In vitro control of phytopathogenic fungi and damping-off of tomato by *Bacillus velezensis* LABIM40 (CMRP 4489)

Controle *in vitro* de fungos fitopatogênicos e tombamento de mudas de tomateiro por *Bacillus velezensis* LABIM40 (CMRP 4489)

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Highlights

Effective antagonism against plant pathogenic fungi.
Establishment of LABIM40 growth curve.
Cell-free supernatant reduces damping-off in tomato plants.

Abstract

The in vitro antagonistic activity of *Bacillus velezensis* LABIM40 (strain CMRP 4489) was assessed against *Alternaria linariae, Botryotinia squamosa, Colletotrichum lindemuthianum, Gibberella zeae,* and *Rhizoctonia solani.* An experiment was conducted using treated seeds under growth chamber conditions to determine the impact of various LABIM40 formulations on tomato seedling growth and the biocontrol of damping-off caused by *R. solani.* The treatments included the use of LABIM40 cell suspension, LABIM40 cell-free supernatant (CFS), 10 times concentrated CFS (10× CFS), commercial products based on *Bacillus amyloliquefaciens* (CP_1) and *Bacillus subtilis* (CP_2), and water. The effects of these products were assessed on tomato seedlings grown in sterile substrate or substrate inoculated with *R. solani.* In a dual culture test, *B. velezensis* LABIM40 inhibited the mycelial growth of the aforementioned fungal pathogens by 46.6%, 67.4%, 64.7%, 49.0%, and 54.4%, respectively. The minimum inhibitory concentration against each fungus was determined using varying concentrations of CFS in potato

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dextrose agar medium, followed by a regression analysis of mycelial growth inhibition. Except for *A. linariae,* the logarithmic model provided the best fit in all cases. Tomato seedlings from seeds treated with 10× CFS in inoculated substrate exhibited a survival rate 57% higher than that exhibited by the control treatment. However, no growth promotion was observed in tomato plants from seeds treated with LABIM40 cells or its CFS metabolites. In summary, these findings highlight the antagonistic activity of *B. velezensis* LABIM40 against *A. linariae, B. squamosa, C. lindemuthianum, G. zeae,* and *R. solani,* as demonstrated by dual culture and CFS diffusion tests. This suggests its potential as a biocontrol agent for damping-off in tomatoes.

Key words: Biological control. Plant-growth promoting rhizobacteria. *Solanum lycopersicum.*

Resumo

O antagonismo do *Bacillus velezensis* LABIM40 (cepa CMRP 4489) foi avaliado in vitro contra *Alternaria linariae, Botryotinia squamosa, Colletotrichum lindemuthianum, Gibberella zeae* e *Rhizoctonia solani.* Com o objetivo de avaliar o efeito de diferentes formulações de LABIM40 no crescimento de mudas de tomateiro e no biocontrole do tombamento causado por *R. solani,* foi conduzido um experimento com sementes tratadas em condições de câmara de crescimento. Os tratamentos foram suspensão de células de LABIM40, LABIM40 SLC, SLC 10 vezes concentrado (10xSLC), produtos comerciais à base de *Bacillus amyloliquefaciens* (PC_1) e *Bacillus subtilis* (PC_2) e água. Os efeitos do produto foram avaliados em mudas de tomate cultivadas em substrato estéril e em substrato inoculado com *R. solani.* No teste de cultura dupla, *B. velezensis* LABIM40 inibiu em 46,6, 67,4, 64,7, 49,0 e 54,4% o crescimento micelial desses fungos fitopatogênicos, respectivamente. A concentração inibitória mínima para cada fungo foi determinada por diferentes concentrações de sobrenadante livre de células (SLC) em meio de batata dextrose ágar (PDA), seguido de análise de regressão da inibição do crescimento micelial. Com exceção de *A. linariae*, em todos os casos o modelo com melhor ajuste foi o logarítmico. A sobrevivência de mudas de tomate de sementes tratadas com 10xSLC em substrato inoculado foi 57% maior do que o tratamento controle. Não foi observada promoção de crescimento em plantas de tomate oriundas de sementes tratadas com células de LABIM40 ou metabólitos de seu SLC. Coletivamente, esses resultados destacam a atividade antagonista de B. velezensis LABIM40 contra *A. linariae, B. squamosa, C. lindemuthianum, G. zeae* e *R. solani* verificada por cultura dupla e testes de difusão de sobrenadante livre de células e seu potencial como agente de biocontrole do tombamento do tomateiro.

Palavras-chave: Controle biológico. Bactérias promotoras do crescimento de plantas (BPCP). *Solanum lycopersicum.*

Introduction

Tomato (*Solanum lycopersicum* L.), one of the most widely consumed vegetables globally, accounted for the second highest production worldwide, with 186.8 million tons produced in 2020 (Food and Agriculture Organization of the United Nations [FAOSTAT], 2022). However, most tomato genotypes are vulnerable to soilborne pathogens, such as *Rhizoctonia solani* (Kühn), which cause seedling diseases like damping-off. This pathogen leads to root rot and stem lesions, often culminating in seedling death and

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subsequent reduction in plant stands. The disease management is challenging due to the fungus's broad host range, long-lasting presence of its survival structures (sclerotia) in the soil, lack of genetic resistance, and limited effectiveness of available chemical fungicides (Zachow et al., 2011). In Brazil, only one chemical fungicide (Naria DM®- BASF S. A.) is registered for damping-off control in tomatoes (Sistema de Agrotóxicos Fitossanitários [AGROFIT], 2022).

The commercial development of biological products for plant disease control is experiencing rapid growth, making it one of the fastest expanding sectors in the global fungicide market. The active ingredients in these products are either beneficial microorganisms or their derivatives. Their effectiveness in managing a variety of plant pathogens without inflicting harm on the host plant has been proven (Huang et al., 2017; Parikh et al., 2018).

The *Bacillus* genus possesses significant biotechnological potential, with numerous species producing biologically active molecules. These molecules are currently utilized in the production of antibiotics, enzymes, and other metabolites by the pharmaceutical, food, and agricultural industries (Abdelnasser et al., 2017; Ayala et al., 2017; Ribeiro et al., 2018). *Bacillus*, a genus of spore-forming, gram-positive, rod-shaped bacteria, includes many species capable of synthesizing antibiotics and secondary metabolites. These metabolites exhibit antagonistic activity against phytopathogenic bacteria and fungi, enabling their use as biocontrol agents for plant diseases (Lanna-Filho et al., 2017; L. Chen et al., 2019; Paz et al., 2018). Furthermore, several *Bacillus* species can safeguard

plants from various pathogens by inducing systemic resistance, competing for invasion sites, or promoting plant growth (Huang et al., 2017; Dhouib et al., 2019; Torres et al., 2020).

Bacillus species, extensively isolated as antagonists of plant pathogens, have been commercialized globally as biocontrol agents and for the use of their secondary metabolites (Palazzini et al., 2016). Recent phylogenetic analysis has led to the reclassification of several *Bacillus* species as *B. velezensis* (Palazzini et al., 2016; Huang et al., 2017; Rabbee et al., 2019). Genomic analysis has revealed that *B. velezensis* possesses strain-specific gene clusters associated with the biosynthesis of secondary metabolites. These metabolites play a crucial role in pathogen suppression, plant growth promotion, and the induction of systemic resistance in plants (Rabbee et al., 2019).

In this study, we investigated *B. velezensis* strain LABIM40 and its cell-free supernatant (CFS) for their potential in controlling *Alternaria linariae, Botryotinia squamosa, Colletotrichum lindemuthianum, Gibberella zeae,* and *R. solani* under in vitro conditions. Additionally, their efficiency in managing damping off, caused by *R. solani,* was examined. The study also explored their impact on the growth of tomato plants.

Materials and Methods

Isolation and identification of B. velezensis LABIM40 strain

The LABIM40 strain was isolated from an *in vitro* culture plate of *Fusarium oxysporum* as an antagonistic contaminant in

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Londrina, Brazil. This strain was subsequently deposited in the Microbial Collection of the Laboratory of Microbial Biotechnology at the State University of Londrina (UEL) and preserved in 20% glycerol at -80 °C. The genome of the strain was sequenced using the MiSeq platform and an MiSeq version 3 reagent kit (600 cycles, Illumina, Brazil) at Embrapa Soja, Londrina. The complete genome was then deposited in DDBJ / EMBL / GenBank under the accession number CP023748 (Baptista et al., 2018). Additionally, LABIM40 was deposited as strain CMRP 4489 in the Coleções Microbiológicas da Rede Paranaense (CMRP) network at the Federal University of Paraná, in Curitiba, Brazil (Baptista et al., 2022). Prior to each experiment, the LABIM40 strain was activated from the cryopreserved stock culture on nutrient agar (NA) plates and incubated at 30 °C with a 12 hour photoperiod until colony formation occurred.

Fungal isolates and growth conditions

The Plant Pathology Laboratory of the Federal University of Viçosa (UFV-MG) provided the *A. linariae* AS94 strain. The *B. squamosa* EITF10 strain, isolated from symptomatic onion leaves in Ituporanga-SC, was supplied by the Agricultural Research and Rural Extension Company of Santa Catarina (EPAGRI). The Phytopathology Laboratory of Instituto de Desenvolvimento Rural do Paraná-IAPAR/Emater provided the *C. lindemuthianum* strain 65, obtained from common bean pods exhibiting typical anthracnose symptoms in Londrina-PR. The *G. zeae* isolate, isolated from commercial corn ears in northern Paraná showing typical rot symptoms, was also included in

the study. Lastly, *R. solani* was isolated from tomato seedlings exhibiting typical damping-off symptoms at the Laboratory of Plant Pathology of UEL. This fungus was identified through microscopic observation (400× magnification) of its right-angle branching hyphae and constriction at the branching area, followed by septum formation.

The fungi were cultivated on Acumedia® potato dextrose agar (PDA) plates, stored in darkness at 6 °C. Prior to each experiment, these cultures were relocated to fresh PDA plates and incubated under a 12-hour photoperiod at 25 °C.

In vitro experiments were conducted using a biochemical oxygen demand incubator at the Microbial Biotechnology Laboratory, while *in vivo* experiments were carried out in a growth chamber located at the Plant Pathology Laboratory of the State University of Londrina.

In vitro experiments

Dual culture test

The antagonistic activity of the LABIM40 strain of *B. velezensis* was assessed *in vitro* against *A. linariae, B. squamosa, C. lindemuthianum, G. zeae,* and *R. solani* using a dual culture test. Mycelial discs, each with a diameter of 7 mm, from each pathogenic fungus were positioned mycelium-side down at the center of PDA plates. The LABIM40 strain was inoculated by touching a sterile platinum loop to 24-hour colonies and was placed 25 mm away from the fungal disc in two perpendicular directions on the same plate. Control plates were prepared with pure cultures of each fungus,

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inoculated under the same conditions. After incubating for 5 days at 25 °C with a 12-hour photoperiod, which is the time necessary for complete fungal growth on the control plates (except for *C. lindemuthianum*, which did not cover the entire plate), the diameter of the colonies was measured using two orthogonal measurements for each colony. The percentage of mycelial growth inhibition (MGI) was calculated using the formula: MGI (%) = 100 - [(diameter of fungal colony in dual culture plate / diameter of fungal colony in control plate) * 100]. The experimental design was completely randomized and included 4 replicates. The experiment was conducted three times. The data from both experiments were subjected to the Kolmogorov-Smirnov test to confirm their normal distribution. The data were then analyzed collectively using the F-test, and when significant, subjected to the Scott-Knott test at a 5% significance level. Statistical analysis was conducted using GraphPad Prism® v.8 software.

Growth curve and production of cell-free supernatant of B. velezensis LABIM40

The antimicrobial activity of the LABIM40 strain was confirmed through a dual culture test, followed by its fermentation. Initially, the LABIM40 strain was activated from the cryopreserved stock culture on a nutrient agar plate (NA) and synchronized after 24 hours by inoculating a colony from the activation plate. The strain concentration was then adjusted to 10^8 CFU mL^{-1} in a saline solution (NaCl 0.85%) using the McFarland scale. A bacterial suspension of forty µL was inoculated into 40 mL of Luria-Bertani (LB) broth and incubated at 25 °C and 125 rpm. After 24 hours, 4 mL (1%) of this culture was transferred into 400 mL of mineral medium (containing 20 g L^{-1} glucose, 5 g L^{-1} NaCl, 0.04 g L^{-1} MnSO$_4$, 1.67 g L^{-1} FeSO$_4$.7 H$_2$O, 1.22 g L^{-1} MgCl. 6 H$_2$O, 1.2 g L^{-1} K$_2$HPO$_4$, and 12.4 g L^{-1} tryptone) and cultivated at 28 °C and pH 7.1 for 72 hours. Every 12 hours, 10 mL aliquots were collected to establish the LABIM40 growth curve via plate counting (MYP agar) and to test the antimicrobial activity. The bacterial cells were separated from the culture broth by centrifugation at 9000 rpm for 10 min at 4 °C, and the supernatant was filtered using a 0.22 µm Millipore syringe. At the end of the process, a portion of the CFS was frozen, while the remainder was used in well diffusion and minimum inhibitory concentration (MIC) tests.

Well diffusion test

The antifungal activity of LABIM40 CFS was investigated by the well diffusion method (Sen & Batra, 2012). The bacterium was activated, synchronized, and adjusted to 10^8 CFU mL^{-1} as previously described. In plates containing 30 mL of solidified PDA medium, four wells were cut out of the agar 20 mm apart from the center with a sterile 1000 µL tip. A mycelial disc (7 mm in diameter) was inoculated in the center of each plate, with the mycelium facing down. CFS, at a volume of 200 µL, was placed into each well. Only fungal disks were cultured in control plates. The plates were incubated under a 12-hour photoperiod at 25 °C until the mycelium in the control plates grew completely. The diameter of the fungal colonies was measured by two orthogonal measurements. The inhibition of mycelial growth was calculated as computed during the dual culture test. The experimental design was completely randomized with four

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replicates, and the experiment was performed in triplicate. The data were subjected to an F-test, and if found significant, they were analyzed via second-degree regression using GraphPad Prism® v.8 software.

MIC test

An MIC test was conducted to ascertain the lowest concentration of CFS that could inhibit 100% fungal growth, utilizing the plate diffusion method. For *B. squamosa* and *R. solani,* CFS was integrated into PDA plates at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, and 0.781 µg mL^{-1}. For *A. linariae, C. lindemuthianum,* and *G. zeae,* the concentrations employed were 600, 500, 400, 200, 100, 50, 25, 12.5, and 6.25 µg mL^{-1}. Plates devoid of CFS served as the control (0.0 µg mL^{-1}). The experimental design was entirely randomized, with four replicates, and the experiment was conducted thrice. Mycelial growth inhibition data were statistically scrutinized through regression analysis and adapted to the most suitable model based on the coefficient of determination (R^2). The data were subjected to an F-test, and if found significant, they were analyzed via second-degree regression using GraphPad Prism® v.8 software.

In vivo experiments

Plant material

Seeds from an indeterminate tomato cultivar (Santa Cruz Kada Gigante' from Top Seed®, lot N. 053735) with a growth cycle of 110-120 days were superficially disinfected through immersion in 70% alcohol for one minute. This was followed by a one-minute immersion in a 1% hypochlorite solution and three rinses with sterile deionized water. The efficacy of the surface disinfestation process was confirmed by spreading 200 µL of the final rinse water on NA plates and incubating at 35 °C for 48 hours.

Seed treatment with biological products

Using a sterile platinum loop, 24-hour colonies of *B. velezensis* LABIM40, grown on NA plates, were transferred to test tubes filled with a 0.85% NaCl solution. These suspensions were then homogenized using a vortex. The bacterial suspension was standardized to approximately 1×108 CFU mL^{-1}, as per the McFarland scale (National Committee for Clinical Laboratory [NCCLS], 2003). Test tubes containing 30 mL of a 24-hour LABIM40 culture in LB were centrifuged at 4 °C and 9000 rpm for a duration of 10 minutes. The final suspension used in the tests was obtained by resuspending the sediment in 3 mL of sterile distilled water. To achieve a ten-fold concentrated CFS (10× CFS), a 200 mL aliquot of crude CFS was concentrated using a Fisatom® evaporator, resulting in a final volume of 20 mL of 10× CFS. The concentration of the commercial B. amyloliquefaciens-based biological product, referred to as "CP_1", was 6 µL g^{-1} of tomato seed.

Germination test

Tomato seeds were treated with various substances: sterile deionized water (control), LABIM40 cell suspension (1x10^8 CFU mL^{-1}), CFS (10 mL g^{-1} seed), 10xCFS (10

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mL g^{-1} seed), and CP_1 (6 µL g^{-1} seed). The treatment process involved immersing the seeds and homogenizing them in a shaker at 125 rpm and 25 ºC for a duration of 10 minutes. However, this process did not apply to CP_2, which was instead applied directly to the substrate.

The seed treatment involved the use of LABIM40 suspensions, crude CFS, 10× CFS, CP_1, and sterile deionized water, which served as the control treatment. The process entailed immersing the seeds and maintaining them in a shaker at 125 rpm and 25 ºC for a duration of 10 minutes.

Seeds were arranged on sterilized filter paper, dampened with sterilized deionized water, and placed in Petri dishes. The dishes were sealed with parafilm and incubated at a temperature of 25 °C under a 12-hour photoperiod. Seven days after sowing (DAS), assessments of germination and growth, specifically the length of the hypocotyl and root, were conducted. Each experimental unit comprised a plate containing 25 seeds. The experimental design was entirely randomized, with four replicates, and the experiment was conducted thrice. The means were compared using the Tukey test, with the significance level set at p < 0.05.

Damping-off and plant growth in tomato seedlings

Commercial vegetable substrate was autoclaved thrice, each time for 40 minutes at 121 °C, with a 24-hour interval between each session. Germination trays, each with 200 cells, were filled halfway with the sterilized substrate. Six days prior to the introduction of tomato seeds, three discs of *R. solani*, each with a diameter of 5 mm and derived from five-day-old cultures, were transferred to each cell. The cells were then filled with substrate, following the methodology employed by Peng et al. (2013). The trays were stored in a growth chamber maintained at 25 °C and 80-90% relative humidity. The moisture content was kept at the maximum water retention capacity.

Tomato seeds (cv. Santa Cruz Kada) were treated as outlined for germination tests and were sown in seedling trays. These trays were filled with a commercial substrate, which was either inoculated with *R. solani* or left uninoculated. The CP_2 treatment, adhering to product guidelines, was directly applied to the substrate. This involved mixing 1 mL of the commercial product with 50 mL of water and administering 5 mL of this solution per tray cell.

Seed germination percentage was evaluated 7 DAS. Assessments were made 21 DAS for seedling survival, stem and root length, and overall plant weight. Following these assessments, the stems and roots were harvested, cleaned, and then dried in a forced-air oven at 65 ºC until a constant mass was achieved.

A completely randomized design was established in a factorial arrangement with six replicates. Each experimental unit comprised 20 seedling tray cells, each containing a single plant. The factorial arrangement was 6 × 2, taking into account six seed treatments (water, LABIM40 cell suspension, CFS, 10× CFS, CP_1, and CP_2) and two culture conditions (sterile and *R. solani* inoculated substrate). The experiment was conducted twice.

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The Kolmogorov-Smirnov test was applied to the data from both experiments to confirm their normal distribution. Subsequently, the data were collectively analyzed using the F-test. If significant, they were further subjected to the Scott-Knott test at a 5% significance level. GraphPad Prism® v.8 software was utilized for the statistical analysis.

Results and Discussion

Dual culture test

LABIM40 demonstrated potent antagonistic activity against *B. squamosa* and *C. lindemuthianum,* inhibiting them at rates of 67.4% and 64.7%, respectively. The inhibition rate of *R. solani* by LABIM40 was recorded at 54.4%, a figure that was statistically distinct from the inhibition rates of *G. zeae* (49.0%) and *A. linariae* (46.6%) (Figure 1A and 1B).
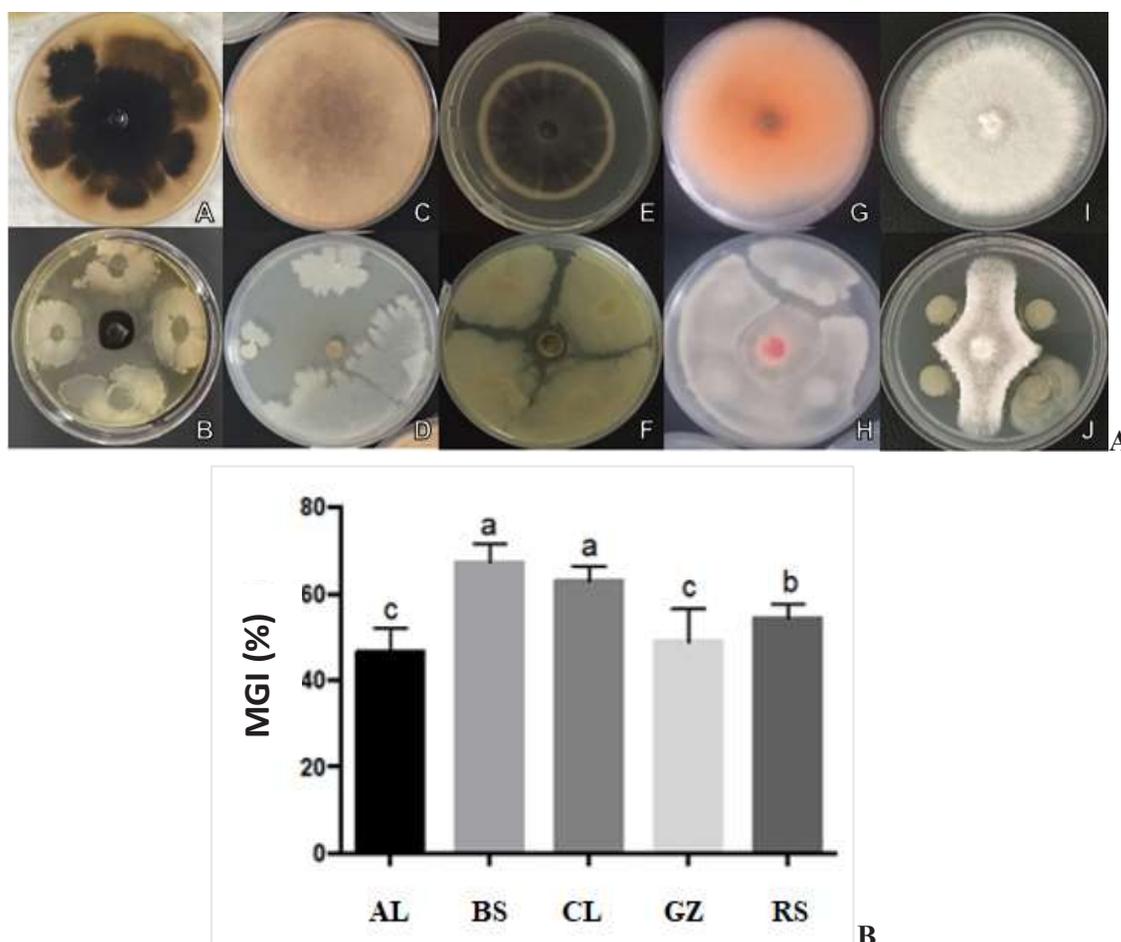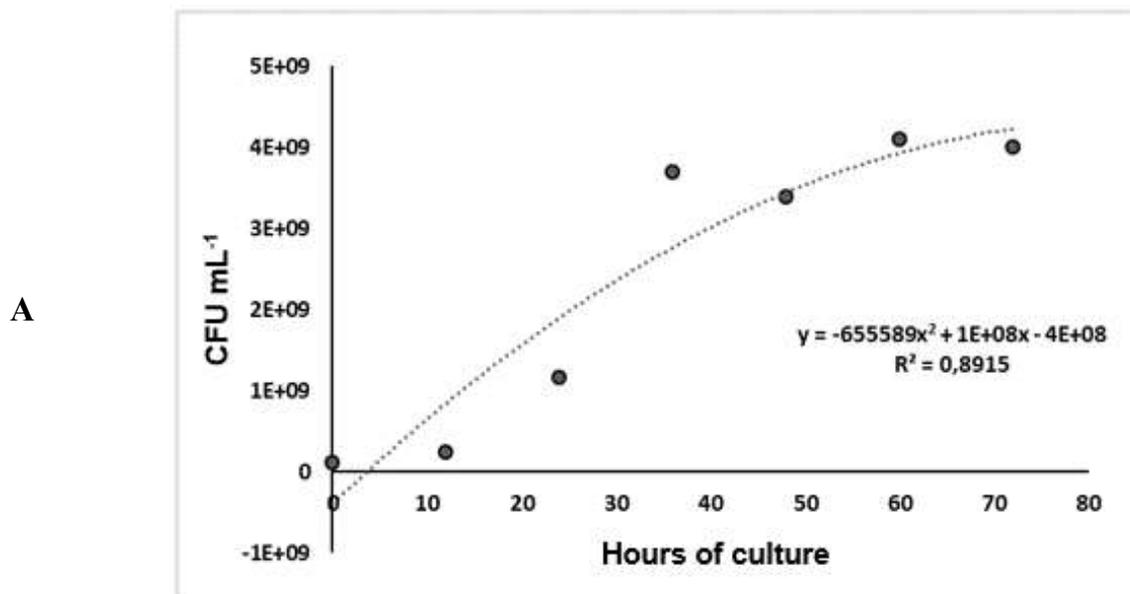

Figure 1. A. Dual culture test. *Bacillus velezensis* LABIM40 co-cultivated with *Alternaria linariae* (AL), *Botryotinia squamosa* (BS), *Colletotrichum lindemuthianum* (CL), *Gibberella zeae* (GZ), and *Rhizoctonia solani* (RS). **B.** Mycelial growth inhibition percentage (MGI %). Error bars represent standard deviation based on four repetitions. Means followed by same letter do not differ by Tukey's test.
A) Control plate of AL; B) AL vs. LABIM40; C) Control plate of BS; D) BS vs. LABIM40; E) Control plate of CL; F) CL vs. LABIM40; G) Control plate of GZ; H) GZ vs. LABIM40; I) Control plate of RS; J) RS vs. LABIM40.

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Ansary et al. (2018) reported similar findings in their study of three endophytic bacterial species' effects on *Sclerotinia sclerotiorum.* They noted that the BCL-1 strain inhibited radial growth by 69.1%, *B. amyloliquefaciens* BDR-2 by 66.7%, and *B. subtilis* BRtL-2 by 54.7%. Cao et al. (2018) also provided evidence of the potent antagonistic activity of *B. velezensis* isolates Y6 and F7 against *Ralstonia solanacearum* and *F. oxysporum* f. sp. *cubense* under both laboratory and greenhouse conditions. Furthermore, these isolates significantly curtailed the growth of *F. oxysporum* f. sp. *cucumerinum* and *Colletotrichum gloeosporioides* in in vitro conditions.

Strains of *B. velezensis* combat plant diseases through the production of metabolites, the induction of systemic resistance in plants, competition for resources with pathogens, and the release of antimicrobial enzymes and volatile compounds. Research supports the effectiveness of LABIM40 and other *B. velezensis* strains as potential biocontrol agents against a variety of plant pathogens, underscoring their potential role in disease management within agricultural systems.

Growth curve and production of cell-free supernatant of LABIM40 strain

The study's results demonstrated a growth curve for a microbial strain during fermentation, which was best described using a second-degree polynomial model. This model, represented by the equation [$y = -655589x^2 + (1 \times 10^8)x - (4 \times 10^8)$], provided an optimal fit for the growth curve. In this equation, 'y' denotes the colony-forming unit (CFU) values, while 'x' signifies the time points measured at 0, 12, 24, 36, 48, 60, and 72 hours (Figure 2A). This model allowed for the estimation of CFU values at each time point (Figure 2B). The growth curve displayed a minimal latency phase, indicating a swift increase in the LABIM40 strain 's cell count owing to the high initial inoculum cell count derived from a 24-hour culture. The equation's maximum value suggested that the maximum CFU count would reach approximately 1.1×10^{10} at 76.26 hours of culture.
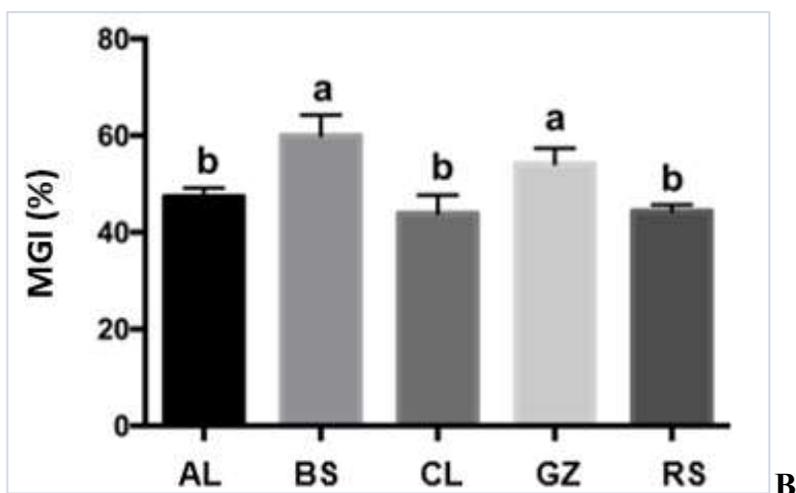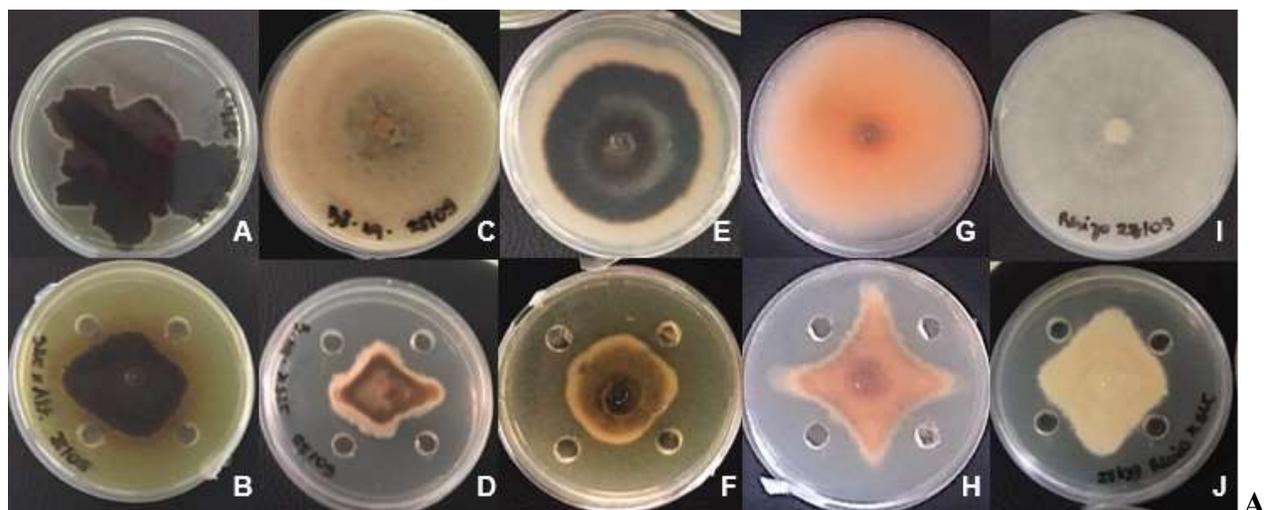
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Figure 2. **A.** Growth curve of *Bacillus velezensis* LABIM40 (CFU mL^{-1}) in 72 h **B.** Number of CFU mL^{-1} obtained and estimated according to y = *-655589x^2 + (1*10^8)x - (4*10^8)*.

Time (hours)	CFU mL^{-1}	
	Obtained	**Estimated**
0	1.2×10^8	4.0×10^8
12	2.4×10^8	8.9×10^8
24	1.2×10^9	2.4×10^9
36	3.7×10^9	4.1×10^9
48	3.4×10^9	5.9×10^9
60	4.1×10^9	7.9×10^9
72	4.0×10^9	10.2×10^9

The cultivation conditions employed in this study, encompassing time, temperature, and pH, mirrored those reported by Vasconcelos (2017) in their research on *Bacillus cereus* strains isolated from dairy products. The congruence in cultivation conditions and CFU/mL values implies that the growth patterns of the LABIM40 strain observed in this study are consistent with prior findings for *B. cereus* strains.

Well diffusion test

Upon exposure to LABIM40 CFS, *B. squamosa* and *G. zeae* demonstrated inhibition rates of 60.0% and 54.2%, respectively. These rates were significantly different from those observed for *A. linariae, R. solani,* and *C. lindemuthianum,* which exhibited inhibition rates of 47.4%, 44.4%, and 43.9%, respectively (Figure 3A and 3B).

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Figure 3. A. Well diffusion test. Cell-free supernatant (CFS) of *Bacillus velezensis* LABIM40 against *Alternaria linariae* (AL), *Botryotinia squamosa* (BS), *Colletotrichum lindemuthianum* (CL), *Gibberella zeae* (GZ) and *Rhizoctonia solani* (RS). **B.** Mycelial growth inhibition percentage (MGI %). Error bars represent standard deviation based on four repetitions. Means followed by equal letters do not differ by Tukey's test.
A) Control plate of AL; B) CFS of LABIM40 strain × AL; C) Control plate of BS; D) CFS of LABIM40 strain × BS; E) Control plate of CL; F) CFS of LABIM40 strain × CL; G) Control plate of GZ; H) CFS of LABIM40 strain × GZ; I) Control plate of RS; J) CFS of LABIM40 strain × RS.

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Jaivel et al. (2019) conducted a study on the antimicrobial activity of the extracellular lipopeptide fraction of B. amyloliquefaciens SR1 against various fungal phytopathogens. These included *R. solani, F. oxysporum, Sclerotium rolfsii, A. solani, Macrophomina phaseolina,* and *Aspergillus niger.* The inhibition zones (measured in cm) for each fungus were 2.33, 1.90, 2.06, 2.23, 1.80, and 2.30, respectively. The antifungal activity of *B. amyloliquefaciens* SR1 was attributed by the authors to the synthesis of surfactin, which is primarily associated with iturine and fengicin. Consistent with these findings, our research also indicates that metabolites from *B. velezensis* LABIM40, purified or otherwise, inhibit phytopathogenic fungi.

Moreover, the distinct composition and concentration of the metabolites or treatments employed could also influence the observed disparities. Various strains or species of *Bacillus* can generate a range of metabolites, each with differing levels of antifungal activity. The existence of specific compounds, such as lipopeptides including surfactin, iturin, or fengycin, as cited by Jaivel et al. (2019), may enhance the antifungal effects.

MIC test

The MIC of LABIM40 CFS necessary to inhibit the mycelial growth of *B. squamosa, C. lindemuthianum, G. zeae,* and *R. solani* was determined using data from three experimental repetitions and regression analysis (Table 1). Interestingly, *A. solani* did not respond to varying CFS concentrations. On average, an increase in CFS dosage led to a higher percentage of mycelial growth inhibition in the other fungi. Full inhibition of mycelial growth was observed in *B. squamosa* and *R. solani* at concentrations of 100 and 400 μg mL^{-1}, respectively. Conversely, *C. lindemuthianum* and *G. zeae* only exhibited a 50% inhibition of mycelium growth at these concentrations, indicating that the tested concentrations were insufficient to achieve complete inhibition.

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Table 1
Minimal inhibitory concentrations (MICs) of *Bacillus velezensis* LABIM40 cell-free supernatant (CFS) to inhibit the mycelial growth of *Alternaria linariae* (AL), *Botryotinia squamosa* (BS), *Colletotrichum lindemuthianum* (CL), *Gibberella zeae* (GZ), and *Rhizoctonia solani* (RS). The test was performed in potato dextrose agar plates amended with increasing concentrations of *B. velezensis* LABIM40 CFS

LABIM40 CFS concentration ($\mu g\ mL^{-1}$)	Mycelial growth inhibition (%)				
	AL	BS	CL	GZ	RS
0	0.00	0.00	0.00	0.00	0.00
0.78	-	3.65	-	-	42.89
1.562	-	24.42	-	-	50.26
3.125	-	48.61	-	-	55.68
6.25	18.28	62.72	1.69	14.75	56.46
12.5	13.81	62.72	14.58	17.70	62.40
25	13.43	62.54	36.27	19.56	66.15
50	7.84	61.40	40.34	19.75	71.58
100	9.70	100.00	51.53	31.47	73.51
200	13.81	100.00	66.10	42.25	84.11
400	19.03	100.00	69.15	49.95	100.00
500	21.64	-	70.17	56.37	-
600	20.15	-	71.53	60.29	-
Model		Y = 14.4622ln(x) + 20.9559	Y = 15.070ln(x) - 19.577	Y = 10.2367ln(x) - 10.8141	Y = 7.4422ln(x) + 43.9101
R2		0.8642	0.8985	0.7879	0.8751
F value		<0.0001	<0.0001	<0.0001	<0.0001

F<0.0001, significant by analysis of variance.

Z. Chen et al. (2020) demonstrated the inhibitory effect of *B. velezensis* ZW-10's cell-free culture filtrate on *Magnaporthe oryzae,* achieving an inhibition rate of 62.9% at a concentration of 12.5 µL mL^{-1} of fermentation broth. Similarly, Calvo et al. (2019) reported comparable results using the purified CFS of *B. amyloliquefaciens* BUZ-14 against *Monilinia fructicola* and *Penicillium expansum.* They identified iturin A, fengicin, and surfactin as the primary metabolite families present in the lipopeptide fraction of the bacterial CFS, with peak concentrations of 407, 853, and 658 µg mL^{-1}, respectively. Given the close genetic similarity between *B. amyloliquefaciens* and *B. velezensis,* as well as the shared nature of the synthesized metabolites, it is reasonable to hypothesize that the use of purified metabolites from the LABIM40 strain, derived from its CFS, could significantly reduce the concentrations needed to inhibit phytopathogenic fungi.

In summary, these findings underscore the suppressive impact of LABIM40 CFS on the mycelial expansion of various phytopathogenic fungi. They also

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propose the potential of purified metabolites from this strain to achieve effective inhibition at reduced concentrations, thereby targeting phytopathogenic fungi in agricultural applications.

Germination test

The use of LABIM40-based biological products and Commercial Product 1 (CP_1) showed no substantial effect on the germination, stem length, and root length of tomato seedlings. However, an exception was observed in the treatment with 10xCFS, which displayed toxicity under the conditions tested. This led to significantly lower germination rates and a reduction in stem and root length (Table 2). Zohora et al. (2016) reported similar findings, noting no statistical difference in the germination of tomato seeds treated with Bacillus subtilis RB14 compared to those that were untreated.

Table 2
Germination rate and radicle and hypocotyl lengths of seedlings from seeds treated with water, LABIM40 cell suspension, LABIM40 cell-free supernatant (CFS), ten times concentrated LABIM40 CFS (10× CFS) and commercial *B. amyloliquefaciens*-based biological product (CP_1) at 7 days after sowing

Treatment	Germination (%)	Radicle (cm)	Hypocotyl (cm)
Water	74.4 a	4.75 a	2.28 a
LABIM40	71.2 a	4.72 a	2.18 a
CFS	71.1 a	4.58 a	2.05 a
10× CFS	23.2 b	0.92 b	0.96 b
CP_1	64.2 a	4.50 a	2.34 a
C.V. (%)	26.22	40.30	40.96

Values denoted by identical letters within the column are not statistically different according to Tukey's test ($p < 0.05$).

The minimal effect of LABIM40-based products and CP_1 on germination, stem length, and root length, except at elevated concentrations, suggests that these treatments may not significantly promote or inhibit growth in tomato seedlings under the conditions tested.

Additional research is required to investigate the fundamental mechanisms responsible for the observed toxicity at elevated concentrations. This will aid in optimizing the use of LABIM40-based products to enhance seed germination and seedling growth, without causing any detrimental effects.

Damping-off and plant growth in tomato seedlings

The factors of seed treatment and the presence or absence of *R. solani* in the substrate demonstrated no interaction in relation to seed germination. The germination percentages were 36.3% and 78.6% in

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the presence and absence of *R. solani,* respectively (data not shown).

The survival of tomato seedlings 21 DAS, originating from seeds treated with biological products or grown in treated substrate (CP_2), varied between treatments within each substrate condition (with or without *R. solani*) (Table 3). Although biocontrol products did not enhance germination in the presence of the fungus, the 10× CFS treatment resulted in a 57% higher survival rate of seedlings at 21 DAS than that of the control seedlings. This outcome demonstrates the efficacy of pathogen control through secondary metabolites from LABIM40. The inferior performance of CP_2 could be attributed to its application method via substrate, which differs from the other treatments that were directly applied to seeds. Comparable results were reported

by Suthin Raj et al. (2020), who conducted research on rice seeds treated with *B. velezensis* Bs-1 against sheath blight, caused by *R. solani,* under field conditions. Seed treatment led to an 83% reduction in disease incidence, with better control observed when seeds were treated and plants were sprayed with the bacteria 20, 40, and 60 days post-transplantation. *B. velezensis* ZW-10 has also been demonstrated to be a potential biological control agent against *Magnaporthe oryzae* (Z. Chen et al., 2020). In this experiment, rice seedlings were sprayed until runoff with the cell-free culture filtrate of *B. velezensis* ZW-10 containing Tween 20. The number of leaf lesions on plants treated with the fermented liquid of ZW-10 and its fermentation broth was reduced by 73.7% and 63.7%, respectively, compared with those treated with water.

Table 3
Survival at 21 days after sowing of tomato seedlings originated from seeds treated with different biological products in substrate inoculated in the absence or presence of *Rhizoctonia solani*

Treatment	Substrate			
	Without *R. solani*	**%**	**With *R. solani***	**%**
Water	78.31 aA	100	27.71 bB	100.0
LABIM40	83.88 aA	107.1	21.80 cB	78.67
CFS	84.98 aA	104.7	28.05 bB	101.2
10xCFS	72.30 bA	92.33	43.52 aB	157.0
CP_1	67.90 cA	86.71	30.71 bB	110.8
CP_2	80.32 aA	102.6	15.16d B	54.71
C.V. (%)	10.89			

Values denoted by identical lower-case letters within columns and identical upper-case letters within rows are not significantly different according to Tukey's test (p < 0.05).

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All treatments demonstrated a significant difference in seedling mass between plants grown with and without *R. solani* (Table 4). Plants grown on a substrate inoculated with *R. solani* exhibited shorter stem lengths than those grown without the fungus. Furthermore, seed treatment with biological products did not promote growth under either cultivation condition (Table 4). This outcome could potentially be attributed to the brief evaluation period for plant growth. Conversely, L. Chen et al. (2019) reported a 12.8% and 25.2% increase in shoot and root lengths, respectively, in peanut seedlings inoculated with *B. velezensis* LD02. They also noted a 16.7% and 18.4% increase in the dry mass of shoots and roots, respectively, compared with those of the control after 14 days of cultivation in pots. Similarly, Wang et al. (2020) found that *B. velezensis* FKM10 significantly enhanced the growth of *Malus hupehensis* Rehd. cultivated for 163 days, resulting in a 17.1% increase in the fresh weight of the aerial part compared with that of the control group.

Table 4
Mass of seedlings (g) and root and stem length (cm) of tomato seedlings originated from seed treated with different biological products in substrate inoculated in the presence or absence of *Rhizoctonia solani (R. s.)*

Treatment	Mass (g)		Root (cm)		Stem (cm)	
	Without *R. s.*	With *R. s.*	Without *R. s.*	With *R. s.*	Without *R. s.*	With *R. s.*
Water	0.44 n.s.	0.34 n.s.	5.2 n.s.	4.5 n.s.	8.7 n.s.	5.8 n.s.
LABIM40	0.48	0.27	5.8	5.1	8.6	5.7
CFS	0.42	0.25	5.5	5.0	8.0	5.0
10xCFS	0.34	0.23	5.7	5.2	8.2	5.3
CP_1	0.32	0.20	5.8	3.8	8.8	6.0
CP_2	0.47	0.25	6.2	5.1	9.0	6.0
Mean	5.70 a	4.78 b	5.70 a	4.78 b	8.55 a	5.63 b
C. V. (%)	42.6		34.7		31.3	

For each variable, means denoted by the same letter do not exhibit significant differences according to ANOVA ($p < 0.05$).

Conclusion

B. velezensis LABIM40 exhibits antagonistic behavior against *A. linariae, B. squamosa, C. lindemuthianum, G. zeae,* and *R. solani,* as evidenced by dual culture and CFS diffusion tests. *In vivo* experiments revealed that tomato seeds, when treated with 10× CFS and planted in a substrate inoculated with *R. solani,* led to a 57% decrease in disease incidence among tomato seedlings.

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Conflict of Interest Statement

This research, aimed to objectively assess the impact of a novel *B. velezensis* strain and its cell-free supernatant on fungal pathogens and tomato damping-off, was partially funded by a grant from Simbiose®, a biological solutions enterprise. We maintain no ongoing commercial or financial relationship with the company.

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