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Isolates of Bacillus spp. to promote growth and protection of garlic (Allium sativum L.) cultivation

Isolados de Bacillus spp. para promoção de crescimento e proteção à cultura do alho (Allium sativum L.)

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Highlights

Four rhizobacteria isolates stimulated garlic growth and bulbification.
Four rhizobacteria isolates reduced the incidence of white rot disease.
The sequencing identified *Bacillus* species as effective for plant stimulation.
There is potential for using *Bacilus* species as bioproducts for garlic.

Abstract

Garlic (*Allium sativum* L.) is among the most widely consumed vegetables in Brazil; however, a significant proportion of the national demand is met through imports. In recent years, domestic garlic production and productivity have improved due to the adoption of advanced agricultural technologies. Nevertheless, certain factors, such as soil-borne diseases caused by pathogenic fungi, continue to hinder further increases in yield. Root diseases such as white rot caused by *Stromatinia cepivora* Berk (sin =. *Sclerotium cepivorum*) is notably difficult to manage and can lead to substantial crop losses. In this context, biological control using rhizobacteria has emerged as a viable and efficient alternative. The present study conducted two greenhouse experiments to evaluate the ability of rhizobacteria isolated from soils of garlic-producing regions. In the first experiment, four isolates were used in a sterilized system, and in the second one, five isolates were inoculated in plants grown in soil. Therefore, the

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isolates EB01, EB15, EB17, and EB21 significantly promoted garlic growth. In the second experiment, the isolates EB01, EB13, EB18, EB19, and EB24 significantly reduced the incidence of *S. cepivorum.* They showed greater similarity, according to the 16S rRNA gene, to *Bacillus velezensis, Bacillus rugosus, Bacillus subtilis, Bacillus tequilensis,* and *Bacillus velezensis,* respectively.
Key words: Alliaceae. Biocontrol agents. Plant growth. Rhizobacteria. *Stromatinia cepivora.*

Resumo

O alho (*Allium sativum* L.) é uma das hortaliças mais consumidas no Brasil, mas a maior parte é importada de outros países. Recentemente, houve aumento na produção e produtividade dessa cultura devido às tecnologias aplicadas à cultura. No entanto, certos fatores, como doenças de solo, especialmente aquelas causadas por fungos, têm limitado o aumento da sua produtividade. Doenças de raízes, tais como podridão branca, causadas por *Stromatinia cepivora* Berk (sin = *Sclerotium cepivorum*) são de difícil controle e causam perdas significativas e o controle biológico por meio de rizobactérias é uma alternativa viável e eficiente. No presente estudo foram realizados dois experimentos em casa de vegetação para testar a capacidade de promoção do crescimento e proteção contra *S. cepivora* por rizobactérias isoladas de solos de regiões produtoras de alho. No primeiro, foram utilizados quatro isolados em sistema esterilizado e, no segundo, cinco isolados foram inoculados em plantas cultivadas em solo esterilizado. No primeiro experimento, os isolados EB01, EB15, EB17 e EB21 promoveram significativamente o crescimento do alho. No segundo experimento, os isolados EB01, EB13, EB18, EB19 e EB24 reduziram significativamente a incidência de S. cepivora. Estes apresentaram maior similaridade, segundo o gene 16S rRNA, com *Bacillus velezensis, Bacillus rugosus, Bacillus subtilis, Bacillus tequilensis* e *Bacillus velezensis,* respectivamente.
Palavras-chave: Agentes de biocontrole. Alliaceae. Crescimento de plantas. Rizobactérias. *Stromatinia cepivora.*

Introduction

Brazil is one of the countries with the highest consumption of garlic (*Allium sativum* L.) (Buta & Silva, 2020). High production costs have been reported due to high demand for labor, agricultural inputs for fertilization, pest, and disease control. Garlic is affected by various foliar and root diseases that reduce its productivity (Lucini, 2004). Root diseases such as white rot, caused by the fungus *Stromatinia cepivora* Berk (sin =. *Sclerotium cepivorum*), have caused significant production declines (Marcuzzo & Luiz, 2017a), which are difficult to control since it requires the use of high-toxicity pesticides, which increases economic costs and environmental risks.

The edaphoclimatic conditions that favor the occurrence of white rot are characterized by cool and humid climates, with temperatures between 50°F and 75°F (10°C and 24°C) and high soil moisture levels. This combination of conditions allows the pathobiome establishment, leading to a significant reduction in garlic productivity (Menezes et al., 1993). In addition, the fungi form resistance structures known as sclerotia that remain viable in the soil for extended

periods, thereby rendering control measures highly challenging and often ineffective (Marcuzzo & Schmoeller, 2017b).

Due to the difficulty in controlling these diseases, alternative methods such as biological control have been used with significant results (Poveda et al., 2022; Zapata-Narváez & Gómez-Marroquín, 2022). Biological control of diseases through plant growth-promoting rhizobacteria (PGPR) has been highlighted as a viable alternative to chemical control (Zapata-Narváez & Gómez-Marroquín, 2022). PGPR exhibits different mechanisms that benefit plant growth and development, including the inhibition of pathogens through the production of secondary metabolites (Vejan et al., 2016; Barbosa et al., 2021). The genus Bacillus spp. is one of the most studied strains since it can survive in soil due to its capacity to generate endospores resistant to a wide range of environmental conditions and different soil types (Elshaghabee et al., 2017). Bacteria from this genus produce compounds that prevent microorganisms that harm agricultural production and have already been used in bioproducts for disease and pest control (Compant et al., 2019).

A collection of twenty-seven *Bacillus* spp. Isolates were examined in previous studies, and thirteen were found to be capable of inhibiting *S. cepivora in vitro* (Balbinot et al., 2020). This finding indicates a significant community among rhizobacteria obtained from the garlic rhizosphere. In this context, the objective of this study was to continue the selection of *Bacillus* spp. isolates for the growth promotion and protection of garlic plants *in vivo* and identify the species of the selected isolates.

Material and Methods

The study was held at the Federal University of Santa Catarina, Santa Catarina State, Curitibanos campus. The isolates belong to the genus *Bacillus* and were obtained from soil and the rhizosphere of a garlic field in Curitibanos, as previously reported (Leôncio & Botelho, 2017). These isolates were previously tested *in vitro* to evaluate their ability to inhibit *S. cepivora* (Balbinot et al., 2020).

The *Bacillus* isolates selected for in planta protection analyses against white rot were obtained from *in vitro* assays of the inhibition of *S. cepivora* (Balbinot et al., 2020).

Chonan class 3 was the garlic variety used, with an average mass of 4 g per clove. The cloves underwent the disinfection process (Silva, 2012) and were dried in a laminar flow cabinet. One hundred mL bottles of liquid LB (Luria Bertani) medium were prepared for each treatment, for the inoculum preparation containing the isolates. They were inoculated with the isolates directly from the stock, which was preserved in glycerol, and incubated at 28°C for 24 hours. Uninoculated media were kept under the same conditions as the control. Thus, in a laminar flow cabinet, 14 garlic cloves were immersed in each bottle (treatment) for 30 minutes. They were then removed and allowed to dry. The treatment with the commercial product followed the same procedure, where this product is ready-to-use, composed of *Bacillus subtilis* strain CCTB04 endospores, water, and additives (a source of nitrogen, vitamins, carbon, and minerals).

All pots received two cloves, corresponding to the sown treatment.

Thinning was performed, leaving one plant per pot after eighteen DAS (days after sowing). The plants were watered with sterilized distilled water for 30 DAS. After this period, they received a weekly application of half-strength Hoagland solution (Hoagland & Arnon, 1950), with a 75% concentration of potassium nitrate. The isolates were re-inoculated at 21 DAS. The procedure for preparing the inoculum was the same as described before. The application was made using a volumetric pipette, adding 10 mL of the bacterial suspension corresponding to the treatment. For the treatment without bacterial inoculation, 10 mL of LB medium was added. After this period, the re-inoculations were performed at 60 and 90 DAS. The plants were collected at 120 DAS to carry out the final evaluations. The data were subjected to ANOVA, and then, to the Scott-Knott test at a 5% error probability using the RStudio program (R Core Team [R], 2019).

The second experiment was conducted using a randomized block design, with nine treatments: control (no fungi and no bacteria), fungi (no bacterial inoculation), inoculation of isolates EB01, EB13, EB18, EB19, EB24, and a product based on *B. subtilis* strain CCTB04. Five-liter pots containing soil from the region, classified as a Haplic Dystrudept with a sandy texture, were used. Each pot, except for the control, was inoculated with 1 cm² of *S. cepivora* growth, obtained from a PDA plate containing 40 fungal sclerotia, and quantified using a Neubauer chamber. The pots were incubated in the greenhouse for 24 hours. The bacterial inocula preparation was carried out as described in the first experiment.

Chonan Cultivar class 3 was the garlic used. All pots received three cloves, corresponding to the sown treatment. At 20 DAS, thinning was performed. Treatment re-inoculations were carried out at 30 and 60 DAS, as described in the first experiment. The protection index (PI) against *S. cepivora* was performed (30 DAS, 60 DAS, and 82 DAS) and was determined by calculating PI = $[(Sh - \bar{x}s) \div \bar{x}t\,] \times 100$, where: Sh is the shoot height of the treatment, $\bar{x}s$ is the mean shoot height of fungus treatment, and $\bar{x}t$ is the mean shoot height of the control.

DNA from the selected isolates used in the *in planta* analyses was extracted and sent to the Laboratory of Developmental Physiology and Plant Genetics at the Federal University of Santa Catarina (UFSC) - Florianópolis for sequencing and genetic identification. The 16S gene of the isolates was sequenced using an Oxford Nanopore Technologies (ONT) Mk1B platform, with the 16S Barcoding Sequencing kit (ONT), in 48-hour sequencing runs (T. Zhang et al., 2023). The reads obtained for each isolate were analyzed using the epi2me software. Reads corresponding to the complete gene (approximately 1500 bases) were then used to generate the consensus sequence in the CLC Genomics software, using the 16S gene sequences of five Bacillus species obtained from GenBank as references.

Gene sequence similarities were assessed using the p-distance (Azevedo et al., 2020). Phylogenetic analysis was performed using 16S rRNA gene sequences from all strains described for the new Subtiles Clade of *Bacillus* spp. (Table 1) and *Metabacillus fastidiosus* NBRC101226T was used as an outgroup (Gupta et al., 2020). The phylogenetic tree was constructed using the Maximum Likelihood algorithm and the General Time Reversible model.

Table 1
GenBank accession numbers of the reference *Bacillus* species and the isolates

Accession Number	Species/Isolates
AB271753	*Bacillus pumilus* NBRC12092T
MT554518	*Bacillus rugosus* SPB7T
KY694464	*Bacillus velezensis* NRRL B-41580T
AB021198	*Bacillus vallismortis* DSM11031T
KJ847721	*Bacillus tequilensis* 10bT
AB042061	*Bacillus subtilis* IAM12118T
MK462260	*Bacillus cabrialesii* TE3T
PP751677	*Bacillus* sp. EB24*
PP813694	*Bacillus* sp. EB19*
PP751629	*Bacillus* sp. EB18*
PP751624	*Bacillus* sp. EB13*
PP751513	*Bacillus* sp. EB01*
AMSH01000114	*Bacillus xiamenensis* HYC-10T
KY643639	*Bacillus siamensis* KCTC13613T
KU836854	*Bacillus nakamurai* NRRL B-41091T
AB021191	*Bacillus mojavensis* ifo15718T
KX785171	*Bacillus licheniformis* DSM13T
MN483266	*Bacillus halotolerans* DSM8802T
KF995513	*Bacillus gobiensis* FJAT-4402T
AB021181	*Bacillus atrophaeus* JCM9070T
AB325583	*Bacillus amyloliquefaciens* NBRC15535T
AB681412	*Metabacillus fastidiosus* NBRC101226T

* *Bacillus* isolated from garlic.

Results and Discussion

First experiment in vivo

In the first greenhouse experiment, the isolates EB01, EB15, EB17, and EB21, selected based on the results of the *in vitro* evaluation of *S. cepivora* growth inhibition reported by Balbinot et al. (2020), were inoculated into the cloves. The colony-forming unit (CFU) for each treatment were as follows: 3.2 x 10^7 (EB01), 3.4 x 10^6 (EB15), 1.1 x 10^7 (EB17), 3.5 x 10^6 (EB21), and 1.0 x 10^8 (*B. subtilis* strain CCTB04). The control treatment was not inoculated and no CFUs were detected.

During thinning, it was possible to observe the fungal mycelium on some plants' roots, and symptoms of white rot were present. However, at 30 DAS, the plants no longer exhibited disease symptoms, and no fungal mycelium was detected in the substrate. The Leonard pot system

possibly limited the fungi's development by making it difficult for them to contact the growing roots, which extended into the lower compartment of the pot. However, a significant difference was observed across all evaluated parameters (Table 2), indicating a positive effect of the tested isolates on plant growth. Plant height was significantly higher in those inoculated with *Bacillus* spp. isolates, with an increase of 15% at all three analysis periods (60, 90, and 120 DAS).

Table 2
Effect of *Bacillus* spp. isolates on the garlic (*Allium sativum* L.) growth cultivated in sterilized substrate

	Plant height (cm)			Shoot (g)		Root (g)		Bulb (g)	
Treatment	60 DAS	90 DAS	120 DAS	FM	DM	FM	DM	FM	DM
S.c	47,00 a	63,54 a	76,72 a	53,74 b	12,41 a	39,12 a	4,80 a	17,40 a	4,49 a
EB01	47,12 a	67,80 a	82,34 a	53,93 b	14,11 a	41,62 a	5,11 a	17,64 a	4,30 a
EB15	47,78 a	67,58 a	82,50 a	61,68 a	16,50 a	37,44 a	5,09 a	18,02 a	4,11 a
EB17	44,82 a	61,92 a	79,56 a	55,33 b	16,01 a	42,08 a	4,71 a	15,69 a	3,11 b
EB21	45,70 a	67,14 a	83,88 a	60,00 a	16,26 a	40,02 a	4,33 a	16,34 a	3,25 b
B. subtilis *	42,70 b	54,88 b	69,02 b	36,13 d	7,10 b	19,13 b	1,63 b	12,54 b	2,64 b
Control	38,50 b	57,40 b	71,58 b	45,12 c	9,06 b	31,54 b	3,08 b	13,56 b	2,43 b
CV (%)	7,23	7,12	7,13	10,49	19,57	16,70	27,34	13,03	27,56

Means followed by the same letter in the column do not differ from each other, according to the Scott-Knott test at a 5% significance level.
S.c – Only *Stromatinia cepivora*
DAS – Days After Sowing
FM - Fresh Mass
DM - Dry Mass
* strain CCTB04.

For shoot fresh mass, the EB15 and EB21 isolates presented the highest values, with increases of 26.85% and 24.8%, respectively, compared to the control (Table 2). Regarding root fresh and dry masses results, all isolates outperformed both the control and *B. subtilis* treatment. Notably, for root dry mass, the EB01 and EB15 isolates stood out, with values of 5.11 g and 5.09 g, respectively, representing an increase of 41.18% relative to the control.

The Leonard pot system probably prevented the phytopathogen from accessing the plant roots during the entire analyzed cycle, thereby precluding direct observation of pathogen infection in the garlic plants. Thus, the present study demonstrated a positive effect of the isolates on garlic growth and development, with an average increase of 15% in plant height and a significant difference observed in the evaluated parameters (Table 2). In

an experiment using the *Bacillus subtilis* Bs10 strain on tomato and lettuce, tomato plants exhibited a 17.5% increase in height compared to the control, while lettuce plants showed a 30.10% increase (Chagas et al., 2023). Similarly, in chickpea, *Bacillus licheniformis* BS 94 inoculation presented a 26.23% height increase compared to the control in a pot experiment (Rathod et al., 2023). These studies and their results support the findings reported in the present work and highlight the potential of Bacillus isolates to stimulate plant growth.

Compared to the control, isolates EB15 and EB21 showed significant increases of 26.85% and 24.8% in shoot fresh mass. Matsumura et al. (2016) evaluated lettuce growth under different conditions using liquid, powder, and *Bacillus amyloliquefaciens* effervescent tablet formulations. In all soil and climate conditions tested, *Bacillus* enhanced the plant's aerial portion growth in all formulations. In the present study, all isolates also outperformed both the control and *B. subtilis* in terms of shoot dry mass (Table 2).

In a previous work by Balbinot et al. (2020), the isolates used in the present study (EB01, EB15, EB17, EB21) produced IAA (Indole Acetic Acid), a plant hormone of the auxin class that plays a key role in root development and overall plant growth promotion. Rhizosphere microorganisms contribute to the assimilation of nutrients and secretion of extracellular molecules such as hormones, antibiotics, and other signaling compounds that support plant growth (Phour et al., 2020). These compounds released by microorganisms act as biostimulants, and several studies have reported that inoculating plants with growth-promoting rhizobacteria is an efficient, biologically based solution for supporting agronomic productivity (Bhat et al., 2020).

For example, an increase in the dry mass of corn roots was observed following inoculation with *Bacillus subtilis* and *Trichoderma* spp. (Chagas et al., 2018). Rhizobacteria stimulated the growth of rice. The isolates BRM32110 (*Bacillus* sp.) and BRM32112 (*Pseudomonas fluorescens*) produced average increases of 24.3 % and 27.1 %, respectively, in root length in the BRS Catiana cultivar (Sousa et al., 2019).

In the present work, the treatments with isolates EB01, EB15, as well as the non-inoculated control, exhibited the highest mean values for clove dry mass. Interestingly, the control treatment showed a significant increase 45.88% in clove dry mass compared to itself in other conditions suggesting that the LB medium may have stimulated the beneficial microbiota associated with the plants. Similar effects have been reported in micropropagated pineapple and bamboo, where inoculation with plant growth-promoting rhizobacteria led to enhanced development (Belincanta et al., 2022).

The isolates tested in the first experiment (EB01, EB15, EB17, and EB21) presented mechanisms to promote plant growth in a previous work (Balbinot et al., 2020); one of these mechanisms is phosphate solubilization. In this assay, all presented phosphate solubilization indices (SI) of calcium phosphate of 0.82, 1.37, and 0.75, except for isolate EB01. All isolates produced IAA in the following concentrations: 10.85 µg. mL^{-1} (EB01), 34.28 µg. mL^{-1} (EB15), 17.26 µg. mL^{-1} (EB17), and

7.56 µg. mL^{-1} (EB21). According to Hartmann et al. (1983), values between 1 to 10 µg. mL^{-1} are classified as medium, and high values are between 11 to 50 mL^{-1}. The *Bacillus* sp. isolates were also characterized for their biological activity in soybean plants (Paula et al., 2021), who reported that IAA production ranged from 8.56 to 31.33 µg. mL^{-1}. The values obtained in the present study are therefore consistent with those previously reported in the literature. Six phosphate-solubilizing *Bacillus* isolates were analyzed for their ability to promote the growth of eggplant, pepper, and tomato plants in pots (Bahadir et al., 2018), and they significantly improved radicle development and increased plant growth. Additionally, they produced IAA in concentrations ranging from 0.80 to 27.22 ug. mL^{-1}, showing their potential as multiple biostimulants.

In the present study, the bacterial isolates significantly enhanced radicle development and overall plant growth. They also produced IAA, ranging from 0.80 to 27.22 µg. mL^{-1}, highlighting their potential as multiple bio-stimulants. The reports corroborate the efficiency of *Bacillus* spp. in promoting the growth and development of various crops.

Second in vivo experiment

In the second experiment, all parameters (plant height, shoot, root, bulb fresh and dry mass) were evaluated at 82 DAS, before the end of the crop cycle, due to a severe fungal outbreak. The CFU/mL numbers were as follows: 1.67 x 10^7 (EB01), 1.4 x 10^7 (EB13), 5.25 x 10^7 (EB18), 3.45 x 10^7 (EB19), 1.27 x 10^7 (EB24), and 1.31 x 10^7 (*B. subtilis* strain CCTB04).

S. cepivora hyphae were observed in the soil and some cloves at 15 DAS. Subsequently, the plants exhibited symptoms of rot, such as yellowing and dead leaves. The height parameters showed a statistical difference among the treatments at 30 and 60 DAS (Table 3). Notably, plants inoculated with *Bacillus* spp. isolates demonstrated improved growth. These results indicate that, except for EB01, the isolates sustained plant development under fungal pressure, maintaining growth levels comparable to the control.

Table 3
Effect of *Bacillus* spp. isolates on the growth of garlic (*Allium sativum* L.) cultivated in soil

	Plant height (cm)			Shoot (g)		Root (g)		Bulb (g)	
Treatment	30 DAS	60 DAS	82 DAS	FM	DM	FM	DM	FM	DM
S.c	26,13 b	50,40 b	50,33 a	8,65 b	1,45 a	5,99 a	1,27 a	5,15 a	1,06 a
EB01	22,23 b	46,33 b	47,03 a	8,04 b	1,30 a	6,16 a	1,16 a	5,61 a	1,25 a
EB13	32,40 a	62,30 a	60,17 a	20,21 a	3,02 a	9,81 a	1,73 a	8,02 a	1,52 a
EB18	36,00 a	60,63 a	61,33 a	19,09 a	3,29 a	10,54 a	1,78 a	7,96 a	1,54 a
EB19	31,60 a	56,80 a	57,30 a	19,43 a	3,20 a	6,64 a	1,04 a	7,85 a	1,68 a
EB24	30,73 a	56,07 a	57,13 a	13,45 b	2,03 a	7,69 a	1,44 a	6,68 a	1,52 a
B. subtilis *	20,78 b	42,93 b	47,03 a	10,91 b	1,53 a	6,18 a	1,06 a	6,18 a	1,25 a
Control	29,87 a	56,00 a	58,03 a	16,65 a	2,37 a	8,55 a	1,81 a	6,34 a	1,23 a
CV (%)	15,06	12,39	13,80	16,67	20,61	16,36	26,44	9,96	15,44

Means followed by the same letter in the column do not differ from each other, according to the Scott-Knott test at a 5% significance level.
S.c – Only *Stromatinia cepivora*
DAS – Days After Sowing
FM - Fresh Mass
DM - Dry Mass
* strain CCTB04.

For the other parameters evaluated, no significant differences were observed (Table 3), except for shoot fresh mass. In this case, the treatments inoculated with isolates EB13, EB18, and EB19 were statistically similar to the control, suggesting a protective effect against the fungus presence.

The protection index (PI) was calculated based on plant height (Table 4), and the statistical results were consistent across the three evaluation periods, as shown in Table 3. The control treatment, which was not exposed to *S. cepivora,* exhibited a PI of 100%. In the second group, the protection index (PI) ranged from 11.67% to 18.67%, corresponding to the treatments inoculated with isolates EB13, EB18, EB19, and EB24. The final group consisted of the treatment with *S. cepivora* alone, as well as those inoculated with isolate EB01 and *B. subtilis* (Table 3), suggesting that the isolates in the second group exhibited a relatively greater protective effect against the pathogen.

Table 4
Protection index (PI) of *Bacillus* spp. isolates against *Stromatinia cepivora* in garlic (*Allium sativum* L.) cultivation in soil

	PI (%)		
Treatment	30 DAS	60 DAS	82 DAS
S.c	0,00 c	0,00 c	0,00 c
EB01	0,00 c	0,00 c	2,67 c
EB13	20,98 b	55,31 b	17,00 b
EB18	33,03 b	18,27 c	18,67 b
EB19	18,30 b	11,42 c	12,00 b
EB24	15,40 b	10,20 c	11,67 b
B. subtilis *	0,00 c	0,00 c	5,67 c
Control	100,00 a	100,00 a	100,00 a
CV (%)	31,98	37,06	29,19

Means followed by the same letter in the column do not differ from each other, according to the Scott-Knott test at a 5% significance level. The data was treated and transformed by statistical analysis.
PI= Protection index
DAS – Days After Sowing
S.c – Only *S. cepivora*
* strain CCTB04.

In the second experiment, it was possible to evaluate both plant growth parameters and protection of bacterial isolates against *S. cepivora* (Tables 3 and 4). Regarding plant height, significant differences were observed among treatments. Plants inoculated with *Bacillus* spp. isolates exhibited growth comparable to the control, except for isolate EB01, which showed a reduced performance.

The potential of *Bacillus* sp. isolates to promote growth and control of *Fusarium verticillioides* in corn was evaluated (Ferreira et al., 2021). These authors reported that the AP-165 isolate differed from the other treatments in terms of plant height as compared to the control. They also observed that the AP512 isolate was the most efficient in inhibiting the mycelial growth.

In an experiment with a *Bacillus subtilis* isolate, Sayago et al. (2020) reported a 57.42% reduction in *S. terrestris* infection in onion plants. Similarly, in studies with grapes, the *Bacillus amyloliquefaciens* strain QST 713 was shown to control 76.7% of black rot in *Muscat Blanc* grape bunches, effectively reducing disease development (Aleinikova et al., 2023).

According to the protection index (Table 4), isolates EB13, EB18, EB19, and EB24 presented control values ranging from 12% to 19% for the phytopathogen. A report used the *Bacillus amyloliquefaciens* YTB1407 strain in sweet potato plants (*Ipomoea batata*) to control fungi in the greenhouse (Wang et al., 2020) and achieved a control level of 49.95 % for *Ceratocystis fimbriata* and 83.33% for *Fusarium solani.*

In studies evaluating the effects of *Bacillus subtilis* isolates against onion white rot over two planting seasons in a greenhouse, all isolates reduced the fungus infection compared to the control. Notably, *B. subtilis* isolate 2 showed the highest level of control, reaching 78.57 and 77.78 % in the first and second planting seasons, respectively.

In a previous study, isolates EB01 and EB13 demonstrated lipase production (Balbinot et al., 2020). The antifungal activity of *Bacillus* spp. is often attributed to the production of extracellular enzymes such as lipases and proteases, which hydrolyze the main components of fungal cell walls (Miljaković et al., 2020). However, the literature on bacterial lipases role in pest and disease control remains limited, indicating a need for further research in this area.

Bacillus isolates identification

According to the phylogenetic tree (Figure 1), *Bacillus* sp. EB01, *Bacillus* sp. EB13, *Bacillus* sp. EB18, *Bacillus* sp. EB19 and *Bacillus* sp. EB24 showed the highest identity in 16S rRNA sequences with their closest neighbors: *B. velezensis* KY694464 (100.0%), *B. rugosus* MT554518 (99.9%), *B. siamensis* KY643639 (99.8%), *B. tequilensis* KJ847721 (99.9%), and *B. velezensis* KY694464 (98.8%).

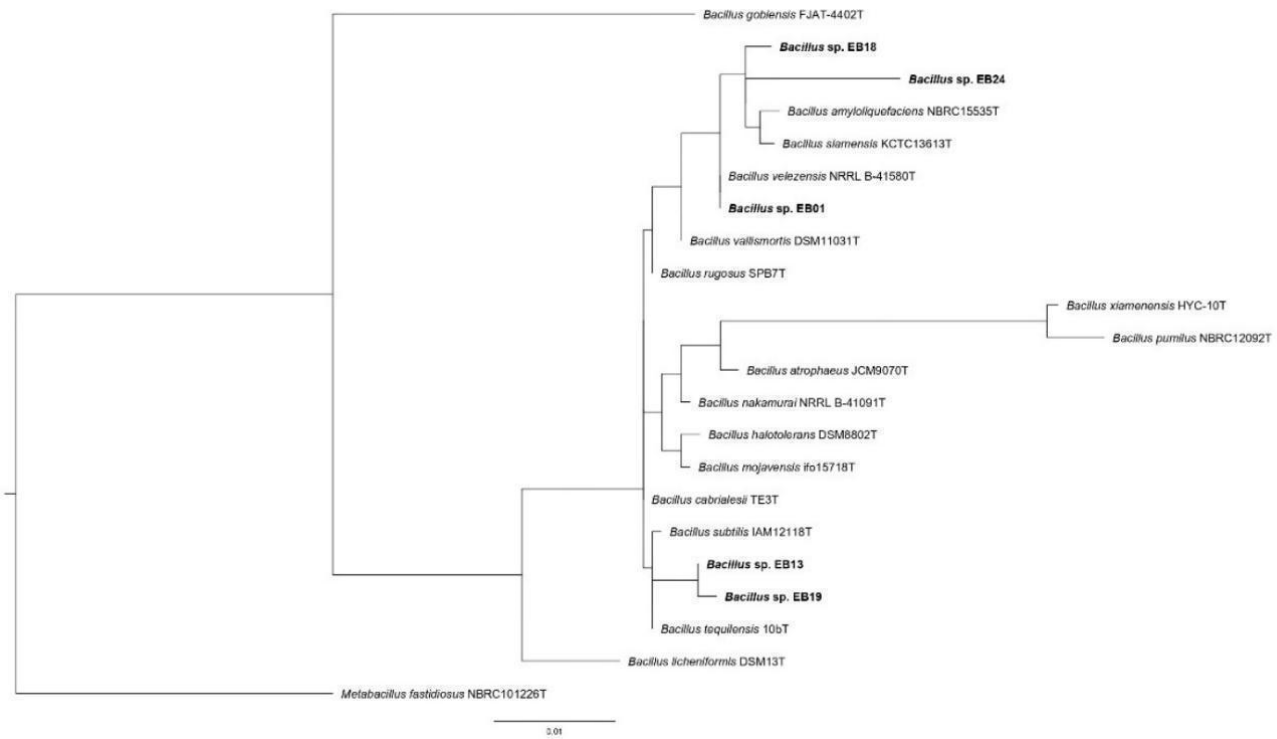

Figure 1. Phylogenetic tree based on 16S rRNA sequence, sixteen isolates from related *Bacillus* species. By employing the Maximum Likelihood method based on the General Time Reversible model, the evolutionary history was inferred using the RAxML-NG software (Kozlov et al., 2019) at the CIPRES Science Gateway (Miller et al., 2010). *Metabacillus fastidiosus* NBRC 101226 T sequence was used as an outgroup.

Phylogeny, together with the moderate identity degree between *Bacillus* sp. EB24 and *B. velezensis* in 16S rRNA (98.8%), suggests this isolate should be investigated in future research as a possible new species.

The *Bacillus* genus has already been the subject of extensive research in the field of biological control. In the present study, the isolates were identified as *B. velezensis, B. rugosus, B. subtilis,* and *Bacillus tequilensis* (Table 1). Among these, *Bacillus velezensis* has been widely referred to as a bio-fungicide for inhibiting the growth of various phytopathogenic fungi. In vitro tests demonstrated that *B. velezensis* LD02 effectively inhibited the growth of A. tenuissima by 78.97%, *A. flavus* (80.77%), *A. niger* (79.74%), *F. oxysporum* (81.03%), *F. moniliforme* (81.28%), *R. solani* (79.23%), and *Rhizopus* sp. (75.64%) (Chen et al., 2019). Additionally, *B. velezensis* ZJ20 has been reported to inhibit the growth of *Cryphonectria parasitica, Helicobasidium purpureum,* and *Cylindrocladium quinqueseptatum in vitro* (Y. Zhang et al., 2022). Furthermore, *B. velezensis* E2 inhibited *Aspergillus westerdijkiae* in pear fruits, reducing the lesion diameter by 73.9% (Xu et al., 2016).

Bacillus subtilis is one of the most extensively studied species in biological control. *B. subtilis* strains inhibited *S. cepivora* (Ocegueda-Reyes et al., 2020), *Sclerotinia sclerotiorum, Sclerotium rofsii,* and *Colletotricum dematium* var truncata *in vitro* (Gabardo et al., 2020). Moreover, *Bacillus subtilis* F62 significantly reduced the incidence of *Fusarium oxysporum* in grapevine rootstocks by 35.4% and 63.6% (Russi et al., 2022).

Recent studies suggested that *B. tequilensis* has the potential to control a range of soil-borne phytopathogens. Karthika et al. (2022) reported that *B. tequilensis* inhibited *Fusarium oxysporum in vitro* growth by 95.33%. Similarly, Li et al. (2018) described that *B. tequilensis* GYLH001 inhibited the growth of *Magnaporthe oryzae* by 61.07%. *B. tequilensis* isolates (B2, B3, and B4) were tested as biocontrol agents against *Fusarium* spp., and they could inhibit the growth of *F. oxysporum, F. culmorum, F. proliferatum,* and *F. verticillioides.* Furthermore, Guerrero-Barajas et al. (2020) reported that B. tequilensis A3 restrained the *in vitro* growth of Colletotrichum gloeosporioides by 60%.

There are limited reports on *B. rugosus* biocontrol potential. Nevertheless, *B. rugosus* NOK47 has been tested as a control for *Fusarium graminearum,* demonstrating antagonistic potential and controlling 69.04% of the pathogen *in vitro* (Kaul et al., 2022).

Currently, several bio-inputs containing *Bacillus* species isolates are commercially available, including *Bacillus subtilis* BV02, *B. velezensis* QST 713, *Bacillus subtilis* UFPEDA 764, and *B. velezensis* D747. These products reinforce the findings of the present study and highlight the potential of the newly identified isolates to contribute to the expanding application of plant growth-promoting microorganisms in sustainable agriculture.

The control of phytopathogens, especially *S. cepivora,* is currently carried out by chemical products. However, these methods have proven to be inefficient, and their excessive use has led to significant

economic and environmental impacts. Therefore, the development of alternative control strategies is essential. In this context, biological control using microorganisms has demonstrated high efficacy (Zapata-Narváez & Gómez-Marroquín, 2022; Elshahawy et al., 2018). This approach not only reduces the damage caused by phytopathogens but also helps maintain ecological balance, contributing to safer and more sustainable agricultural production.

Conclusion

Even in the absence of white rot symptoms (*S. cepivora*), *Bacillus* spp. isolates EB01, EB15, EB17, and EB21 were able to stimulate garlic plant growth, indicating their potential as plant growth promoters.

When the disease was present, isolates EB13, EB18, EB19, and EB24 effectively reduced disease symptoms, indicating potential for biological control. These isolates were identified as *Bacillus velezensis, Bacillus rugosus, Bacillus subtilis, Bacillus tequilensis,* and *Bacillus velezensis,* respectively. Some of these species are already reported as biological control agents, and others are, nowadays, used as commercial bio-products.

Statements and Declarations

The authors declare they have no competing interests related to the content of this article.

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