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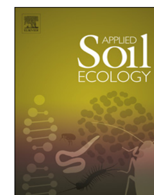
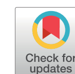


Growth promotion on horticultural crops and antifungal activity of *Bacillus velezensis* XT1



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ABSTRACT

Bacillus velezensis XT1 is a halotolerant bacterium isolated from a saline habitat in Spain. The objective of this work was to evaluate its plant growth promotion capacity on horticultural crops and its *in vitro* antifungal activity. The biochemical properties related to both of these characteristics were identified. When applied directly in the soil, strain XT1 significantly increased aerial fresh weight of tomato, pepper, pumpkin and cucumber plants by 53%, 63.6%, 129.2% and 100.8%, respectively, as compared to the control. Plant height, number of flowers and number of fruits increased more significantly in tomato plants treated with strain XT1. Under sterile conditions, strain XT1 significantly increased shoot and root biomass, height and number of leaves of tomato plants showing that its plant growth-promoting properties were independent of soil microbiota. In greenhouse and field experiments, addition of strain XT1 increased aerial biomass between 37 and 43.8% and height of tomato plants by 20.1 and 22.2%, respectively. Metabolic features associated with the properties of strain XT1 included nitrogen-fixing capacity; organic and inorganic phosphate solubilization; siderophore production, enzymes (*e.g.* urease and 1-aminocyclopropane-1-carboxylate deaminase) and volatile metabolites (such as acetoin and 2,3-butanediol). It produced endospores, had a high motility, synthesized high yields of exopolysacharides and formed biofilms. Strain XT1 displayed *in vitro* antifungal activity (over 40% of mycelium inhibition) against the phytopathogens *Alternaria alternata, Fusarium oxysporum, Monilinia fructicola, Magnaporthe oryzae, Thanatephorus cucumeris* and *Sclerotinia sclerotiorum*. All the characteristics described above showed a high potential of *Bacillus velezensis* XT1 to be used in agriculture.

1. Introduction

Over the past decades, management practices in agriculture have mainly been based on the use of synthetic chemical compounds, whose abuse has caused severe environmental and health problems (Aktar et al., 2009). Nowadays, agrochemicals have a very important role in crop enhancement, both at the quality and quantity levels. Still, during the last years, there has been a change in attitude in the society towards chemical-related products due to the emerging risks to the environment and human health. Consequently, more strict legislation is being imposed regarding the utilization of some chemical-based fertilizers and pesticides. As a result, novel alternative strategies to control plant diseases are urgently sought by scientists in order to maintain the rapidly growing world population, to produce good quality and abundant food and to avoid enormous economic losses in the agriculture sector (FAO, 2017; Reeves et al., 2019). The growing demand for healthy food supplies has had, as a consequence, an urgent need to progressively replace current control procedures with safe, eco-friendly approaches (Rouphael and Colla, 2018). Global efforts worldwide are focusing on seeking novel strategies to be used alone or in combination with agrochemicals in order to render the agriculture industry more sustainable and to maintain a healthy environment.

One of the most important strategies used in the agriculture industry is the use of formulations based on plant growth-promoting bacteria (PGPB), often referred to as plant growth-promoting rhizobacteria (PGPR) (Borriss, 2011). They are beneficial microorganisms which colonize the plant rhizosphere soil, compete with pathogens and act as fertilizers through different mechanisms (Richardson et al., 2009; Vejan et al., 2016). Thus, PGPB are able to stimulate plant growth through direct and indirect mechanisms. First, direct stimulation mechanisms

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include nitrogen fixing (Sessitsch et al., 2002); production of hormones such as auxins, gibberellins and cytokinins which increase root elongation, division and size (García de Salamone et al., 2001; Perrine et al., 2004); phosphate solubilization (Rodríguez and Fraga, 1999); and siderophore secretion (Kloepper et al., 1980). Second, indirect plant growth stimulation includes various biocontrol mechanisms against phytopathogenic agents, such as bacteria, fungi, arthropods and nematodes. These mechanisms include competition for ecological niches and/or substrates; production of hydrolytic enzymes (such as proteases, lipases and glucanases) and secondary metabolites (such as surfactin); root colonization and production of siderophores and volatile compounds (such as acetoin and 2,3-butanediol). All of them increase plant resistance to infections, which is also called induced systemic resistance (ISR; Wei et al., 1991; Borriss et al., 2019). In addition, some PGPB produce antimicrobial compounds, such as lipopeptides, that improve plant health acting against fungi and bacterial pathogens by reducing the number of root-colonizing phytopathogens and deleterious soil bacteria (Ongena and Jacques, 2008).

Members of the genus *Bacillus* are PGPB commonly used in agricultural systems, where they are preferred because of their ability to produce endospores, which allows them to survive to stress conditions. Additionally, *Bacillus* spp. are common inhabitants of the soil microbiota and they seem to not negatively affect native microbial diversity (Chowdhury et al., 2013; Qiao et al., 2017). Within the genus *Bacillus*, the species classically used as PGPB and biocontrol agents are *Bacillus subtilis*, *B. thuringiensis*, *B. amyloliquefaciens* and *B. megaterium* (Borriss, 2015). Nowadays, many registered strains of these species are commercially available (Parnell et al., 2016; Rabbee et al., 2019). In the last decades, the species *B. velezensis* (Ruiz-García et al., 2005) has joined the group of the most commonly used PGPB, being considered by some authors as being ahead of this list (Fan et al., 2018).

The type strain *Bacillus velezensis* CR-502^T was isolated from the river Vélez in Málaga, Spain (Ruiz-García et al., 2005). To date, this species has been isolated from many different habitats, such as water, soil, air and fermented food (Ruiz-García et al., 2005; Lee et al., 2017; Lim et al., 2018). *B. velezensis* has shown to have applications in sectors such as aquaculture, what makes it an economically relevant *Bacillus* species (Yi et al., 2018; Thurlow et al., 2019). However, its main use is in agriculture, where *B. velezensis* is one of the main sources of commercial bacterial inoculants (Fan et al., 2018). Although several strains of *B. velezensis* with biocontrol and/or plant growth-promoting ability have been identified, the activity of the different isolates can vary due to the presence of strain-specific clusters of genes, and therefore due to the production of particular enzymes and metabolites which play significant roles in both pathogen suppression and growth promotion (Cao et al., 2018; Rabbee et al., 2019).

The objective of this study was to demonstrate the plant growth-promoting potential of *Bacillus velezensis* XT1, a novel strain isolated from a saline habitat, and the *in vitro* antifungal activity of the strain.

2. Materials and methods

2.1. Bacterial strain

Bacillus velezensis strain XT1 was isolated in 2001 from the rhizosphere of a *Juncus effusus* (soft rush), which was grown in a saline soil (histosol; Soil Survey Staff, 2010) adjacent to the Capacete lagoon (Málaga, Spain; 37°01′33.3″N 4°49′38.2″W). Composite soil sample was stored at 4 °C for 2 h and transported to the laboratory for processing. Soil characteristics were pH 7.7, electroconductivity 0.93 S m^{-1} and 5.8 g L^{-1} salinity, indicating a saline soil. The isolation medium was MY (malt extract-yeast extract) (Moraine and Rogovin, 1966) modified with a balanced mixture of 7.5% (w/v) sea salts (Rodríguez-Valera et al., 1981). If not other stated, strain XT1 was grown on tryptic soy agar (TSA) at 32 °C.

2.2. Characterization of strain XT1

2.2.1. Genetic characterization

Genomic DNA of strain XT1 was extracted from a 5-mL overnight culture in tryptic soy broth (TSB) using the XDNA purification kit (Xtrem Biotech, Granada, Spain). 16S rRNA gene was amplified using primers 16F27 and 16R1488 (Brosius et al., 1978) and standard protocols (Saiki et al., 1988). An intermediate primer annealing at positions 401–421, 5′-CGGATCGTAAAGCTCTGTTG-3′, was designed using the Primer3 software (Untergasser et al., 2012). The 16S rRNA fragments obtained by PCR were cloned into the pGEM-T vector (Promega, Madison, USA) and transformed into *Escherichia coli* DH5α in order to obtain the complete sequence. The sequence obtained (1502 bp) was compared to reference 16S rRNA gene sequences available at the NCBI database using BLAST search tool (Altschul et al., 1990) and EzTaxon-e EzBioCloud program (Yoon et al., 2017).

2.2.2. Morphological, physiological and biochemical characterizations

Bacterial growth at ranges of temperature from 4 to 45 °C and pH values between 4 and 10 (1-steps) were determined on TSA by cell counts. Optimal growth and tolerance to salt stress were tested on TSA supplemented with 0 to 15% (w/v) NaCl (1%-steps) and determined visually. Gram staining and spores were observed by optical microscopy (Coico, 2005). Cell morphology was observed in a CrossBeam NVision40 field emission scanning electron microscope integrated with a focused Gallium ion beam microscope (Carl Zeiss, Oberkochen, Germany). Motility was determined by the hanging drop method using optical microscopy (Barrow and Feltham, 1993). Assays were carried out in triplicates.

The use of carbohydrates as sole carbon and energy sources was evaluated with an API 50CH system (BioMérieux, Marcy-l'Étoile, France) according to the manufacturer's protocol and Logan and Berkeley (1984). Growth in nitrogen-free medium was determined in Weinberg tubes containing Burk's medium with 0.5% (w/v) agar (Stella and Suhami, 2010). Growth with oxygen and with nitrate and nitrite under aerobic and anaerobic conditions, 2,3-butanediol fermentation (Voges-Proskauer test) and production of alkaline phosphatase, urease, glucanase (starch hydrolysis), proteases (gelatin and casein hydrolysis) and lipases (lecithin, Tween 20 and Tween 80 hydrolysis) were assayed according to Barrow and Feltham (1993) and Mata et al. (2002). The production of pectinases was determined according to Banakar and Thippeswamy (2012). Acid phosphatase was assessed using Pikovskaya's medium (Pikovskaya, 1948). Cellulase production was tested in TSA after replacing glucose with 1% (w/v) cellulose. ACC (1-aminocyclopropane-1-carboxylate) deaminase was analyzed according to the method described by Poonguzhali and Madhaiyan (2006). Siderophore production was evaluated with an overlay of chrome azurol S on cultures grown on TSA (Pérez-Miranda et al., 2007). Assays were conducted in triplicates.

Biofilm formation was determined using a crystal violet assay and by measuring absorbance at 540 nm (O'Toole and Kolter, 1998). Production of exopolysaccharides (EPS) was determined in Cooper medium (Cooper et al., 1981) supplemented with 1% (w/v) NaCl and 4% (w/v) sucrose following to the method described by Quesada et al. (1993). Total carbohydrate content was analyzed according to Dubois et al. (1956). In all tests, non-inoculated sterile medium was used as negative control. Assays were carried out in triplicates.

2.3. Growth promotion of horticultural crops

Tomato (*Solanum lycopersicum* var. *lycopersicum*), pepper (*Capsicum annuum* var. *glabriusculum)*, pumpkin (*Cucurbita pepo* subsp. *pepo* var. *styriaca*) and cucumber (*Cucumis sativus* var. *sativus*) plants were used for the outdoor experiments, which were conducted in spring (April–May) in Granada, Spain (37°05′49.6″N 3°35′58.7″W) at environmental temperatures ranging from 15 to 32 °C. Ten-cm high

seedlings of each plant species were transferred to pots (20 × 20 × 20 cm) filled with non-sterile potting soil (Compo, Münster, Germany). Twenty pots per plant species were placed randomly and watered every 48 h with tap water (~100 mL). Every 7 days, instead of watering, 5 mL of a culture (10^6 CFU mL^{-1}) of strain XT1 in Schaeffer's-glucose (SG) medium (Schaeffer et al., 1965) were diluted in ~100 mL of tap water and added to ten pots of each type of plant. An equal volume of tap water with 5 mL of non-inoculated SG was used to water the negative controls. After 50 days, number of leaves, flowers and fruits was counted, and height of the stem (tomato and pepper) or wine (pumpkin and cucumbers) was measured. Aboveground part of each plant was cut and its fresh weight was determined.

2.4. Growth responses of tomato plants to strain XT1 inoculation

2.4.1. In vitro experiments

Tomato seeds were surface-sterilized with a 10% (*v*/v) NaOCl solution for 10 min and 70% (v/v) ethanol for 8 min, rinsed with sterile distilled water and sown individually in twenty 25-cm high glass tubes containing 20 mL of ¼ sucrose-free Murashige-Skoog (MS) medium with 0.3% (w/v) agar (Murashige and Skoog, 1962). Ten tubes were inoculated with 100 μL of a culture of strain XT1 in SG (10^6 CFU mL^{-1}). Ten tubes supplemented with sterile non-inoculated SG were used as negative controls. All tubes were incubated at 25 °C and exposed to 16 h of light per day. After 30 days, plants were harvested and total aerial and root weight, height and number of leaves of each plant were recorded. Assays were carried out in triplicates.

2.4.2. Greenhouse experiments

Experiments in sterile conditions were done with tomato seeds which were surface-sterilized as explained above. Seeds were sown individually in sixteen 20 × 20 × 20-cm glass pots containing sterile potting soil (Compo, Münster, Germany), which had previously been autoclaved three times at 121 °C for 60 min, with 24 h-incubation at 28 °C between sterilizations (Trevors, 1996). The experiment was conducted once the plant height reached ~20 cm.

Experiments in non-sterile conditions were conducted with 20-cm (± 3.1 cm) tomato plants in pots (20 × 20 × 20 cm) filled with non-sterile potting soil. Fifty two plants were divided in two replicates and placed in two different greenhouse compartments. The plants were watered every 48 h with ~100 mL tap water.

Plants in both the sterile and the non-sterile experiments were watered every 48 h with ~100 mL sterile tap water. Every 7 days, instead of watering, 5 mL of a culture (10^6 CFU mL^{-1}) of strain XT1 in SG were diluted in ~100 mL sterile water and added to half of the pots of each replicate. An equal volume of water with 5 mL of sterile non-inoculated SG was used to water the negative controls. Assays were performed in winter (February–March) in a greenhouse located in Granada (Spain; 37°11′42.8″N 3°35′47.6″W) at 22–25 °C and with 16 h of light per day. After 35 days, aerial fresh weight and plant height were determined.

2.4.3. Field experiments

Assays were performed with 35-cm (± 4.7 cm) high tomato plants in summer (June–July) in a field in Almería, Spain (36°53′37.9″N 2°22′26.3″W) at ~20–32 °C and ~16 h of light per day, during 60 days. Two hundred and fifty two plants were divided into three replicates and planted directly in the soil in different parcels (84 plants per parcel). All plants were watered every 24 h with a similar amount of tap water (~200 mL) through a drip irrigation system.

Half of the plants in each parcel (42 plants) were treated with strain XT1. Plants were immersed for 5 min in a culture of XT1 in SG (10^7 CFU mL^{-1}) and then transferred to the field. After 12, 22 and 49 days, they were watered with 4 mL culture of XT1 (10^6 CFU mL^{-1}) diluted in 200 mL of a liquid nutrient solution prepared with 250 mg mL^{-1} of the fertilizer Herocris Micro Extra (Herogra Especiales, Granada, Spain). Equal volumes of non-inoculated SG and nutrient solution were used for negative control plants. Plant height and stem diameter were measured and number of leaves were counted at transplantation and at the end of the experiment. Aerial and root fresh biomass was weighed at the end of experiment.

2.5. In vitro antifungal activity

The antifungal activity of *Bacillus velezensis* XT1 was tested in two assays against six phytopathogenic fungi species: *Alternaria alternata* CECT 20560, *Fusarium oxysporum* CECT 2159, *Monilinia fructicola* CECT 21105, *Magnaporthe oryzae* CECT 20276, *Sclerotinia sclerotiorum* CECT 2769 and *Thanatephorus cucumeris* CECT 2813. All pathogenic fungi were cultivated in potato dextrose broth (PDB) at 25 °C with 100-rpm rotary shaking and maintained on potato dextrose agar (PDA).

2.5.1. Assay on solid media

Mycelium inhibition was measured on a solid medium (Ji et al., 2013). First, 10 μL of an overnight culture of XT1 in TSB (10^8 CFU mL^{-1}) were spread with a cotton swab over a ~1-cm^2 area on a PDA plate. Then, an 8-mm agar plug of the fungal mycelium was deposited on the opposite side of the plate. After a 20-day incubation period at 25 °C, the mycelium inhibition rate [IR% = (A − B) / A × 100] was calculated considering A as the maximum and B the minimum values of the mycelium radius. Non-inoculated TSB was used as a negative control. Mean value and standard deviations of the measurements were obtained based on three replicates of the assay.

2.5.2. Assay in liquid media

Antifungal activity was evaluated to determine the inhibition of fungal spore germination (and posterior mycelium formation) by the supernatant of a liquid culture of strain XT1 (Frikha-Gargouri et al., 2017; Toral et al., 2018). Briefly, 15-day cultures of each fungus were crushed and filtered through a 80-μM pore gauze under sterile conditions. This spore suspension was adjusted to a concentration of 10^7 conidia mL^{-1}, and was treated with 2.5 mg mL^{-1} of benzylpenicillin (Sigma-Aldrich, St. Louis, USA) and 10 mg mL^{-1} of streptomycin (Sigma-Aldrich, St. Louis, USA) to avoid bacterial contamination. Strain XT1 was cultivated in SG (10^9 CFU mL^{-1}) for 48 h at 32 °C with 100-rpm rotary shaking. The culture was centrifuged at 10,000 ×*g* for 15 min, and the supernatant was filtered through a 0.22 μM-pore membrane filter. A mix of 900 μL of the spore suspension and 300 μL of the filtered supernatant of strain XT1 were placed in the wells of a 48-well culture plate. The same volume of spore suspension supplemented with 50 μg mL^{-1} of cycloheximide (Sigma-Aldrich, St. Louis, USA) was used as a positive control of growth inhibition, while the spore suspension alone was used as a negative control of fungi inhibition. After 30 days of incubation at 25 °C with 100-rpm rotary shaking, the growth of the pathogenic fungi was assessed visually and spectrophotometrically (OD_{600}). Assays were carried out in triplicates.

2.6. Statistical analyses

Statistical analyses were conducted using the R software (R Core Team, 2017). The global effect of strain XT1 on different variables in each experiment was assessed by a two-way ANOVA. A normal distribution and identity link function for variables measured (such as height and fresh weight), as well as a quasi-poisson distribution and square root link function for the variables counted (such as number of fruit, leaves and flowers) were assumed. The effect of treatment under each condition was then assessed by *post-hoc* Tukey analysis.

3. Results

3.1. Characterization of strain XT1

With the objective of genetically identifying strain XT1, its complete

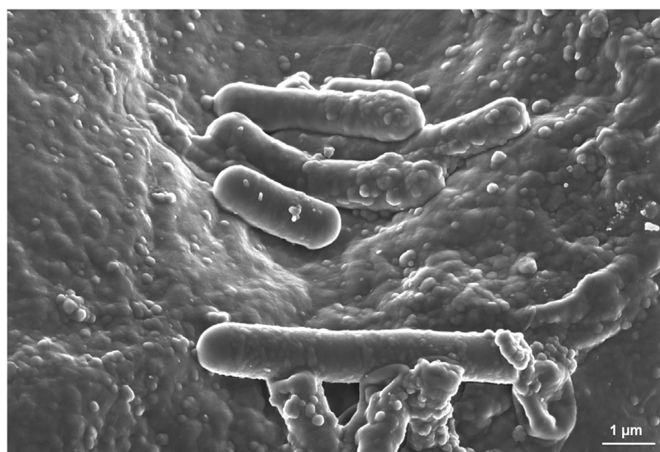

Fig. 1. Scanning electron microscopy of *Bacillus velezensis* XT1. Exopolysaccharideis observed surrounding the rod cells.

16S rRNA gene (1502 bp) was sequenced. The analysis showed that strain XT1 had 99.7% sequence similarity to type strain *Bacillus velezensis* CR-502^T (Ruiz-García et al., 2005).

In order to determine the properties related with plant growth-promoting activity of strain XT1, morphological, physiological and biochemical studies were conducted. Briefly, *B. velezensis* XT1 was an extremely motile, sporulating, gram-positive and halotolerant rod (Fig. 1). It had the ability to grow in a wide range of salt concentrations [0–12% (w/v) NaCl], temperature (15–40 °C) and pH (pH 5–10), with optimum growth conditions at 0.5% (w/v) NaCl, 32 °C and pH 7. Strain XT1 did not have any special nutritional requirements; it was able to fix nitrogen and to grow with several organic compounds (such as glucose, fructose, mannose, glycerol, sucrose, cellobiose and arabinose) as sole carbon and energy sources. It could grow under aerobic conditions, with oxygen as electron acceptor, as well as under anaerobic conditions using nitrate or nitrite as terminal electron acceptor. Moreover, strain XT1 produced acetoin and 2,3-butanediol during glucose fermentation and it was able to hydrolyze pectin, starch, gelatin, casein and lecithin. However, it did not hydrolyze cellulose, Tween 20 and Tween 80. Strain XT1 also synthesized enzymes such as alkaline and acid phosphatases, ureases and ACC deaminase. It produced siderophores and formed biofilm. Regarding the production of EPS, strain XT1 yielded 1.5 g L^{-1} of EPS with 40% (*w*/w) of carbohydrate content after 5 days of incubation. This EPS was easily observed surrounding the cells by microscopy (Fig. 1).

3.2. Growth promotion of horticultural crops

The results obtained in the outdoor experiment showed very significant differences between the non-inoculated and the XT1-treated plants ($P < 0.001$) with respect to most of the parameters evaluated. These differences were observed in the four types of plant tested (Fig. 2a; Table 1). Fresh weight of the aerial part increased by 129.2, 100, 63.6 and 53% in pumpkin, cucumber, pepper and tomato plants, respectively, when treated with strain XT1. Number of leaves increased significantly in pumpkin, cucumber and pepper ($P < 0.01$) by 56.2, 100 and 16.7%, respectively. However, this parameter was not measured in tomato plants. Plant height of pumpkin, cucumber, pepper and tomato rose by 9.8, 6.5, 22.1 and 13.7%, respectively, with significant differences ($P < 0.05$) being for all crops except for cucumber plants. Number of flowers and fruits increased significantly ($P < 0.05$) for all the plants treated with strain XT1.

Tomato plants showed the most significant differences ($P < 0.001$) in all the parameters tested between the non-treated and the XT1-treated plants. Therefore, this type of horticultural crop was chosen for the rest of the plant experiments.

3.3. Growth promotion of tomato plants

3.3.1. In vitro experiments

Tomato plants inoculated with strain XT1 showed longer shoots (aerial part) than the non-treated plants, with an increase of 12.1% in height. Fresh weight (aerial and root weight) of the tomato plants was increased by 38.6% by strain XT1. Number of leaves increased by 38.5% (Table 2). Differences observed were significant ($P < 0.05$).

3.3.2. Greenhouse experiments

Tomato plants cultivated in pots with sterile substrate and inoculated with strain XT1 exhibited a significantly higher ($P < 0.05$) stem than the non-treated plants, showing an increase of ~10% in height. Regarding the total fresh weight, it was increased by 32% ($P < 0.05$).

In the assays conducted in pots with non-sterile substrate, the total fresh weight and the height of the tomato plants treated with *B. velezensis* XT1 displayed an increase of 37% and 20.1%, respectively, as compared to the non-treated plants. The differences observed were statistically significant ($P < 0.05$) for both.

3.3.3. Field trials

Aerial weight of tomato plants was increased by 43.8%, stem diameter by 9.3% and plant height by 22.2% in comparison with non-treated plants (Fig. 2b). These three parameters differed significantly ($P < 0.01$) with the control plants. However, differences observed in root weight and number of leaves were not considered significant ($P > 0.05$).

3.4. In vitro antifungal activity

The assay in solid media demonstrated that strain XT1 had *in vitro* antagonistic effect and it reduced significantly ($P < 0.05$) by over 40% the mycelia of the pathogenic fungi tested after 20 days of incubation (Fig. 3). The maximum levels of antagonist activity (> 80%) were reached against the pathogen *Monilinia fructicola*.

In the liquid media assay, the supernatant of strain XT1 completely inhibited spore germination and therefore later mycelium formation (100% inhibition) of *Magnaporthe oryzae, Thanatephorus cucumeris, Sclerotinia sclerotiorum* and *Monilinia fructicola* after 30 days. Antifungal activity against *Alternaria alternata* and *Fusarium oxysporum* was lower (~80% inhibition), as both showed a very subtle turbidity (OD_{600} 0.09) in the wells due to fungal growth. Differences to non-treated plants were significant ($P < 0.05$) in all cases.

4. Discussion

Strain XT1, isolated from a rhizosphere sample taken from a saline soil, belongs to the *Bacillus velezensis* species. Interestingly, although many strains of *B. velezensis* have been identified to date, only a few have been isolated from saline environments (Marasini et al., 2017; Zhu et al., 2019). Soil salinization and desertification are upcoming problems in agriculture due to climate change. Given the rapid increase in the area of arable land affected, salt-tolerant PGPB appear to be an appropriate tool to deal with the problem of productivity (Shrivastava and Kumar, 2015; Forni et al., 2017).

Regarding the biochemical explanation for the activity of *B. velezensis* XT1, the following features may explain its role in plant growth promotion and therefore its potential use in agriculture, as it has been confirmed in other PGPB. Strain XT1 produces endospores and it is highly resistant to physical and chemical stressors such as salinity, temperature and pH. This suggests that this bacterium can survive under stress conditions, such as acid, basic and saline soils, as well as cold and hot climates. In addition, its high motility and EPS production could favor habitat colonization. EPS, which surround the bacterial cells and are involved in biofilm formation, are described to favor plant

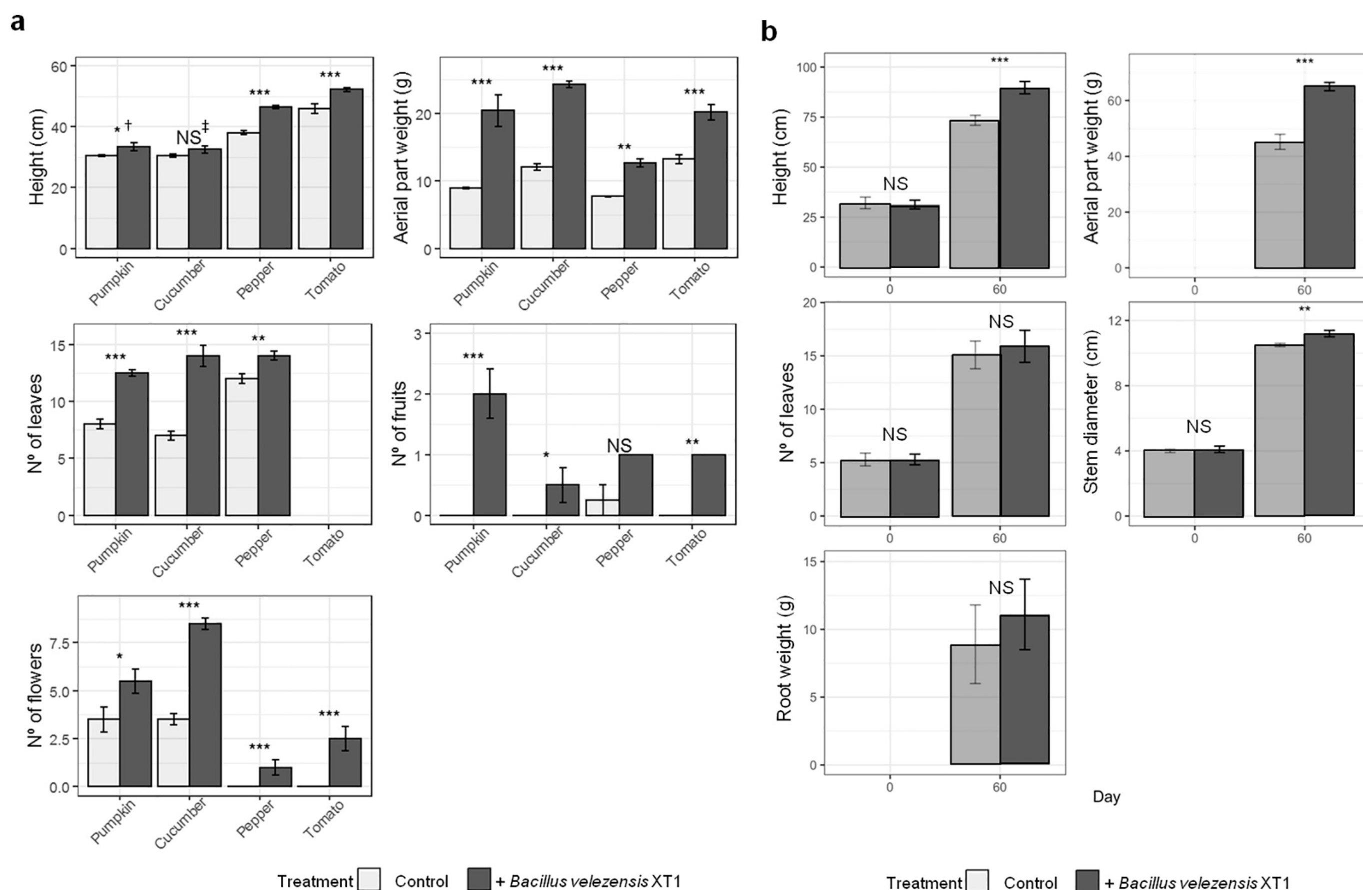

Fig. 2. Growth of horticultural crops with and without addition of *Bacillus velezensis* XT1. (a) Pumpkin, cucumber, pepper and tomato plants in the outdoor experiment. (b) Tomato plants in the field trial. †Significant differences between treatments within each species are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). ‡NS: not significant.

colonization by improving adhesion to roots (Al-Ali et al., 2017). They also protect both the plant and the bacteria against chemical compounds commonly used in agriculture and stress conditions such as salinity (Ashraf et al., 2004).

Another reason to consider the potential of strain XT1 in agriculture is its versatile metabolism. It is able to grow using a broad range of organic compounds as carbon sources and it performs different types of respiration and fermentation. XT1 is facultative anaerobic, which allows it to grow on both soil surface and in the rhizosphere under anaerobic conditions. Moreover, it could also perform butanediol fermentation. 2,3-butanediol and its precursor acetoin, formed during this fermentation, are reported to produce an increase in plant resistance to

Table 1
Horticultural crops in outdoor conditions inoculated with *Bacillus velezensis* XT1.

	Height	Aerial part weight	Leaves number	Fruits number	Flowers number
	cm	g			
Pumpkin					
Non-inoculated	30.5 ± 0.7	8.9 ± 0.2	8.0 ± 0.8	0.0 ± 0.0	3.5 ± 1.3
Strain XT1	33.5 ± 2.7*[†]	20.4 ± 4.7***	12.5 ± 0.6***	2.0 ± 0.8***	5.5 ± 1.3*
% increase	9.8	129.2	56.2	ND[b]	ND
Cucumber					
Non-inoculated	30.5 ± 1.1	12.1 ± 0.9	7.0 ± 0.8	0.0 ± 0.0	3.5 ± 0.5
Strain XT1	32.5 ± 2.2	24.3 ± 1.0***	14.0 ± 1.8***	0.5 ± 0.5***	8.5 ± 0.5***
% increase	6.5	100.0	100.0	ND	ND
Pepper					
Non-inoculated	38.1 ± 1.1	7.7 ± 0.12	12.1 ± 0.8	0.2 ± 0.5	0.0 ± 0.0
Strain XT1	46.5 ± 1.1***	12.6 ± 1.2**	14.1 ± 0.8**	1.0 ± 0.0**	1.0 ± 0.8***
% increase	22.1	63.6	16.7	ND	ND
Tomato					
Non-inoculated	46.1 ± 3.1	13.2 ± 1.2	ND	0.0 ± 0.0	0.0 ± 0.0
Strain XT1	52.3 ± 1.3***	20.1 ± 2.3***	ND	1.0 ± 0.0***	2.5 ± 1.3***
% increase	13.7	53.1	ND	ND	ND

[†] Significant differences between mean values of strain XT1-treated and non-inoculated plants within each horticultural crop are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
[a] Not determined.

Table 2
Tomato plants in sterile conditions inoculated with *Bacillus velezensis* XT1.

	Height	Aerial and root weight	Leaves number
	cm	mg	
Non-inoculated	8.2 ± 0.48a[†]	60.0 ± 3.7a	2.6 ± 0.24a
Strain XT1	9.2 ± 0.37b	83.2 ± 3.3b	3.6 ± 0.24b
% increase	12.1	38.6	38.5

[†] Mean values within a column followed by different lowercase letters indicate that they are significantly different ($P \leq 0.05$).

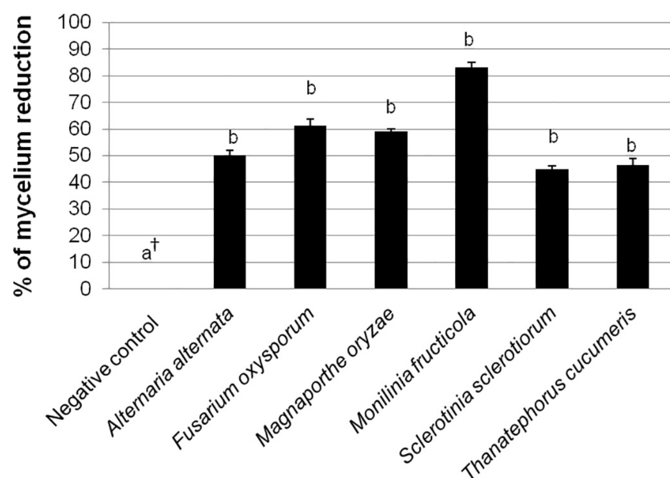

Fig. 3. Antifungal activity of *Bacillus velezensis* XT1. Percentage of mycelium reduction of *Alternaria alternata, Fusarium oxysporum, Magnaporthe oryzae*, *Monilinia fructicola*, *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* in the presence of *Bacillus velezensis* XT1 as assessed in the *in vitro* solid antifungal test. Negative control consists of non-inoculated tryptic soy broth. Error bars represent standard deviations. †Different letters above the bars indicate that the means differ significantly ($P < 0.05$) with respect to the non-treated negative control.

infections, which is called ISR (Wei et al., 1991). Strain XT1 could also grow in soils with low levels of nitrogen compounds due to its nitrogen-fixing capacity, a property which is typically found in many free-living rhizobacteria that live outside plant cells. These bacteria fix atmospheric nitrogen in soil, providing it to plants (Riggs et al., 2001).

Strain XT1 synthesizes enzymes such as pectinases, glucanases, proteases and lipases, which enable the utilization of a wide range of molecules to be used as carbon sources (Jadhav and Sayyed, 2016). Other enzymes produced by strain XT1, such as phosphatases, ureases and ACC deaminase, are closely associated with plant growth promotion. Firstly, phosphate, which is mainly found in a non-soluble inorganic or organic form in soils, is often unavailable to plants. Strain XT1 produced alkaline phosphatases, which release phosphate from organic compounds, and acid phosphatases, which solubilize inorganic phosphate (Rodríguez and Fraga, 1999). By improving phosphorous uptake by the plant, these enzymes are described to play an important role in crop production (Khan et al., 2009). Secondly, urease activity in strain XT1 enables ammonium to be liberated from urea, a nitrogen source for plants and thus an important metabolite associated with plant growth promotion (Witte, 2011). Thirdly, the production of ACC deaminase has been described to increase plant resistance to several stresses. This enzyme reduces ethylene levels in plants, which has been related to various stress conditions such as salinity, drought, flooding, flower wilting, metals, organic contaminants and pathogens (Glick, 2014). Finally, strain XT1 also produced siderophores which on one hand can provide plants with soluble iron and on the other hand contribute to disease suppression by chelating iron, which is essential for the survival of microbial pathogens (Kloepper et al., 1980).

When applied into the soil, strain XT1 stimulated the growth of tomato, pepper, cucumber and pumpkin crops, being tomato plants those that showed the most significant differences to non-treated control plants in all the growth parameters tested. Differences between field and greenhouse experiments arise from the different assay conditions and development stages of the test plants.

Experiments with sterile substrate were conducted to confirm the plant growth-promoting effects to be attributable to strain XT1 and to exclude synergic effects of other plant-associated or soil bacteria. Since no significant differences in growth promotion by strain XT1 using sterile and non-sterile conditions were observed, plant growth-promoting effects were proved to be due to XT1. However, the increase observed is less pronounced than the recorded in non sterile soil, but the assay conditions and plant development stage where not the same. Similar differences in the results obtained were also reported for other PGPB (Gholami et al., 2009), suggesting that plant growth promotion due to indirect interaction with native soil or root microorganisms cannot be disregarded (Kloepper and Schroth, 1981). Related to this, several authors have determined that different plant growth-promoting *Bacillus* species do not negatively affect the native rhizosphere microbial community (Chowdhury et al., 2013; Qiao et al., 2017), indicating the importance of a functional microbial rhizosphere community.

Several strains of *B. velezensis* have been described as PGPB and were evaluated *in vitro* or in pot experiments under greenhouse controlled conditions. For instance, the addition of *B. velezensis* strain BAC03 enhanced the growth of nine different types of plants, leading to an increase in biomass, although this is not specified (Meng et al., 2016). Palencia et al. (2015) studied the positive effect of treatment with *B. velezensis* IT45 on strawberry plants, although growth promotion was low in comparison with the non-treated control. On the other hand, Madhaiyan et al. (2010) demonstrated that *B. velezensis* CBMB205, increased the root length of tomato, red pepper and canola plants by 38.3, 4.2 and 22.4%, respectively, but data regarding plant height and aerial weigh were not given. Finally, Hassan et al. (2019) studied the effect of the addition of several *B. velezensis* strains on soy shoot length, but the differences were not significant. On the one hand, the data obtained by other authors were not as high and promising as the results obtained with strain XT1. On the other hand, although studies *in vitro* or in pots exist, extensive studies such as the one conducted in this work are scarce (Chowdhury et al., 2013; Wang et al., 2016).

With regard to fungal mycelium growth inhibition, several strains of *B. velezensis* have been reported to suppress the growth of fungal phytopathogens such as *Trichoderma, Fusarium, Alternaria* and *Monilinia* (Grady et al., 2019; Luo et al., 2019; Pandin et al., 2019). Strain XT1 showed high antifungal activity against six plant pathogenic fungi, some of which have a very important economic impact (Dean et al., 2012). Although several studies have demonstrated the antifungal activity of *B. velezensis* strains, the different methodology used in each case makes comparisons with our results difficult. For instance, the supernatant of *B. velezensis* SYBCH47 produces a 20% inhibition of *F. oxysporum* in a liquid test (Li et al., 2016), while strain XT1 produces a reduction of ~80%. Lim et al. (2017) demonstrated that *B. velezensis* SGAir0473 had activity against *Magnaporthe oryzae* and *Sclerotinia sclerotiorum* in a solid test, but mycelium growth inhibition was not quantified. Grady et al. (2019) showed the antifungal activity of *B. velezensis* 9D-6 against *M. fructicola* in a solid test, but the authors did not quantify the fungal inhibition of the strain.

Biocontrol microorganisms are able to inhibit fungal mycelium growth by diverse mechanisms such as the production of lipopeptides (Ongena and Jacques, 2008), siderophores (Yu et al., 2011), proteases (Zhang et al., 2012) and volatile compounds (Gao et al., 2018), amongst others. Strain XT1 has been described to produce siderophores, proteases and large amounts of lipopeptides (10 g L^{-1}). Toral et al. (2018) identified the antifungal lipopeptides surfactin, bacillomycin and fengycin from culture filtrates of strain XT1, which inhibit the growth of

the fungus *Botrytis cinerea*. In the past years, other strains of *B. velezensis* have been identified as producers of lipopeptides, which have proven to be effective against certain pathogenic fungi (Dimkić et al., 2017; Cao et al., 2018). The lipopeptides produced by *B. velezensis* XT1 (Toral et al., 2018), together with the metabolites and enzymes described in this work, are probably responsible for its wide antifungal activity.

5. Conclusions

Bacillus velezensis XT1 showed great plant growth-promoting potential, which was demonstrated by *in vitro* and *in vivo* plant experiments under sterile and non-sterile conditions. In addition, strain XT1 exhibited high *in vitro* antifungal activity against plant pathogenic fungi. These growth-promoting and antagonistic features are related to the strains' biochemical and physiological characteristics. The results obtained suggest that *Bacillus velezensis* XT1 has great potential as a biotechnological commercial inoculant in the agriculture sector.

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Declaration of competing interest

All the authors declare that the research was conducted in the absence of any competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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