



## Potential of a novel endophytic *Bacillus velezensis* in tomato growth promotion and protection against *Verticillium* wilt disease

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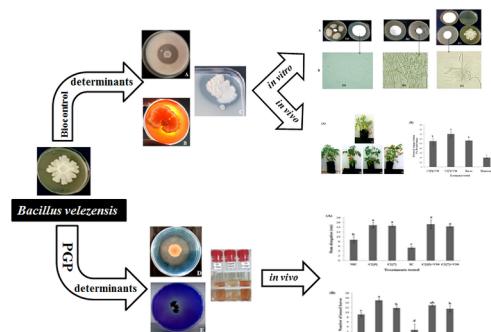
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### GRAPHICAL ABSTRACT



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### ABSTRACT

Vascular wilts are one of the major threats to the growth and the productivity of crop plants. Repeated use of chemical fungicides to manage vascular wilt diseases are raising concerns. Other strategies including biocontrol using endophytic bacteria have been considered. To investigate the role of endophytic bacteria on *Verticillium* wilt, a novel bacterium C2, isolated from the crown tissue of tomato, was evaluated, *in vitro* and *in vivo*, against tomato wilt disease caused by *Verticillium dahliae*. C2 was identified by 16S rRNA gene sequencing as *Bacillus velezensis*. This strain exhibited, *in vitro*, significant antifungal activity against *V. dahliae*. This strain harbored genes involved in synthesis of lipopeptides (bacillomycin, fengycin and surfactin), polyketides (macrolactin, bacillaene and difficidin) and the dipeptide bacilysin. Moreover, GC-MS analysis of C2 showed the presence of volatile metabolites known by their antifungal activity, namely tetradecane, benzeneacetic acid, benzaldehyde, 1-decene and phenylethyl alcohol. Furthermore, the strain was able to produce lytic enzymes (protease, chitinase and  $\beta$ -glucanase), siderophore and indole-3-acetic acid and to solubilize inorganic phosphate. Result of *in vivo* experiments indicated that the application of C2 at  $10^7$  CFU/mL, reduced significantly the *Verticillium* wilt incidence in tomato plant by  $70.43 \pm 7.08\%$ , compared to the untreated control. Besides protection, C2 improved markedly tomato plant growth which was evidenced by stem elongation and leaves number. The overall results of this study showed that *B. velezensis* C2 has a great potential to be commercialized as a biostimulant and biocontrol agent to manage effectively tomato *Verticillium* wilt disease.

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## 1. Introduction

*Verticillium dahliae* is a soil-borne fungus that can cause significant crops losses (Hollensteiner et al., 2017). *V. dahliae* invades the host through roots and then attacks xylem vessels which plug the vascular system. This results in wilting, vascular discoloration, early senescence and ultimately the death of the infected plant (Fradin and Thomma, 2006; Pegg and Brady, 2002). The control of *V. dahliae* is difficult because it can survive in soil for long periods of time in the form of resting structures (microscleroties) (Schnathorst, 1981). Soil fumigants used to control *Verticillium* wilt have limited efficiency and caused undesirable effects on non-targeted organisms and environment (Depotter et al., 2016; Frank, 2003). Ecological strategies such as solarization, crop rotation and the use of resistant plant species were also available and aimed at reducing initial soil inocula. However, all these strategies have only a limited efficiency (Depotter et al., 2016; Haas and Défago, 2005).

According to present regulations, which consider that treatment must prevent environment pollution and adverse effects on human health, biological control, through the use of plant growth promoting bacteria (PGPB), represents a potential alternative for several fungal disease management (Compan et al., 2010). Some of these PGPB have the ability to live inside plant tissues without causing any disease and are referred as plant growth-promoting bacterial endophytes (PGPE) (Reinhold-Hurek and Hurek, 2011). The potential of bacterial endophytes as biocontrol agents of vascular wilt has been revised by Eljounaidi et al. (2016). Endophytes confer plant protection against diseases by the induction of plant defense mechanisms, and the production of pathogen-antagonistic substances including antibiotics (Alina et al., 2015) and lytic enzymes (Chernin and Chet, 2002), or through the competition for colonization sites and nutrients (Compan et al., 2010). These bacteria colonize an ecological niche similar to plant pathogen, especially vascular wilt pathogens, which might favor them as potential candidates, not only for biocontrol but also for plant growth promotion (Bressan and Borges, 2004). Endophytes promote plant growth thanks to several mechanisms that include nitrogen fixation, phosphate solubilization, siderophores and phytohormones production such as auxins, cytokinins and gibberellins; and stress alleviation through the modulation of ACC deaminase expression. *Bacillus* species were reported as PGPE with a high potential as fungal antagonist (Hollensteiner et al., 2017). Moreover, compared to other biocontrol agents, several advantages are provided by *Bacillus* such as: (i) spore-forming ability that resists adverse environmental conditions (Nicholson et al., 2000), (ii) dry-product formulation which contains a lower contamination rate, (iii) establishment of a large-scale treatment and (iv) guarantee of an easy usage and cheap treatment (Fravel, 2005).

Looking at the great role of endophytic bacteria to control vascular diseases, the current study aims to: (i) evaluate, *in vitro*, the antagonistic and plant growth promotion activities of a new endophytic strain through the screening of some biocontrol and PGP determinants, and (ii) assess, *in vivo*, its plant growth promotion effects and its ability to suppress *Verticillium* wilt in tomato plants.

## 2. Material and methods

### 2.1. Bacterial strain, growth conditions and spore suspension preparation

C2 strain was originally isolated from surface-sterilized crown of tomato from Tunisian soil. The strain was selected among more than a hundred isolates belonging to our laboratory bacterial collection based on its endophytic behavior and potent antifungal activity, *in vitro*, against several fungi (data not published). It was routinely maintained on Lauria-Bertani (LB) (Miller, 1996) at 4 °C. For long-term storage, the bacterial strain was transferred to 15% glycerol and kept at -80 °C.

C2 strain was cultured, in an optimized medium (OM) for antifungal metabolite production (Mezghanni et al., 2012), at 30 °C and 200 rpm for 48 h. For spore suspension preparation, 72 h old culture of C2 was centrifuged at 6000 rpm for 20 min and the pellet was resuspended in sterile saline water (0.9% NaCl).

### 2.2. Identification of bacterial strain

C2 strain was characterized on the basis of morphological and physiological attributes as well as 16S rDNA sequencing. PCR was carried out using universal primers Fd1 (5'-AGAGTTGATCCTGGCT CAG-3') and Rd1 (5'-AAGGAGGTGATCCAGCC-3'). The genomic DNA of C2, extracted by standard protocols (Sambrook and Russell, 2001), was used as a template for PCR amplification that consisted of 30 cycles, with denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min and extension at 72 °C for 2 min and with a final extension of 1 cycle of 72 °C for 7 min. The amplified 1.5 kb product was purified from agarose gel, and then sequenced in an automatic sequencer (Avant Genetic analyzer, 3100 model). The obtained sequence was compared with reference strains in the National Center for Biotechnology Information (NCBI) database.

### 2.3. Fungal pathogen and spore preparation

The pathogenic fungal *V. dahliae* (strain V30) was provided by the Olive Institute of Sfax, Tunisia. It was maintained on potato dextrose agar (PDA) medium at 25 ± 2 °C in darkness and thereafter stored at 4 °C. The V30 spore suspension was prepared by incubating V30 in potato dextrose broth (PDB) supplemented with chloromphenicol (Cm) at 120 rpm and 25 ± 2 °C during 6 days. The spore suspension concentration was adjusted to 10<sup>5</sup> spores/mL.

### 2.4. In vitro antagonist assay of C2 metabolites against *V. dahliae* V30

The *in vitro* antagonistic activity of C2 strain against *V. dahliae* V30 was determined by dual culture, diffusible and volatile metabolites assays. For dual culture assay, a 5 mm diameter disk of actively grown V30 was placed in the center of a PDA plate, and then C2 was inoculated in four symmetrical spots. The plates were incubated at 25 ± 2 °C for seven days and the growth of the pathogen was measured and compared with the control. For the assessment of diffusible anti-fungal metabolites assays, well diffusion technique was used. A 5 mm disk of V30 was placed in the center of the Petri plate. Wells (6 mm in diameter) were made at 20 mm from the fungal plug and a 100 µL aliquot of cell-free supernatant previously filtered (0.22 µm sterilized millipore membrane filter) was pipetted into the wells. Plates were kept at 5 °C for 2 h to allow diffusion of the samples and then were incubated at 25 ± 2 °C for 7 days.

For volatile metabolites detection, the sealed plate method was used (Chaurasia et al., 2005). C2 was cultured on LB plate by spreading 50 µL of liquid culture. A mycelial plug (5 mm diameter) of V30 was placed to the center of PDA plate that was inverted and placed over the bacterial culture. The two culture plates were placed face-to-face preventing any physical contact and sealed together with double layer of paraffin film to prevent the loss of volatiles produced. As positive control LB plate without bacterial culture was covered and sealed with PDA plate inoculated with *V. dahliae* V30. The plates were incubated at 25 ± 2 °C for seven days and the pathogen growth was measured. Each assay was carried in triplicate. The fungal growth inhibition (GI) was calculated using the formula as follows: GI = [(C - T)/C] × 100, where: C = mycelia growth in control and T = mycelia growth in treatment.

The effect of C2 metabolites on fungal mycelium aspects was examined. Hyphae of *V. dahliae* were taken after 7 days incubation with

C2, from diffusible and volatile metabolites assays and observed using optical transmission microscopy (Zeiss LSM 3100). Samples from fungal control plates were also collected and observed. This experiment was repeated twice.

#### 2.5. Determination of inhibition zone, MIC/MFC of C2 extract

Fungal inhibition zone, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined for C2 extract obtained by the acid precipitation method. The antifungal substances were precipitated with 12-N HCl (pH 2.0) and the precipitate was extracted with minimal amount of methanol. The sample was dried and dissolved in tris HCl buffer (50 mM, pH 7.5). Antifungal activity was performed by the agar well diffusion method. One hundred  $\mu$ L of freshly prepared cell suspension of V30, adjusted to  $10^5$  spores/mL, were inoculated onto the surface of PDA plates. Thereafter, wells with 6 mm in diameter were punched in the inoculated agar medium with sterile Pasteur pipettes and 50  $\mu$ L of extracts (50 mg/mL) was added to each well. The plates were then incubated at  $25 \pm 2$  °C for 7 days and the diameters of fungal inhibition zone were measured. MIC was determined according to Eloff (1998) in sterile 96-well microplates with a final volume in each microplate well of 100  $\mu$ L. A twofold serial dilution of the extract was prepared in the microplate wells over the range 0.019–10 mg/mL. To each test well, 10  $\mu$ L of V30 cell suspension ( $10^5$  spores/mL) was added. The plates were then incubated at  $25 \pm 2$  °C for 7 days. The MIC was defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth after incubation. The MFC was determined by plating 10  $\mu$ L of liquid from each well that showed no fungal growth on PDA agar and incubated at  $25 \pm 2$  °C for 7 days. The lowest concentration that yielded no growth after this sub-culturing was taken as the MFC, indicating that > 99.9% of the original inoculum was killed. Three replicates were maintained to confirm the antifungal activity.

#### 2.6. PCR detection of antibiotic biosynthesis genes

For detection of lipopeptide, polyketide and dipeptide biosyntheses genes, primers listed in Table 1 were used for the PCR amplifications. The reaction mixture (total volume of 50  $\mu$ L) contained 10  $\mu$ L of 5  $\times$  PCR buffer, 4  $\mu$ L of 25 mmol/L MgCl<sub>2</sub>, 5  $\mu$ L of dNTP-mix (0.2 mmol/L), 5  $\mu$ L of each forward and reverse primer (10 mmol/L), 2 U of Taq DNA polymerase (GoTaq, Promega, Madison, WI, USA) and 50 ng of template DNA. Thermal cycling conditions included an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, 50–58 °C primer annealing for 1 min, extension at 72 °C

for 1.5 min and a final extension step at 72 °C for 7 min.

#### 2.7. Identification of C2 volatile compounds

Volatile compounds (Vocs) produced by C2 strain were analyzed by solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) on Agilent 7820A GC system coupled to Agilent 5975 series quadrupole mass spectrometer working in EI mode. The GC-MS was equipped with Thermo HP-5MS column (30 m  $\times$  250  $\mu$ m  $\times$  0.25  $\mu$ m) (J and W Scientific, USA). Operating conditions consisted of Helium as carrier gas (1.2 mL/min) and a temperature detector of 250 °C. The column was held for 5 min at 50 °C, then programmed to increase at rate of 4 °C per minute to a final temperature of 180 °C. The compounds were identified using NIST 11 library of mass spectra on Agilent ChemStation software.

#### 2.8. Lytic enzyme production

Protease and chitinase activities were evaluated by culturing bacterial strain on skim milk agar plate (Brown and Foster, 1970) and colloidal chitin agar plate (Wirth and Wolf, 1990), respectively. A clear zone around the colony after two days of incubation at 30 °C indicated the enzymatic degradation. To test  $\beta$ -glucanase activity, the bacterial strain was cultured in barley flour agar plate at 30 °C for two days and the plates were flooded with congo red solution (0.1%) for 15 min. Appearance of halo zone was considered to be a positive response for  $\beta$ -glucanase activity (Teather and Wood, 1982).

#### 2.9. Identification of plant growth promoting determinants

The strain C2 was analyzed for diverse plant growth promotion traits. Phosphate solubilization was assessed using NBRI-BPB medium (Ji et al., 2014), while Chrome Azurol S medium (CAS-medium) was used to detect siderophore production (Schwyn and Neildans, 1987).

Indole-3-acetic acid (IAA) production was analyzed following the method of Bric et al. (1991). Bacterial cells were grown on LB broth supplemented with or without tryptophan (1 mg/mL) and incubated at 30 °C for 72 h at 120 rpm. After incubation, the cells were centrifuged at 13,000 rpm for 15 min. Supernatant (1 mL) was mixed with two drops of orthophosphoric acid and 4 mL of Salkowski's reagent and kept for 25 min at 30 °C in the dark. IAA production was indicated by the development of a pink color. Spectroscopic analysis was done at 530 nm and quantified using an IAA (Sigma-Aldrich) standard curve. All experiments described in this section were performed in triplicate.

**Table 1**

Primers used for the detection of lipopeptide, polyketide and dipeptide biosyntheses genes in *Bacillus velezensis* C2.

Lipopeptides	Gene	Primers	Sequences (5'-3')	PCR products size (bp)	References
Bacillomycin	bmyB	BMYB-F BMYB-R	GAATCCCGTTCTCCAAA GCGGGTATTGAATGCTTGT	370	Mora et al. (2011)
Fengycin	fend	FEND-F FEND-R	GGCCCGTTCTCAAATCCAT GTCTATGCTGACGGAGAGCAA	269	Mora et al. (2011)
Surfactin	srfAA	SRFA-F SRFA-R	TGGGGACAGGAAGACATCAT CCACTCAAACGGATAATCCTGA	201	Mora et al. (2011)
Macrolactin	mlnA	mlnA-F mlnA-R	CCGTGATCGGACTGGATGAG CATCGCACCTGCCAACATCG	668	Compaoré et al. (2013)
Bacillaene	baeA	baeA-F baeA-R	ATGTCAGCTCAGTTCCGCA GATGCCGTCTCAATTGCC	688	Compaoré et al. (2013)
Difficidin	dfnA	dfnA-F dfnA-R	GGATTCAAGGAGGCATACCG ATTGATTAAACGGCGGAGC	653	Compaoré et al. (2013)
Bacilysin	BacA	BacA-F BacA-R	CAGCTCATGGGAATGCTTT CTCGGTCTGAAGGGACAAG	498	Mora et al. (2011)

## 2.10. Plant material, growth condition and treatments

One-month-old tomato plants (*L. esculentum*) were used for bioassays. These plants were placed in polyethylene bags (diameter 20 cm) filled with 500 g of sterile mixture of soil and peat (1:1). Treatments with spore suspension of C2 (prepared as described above and adjusted to a final concentration of  $10^5$  and  $10^7$  CFU/mL) were performed by soil drench each 15 days for four times in total. Soil drench with Prev-Am (active ingredient: Borax 0.99%) and Tachigazol (active ingredient: Hymexazol) served as biological and chemical controls, respectively. Fifteen days after first treatments, tomato plants were infected by incorporating  $10^5$  spores/mL of *V. dahliae* microsclerotia.

The following treatments were applied on tomato plants *in vivo*: (i) C2 alone at  $10^5$  CFU/mL, (ii) C2 alone at  $10^7$  CFU/mL, (iii) C2 at  $10^5$  CFU/mL with *V. dahliae*, (iv) C2 at  $10^7$  CFU/mL with *V. dahliae*, (v) Borax with *V. dahliae*, (vi) Hymexazol with *V. dahliae*, (vii) NIC: untreated non-infected control (only treated with water) as negative control, (viii) IC: untreated infected control with *V. dahliae* as positive control. Each treatment included three replicates with fifteen plants per replicate arranged in a complete randomized design.

## 2.11. Assessment of C2 effect on *Verticillium* wilt *in vivo*

To assess the efficacy of C2 strain in controlling *Verticillium* wilt, typing wilting symptoms were observed on all tomato plants infected by the pathogen. *Verticillium* wilt severity was recorded for nearly 6 weeks post infection via disease index (DI) calculation. DI is expressed as the number of diseased leaves over the total number of leaves per plant. The DI was determined based on a 0–4 scale with 0 = no discoloration (healthy leaf), 1 = 1–25% discoloration, 2 = 26–50% discoloration, 3 = 51–75% discoloration and 4 = 76–100% discoloration and the percentage of protection (%) for each treatment was calculated using formula: Protection (%) = [(DI of untreated infected tomato plant – DI of treated infected tomato plant)/DI of untreated infected tomato plant] × 100.

For the pathogen detection inside infected tomato plant, re-isolation of *V. dahliae* from fragments of roots, crown, stem and leaves was performed on PDA after 4, 7 and 10 weeks post infection. Each organ was superficially disinfected with 70% alcohol for 1 min as well as 1%

sodium hypochlorite for 1 min and washed 3 times in sterile distilled water. The samples were inoculated on the surface of PDA agar plates and incubated at  $25 \pm 2$  °C for seven days. The presence of V30 in the different plant parts was determined based on its morphology. Three replications were maintained for each treatment.

## 2.12. Plant growth measurement

To assess the capability of C2 to promote tomato plants growth, some biometrical parameters were recorded on plants belonging to the following treatments (i) C2 alone at  $10^5$  CFU/mL, (ii) C2 alone at  $10^7$  CFU/mL, (iii) C2 at  $10^5$  CFU/ml with *V. dahliae*, (iv) C2 at  $10^7$  CFU/mL with *V. dahliae*, (v) NIC: untreated non-infected control as negative control, (vi): IC untreated infected with *V. dahliae* as positive control. The effect of C2 on tomato growth promotion was measured in term of evolution of plant height and leaves number in the end of experiments.

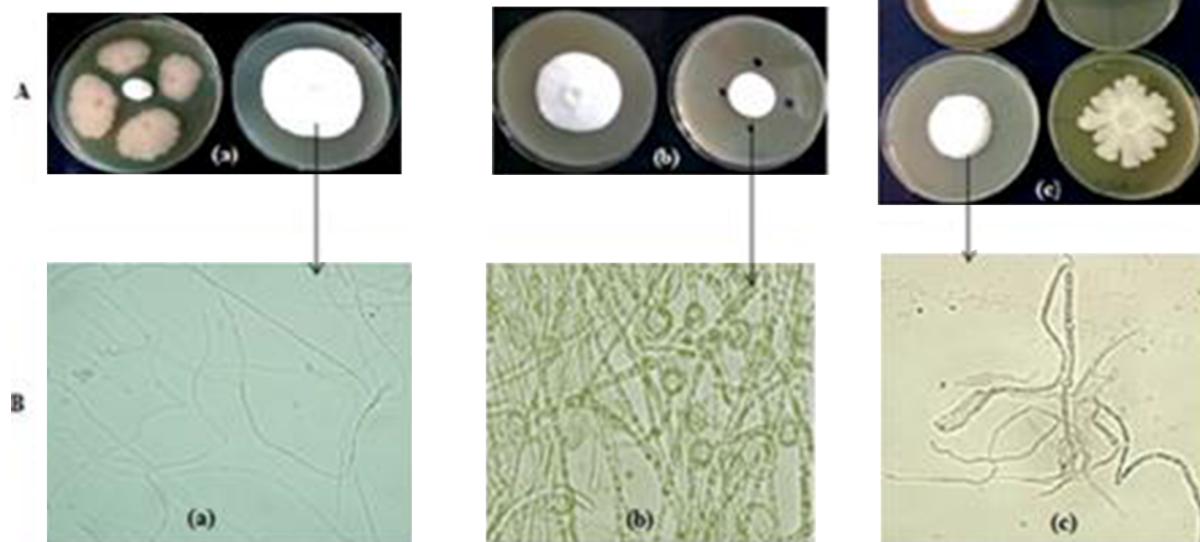
## 2.13. Statistical analysis

The data were subjected to variance analysis using the Statistical Package for the Social Sciences (SPSS V.17; SPSS Inc., Chicago, IL, USA). The mean values among the treatments were compared using the Duncan test at the 5% level of significance ( $p < 0.05$ ).

## 3. Results

### 3.1. C2 identification

C2 strain used in the present study was originally isolated from internal tomato crown. Phenotypic characteristics identification revealed that C2 is a Gram-positive, aerobic, motile, rod shaped and endospore forming bacterium with positive reaction for oxidase and catalase activities. In a molecular approach, the genomic DNA of this strain was used as template to amplify a 1450 bp PCR-fragment coding its 16S rRNA. Then, it was purified and sequenced. Blast results showed 100% identity with *Bacillus velezensis* NY12-2 (CP033576.1). The accession number obtained from GenBank for the partial 16S rRNA nucleotide sequence of *B. velezensis* strain C2 was KX931018.



**Fig. 1.** (A) Growth inhibition of *V. dahliae* by strain C2 using different *in vitro* assays: (a) Dual culture, (b) diffusible metabolites and (c) volatile metabolites. (B) Morphological effect of *B. velezensis* C2 on *V. dahliae*: (a) Control mycelium of *V. dahliae*, (b) damaged mycelia of *V. dahliae* by diffusible metabolites (c) damaged mycelia of *V. dahliae* by volatiles metabolites.

### 3.2. In vitro assessment of antifungal activity of C2 against *V. dahliae*

Direct confrontation assays (dual culture plate, diffusible and volatile metabolites assays) were used to evaluate the antagonist activity of C2 strain against *V. dahliae*, *in vitro*. As shown in Fig. 1 A, a significant inhibitory activity against the fungus was observed in the different assays. Fungal growth was reduced about  $59.13 \pm 0.9\%$  in dual culture assay by antagonist C2; and  $43.39 \pm 1.4\%$  and  $60.32 \pm 1.53\%$  by diffusible and volatile metabolites, respectively, compared with the controls.

The effect of C2 strain on hyphae of *V. dahliae* was examined. Microscopic observations showed clear differences in hyphae morphology. Malformations, vacuulations and swellings occurred in hyphae treated with C2 volatile or non-volatile metabolites. However, the mycelia from the control were intact, smooth with no swellings or vacuolation (Fig. 1B).

Fungal inhibition zone, MIC and MFC of C2 extract were determined against V30 (Table 2). The inhibition zone was around 32 mm. The MIC and the MFC values indicated that C2 extract has a fungicidal effect against V30 since the MFC/MIC ( $78/78 = 1$ ) ratio was less than 4 (Table 2).

### 3.3. Detection of metabolites potentially related to antimicrobial activity of C2

Potential biocontrol determinants, such as exoenzymes production, antibiotics gene presence and VoCs emission, were analyzed in C2. This strain showed positive reactions for protease (Fig. 2A),  $\beta$ -glucuronidase (Fig. 2B) and chitinase (Fig. 2C) activities. PCR were performed to determinate the presence of genes involved in the biosynthesis of cyclic lipopeptides (bacillomycin, fengycin and surfactin), polyketides (difficidin, bacillaene, macrolactin) and dipeptide (bacilysin) by C2 strain. The obtained PCR products were of expected sizes: 370, 269, 201 bp coding for bacillomycin, fengycin and surfactin respectively (Fig. 3A). These results showed that C2 could produce different types of antimicrobial lipopeptides. Alongside lipopeptides, genes involved in the dipeptide bacilysin (*bacA*) and the polyketides macrolactin (*mlnA*), bacillaene (*baeA*) and difficidin (*dfnA*) syntheses were also detected in C2 (Fig. 3B).

The VoCs emitted by strain C2 were analyzed by SPME-GC-MS. Thirty three known compounds and 6 unknown molecules were detected. The identified VoCs included 6 alcohols, 2 benzenes, 3 alkyls, 2 ketones, 1 aldehyde, 1 alkene, 1 ether and 17 terpenes, (Table 3).

### 3.4. Characterization of C2 for plant growth promoting traits

Several traits potentially involved in plant growth promotion were examined, *in vitro*. The strain C2 was positive for siderophore production, forming an orange halo ( $25.5 \pm 0.5$  mm) around bacterial colony on the CAS agar medium (Fig. 2D). The phosphate solubilization was observed by the presence of a clear halo around the colony on NBRI-BPB medium (Fig. 2E). Moreover, the IAA production by C2 was highlighted by the Salkowski assay in liquid medium with or without L-tryptophan induction. The IAA was produced at the respective concentrations of  $38.85 \pm 1.6$  and  $12.14 \pm 0.8$   $\mu\text{g/mL}$ .

### 3.5. Biocontrol efficacy of C2 against *Verticillium* wilt *in vivo*

In order to evaluate the antagonistic activity of C2, *in vivo* trials were carried out against *V. dahliae*, on tomato plants. Disease severity was assessed through the index of leaf damage, 6 weeks after infection and compared with chemical (hymexazol) and biological (borax) fungicides (Fig. 4). Severe *Verticillium* symptoms with chlorosis of the leaves, necrosis, wilt, and defoliation were noted in plants inoculated with *V. dahliae*. By comparison, plants treated with the antagonistic bacterium C2, borax or hymexazol showed lower wilt symptoms.

Otherwise, hymexazol provided a little protection effect (20.2%). However, the controlling efficacy of C2 at both concentrations ( $10^5$  CFU/mL and  $10^7$  CFU/mL) and borax were more than 50%. Particularly, C2 at  $10^7$  CFU/mL exhibited the best protection with 70.43% against 55.42% and 56.35% for C2 at  $10^5$  CFU/mL and borax respectively.

The presence of the pathogen inside the infected tomato plants (with or without treatments), was verified by the re-isolation of *V. dahliae* from the different organs at 4, 7 and 10 weeks post infection. The results, presented in Table 4, showed that the pathogen colonized the aerial part of infected plants after 7 weeks of infection. Plant treatment with either C2 at  $10^5$  CFU/mL or borax resulted in a slower progression of the fungus that colonized the aerial parts within 10 weeks. Interestingly, the application of C2 at  $10^7$  CFU/mL hampered the progression of the fungus to the aerial parts which remained localized in the root system.

### 3.6. Plant growth promotion by C2

The growth promoting activity of *B. velezensis* strain C2 was studied on tomato plants. Stem elongation and issued leaves number were assessed. The treatment of plants with C2 ( $10^5$  or  $10^7$  CFU/mL), whether infected with *V. dahliae* or not, significantly increased plants height by about 15 cm as compared to the control ones ( $8.7 \pm 1.2$  cm for NIC and  $5.37 \pm 1.77$  cm for IC) (Fig. 5A). Similarly, the number of issued leaves of the non-infected plants, as well as that of the infected ones, was significantly higher than those of the control plants, mainly when C2 was used at  $10^5$  CFU/mL (Fig. 5B).

## 4. Discussion

Vascular fungi, including *V. dahliae*, are generally devastating pathogens for which no curative control measures exist. *V. dahliae* is the causal agent of vascular wilt in many economically important crops worldwide including tomato. Current efforts to isolate, characterize and select potential bacterial antagonists to control phytopathogens have been reported (Rajaofera et al., 2017). The genus *Bacillus*, particularly, has been attracting attention due to its capacity to control *Verticillium* wilt (Danielsson, 2008; Han et al., 2015; Li et al., 2013; Tjamos et al., 2004; Yang et al., 2014). Here, the beneficial effect of an endophytic bacterial strain, isolated from the crown tissue of tomato and named C2, was investigated on tomato protection against *Verticillium* wilt; as well as on tomato growth promotion. According to morphological tests and 16S rDNA sequencing, C2 was identified as *Bacillus velezensis*. Potentiality of C2 as biocontrol agent against V30, was assessed via *in vivo* trials. Results demonstrated that tomato plants co-inoculated with *V. dahliae* and C2 at  $10^7$  CFU/mL had significantly less symptoms of *Verticillium* wilt. C2 reduced the disease incidence by  $70.43 \pm 7.08\%$  and had better biocontrol efficacy than borax and hymexazol. Likewise, Tjamos et al. (2004), showed that *B. amyloliquefaciens* strain 5–127 at  $10^7$  CFU/mL reduced disease by 40–70% in eggplant. Otherwise, pathogen re-isolation from different organs of infected treated plants confirmed that C2 inhibited *V. dahliae* V30 invading and spreading which remained localized in the root system without reaching the stem.

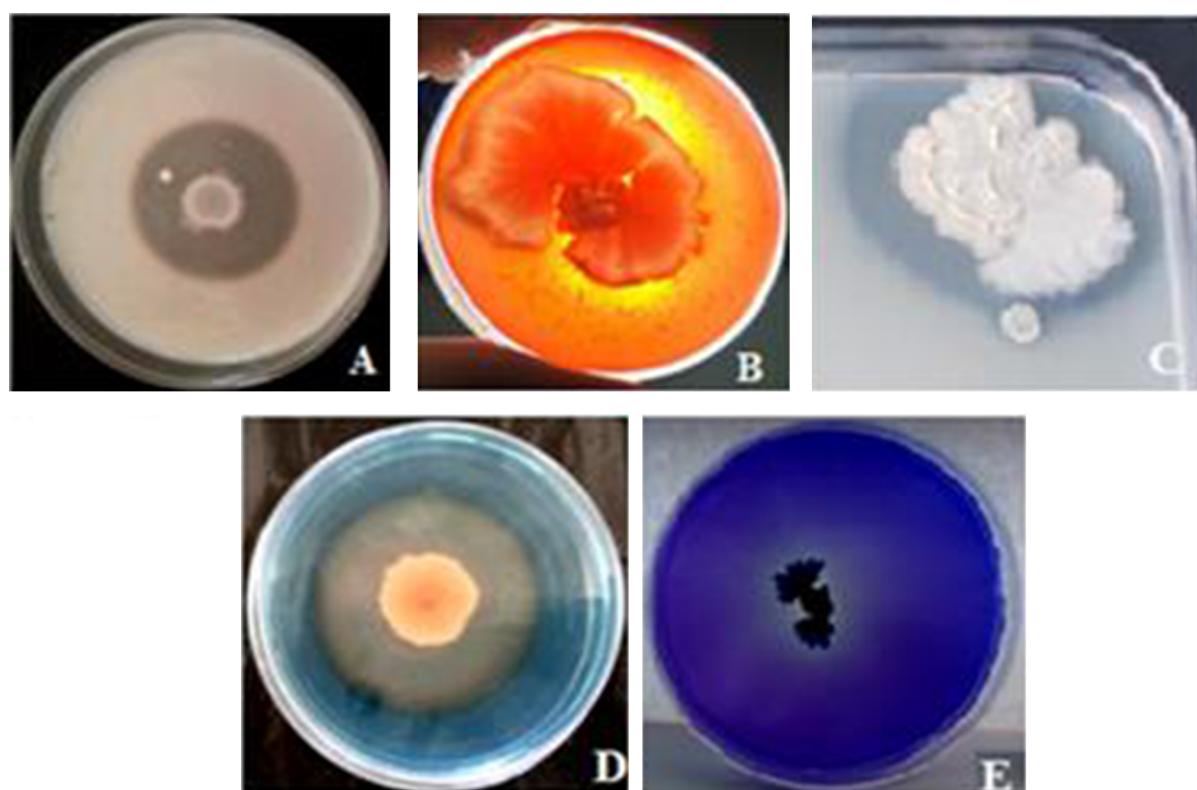
**Table 2**  
Antifungal activity of C2 extract.

Strain	C2 extract			
	IZ	MIC	MFC	MFC/MIC
<i>Verticillium dahliae</i>	$32 \pm 2$	78	78	1

IZ: Diameter of inhibition zone in mm. Each value is the mean  $\pm$  SD of three replicates.

MIC: The minimum inhibitory concentrations (as  $\mu\text{g/mL}$ ).

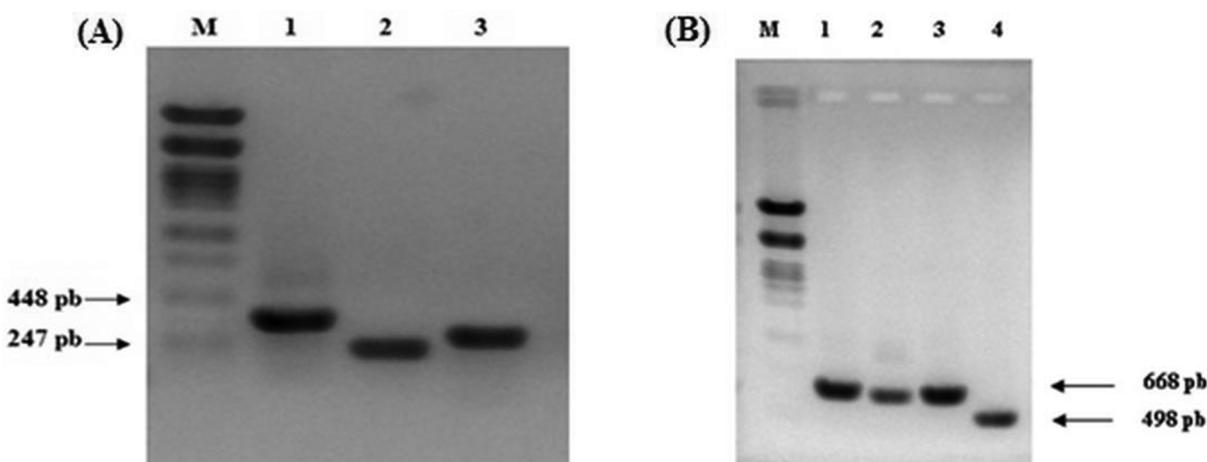
MFC: The minimum fungicidal concentration (as  $\mu\text{g/mL}$ ).



**Fig. 2.** Detection of biocontrol and plant growth-promoting determinants in strain C2 (A) Protease activity; (B)  $\beta$ -glucanase activity, (C) Chitinase activity, (D) Siderophore production and (E) Phosphate solubilization.

The biocontrol potential of C2 would be due mainly to its capacity to produce a wide variety of metabolites. Indeed, biocontrol agents revealed their antagonistic activity by the excretion of cell wall degrading enzymes, release of volatile compounds and secretion of extracellular antibiotics. Besides their direct antipathogenic action, antibiotics act as determinants in triggering induced systemic resistance (ISR) in the plant system and contribute to disease suppression by conferring a competitive advantage to biocontrol agents (Van Loon, 2007). Here, C2 exhibited *in vitro*, high antagonistic activity against *V. dahliae* and caused clear morphological distortions on fungal hyphae. In addition, C2 produced cell-wall degrading hydrolases namely protease, chitinase and  $\beta$ -glucanase and volatile compounds. The production of lytic

enzymes is an important trait in the selection of bacterial isolates as biological control agents. These lytic enzymes are able to inhibit growth of fungi by degrading their cell walls constituted mainly by protein,  $\beta$ -glucan and chitin (Maksimov et al., 2011). Volatile compounds, including aldehydes, ketones, alcohols, aliphatic alkenes, organic acids and sulphides are endowed with antifungal activity (Alina et al., 2015; Fernando et al., 2005; Sanzani et al., 2009) and were reported to protect tomato against the fungal pathogens *Alternaria solani* and *Botrytis cinerea* (Gao et al., 2017). Among VOCs released by C2, Tetradecone, 1-Decene, Benzaldehyde, Benzeneacetic acid and Phenylethyl Alcohol were described for their antifungal activities (Gu et al., 2007; Jasim et al., 2016; Wan et al., 2008; Yuan et al., 2012; Zouet et al., 2007). Thus,



**Fig. 3.** Agarose gel-electrophoresis of PCR fragments of antibiotic biosynthesis genes from *B. velezensis* C2: (a): lipopeptide genes: Lane M,  $\lambda$ -PstI molecular weight marker; Lane 1, Bacillomycin gene *bmyB*; Lane 2, Surfactin gene *srfAA*; Lane 3, Fengycin gene *fengD*. (b): polyketide and dipeptide genes: Lane M,  $\lambda$ -PstI molecular weight marker; Lane 1, Bacillaene gene *baeA*; Lane 2, Difficidin gene *dftA*; Lane 3, Macrolactin gene *mhnA* and Lane 4: Bacylysin gene *bacA*.

**Table 3**Volatile organic compounds released from *Bacillus velezensis* strain C2 detected by gas chromatography–mass spectrometry.

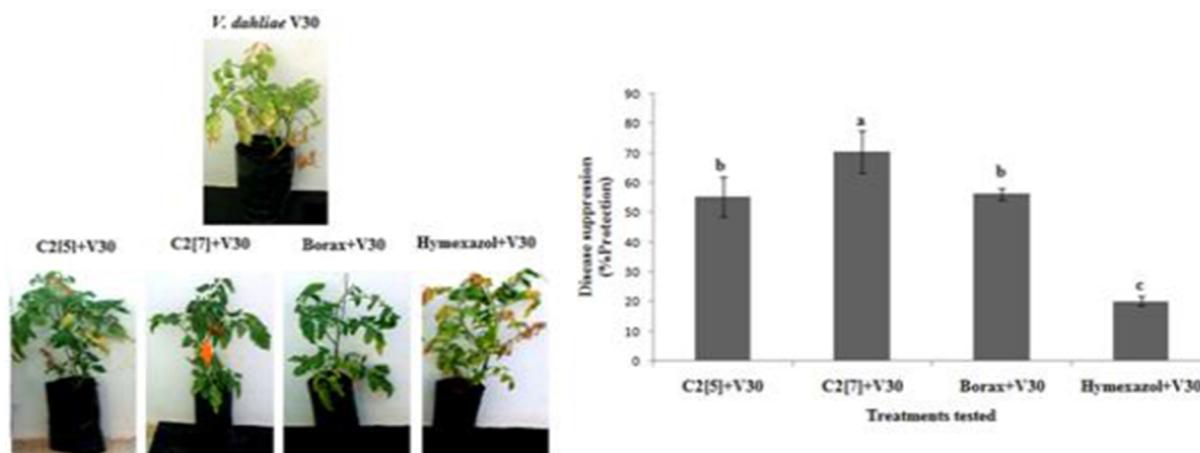
Compounds	Kovat's Index	Chemical formula	Role	References
<i>Alcohol</i>				
Phenylethyl Alcohol	1072	C <sub>8</sub> H <sub>18</sub> O	Antifungal activity	Wan et al. (2008)
trans-Sabinene	1051	C <sub>10</sub> H <sub>18</sub> O		
cis-p-mentha-2,8-diene-1-ol	1140	C <sub>10</sub> H <sub>16</sub> O		
Borneol	1158	C <sub>10</sub> H <sub>16</sub> O		
Isopinocarveol	1176	C <sub>10</sub> H <sub>16</sub> O		
α-terpineol	1185	C <sub>10</sub> H <sub>18</sub> O	Nematicidal activity	Gu et al. (2007)
trans-p-mentha-1(7),8-dien-2-ol	1202	C <sub>10</sub> H <sub>16</sub> O		
<i>Ketones</i>				
Pyranone	1119	C <sub>5</sub> H <sub>4</sub> O <sub>2</sub>		
2-undecanone	1298	C <sub>11</sub> H <sub>22</sub> O		
<i>Alkane</i>				
Decane	1000	C <sub>10</sub> H <sub>22</sub>	Induced Systemic Resistance (ISR)	Lee et al. (2012)
Undecane	1115	C <sub>11</sub> H <sub>24</sub>	Induced Systemic Resistance (ISR)	Lee et al. (2012)
Tetradecane	1400	C <sub>14</sub> H <sub>30</sub>	Antifungal activity against <i>F. oxysporum</i>	Yuan et al. (2012)
<i>Benzene</i>				
Ethyl benzene	854	C <sub>8</sub> H <sub>10</sub>	Used as pesticides	Xu et al. (2003)
<i>Epoxide</i>				
Cyclohexene oxide	731	C <sub>6</sub> H <sub>10</sub> O		
<i>Alkene</i>				
1-decene	987	C <sub>10</sub> H <sub>20</sub>	Antifungal activity against <i>aecillomyces lilacinus</i> and <i>Pochonia chlamydosporia</i>	Zouet al. (2007)
<i>Ether-oxide</i>				
Tetrahydrofuran	617	C <sub>4</sub> H <sub>8</sub> O		
<i>Acid</i>				
Benzeneacetic acid	1259	C <sub>4</sub> H <sub>8</sub> O	– An active auxin (a type of plant hormone), naturally produced by the metapleural gland of most ant species and used as an antimicrobial. – Volatile antifungal compound produced by <i>B. Amyloliquefaciens</i> strain BmB1	Wightman and Lighty (1982) Jasim et al. (2016)
<i>Aldehyde</i>				
Benzaldehyde	927	C <sub>7</sub> H <sub>6</sub> O	Antifungal activity against <i>Paecillomyces lilacinus</i> and <i>Androchonia chlamydosporia</i>	Guet al. (2007)
<i>Terpene</i>				
Camphepane	942	C <sub>10</sub> H <sub>16</sub>		
β-pinene	975	C <sub>10</sub> H <sub>16</sub>		
P-cymene	1011	C <sub>10</sub> H <sub>14</sub>		
d-Limonene	1018	C <sub>10</sub> H <sub>16</sub>	Antimicrobial effects against human pathogenic fungi and bacteria	Aggarwalet al. (2002)
Dimethyl styrene	1067	C <sub>10</sub> H <sub>12</sub>		
3-Methylene-p-menth-8-ene	1079	C <sub>10</sub> H <sub>18</sub>		
α-Terpinolene	1083	C <sub>10</sub> H <sub>16</sub>		
β-Elemene	1388	C <sub>15</sub> H <sub>24</sub>		
trans-Caryophyllene	1414	C <sub>15</sub> H <sub>24</sub>	– Anti-inflammatory – Local anaesthetic – Antifungal properties – Potent cytotoxic activity – Anti-oxidant – Antibacterial activity (slows bacterial growth)	Russo (2011)
γ-Muurolene	1474	C <sub>15</sub> H <sub>24</sub>		
Germacrene D	1477	C <sub>15</sub> H <sub>24</sub>	Antimicrobial and insecticidal proprieties	Arimura et al. (2004)
β-Selinene	1482	C <sub>15</sub> H <sub>4</sub> O <sub>2</sub>		
7-Epi-cis-sesquisabinene	1523	C <sub>15</sub> H <sub>26</sub> O <sub>2</sub>	Anti-inflammatory and anti-diarrheal	Kamal et al. (2015)
Geranyl isovalerate	1573	C <sub>15</sub> H <sub>26</sub> O <sub>2</sub>	Anti-inflammatory, antioxidant and anti-viral activities	Kamal et al. (2015)

these compounds could contribute to the potent antifungal activity of C2. Furthermore, VOCs have good potential for use as biostimulants to improve plant health (Sharifi and Ryu, 2018).

Otherwise, C2 was shown to harbor genes involved in biosynthesis of polyketide, bacilysin and lipopeptides (surfactin, fengycin and bacillomycin). Polyketides present a large family of secondary metabolites known by their antimicrobial activities (Arguelles-Arias et al., 2009). Different *Bacillus* strains were characterized by the production of these polyketides (Chen et al., 2006; Schneider et al., 2007). Bacilysins were also described for their antifungal and antibacterial activities (Arguelles-Arias et al., 2009). The fungicidal activities of lipopeptides have been reported against a large number of fungi (Ongena and Jacques, 2008). Fengycins, as well as Bacillomycin D, a member of the iturin family, display strong fungitoxic activity against filamentous and

soil-born fungi (Mardanova et al., 2017; Mora et al., 2011; Xu et al., 2013) and a limited antibacterial activity (Moyné et al., 2001; Ramarathnam et al., 2007). Kounoutsi et al. (2004) noticed the presence of synergistic action between these two lipopeptides. Surfactin is considered as a potent antifungal biosurfactant (Ben Abdallah et al., 2015; Peypoux et al., 1999; Tendulkar et al., 2007) and it is known by its ability to form stable biofilms and to promote plant tissue colonization by the antagonist (Cawoy et al., 2015). Besides, lipopeptides are reported to induce systemic resistance in host plant (Raaijmakers et al., 2010).

In addition to its biocontrol activity, the effect of C2 on growth promotion of tomato plants, *in vivo*, with or without Verticillium, was evidenced by an enhancement in the stem elongation and number of issued leaves, particularly at 10<sup>5</sup> CFU/mL. Results showed, also, that



**Fig. 4.** Biocontrol efficacy of strain C2 against *Verticillium* wilt on tomato *in vivo*. Biocontrol was assessed on tomato plants infected by V30 after 2 weeks of the following treatments: C2[5] + V30: C2 at  $10^5$  CFU/mL; C2[7] + V30: C2 at  $10^7$  CFU/mL; Borax + V30: biofungicide Prev-Am; Hymexazol + V30: chemical fungicide Tachigazol. Bars with the same letters indicate no significant differences between means as determined by the least significant difference test ( $p \leq 0.05$ ). Error bars indicate  $\pm$  SD of three replicates.

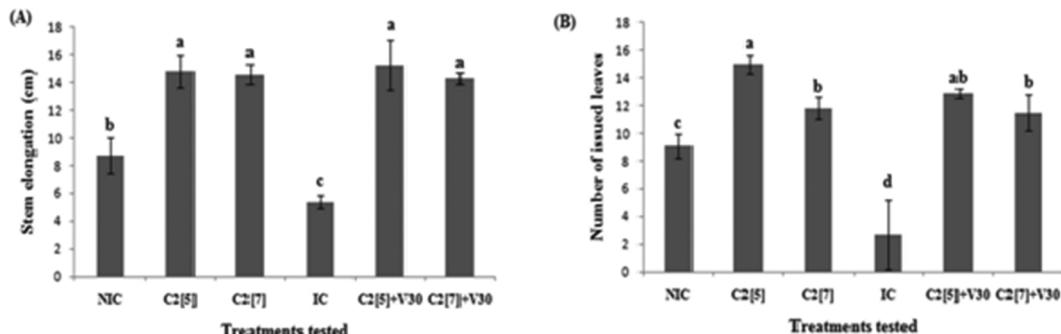
**Table 4**

Re-isolation of pathogen V30 on PDA from different parts of infected plants.

Treatment	Re-isolation time (Weeks after infection)	Tomato parts				
		Primary root	Secondary root	Crown	Stem	Leaves
IC	4	+	+	–	–	–
	7	+	+	+	+	–
	10	+	+	+	+	–
C2 [1 0 <sup>5</sup> ] + V30	4	+	+	–	–	–
	7	+	+	–	–	–
	10	+	+	+	+	–
C2 [1 0 <sup>7</sup> ] + V30	4	+	+	–	–	–
	7	+	+	–	–	–
	10	+	+	–	–	–
Borax	4	+	+	–	–	–
	7	+	+	–	–	–
	10	+	+	+	–	–
Hymexazol	4	+	+	–	–	–
	7	+	+	–	–	–
	10	+	+	+	+	–

+: presence of pathogen V30 / -: absence of pathogen V30.

IC: infected with the pathogen and untreated controls, C2[5] + V30: infected with V30 and treated with C2 at  $10^5$  CFU/mL, C2[7] + V30: infected with V30 and treated with C2 at  $10^7$  CFU/mL.



**Fig. 5.** Effects of strain C2 on tomato plant growth. (a) Effect of C2 treatments on stem elongation of tomato and (b) Effect of C2 treatments on number of issued leaves of tomato. NIC: untreated non infected control; IC: untreated infected with *V. dahliae*; C2[5]: C2 at  $10^5$  CFU/mL alone; C2[7]: C2 at  $10^7$  CFU/mL alone; C2[5] + V30: C2 at  $10^5$  CFU/mL with V30 and C2[7] + V30: C2 at  $10^7$  CFU/mL with V30. Bars with the same letters indicate no significant differences between means as determined by the least significant difference test ( $p \leq 0.05$ ). Error bars indicate  $\pm$  SD of three replicates.

high concentrations were not recommended for plant growth promotion. Similarly, Poupin et al. (2013) showed that when different doses (ranging from  $10^2$  to  $10^6$  CFU/mL of *Burkholderia phytofirmans* PsJN) were tested, plant growth parameters were significantly increased only with  $10^4$  CFU/mL.

The great potential of C2 for plant growth promotion could be related to the ability of this bacterium to produce multiples PGP determinants. *In vitro* investigation showed that C2 produced IAA, phosphatases and siderophores. Auxin phytohormone IAA, is the main auxin in plants that improves the fitness of the plant-bacteria interaction (Spaepen et al., 2007; Souza et al., 2015). Generally, IAA was implicated in the control of many physiological processes of vegetative growth, plant cell division, extension and differentiation, development of root system and enhancement of xylem rate (Etesami et al., 2015). Barazani and Friedman (1999) reported that bacteria producing 76.6 µg/mL of IAA caused root growth suppression; whereas a lower level of IAA (16.4 µg/mL) had a positive effect on root growth. This could explain the positive effect of C2 on tomato growth. Phosphorus solubilizing bacteria secrete organic acids and phosphatases which increases its uptake by the plant (Ji et al., 2014). Chen et al. (2014) reported that endophytic bacteria were able to solubilize phosphate and suggested that the inoculation of soil by endophytic bacteria improved not only phosphate availability but also, improve their adaptation to the soil environment. Siderophores are known by their positive effects on plant growth. The excretion of siderophores by bacteria provides plants with Fe nutrition which enhances their growth when its bioavailability is low. In addition, production of siderophores improves the colonization of the plants' tissues by the endophytic bacteria (Loaces et al., 2011). Siderophores act, also, as biocontrol agents by sequestering the limited iron in the rhizosphere and reducing its availability for the growth of pathogens (Ahmed and Holmström, 2014; Meziane et al., 2005; Yu et al., 2011).

Thus, the obtained results suggest a causal relationship between the wide variety of the beneficial traits produced *in vitro* and the biocontrol and growth promotion capacity of C2 *in vivo*. Further investigations are required to more understand the nature of interaction between antagonist, pathogen and host plant.

## 5. Conclusion

In conclusion, the current study reveals the bioactive potential of the endophytic *B. velezensis* strain C2 isolated from the crown tissue of tomato. *In vitro* investigations through lipopeptide biosynthesis gene detection, GC/MS analysis and PGP trait determination proclaim that the strain is a promising producer of a variety of compounds for plant growth promotion and fungal pathogen biocontrol. *In vivo* trials revealed that C2 seems to be an excellent candidate to be exploited as biocontrol agent against vascular diseases, particularly *Verticillium* wilt, as well as biofertilizer. Further studies concerning field applications and commercialization are in progress.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Author statement

S. Tounsi, M.A. Triki and H. Dhouib planned and designed the research.

H. Dhouib, I. Zouari, D. Ben Abdallah, L. Belbahri and W. Taktak performed experiments.

H. Dhouib and S. Tounsi analyzed data.

H. Dhouib, I. Zouari and S. Tounsi wrote the manuscript.

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