilt failed to successfully suppress disease due to the emergence of new physiological races of FOL [3].

Biocontrol using endophytic microorganisms (bacteria, actinomycetes or fungi) is a potentially attractive and eco-friendly alternative since endophytes could better limit disease incidence and severity through inhibition of fungus vascular progress [4-6]. These endophytes are known by their beneficial effects on plant growth promotion [7]. Several mechanisms are displayed by these bacteria towards target pathogens including antibiosis, competition for nutrients and minerals and/or inducing systemic resistance [8]. Furthermore, the beneficial effects of endophytic bacteria on plant growth are mainly attributed to their capacity to produce indole-3-acetic acid (IAA) and siderophores, to solubilize phosphates and to fix nitrogen [7].

Almost all plants harbor various endophytic bacteria within their intercellular spaces and xylem vessels and some of them are also to colonize the reproductive organs of plants i.e. flowers, fruits and seeds [9].

Various wild Solanaceae plants have been exploited as potential sources of antimicrobial metabolites i.e. *Datura stramonium* [10], *D. metel* [11], *W. somnifera* [12] and as biocontrol agents, especially endophytic bacteria, i.e. *Nicotiana attenuata* [13], *Solanum trilobatum* [14], *S. melongena,* and *S. torvum* [15].

W. somnifera is a wild solanaceous species known by its pharmaceutical and medicinal features [16]. Several previous studies had valorized this plant as natural source of bioactives metabolites with antibacterial and antifungal activities [17,18]. However, this wild species was not widely reported and exploited as natural source of isolation of endophytic microorganisms such as fungi [19] and/or bacteria [20]. Moreover, the isolated agents were not assessed for their antimicrobial activity.

In this study, four putative endophytic bacterial isolates, recovered

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from surface-sterilized stem and fruit tissues of native *W. somnifera* plants were assessed for their antifungal potential toward FOL and for tomato growth-promoting features.

Materials and Methods

Preparation of tomato plants

Seedlings of tomato cv. Rio Grande, known to be susceptible to FOL races 2 and 3 [21] were grown under greenhouse with at 16 h photoperiod, 60-70% relative humidity and air temperatures ranging between 20 and 30°C. A sterilized peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg) was used as a substrate. Tomato seedlings at the two-true-leaf growth stage were used in all bioassays.

Fungal culture

F. oxysporum f. sp. *lycopersici*, previously isolated from tomato stems showing vascular discoloration [22], was used in this study. The pathogen was re-isolated from artificially inoculated plants, fulfilling Koch's postulates, and identified based on wilting pattern and pathogen morphological and cultural traits on Potato Dextrose Agar (PDA) medium [23]. FOL cultures previously grown for 7 days on PDA and incubated at 25°C were used for *in vitro* and *in vivo* bioassays.

Withania somnifera sampling and isolation of endophytic bacteria

Endophytic bacteria, used in this study, were isolated from stems and fruits of healthy *W. somnifera* plants sampled on April 2013 from Teboulba, Tunisia (N35°38'38.256"; E10°56'48.458") (Table 1). Stem and fruit samples were individually disinfected by soaking in 70% ethanol for 1 min, immersion in 1% sodium hypochlorite for 10 min then in 70% ethanol for 30 s. They were rinsed three times in sterile distilled water (SDW) and air-dried on sterile filter papers. Twenty surface-sterilized stem and fruit pieces, of about 1 cm in length, were aseptically transferred onto Nutrient Agar (NA) medium. Each sample was checked for the efficiency of the disinfection process [9]. Plates were incubated at 25°C for 48 h. Bacterial colonies were previously cultured onto NA and incubated at 25°C for 48 h before being used in the different bioassays.

Endophytic ability test

Four (4) bacterial isolates were grown onto NA amended with two antibiotics: streptomycin sulfate and rifampicin used at 100 µg/mL (w/v) each [24]. Double-resistant isolates were selected and the wild-type ones were used for inoculation of tomato cv. Rio Grande seedlings (two-true-leaf stage). Seedlings were dipped for 30 min into bacterial cell suspensions (10^8 cells/mL) [25]. SDW was used for treatment of control seedlings. Five plant replications were used for each individual treatment. Seedlings were grown for 60 days under greenhouse conditions as previously described.

Bacterial isolates were re-isolated from tomato stems onto NA medium supplemented with both antibiotics (streptomycin sulfate and rifampicin) and incubated at 25°C for 48 h. Bacterial colonies similar to the wild-type ones were considered as endophytes and subjected to further screening assays.

Isolate	Source of bacterial isolation	Locality	GPS locality	Year of isolation
S7	Fruit	Teboulba, Tunisia	N35°38'38.256"; E10°56'48.458"	April 2013
S8	Fruit			
S9	Fruit			
S15	Stem			

Table 1: Endophytic bacterial isolates recovered from *Withania somnifera* plants and their isolation sources.

In vivo test of plant growth-promoting ability

Four putative endophytic isolates were tested for their potential to promote tomato growth under greenhouse conditions. Seedlings (two-true-leaf stage) were soaked for 30 min into water bacterial suspensions (~10^8 cells/mL) [26] and control ones were dipped into SDW only. Five replications were used for each individual treatment. Growth parameters (plant height, fresh weight of the aerial parts and roots, and maximum root length) were noted after 60 days of culture.

In vivo screening of the antifungal potential of the endophytic isolates toward *Fusarium oxysporum* f. sp. *lycopersici*

Tomato cv. Rio Grande seedlings (two-true-leaf stage) were treated with the four bacterial isolates separately by drenching 25 mL of a water bacterial cell suspension (10^8 cells/mL) into the culture substrate near the collar level [27]. Twenty five mL of the FOL conidial suspension (10^6 conidia/mL) were also applied as substrate drenching 6 days after bacterial treatment [25]. Negative control seedlings were uninoculated with FOL and treated with SDW only. FOL-inoculated seedlings treated with SDW only were used as positive control. Each individual treatment was replicated five times.

Assessment of wilt severity was performed, 60 days post-inoculation (DPI), on tomato plants inoculated with FOL based on intensity of leaf yellowing and necrosis using the following 0-4 scale: where 0 = no disease symptoms (healthy leaves in the whole plant) and 4 = 76-100% of leaves with yellowing and/or necrosis [28]. Furthermore, wilt severity was assessed based on the extent, from collar, of the vascular browning after performing longitudinal sectioning of stems. Pathogen re-isolation frequency was calculated [1] as the percentage of FOL colonization of stem sections. Growth parameters such as plant height and fresh weight of whole plant were also noted in tomato plants challenged or not with FOL.

The most effective isolates in suppressing Fusarium wilt disease were further subjected to morphological and biochemical characterization and identification.

Characterization and hypersensivity test of the selected endophytic isolates

Colonies of the two most effective bacterial isolates were morphologically characterized as previously described [29], and the Gram's staining was also assessed. Biochemical tests were also performed for the two selected isolates using standard protocols of Schaad [30].

Tobacco plants were used for the hypersensitivity test of the two bacterial isolates. This test was performed by injecting 10 µL of a water bacterial cell suspension (~10^8 cells/mL) into tobacco leaves. SDW was used for inoculation of control leaves. After incubation at room temperature for 24 h, the development of chlorotic and/or necrotic zones around bacteria-inoculated points was checked. Positive isolates were considered as pathogenic and were excluded from further assays of their biocontrol ability [31].

Identification of the selected endophytic isolate by 16S rDNA gene sequencing and phylogenetic analysis

Molecular identification of one bacterial isolate was performed after extraction of the genomic DNA using the method

described by Chen and Kuo [32] for Gram- bacteria. The 16S rDNA was amplified using the universal eubacterial primers 27f (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and 1492r (5'-TACGG(C/T)TACCTTGTTACGACTT-3') according to Moretti et al. [1] protocol. Amplifications were carried out in Thermal Cycler® (CS Cleaver, Scientific Ltd., TC 32/80). PCR cycling conditions were 94°C for 4 min, followed by 40 cycles at 94°C for 30 s, 45°C for 30 s, 72°C for 45 s, and then 72°C for 7 min. The most related species to the bacterial isolate (namely S8) were obtained using BLAST-N program from Genbank database (http: www.ncbi.nlm.gov/BLAST/). The culturable endophytic bacterium (isolate S8) sequence was submitted to GenBank under the accession number KR818077. The second endophytic bacterium shown to be effective in suppressing Fusarium wilt severity (namely S15) was unidentified.

Alignment of partial 16S rDNA sequences was achieved using the ClustalX (1.81). The phylogenetic tree was constructed based on neighbor joining (NJ) method with 1000 bootstrap sampling.

Effect of the selected endophytic isolates on germination of tomato seeds

Tomato seeds cv. Rio Grande were disinfected with sodium hypochlorite (5%) for 3 min and then they were rinsed six times with SDW. The disinfected seeds were subsequently inoculated with bacterial suspensions (~10^8 cells/mL) using 20 µL per seed. A same volume of SDW was used for treatment of control seeds. Two replications were performed for each individual treatment. Treated and untreated seeds were placed in Petri plates containing sterile filter paper moistened with SDW (10 seeds/Petri dish) and incubated at 25°C in the dark. The percentage of germination was noted after 2 and 5 days of incubation [27].

Antifungal activity test of the selected endophytic isolates

Streak method: The antifungal potential of the two selected endophytic isolates toward FOL was assessed using the streak method on PDA medium according to Sadfi et al. [33] protocol. Control Petri plates were streaked with SDW only. Four replications were performed for each individual treatment. Cultures were incubated at 25°C for 4 days and the diameter of FOL colony was measured. The growth inhibition rate of FOL was calculated using the following formula IR% = [(C2-C1) / C2] × 100 where C2: colony diameter of FOL in control plates and C1: colony diameter of FOL in treated plates [34].

Disc diffusion method: The antagonistic potential of the two selected endophytic bacterial isolates was also tested on PDA medium using the disc diffusion method according to Vethavalli and Sudha [35] protocol. Four replications were performed for each individual treatment. The diameter of the inhibition zone was measured after incubation at 25°C for 4 days.

Sealed plate method: In order to assess the antifungal activity of volatile metabolites produced by the bacterial isolates tested against FOL, a sealed plate method was used according to Chaurasia et al. [36] protocol. Three replications were performed for each individual treatment. The inhibition rate of FOL growth was calculated as previously described [34] after 7 days of incubation at 25°C.

Chitinase production

Chitinase production of the two selected bacterial isolates was checked according to Tiru et al. [34] on minimum-medium supplemented with chitin® (MP Biomedicals, LLC, IIIKrich, France). Water bacterial suspensions (~10^8 cells/mL) were streaked onto sterilized chitin-agar medium (0.5% w/v). Chitin-agar medium plates were used as control. Treatments were replicated thrice. After incubation at 28 ± 2°C for 72 h, the presence of clearing zones around bacterial colonies was noted.

Protease production

The two endophytic bacterial isolates were assessed for their ability to produce proteolytic enzymes onto skim milk agar or SMA (3% v/v) medium [34]. Control plates containing SMA only. Treatments were performed in triplicate. The diameter of the clear zone formed around the bacterial colonies was measured after 48 h of incubation at 28 ± 2°C.

Pectinase production

Pectinase production was detected according to Tiru et al. [34] by streaking individually the two bacterial suspensions (~10^8 cells/mL) onto NA-pectin® (ICN Biomedicals, Inc, Germany) medium (0.5%) (w/v). Plates containing the NA-pectin medium only were used as control. Treatments were performed in triplicate. Cultures were incubated at 28 ± 2°C for 48 h. The presence or the absence of clear zones around bacterial colonies was noted.

HCN production ability

The hydrogen cyanide (HCN) production ability was assessed qualitatively following Lorck [37] method. Selected endophytic bacterial isolates were inoculated individually onto NA medium amended with glycine (4.4 g/L) (w/v). Uninoculated controls were used for comparison. Treatments were performed in triplicate. The plates were incubated at 25°C for 4 days. Change in color from yellow to light-reddish brown marked a positive production of HCN.

Phosphate solubilization activity

Phosphate solubilization activity was tested ccording to Sgroy et al. [38] protocol where the two bacterial isolates colonies were individually deposited onto Pikovskaya agar medium. Uninoculated plates were used for comparison. Treatments were performed in triplicate. The clearing zone observed around bacterial colonies was measured after 7 days of incubation at 28 ± 2°C.

Indole-3-acetic acid (IAA) production

Ability of the the two selected endophytic bacterial isolates to produce IAA were checked according to Sgroy et al. [38] protocol. Isolates were cultivated into LB-L-tryptophan (50 µg/mL w/v) medium under continuous shaking for 2 days in the dark. The negative control was uninoculated growth medium. Treatments were performed in triplicate. Absorbance was read at 530 nm. IAA concentration was determined using a standard curve prepared from IAA dilution series at 100 µg/mL (w/v) in LB medium.

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows. For all the *in vitro* antifungal potential bioassays and the *in vitro* tests of enzymes, IAA and HCN production and phosphate solubilization activity, each treatment was replicated three to four times. In the seed germination test, each individual treatment was replicated two times. Data analysis was carried out according to a completely randomized design. All the *in vivo* bioassays were analyzed in a completely randomized model and each treatment was replicated five times. Each of the *in vitro* or *in vivo* experiments was repeated twice. For the *in vitro* germination of tomato seeds, means were separated using Student t test at $P \leq 0.05$. For the rest of bioassays,

means were separated using Student-Newman-Keuls test at $P \leq 0.05$. Correlations analyses between Fusarium wilt severity and plant-growth parameters were performed using bivariate Pearson's test (at $P \leq 0.05$).

Results

Confirmation of endophytic behavior of bacteria isolated from *Withania somnifera*

Four (4) bacterial isolates, recovered from *W. somnifera* fruits and stems, were found to be double-resistant to streptomycin sulfate and rifampicin (100 µg/mL w/v). Their presence within tomato cv. Rio Grande stems was confirmed through their successful re-isolation from the internal tissues stems of inoculated tomato plants and by the development of typical colonies similar to the wild ones on NA medium supplemented with both antibiotics.

These four endophytic bacterial isolates (S7, S8, S9, and S15) were assessed *in vivo* and *in vitro* for their antifungal potential toward FOL and their growth-promoting effects on tomato seedlings.

Assessment of plant growth-promoting (PGP) potential

Tomato plants cv. Rio Grande, inoculated using the root dipping technique, with the four endophytic isolates separately remained healthy until the end of the experiment (60 days post-treatment). These non pathogenic isolates were evaluated for their plant growth-promoting effects when challenged to pathogen-free tomato plants. Analysis of variance exhibited a significant (at $P \leq 0.05$) variation, noted 60 days post-treatment, in all plant growth parameters (plant height, maximum root length, aerial part and root fresh weight) depending on tested bacterial treatments.

As shown in Table 2, tomato plants treated with isolates S7, S8, S9, and S15 were significantly ($P = 0.002$) 21.1-31.3% taller than the untreated controls. A significant increment ($P = 1.53$ E-6) in the aerial part fresh weight, varying from 52.2 to 66.2% depending on isolates, was induced by the four tested isolates as compared to the control. The highest weight increment (66.2%) was achieved using S9 and at a lesser extent S8 and S15 isolates (62.9 and 59.6%, respectively) compared to 52.2% recorded using the isolate S7 (Table 2).

Plant growth promoting ability, as estimated by the maximum root length, was also significantly ($P = 0.001$) increased by 25.1 to 31.8% with the four tested isolates compared to the untreated control (Table 2). A significant ($P = 7.37$ E-5) enhancement in the root fresh weight, relative to the untreated control, was registered using S9-, S15- and S8-based treatments. The recorded root fresh weight increment ranged from 28.5 to 39.8%, respectively (Table 2).

Assessment of Fusarium wilt bio-suppression potential

The four endophytic bacterial isolates were tested on tomato cv. Rio Grande plants challenged with FOL. Sixteen days post-inoculation with FOL, performed analysis of variance revealed a significant decrease (at $P \leq 0.05$) in Fusarium wilt severity depending on tested bacterial treatments. Data given in Table 3 showed that the four isolates led to a significant decrease in the leaf damage index (yellowing and/or necrosis) and in the vascular browning extent relative to the FOL-inoculated and untreated control. Interestingly, FOL-inoculated and treated tomato plants with S15 and S8 isolates exhibited 94.1% less wilting severity ($P = 1.61$. E-10) and 92.6-96.3% lower vascular browning extent ($P = 1.31$ E-11) compared to control (Table 3). Furthermore, S8- and S15-based treatments had significantly similar effects as the disease free control.

The four endophytic bacterial isolates had significantly (at $P \leq 0.05$) enhanced plant height and plant fresh weight in tomato plants challenged with FOL as compared to the control ones inoculated with FOL and untreated. The increment in plant height ranged significantly ($P = 7.03$ E-10) between 24 and 56.3% and the highest enhancement, by 52.4-56.3%, was achieved using S8 and S15 isolates, respectively. As compared to the pathogen-free control, significant improvement, by 20.4 and 27%, in plant height was induced by S8 and S15 isolates, respectively (Table 3).

Assessed for their effects on whole plant fresh weight, S7-, S9, S8- and S15- based treatments led to a significant ($P = 4.52$ E-4) improvement in this parameter by 40.8 to 53.4% relative to FOL-inoculated and untreated control plants, respectively. The plant weight of FOL-inoculated tomato plants and treated separately with these four bacterial isolates was significantly similar to that of disease-free control ones (Table 3).

Pathogen re-isolation performed from of all tomato stem tissues varied depending on tested bacterial treatments. As illustrated in Table 3, a decrease in FOL re-isolation frequency, by 50 to 90% versus the untreated control, was recorded in FOL-inoculated tomato plants and treated with all bacterial isolates. S8- and S15- based treatments induced a decrease, of about 90%, in FOL re-isolation frequency.

Correlation analysis between Fusarium wilt severity and plant growth parameters

Pearson's analysis demonstrated that decreased Fusarium wilt severity as estimated by leaf yellowing intensity and vascular browning extent led to increment in plant growth parameters. In fact, plant height was significantly and negatively correlated to the leaf damage index ($r = -0.89$; $P = 0.01$) (Figure 1a) and the vascular browning extent ($r = -0.89$; $P = 0.01$) (Figure 1b). Furthermore, the plant fresh weight was significantly and negatively correlated to foliar disease severity score

Bacterial treatment	Plant Height (cm)	Aerial part fresh weight (g)	Maximum root lenght (cm)	Root fresh weight (g)
NIC	20.04 ± 0.5^{b}	8.64 ± 0.4^{c}	18.2 ± 0.3^{b}	5.43 ± 0.2^{c}
S7	25.4 ± 0.2^{a}	18.08 ± 0.9^{b}	24.3 ± 1.5^{a}	6.58 ± 0.1^{bc}
S8	29.2 ± 0.6^{a}	23.3 ± 1.7^{ab}	26.4 ± 1.7^{a}	9.02 ± 0.4^{a}
S9	27.5 ± 1.6^{a}	25.6 ± 1.5^{a}	26.7 ± 0.6^{a}	7.6 ± 0.2^{ab}
S15	26.24 ± 1.3^{a}	21.4 ± 0.6^{ab}	26.2 ± 1^{a}	9 ± 0.5^{a}

Table 2: Comparative plant growth-promoting ability of endophytic bacterial isolates recovered from *Withania somnifera* on tomato cv. Rio Grande plants noted 60 days post-treatment. S7, S8 and S9: Isolates from fruits; S15: Isolate from stems; NIC: Uninoculated with the pathogen and untreated control. For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$.

Bacterial treatment	Disease severity (0-4)	Vascular browning extent (cm)	Plant Height (cm)	Plant fresh weight (g)	FOL re-isolation (%)
NIC	0 ± 0^{c}	0 ± 0^{c}	26.4 ± 0.2^{b}	8.79 ± 0.1^{a}	0
IC	3.4 ± 0.2^{a}	8.2 ± 0.6^{a}	15.8 ± 0.2^{d}	4.4 ± 0.4^{b}	90
S7	2.2 ± 0.2^{b}	5.9 ± 0.2^{b}	20.8 ± 0.3^{c}	7.436 ± 0.4^{a}	50
S8	0.2 ± 0.1^{c}	0.3 ± 0.2^{c}	33.2 ± 0.6^{a}	9.252 ± 0.8^{a}	10
S9	2.4 ± 0.2^{b}	5.8 ± 0.1^{b}	21.2 ± 1.7^{c}	7.782 ± 0.1^{a}	80
S15	0.2 ± 0.1^{c}	0.6 ± 0.4^{c}	36.2 ± 1.6^{a}	9.462 ± 0.7^{a}	10

Table 3: Effects of endophytic bacterial isolates recovered from *Withania somnifera* on Fusarium wilt severity, plant growth parameters and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) re-isolation frequency from tomato cv. Rio Grande plants as compared to controls. S7, S8 and S9: Isolates from fruits; S15: Isolates from stems; NIC: Uninoculated with the pathogen and untreated control. IC: Inoculated with FOL and untreated control. For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$.

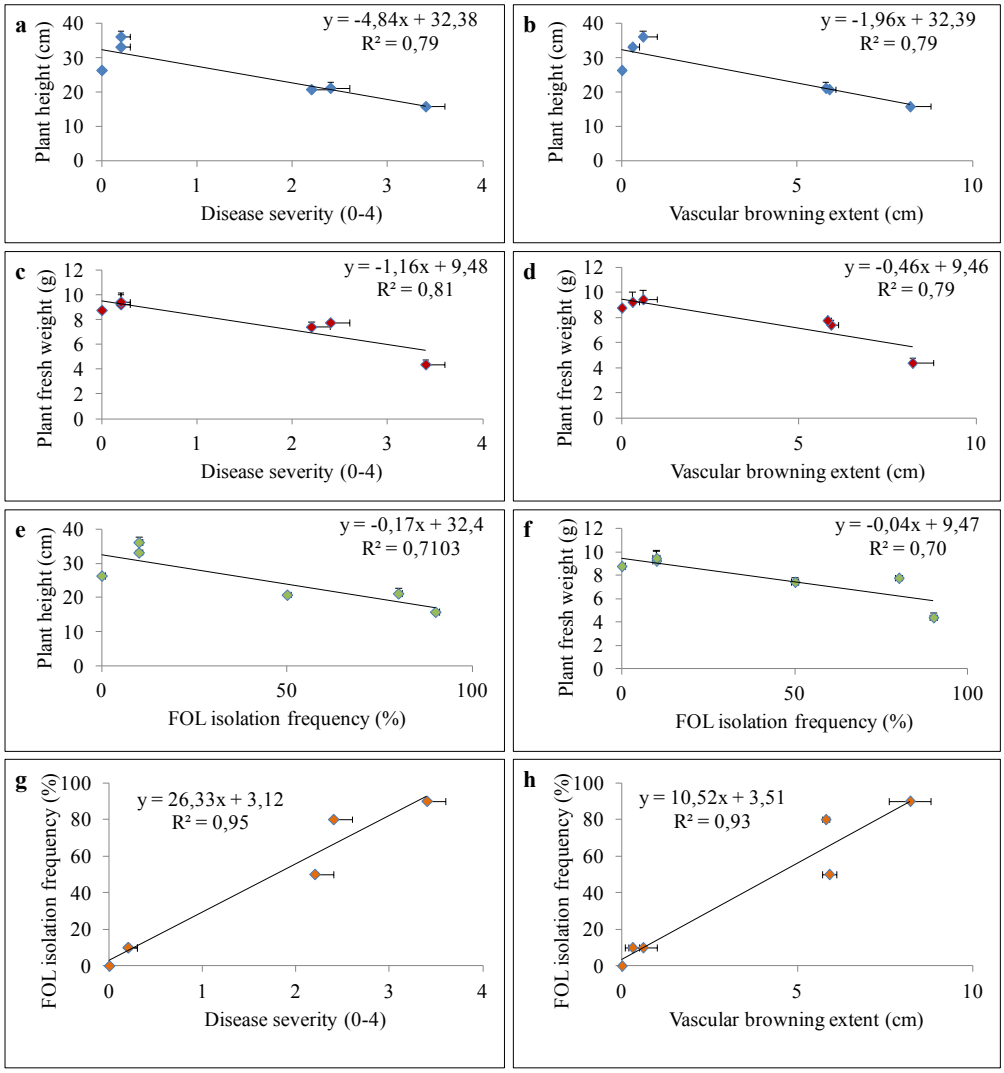

Figure 1: Correlation between Fusarium wilt severity and plant growth parameters (a, b, c, d, e, f) and between FOL isolation frequency and Fusarium wilt severity parameters (g, h). Correlation analysis was performed using bivariate Pearson's test at $P \leq 0.05$.

(r = -0.90; $P = 0.01$) (Figure 1c) and the vascular browning extent (r = -0.89; $P = 0.01$) (Figure 1d).

Pearson's analysis indicated that lowered Fusarium wilt severity led to decrease in tomato stem colonization by FOL and consequently growth promotion. Also, FOL re-isolation frequency was significantly and negatively correlated to plant height (r = -0.84; $P = 0.03$) (Figure 1e) and to the whole plant weight (r = -0.84; $P = 0.03$) (Figure 1f). Moreover, pathogen re-isolation frequency was positively correlated to leaf damage index (r = 0.97; $P = 7.23$ E-4) (Figure 1g) and to the vascular browning extent (r = 0.96; $P = 0.001$) (Figure 1h).

The two endophytic bacterial isolates (S8 and S15) were selected for further elucidation of their mechanisms of action involved in the recorded disease suppression and growth promotion (Figure 2). The two selected isolates will be characterized based on morphological and biochemical traits and only the isolate S8 will be molecularly identified.

Assessment of hypersensitivity reaction and characterization of the selected endophytic isolates

The two selected isolates (S8 and S15) were found to be nonpathogenic as indicated by the absence of development of chlorotic and/or necrotic areas on tobacco leaves after 24 h of incubation as compared to the uninoculated control ones (Table 4).

These two selected isolates were characterized and their main morphological and biochemical traits were summarized in Table 4. Only the isolate S8 was identified using 16S rDNA gene sequencing. Blast-N analysis of sequenced 16S rDNA gene homology revealed that the isolate S8 belonged with 99.8% of similarity to *Alcaligenes faecalis* subsp. *faecalis* strain SK12, with 99.6% of similarity to *Alcaligens faecalis* strain CD205 and *Alcaligenes* sp. strain CRRI 27 and with 99.5% of similarity to *Bacterium* strain KKCSSM (Table 4). The phylogenetic tree distance analysis revealed the short distance between the isolate S8 and *Alcaligenes faecalis* subsp. *faecalis* strain SK12 (Figure 3). The accession number for the partial 16S rDNA gene of the isolate S8 was KR818077.

Effect of the two selected endophytic isolates on germination of tomato seeds

Tomato seeds cv. Rio Grande bacterized by the isolate S15 have

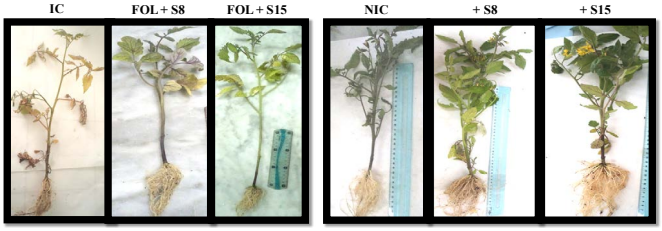

Figure 2: Effect of endophytic bacteria (S8 and S15 isolates) recovered from *Withania somnifera* on Fusarium wilt severity and growth promotion of tomato cv. Rio Grande plants compared to the untreated controls. NIC: Uninoculated with the pathogen and untreated control. IC: Inoculated with FOL and untreated control. S8: Isolate from fruit. S85: Isolate from stem.

germinated after 2 days of incubation, compared to the untreated control seeds, with a significant ($P = 0.01$) percentage of germination of about 30% (Figure 4). After 5 days of incubation, all seeds have germinated and the percentage of germination ranged between 75 and 95%. The highest percentage of germination reached, induced by the unidentified bacterium str. S15, was of about 95% and was significantly higher than that recorded on the untreated seeds (60%) (Figure 4).

In vitro assessment of the antifungal activity of the two selected endophytic bacterial isolates

Tested using the streak method, the two tested bacterial isolates had significantly ($P = 0.01$) reduced the diameter of FOL colony relative to the untreated control, after 4 days of incubation at 25°C (Table 5). FOL mycelial growth was lowered by 16.8 and 10.7% with *A. faecalis* subsp. *faecalis* str. S8 and the unidentified bacterium str. S15, respectively (Figure 5a). Moreover, S8 and S15 isolates formed ($P = 1.6$ E-11) an inhibition zone (8.25 to 8.5 mm, respectively) against FOL when tested using the disc diffusion method on PDA medium (Table 5 and Figure 5b).

Using the sealed plate method, the unidentified bacterium str. S15 exhibited a significant inhibitory effect ($P = 0.01$) against the pathogen expressed by about 53.8% lowered FOL growth versus the untreated control. Furthermore, the antifungal effect displayed by *A. faecalis* subsp. *faecalis* str. S8 was expressed by 20.7% decrease in FOL growth relative to control (Table 5). These inhibition rates revealed the ability of the two endophytic isolates S8 and S15 to inhibit pathogen growth even at distance through their antifungal volatile compounds (Figure 5c).

Bacterial isolates		
Morphological characterization	**S8**	**S15**
Size	Medium	Small
Form	Circular	Circular
Margin	Entire	Entire
Elevation	Elevated	Elevated
Surface	Smooth	Smooth
Opacity	Translucent	Opaque
Color	White	Cream
Gram staining	Negative	Negative
Biochemical characterization		
King A	-	-
Catalase	+	+
Urease	+	-
Lecithinase	-	-
Nitrate reductase	+	+
Tryptophane deaminase	-	-
Lysine decarboxylase	-	+
Mannitol	+	+
Simmons citrate	+	+
Indole	+	+
Red of Methyl	-	-
Voges-Proskauer	+	+
Hydrogen sulfide	-	-
Hypersensivity reaction	-	-
16S rDNA sequencing gene		
Most related species	SK12, *Alcaligenes faecalis* subsp. *faecalis* (99.8)	Unidentified
	CD205, *A. faecalis* (99.6)	
	CRRI 27, *Alcaligenes* sp. (99.6)	
	KKCSSM, *Bacterium* (99.5)	
Accession number	KR818077	

Table 4: Characterization and identification of the bioactive endophytic bacterial isolates recovered from *Withania somnifera*. +: Positive test; -: Negative test. Numbers in parenthesis indicate the percentage (%) of sequence homology obtained from Blast-N analysis.

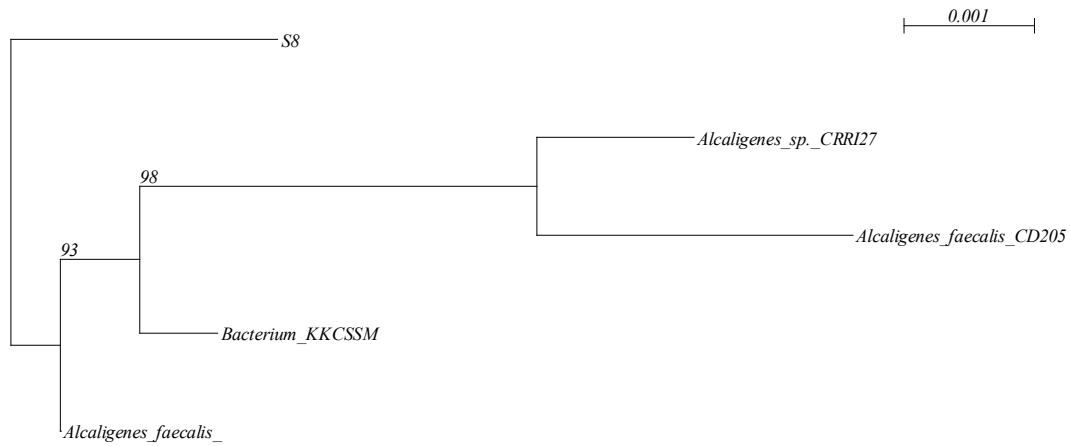

Figure 3: Neighbor-joining phylogenetic tree of partial 16S rDNA sequence of the endophytic bacterial isolate S8 recovered from *Withania somnifera* fruits and their closest phylogenetic relatives. The nucleotide sequences used of representative strains were obtained from Genbank database under the following accession numbers: KC790302 (*Alcaligenes faecalis* subsp. *faecalis* strain SK12), JN256919 (*Alcaligenes* sp. strain CRRI 27), JX871323 (*A. faecalis* strain CD205), JN792202 (*Bacterium* strain KKCSSM), and for the tested bacterial isolate: KR818077 (S8). The tree topology was constructed using ClustalX (1.81).

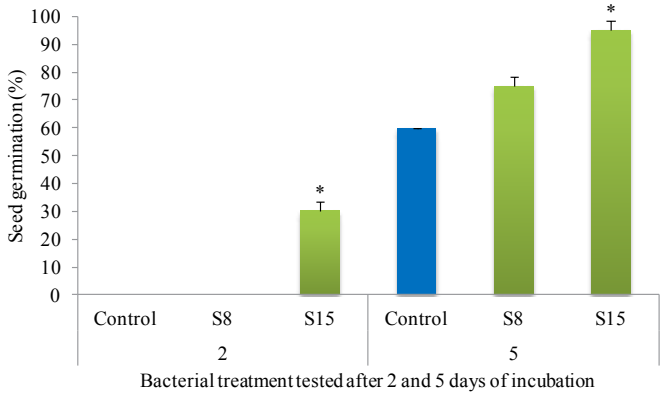

Figure 4: Effect of *Alcaligenes faecalis* subsp. *faecalis* str. S8 and unidentified bacterium str. S15 on germination of tomato cv. Rio Grande seeds noted after 2 and 5 days of incubation at 25°C in the dark as compared to the untreated control. S8 and S15: Isolated from fruits and stems of *Withania somnifera*, respectively. For each incubation period, bars with asterisk are significantly different to the control according to test-t of Student at $P \leq 0.05$.

Bacterial treatment	Streak method[a]	Sealed plate method[b]	Disc diffusion method[c]
	Colony diameter (cm) and growth inhibition of FOL (%)		Inhibition zone (mm)
Untreated control	3.71 ± 0.08[a]	6.41 ± 0.03[a]	0 ± 0[b]
Alcaligenes faecalis subsp. *faecalis* str. S8 (KR818077)	3.08 ± 0.06[b] (16.8)	5.08 ± 0.1[b] (20.7)	8.25 ± 0.1[a]
Unidentified bacterium str. S15	3.31 ± 0.1[b] (10.7)	2.96 ± 0.1[c] (53.8)	8.5 ± 0.1[a]

Table 5: Antifungal activity of *Alcaligenes faecalis* subsp. *faecalis* str. S8 and unidentified bacterium str. S15, recovered from *Withania somnifera*, toward *Fusarium oxysporum* f. sp. *lycopersici*. For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$. Values in parentheses indicate the percentage (in %) of the mycelial growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* as compared to the untreated control.
[a, c]Noted after 4 days of incubation at 25°C.
[b]Noted after 7 days of incubation at 25°C.

Hydrolytic enzymes and HCN production

The two tested isolates showed clear zones around colonies on chitin-, pectin-, and milk-agar media. Thus, *A. faecalis* subsp. *faecalis* str. S8 and the unidentified S15 isolate were found able to produce chitinase, pectinase and protease, respectively (Table 6). Furthermore, these two isolates (S8 and S15) were able to release the volatile antibiotic, HCN, on NA supplemented with glycine (Table 6).

IAA production

The two selected bacterial isolates, *A. faecalis* subsp. *faecalis* str. S8 and the unidentified bacterium str. S15, were able to produce IAA, involved in plant growth promotion, after 48 h of incubation (Table 6). The highest production of IAA, of about 33.91 µg/mL, was obtained after 48 h of incubation using *A. faecalis* subsp. *faecalis* str. S8.

Phosphate solubilization ability

A. faecalis subsp. *faecalis* str. S8 and the unidentified bacterium str. S15 formed a clear zone of about 11.33 and 19.67 mm in diameter around their colonies, respectively. Thus, both isolates had the ability to solubilize phosphate (Table 6).

Discussion

Wild indigenous plants are able to survive under extreme stress conditions. Therefore, they may be valorized and exploited for various features such as isolation and development of endophytic biocontrol agents. In the present study, native *W. somnifera* plants were exploited for isolation of endophytic bacterial isolates for bio-suppression of tomato Fusarium wilt disease and improvement of plant growth. Previous studies have identified other potential uses for *W. somnifera* where their methanolic leaf and/or root extracts have shown antifungal activity against *Ascochyta rabiei* [39] and *F. oxysporum* f. sp. *cepae* [40]. Leaf aqueous extracts also exhibited antifungal potential against *Sclerotinia sclerotiorum* [41]. Nefzi et al. [12] also recorded a strong inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* growth using butanolic extracts from *W. somnifera* leaves, stems and fruits compared to ethyl acetate and chloroform extracts. Moreover, Khan et al. [19] had exploited this plant for isolation of endophytic fungi i.e. *Fusarium* spp., *Aspergillus* spp., *Alternaria alternata*, *Penicillium* spp., *Curvularia oryzae*, *Myrothecium roridum*, *Drechslera australiensis*, *Cladosporium cladosporioides*, *Chaetomium bostrycode*, *Eurotium rubrum*, *Phoma* sp., and *Melanospora fusispora*. However, no antimicrobial activity against plant pathogens was previously reported. Hence, and to the best of our knowledge, this study reported for the first time the potential use of *W. somnifera* as source of endophytic bacteria displaying antagonistic potential towards FOL and exhibiting plant growth promotion ability in tomato plants.

In this study, bacterial endophytic progress within tomato stems

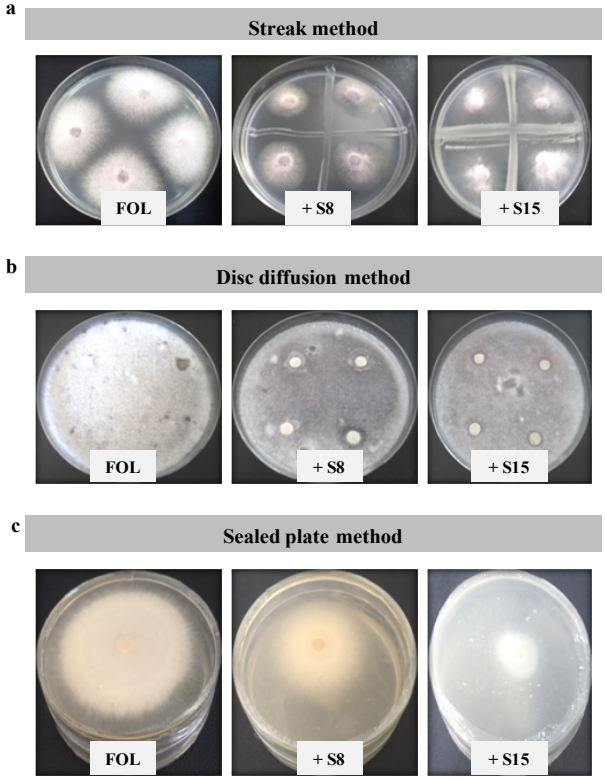

Figure 5: Inhibition of *Fusarium oxysporum* f. sp. *lycopersici* mycelial growth induced by *Alcaligenes faecalis* subsp. *faecalis* str. S8 (KR818077) and unidentified bacterium str. S15, recovered from *Withania somnifera*, as compared to the untreated controls. a and b: Noted after 4 days of incubation at 25°C on Potato Dextrose Agar (PDA) medium; c: Noted after 7 days of incubation at 25°C on PDA.

Bacterial isolate	Antifungal activity				PGP ability	
	Chitinase[a]	**Protease[b]**	**Pectinase[c]**	**HCN production[d]**	**Phospahate solubilzation[e]**	**IAA production[f]**
Alcaligenes faecalis subsp. *faecalis* str. S8 (KR818077)	+	+[u]	+	+	+[w]	+[y]
Unidentified bacterium str. S15	+	+[v]	+	+	+[x]	+[z]

Table 6: Antifungal and plant growth-promoting (PGP) mechanisms displayed *Alcaligenes faecalis* subsp. *faecalis* str. S8 and unidentified bacterium str. S15 recovered from *Withania somnifera* fruits and stems, respectively.
[a]Tested on chitin-agar (0.5 % w/v) medium and incubated at 28 ± 2°C for 72 h; +: Presence of clear zone.
[b]Tested on skim milk agar (3% v/v) medium and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone (u and v: 24.67 ± 0.02 and 24.17 ± 0.03 mm in diameter, respectively).
[c]Tested on pectin-agar (0.5% w/v) medium and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone.
[d]HCN: Hydrogen cyanide production on glycine-agar (4.4 g/L w/v) medium and incubated at 25°C for 4 days; +: Modification on the filter paper color (light-reddish color) compared to the control (yellow).
[e]Tested on Pikovskaya agar medium and incubated at 28 ± 2°C for 7 days; +: Presence of clear zone (w and x: 11.33± 0.06 and 19.67 ± 0.01 mm in diameter).
[f]IAA: Indole-3-acetic acid production after 48 h of incubation at 28 ± 2°C in Luria-Broth medium; +: Production of IAA (y and z: 33.91 ± 0.14 and 14.6 ± 0.1 µg/mL, respectively).

tissues was confirmed for 4 non pathogenic isolates which were double-resistant to streptomycin and rifampicin. Mutants combining resistance to rifampicin and streptomycin were also used by Chen et al. [24] for confirmation of the endophytic behavior of their isolates.

These endophytic bacteria, recovered from *W. somnifera*, were assessed for their ability to promote tomato growth onto plants uninoculated with FOL under greenhouse condition and results clearly showed an improvement in all growth parameters, ranging from 23.6 to 66.2% relative to the untreated control, which was induced by the four tested endophytic bacterial isolates. Similar results have confirmed the plant growth-promoting ability of endophytic bacteria obtained from *Prosopis stormbulifera* roots [38] and *Zingiber officinale* rhizomes [42]. In Dong et al. [43] study, an endophytic bacterium namely *Klebsiella pneumoniae* was isolated from roots of coffee plants and corn which were shown able to promote growth of *Arabidopsis* and *Triticum* when applied at the root level. In this regard, Zhu et al. [44] reported that *Stenotrophomonas maltophilia*, recovered from rice root, is an endophyte bacterium with bio-fertilizing properties.

Assessed on FOL-inoculated tomato plants, the two bacterial isolates S15 and S8, recovered from *W. somnifera* stems and fruits, exhibited strong disease suppressive effects, by reducing wilt severity and the vascular browning extent (by 94% and 92-96%, respectively) relative to the control inoculated with FOL and untreated. Tomato Fusarium wilt severity has been also lowered at least by 75% in plants inoculated with FOL and treated using two unidentified endophytic bacteria PA and PF, isolated from wild and cultivated young oilseed rape plants [27]. In the current study, the reduction in tomato Fusarium wilt severity was associated to lowered FOL colonization of vascular tissues, thus, leading to the recorded growth promotion. In the same sense, previous findings revealed that disease-suppressive effects, by 68.4%, displayed by an endophytic bacterium *B. subtilis* str. EPC016, isolated from cotton plants, enhanced consequently plant growth and fruit yield of tomato compared to control [45]. Moreover, growth enhancements were also achieved on maize plants treated with an endophytic *B. mojavensis* and grown in presence of pathogenic isolates of *F. verticillioides* [6]. In our recent studies, a strong decrease in Fusarium wilt severity was achieved using various endophytic bacteria including *B. cereus* str. S42, *A. faecalis* str. S18, *Stenotrophomonas* sp. S33, *Pseudomonas* sp. S85, *B. mojavensis* str. S40, and *S. maltophilia* str. S37, recovered from other wild Solanaceae species namely *Nicotiana glauca*, *Datura stramonium*, and *D. metel*, respectively. These endophytic isolates were also shown effective in enhancing tomato growth in plants inoculated or not with FOL [23,46,47].

The two best antagonistic bacterial isolates, S15 and S8, were macro-morphologically and biochemically characterized and only the isolate S8 was molecularly identified by 16S rDNA gene sequencing as *Alcaligenes feacalis* subsp. *faecalis* str. S8 (KR818077). This species has been isolated from mangrove (*Avicennia nitida*) [48] and also from crape jasmine (*Tabernaemontana divaricata*) leaves [49]. Several investigations reported that *A. faecalis* displayed an antifungal activity against *Aspergillus niger*, *A. flavus*, *Paecilomyces variotii*, *Candida albicans*, *A. alternata*, *Cercospora arachicola*, *Rhizoctonia solani*, and *F. oxysporum* [50-52]. *A. faecalis* str. AF3, a rhizobacterium associated to maize, was also shown able to promote growth of maize seedlings under conventional conditions and under drought stress [53]. It should be indicated that the second unidentified S15 isolate, selected in the current study, belonged to Gram-negative bacteria. Several previous studies demonstrated that Gram-negative bacteria belonging to genera *Pseudomonas*, *Serratia*, *Enterobacter* [54], and *Stenotrophomonas* [55] displayed an endophytic behavior. Furthermore, endophytic *S. maltophilia* isolates exhibited antimicrobial potential toward plant pathogenic fungi, bacteria (such as *Ralstonia solanacearum*) and the plant-parasitic nematode *Meloidogyne incognita* [56,57]. In the same way, *P. brenneri* str. YC6890 isolated by Bibi et al. [58] showed antagonistic activity against oomycetes plant pathogens such as *Pythium ultimum* and *Phytophthora capsici*. Cyclamen plants treated with *Serratia marcescens* str. B2 and inoculated with sclerotia of *Rhizoctonia solani* and/or conidia of *Fusarium oxysporum* f. sp. *cyclaminis* showed lowered disease severity [59].

Bacterial isolates selected in this study (S8 and S15) had improved germination of tomato cv. Rio Grande seeds by 75-95% after 5 days of incubation with isolate S15 being the most effective (95%). This unidentified bacterium had also induced an earlier germination of tomato seeds, by 30%, after 2 days of incubation. In Sundaramoorthy and Balabaskar [60] study, two endophytic *B. subtilis* isolates, recovered from coconut and cotton, and three rhizospheric isolates of *P. fluorescens* had enhanced germination of tomato seeds by about 88-93% which was higher than the untreated control (80%) after 10 days of incubation. The highest percentage of germination (96%) was noted on seeds treated with a combination of *B. subtilis* str. EPCO16 and *P. fluorescens* str. Pf 1.

In the current study, *A. faecalis* subsp. *faecalis* str. S8 and the unidentified bacterium str. S15, assessed *in vitro* for their antifungal activity against the pathogen, had inhibited FOL mycelial growth by both diffusible and volatiles metabolites and induced an antibiosis zone. Similar inhibitory effects of *F. oxysporum* were demonstrated by

Nandhini et al. [61] and Patel et al. [29] using endophytic *Pseudomonas aeruginosa* HR7, *Pseudomonas* sp. TEP3, *Bacillus* sp. TEB6, *Citrobacter* sp., and *Klebsiella* sp. TEK1 isolates recovered from *S. lycopersicum*. Volatile metabolites from the two tested S8 and S15 isolates caused greater growth inhibition of FOL (20.7 to 53.8%) than the diffusible compounds (10.7 to 16.8%). Similar finding was reported in Chaurasia et al. [36] study where volatile compounds from *B. subtilis* were more effective than the diffusible ones when tested against *F. oxysporum*, *Alternaria alternata*, *Cladosporium oxysporum*, *Paecilomyces lilacinus*, *P. variotii*, and *Pythium afertile*. However, diffusible metabolites produced by endophytic *Bacillus* spp., recovered from wild Solanaceae stems, were shown to be more active against FOL than volatile compounds [22].

Both isolates were elucidated *in vitro* for their properties deployed in the registered antifungal effect. In fact, both isolates S15 and *A. faecalis* subsp. *faecalis* str. S8 were shown able to produce chitinase, protease and pectinase enzymes as shown on chitin-, milk-, and pectin-agar media, respectively. Therefore, these isolates had inhibited FOL growth through, among others, the biosynthesis of lytic enzymes such as chitinases, pectinases and/or proteases. Several genera of endophytic bacteria such as *Bacillus, Burkholderia*, *Serratia*, *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Micrococcus,* and *Microbacteruim* were positive for chitinase, protease and β-1,3-glucanase production involved in cell-wall degradation of various pathogens [54,58]. Moreover, colonization of plant tissues by endophytic bacteria was facilitated by their pectinase and cellulase activities [9]. These enzymes may be also involved in the enhancement of tomato growth as previously reported by Baldan et al. [62].

Furthermore, the production of HCN has been shown to be involved in the effective pathogen inhibition. The isolate S8 of *A. faecalis* subsp. *faecalis* and the unidentified bacterium str. S15 were found to be HCN-producing agents. This volatile antibiotic was frequently produced by Gram negative bacteria like *Chromobacterium violaceum* and *Pseudomonas fluorescens* [63,64] and was shown to be involved in *Sclerotium rolfsii* [65] and *R. solani* [64] biocontrol. However, two endophytic *S. maltophilia* isolates, namely TEM56 and PM22 recovered from *Amaranthus hybridus* and *Cucurbita maxima*, were unable to produce HCN [66]. In our other recent studies, Gram-negative bacterial isolates such as *A. faecalis* str. S8 and *S. maltophilia* str. S37, isolated from *N. glauca* and *D. stramonium* stems, respectively, were capable to produce this volatile antibiotic [46,47] whereas *Stenotrophomonas* sp. str. S33 and *Pseudomonas* sp. str. S85, recovered from *D. metel* stems and roots, respectively, were not HCN-producing agents [23].

The two selected endophytic bacteria (*A. faecalis* subsp. *faecalis* str. S18 and the unidentified bacterium str. S15) were found able to produce indole-3-acetic acid (IAA) and phosphatase as allelochemicals involved in the recorded increment of tomato growth relative to the untreated control. In fact, our isolates S8 of *A. faecalis* subsp. *faecalis* and the unidentified bacterium str. S15 had produced 33.91 and 14.6 µg IAA/mL, respectively. The IAA amount released by *A. faecalis* subsp. *faecalis* str. S8 is interestingly higher when compared to 16.4 µg/ml produced by *A. piechaudii* in another study [67]. IAA production was due to the catalyse of the indole acetonitrile (IAN) using nitrilase enzyme as shown for two *A. faecalis* isolates i.e. ATCC 8750 and JM3[68]. Thus, IAA producing ability detected in *A. faecalis* subsp. *faecalis* str. S8 may be attributed to nitrilase activity. Furthermore, endophytic *Pseudomonas* sp. JDB3, JDB5 and JDB6 strains isolated from soybean plants [69], and endophytic *S. maltophilia* TEM56 and PM22 strains recovered from *A. hybridus* and *Cucurbita maxima*, respectively, were found able to produce IAA but with slight amounts in both last strains (0.32 and 0.49 mg/L, respectively) [66].

Phosphate solubilization ability was also assessed and confirmed for *A. faecalis* subsp. *faecalis* str. S8 and the unidentified bacterium str. S15. The potential to solubilize phosphate displayed by endophytic bacteria such as *Pseudomonas* spp., *Serratia* spp., *Enterobacter asburiae*, *Rahnella aquatilis*, *Ewingella americana*, and *Yokenella regensburgei* was mentioned in Ngamau et al. [70] study. The phosphatase activity of *A. faecalis* subsp. *faecalis* str. S8 and unidentified bacterium str. S15 was indicated by the presence of clear zone of 11.33 and 19.67 mm. Patel et al. [29] reported that some unidentified endophytic bacterial isolates and *P. aeruginosa* str. HR7, recovered from tomato plants, formed a clear zone of 8-31 mm in diameter.

Conclusion

W. somnifera was firstly reported in the current study as a potential source of biocontrol and plant growth-promoting bacterial agents. Benefits of selected endophytic bacterial isolates as biofertilizers were clearly demonstrated onto uninoculated and inoculated tomato plants. Their screening for their antifungal potential towards FOL led to the selection of two promising biocontrol agents, the first one was identified as *Alcaligenes faecalis* subsp. *faecalis* str. S8 (KR818077) using 16S rDNA gene sequencing and the second one, a Gram-negative bacterium str. S15, was still unidentified. These two selected isolates were found to be potential sources of non volatile and/or volatile bioactive metabolites effective against FOL growth. Interesting enzymatic activity (chitinase, protease and/or pectinase) and HCN production were elucidated in the recorded antifungal activity displayed against FOL. Furthermore, plant growth-promoting traits were assessed through the phosphate solubilization ability and IAA production.

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