Use of Endophytic Bacteria Naturally Associated with *Cestrum nocturnum* for Fusarium Wilt Biocontrol and Enhancement of Tomato Growth

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ABSTRACT

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Three endophytic bacterial isolates, recovered from *Cestrum nocturnum* (night blooming jasmine) leaves and stems, were assessed for their ability to suppress tomato Fusarium wilt disease, caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), and to improve growth of tomato plants. Isolates tested had significantly decreased disease severity by 46.6-97.7% compared to FOL-inoculated and untreated control. The isolate C4 was found to be the most effective in decreasing leaf damage by 86.6% and the vascular browning extent by 97.7% relative to control. A significant increment by 39-41.6%, compared to pathogen-inoculated and untreated control, was recorded in tomato growth parameters. Moreover, the isolate C4 had significantly enhanced plant growth by 24.5-53.3% over pathogen-free and untreated control. This isolate C4 was morphologically and biochemically characterized and identified using 16S rDNA sequencing genes as *Serratia* sp. (KX197201). Screened in vitro for its antifungal activity against FOL, *Serratia* sp. C4 led to 19.52% decrease in pathogen radial growth and to the formation of an inhibition zone of 8.62 mm in diameter. Cell-free culture filtrate of *Serratia* sp. C4, supplemented to PDA medium at 20% (v/v), had lowered pathogen growth by 23% as compared to 21.7 and 9.2% recorded after heating at 50 and 100°C, respectively. Chloroform and *n*-butanol extracts from *Serratia* sp. C4, applied at 5% (v/v), displayed antifungal potential against FOL expressed as growth inhibition by 54.6-66.5% compared to untreated control which was higher than that achieved using two commercial pesticides i.e. Bavistin® (50% carbendazim, chemical fungicide) and Bactospeine® (16000UI/mg, *Bacillus thuringiensis*-based biopesticide). *Serratia* sp. C4 was found to be a chitinase-, pectinase-, and protease-producing agent and was able to produce the indole-3-acetic acid and to solubilize phosphate.

Keywords: Antifungal activity, *Cestrum nocturnum*, endophytic bacteria, *Fusarium oxysporum* f. sp. *lycopersici*, growth promotion, secondary metabolites, tomato

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Tomato Fusarium wilt incited by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a destructive disease infecting

tomato. Fusarium wilt causes important losses of tomato crops grown both in open field and under greenhouses (Ignjatov et al. 2012; Moretti et al. 2008). Diseased plants exhibit yellowing and wilting of the foliage, vascular discoloration, stunting and eventual death of the whole plant (Lim et al. 2006).

Control of tomato Fusarium wilt is difficult due to the pathogen survival structures (chlamydospores) in soil for many years without a host and due to its progress within vascular tissues (Ignjatov et al. 2012). Moreover, chemical and genetic control failed to successfully suppress disease due to fungicide resistance development and to emergence of new physiological races of FOL (Ge et al. 2004). Given the internal progress of the pathogen within vascular tissues, the use of endophytic fungi (Mahdi et al. 2014) and bacteria (Goudjal et al. 2014; Kalai-Grami et al. 2014) may be effective in biologically controlling disease.

These endophytic microorganisms are known to colonize plant tissues without causing any harmful effects on their host plants. They may remain at their entry points or spread throughout the plant (Hallmann et al. 1997). Endophytes are excellent plant growth promoters and/or sources of biocontrol agents (Strobel, 2006). Worldwide, endophytic bacteria have been used for the control of some pathogens causing vascular diseases on various plants such as *F. oxysporum* f. sp. *vasinfectum* on cotton (Chen et al. 1995) and *Verticillium dahliae* on rapeseed (Alström, 2001), eggplant, and potato (Eleftherios et al. 2004). Several previous studies have demonstrated the growth-promoting effect induced by endophytic bacteria on treated plants. Indeed, *Burkholderia caribensis*, *Kosakonia oryzae, Pectobacterium* sp., *Enterobacter asburiae*, *E. radicincitans*, *Pseudomonas fluorescens,* and *E. cloacae*, isolated from sugarcane roots and stems, have improved the development of this plant (Marcos et al. 2016). Four endophytic bacteria namely *Azospirillum brasilense*, *Burkholderia ambifaria*, *Gluconacetobacter diazotrophicus,* and *Herbaspirillum seropedicae* were shown able to colonize the internal tissues of roots, stems and leaves of *Solanum lycopersicum* var. *lycopersicum* and to stimulate its growth (Botta et al. 2013). Endophytic bacteria inhibit pathogen growth through the production of antibiotics, cell wall-degrading enzymes, competition for nutrients and minerals, and/or via the induction of systemic resistance (Lugtenberg et al. 2013). Plant growth promotion may be achieved through indole-3-acetic acid (IAA) and siderophore production, phosphate solubilization and nitrogen fixation (Rosenblueth and Martínez-Romero, 2006).

Several previous studies have shown that cultivated *Solanaceae* species may be useful as potential sources of bioactive molecules i.e. *Cestrum* spp. (*C. parqui*, *C. diurnum* and *C. sendtenerianum*) (Ahmad et al. 1993; Chaieb et al. 2007; Haraguchi et al. 2000) and as biocontrol agents, especially endophytic bacteria, i.e. *Capsicum annum* (Paul et al. 2013), *Solanum tuberosum* (Sturz et al. 2002), and *S. lycopersicum* (Ramyabharathi and Raguchander 2014).

Cestrum nocturnum is a cultivated *Solanaceae* species used as an ornamental plant; its flowers exude a special sweet fragrance at night, the main reason for its folk names night cestrum, lady of the night and night blooming jasmine (Sharif et al. 2009). Several previous studies have valorized this plant as natural source of bioactive metabolites with insecticidal (Jawale and Dama 2010; Patil et al. 2011; Yogalakshmi et al. 2014), antibacterial,

and antifungal potential (Khan et al. 2011; Prasad et al. 2013; Sharif et al. 2009). However, *C. nocturnum* was only reported as source of isolation of endophytic fungi without assessment of its antimicrobial activity (Huang et al. 2008). Moreover, to our knowledge, this ornamental species was not yet explored as natural source of isolation of endophytic bacteria.

In this study, three endophytic bacterial isolates, recovered from surface-sterilized stems and leaves of *C. nocturnum* plants were assessed for their antifungal potential toward FOL and for their growth-promoting traits on tomato plants.

MATERIALS AND METHODS

Tomato seedling preparation.

Tomato cv. Rio Grande, known to be susceptible to FOL races 2 and 3 (Barker et al. 2005), was used in this study. Seedlings were kept under greenhouse with 16 h light and 8 h dark, 60-70% relative humidity and air temperatures ranging between 20 and 30°C, and grown until reaching the two-true-leaf growth stage. A sterilized peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg) was used as culture substrate.

Pathogen culture.

F. oxysporum f. sp. *lycopersici* isolate used in this study was originally recovered from tomato stems showing vascular discoloration (Aydi Ben Abdallah et al. 2016a) and maintained in the fungal culture collection at the Laboratory of Plant Pathology, *Centre Régional des Recherches en Horticulture et Agriculture Biologique* (CRRHAB), Chott-Mariem, Tunisia. It was cultured on Potato Dextrose Agar (PDA) and incubated at 25°C for 7 days before use.

***Cestrum nocturnum* sampling and isolation of endophytic bacteria.**

Endophytic bacteria, used in this study, were isolated from leaves (C1 and C2) and stems (C3 and C4) of healthy *C. nocturnum* plants sampled on April 2013 from Chott-Mariem (N35°56'20.451"; E10°33'32.028"), Tunisia.

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. Each sample was checked for disinfection process efficiency based on Hallmann et al. (1997) protocol. In fact, 100 µl of the SDW used in the last rinse were injected onto Nutrient Agar (NA) medium. After 48 h of incubation at 25°C, if no microbial growth was observed on medium, the surface disinfection procedure was considered as succeeded.

Twenty surface-sterilized stem and leave pieces, of about 1 cm length, were cut longitudinally with a sterile scalpel and aseptically transferred onto NA medium with the longitudinal sectioning surface placed directly in contact with medium. Plates were incubated at 25°C for 48 h. For each sampled organ, bacterial colonies exhibiting morphological diversity were picked separately onto NA and purified.

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

Test of endophytic colonization ability.

The endohytic colonization ability of the four bacterial isolates collected was tested according to Chen et al. (1995) method. Isolates were grown onto NA

amended with streptomycin sulfate (100 µg/ml w/v) and rifampicin (100 µg/ml w/v). Only double-resistant isolates were selected in order to follow their presence in tomato stems after re-isolation in NA medium amended with these both antibiotics and the wild-type ones (the original isolates) were used for inoculation of tomato. Seedlings were dipped for 30 min into bacterial cell suspensions adjusted to 10^8 cells/ml using a hemocytometer (Botta et al. 2013). SDW was used for treatment of control seedlings. Five seedlings were used for each individual treatment. Seedlings were transplanted into individual pots (12.5 × 14.5 cm) containing commercialized peat and grown for 60 days under greenhouse conditions as previously described. Bacterial isolates were re-isolated from tomato stems onto NA medium supplemented with streptomycin sulfate and rifampicin (100 µg/ml (w/v) each) and incubated at 25°C for 48 h. Bacterial colonies similar to the wild-type ones were considered as endophytes (Hallmann et al. 1997) and subjected to further screening bioassays.

Test of plant growth-promoting ability.

Three endophytic isolates were tested for their potential to promote tomato growth under greenhouse conditions. Healthy tomato seedlings (cv. Rio Grande), at two-true-leaf stage, were carefully removed from alveolus plates and soaked for 30 min into water bacterial suspensions adjusted to 10^8 cells/ml (Botta et al. 2013). Control seedlings were dipped into SDW only. Inoculated and control seedlings were transplanted into individual pots (12.5 × 14.5 cm) containing sterilized peat. Five replications were used for each individual treatment. At 60 days post-treatment, plants were carefully uprooted and their roots were washed under running water to remove peat. Growth parameters noted were plant height, fresh weight of the aerial parts and roots, and maximum root length.

Test of disease-suppressive ability.

Tomato cv. Rio Grande seedlings were treated with the three bacterial isolates separately by drenching 25 ml of bacterial suspensions into culture substrate near the collar level (Nejad and Johnson, 2000). Six days after bacterial treatment, 25 ml of FOL conidial suspension (10^6 conidia/ml) were applied as substrate drenching (Fakhouri and Buchenauer 2002). Negative control seedlings were not inoculated with FOL and treated with SDW only. Positive control seedlings were pathogen-inoculated and treated with SDW. Each individual treatment was replicated five times.

Assessment of Fusarium 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 (Amini 2009). Furthermore, wilt severity was assessed based on the extent of the vascular browning (from collar) after performing longitudinal stem sectioning. Pathogen re-isolation frequency was calculated for five plants per individual treatment as the percentage of FOL colonization of stem sections on PDA (Moretti et al. 2008). Growth parameters such as plant height and fresh weight of whole plant were also noted for all tomato plants challenged or not with FOL.

The most effective isolate in suppressing Fusarium wilt severity and in promoting plant growth was further subjected to morphological and

biochemical characterization and molecular identification.

Characterization of the most active endophytic isolate.

Morphological characterization. Colonies of the most active isolate were morphologically characterized based on their size, shape, margin, elevation, texture, opacity, consistency and pigmentation on NA medium (Patel et al. 2012). Gram's staining was performed using light microscopy.

Biochemical characterization. The most bioactive isolate was also characterized using conventional biochemical tests according to Schaad et al. (2001) protocols. The biochemical tests performed in this study include catalase, Red of Methyl (RM), Vosges Proskauer (VP), mannitol, lecithinase, urease, indole, tryptophan deaminase, Simmons citrate, hydrogen sulfide, nitrate reductase, lysine decarboxylase, and pyocyanin on King A medium.

Molecular characterization. Molecular characterization of the selected isolate was performed after extraction of genomic DNA according to Chen and Kuo (1993) for the Gram negative bacteria. The 16S rDNA was amplified using the universal eubacterial primers 27f (5'-AGAGTTTGATC(A/C)TGGCTC AG-3') and 1492r (5'-TACGG(C/T)TAC CTTGTTACGACTT-3') (Moretti et al. 2008). The PCR conditions were as follows: one denaturing cycle at 94°C for 4 min, followed by 40 cycles of denaturing at 94°C for 30 s, annealing at 45°C for 30 s, and polymerization at 72°C for 45 s, then an extension cycle at 72°C for 7 min. Amplifications were carried out in Thermal Cycler® (CS Cleaver, Scientific Ltd., TC 32/80).

The homology of the 16S rDNA sequence of the given isolate was performed using BLAST-N program from GenBank database (http: www.ncbi.nlm.gov/BLAST/). Alignment of sequences was performed using the ClustalX (1.81). The phylogenetic analysis for the aligned sequences was performed using the Kimura two-parameter model (Kimura 1980). The phylogenetic tree was constructed based on neighbor joining (NJ) method with 1000 bootstrap sampling. The bioactive endophytic bacterium (isolate C4) sequence was submitted to GenBank and assigned the following accession number: KX197201.

Hypersensitivity test. Hypersensitivity test of the selected isolate was performed on tobacco plants. Ten microliters (10 µl) of water bacterial cell suspension (~10^8 cells/ml) were injected to tobacco leaves using a sterile microsyringe. Leaves injected with the same volume of SDW were used as negative control. All tobacco plants were incubated at room temperature for 24 h. After incubation, inoculated leaf areas were checked for the presence of chlorotic and/or necrotic zones indicating that the tested isolate is phytopathogenic and should be excluded from further biocontrol trials (Nawangsih et al. 2011).

Hemolytic test. Bacterial cell suspensions (~10^8 cells/ml, 100 µl) of the selected isolate were transferred on Blood Agar® (HiMedia, India) medium to test its ability to degrade hemoglobin. Bacterial cultures were incubated at 25°C for 48 h. Positive hemolytic activity is indicated by the formation of clear zones around bacterial colonies (Murray et al. 2003). Thus, the tested isolate will be considered to be pathogenic to humans and excluded from the following tests.

Assessment of the antifungal activity of *Serratia* sp. C4.

Streak method. Bacterial suspension of *Serratia* sp. C4 (~10^8 cells/ml) were streaked across the center and perpendicularly to the first streak on the surface of PDA poured in Petri plates (9 cm in diameter). Four agar plugs (6 mm in diameter), removed from 7 day-old cultures of FOL, were placed at each side of the streaked bacterial suspensions (Sadfi et al. 2001). Control plates were streaked with SDW only. Each individual treatment was repeated four times. After 4 days of incubation at 25°C, pathogen colony diameter was noted. The inhibition rate (IR) of FOL mycelial growth was calculated using Tiru et al. (2013) formula as follows: IR% = [(D2-D1) / D2] × 100 where D2: Diameter of pathogen colony in control plates and D1: Diameter of pathogen colony co-cultured with the tested bacterial isolate.

Disc diffusion method. The antifungal activity of the selected bacterial isolate was also evaluated on PDA using the disc diffusion method. FOL was incorporated into molten PDA and after medium solidification, 20 µl droplets of bacterial suspensions (~10^8 cells/ml) were deposited on Whatman No. 1. filter paper discs (6 mm in diameter). Four discs were used per plate (Vethavalli and Sudha 2012). For control plates, paper discs were treated with a same volume of SDW. Each individual treatment was repeated four times. After four days of incubation at 25°C, the diameter of the inhibition zone was noted.

Activity of cell-free culture filtrates. *Serratia* sp. C4 colonies were grown in Luria-Bertani broth (LB) at 28 ± 2°C for 3 days and under continuous shaking at 150 rpm. Two ml of the obtained liquid culture were centrifuged for 10 min at 10,000 rpm. The centrifugation was repeated three times. Cell-free culture filtrate was sterilized by filtration through a 0.22 µm pore size filter. To determine the stability of extracellular metabolites produced by this isolate, filtrate was incubated at 50 or 100°C for 15 min (Romero et al. 2007). Antifungal activity of cell-free cultures, untreated and heated, used at 20% (v/v) was assessed according to Karkachi et al. (2010). Control cultures contained LB filtrate only. Each individual treatment was replicated three times. After four days of incubation at 25°C, the diameter of FOL colony was measured and the mycelial growth inhibition rate was calculated (Tiru et al. 2013).

Activity of *Serratia* sp. C4 organic extracts.

Extraction of secondary metabolites. Two types of extraction were carried out to extract the antifungal metabolites produced by *Serratia* sp. C4. The first one was performed using chloroform (Bhoonobtong et al. 2012) and the second one with *n*-butanol (Romero et al. 2007). Sixty milliliters (60 ml) of cell-free culture filtrate of *Serratia* sp. C4, prepared as described above, were placed in a separating funnel. Then, 60 ml of the 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) were collected. The aqueous phase was replaced in the funnel and the extraction was repeated two other times as described. The solvent was evaporated in a rotary evaporator at 35°C for chloroform and at 75°C for *n*-butanol with a slight rotation at 150 rpm.

Testing of antifungal activity of organic extracts. Obtained organic extracts were assessed for their biological activity against FOL. Each extract was suspended in ethanol (1/1) (w/v) and added to Petri plates containing 10 ml of molten PDA amended with streptomycin sulfate (300 mg/l) (w/v) at two concentrations 2.5 and 5% (v/v). Control cultures were treated with ethanol also tested at 2.5 and 5% (v/v). The antifungal activity of secondary metabolites released by *Serratia* sp. C4 was compared to two commercial products i.e. Bavistin® (50%, chemical fungicide with carbendazim as active ingredient) and Bactospeine® (16000UI/mg, *Bacillus thringiensis*-based biopesticide). After solidification of the mixture, an agar plug (6 mm in diameter), removed from FOL culture previously grown at 25°C for 7 days, was placed at the center of each plate. After seven days of incubation at 25°C, FOL colony diameter was measured and the inhibition rate was calculated (Tiru et al. 2013).

Assessment of *Serratia* sp. C4 enzymatic activity.

Chitinase production. Chitinase production ability of *Serratia* sp. C4 was checked according to Tiru et al. (2013) on minimum-medium supplemented with chitin® (MP Biomedicals, LLC, IIIKrich, France) by streaking bacterial suspensions (~10^8 cells/ml) onto sterilized chitin-agar medium (0.5% w/v). Chitin-agar medium plates non-streaked with bacterial suspensions were used as control. Treatments were replicated thrice. After 72 h of incubation at 28 ± 2°C, the presence of clearing zones around bacterial colonies was noted.

Protease production. *Serratia* sp. C4 was assessed for its potential to release protease onto skim milk agar or SMA (3% v/v) medium (Tiru et al. 2013). Plates containing SMA only were used as control. 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. Pectinase production ability of *Serratia* sp. C4 was detected according to Tiru et al. (2013) method. Water bacterial suspensions (~10^8 cells/ml) were streaked 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. After 48 h of incubation at 28 ± 2°C, the presence or the absence of clear zones around bacterial colonies was noted.

Phosphate solubilization. Phosphate solubilization activity of the selected bacterial isolate was evaluated qualitatively according to Katzenlson and Bose (1959) method with some modifications. An agar plug (6 mm in diameter) containing *Serratia* sp. C4 colonies, previously grown on NA during 48 h, was putted onto Pikovskaya agar medium. Un-inoculated plates were used as control. Experiments were performed in triplicate. After seven days of incubation at 28 ± 2°C, clear zones formed around colonies, due to the utilization of tricalcium phosphate present in the medium, were measured.

Production of indole-3-acetic acid (IAA). The ability of *Serratia* sp. C4 to produce IAA was checked using the colorimetric method described by Glickmann and Dessaux (1995) with some modifications. *Serratia* sp. C4 was cultivated into LB medium supplemented with L-tryptophan (50 µg/ml) under continuous shaking at 150 rpm for 2 days

in the dark. The bacterial suspension was centrifuged at 10,000 rpm for 10 min. One ml of the culture supernatant was mixed with 2 ml of Salkwoski's reagent and 2-3 drops of orthophosphoric acid. Un-inoculated LB medium was used as negative control. Absorbance was measured daily at 530 nm. The concentration of IAA was determined and compared to a standard curve prepared from IAA dilution series at 100 µg/ml in LB medium.

Statistical analysis.

Data were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0. For all the in vitro antifungal potential bioassays and the in vitro tests of enzymes and IAA production and phosphate solubilization ability, each treatment was replicated three to four times. For cell-free culture filtrate tests, data were analyzed according to a completely randomized factorial design with two factors (Heating and bacterial treatment). The in vitro assay of organic extracts was analyzed according to a completely randomized factorial model with two factors (treatments and concentrations). For the remaining in vitro bioassays, data were analyzed 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. For the in vitro antifungal activity tests using the streak and the disc diffusion methods, means were separated using Student t test at $P \leq 0.05$. For the remaining bioassays, means were separated using LSD test for the in vitro antifungal activity test of cell-free cultures and organic extracts and using Duncan Multiple Range test for the others to identify significant pair-wise differences 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 ability of bacterial isolates recovered from *Cestrum nocturnum*.

Four bacterial isolates exhibiting macro-morphological diversity on NA medium were selected among twenty others recovered from stems and leaves of *C. nocturnum* plants. The four selected isolates were found to be resistant to streptomycin and rifampicin (100 µg/ml) and only three were successfully re-isolated from the internal stem tissues of tomato cv. Rio Grande plants on NA medium amended with these antibiotics. These three endophytic isolates (namely C1, C3, and C4) were further assessed in vivo and in vitro for their antifungal potential toward FOL and for their growth-promoting effects on tomato seedlings.

Plant growth-promoting ability displayed by the selected endophytic isolates.

The three endophytic bacterial isolates (C1, C3 and C4) were screened for their growth-promoting potential onto pathogen-free tomato plants. ANOVA analysis revealed that all plant growth parameters (plant height, aerial part fresh weight, maximum root length, and root fresh weight), noted 60 days post-treatment, varied significantly (at $P \leq 0.05$) depending on bacterial treatments tested.

Data given in Table 1 revealed that, C1- and C4-based treatments led to significant ($P \leq 0.05$) increase in plant height by 18.7 and 31.9%, respectively, compared to the untreated control. The highest plant height improvement (by

31.9% over control) was achieved using C4 isolate.

The aerial part fresh weight was significantly enhanced by 50% using C4-based treatment compared to control (Table 1). As estimated by the maximum root length, a significant ($P \leq 0.05$) improvement (by 24.6% over control) was recorded on tomato plants treated with C4. Similar trend (53.3%) was noted based on root fresh weight (Table 1).

Table 1. Comparative plant growth-promoting ability of endophytic bacterial isolates recovered from *Cestrum nocturnum* on tomato cv. Rio Grande plants noted 60 days post-treatment

Bacterial treatment*	Plant Height (cm)	Aerial part fresh weight (g)	Maximum root length (cm)	Root fresh weight (g)
NIC	20 ± 0 c	8 ± 0.1 b	17.2 ± 0.6 b	4.2 ± 0.1 b
C1	24.6 ± 0.3 b	6.8 ± 0.2 b	19 ± 0.5 b	5.8 ± 0.2 b
C3	21.6 ± 0.5 c	7 ± 0.5 b	18.8 ± 0.8 b	5.8 ± 0.2 b
C4	29.4 ± 0.8 a	16 ± 0.1 a	22.8 ± 0.5 a	9 ± 0.7 a

*C1: Isolate from *C. nocturnum* stem; C3 and C4: Isolates from *C. nocturnum* leaves; NIC: Un-inoculated with the pathogen and untreated control. For each column, values followed by the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

Fusarium wilt suppression by the selected endophytic isolates.

The three selected endophytic bacterial isolates were tested on tomato cv. Rio Grande plants challenged with FOL. ANOVA analysis revealed that Fusarium wilt severity, noted on tomato plants 60 DPI, varied significantly ($P \leq 0.05$) depending on bacterial treatments tested. A significant ($P \leq 0.05$) decrease in leaf damage index (yellowing and/or necrosis), by 46.6 to 86.6% compared to pathogen-inoculated and untreated control, was noted on tomato plants already challenged with FOL and treated using the three tested isolates (Table 2). The reduction of the vascular browning extent was significant, by 55.5 to 97.7% compared to control, using C1-, C3-, and C4-based treatments. The isolate C4 was found to be the most effective in suppressing leaf yellowing and wilt symptoms (86.6%) and in reducing the vascular browning extent (97.7%) relative to FOL-inoculated and untreated control. Furthermore, C4-treated plants behaved significantly similar to the un-inoculated (disease-free) and untreated ones based on both disease severity parameters (Table 2).

Growth parameters of tomato plants (plant height and fresh weight), noted 60 DPI with FOL, varied significantly ($P \leq 0.05$) depending on treatments tested. The increment in plant height ranged significantly between 12.6 and 39% over FOL-inoculated and untreated control using C1-, C2-, and C4-based treatments and the highest enhancement (of about 39%) was achieved using C4 isolate. It should be also highlighted that tomato plants infected with FOL and treated with C4 exhibited a significantly higher (by 12.7%) plant height than disease-free and untreated ones.

All bacterial isolates tested had significantly ($P \leq 0.05$) increased plant fresh weight by 23.2 to 41.7% over FOL-inoculated and untreated control and the highest increment (41.7%) was induced by the isolate C4. The fresh weight of

FOL-inoculated tomato plants treated with C4 isolate was significantly similar to that of disease-free control ones (Table 2).

FOL re-isolation frequency from tomato stems varied depending on bacterial treatments tested. A decrease in pathogen isolation frequency, ranging between40 and 90% relative to the untreated control, was recorded from tomato plants already infected with FOL and treated with the three enpdophytic isolates tested. The highest decrease in FOL re-isolation frequency was achieved by using the isolate C4 (90%) (Table 2).

Table 2. Effects of endophytic bacterial isolates recovered from *Cestrum nocturnum* 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

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	24.8 ± 0.4 b	7.05 ± 0.2 ab	0
IC	3 ± 0.1 a	9 ± 0.5 a	17.3 ± 1 d	4.2 ± 0.1 d	100
C1	1.2 ± 0.1 b	4 ± 0.1 b	21 ± 0.5 c	6.25 ± 0.1 bc	50
C3	1.6 ± 0.2 b	4 ± 0.1 b	19.8 ± 0.4 c	5.47 ± 0.3 c	60
C4	0.4 ± 0.2 c	0.2 ± 0.1 c	28.4 ± 0.3 a	7.21 ± 0.2 a	10

*C1: Isolate from *C. nocturnum* stem, C3 and C4: Isolates from *C. nocturnum* leaves; NIC: Un-inoculated with the pathogen and untreated control. IC: Inoculated with FOL and untreated control.
**The re-isolation of FOL was carried out from stems of five tomato plants cv. Rio Grande at 0-15 cm high from the collar. Ten (10) stem fragments were plated onto PDA medium and incubated at 25°C for 4 days. After incubation, the percentage of FOL colonization of stems sections was calculated.
For each column, values followed by the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

Correlation analysis between Fusarium wilt severity and plant growth parameters.

Pearson's analysis revealed that decreased Fusarium wilt severity as estimated by leaf damage index (and/or necrosis) and vascular browning extent led to increment in all plant growth parameters. In fact, plant height was significantly and negatively correlated to the leaf damage index (r = -0.874; P = 0.053) (Fig. 1A) and to the vascular browning extent (r = -0.909; P = 0.033) (Fig. 1B). Furthermore, the plant fresh weight was significantly and negatively correlated to leaf yellowing score (r = -0.981; P =0.003) (Fig. 1C) and to the vascular browning extent (r = -0.973; P = 0.005) (Fig. 1D).

Pearson's analysis demonstrated that lowered Fusarium wilt severity led to decrease in tomato stem colonization by FOL and consequently growth promotion where significant and negative correlations were also detected between FOL re-isolation frequency, plant height (r = -0.914; P = 0.03) (Fig. 1E), and whole plant fresh weight (r = -0.975; P = 0.005) (Fig. 1F). Moreover, pathogen re-isolation frequency was positively correlated to leaf damage index (r = 0.991; P = 0.001) (Fig. 1G) and to the vascular browning extent (r = 0.987; P = 0.002) (Fig. 1H).

The endophytic bacterial isolate C4 shown to be effective in suppressing Fusarium wilt severity and in promoting growth of tomato plants inoculated or not

with FOL (Fig. 2) was selected for further characterization, identification and elucidation of its mechanisms of action involved in those both effects.

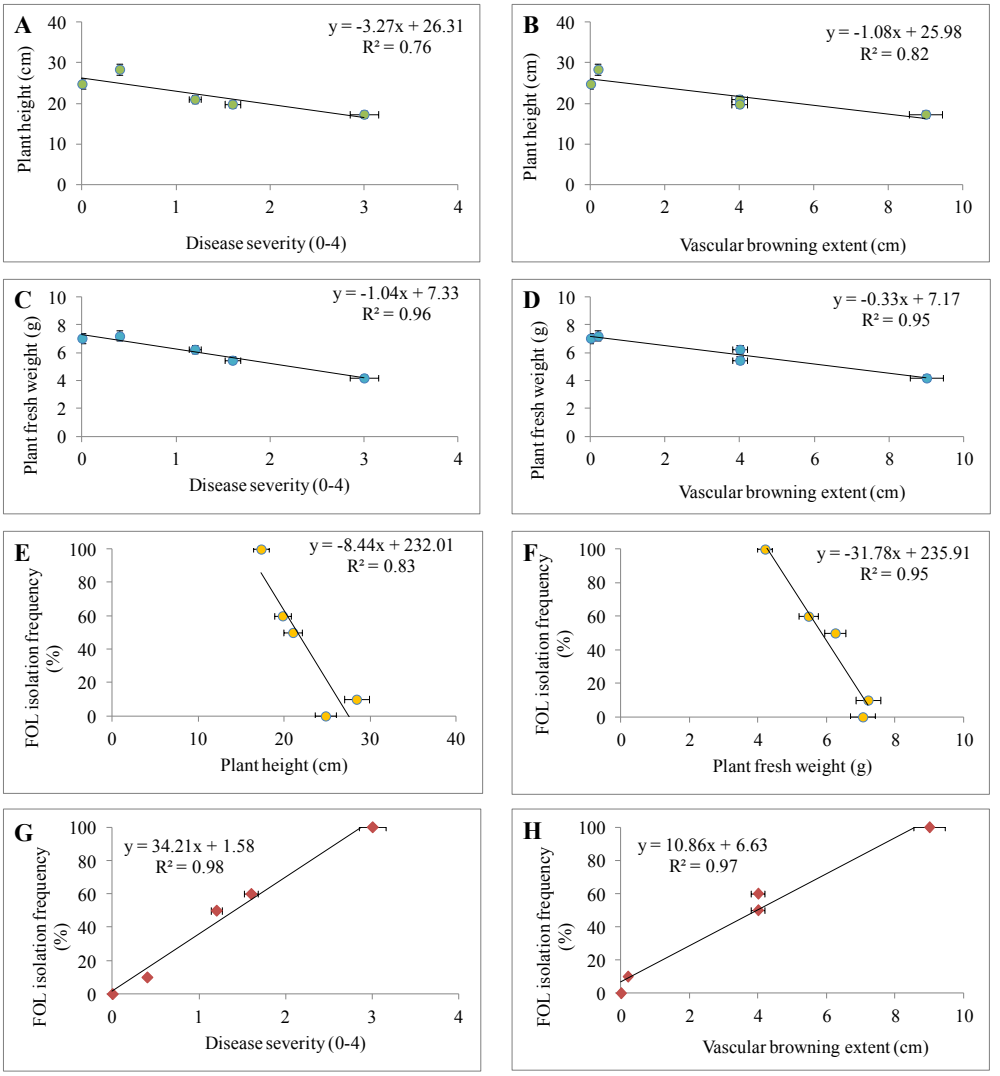

Fig. 1. Correlation between Fusarium wilt severity and plant growth parameters (A, B, C, D, E, F) and between FOL isolation frequency and Fusarium wilt severity parameters (G, H). Correlation analysis was performed using bivariate Pearson's test at $P \leq 0.01$.

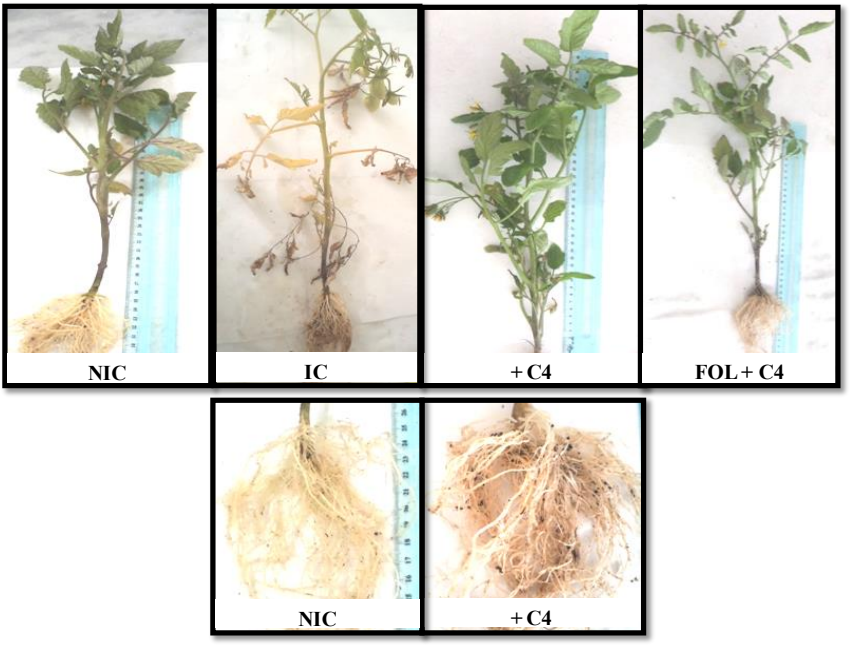

Fig. 2. Effect of endophytic bacterial isolate C4 recovered from *Cestrum nocturnum* on Fusarium wilt severity and growth promotion of tomato cv. Rio Grande plants compared to the untreated controls. NIC: Un-inoculated with the pathogen and untreated control. IC: Inoculated with FOL and untreated control; C4: Isolate from *Cestrum nocturnum* leaves.

Morphological, biochemical and molecular characterization of the selected bacterial isolate.

The colony morphology of the selected isolate C4 showed a small size and translucent colonies with circular form, an entire margin and plane elevation, smooth surface and cream color on NA medium (Table 3). C4 was found to be a Gram negative strain.

C4 was able to produce catalase, lecithinase, lysine decarboxylase, nitrate reductase, and indole. The isolate C4 cannot produce urease, tryptophane desaminase, hydrogen sulfide, and pyocyanin on King A medium. Simmons citrate and mannitol were not used by C4 colonies as carbon sources. C4 cannot ferment glucose through the mixed acid (MR-) but by using the glycol butylene path (VP +) (Table 3).

Blast-N analysis of sequenced 16S rDNA gene homology and the phylogenetic analysis based on neighbor joining (NJ) method with 1000 bootstrap sampling revealed that the isolate C4 belonged with 100% of similarity to Uncultured *Serratia* sp. strain CTL-81 and *Serratia proteamaculans* strain AP-CMST (Table 3; Fig. 3). The accession number of *Serratia* sp. strain C4 deposed in GenBank was KX197201 (Table 3).

Table 3. Characterization and molecular identification of the selected endophytic bacterial isolate C4 recovered from *Cestrum nocturnum* leaves

Morphological characterization	
Size	Small
Form	Circular
Margin	Entire
Elevation	Plane
Surface	Smooth
Opacity	Translucent
Color	Cream
Gram staining	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	-
Molecular characterization	
Most related species	CTL-81, Uncultured *Serratia* sp. (100)
	AP-CMST, *Serratia proteamaculans* (100)
Accession number GenBank	KX197201
Hypersensivity reaction	
-	
Hemolytic activity	
-	

+: Positive test; -: Negative test. Numbers in parenthesis indicate the percentage (in %) of sequence homology obtained from Blast-N analysis from GenBank database (http: www.ncbi.nlm.gov/BLAST/).

The nucleotide sequences used of representative strains were obtained from Genbank database under the following accession numbers: FJ752236 (*Serratia proteamaculans* AP-CMST), JQ798999 (Uncultured *Serratia* sp. CTL-81), KU682855 (*Serratia* sp. PKL:12), KJ922535 (*Shewanella* sp. XH39), JX162043 (*Enterobacteriaceae bacterium* Pokym2-b), NR_037112 (*Serratia quinivorans* 4364), KU999993 (*Serratia liquefaciens* strain ZMT-1), KM187235 (*Gluconacetobacter* sp. CC3H1), KM453915 (Uncultured bacterium D05 placa2), NR_113616 (*Serratia grimesii* NBRC 13537), and for the bacterial isolate tested: KX197201 (C4). The tree topology was constructed using ClustalX (1.81).

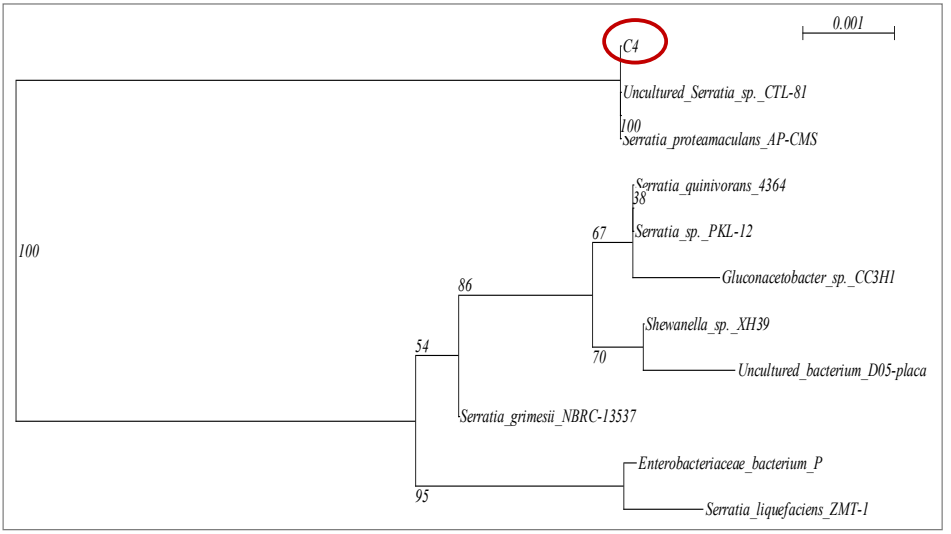

Fig. 3. Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of the best antagonistic and plant growth promoting endophytic bacterial isolate C4 recovered from *Cestrum nocturnum* and their closest phylogenetic relatives.

Hypersensivity reaction and hemolytic activity of *Serratia* sp. C4.

No hypersensitive reaction (HR) (chlorotic or necrotic zone) was detected on inoculated tobacco leaf areas as compared to control ones after 24 h of incubation. Thus, the isolate C4 was found to be non phytopathogenic and was selected for further screenings.

No hemolytic activity was displayed by the isolate C4 expressed by the absence of clear zones around its colonies grown on Blood Agar medium after 48 h of incubation at 25°C. Thus, this isolate was nonpathogenic to humans and it can be used in the following tests (Table 3).

Antifungal activity of *Serratia* sp. C4 against *Fusarium oxysporum* f. sp. *lycopersici*.

Activity of whole culture. The endophytic bacterial isolate *Serratia* sp. C4, tested using the streak method, induced a significant ($P \leq 0.05$) decrease, by 19.5% compared to control, in FOL mycelial growth noted after 4 days of incubation at 25°C (Table 4; Fig. 4 A).

Tested using the disc diffusion method on PDA medium, *Serratia* sp. C4 formed an inhibition zone of about 8.62 mm in size around FOL colony after 4 days of incubation at 25°C (Table 4; Fig. 4B).

Table 4. Antifungal activity of *Serratia* sp. C4 against *Fusarium oxysporum* f. sp. *lycopersici* (FOL)

Bacterial treatment	Diameter of FOL colony (cm)[a]	Inhibition zone (mm)[b]
Untreated control	3.71 ± 0.08	0 ± 0
Serratia sp. C4 (KX197201)	2.98* ± 0.04	8.62* ± 0.5

Values with asterisk indicate a significant difference with the control (t-test at $P \leq 0.05$).
[a] Tested using the streak method on PDA medium and incubated at 25°C for 4 days.
[b] Tested using the disc diffusion method on PDA medium and incubated at 25°C for 4 days.

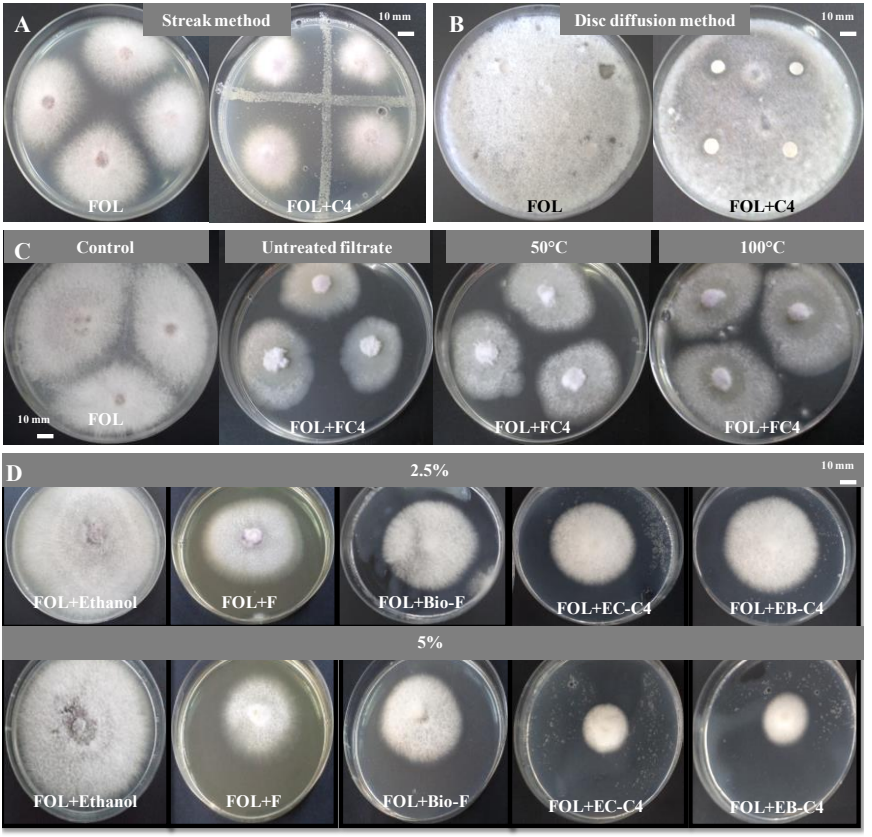

Fig. 4. Antifungal activity of endophytic *Serratia* sp. C4 against *Fusarium oxysporum* f. sp. *lycopersici* (FOL) using whole culture (A and B), untreated cell-free culture and filtrates heated at 50 and 100°C (C) and organic extracts tested at two concentrations (D) as compared to controls.
C4: Whole culture of *Serratia* sp. C4; FC4: Cell-free culture filtrate from *Serratia* sp. C4; Ethanol: Negative control; F: Positive control (Bavistin®, chemical fungicide with carbendazim as active ingredient); Bio-F: Positive control (Bactospeine®, *Bacillus thuringiensis*-based biopesticide); EC-C4: Chloroform extract from *Serratia* sp. C4; EB-C4: n-butanol extract from *Serratia* sp. C4.

Activity of cell-free culture filtrate. Analysis of variance revealed significant ($P \leq 0.05$) variation in pathogen colony diameter depending on treatments tested and a significant interaction was recorded between cell-free treatments and heating. A significant decrease in FOL colony diameter, 23% versus the un-inoculated control, was induced by the untreated cell-free culture of *Serratia* sp. C4 (Fig. 5). The filtrate heated at 50°C for 15 min had also significantly ($P \leq 0.05$) reduced FOL growth by 21.7% (Fig. 5). However, heating treatment at 100°C for 15 min decreased the antifungal activity of the tested cell-free culture supernatant toward FOL where pathogen growth was inhibited by 9.3% relative to 21.7 and 23% recorded using filtrate heated at 50°C and unheated one, respectively (Figs. 4C and 5).

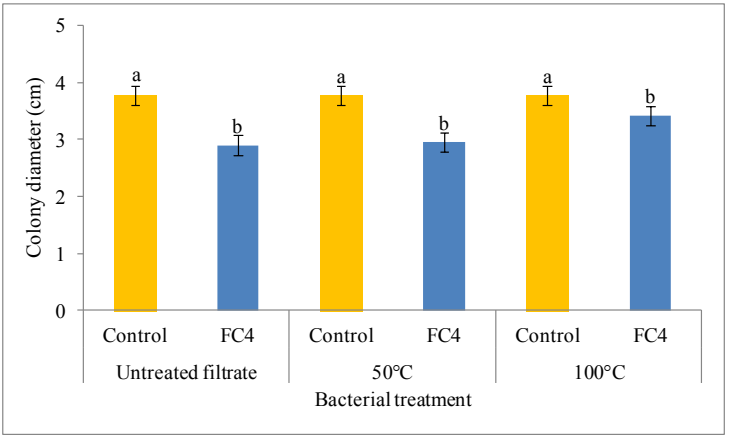

Fig. 5. Effect of heating of the cell-free culture filtrate of *Serratia* sp. C4 on its antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (FOL) noted after 4 days of incubation at 25°C as compared to controls.
FC4: Cell-free culture filtrate from *Serratia* sp. C4 (KX197201) isolated from surface sterilized *Cestrum nocturnum* leaves. Control: Luria-Bertani broth filtrate. LSD (Bacterial treatment × Heating): 0.33 cm at $P \leq 0.05$. For each heating treatment, bars with the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

Activity of organic extracts. ANOVA analysis revealed a significant ($P \leq 0.05$) variation in the mean colony diameter of FOL depending on organic extracts (chloroform and *n*-butanol extracts) tested, concentrations used, and their interactions. Chloroform and *n*-butanol extracts from *Serratia* sp. C4, applied at 1 mg/ml, had inhibited FOL growth by 27.6 to 66.5% as compared to ethanol control whatever the concentration tested (Fig. 6).

Both organic extracts from *Serratia* sp. C4 were found to be more active at the concentration 5% than at 2.5% (v/v) where pathogen growth was inhibited by 54.6-66.5% and 27.6-31.3%, respectively (Fig. 6). In fact, chloroform extract from *Serratia* sp. C4 decreased FOL growth by 54.6% when applied at

5% (v/v), compared to 31.3% recorded at 2.5% (v/v). In addition, *n*-butanol extract from this isolate, applied at 5% (v/v), had inhibited pathogen growth by 66.5% compared to 27.6% recorded at 2.5% (v/v) (Figs. 4D and 6).

It should be highlighted that the decrease in FOL growth was higher using *Serratia* sp. C4 organic extracts at 5% (v/v) (54.6-66.5%) than that achieved using Bavistin® (31.3-39.5%) and Bactospeine® (40.9-43.2%) whatever the concentration used (Figs. 4D and 6).

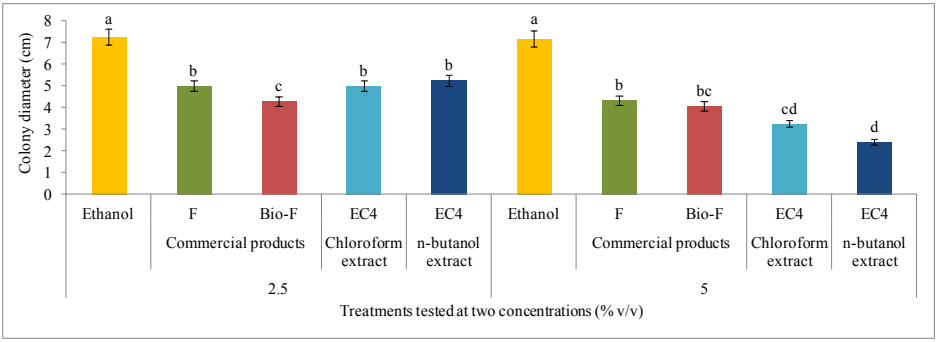

Fig. 6. Effect of chloroform and *n*-butanol extracts from endophytic *Serratia* sp. C4 tested at two concentrations against *Fusarium oxysporum* f. sp. *lycopersici* noted after 7 days of incubation at 25°C as compared to controls. EC4: Organic extract from *Serratia* sp. C4 (KX197201) isolated from surface sterilized *Cestrum nocturnum* leaves. Control: Ethanol control. F: Bavistin® (Chemical fungicide, carbendazim); Bio-F: Bactospeine® (*Bacillus thuringiensis*-based biopesticide). LSD (Treatments tested × Concentrations used): 0.86 cm at $P \leq 0.05$. For each concentration, bars sharing the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

Production of chitinase, protease and pectinase by *Serratia* sp. C4

Serratia sp. C4 formed clear zones around its colonies when grown onto chitin-, pectin- and milk-agar media. This indicates that *Serratia* sp. C4 is able to produce cell-wall degrading enzymes i.e chitinase-, pectinase and protease, respectively (Table 6).

Phosphate solubilization and IAA production ability of *Serratia* sp. C4

Serratia sp. C4 was able to solubilize phosphate as indicated by the formation of a clear zone of about 10.33 mm width around its colonies when grown on Pikovskaya agar medium (Table 5).

The selected endophytic isolate, *Serratia* sp. C4, was able to produce the indole-3-acetic acid (IAA), involved in plant growth promotion, estimated at 29.52 µg/ml after 48 h of incubation compared to 0.3 µg/ml recorded after 24 h (Table 5).

Table 5. Production of cell-wall degrading enzymes and plant growth-promoting compounds by endophytic *Serratia* sp. C4 recovered from *Cestrum nocturnum* leaves

Isolate	Cell-wall degrading enzymes			Plant growth-promoting compounds		
	Chitinase[a]	Protease[b]	Pectinase[c]	Indole-3-acetic acid (µg/ml)[d]		Phosphate solubilization[e]
				24 h	48 h	
***Serratia* sp. C4 (KX197201)**	+	+	+	+	++	+

[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 (14.5 ± 0.03 mm in diameter).
[c] Tested on pectin-agar (0.5 % w/v) medium and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone.
[d] Indole-3-acetic acid (IAA) compounds production after 24 and 48 h of incubation at 28 ± 2°C in Luria-Bertani broth medium; +: Production of IAA compounds (0.3 ± 0 µg/ml) ++: Production of IAA compounds (29.52 ± 0.05 µg/ml).
[e] Tested on Pikovskaya agar medium and incubated at 28 ± 2°C for 7 days; +: Presence of clear zone (10.33 ± 0.01 mm in diameter).

DISCUSSION

Endophytic bacteria isolated from cultivated plants i.e. tomato and colza were successfully used as biocontrol agents against Fusarium wilt of tomato (Nandhini et al. 2012; Nejad and Johnson 2000; Ramyabharathi and Raguchander 2014). In view of previous studies, the present investigation focused on potential use of endophytic bacteria recovered from *C. nocturnum* grown in Tunisia (Chott-Mariem, Sousse) for controlling tomato Fusarium wilt.

C. nocturnum was successfully used as a natural source of bioactive metabolites with antifungal activities. In fact, ethanol, methanol and butanol extracts from *C. nocturnum* were explored for their antifungal activity against *Trichoderma* sp. and *Aspergillus* sp. (Prasad et al. 2013). Furthermore, aqueous extracts and chloroform, ethyl acetate and *n*-butanol extracts from *C. nocturnum* showed an inhibitory effect against *Candida albicans*, *Microsporum canis*, *Candida glaberata* growth ranging between 30 and 65% (Khan et al. 2011). Few data were available on *C. nocturnum* use as potent source of isolation of microorganisms such as fungi i.e *Alternaria* spp., *Aspergillus* spp., *Colletotrichum* spp., *Fusarium* spp., *Gliocladium* sp., *Phoma* spp., *Phomopsis* spp., *Phyllosticta* sp., *Torula* sp. and Xylariales sp. (Huang et al. 2008). To our Knowledge, no data had previously valorized this species as natural source of isolation of bacterial biocontrol agents. Hence, this is the first study reporting on possible exploration of *C. nocturnum* as potential source of endophytic bacteria exhibiting antifungal activity against Fusarium wilt and growth-promoting potential onto tomato seedlings.

In the current study, four bacterial isolates were collected among many others based on macro-morphology diversity onto NA medium and were found to be resistant to streptomycin and rifampicin (100 µg/ml). Endophytic

progress within tomato stems was confirmed for three isolates among the four tested after re-isolation onto NA medium amended with these both antibiotics. These three bacterial isolates recovered from *C. nocturnum* and exhibiting endophytic behavior on tomato plants, were assessed for their ability to control Fusarium wilt disease and to enhance growth of tomato seedlings. In fact, assessed on seedlings un-inoculated with FOL, the current results clearly demonstrated that the bacterial isolate C4 had significantly stimulated the growth of the aerial plant parts by 31.9-50% and root development by 24.6 to 53.3% compared to the untreated control. Hence, the isolate C4, recovered from *C. nocturnum* leaves, exhibited bio-fertilizing properties. Similar results on plant growth-promoting potential (PGPB) were reported on wheat seedling treated with *Serratia marcescens* KR-4, *B. thuringiensis* KR-1 and *Enterobacter asburiae* KR-3, originally recovered from nodule of *Pueraria thunbergiana* (Selvakumar et al. 2008a). When assessed on tomato seedlings inoculated with FOL, the strongest suppressive effect of disease severity was also displayed by the isolate C4. This ability to suppress Fusarum wilt was expressed by more than 86.6% decrease in leaf damage index and 97.7% in vascular browning extent resulting in a significant decrease in FOL colonization of stem tissues. Nejad and Johnson (2000) also observed a decrease by at least 75% in tomato Fusarium wilt severity using two unidentified endophytic isolates PA and PF, issued from healthy cultivated oilseed rape plants. The selected C4 isolate was also able to enhance plant growth on tomato plants inoculated or not with FOL. Furthermore, Pearson correlation analysis indicates that Fusarium wilt severity decrease was found to be related to the registered increment in all plant growth parameters. Ramyabharathi and Raguchander (1994) also found that tomato Fusarium wilt-suppressive effect, by 68.4%, displayed by an endophytic bacterium *B. subtilis* str. EPC016, isolated from cotton plants, was associated to promotion of plant growth and fruit yield in tomato compared to control. In our recent studies, a strong decrease in Fusarium wilt severity was also achieved using various endophytic bacteria including *Stenotrophomonas* sp. S33, *Pseudomonas* sp. S85, *B. mojavensis* S40, *S. maltophilia* S37, *B. tequilensis* SV104, *Bacillus* sp. SV101, *Alcaligenes feacalis* subsp. *faecalis* S8, *B. cereus* S42 and *A. faecalis* S18, originally recovered from wild *Solanaceae* species namely *Datura metel, D. stramonium*, *Solanum elaeagnifolium*, *Withania somnifera*, and *Nicotiana glauca*, respectively. These endophytic isolates were also shown effective in enhancing tomato growth in plants inoculated or not with FOL (Aydi Ben Abdallah et al. 2016a-e).

The best antagonistic and plant growth-promoting isolate (C4) was macro-morphologically and biochemically characterized and molecularly identified by 16S rDNA gene sequencing as *Serratia* sp. C4 (KX197201).Gyaneshwar et al. (2011) used an isolate of *Serratia marcescens*, showing an endophytic ability on rice plants, which significantly stimulated plant growth as estimated by the length of roots and the dry weight of the whole plant. Similarly, plant height and fresh weight, leaf dry weight and the number of fruits per plant were also improved in cultivated tomato using various species of rhizospheric and/or plant-associated bacteria belonging to *Pseudomonas putida*, *P. fluorescens*, *S. marcescens*, *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* (Almaghrabi et al. 2013). Furthermore, *S. marcescens* B2, applied

to cyclamen plants inoculated with sclerotia of *Rhizoctonia solani* and/or conidia of *F. oxysporum* f. sp. *cyclaminis*, was found to be effective in suppressing both diseases induced by these pathogens (Someya et al. 2000).

In the current study, *Serratia* sp. C4, evaluated in vitro for its antagonistic potential toward FOL, had reduced pathogen mycelial growth and formed an inhibition zone. Similar effects were reported by Patel et al. (2012) using endophytic bacteria from cultivated tomato, identified as *Pseudomonas aeruginosa* str. HR7, for *F. oxysporum* biocontrol. The cell-free culture filtrate of *Serratia* sp. C4, from 3-day-old culture, tested in the current study had also significantly reduced FOL growth compared to control even if heated at 50, 100°C for 15 min and/or unheated. However, the antifungal potential of *Serratia* sp. C4 cell-free culture filtrate significantly declined, as compared to control filtrate, after heating at 100°C for 15 min. In the same way, antagonistic potential of culture filtrate from *Streptomyces hygroscopicus* toward *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* declined heating at 100°C for 45 min (Prapagdee et al. 2008). However, the antifungal activity of culture filtrate from *Bulkhorderia cepacia* toward *C. gloeosporioides* was similar to that expressed by the untreated filtrate even if heated at 50 or 100°C and autoclaved at 121°C for 20 min (Kadir et al. 2008).

In this study, *Serratia* sp. C4 cell-free culture filtrate was also subjected to an extraction with chloroform and *n*-butanol to elucidate the antifungal potential of its extracellular metabolites. Results showed that both organic extracts from *Serratia* sp. C4, used at 1 mg/ml, had reduced FOL growth by 27.6 to 66.5%, compared to ethanol control. Biologically active metabolites extracted with ethyl acetate, methanol and chloroform from *Serratia* sp., isolated from the coralline red algae *Amphiroa anceps*, inhibited the mycelial growth of several pathogenic bacteria and fungi (Karkachi et al. 2010). The chloroform-methanol and diethyl ether extracts from *S. marcescens* S10, isolated from the gut of the insect American cockroach showed antimicrobial potential toward various bacterial and fungal agents namely *Lesteria* spp., *Salmonella* spp., *Klebsiella* spp., *Staphylococcus aureus*, *C. albicans, Aspergillus niger*, and *Geotricum* spp. (Ahmed and Hassen 2013).

Chloroform and *n*-butanol extracts from *Serratia* sp. C4 were found to be more effective at the concentration of 5 than at 2.5% (v/v). The highest antifungal potential toward FOL growth (66.5%) was achieved using the *n*-butanol extract from the tested isolate used at 5% (v/v) versus the ethanol control and the two commercial products used i.e. Bavistin® (50%, carbendazim) and Bactospeine® (16000UI/mg, biopesticide). These results are in agreement, in part, with Aydi Ben Abdallah et al. (2015) findings using chloroform and *n*-butanol extracts from six endophytic *Bacillus* spp. isolates, recovered from wild Solanaceae plants (namely *D. metel*, *S. nigrum*, *S. elaeagnifolium* and *N. glauca*), where the two types of organic extracts from *Bacillus* spp. exhibited an interesting antifungal potential toward FOL which was higher than that induced by the same two commercial products used in the present study whatever the concentrations used. Furthermore, chloroform and *n*-butanol extracts from *Bacillus* spp. were found to be more effective at the concentration of 5 than at 2.5% (v/v) toward FOL (Aydi Ben Abdallah et al. 2015).

Serratia sp. C4 was elucidated in vitro for its properties deployed in the observed antifungal effect. In fact, this isolate was shown able to produce chitinase, protease and pectinase enzymes as shown on chitin-, milk-, and pectin-agar media, respectively. Several genera of endophytic bacteria such as *Serratia, Bacillus, Burkholderia*, *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Micrococcus,* and *Microbacteruim* were found able to produce chitinase, protease and β-1,3-glucanase involved in cell-wall degradation of various pathogens (Berg et al. 2005; Bibi et al. 2012). Moreover, pectinase and cellulase were previously reported by Hallmann et al. (1997) as essential enzymes for colonization of plant tissues. These enzymes may be also involved in the enhancement of tomato growth (Baldan et al. 2003). As metabolites involved in the recorded increment of tomato growth relative to the untreated control, in the present study, the selected isolate *Serratia* sp. C4 was found able to produce indole-3-acetic acid (IAA). The IAA amount released by our isolate *Serratia* sp. C4 (29.52 µg/ml, after 48 h of incubation) is interestingly higher when compared to 11.1 µg/ml produced by *S. marcescens* SRM, recovered from flowers of *Cucurbita pepo*, in Selvakumar et al. (2008b) study but lower than the amount produced by *Serratia nematodiphila* (58.9 µg/ml) isolated from forest soil (Dastager et al. 2011).

Phosphate solubilization ability was also assessed, in this study, and confirmed for *Serratia* sp. C4. Ngamau et al. (2012) study revealed that endophytic bacteria such as *Pseudomonas* spp., *Serratia* spp., *Enterobacter asburiae*, *Rahnella aquatilis*, *Ewingella americana*, and *Yokenella regensburgei* were able to solubilize the phosphate. Furthermore, plant growth promotion attributes such as phosphate solubilization, IAA production, hydrogen cyanide production and N-fixation were found in *S. marcescens* KR-4 recovered from *Pueraria thunbergiana* (Selvakumar et al. 2008a), *S. marcescens* KiSII isolated from the rhizosphere of coconut palms (George et al. 2013) and *S. nematodiphila* issued from forest soil (Dastager et al. 2011). The phosphatase activity of our endophytic isolate *Serratia* sp. C4 was indicated by the presence of clear zone of about 10.33 mm. Phosphate solubilization ability of unidentified endophytic bacterial isolates and *P. aeruginosa* HR7, recovered from tomato plants, was also expressed by the formation of a clear zone of about 8 to 31 mm around their colonies (Patel et al. 2012).

The chemical identification of *Serratia* sp. C4 extracellular metabolites separated in the different organic extracts tested will give additional information on the nature of bioactive molecules involved in the recorded disease-suppressive potential of this endophytic isolate.

RESUME

Aydi Ben Abdallah R., Mejdoub-Trabelsi B., Nefzi A., Jabnoun-KhiareddineH. et Daami-Remadi, M. 2017. Utilisation de bactéries endophytes naturellement associées à *Cestrum nocturnum* pour la lutte biologique contre la flétrissure fusarienne et l'amélioration de la croissance de la tomate. Tunisian Journal of Plant Protection 12: 15-40.

Trois isolats bactériens endophytes, isolés à partir des feuilles et des tiges de *Cestrum nocturnum* (jasmin de nuit), ont été évalués pour leur aptitude à supprimer la fusariose vasculaire, causée par

Fusarium oxysporum f. sp. *lycopersici* (FOL), et à améliorer la croissance des plants de tomate. Les isolats testés ont significativement diminué la sévérité de la maladie de 46,6 à 97,7% par rapport au témoin inoculé par FOL et non traité. L'isolat C4 s'est révélé le plus efficace dans la réduction des altérations foliaires de 86,6% et de la hauteur du brunissement vasculaire de 97,7% par rapport au témoin inoculé par FOL et non traité. Une augmentation significative, de 39 à 41,6% par rapport au témoin inoculé par le pathogène et non traité, a été enregistrée au niveau des paramètres de croissance de la tomate. De plus, l'isolat C4 a significativement augmenté les paramètres de croissance de 24,5-53,3% par rapport aux plants témoins non inoculés par le pathogène et non traités. Cet isolat a été morphologiquement et biochimiquement caractérisé et identifié en utilisant le séquençage du gène 16S ADNr comme *Serratia* sp. (KX197201). Criblé *in vitro* pour son activité antifongique contre FOL, *Serratia* sp.C4 a induit une diminution de 19,52% de la croissance radiale du pathogène et la formation d'une zone d'inhibition de 8,62 mm de diamètre. Le filtrat de culture de *Serratia* sp. C4, additionné au milieu PDA à raison de 20% (v/v), a réduit la croissance radiale du pathogène de 23% comparé aux 21,7 et 9,2% enregistrés après son chauffage à 50 et 100°C, respectivement. L'extrait *n*-butanolique de *Serratia* sp. C4, appliqué à 5% (v/v), a présenté un potentiel antifongique contre FOL traduit par une inhibition de la croissance de 66,5% par rapport au témoin non traité et qui a été supérieure à celle obtenue moyennant deux pesticides commerciaux, à savoir Bavistin® (carbendazime à 50%, fongicide chimique) et Bactospeine® (16000 UI/mg, biopesticide à base de *Bacillus thuringiensis*). *Serratia* sp. C4 s'est montré un agent producteur de chitinase, de pectinase et de protéase et capable de produire l'acide indole-3-acétique et de solubiliser le phosphate.

Mots clés: Activité antifongique, bactéries endophytes, *Cestrum nocturnum*, *Fusarium oxysporum* f. sp. *lycopersici*, métabolites secondaires, promotion de la croissance, tomate

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ملخص

العايدي-بن عبد الله، رانية وبثينة مجدوب-طرابلسي وأحلام النفزي وهيفاء جبنون-خيار الدين، و ماجدة الدعمي-الرمادي. 2017. استخدام البكتيريا الداخلية المرتبطة طبيعيا بنبتة مسك الليل (*Cestrum nocturnum*) للمكافحة الحيوية للذبول الفوزاري وتحسين نمو الطماطم. Tunisian Journal of Plant Protection 12: 15-40.

تمّ تقييم قدرة ثلاث عزلات بكتيرية داخلية نباتية، عزلت من أوراق وسيقان مسك الليل(*Cestrum nocturnum*)، على الحدّ من الذبول الفوزاري للطماطم الناتج عن الفطر *Fusarium oxysporum* f. sp. *lycopersici* (FOL) وعلى تحسين نموّ نباتات الطماطم. خفّضت العزلات بشكل ملحوظ من شدة المرض بنسبة تراوحت بين 46,6 و 97,7% مقارنة مع الشاهد الملقّح بـ FOL والغير معامل. تم انتقاء العزلة C4 بصفتها الأكثر فعالية في الحدّ من إتلاف الأوراق بـ 86.6% ومن ارتفاع اسمرار الأوعية بـ 97.7% مقارنة مع الشاهد الملقّح بـ FOL والغير معامل. سجّلت زيادة معتبرة، من 39 إلى41.6 %، مقارنة مع الشاهد الخالي من العنصر المسبب للمرض والغير معامل، في معالم نمو الطماطم. إضافة على ذلك، مكّنت العزلة C4 من الزيادة في معالم النمو بنسبة 24.5إلى53.3%مقارنة مع النباتات الشواهد الغير ملقّحة والغير معاملة. تمّ تشخيص هذه العزلة ماكرومورفولوجيا وبيوكيميائيا وتمّ تحديد انتمائها باستخدام تسلسل جينات الحمض النووي S 16 إلى نوع من الجنس البكتيري *Serratia* sp. (KX197201). عند اختبار الفعاليّة المضادة في المخبر ضد FOL، تمكّنت *Serratia* sp. C4 من التخفيض من نمو العنصر المسبب للمرض بـ 19.52% ومن تشكيل منطقة التثبيط بـ 8.62 مم من قبس القطر. وتمكنت الرّواشح الزراعية لـ *Serratia* sp. C4، المضافة للوسط الإنمائي PDAبنسبة 20% (سعة/سعة)، من التخفيض من نمو العنصر المسبب للمرض بنسبة 23% مقارنة مع 21.7 و 9.2% المسجلة بعد التسخين في 50 و 100°س، على التوالي. أظهر مستخلص ن-بيوتانول لـ *Serratia* sp. C4، عند استعماله لجرعة بنسبة 5% (سعة/سعة)، فعالية مضادة ضد FOL بالحدّ من النمو بـ66.5%، مقارنة مع الشاهد الغير معامل، والتي كانت أعلى من تلك التي تم الحصول عليها باستخدام اثنين من المبيدات التجارية Bavistin® (50% Carbendazim، مضاد فطري كيميائي) و®Bactospeine (16000 UI/مغ، مبيد حيوي متكون من *Bacillus thuringiensis*). تميزت *Serratia* sp. C4 كذلك كمنتج للكيوتيناز والبيكتيناز والبروتاييناز والقادر على إنتاج حمض الأندول-3-أساتيك وعلى إذابة الفوسفور.

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