Endophytic Bacteria from *Solanum nigrum* with Plant Growth-Promoting and Fusarium Wilt-Suppressive Abilities in Tomato

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ABSTRACT

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Fifteen endophytic bacterial isolates from *Solanum nigrum* and *S. nigrum* var. *villosum* stems were screened for their plant growth-promoting potential and antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Isolates SV65, SV68 and SV109 were the most efficient in controlling the development of the disease (77-92%) and in improving tomato growth (32-62%) compared to the controls. They were characterized and identified by using 16S rDNA sequencing genes as being *Bacillus amyloliquefaciens* subsp. *plantarum* for the strain SV65 (KR818073) and *B. methylotrophicus* for the two strains SV68 (KR818074) and SV109 (KR818076). Gas Chromatography-Mass Spectrometry analysis of the n-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 matched phthalic acid, mono(2-ethylhexyl)ester as major compound. The bacterium *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV109 were shown to be chitinase-, protease-, pectinase-, phosphatase-, and indole 3-acetic acid (IAA)-producing agents. Furthermore, *B. methylotrophicus* SV68 produced chitinase, pectinase, and IAA (28.49 µg/ml), and *B. amyloliquefaciens* subsp. *plantarum* SV65 excreted siderophores andoxalic and malic acids. This study demonstrates that *S. nigrum* and *S. nigrum* var. *villosum* can be potential plant species for isolation of endophytic bacteria serving as biocontrol and biofertilizing agents for the improvement of production of tomato grown in FOL infested and non-infested soils.

Keywords: Bacillus spp., biocontrol, *Fusarium oxysporum* f. sp. *lycopersici*, metabolites, *Solanum nigrum*, tomato growth

After potato, tomato (*Solanum lycopersicum*) is one of the most widely grown and consumed vegetable species all over the world (Olaniyi et al. 2010). However, this crop is highly susceptible to various pathogens such as viruses, bacteria and fungi. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), is one of the most devastating soil-borne disease occurring in major tomato-producing areas worldwide (Moretti et al. 2008). This phytopathogen is responsible for heavy yield losses both in open field (season crop) and all year under greenhouse crops (McGovern 2015).

Disease control is a difficult task due to the limited range of effective fungicides, the colonization ability of the

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causal agent within the vascular tissues, and to the survival of its resting structures (chlamydospores) in the soil for many years (Vethavalli and Sudha 2012). In addition, using tomato cultivars resistant to FOL race 1 and race 2 failed to control disease in the presence of race 3 emerging in several countries (Reis et al. 2005).

Biological control of Fusarium wilt, using non-pathogenic microbial agents, remains the most promising and environmentally safe method (Moretti et al. 2008). These microbial agents are associated with various plant species and are commonly present in many environments. Associations between microorganisms and botanical species play an important role in plant surviving in diverse environmental conditions either under abiotic or biotic stresses (Andreolli et al. 2017; Compant et al. 2005). Potential uses of plant-associated bacteria as plant growth-stimulating agents and for managing soil and plant health was largely investigated (Botta et al. 2013; Chen et al. 1995; Eleftherios et al. 2004; Hallman et al. 1997; Marcos et al. 2016; Sturz et al. 2000; Welbaum et al. 2004).

The widely recognized mechanisms of biocontrol mediated by plant-associated bacteria are competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants toward a broad spectrum of pathogens (Compant et al. 2005). Microbial-derived allelochemicals included iron-chelating siderophores (Bar-Ness et al. 1992), cell wall-degrading enzymes (Berg et al. 2005), lipopeptide antibiotics (Han et al. 2015) and/or other compounds belonging to the family of phthalic acids (Ramyabharathi and Raguchander 2014). Moreover, endophytic bacteria stimulate plant growth via indole-3-acetic acid (IAA) production, phosphate solubilization, nitrogen fixation (Gaiero et al. 2013) and/or indirectly through inducing systemic resistance (Compant et al. 2005).

Solanum genus has been explored as a rich source of antimicrobial metabolites (Lin et al. 2011; Nefzi et al. 2016; Tuba et al. 2016). Furthermore, *S. trilobatum*, *S. melongena* and *S. torvum* are known to harbor various endophytic bacteria which were not previously assessed for their antifungal activity or their plant growth-promoting ability (Achari and Ramesh 2014; Bhuvaneswari et al. 2013).

S. nigrum, commonly known as Makoi or black nightshade, is a medicinal plant from the Solanaceae family. This wild plant was widely used in traditional medicine due to its antitumorigenic, antioxidant, anti-inflammatory, hepato-protective, diuretic and antipyretic properties (Jain et al. 2011). This plant was also explored as potential source of isolation of endophytic fungi with potent plant growth promoting properties as is the case of *Fusarium tricinctum* and *Alternaria alternate* (Khan et al. 2015a). *S. nigrum* has been also used for isolation of endophytic bacteria such as *Serratia* sp. displaying the ability to enhance plant growth and phytoremediation potential under cadmium stress (Khan et al. 2015b). In addition, endophytic *Streptomyces* strains isolated from *S. nigrum* have been used for the biocontrol of *Rhizoctonia solani* damping-off and the promotion of tomato growth (Goudjal et al. 2014).

In this study, healthy *S. nigrum* and *S. nigrum* var. *villosum* plants were explored for the isolation of endophytic bacteria with the ability to suppress Fusarium wilt severity and to stimulate growth of tomato plants under greenhouse conditions. The most effective isolates were screened for their in vitro antifungal potential toward FOL. Antimicrobials

involved in disease suppression and plant growth promotion were also elucidated.

MATERIALS AND METHODS

Preparation of tomato seedlings.

Tomato cv. Rio Grande seeds® (BADDAR semences · seeds), used in this study, were gratefully provided by the Regional Center of Research on Horticulture and Organic Agriculture at Chott-Mariem, Sousse, Tunisia. This cultivar is known by its susceptibility to Fusarium wilt disease incited by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) races 2 and 3 (Barker et al. 2005). Peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg) was sterilized into individual batches (80 × 120 cm) at 120°C, 1 bar pressure for 30 min before use. Seedlings were grown in alveolus plates (7 × 7 cm) filled with sterilized peat under greenhouse with 16 h photoperiod, 60-70% relative humidity and air temperatures ranging between 20 and 30°C. They were watered regularly until reaching the two-true-leaf growth stage. Seedlings with approximately similar heights were used for all the in vivo bioassays.

Phytopathogen culture.

F. oxysporum f. sp. *lycopersici* (FOL) strain used in this study was originally isolated from tomato plants showing typical Fusarium wilt symptoms. Isolation was performed from stem tissues exhibiting vascular discoloration. The phytopathogen was re-isolated from artificially infected tomato plants, fulfilling Koch's postulates, and previously identified based on wilting pattern and phytopathogen morphological and cultural traits on Potato Dextrose Agar (PDA) medium (Aydi Ben Abdallah et al. 2016a).

FOL strain used for in vitro and in vivo bioassays was previously grown for 7 days on PDA medium supplemented with streptomycin sulfate (300 mg/ml) (w/v) and incubated at 25°C.

Solanum nigrum sampling and isolation of endophytic bacteria.

Healthy wild *S. nigrum* and *S. nigrum* var. *villosum* plants were used for isolation of endophytic bacteria. Plants were sampled, at the fruiting stage, from Chott-Mariem, Tunisia (N35°56'20,451"; E10°33'32,028").

Five stem samples per plant were individually disinfected by soaking in 70% ethanol for 1 min, immersion in 1% sodium hypochlorite solution for 10 min, and then in 70% ethanol for 30 s. They were rinsed thrice in sterile distilled water (SDW) and air-dried on sterile filter papers. Three stem pieces (1 cm in length) were pierced with a sterile-nipper and the liquid exuding from the internal tissues was streaked on Nutrient Agar (NA) medium. The efficiency of surface sterilization of stem sections was determined according to Hallmann et al. (1997) procedure. Plates were incubated at 25°C for 48 h. Bacterial colonies with morphological diversity were selected and conserved in Nutrient Broth (NB) supplemented with 40% glycerol at -20°C. Before being used in the different bioassays, stock cultures were grown onto NA and incubated at 25°C for 48 h.

Evaluation of endophytic colonization ability.

The endophytic colonization ability of bacterial isolates recovered from *S. nigrum* plants was checked according to Chen et al. (1995). Twenty isolates were grown on NA supplemented with streptomycin sulfate and rifampicin (applied at 100 µg/ml each). Isolates exhibiting resistance to these antibiotics were selected and the wild types were used for the inoculation of tomato cv. Rio

Grande seedlings (at the two-true-leaf stage). Seedlings were soaked for 30 min in a suspension of bacterial cells (~10^8 cells/ml) prepared by suspending colonies growing in NA after 48 h of incubation in SDW. Control seedlings were dipped in SDW only. Seedlings were transplanted into individual pots (12.5 × 14.5 cm) filled with sterilized peat. Five seedlings were used for each individual treatment. Seedlings were grown for 60 days under greenhouse conditions as previously described. Re-isolation of bacterial isolates from the internal stem tissues was performed on NA amended with both antibiotics (100 µg/ml) as described above. After incubation at 25°C for 48 h, growing bacterial colonies similar to the wild type ones were considered as endophytes.

Evaluation of the hypersensitivity reaction.

Fifteen isolates that shown to be able to colonize the internal stem tissues of tomato cv. Rio Grande seedlings were used for the hypersensitivity test.

Tobacco (*Nicotiana tabacum*) seeds were provided by the laboratory of phytopathology of the Regional Research Center on Horticulture and Organic Agriculture at Chott-Mariem, Sousse, Tunisia. Seeds were disinfected with 0.2% sodium hypochlorite for 3 min, washed several times with SDW and then sown into individual pots (12.5 × 14.5 cm) filled with sterilized peat. Tobacco seedlings were grown in a growth chamber at 24-26°C with 12 h photoperiod and 70% humidity for 15 days and watered regularly until reaching the two-true-leaf growth stage. A volume of 10 µl of bacterial cell suspension (~10^8 cells/ml) was injected to tobacco leaves using a microsyringe (1 ml U-100 BD Micro-Fine™ Plus - 0.33 mm (29 G) × 12.7 mm). Negative control leaves were treated similarly using an equal volume of SDW. Tobacco plants (inoculated and non-inoculated) were incubated under ambient room conditions (~27°C) for 24 h. Isolates inducing the formation of chlorotic and/or necrotic zones on inoculated leaf areas were considered as phytopathogens and they were excluded from further biocontrol trials (Nawangsih et al. 2011).

Evaluation of the hemolytic activity.

Fifteen endophytic isolates were tested for their ability to degrade hemoglobin according to Murray et al. (2003). In fact, 100 µl of each bacterial cell suspensions (~10^8 cells/ml) were transferred on the Blood Agar® (HiMedia, India) medium and incubated at 25°C for 48 h. Bacterial isolates with positive hemolytic activity, expressed by the formation of clear zones around their colonies, were considered as pathogenic to humans and excluded from the following trials.

Evaluation of the plant growth-promoting ability.

Fifteen endophytic bacterial isolates were screened in vivo for their ability to enhance tomato growth based on Botta et al. (2013) method. Roots of tomato cv. Rio Grande seedlings (at the two-true-leaf stage) were soaked for 30 min in bacterial suspensions adjusted at 10^8 cells/ml. Control seedlings were treated similarly with SDW.

Treated and non-treated (control) seedlings were transferred to individual pots (12.5 × 14.5 cm) filled with sterilized peat. Five seedlings were used for each individual treatment. Growth conditions were similar to those described above. At 60 days post-planting, plant height, fresh weight of the aerial parts and roots, and the maximum root length were noted.

Evaluation of Fusarium wilt-suppressive ability.

Seedling bacterization test was performed for fifteen endophytic isolates according to Nejad and Johnson (2000). Tomato cv. Rio Grande seedlings were separately drenched with 25 ml of bacterial suspensions (10^8 cells/ml). Six days post-bacterial treatment, they were drenched with 25 ml of FOL conidial suspension adjusted at 10^6 conidia/ml using a hemocytometer. Negative control seedlings were non-inoculated with FOL and watered with equal volume of SDW. Positive control seedlings were inoculated with FOL and treated similarly with SDW. Each individual treatment was replicated five times (five seedlings per treatment).

At 30 days post-inoculation (DPI) with FOL, disease severity was rated on 0-4 scale (Amini 2009), where 0 = no symptoms and 4 = 76-100% of leaves showing chlorosis and/or necrosis, and the vascular browning extent (from collar). Plant height, fresh weight of the whole plant and the percentage of FOL re-isolation frequency from stems sections onto PDA were also noted. Isolation frequency was calculated using formula: IF (%) = f/F ×100, where f = number of stem fragments showing growing FOL colonies and F = total number of stem fragments plated onto PDA (Moretti et al. 2008).

The most efficient bacterial isolates in suppressing Fusarium wilt severity and in improving growth of tomato plants challenged or not with FOL were selected for further characterizations, and identification of their metabolites involved in both effects.

Characterization and molecular identification of the most efficient antagonist isolates.

Morphological, biochemical and molecular characterizations were performed for the most effective bacterial isolates in reducing tomato Fusarium wilt severity and in improving tomato growth. Colonies were characterized macro-morphologically based on their form, margin, elevation, surface, opacity and pigmentation on NA medium (Patel et al. 2012). Gram staining was performed using Gram procedure and colonies were observed using light microscopy® (LABORLUX S, Leitz). Biochemical characterization was performed using conventional tests according to Schaad et al. (2001) protocols.

Molecular identification was carried out by 16S rDNA sequencing genes and similarity analysis. Identification was performed after extraction of genomic DNA using the method described by van Soolingen et al. (1994) for Gram positive bacteria. PCR conditions and the two universal eubacterial primers 27f(5′-AGAGTTT GATCMTGGCTCAG-3´) and 1492r(5′-TACCTTGTTACGACTT-3´) used for amplification of 16S rDNA gene were detailed in Aydi Ben Abdallah et al. (2016b). The similarity of the 16S rDNA sequence of a given isolate was performed using BLAST-N program from GenBank database (http:www.ncbi.nlm.gov/BLAST/) and Ez-Taxon-e-server (http://eztaxon-e.ezbiocloud.net/). Alignment of the sequences was performed using ClustalX (1.81). Phylogenetic analysis for the aligned sequences was performed using the Kimura two-parameter model (Kimura 1980). The phylogenetic tree was constructed based on the neighbor joining (NJ) method with 1000 bootstrap sampling.

Evaluation of the antifungal activity.

Antifungal activity of the selected endophytic isolates was performed using their whole cell cultures,

cell-free culture filtrates and organic extracts.

Evaluation of the antifungal activity of whole cells.

The antifungal activity of the whole cell suspensions of selected isolates was carried out using the streak method (Sadfi et al. 2001) and the disc diffusion method (Vethavalli and Sudha 2012). Bacterial suspensions (~10^8 cells/ml) were streaked along two perpendicular lines crossing the center of a Petri plate (9 cm in diameter) containing PDA medium. Four agar plugs (6 mm in diameter), cut from the actively growing edge of a 7 day-old culture of FOL, were placed at each side of the tested bacterial isolate. Control plates were similarly streaked using SDW. Each individual treatment was replicated four times. After 4 days of incubation at 25°C, diameter of FOL colony was noted. The inhibition rate (IR) of pathogen mycelial growth was calculated according to Tiru et al. (2013) formula: IR% = [(C2-C1) / C2] × 100, where C2: diameter of pathogen colony in control plates and C1: diameter of pathogen colony in treated plates.

For the disc diffusion method, FOL suspension (10^6 conidia/ml) was added (at 1% v/v) to molten PDA pre-cooled to around 45°C. Bacterial cell suspensions (~10^8 cells/ml) were applied as 20 µl droplets injected onto sterile Whatman No. 1. filter paper discs (6 mm in diameter). Four paper discs were used per plate. In control plates, paper discs were treated similarly with SDW. Each individual treatment was replicated four times. After incubation at 25°C for 4 days, the inhibition zones formed around bacterial colonies were measured.

Evaluation of the antifungal activity of cell-free culture filtrate.

The antifungal activity of extracellular metabolites in cell-free culture filtrates of the selected bacterial isolates was assessed according to Karkachi et al. (2010) protocol. Liquid cultures, obtained from bacterial suspension (~10^8 cells/ml) previously grown for 3 days in NB medium at 28 ± 2°C under continuous shaking at 150 rpm, were centrifuged at 10.000 rpm for 10 min. The centrifugation was repeated three times. The cell-free filtrates were sterilized by filtration through a 0.22 µm pore size filter. NB filtrate was used as control treatment. Filtrates were aseptically added (at 20% v/v) to Petri plates containing molten PDA pre-cooled to around 45°C and supplemented with streptomycin sulfate (300 mg/l). This concentration of filtrates was chosen based on previous screening tests in our preliminary experiments (data not shown). After solidification of the mixture, three agar plugs of the phytopathogen (6 mm in diameter) were plated equidistantly in each plate. Each individual treatment was replicated three times. After incubation at 25°C for 4 days, the diameter of FOL colony was measured and the inhibition rate (IR) was calculated as described above.

Evaluation of the antifungal activity of organic extracts.

Organic extraction was performed for only one isolate being the most effective using chloroform (Bhoonobtong et al. 2012) and *n*-butanol (Romero et al. 2007). Sixty milliliters of cell-free culture filtrate of *B. amyloliquefaciens* subsp. *plantarum* SV65 were poured in a separating funnel and 60 ml of solvent (chloroform or *n*-butanol) were added carefully. The funnel was reversed several times by degassing from time to time. The mixture was allowed to settle for few minutes with the

cap open. The organic phase (the lower phase for extraction with chloroform and the upper one with *n*-butanol) was recovered. The aqueous phase was replaced in the funnel and the extraction was repeated two other times. The solvent was evaporated in a rotary evaporator at 35°C for chloroform and 75°C for *n*-butanol with a slight rotation at 150 rpm. The weight of crude extracts obtained using chloroform and *n*-butanol solvents were measured and the yield of each extraction was determined (in mg/ml).

To assess their antifungal activity against FOL, chloroform and *n*-butanol extracts were suspended in ethanol (1:1) (mg/ml) and added separately at two concentrations (2.5 and 5% v/v) to molten PDA medium pre-cooled to about 45°C and amended with streptomycin sulfate (300 mg/l) before being poured in Petri plates. Negative control cultures were treated with similar concentrations of ethanol. Two positive controls carbendazim (Amini and Sidovich, 2010) and *Bacillus thuringiensis* (Choi et al. 2007) were also included in the bioassay used at 2.5 and 5% (v/v) each. After solidification of the mixture, an agar plug (6 mm in diameter) colonized by FOL, removed from 7-day-old cultures, was placed at the center of each plate. Each individual treatment was replicated three times. After incubation for 7 days at 25°C, the diameter FOL colony was measured and the inhibition rate (IR) was calculated as described above.

GC-MS analysis of the most effective organic extract.

The major compounds present in the most bioactive extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 (*n*-butanol extract) were identified using Gas Chromatography coupled to Mass Spectrometry (GC-MS) analysis. This analysis was performed with an Agilent 7890A gas chromatograph equipped with a HP-5MS column (30 m × 0.25 mm; 0.25 µm film thickness), interfaced with an Agilent mass selective detector 5975C inter MSD. Oven temperature program was from 60 to 240°C at 4°C/min; injector temperature was 250°C; helium at 0.8 ml/min was used as carrier gas and interface temperature was 280°C; MS source temperature was 230°C; MS quadrupole temperature was 150°C; mass scan range from 50 to 550 amu at 70 eV; scan velocity was 2.91 scans/s. One microliter of sample was injected. The identification of compounds was performed by comparing the mass spectra with the data from the National Institute of Standards and Technology (NIST) library.

Enzymatic activity of selected endophytic isolates.

The ability of three selected endophytic isolates to produce chitinase, protease and pectinase was checked onto agar plates according to Tiru et al. (2013). Briefly, chitinase activity was assessed on minimum-medium supplemented with chitin® (MP Biomedicals, LLC, IIIKrich, France) by streaking the water bacterial suspensions (~10^8 cells/ml) onto sterilized chitin-agar medium 0.5% (w/v). Control plates contained chitin-agar medium only. Three plates were used for each individual treatment. After 72 h of incubation at 28 ± 2°C, the presence of clearing zones around bacterial colonies was noted.

For the test of protease activity, isolates were streaked on sterilized skim milk agar (SMA, 3% v/v) medium. Control plates contained SMA only. Treatments were performed in triplicate. The diameter of the clear zone formed around the bacterial spots was measured after 48 h of incubation at 28 ± 2°C.

Pectinase production ability was tested by streaking bacterial suspensions (~10^8 cells/ml) onto sterilized NA-pectin®

(ICN Biomedicals, Inc, Germany) medium 0.5% (w/v). Control plates contained the NA-pectin medium only. Treatments were performed in triplicate and after 48 h of incubation at 28 ± 2°C, the presence or the absence of clear zones around bacterial colonies was noted.

Characterization of plant growth-promoting traits.

Indole-3-acetic acid (IAA) production. The ability of the three selected isolates to produce IAA was checked using the colorimetric method described by Glickmann and Dessaux (1995) with some modifications. Liquid cultures, obtained from bacterial suspensions previously grown in Luria-Bertani (LB) medium supplemented with L-tryptophan 50 µg/ml and incubated for 48 h under continuous shaking, were centrifuged at 10.000 rpm for 10 min. Two milliliters of Salkwoski's reagent and 2-3 drops of orthophosphoric acid were added to 1 ml of the culture supernatant. Non-inoculated growth medium was used as negative control. Absorbance was read at 530 nm. Treatments were performed in triplicate. The concentration of IAA was determined and compared to a standard curve prepared from IAA dilution series at 100 µg/ml in LB medium.

Phosphate solubilization ability. The phosphate solubilization ability of the selected bacterial isolates was assessed according to Katzenlson and Bose (1959) with some modifications. An agar plug (6 mm in diameter) removed from bacterial cultures previously grown for 48 h on NA medium, was deposited onto Pikovskaya agar medium. Non-inoculated plates were used as control. Treatments were performed in triplicate. After 7 days of incubation at 28 ± 2°C, the clearing zone formed around colonies was measured.

Siderophore production. The siderophore production ability of the three selected isolates was checked qualitatively according to Lacava et al. (2008). An agar plug (6 mm in diameter) removed from bacterial cultures previously grown for 48 h on NA medium, was plated onto chrome azurol S (CAS) agar medium. Control cultures contained the CAS agar medium only. Three plates were used for each individual treatment. After 5 days of incubation at 28 ± 2°C, the clearing zone (yellow halo) formed around colonies was measured.

Organic acids production.

Preparation of root exudates of tomato. B. amyloliquefaciens subsp. *plantarum* SV65, which had completely suppressed Fusarium wilt and stimulated the growth of tomato plants challenged or not with FOL using their cells suspensions and filtrates cultures (data not shown), was used for this trial.

Tomato seeds cv. Rio Grande were disinfected with sodium hypochlorite (5% v/v) for 3 min and rinsed six times with SDW. Then, they were placed on sterile filter paper moistened with 2 ml of SDW and incubated in the dark at 26°C for 4 days. After germination, homogeneous seedlings were selected and cultured in 27 ml of Hoagland's nutrient solution (50% v/v) supplemented with the bacterial suspension (adjusted at ~10^4 cells/ml), issued from cultures previously grown in LB medium for 48 h. Control seedlings were cultivated in the Hoagland nutrient solution 50% (v/v). Bacterized and control seedlings were grown for 14 days in a growth chamber adjusted at 16 h photoperiod, 65% of relative humidity and air temperatures ranging between 20 and 25°C temperatures (Lugtenberg et al.1999).

Two elementary treatments were tested: (I) seedlings untreated with *B.*

amyloliquefaciens subsp. *plantarum* SV65, and (ii) seedlings treated with *B. amyloliquefaciens* subsp. *plantarum* SV65. Each treatment was replicated 20 times.

Analysis of organic acids by HPLC. The Hoagland's nutrient solution, in which the tomato seedling was grown, was centrifuged at 5000 rpm for 20 min. Root exudates collected from the supernatant were analyzed by high performance liquid chromatography (HPLC). The organic acids used as standards for HPLC analysis are oxalic acid, malic acid, citric acid, and succinic acid. The mobile phase consisted of 90% H_2O and phosphoric acid (H_3PO_4) (pH 2.6) and 10% methanol (MeOH). After the initial equilibration of the column for 1 h, the samples (20 µl each) were eluted for 10 min each at a flow rate of 0.8 ml/min and detected at 216 nm (Kamilova et al. 2006). The standard curve was prepared by a mixture of four organic acids with concentrations ranging from 0.5 to 250 mg/l. The organic acids from each sample were identified by comparing their retention times and peak areas with the acids used as standards.

Data analysis.

Data were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0. Each in vitro and/or in vivo experiment was conducted twice yielding similar results. The in vitro assay of organic extracts was analyzed according to a completely randomized factorial model with two factors (treatments and concentrations). For the other in vitro bioassays, data were analyzed according to a completely randomized design. All in vivo trials were performed following a completely randomized model. Means were separated using LSD or Student Newman Keuls tests to identify significant pair-wise differences at $P \leq 0.05$. For the test of organic acid analysis in root exudates of tomato, means were separated using test-t of Student at $P \leq 0.05$. Correlations between Fusarium wilt severity and plant growth parameters were analyzed using bivariate Pearson's test at $P \leq 0.01$.

RESULTS

Endophytic colonization ability of bacterial isolates recovered from *Solanum nigrum* stems.

Twenty bacterial isolates, exhibiting diversity in their macro-morphological traits on NA medium, were recovered from the internal stem tissues of *S. nigrum* and *S. nigrum* var. *villosum* plants. Among them, 16 isolates were found to be resistant to streptomycin and rifampicin (when grown on NA amended with these antibiotics). Challenged to tomato cv. Rio Grande seedlings, 15 isolates were successfully re-isolated from the internal stem tissues when plated on NA medium amended with these antibiotics. They were classified as endophytes and retained for further screening of their plant growth-promoting and Fusarium wilt-suppressive abilities on tomato plants cv. Rio Grande.

Plant growth-promoting ability displayed by *S. nigrum*-associated endophytic bacteria.

When tested onto pathogen-free tomato seedlings, bacterial treatments had significantly (at $P \leq 0.05$) affected growth parameters (plant height, aerial part fresh weight, maximum root length and root fresh weight) noted 60 days post-treatment. As shown in Table 1, SV60-, SV64-, SV109-, SV68- and SV65- based treatments led to significant increment in plant height over the untreated control

ranging from 29.1 to 37.8 %. The aerial part fresh weight was significantly enhanced from 33.7 to 46.1% by the isolates SV61, SV64, SV60, SV109, SV65 and SV68 in comparison to control.

Assessed for their ability to promote root growth, SV64, SV65, SV68 and SV109 based-treatments had induced significant increase in the maximum root length which ranged from 27.2 to 43.6% compared to control. Three isolates namely SV65, SV68 and SV109 out of the 15 tested had significantly enhanced the root fresh weight by 50.9-54.1% versus control (Table 1).

Table 1. Comparative plant growth-promoting ability of endophytic bacterial isolates recovered from *Solanum nigrum* stems on tomato cv. Rio Grande growth parameters noted 60 days post-treatment

Plant source	Bacterial isolate	Plant height (cm)	Aerial part fresh weight (g)	Maximum root length (cm)	Root fresh weight (g)
	Control	20 ± 0.7 e	11.08 ± 0.4 e	15 ± 0.3 c	4.2 ± 0.2 b
S. nigrum	SV59	22.4 ±1.6 de	13.99 ± 0.7 de	18.8 ± 0.3 bc	5.16 ± 0.5 b
	SV60	28.2 ± 1.1 abcd	17.85 ± 0.3 abcd	20 ± 0.3 bc	5.44 ± 0.5 b
	SV61	25 ± 1.2 bcde	16.72 ± 0.7 abcd	19 ± 1.2 bc	4.64 ± 0.2 b
	SV62	22.2 ± 0.8 de	15.11 ± 0.3 cde	17.6 ± 0.6 bc	4.45 ± 0.2 b
	SV63	23.2 ± 0.8 cde	15.72 ± 0.6 bcde	17 ± 1.4 bc	4.05 ± 0.3 b
	SV64	28.2 ± 1.1 abcd	17.06 ± 0.6 abcd	20.6 ± 1.1 b	5.73 ± 0.3 b
	SV65	32.2 ± 0.8 a	19.90 ± 1 ab	25 ± 0.8 a	8.54 ± 0.3 a
	SV66	22.4 ± 0.6 de	14.97 ± 0.4 cde	16.6 ± 0.7 bc	4.99 ± 0.1 b
	SV68	30.6 ± 1.2 ab	20.57 ± 1.2 a	26.6 ± 0.3 a	8.85 ± 0.3 a
	SV69	24.4 ± 1.3 bcde	15.56 ± 0.4 bcde	18.6 ± 0.3 bc	4.47 ± 0.2 b
	SV70	20.4 ± 0.5 e	13.54 ± 1.1 de	16.4 ± 0.6 bc	4.35 ± 0.5 b
S. nigrum* var. *villosum	SV105	24.4 ± 0.3 bcde	15.46 ± 0.3 bcde	17.6 ± 0.2 bc	5 ± 0.5 b
	SV106	24.4 ± 0.2 bcde	14.75 ± 0.2 cde	17 ± 0.5 bc	4.96 ± 0.2 b
	SV109	29.6 ± 0.4 abc	19.01 ± 0.3 abc	24.8 ± 0.2 a	9.15 ± 0.4 a
	SV111	23.6 ± 1 cde	14.28 ± 0.4 cde	15.8 ± 1.1 bc	4.37 ± 0.3 b

Control: Non-inoculated with pathogen and untreated. For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$. SD: Standard deviation.

Fusarium wilt-suppressive ability displayed by *S. nigrum*-associated endophytic bacteria.

Fusarium wilt severity, noted on tomato plants 30 DPI with FOL, varied significantly (at $P \leq 0.05$) depending on bacterial treatments tested. Significant decreases by 77.3 to 88.9 % in the leaf damage index (chlorosis and/or necrosis) and by 88 to 92% and in the vascular browning extent were noted on tomato plants already challenged with FOL and treated with the three isolates SV109, SV68 and SV65 as compared to FOL-inoculated and untreated control (Table 2).

Also, FOL re-isolation frequency onto PDA medium was lowered by 20 to 100% relative to positive control when performed for stems tissues issues from tomato plants challenged with FOL and treated with bacterial isolates. The highest decrease (100%) in this parameter was achieved using the isolates SV65, SV68 and SV109 (Table 2).

Furthermore, as given in Table 2, SV109-, SV68- and SV65-based treatments had significantly improved plant height and plant fresh weight by 36.6-38.1% and 61.1-62.4%, respectively, if compared to the FOL-inoculated and

untreated control. It should be also highlighted that tomato plants infected with FOL and treated with these isolates showed significantly similar growth as disease-free and untreated control ones.

Correlation analysis between Fusarium wilt severity and plant growth parameters.

Pearson's correlation analysis revealed that the decrease in Fusarium wilt severity, as estimated by the leaf damage index (and/or necrosis) and the vascular browning extent, led to an increment in all plant growth parameters. In fact, plant height was significantly and negatively correlated to the leaf damage index (r = -0.832, P = 3.444 E-5) and to the vascular browning extent (r = -0.904, P = 6.182 E-7). Furthermore, the plant fresh weight was significantly and negatively linked to leaf chlorosis score (r = -0.928, P = 7.464 E-8) and to the vascular browning extent (r = -0.811, P = 7.55 E-5). Similar trend was observed between FOL re-isolation frequency and plant growth parameters where significant and negative correlations were recorded between FOL re-isolation frequency, plant height (r = -0.739, P = 0.0006), and whole plant fresh weight (r = -0.877, P = 3.806 E-6).

Table 2. Effects of endophytic bacterial isolates recovered from *Solanum nigrum* stems on Fusarium wilt severity, plant growth parameters and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) re-isolation frequency from tomato cv. Rio Grande plants, recorded 30 days post-inoculation with FOL as compared to controls.

Plant source	Bacterial isolate	Disease severity (0-4)	Vascular browning extent (cm)	Plant height (cm)	Plant fresh weight (g)	FOL re-isolation* (%)
	NIC	0 ± 0 c	0 ± 0 b	8 ± 0.3 abc	3.43 ± 0.1 a	0
	IC	3.6 ± 0.2 a	5 ± 0.4 a	5.2 ± 0.4 e	1.30 ± 0 d	100
Solanum nigrum	SV59	3 ± 0.3 a	4.8 ± 0.2 a	4.8 ± 0.1 e	1.65 ± 0.1 cd	60
	SV60	2.8 ± 0.4 a	5.4 ± 0.1 a	5.6 ± 0.5 de	2.82 ± 0.1 abc	70
	SV61	2.2 ± 0.4 ab	5 ± 0.1 a	5.2 ± 0.6 e	2.51 ± 0.2 abcd	80
	SV62	2.6 ± 0.3 a	3.9 ± 0.3 a	6.1 ± 0.3 bcde	2.28 ± 0.2 abcd	70
	SV63	2.6 ± 0.5 a	5 ± 0.4 a	6.2 ± 0.2 bcde	2.37 ± 0.1 abcd	80
	SV64	4 ± 0 a	4.6 ± 0.5 a	5.9 ± 0.5 cde	1.29 ± 0.2 d	70
	SV65	0.4 ± 0.2 c	0.4 ± 0.2 b	8.4 ± 0.2 a	3.35 ± 0 ab	0
	SV66	2.2 ± 0.2 ab	4.7 ± 0.2 a	6 ± 0.2 bcde	1.99 ± 0.1 bcd	70
	SV68	0.6 ± 0.2 c	0.6 ± 0.3 b	8.2 ± 0.2 abc	3.47 ± 0.1 a	0
	SV69	2.6 ± 0.3 a	5.4 ± 0.2 a	6.6 ± 0.4 abcde	2.12 ± 0.3 abcd	60
	SV70	2.6 ± 0.5 a	3.8 ± 0.2 a	6 ± 0.1 bcde	2.45 ± 0.1 abcd	50
S. nigrum* var. *villosum	SV105	3.4 ± 0.4 a	5.2 ± 0.2 a	5.8 ± 0.4 cde	1.96 ± 0.2 bcd	50
	SV106	4 ± 0 a	5.2 ± 0.4 a	5.8 ± 0.3 cde	1.26 ± 0.2 d	40
	SV109	0.8 ± 0.2 bc	0.6 ± 0.3 b	8.3 ± 0.2 ab	3.36 ± 0.1 ab	0
	SV111	4 ± 0 a	5.5 ± 0.1 a	6 ± 0.1 bcde	1.65 ± 0.3 cd	80

NIC: Non-inoculated with 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 $P \leq 0.05$. SD: Standard deviation.

*The re-isolation of FOL was carried out from the stems of tomato plants cv. Rio Grande at 0-15 cm height from the collar. Ten stem fragments were plated on Potato Dextrose Agar (PDA) medium and incubated at 25°C for 4 days.

Characterization and identification of the bioactive bacterial isolates.

The three bioactive isolates selected above (SV65, SV68, and SV109) based on their effectiveness in suppressing Fusarium wilt severity and in improving tomato growth even in plants challenged with FOL were morphologically and biochemically characterized (Table 3). These isolates were also checked for their hypersensitivity reaction and their hemolytic activity and were found negative for these both tests (Table 3).

Table 3. Characterization of the most effective endophytic bacterial isolates from *Solanum nigrum* stems on Fusarium wilt suppressive and plant growth-promoting.

Bacterial characterization	Bacterial isolates		
	SV65	SV68	SV109
Morphological characterization			
Form	Irregular	Irregular	Circular
Margin	Lobed	Irregular	Undulate
Elevation	Humped	Convex	Humped
Surface	Rough	Rough	Smooth
Opacity	Opaque	Opaque	Opaque
Color	White	White	White
Gram's staining	Positive	Positive	Positive
Biochemical characterization			
King A	-	-	-
Catalase	+	+	+
Urease	-	-	+
Lecithinase	-	-	+
Nitrate reductase	+	+	+
Tryptophane deaminase	-	+	-
Lysine decarboxylase	-	-	-
Mannitol	+	+	-
Simmons citrate	-	+	+
Indole	+	+	+
Red of Methyl	-	-	-
Vosges-Proskauer	+	+	+
Hydrogen sulfide	-	-	-
Gaz production	+	+	+
Lactose	-	+	-
Glucose	-	-	-
Hypersensitivity reaction			
	-	-	-
Hemolytic activity			
	-	-	-

-: Negative test; +: Positive test; Lactose (+/-): (fermentation/utilization); Glucose (+/-): (fermentation/utilization).

Blast-N analysis of sequenced 16S rDNA gene similarity revealed that the three endophytic isolates belonged to the genus of *Bacillus* with 99.6% of similarity to *B. amyloliquefaciens* subsp. *plantarum* Hs-18, 99.8% to *B. methylotrophicus* Ha13 and 100 % of similarity to *B. methylotrophicus* Nk5-1with isolates SV65, SV68 and SV109, respectively (Table 4). The phylogenetic

analysis of the aligned 16S rDNA sequences of isolates SV65, SV68 and SV109 and the most related species revealed a short distance between the tested isolates and *Bacillus* species indicated above (Fig. 1). Sequences of SV65, SV68 and SV109 were submitted to GenBank and assigned the following accession numbers: KR818073, KR818074 and KR818076, respectively (Table 4). Based on EzTaxon, the strain SV65 showed a similarity of 99.45% with *Bacillus siamensis* KCTC13613(T) and the strains SV68 and SV109 belonged to *Bacillus velezensis* CR502(T) with 99.62 and 99.27% of similarity, respectively.

Table 4. Identification of the most effective endophytic bacterial isolates from *Solanum nigrum* stems by 16S rDNA sequencing genes

Isolate	Accession number	Most related species	Sequence similarity (%)
SV65	KR818073	*Bacillus amyloliquefaciens* subsp. *plantarum* Hs-18	99.6
SV68	KR818074	*Bacillus methylotrophicus* Ha13	99.8
SV109	KR818076	*Bacillus methylotrophicus* Nk5-1	100

SV65 and SV68: Bacterial isolates recovered from internal stem tissues of *Solanum nigrum*.
SV109: Bacterial isolate issued from internal stem tissues of *S. nigrum* var. *villosum.*

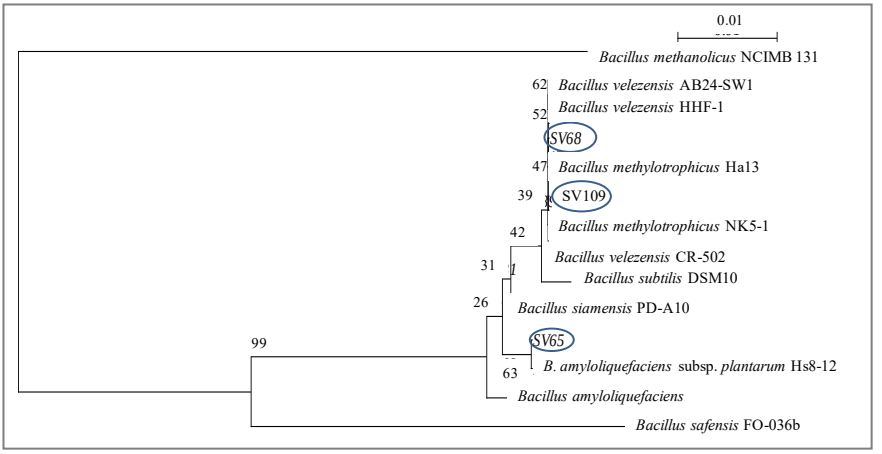

Fig. 1. Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of the endophytic bacterial isolates SV65, SV68 and SV109 recovered from *Solanum nigrum* and *S. nigrum* var. *villosum* and their closest phylogenetic relatives.
The nucleotide sequences used of representative strains were obtained from Genbank database under the following accession numbers: JF899271 (*B. amyloliquefaciens* subsp. *plantarum* Hs8-12), JF899290 (*B. methylotrophicus* Ha13), HQ831395 (*B. methylotrophicus* Nk5-1),AY603658 (*B. velezensis*CR-502), AB112727 (*B. methanolicus* NCIMB), AB006920 (*B. amyloliquefaciens*), GQ281299 (*B. siamensis* PD-A10), AJ276351 (*B. subtilis* DSM10), AF234854 (*B. safensis* FO-036b), MG757677 (*B. velezensis* HHF-1), MG890226 (*B. velezensis* AB24-SW), and for the bacterial isolates tested: KR818073 (SV65), KR818074 (SV68) and KR818076 (SV109). The tree topology was constructed using ClustalX (1.81).

Mycelial growth inhibitory ability displayed by *Bacillus* spp. associated to *S. nigrum*.

Antifungal activity of* Bacillus *spp. whole cell suspensions. Tested using the streak method, metabolites present in the whole cell suspension of tested *Bacillus* spp. isolates induced a significant ($P \leq 0.05$) decrease in FOL mycelial growth, noted after 4 days of incubation at 25°C, as compared to the untreated control (Table 5). Phytopathogen growth was lowered by 40.404 to 55.555%, relative to control, when dual cultured with the three isolates of *B. methylotrophicus* SV109, *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV68, respectively. Tested using the disc diffusion method, the three isolates of *Bacillus* formed, after 4 days of incubation at 25°C, an inhibition zone against FOL growth of about 12-12.62 mm (Table 5).

Antifungal activity of* Bacillus *spp. cell-free culture filtrates. ANOVA revealed a significant ($P \leq 0.05$) variation in the colony diameter of FOL depending on cell-free cultures filtrates of *Bacillus* spp. tested and used at 20% (v/v). The FOL growth inhibition (58.7-62%) was obtained using the extracellular metabolites from *B. methylotrophicus* SV68, *B. methylotrophicus* SV109 and *B. amyloliquefaciens* subsp. *plantarum* SV65 (Table 5).

Table 5. Antifungal activity of *Bacillus* spp. isolates and their extracellular metabolites against *Fusarium oxysporum* f. sp. *lycopersici* noted after 4 days of incubation at 25°C

Bacterial treatment	Whole cell suspension (~10^8 cells/ml)		Extracellular metabolites (20 % v/v)
	Colony diameter (cm) and growth inhibition of FOL (%)	Inhibition zone (mm)	Colony diameter (cm) and growth inhibition of FOL (%)
Untreated control	3.71 ± 0.08 a	0 b	3.63 ± 0.02 a
***B. amyloliquefaciens* subsp. *plantarum* SV65 (KR818073)**	1.98 ± 0.1 bc (46.5)	12.62 ± 1.4 a	1.38 ± 0.08 b (62)
***B. methylotrophicus* SV68 (KR818074)**	1.65 ± 0.08 c (55.5)	12.5 ± 0.7 a	1.5 ± 0.1 b (58.7)
***B. methyoltrophicus*SV109 (KR818076)**	2.21 ± 0.09 b (40.4)	12 ± 0.3 a	1.45 ± 0.2 b (60)

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 parenthesis indicate the percentage (in %) of the mycelial growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* as compared to the untreated control.

Antifungal activity of organic extracts from *Bacillus amyloliquefaciens* subsp. *plantarum* SV65.

The *n*-butanol extraction performed for the isolate *B. amyloliquefaciens* subsp. *plantarum* SV65 yielded 373 mg of dry residues (estimated at 6.22 mg/ml) compared only to 26 mg (0.43 mg/ml) using the chloroform extraction.

Analysis of variance revealed a significant ($P \leq 0.05$) variation in the average diameter of FOL colonies depending on organic extracts tested (chloroform and *n*-butanol extracts) and concentrations used, and their interactions.

Results shown in Fig. 2 indicated that chloroform and *n*-butanol extracts from *B. amyloliquefaciens* subsp. *plantarum* SV65, used at 1 mg/ml, had inhibited FOL growth by 43.4-73% and 61.1-90% as compared to ethanol-treated control whatever the concentration used, respectively. Both organic extracts from *B. amyloliquefaciens* subsp. *plantarum* SV65 were more active when used at 5% than at 2.5% (v/v) which reduced FOL growth was reduced by 43.3-61.1% and 73-90%, respectively (Fig. 2). The highest inhibition, of about 90%, was achieved using the *n*-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 at 5% (v/v).

The decrease in FOL growth was significantly higher with the chloroform and *n*-butanol extracts from *B. amyloliquefaciens* subsp. *plantarum* SV65 used at 5% (v/v) (73 and 90%, respectively) compared to carbendazim- (39.5%) and *B. thuringiensis*-based treatments (43.2%). Tested at 2.5% (v/v), the *n*-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 was more effective (61.1%) than the two commercial used products (31.3-40.9%). The chloroform extract from *B. amyloliquefaciens* subsp. *plantarum* SV65, used at 2.5% (v/v), had significantly reduced FOL radial growth by 43.4% and was more effective than the chemical fungicide (31.3%) (Fig. 2).

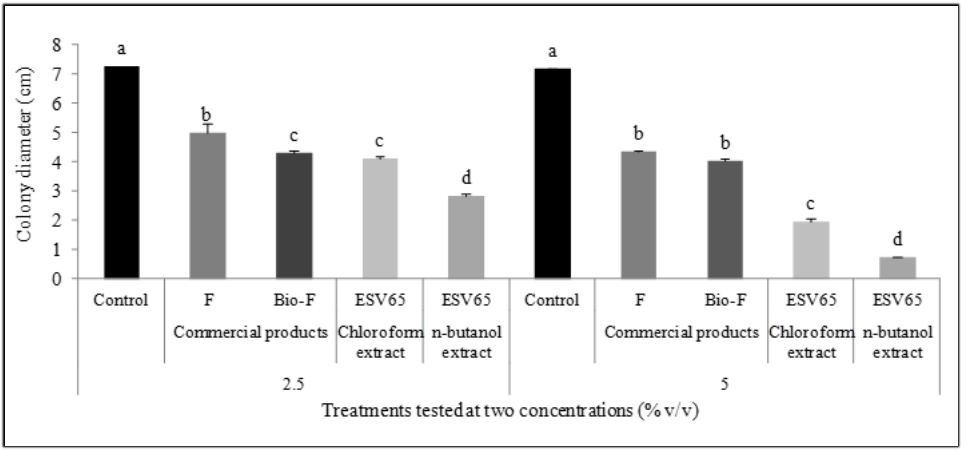

Fig. 2. Effect of chloroform and *n*-butanol extracts from endophytic *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 tested at two concentrations on *Fusarium oxysporum* f. sp. *lycopersici* mycelial growth noted after 7 days of incubation at 25°C as compared to positive and negative controls.
ESV65: Organic extract from *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 (KR818073) isolated from internal tissues stems of *Solanum nigrum*. Control: Ethanol control. F: Carbendazim used at 2.5 and 5% (v/v); Bio-F: *Bacillus thuringiensis* used at 2.5 and 5% (v/v). LSD (Treatments tested × Concentrations used): 0.74 cm at $P \leq 0.05$. For each concentration, bars sharing the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$.

GC-MS analysis of the *n*-butanol extract from *Bacillus amyloliquefaciens* subsp. *plantarum* SV65.

GC-MS analysis of the most bioactive extract (*n*-butanol) from *B. amyloliquefaciens* subsp. *plantarum*SV65 detected 9 compounds. The peak noted at

the retention time 47.398 min with high percentage area of about 33.2% corresponded to the phthalic acid, mono(2-ethylhexyl)ester (Table 6). The other compounds identified, including 2-methyl butanoic acid, 3-(P-T-butyl)-2-methyl propane-1-thiol, tetradecamethyl-heptasiloxane, tetracosamethyl-cyclododecasiloxane, *n*-benzyl-*n*-ethyl-4-isopropylbenzamide, pentanoic acid, silicate anion tetramer and 3-isobutylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione were detected at 15.47, 10.18, 10.02, 8.76, 6.67, 5.47, 3.55 and 3.69%, respectively (Table 6).

Table 6. Major compounds identified in the *n*-butanol extract from the endophytic *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 (KR818073) isolated from *Solanum nigrum* stems by gas chromatography-mass spectrometry analysis

Peak	Retention time (min)	Area (%)	Name
1	3.527	5.47	Pentanoic acid
2	3.711	15.47	2-Methyl butanoic acid
3	34.077	3.55	3-Isobutylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione
4	38.924	3.69	Silicate anion tetramer
5	42.116	6.67	n-Benzyl-n-ethyl-4-isopropylbenzamide
6	45.052	8.76	Tetracosamethyl-cyclododecasiloxane
7	47.398	33.20	Phthalic acid, mono (2-ethylhexyl) ester
8	47.821	10.18	3-(P-T-butyl)-2-methyl propane-1-Thiol
9	51.020	10.02	1,1,3,3,5,5,7,7,9,9,11,11,13,13-Tetradecamethyl - heptasiloxane

Enzymatic activity of endophytic *Bacillus* spp. associated to *Solanum nigrum*.

B. amyloliquefaciens subsp. *plantarum* SV65, *B. methylotrophicus* SV68 and *B. methylotrophicus* SV109 formed clear zones around their colonies when grown on chitin- and pectin-agar medium. This indicates that the three isolates of *Bacillus* spp. tested are able to produce chitinase and pectinase, respectively (Table 7).

Transferred on skim milk-agar medium, clear zones (of 9 and 36.33 mm in diameter) were formed around *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV109 colonies, respectively. Thus, these two *Bacillus* strains were found to be protease-producing agents. However, *B. methylotrophicus* SV68 cannot produce protease as no clear zones were observed (Table 7).

Plant growth-promoting traits expressed in *Bacillus* spp. associated to *Solanum nigrum*.

Indole-3-acetic acid production. When assessed for their ability to produce phytohormones, the three selected *Bacillus* spp. isolates were shown able to produce the indole-3-acetic acid (IAA), involved in plant growth promotion, after 24 and 48 h of incubation (Table 7). This production of IAA rose from 0.11-2.28 µg/ml after 24 h of incubation to 15.48-28.49 µg/ml after 48 h. The highest production of IAA, estimated at 28.49 µg/ml, was recorded after 48 h of incubation using *B. methylotrophicus* SV68.

Phosphate solubilization ability. Data shown in Table 7 indicated the ability of *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV109 to solubilize phosphate as expressed by the formation of a clear zone of about 9.5 and 10.5 mm in diameter around their colonies, respectively. However, *B. methylotrophicus* SV68 did not produce phosphatase on Pikovskaya agar medium.

Siderophores production. Regarding their capacity to produce siderophores, *B. amyloliquefaciens* subsp. *plantarum* SV65 grown in CAS agar medium for 5 days showed ability to synthesize these compounds (Table 7) as indicated by the presence of yellow color of about 23.33 mm in diameter around its colonies.

Table 7. Enzymatic activity and plant-growth promoting traits of endophytic *Bacillus* spp. Isolates recovered from *Solanum nigrum* stems

Isolate	Enzymatic activity			PGPB ability		
	Chitinase[a]	Protease[b]	Pectinase[c]	Siderophore production[d]	IAA production[e]	Phosphate solubilization[f]
***B. amyloliquefaciens* subsp. *plantarum* SV65 (KR818073)**	+	+	+	+	+[v]	+[y]
***B. methylotrophicus* SV68 (KR818074)**	+	-	+	nt	+[w]	-
***B. methylotrophicus* SV109 (KR818076)**	+	++	+	nt	+[x]	+[z]

-: No metabolite production; +: Metabolite production; nt: Not tested.

[a] Tested on chitin-agar (0.5 % w/v) medium and incubated at 28 ± 2 °C for 72 h.

[b] Tested on skim milk agar (3 % v/v) medium and incubated at 28 ± 2 °C for 48 h; +: Presence of clear zone (9 mm in diameter), ++: Presence of clear zone (36.33 mm in diameter).

[c] Tested on pectin-agar (0.5 % w/v) medium and incubated at 28 ± 2 °C for 48 h.

[d] Tested on Chrome Azurol Sulfonate (CAS) agar medium and incubated at 28 ± 2 °C for 5 days; +: Presence of yellow color: 23.3 mm.

[e] IAA (Indole-3-acetic acid) production after 24 and 48 h of incubation at 28 ± 2 °C in Luria-Broth medium; [v,w, x]: Production of IAA (2.28 and 18.11, 0.62 and 28.49, 0.11 and 15.48 µg/ml, respectively).

[f] Tested on Pikovskaya agar medium and incubated at 28 ± 2 °C for 7 days; [y, z]: Presence of clear zone (9.5, 10.5 mm in diameter, respectively).

Organic acid production. HPLC analysis performed for root exudates from tomato plants treated with *B. amyloliquefaciens* subsp. *plantarum* SV65 showed the presence of oxalic acid and malic acid (Table 8).

A significant production (about 4448.75 mg/l) of oxalic acid was recorded in the root exudates of plants treated with *B. amyloliquefaciens* subsp. *plantarum* SV65 as compared to the untreated ones (3587.58 mg/l). The production of malic acid in root exudates of treated tomato plants was of about 18.03 mg/l while no production of this acid was detected in root exudates from untreated control plants. Succinic acid and citric acid were not released by roots of all tomato plants treated or not with *B. amyloliquefaciens* subsp. *plantarum* SV65 (Table 8).

Table 8. Amounts of organic acids detected in root exudates[x] of tomato cv. Rio Grande treated with endophytic *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 recovered from *Solanum nigrum* stems compared to the untreated control

Treatment tested	Oxalic acid (mg/l)	Malic acid (mg/l)	Citric acid (mg/l)	Succinic acid (mg/l)
Control	3587.58 ± 126.66	0 ± 0	0	0
***B. amyloliquefaciens* subsp. *plantarum* SV65**	4448.73* ± 56.24	18.03* ± 4.46	0	0

[x] Root exudates were collected after 14 days from tomato plants cultivated in Hoagland solution (50 %). Results are the mean of two high-performance liquid chromatography analyses. SD = Standard deviation. Values with asterisk are significantly different to the control according to test-t of Student at $P \leq 0.05$.

DISCUSSION

Endophytic bacteria have been widely explored as potent biocontrol agents effective against vascular wilt diseases on various crops (Chen et al. 1995; Eleftherios et al. 2004; Ramyabharathi and Raguchander 2014). Furthermore, they were found to be excellent plant growth promoters on several crops such as sugarcane (Marcos et al. 2016) and tomato (Botta et al. 2013).

In the current study, out of 20 isolates recovered from *S. nigrum* and *S. nigrum* var. *villosum* stems, 15 isolates formed endophytic populations on tomato plants cv. Rio Grande. The present study clearly demonstrated the strong Fusarium wilt suppressive ability displayed by three isolates namely SV65, SV68 and SV109. Blast similarity analyses of 16S DNAr sequencing gene revealed that SV65 belonged to *B. amyloliquefaciens* subsp. *plantarum* with 99.6% of similarity compared to 99.45% of similarity to *B. siamensis* noted using Ez-cloud taxon server. Also, based on NCBI database, SV68 and SV109 isolates showed 99.8 and 100% of similarity with *B. methylotrophicus* compared to 99.62 and 99.27% of similarity with *B. velezensis* recorded using Ez-cloud taxon server.

The three selected isolates were also shown to be able to enhance plant growth on tomato plants inoculated or not with FOL. In other studies, various endophytic *Bacillus* species were recovered from *N. attenuata*, *S. trilobatum* (Bhuvaneswari et al. 2013; Santhanam et al. 2014), *S. melongena* and *S. torvum* (Achari and Ramesh 2014) but they were not tested against FOL nor for their plant growth promoting ability onto tomato plants. In Nawangsih et al. (2011) study, an endophytic *B. amyloliquefaciens* JK-SD002 strain, recovered from tomato stems, was shown to be able to improve height of inoculated tomato plants. Similarly, Algam et al. (2005) found that endophytic *Brevibacillus brevis* B2 and *B. subtilis* (B5, B7 and B8), originally isolated from tomato rhizosphere, had enhanced growth of tomato and had successfully controlled the bacterial wilt disease caused by *Ralstonia solanacearum*. As confirmed through Pearson's correlation analysis, the decrease in Fusarium wilt severity was related to the lowered FOL colonization of vascular tissues leading to the enhancement of plant growth. In the same regards, Ramyabharathi and Raguchander (2014) found that *Bacillus subtilis* EPC016-treated tomato plants grown in presence of FOL showed lowered infection and enhanced growth. In our previous studies, Fusarium wilt-suppressive effect was associated to tomato growth promotion using *Bacillus*, *Stenotrophomonas*, *Pseudomonas* and *Alcaligenes* species recovered from the explored wild Solanaceae plants (Aydi

Ben Abdallah et al. 2016a, 2016b, 2016c, 2016d, 2016e).

The three endophytic *Bacillus* spp. isolates selected in the present study (*B. amyloliquefaciens* subsp. *plantarum* SV65, *B. methylotrophicus* SV68, and *B. methylotrophicus* SV109) were assessed in vitro for their antifungal activity towards FOL. In fact, when tested as whole cell cultures, the three *Bacillus* spp. isolates had inhibited FOL mycelial growth and induced the formation of an inhibition zone. Similar findings were reported for various endophytic *B. mojavensis* isolates inhibiting *F. moniliforme* radial growth through the formation or not of an inhibition zone depending on isolates (Bacon and Hinton 2002). Extracellular metabolites present in the cell-free culture filtrates from *B. amyloliquefaciens* subsp. *plantarum* SV65, *B. methylotrophicus* SV68, and *B. methylotrophicus* SV109 and other *Bacillus* species (*Bacillus* sp. SV101, *B. cereus* S42 and *B. tequilensis* SV104) (Aydi Ben Abdallah et al. 2016b, 2016f) were found to be effective in suppressing FOL in vitro growth. Other studies also reported the inhibitory effects of secondary metabolites from endophytic *Bacillus* spp. being active against *Fusarium* spp. and other plant pathogens (Li et al. 2012).

The inhibitory effect on FOL radial growth may be due in part to the production of hydrolytic enzymes such as chitinases and/or proteases as shown on chitin- and skim milk- agar media. Ability to synthesize hydrolytic enzymes seemed to vary depending on isolates as shown for *B. methylotrophicus* SV68 which failed to produce proteases. Sgroy et al. (2009) also demonstrated variation in protease production between endophytic *Bacillus* species. They found that *B. licheniformis* Ps14 was negative for protease production whereas *B. subtilis* Ps8 and *B. pumilus* Ps19 were positive. Chitinases may be released by various endophytic *Bacillus* spp. isolates (Berg et al. 2005). However, endophytic *B. velezensis*, *B. mojavensis*, *B. amyloliquefaciens*, and *B. methylotrophicus* isolates recovered from *Citrus* plants were negative for the chitinase activity (Kalai-Grami et al. 2014).

In the present study, we investigated the chemical composition of the most bioactive organic extracts against FOL in order to search for new bioactive metabolites from endophytic *Bacillus* isolates. Chloroform and *n*-butanol extractions were performed for extracellular metabolites present in the cell-free culture of *B. amyloliquefaciens* subsp. *plantarum* SV65. The *n*-butanol extract was found to be more effective than the chloroformic one where FOL growth was inhibited by 61-90% whatever the concentration used. In the same sense, the *n*-butanol extract from endophytic *B. subtilis* ZZ120 (applied at 1 mg/ml) led to 61.4% decrease in *F. graminearum* growth (Li et al. 2012). Identification of the chemical compounds in the *n*-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 led to the detection of phthalic acid, mono (2-ethylhexyl) ester as major compound. A similar compound was also excreted by an endophytic fungus *Aspergillus flavipes* which displayed antifungal activity against *Sclerotinia sclerotiorum* (Verma et al. 2014). The phthalic acid, bis (2-ethylhexyl) was also produced by *Tsukamurella inchonensis* and *Corynebacterium nitrilophilus* exhibiting antifungal potential toward *Alternaria solani*, *F. oxysporum* and *Penicillium digitatum* (El-Mehalawy et al. 2008). Furthermore, Aydi Ben Abdallah et al. (2016f) reported the presence of phthalic acid dibutyl ester in the chloroform extract from endophytic *B. cereus* S42 isolated from *N. glauca* stems.

Similarly, phthalic acid was also detected in filtrates of an endophytic bacterium (*B. cereus*) isolated from *Azadirachta indica* (Kumar et al. 2015). Indeed, the phthalic acid dibutyl ester and fatty acids are produced by endophytic *B. subtilis* recovered from cotton and exhibiting antifungal activity against FOL (Ramyabharathi and Raguchander 2014). In the current study, the other compounds identified in the *n*-butanol extract from *B. velezensis* SV65 included 2-methyl butanoic acid, 3-(P-T-butyl)-2-methyl propane-1-thiol, tetradecamethyl-hepta siloxane, tetracosamethyl-cyclododeca siloxane, n-benzyl-n-ethyl-4-isopropyl benzamide, pentanoic acid, silicate anion tetramer and 3-isobutylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione. These secondary metabolites may be also involved in the antifungal activity recorded towards FOL. In other previous works, *Bacillus* spp. extracts showed the presence of chemical substances belonging to the families of aldheydes, ketones, benzenes (Yuan et al. 2012) and dimethyl disulfide (Huang 2012).

The ability to produce siderophores has been widely reported to be involved in antagonism through competition for iron with phytopathogenic agents during their progression within host tissues (Bacon and Hinton 2011) and/or stimulation of plant growth through iron supply (Bar-Ness et al. 1992). *B. amyloliquefaciens* subsp. *plantarum* SV65 was able to produce siderophores. Several *Bacillus* species including *B.amyloliquefaciens* isolated from three *Citrus* species are also capable to produce siderophores (Kalai-Grami et al. 2014). In addition, *B. tequilensis* has been demonstrated by Verma *et al*. (2014) as a siderophore-producing agent, by forming a clear zone of 22.5 mm in size compared to 23.3 mm induced by our isolate *B. amyloliquefaciens* subsp. *plantarum* SV65.

Dastager et al. (2011) found that black pepper seeds treated with *B. tequilensis* NII-0943, a siderophore and IAA producing agent, showed higher root initiation in inoculated plants compared to untreated ones. *B. methylotrophicus* SV109, *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV68 selected in the present study as the most active plant growth promoting agents were found able to produce IAA at 15.48, 18.11 and 28.49 µg/ml, respectively. These amounts are higher than those produced by *B. thuringiensis*, *B. subtilis*, *B. arbutinivorans* and *B. fusiformis* (ranging from 4 to 14.46 mg/l) but less than that released by *B. megateruim* i.e. > 50 mg/l (Wang et al. 2013).

Solubilization of bound soil phosphorus and plant supply with phosphate are also involved in plant growth-promoting traits. In the current study, phosphate solubilization ability was confirmed for *B. methylotrophicus* SV109 and *B. amyloliquefaciens* subsp. *plantarum* SV65. Some endophytic bacteria recovered from *Citrus* species namely *B. methylotrophicus*, *B. velezensis* and *B. mojavensis* were also able to solubilize phosphate (Kalai-Grami et al. 2014).

The three selected endophytic *Bacillus* spp. isolates were shown to be able to produce pectinase and this potential may be also involved in the recorded enhancement of tomato growth as reported by Baldan et al. (2003) and in the endophytic colonization of host plant (Hallmann et al. 1997). It should be also mentioned that pectinolytic enzymes act normally as virulence factors for plant pathogenic bacteria but in case of endophytic microorganisms, they might play a role in invasion of host plants by endophytes as demonstrated for *B. cereus*,

B. subtilis and *B. stearothermophilus* (Torimiro and Okonji 2013).

Other substances are detected in root exudates such as organic acids, sugars and amino acid. These allelochemicals may be involved in the promotion of plant growth (Kamilova et al. 2006). Composition of root exudates induced chemotactic responses for endophytic bacteria for early colonization of plant tissues (Bacilio-Jiménez et al. 2003). In the current study, chemical analysis of root exudates from tomato plants treated by *B. amyloliquefaciens* subsp. *plantarum* SV65 revealed the presence of oxalic acid and malic acid which may play a key role in plant colonization. In fact, chemotaxis through root exudates such as malic acid and citric acid is crucial for the colonization of tomato roots by *Pseudomonas* (De Weert et al. 2007). Yuan et al. (2015) assessed the role of banana root exudates (especially oxalic acid, malic acid and fumaric acid) in the colonization of *B. amyloliquefaciens* NJN-6.

In conclusion, *S. nigrum* and *S. nigrum* var. *villosum* were potentially important sources for isolation of endophytic bacteria serving as biocontrol and biofertilizing agents in the production of tomato on both FOL infested and non-infested soils. The most effective agents were identified as *B. amyloliquefaciens* subsp. *plantarum* SV65 (KR818073), *B. methylotrophicus* SV68 (KR818074) and *B. methylotrophicus* SV109 (KR818076). These isolates displayed interesting enzymatic activity. Their plant growth-promoting traits and colonization ability were achieved through IAA and siderophores production, phosphate solubilization, and release of organic acids in root exudates. *B. amyloliquefaciens* subsp. *plantarum* SV65, recovered from *S. nigrum* stems, was considered as a novel biologically source of phthalic acid, mono (2-ethylhexyl) ester. The extracellular metabolites from these *Bacillus* spp. isolates will be further tested for their in vivo antifungal activity against FOL and probably their ability to induce systemic resistance and to promote growth.

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RESUME

Aydi-Ben Abdallah R., Jabnoun-Khiareddine H., Nefzi A., Ayed F. et Daami-Remadi, M. 2018. Des bactéries endophytes issues de *Solanum nigrum* ayant des capacités promotrices de la croissance des plantes et suppressives de la fusariose vasculaire de la tomate. Tunisian Journal of Plant Protection 13 (2): 157-182.

Quinze isolats bactériens endophytes issus des tiges de *Solanum nigrum* et *S. nigrum* var. *villosum* ont été criblés pour leur potentiel promoteur de la croissance et leur activité antifongique contre *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Les isolats SV65, SV68 et SV109 se sont montrés les plus efficaces dans le contrôle de la maladie (77-92%) et dans l'amélioration de la croissance de la tomate (32-62%)

comparés aux témoins. Ils ont été caractérisés et identifiés après séquençage de leurs gènes d'ADNr 16S comme étant *Bacillus amyloliquefaciens* subsp. *plantarum* pour l'isolat SV65 (KR818073), *B. methylotrophicus* pour les deux isolats SV68 (KR818074) et SV109 (KR818076). L'analyse par chromatographie en phase gazeuse et spectrométrie de masse de l'extrait butanolique de *B. amyloliquefaciens* subsp. *plantarum* SV65 a montré que l'ester mono (2-éthylhexyl) de l'acide phtalique est un composé majeur. Les bactéries *B. amyloliquefaciens* subsp. *plantarum* SV65 et *B. methylotrophicus* SV109 se sont révélés être des agents productifs des chitinases, des protéases, des pectinases, des phosphatases et de l'acide indole 3-acétique (AIA). *B. methylotrophicus* SV68 a produit des chitinases, des pectinases et de l'AIA (28,49 µg/ml), et *B. amyloliquefaciens* subsp. *plantarum* SV65 a excrété des sidérophores, de l'acide oxalique et de l'aide malique. Cette étude a démontré que *S. nigrum* et *S. nigrum* var. *villosum* peut être de bonne espèce de plante pour l'isolement de bactéries endophytes servant d'agents de lutte biologique et de biofertilisant pour l'amélioration de la croissance et la production de tomates cultivées dans des sols infestés et non infestés par FOL.

Mots-clés: *Bacillus* spp., biocontrôle, *Fusarium oxysporum* f. sp. *lycopersici*, *Solanum nigrum*, métabolites, croissance de la tomate

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ملخص

العايدي-بن عبد الله، رانية وهيفاء جبنون-خيار الدين وأحلام النفزي وفاخر عياد وماجدة الدعمي-الرمادي. 2018. بكتيريا داخلية نباتية مجمّعة من نبتة *Solanum nigrum* ذات قدرة على تحثيث نمو النباتات وعلى الحدّ من الذبول الفوزاري على الطماطم. Tunisian Journal of Plant Protection 13 (2): 157-182.

تمّ تقييم قدرة خمسة عشرعزلة بكتيرية داخلية نباتية، مجمّعة من جذوع نبتتيْ *Solanum nigrum* و *S. nigrum* var. *villosum*، على تحثيث نموّ النباتات وعلى المكافحة الحيوية ضد *Fusarium oxysporum* f. sp. *lycopersici* (FOL). كانت العزلات SV65، SV68 وSV109 الأكثر فعالية في السيطرة على المرض (77-92%) وتحثيث نمو الطماطم (32-62%) مقارنة بالشواهد. تمّ تشخيص هذه العزلات وتمّ تحديد انتمائها باستخدام تسلسل جينات الحمض النووي 16S إلى *Bacillus amyloliquefaciens* subsp. *plantarum* بالنسبة للعزلة SV65 (KR818073) و *B. methylotrophicus* للعزلتين SV68 (KR818074) و SV109 (KR818076). وأظهر التحليل الكروماتوغرافي الغازي ومطياف الكتلة للمستخلص البيتانولي من SV65 *B. amyloliquefaciens* subsp. *plantarum* أنّ ester mono (2-éthylhexyl) من حمض الفتاليك هو من أكثر المركبات. تمكنت *B. amyloliquefaciens* subsp. *plantarum* SV65 وSV109 *B. methylotrophicus* من إنتاج الكايتيناز والبروتياز والبكتيناز والفوسفاتاز وحمض الأندول-3-أساتيك. أظهرت العزلة SV68 *B. methylotrophicus* قدرتها على إنتاج الكايتيناز والبيكتيناز وحمض الأندول-3-أساتيك (28،49 مكغ/مل) والعزلة SV65 *B. velezensis* على إنتاج السيداروفور وحمض الأكساليك وحمض الماليك. بينت هذه الدراسة أن نيتتيْ *S. nigrum* و *S. nigrum* var. *villosum* يمكن أن تكونا نوعا من النباتات الجيدة لعزل البكتيريات الداخلية النباتية بمثابة عناصر للمكافحة البيولوجية والتسميد الحيوي الفعالة في تحثيث نمو وإنتاج الطماطم المزروعة في التربة السليمة والملوثة بالفطر FOL.

كلمات مفتاحية: إفرازات، طماطم، مكافحة حيوية، نمو، *Bacillus* spp.، *Fusarium oxysporum* f. sp. *lycopersici*، *Solanum nigrum*

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