



Bacillus subtilis ELI239 and *B. amyloliquefaciens* ELI203 improve plant growth and control phytopathogens through their metabolites

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Abstract

Plant growth-promoting microorganisms such as *Bacillus* sp. can be applied such as microbial biocontrol agents to promote sustainable agriculture and reduce dependency on chemical pesticides. *B. subtilis* group species have been employed to enhance plant growth and promotion or to control pathogens of some crops due to their capacity to produce a wide array of secondary metabolites with different biological activities. However, not all these species have the same ability to stimulate plant growth or the same antifungal activity. The objective of this study was to conduct a comparative analysis of the plant growth-promoting properties of *B. subtilis* ELI204, *B. subtilis* ELI239, *B. velezensis* ELI71, and *B. amyloliquefaciens* ELI203, all belonging to the *B. subtilis* group, while also comparing their antifungal activity. This comparison can provide insight into which metabolites are responsible for their function in plants. The data showed that the crude extract of *B. subtilis* ELI239 and *B. amyloliquefaciens* ELI203 inhibited various fungi at rates ranging from 67% to 97%, depending on the fungi. The four strain crude extracts were probed in four plants such as, chickpea (*Cicer arietinum* L.), lentils (*Lens culinaris*), maize (*Zea mays* var. *amylacea*) and radish (*Raphanus sativus*), both in germination and plant growth development stages, identifying which strain is the best to apply on plants. Pot experiments demonstrated that *B. subtilis* ELI239 and *B. amyloliquefaciens* ELI203 significantly enhanced plant growth. The capacity of these species to produce natural compounds was studied to analyze the chemical profile of each species and what relations had with their role in plants. *B. subtilis* ELI239 and *B. amyloliquefaciens* ELI203 produced macrolactin A, which can be involved in the strong antifungal activity of both strains.

Keywords *Bacillus subtilis* · Natural compounds · Chemical profile · Plant growth · Antifungal activity

Introduction

Agriculture crops are susceptible to various diseases caused, among other factors, by plant-pathogenic fungi, which contributes to a decline in crop quality and productivity. Chemical pesticides have been used for a long time; however, their use poses several problems, such as those related to health or the environment (48). Furthermore, crop production is linked to soil quality, which is why fertilization treatments are used in agricultural crops. Microbial biological control agents are essential for promoting sustainable agriculture and reducing dependence on chemical pesticides (29; 5). The plant-growth-promoting rhizobacteria (PGPRs) are used to promote plant growth and control pathogens (8; 15). In this regard, *Bacillus* sp., represent a promising solution (3; 27). Furthermore, *Bacillus* is a genus that has been extensively studied for its use as a biological control agent and plant growth promoter (37) and secretes a wide range of

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metabolites which can increase defenses and plant growth and development (24).

The *B. subtilis* group includes several original phylogenetically and phenotypically homogeneous species such as *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. velezensis* and *B. pumilus* (10; 2; 41; 28). In recent years, *B. subtilis* and its closest relatives *B. amyloliquefaciens*, *B. velezensis*, and *B. licheniformis* have been widely used as biofertilizers and biofungicides (14).

However, not all species are suitable for promoting plant growth or for agricultural applications; in this regard, it is important to study which species in this group are the most effective plant growth promoters.

The most important characteristic of this group of species is their ability to produce a wide range of secondary metabolites with antibacterial and antifungal activities (17; 28). Several species in this group have been used to enhance the growth of certain crops through direct mechanisms such as nitrogen fixation and the solubilization of certain nutrients, or through indirect mechanisms that produce substances acting as pathogen antagonists or inducing resistance in plants (1; 22). Species in the *B. subtilis* group have significant biotechnological applications as microorganisms used for the biological control of plant pathogens due to their extensive machinery for producing natural compounds (13; 35; 20). *B. subtilis* is one of the most studied species as a pathogen control agent, plant growth promoter, and soil improver (44; 12). It has been applied to promote the growth of tomato plants among other crops (30).

B. subtilis inhibits growth of *Sclerotinia sclerotiorum*, in soybean causing severe oxidative stress to fungal hyphae and inhibits melanin synthesis in the sclerotia (4). *B. velezensis* can inhibit germination, conidia production, and mycelial growth of *F. oxysporum* effectively controlling soybean root rot (36). However, few studies have been published on the ability of other species in the *B. subtilis* group to promote plant growth or exert biological control against plant pathogens, compared to the well-studied *B. subtilis*.

Based on this premise, the aim of this work was to conduct a comparative analysis of the plant growth-promoting properties of several strains belonging to the *B. subtilis* group such as, *B. subtilis* ELI204, *B. subtilis* ELI239, *B. velezensis* ELI71 and *B. amyloliquefaciens* ELI203, including their antifungal activity. To study the role of these strains in plants, crude extracts from the four strains were evaluated into four plant species, including chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris*), maize (*Zea mays* var. *amylacea*) and radish (*Raphanus sativus*), both in germination and plant growth development stages, to identify which strain is most suitable for application to plants. The ability of these species to produce natural compounds was studied to analyze the chemical profile of each and determine how it relates to their function in plants.

Materials and methods

Microorganisms and growth conditions

Bacillus spp. isolates were previously characterized as *B. subtilis* ELI204, *B. subtilis* ELI239, *B. velezensis* ELI71, and *B. amyloliquefaciens* ELI203, using genetic tools (Figure S1). The molecular characterization of these strains was done as described in supporting information. These strains were cultured on Luria Bertani (LB) plates and maintained for the next studies.

Isolation and elucidation of natural compounds from Bacillus strains.

Bacillus strains were inoculated into 250 mL of trypticase soy broth medium with amberlite® XAD-16 adsorbent resin (1.25 g) incubating, at 29 °C for 7 days. A total of 4 L were incubated for each *Bacillus* strain. The adsorbent resin was recovered and washed with 60 mL of methanol. The methanol was evaporated to achieve the crude extracts with yields from 1.08 g/L to 0.7 g/L that were retained for further studies. The crude extracts were accumulated from several liters, until the quantity reached 3.5 g, which were dissolved gradually with methanol until the solid was dissolved and is mixed thoroughly with the silica gel using the mortar. When the methanol was evaporated the mix of the extract with silica was applied to the column chromatography using silica gel (70–230 mesh) as a stationary phase and different solvents as eluents. The eluent systems that were used are indicated in the next Table 1 using two volumes of 100 ml of each solvent mixture to achieve twelve fractions. Each fraction was analyzed by thin layer chromatography and purified to yield different compounds.

Table 1 Eluent systems used on the elution procedure

FRACTIONS	ELUENT SYSTEM (200 ml)
1	Hexane 100%
2	Hexane 90% – AceOEt 10
3	Hexane 75% – AceOEt 25%
4	Hexane 50% – AceOEt 50%
5	AcOEt 100%
6	AcOEt 98% - MeOH 2%
7	AcOEt 95% - MeOH 5%
8	AcOEt 90% - MeOH 10%
9	AcOEt 80% - MeOH 20%
10	AcOEt 50% - MeOH 50%
11	AcOEt 20% - MeOH 80%
12	MeOH 100%

Antifungal activity assay using microplates

Commercially available 12-well microtiter plates (Corning Inc., Corning, N.Y.) were used for this assay. The microplates were filled with PDA (potato dextrose agar) medium containing 30 µg of crude extracts (1 mg/mL). Then a 5 mm diameter disk with phytopathogenic fungus impregnated is placed on filled microplates and incubated at 29 °C for 48 h reading the results at 48 h with CanoScan LiDE200 (Canon) scanner and ImageJ 1.51 k program. The inhibition percentage (%) of each compound was calculated following the equation below. The positive control was the medium without any compound and then phytopathogenic fungus was added. The assay was performed in triplicate.

$$\text{Inhibition percentage (\%)} = ((D0 - Dx)/D0) \times 100$$

where D0=diameter r of fungus alone and Dx=diameter of fungus in presence of tested compound.

Cell damage study under phase-contrast microscopy

The complete methodology was described by Salazar et al. (2020). Briefly, a bit of mycelia in the inhibition zone caused by the extracts was taken with a sterile, transparent adhesive tape and put on a slide under Phase-Contrast Microscopy. Lactophenol cotton blue stain was used to contrast the fungal structures. Finally, the damage to the cytoplasm or spores was observed at 40X and compared with a control of the same fungus. The control was prepared and processed under identical conditions by taking a bit of mycelia from a fungus grown normally in same conditions but without any compound.

Germination experiment

The studies were conducted on chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris*), maize (*Zea mays* var. *amylacea*) and radish (*Raphanus sativus*). For laboratory experiments, the seeds were sterilized adding a 1% sodium hypochlorite solution (covering the seeds) and shaking 10 times for 5 min. Immediately the liquid was decanted, and 70% ethyl alcohol was added, shaking 10 times for 2 min. Then the liquid was removed by decanting. Finally, seeds were washed with sterile distilled water.

The sterilized seeds were placed in a sterilized beaker containing a cotton bed (six same seeds for each beaker). The seeds were watered from the first day and every day during the three days with 600 µl of sterile distilled water or with a solution of crude extract depending on whether the seeds are going to be used for micropropagation in pots or if seeds are only to measure germination, respectively. Four

concentrations were used to see the impact of the concentration of each crude extract as follows: 0.125 mg per mL, 0.25 mg per mL, 0.5 mg per mL and 1 mg per mL. The seeds should be kept at room temperature under a 16/8 h photoperiod and were left for 3 days to germinate. After this time, each seed is removed from the cotton wool with tweezers, and the root is measured individually. The experiment was done for quadrupled for each type of seed and each treatment. The relative growth (% RG) for each crude extract concentration was calculated following the equation below:

$$\% RG = [(treatment\ length - control\ length) / control\ length] \times 100$$

Micropropagation in pots

A mixture of commercial soil, which was sieved, was added to plastic pots (6.5×9×10 cm). These pots with soil were sterilized three times. The soil was subjected to a sterility control test ensuring sterility. The micropropagation in pots was performed under greenhouse conditions in a completely randomized design. Uniformly germinated seedlings were selected and transplanted into sterilized pots, (for sextupled), covering the seeds lightly with soil from the pot. The pots are transferred to the greenhouse and watered either with 30 ml of water (untreated control) or with solutions of the crude extracts (dissolved in 30 ml distilled water) isolated from *Bacillus* strains. Four concentrations were used to see the impact of the concentration of each crude extract as follows: 0.125 mg per mL, 0.25 mg per mL, 0.5 mg per mL and 1 mg per mL. It should be noted that the solutions with the crude extracts to be tested are only used for watering on the first day of micropropagation. On subsequent days, they are watered only with 30 mL of sterile distilled water. Watering is carried out every 3 days. Plants grew with an average temperature of 26°C during the day (14 h) and 16°C during the night under a 16/8 h photoperiod.

The initial data were acquired on the seventh day of micropropagation with the half of the plants (three for each plant), measuring the following parameters: root length, stem length, node length, and root hair length and number. The next data were acquired on the tenth day of micropropagation with the rest of the plants, measuring the same parameters. The relative growth (% RG) for each crude extract concentration was calculated following the equation below:

$$\% RG = [(control\ length - treatment\ length) / control\ length] \times 100.$$

Inoculation of plants with *Bacillus* sp

An inoculum of the corresponding strains (*B. subtilis*, *B. amyloliquefasciens*, or *B. velezensis*) is added to 3 mL of LB broth, incubating at 30 °C with agitation at 200 rpm for

24 h. To inoculate each pot, 1 ml of the bacterial suspension (broth with the *Bacillus* strain or combination of strains) is added just below the stem, as close to the root as possible without touching the soil or the seedling. For watering the plants in the next days 30 ml of water will be used. The plants were growth under a 16/8 h photoperiod.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For bacteriocin analysis, the fractions that contained this type of compounds were analyzed by SDS PAGE using protocol described in (38). Gel analyzer program was used to allow for accurate determination of the molecular mass of the bacteriocins.

Chemical structure elucidation

The structures of these compounds were completely elucidated by using extensive spectroscopic methods, such as 1D NMR, 2D NMR, mass spectroscopy and IR (Supporting information). ^1H and ^{13}C NMR spectra and ^1H - ^1H COSY, DEPT, and HSQC experiments were measured with a FTNMR Bruker spectrometer of 500 MHz using deuterated chloroform (CDCl_3) as the solvent and tetramethylsilane Me_4Si (TMS) as the internal standard. ^1H NMR chemical shifts (δ) are reported in parts per million (ppm) relative to Me_4Si ($\delta=0.0$ ppm) with coupling constants (J) reported in Hertz (Hz). Multiplicities are reported as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quint), Sextet (s), multiplet (m), broad singlet (bs). ^{13}C NMR spectra are reported using 77.0 ppm (CDCl_3) as internal reference. The number of scans were adjusted based on concentration of the compounds, normally 64 scans were used to achieve the ^1H NMR spectra and from 256 to 1024 scans to acquire the ^{13}C NMR spectra.

Statistical data analysis

Data were subjected to analysis of variance using BM SPSS software (SPSS Inc., Chicago, IL, USA). Data were

Table 2 Inhibition percentage (%) of the crude extracts against target phytopathogens after 48 h

Extract <i>Bacillus</i> / Fungus	<i>F. solani</i>	<i>F. oxysporum</i>	<i>Mucor</i> sp.	<i>L.theobromae</i>
<i>B. amyloliquefaciens</i> (ELI 203)	79	89	85	87
<i>B.subtilis</i> (ELI 204)	49	61	6	87
<i>B.subtilis</i> (ELI 239)	67	97	66	85
<i>B.velezensis</i> (ELI 71)	19	11	0	32

expressed as mean $\pm$ standard deviation (SD) for descriptive purposes. Standard deviation was calculated using casio graphing calculator like the fx-CG50 which uses a high-performance SuperH (SH-4 A) 32-bit RISC core processor.

Results

First, the ability of the strains *B. subtilis* ELI204, *B. velezensis* ELI71 and *B. amyloliquefaciens* ELI 203 to enhance the growth of various vegetable crops was studied. The strains were cultured and inoculated into plants such as chickpea (*Cicer arietinum* L.), lentils (*Lens culinaris*), maize (*Zea mays* var. *amylacea*) and radish (*Raphanus sativus*); including the application of these strain mixtures to study the effect of co-inoculation of multiple strains in a consortium (16), as shown in Table S1 and Figure S2.

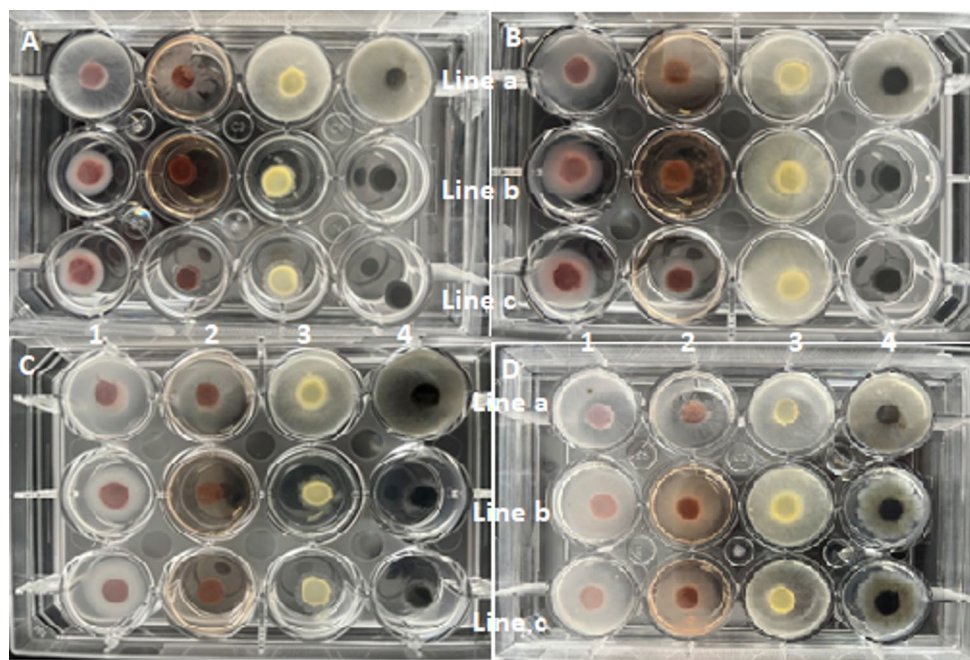
In most plants, co-inoculation with a mixture of *B. subtilis* and *B. amyloliquefaciens* promotes plant growth, except in radish plants, where inoculation of *Bacillus* mixtures hinders growth. *B. velezensis* inhibited plant growth in all cases. These results suggest that *B. subtilis* and *B. amyloliquefaciens* have a positive effect by promoting growth, while *B. velezensis* inhibits growth in all plants.

To evaluate the antifungal activity of the crude extract from each strain, microplate method was followed against four fungi such as *Fusarium solani*, *Fusarium oxysporum*, *Mucor* sp. and *Lasidiopodia theobromae* as shown in Table 2; Fig. 1.

The results indicate that *B. amyloliquefaciens* ELI203 exhibits the strongest antifungal activity, followed by *B. subtilis* ELI 239 and *B. subtilis* ELI 204. There are several cases in which *Bacillus* extracts almost completely inhibit the growth of the analyzed fungi; this is observed in the case of *F. oxysporum*, where *B. subtilis* ELI239 and *B. amyloliquefaciens* ELI203 inhibit growth by 97% and 89% respectively, highlighting that both strains possess potent antifungal activity. In addition, a study of cellular damage was conducted to observe the damage caused by the *B. amyloliquefaciens* extract, as shown in Fig. 2.

Figure 2A shows the control sample of *F. oxysporum*, with abundant small, uniform and curved microconidia and hyaline, septate hyphae; whereas the same fungus treated with *B. amyloliquefaciens* extract exhibits fewer thin, vacuolated, and curled hyphae with indistinct septa, and no spores are observed. Figure 2B shows a control of *L. theobromae* with numerous septate hyphae and conidia. In the corresponding treated fungus, fewer thin, vacuolated, and curled hyphae are observed. Figure 2C shows the *Mucor* control with several septate hyphae and conidia; however, neither hyphae nor conidia are observed in the treated fungus. In Fig. 2D, the *F. solani* control shows septate hyphae

Fig. 1 Microplates assay showing the four tested phytopathogens in each column. The first line (line a) corresponds to the growth of each fungus alone and the two other lines (Lines b and c) correspond to the fungus with the extract. Panel **A**) *B. amylo-liquefaciens* (ELI 203); Panel **B**) *B. subtilis* (ELI 204); Panel **C**) *B. subtilis* (ELI 239); Panel **D**) *B. velezensis* (ELI 71). The numbers indicate the tested fungi *Fusarium solani*, *Fusarium oxysporum*, *Mucor* sp. and *Lasiodiplodia theobromae*



and uniform microconidia; in contrast, neither is observed in the treated fungus. Clearly, the cellular damage caused by the *B. amyloliquefaciens* extract is evident, affecting both the hyphae and spore formation.

After evaluating antifungal activity, it was decided to analyze the growth-promoting activity in plants. Therefore, the crude extracts of each strain were evaluated on four plant species: chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris*), maize (*Zea mays* var. *amylacea*) and radish (*Raphanus sativus*), assessing both germination and plant growth using different extract concentrations.

In the germination experiment, the data obtained were significant. The optimal concentration for almost all crude extracts was 0.125 mg/ml; in some cases, even 0.25 mg/ml proved to be optimal. In contrast, concentrations of 0.5 and 1 mg/ml yielded the worst results as they inhibited seed growth. Two different strains of *B. subtilis* were analyzed, and surprisingly, the results obtained for each were very different, with *B. subtilis* ELI204 being the strain that best promoted germination (Table S2; Figs. 3 and 4). In both maize and radish plants, germination increased with *B. subtilis* ELI204 compared to the other three *Bacillus* strains. However, *B. subtilis* ELI239 improved germination in lentil seeds. In contrast, in chickpea plants, germination increased with the *B. amyloliquefaciens* ELI203 strain, although with the rest of the seeds this strain inhibited the germination process.

Regarding plant growth, stem and roots length were measured on the seventh and tenth days, along with the number of nodes, leaves and root hairs in the treatments with crude extracts from the four strains, compared to the control, in

maize, lentil, chickpea and radish, as shown in Tables S3-S6 and Figs. 5 and 6, respectively.

In maize plants (Fig. 5I) the crude extract of *B. subtilis* ELI204 yielded better results in terms of plant growth than the extract of *B. subtilis* ELI239, as measurements of the stem and roots, as well as the number of nodes, leaves and root hairs improved with *B. subtilis* ELI 204, with 0.25 mg/ml being the best concentration to apply, followed by 0.5 mg/ml, while 1 mg/ml is the worst concentration, as it inhibits plant growth. *B. amyloliquefaciens* ELI203 at 0.25 mg/ml yielded moderate results. In lentil plants (Fig. 5II) the crude extract of *B. subtilis* ELI239 and *B. amyloliquefaciens* ELI203 yielded the best results at 0.5 mg/ml and 0.25 mg/ml, respectively.

In chickpea plants (Fig. 6I), crude extracts of *B. subtilis* ELI204 and *B. subtilis* ELI239 at a concentration of 0.5 mg/ml yielded the best results. In radish plants (Fig. 6II), crude extracts of *B. subtilis* ELI239 and *B. amyloliquefaciens* ELI203 yielded the best results at 0.25 mg/ml and 0.5 mg/ml, respectively.

The data obtained showed that, in general *B. subtilis* and *B. amyloliquefaciens* yielded the best results for promoting growth in most plants. These results may be related to the chemical compounds present in each crude extract. With this idea in mind, these crude extracts were subjected to purification to isolate different compounds.

Di[(R)-2-ethylhexyl] Phthalate was recovered using a hexane/chloroform (90:10) elution system as a colorless waxy liquid (7, 7). The 2,3-dihydroxypropyl stearate compound was recovered using a hexane/ethyl acetate (30:70) elution system as white solids (6). Macrolactins were

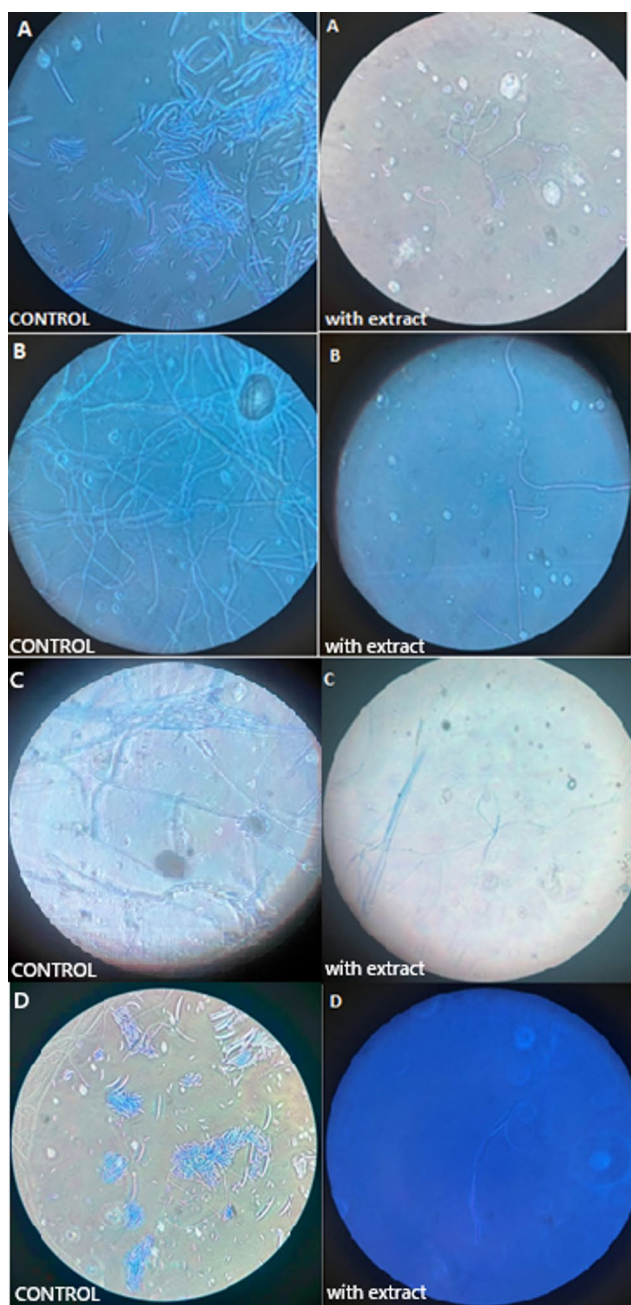


Fig. 2 Phase-contrast microscopy of the cell damage produced by crude extract of *B. amyloliquefaciens* in (A) *Fusarium oxysporum*; (B) *L. theobromae*; (C) *Mucor* sp.; (D) *Fusarium solani*, fungi compared with their control

obtained using a EtOAc (100%) elution system (32). The indole compounds were eluted using a hexane/ ethyl acetate (50:50) elution system for indole, dichloromethane/methanol (99:1) for Phenethylbutanamide and an ethyl acetate/methanol (95:5) system for N-acetyltryptamine (39). The diketopiperazines were recovered using an ethyl acetate/methanol (90:10) elution system (33).

The chemical structures of these isolated compounds (Table 3) were fully determined as described in the experimental section and in the supplementary information (Figures S3-S53).

Table 3 shows that some compounds are very widespread in all species of the *B. subtilis* group, such as diketopiperazines, (33) or Di[(R)-2-ethylhexyl] Phthalate, (Fig. 7).

Some compounds are present in certain strains but not in all. For example, 2,3-dihydroxypropyl stearate, a mono-acylglycerol, was found only in *B. subtilis* ELI204, but not in the other *Bacillus* species. Similarly, indole compounds are found only in *B. velezensis*, but not in the other *Bacillus* species. Macrolactins were isolated from both *B. amyloliquefaciens* and *B. subtilis* ELI239.

Lipopeptides are always isolated from *Bacillus* and exhibit antifungal activity against various plant-pathogenic fungi. Finally, bacteriocins are peptide compounds isolated from all *Bacillus* species that possess antibacterial activity. However, the isolated bacteriocins differ in weight and quantity, with *B. subtilis* ELI239 being the strain that produced the most abundant and largest bacteriocins compared to the other *Bacillus* species.

The results indicated that *B. amyloliquefaciens* ELI203 and *B. subtilis* ELI239 produce macrolactins. Accordingly, both strains showed the best results against certain plant pathogens and yielded the best results in plant growth, particularly in lentil and radish plants.

Discussion

In recent years, *B. subtilis*, *B. velezensis* and *B. amyloliquefaciens* have been recognized as broad-spectrum biological control agents capable of effectively inhibiting various plant pathogens (42; 23; 21; 25; 44). *B. subtilis* strains improved maize growth and suppressed seed-borne fungi by activating defenses and colonizing the rhizosphere (9). In Chickpea *Bacillus subtilis* FJ3 confirms its potential for biological control and plant growth promotion (18). There are recent reports on the positive impact of *B. velezensis* on grain yield, phosphate content, and soybean weight (40) and the positive effects of *B. subtilis* on soybean production (26, 26). There is a report on plant growth promotion and the suppression of plant diseases induced by GD4a *B. amyloliquefaciens* strain (45). A potent strain identified as *B. amyloliquefaciens* promotes the growth of chilli peppers (*Capsicum annuum* L.) and exhibits good inhibition against *Agroathelia rolfii* a chilli pepper pathogen (34, 34). There are several reports on the ability of *B. velezensis* to act as a biological control agent against pathogenic fungi and as a plant growth promoter (47; 11, 11; 19; 14).

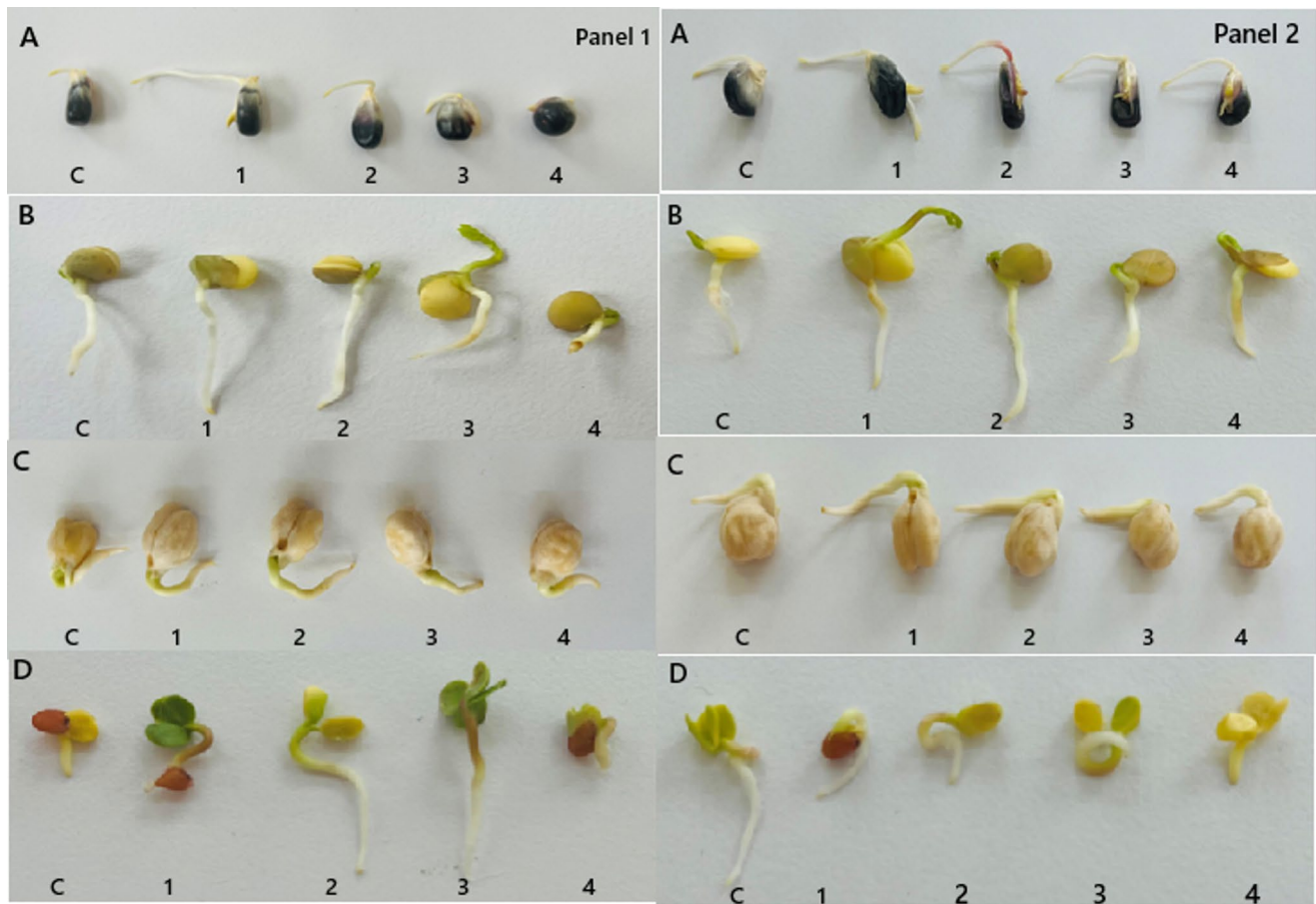


Fig. 3 Photographs of the root length in germination step. Panel 1: *B. subtilis* ELI204 and panel 2: *B. subtilis* ELI239. **A)** maize seeds; **B)** lentil seeds; **C)** chickpea seeds; **D)** radish seeds. The legends below

the seeds are as follows: C=control (distilled water), 1=0.125 mg/ml, 2=0.25 mg/ml, 3=0.5 mg/ml, 4=1 mg/ml

Fig. 4 Photographs of the root length in germination step. Panel 1: *B. velezensis* ELI171 and panel 2: *B. amyloliquefaciens* ELI203. **A)** maize seeds; **B)** lentil seeds; **C)** chickpea seeds; **D)** radish seeds. The legends below the seeds are as follows: C=control (distilled water), 1=0.125 mg/ml, 2=0.25 mg/ml, 3=0.5 mg/ml, 4=1 mg/ml

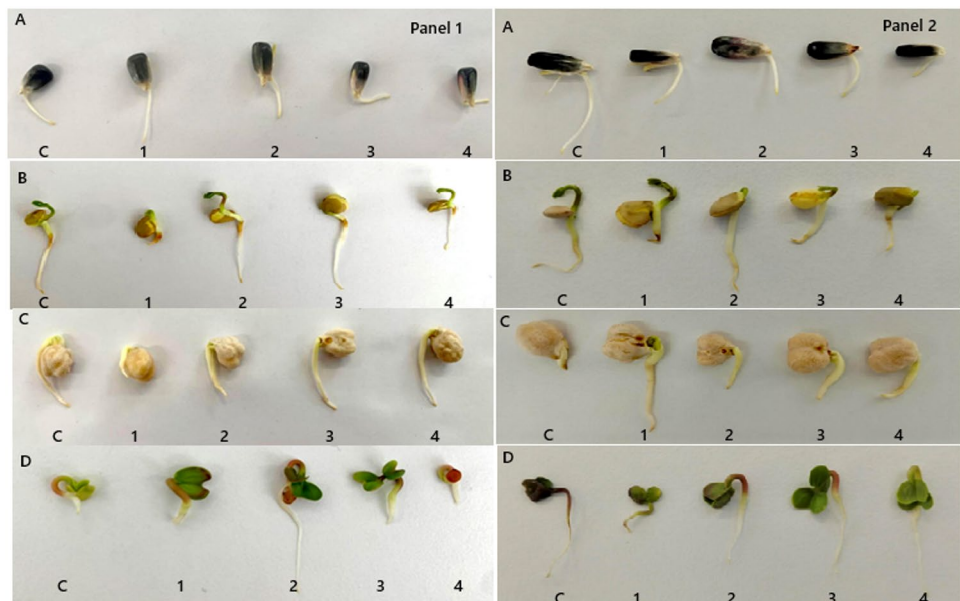


Fig. 5 Photographs of the Maize (Panel I) and lentil plants (Panel II) with the crude extracts of every *Bacillus* sp. on seventh and tenth days. **A)** *B. subtilis* ELI204; **B)** *B. subtilis* ELI239; **C)** *B. velezensis* ELI71; **D)** *B. amyloliquefaciens* ELI203. The legends below the plants represent the concentrations as follows: C=control (distilled water), 1=0.125 mg/ml, 2=0.25 mg/ml, 3=0.5 mg/ml, 4=1 mg/ml

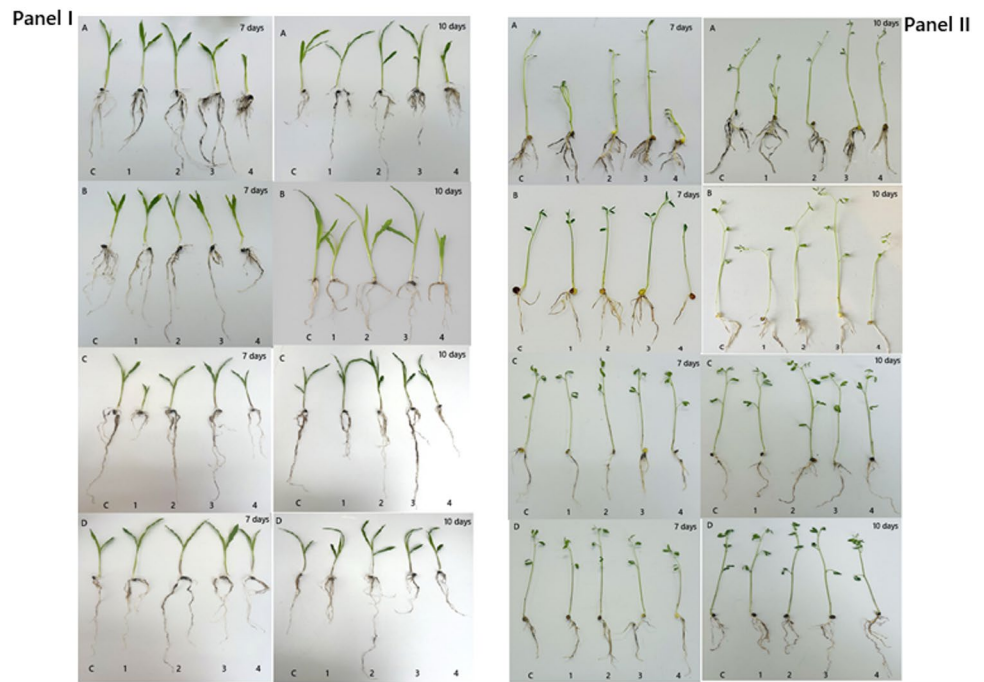
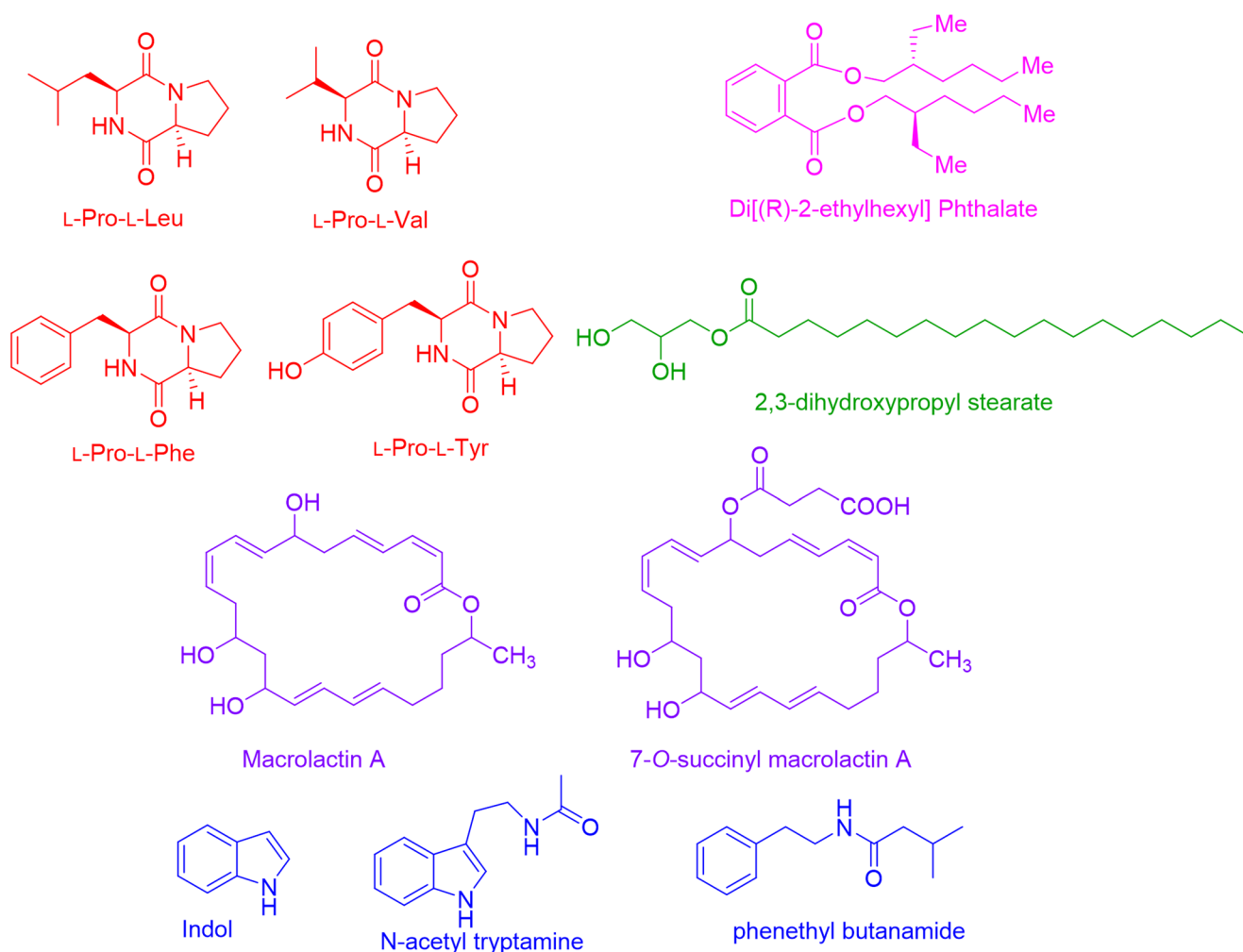


Fig. 6 Photographs of the chickpea (Panel I) and radish (Panel II) plants with the crude extracts of every *Bacillus* sp. on seventh and tenth days. **A)** *B. subtilis* ELI204; **B)** *B. subtilis* ELI239; **C)** *B. velezensis* ELI71; **D)** *B. amyloliquefaciens* ELI203. The legends below the plants represent the concentrations as follows: C=control (distilled water), 1=0.125 mg/ml, 2=0.25 mg/ml, 3=0.5 mg/ml, 4=1 mg/ml

B. subtilis ELI204; **B)** *B. subtilis* ELI239; **C)** *B. velezensis* ELI71; **D)** *B. amyloliquefaciens* ELI203. The legends below the plants represent the concentrations as follows: C=control (distilled water), 1=0.125 mg/ml, 2=0.25 mg/ml, 3=0.5 mg/ml, 4=1 mg/ml

Table 3 Chemical compounds that were isolated from *B. subtilis* ELI204, *B. subtilis* ELI239, *B. velezensis* ELI71, and *B. amyloliquefaciens* ELI203

<i>B. subtilis</i> ELI204	<i>B. subtilis</i> ELI239	<i>B. velezensis</i> ELI71	<i>B. amyloliquefaciens</i> ELI203
Di[(R)-2-ethylhexyl] Phthalate	Di[(R)-2-ethylhexyl] Phthalate	Di[(R)-2-ethylhexyl] Phthalate	-
2,3-dihydroxypropyl stearate	2-(2-Butoxyethoxy)-ethanol	Indol	Macrolactin A
2-(2-Butoxyethoxy)-ethanol	Macrolactin	Phenetyl butanamide	Succinyl macrolactin A
		N-acetyl tryptamine	
Diketopiperazines	Diketopiperazines	Diketopiperazines	Diketopiperazines
- L-Pro-L-Phe	- L-Pro-L-Phe	- L-Pro-L-Phe	- L-Pro-L-Tyr
- L-Pro-L-Tyr	- L-Pro L-Tyr	- L-Pro-L-Tyr	- L-Pro-L-Val
- L-Pro-L-Leu		- L-Pro-L-Val	
Lipopeptides	Lipopeptides	Lipopeptides	Lipopeptides
Bacteriocin 1.7 kDa	Bacteriocin 3.5 kDa	Bacteriocin 5.3 kDa	Bacteriocin
	Bacteriocin 14.4 kDa		2.6 kDa
	Bacteriocin 4.7 kDa		Bacteriocin
	Bacteriocin 7.7 kDa		4.1 kDa

**Fig. 7** Chemical structures of the compounds that were isolated from *B. subtilis* group species

Antifungal activity has been linked to lipopeptide compounds, which inhibit phytopathogenic fungi (31), including, in some cases, volatile organic compounds (VOCs) (46). Our research group had previously reported that macrolactins possess strong antifungal activity (32).

In this study, it has been demonstrated that the strains *B. subtilis* ELI 239 and *B. amyloliquefaciens* ELI203 separately or co-inoculated promote plant growth, which is consistent with the available literature. Furthermore, both strains had antifungal activity against several plant pathogens. In

contrast, *B. velezensis* did not exhibit any positive effect on plant growth or any biological control capacity, unlike existing literature that reports the capacity of *B. velezensis* to be a good biological control agent and plant growth promoter. This result may be related to the chemical profile of these strains. *B. subtilis* ELI 239 and *B. amyloliquefaciens* ELI203 strains produce macrolactins, unlike *B. velezensis*, which does not produce these compounds. The results presented in this study suggest that macrolactins may play a positive role in plants, helping to inhibit plant pathogens and improving plant growth.

Conclusion

In summary, the crude extracts of *B. amyloliquefaciens* ELI203 and *B. subtilis* ELI239 exhibited the best antifungal activity against certain plant pathogens. At the same time, the crude extracts of *B. amyloliquefaciens* ELI203 and *B. subtilis* ELI239 promoted the growth of most of the plants analyzed. Regarding the isolation of chemical compounds from each strain, it can be summarized that some compounds are widely distributed across different species; however, macrolactins were isolated only from *B. subtilis* ELI239 and *B. amyloliquefaciens* ELI203. These results may be related to the ability of these two strains to both exhibit antifungal activity and promote plant growth.

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Author contributions A. C. and J.V. did experimental parts in the lab. U.A. revised the manuscript. A.O and E.S wrote the main manuscript and prepared the figures. All authors reviewed the manuscript.

Data availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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