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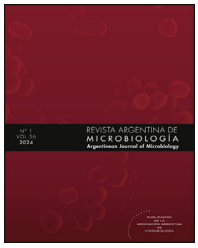

ORIGINAL ARTICLE

Inhibitory effect of the lipopeptide extract from *Bacillus amyloliquefaciens* B17 on the mycelial growth and spore germination of *Gilbertella persicaria*

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KEYWORDS
Antifungal activity;
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Lipopeptides

Abstract *Bacillus* species are of great interest due to their ability to produce lipopeptides with antifungal properties against various plant pathogenic fungi. The aim of this study was to evaluate the inhibitory effect of a lipopeptide extract from *Bacillus amyloliquefaciens* B17 on the mycelial growth and spore germination of the fungus *Gilbertella persicaria*, as well as to identify the compounds responsible for the antifungal activity. Scanning electron microscopy revealed that the lipopeptide extract caused abnormal bulges in the hyphae and malformations in the germ tubes. Additional analyses showed increased permeability in both the mycelium and spores, attributed to the action of the lipopeptides. Thin-layer chromatography (TLC) combined with mass spectrometry (ESI-MS) identified a total of eleven homologs corresponding to fengycin A, fengycin B, bacillomycin D, and surfactin in the extract. These findings suggest that the lipopeptide extract from *B. amyloliquefaciens* B17 represents a promising tool for the biological control of *G. persicaria*.

PALABRAS CLAVE
Actividad antifúngica;
Bacillus amyloliquefaciens;
Gilbertella persicaria;
Lipopéptidos

Efecto inhibitorio del extracto de lipopéptidos de *Bacillus amyloliquefaciens* B17 sobre el crecimiento micelial y la germinación de esporas de *Gilbertella persicaria*

Resumen Las especies de *Bacillus* son de gran interés debido a su capacidad para producir lipopéptidos con propiedades antifúngicas contra varios hongos fitopatógenos. Los objetivos de este estudio fueron evaluar el efecto inhibidor de un extracto de lipopéptidos de *Bacillus amyloliquefaciens* B17 sobre el crecimiento micelial y la germinación de esporas del hongo

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Gilbertella persicaria e identificar los compuestos responsables de la actividad antifúngica. Mediante microscopía electrónica de barrido se reveló que el extracto de lipopéptidos generó abultamientos anormales en las hifas, así como malformaciones en los tubos germinativos. Análisis complementarios indicaron un aumento de la permeabilidad del micelio y de las esporas de *G. persicaria*, atribuido a la acción de los lipopéptidos. La cromatografía en capa fina (TLC) combinada con espectrometría de masas (ESI-MS) identificó un total de 11 homólogos correspondientes a fengicina A, fengicina B, bacilomicina D y surfactina como los componentes del extracto. Estos hallazgos sugieren que el extracto de lipopéptidos de *B. amyloliquefaciens* B17 representa una herramienta prometedora para el control biológico de *G. persicaria*.

Introduction

Diseases caused by plant pathogenic fungi lead to significant losses in the agricultural sector, as they affect various economically important crops[15]. Several plant pathogenic fungi have been identified as contributors to the deterioration observed during the production, processing, and marketing of a wide range of crops. Among the diverse groups of plant pathogenic fungi, those belonging to the order Mucorales cause notable losses in cereals, fruits and vegetables. These fungi are predominantly considered saprophytic, as they inhabit the soil and feed on decomposing plant residues[25].

Gilbertella persicaria is a plant pathogenic fungus from the order Mucorales, recognized for its rapid growth and ability to produce coenocytic mycelium. This fungus is distinguished by the formation of globose sporangia, which, once mature, release hundreds of sporangiospores[6,25]. *G. persicaria* is primarily found in tropical and subtropical regions and is the causal agent of soft rot in various fruits, including tomato (*Lycopersicon esculentum* Mill.)[23,33], peach (*Prunus persica* (L.) Batsch)[23], pear (*Pyrus communis* L.)[23], apple (*Malus domestica* Borkh.)[23], pitahaya (*Hylocereus* spp.)[11], jambolan (*Syzygium cumini*)[38], papaya (*Carica papaya* L.)[16], eggplant (*Solanum melongena*)[47] and strawberry (*Fragaria ananassa* Duch.)[51].

Although chemical control is vital to mitigate the rot caused by *G. persicaria*, the overuse of chemical fungicides has resulted in fungicide resistance, ecosystem contamination, and adverse effects on human health[23,54]. Therefore, it is crucial to prioritize the development of sustainable and innovative strategies for controlling plant pathogenic fungi, ensuring minimal repercussions on ecological integrity and human well-being[39,55].

Biological control is among the alternatives to chemical control, which involves the use of microorganisms or their metabolites to manage diseases caused by various plant pathogens. Bacteria from the genus *Bacillus* play a significant role, utilizing diverse mechanisms of action such as substrate colonization and competition with pathogens, production of volatile organic compounds, synthesis of lytic enzymes, stimulation of plant defense response, and secretion of antimicrobial compounds[7,12,36,37].

Among the antimicrobial compounds produced by *Bacillus*, lipopeptides are of particular importance because these molecules are bioactive against plant pathogenic agents (bacteria, fungi, and oomycetes)[37]. Lipopeptides consist of a cyclic peptide linked to a fatty acid chain and can be mainly classified into three groups or families: surfactin, iturin, and fengycin[9,20]. Surfactin family members are heptapeptides characterized by their linkage to a fatty acid chain (β-hydroxy) of between 12 and 16 carbon atoms[9,14]. On the other hand, compounds of the fengycin family are decapeptides linked to a fatty acid (β-hydroxy) with a chain length between 14 and 18 carbon atoms. These compounds exhibit strong antifungal activity, especially against filamentous fungi, since they can interact with sterol and phospholipid molecules, thus altering the structure and permeability of fungal membranes[19]. Compounds of the iturin family are characterized as cyclized heptapeptides linked to a β-amino fatty acid containing between 14 and 17 carbon atoms. These compounds exhibit strong antifungal activity against a wide variety of plant pathogenic fungi due to their ability to form ion-conducting pores in membranes, generating osmotic disturbances that result in the disruption of fungal cells[1,35,50].

This study aimed to evaluate the inhibitory effect of a lipopeptide extract from *Bacillus amyloliquefaciens* B17 on the mycelial growth and spore germination of *G. persicaria*, as well as to identify the compounds responsible for the antifungal activity.

Materials and methods

Reagents and materials

The culture media used were potato dextrose agar (PDA, BD Bioxon™), nutrient agar (AN, BD Bioxon™), potato dextrose broth (PDB, Difco™), and Luria Bertani broth (LB, Sigma™). Phosphate buffer solution (PBS, pH 7, Baker™) and trypan blue dye (Golden Bell Reagents™) were used. Aluminum plates (10 cm × 10 cm) for thin-layer chromatography (TLC) coated with silica gel 60 F254 were purchased from Merck™. All solvents used (chloroform, methanol, and water) were

HPLC grade (Baker®). Fengycin (purity >90%), iturin A (purity >95%) and surfactin (purity >98%) standards were purchased from Sigma-Aldrich™.

Microorganisms and culture conditions

The microorganisms used in this study were provided by the Plant Pathology Laboratory at the Center for Food and Development Research (Culiacan Branch). *B. amyloliquefaciens* B17 (GenBank accession number: KX953161 for the 16S ribosomal region), isolated from the rhizosphere of tomato crops in Sinaloa, Mexico, was maintained on nutrient agar (NA) at 27 °C. *G. persicaria* HP15 (accession numbers: KR076758 and KR076761 for the ITS and 28S ribosomal regions, respectively), isolated from papaya fruit grown in Colima, Mexico, was cultured on potato dextrose agar (PDA) in Petri dishes at 27 °C.

Inoculum preparation

B. amyloliquefaciens B17 was cultured in LB medium by transferring a colony from an NA plate into a 250 mL flask containing 120 mL of LB broth. The culture was incubated for 24 h at 27 °C and 150 rpm until reaching 1.2×10^9 CFU/mL, based on the McFarland scale[41].

Procedure for lipopeptide extraction

The lipopeptide extract with antifungal activity was produced under fermentation conditions optimized for the *B. amyloliquefaciens* strain B17 by Rivera-Salas et al.[41]. The process involved adding 8 mL of inoculum to a 1 L flask containing 200 mL of Landy medium. The Landy medium (glucose, 20 g/L; L-glutamic acid, 5 g/L; yeast extract, 1 g/L; K_2HPO_4, 1 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; KCl, 0.5 g/L; $CuSO_4 \cdot 5H_2O$, 1.6 mg/L; $Fe_2(SO_4)_3 \cdot 7H_2O$, 0.4 mg/L; $MnSO_4 \cdot H_2O$, 1.2 mg/L) was adjusted to pH 7 prior to sterilization[56]. Fermentation was performed at 26.8 °C for 158.6 h with constant stirring at 230 rpm. Lipopeptides were extracted from the cell-free supernatant by acid precipitation (pH 2) for 12 h at 4 °C using 6N HCl, followed by alkaline solubilization (pH 8) with 0.5 M NaOH. The alkaline solution was filtered using Whatman syringe filters with a nylon membrane of 0.45 μm pore size to obtain the lipopeptide extract, which was lyophilized and stored at 4 °C for further analysis.

Antifungal activity of the lipopeptide extract against *G. persicaria*

Effect of the lipopeptide extract on mycelial growth

Different concentrations of the lyophilized extract (15, 30, 36, 45, 90, 120, and 150 μg/mL) were dissolved in methanol and added to a sterile PDA medium before being poured into Petri dishes. PDA medium without the extract was used as a control. Discs of 6 mm in diameter containing mycelium from the 7-day-old *G. persicaria* fungus were placed at the center of the plates containing PDA with different concentrations of the extract and on the control plates. After incubating the plates at 27 °C for 48 h, the radial mycelial growth of the fungus was measured, and the percentage of inhibition was calculated using the following formula[40]:

$$\%\,\text{Inhibition} = \frac{\text{mycelial diameter on control plates} - \text{mycelial diameter on extract-treated plates}}{\text{mycelial diameter on control plates}} \times 100$$

Effect of the lipopeptide extract on sporangia maturation

After evaluating the effect of the lipopeptide extract on *G. persicaria* mycelial growth, 1 cm diameter agar discs were cut from both the control plates and those where mycelial growth was inhibited, with no sporangia observed macroscopically. Their morphology was then examined using scanning electron microscopy.

Effect of the lipopeptide extract on spore germination

Different concentrations of the lyophilized extract (75, 150, 300, 450, 600, 750, 900, 1050, and 1200 μg/mL) were dissolved in methanol and incorporated into a sterile PDA medium prior to its distribution into Petri dishes. PDA medium without the extract served as the control. Once the medium had solidified, 100 μL aliquots of the *G. persicaria* spore suspension (1×10^7 sporangiospores/mL) were evenly spread onto the agar surface. The plates were incubated at 27 °C for 48 h. Subsequently, 1 cm agar discs were cut for morphological observation through scanning electron microscopy.

Scanning electron microscopy (SEM)

Agar discs intended for SEM were fixed with 2.5% (v/v) glutaraldehyde and progressively dehydrated using ethanol solutions of increasing concentration (10%, 30%, 50%, 70%, 96%, and 100%), following the methodology described by Torres et al.[46]. The dehydrated discs were observed using an EVO-50 scanning electron microscope (Zeiss, Germany) under high vacuum conditions, equipped with a secondary electron detector (SE1) and operated at an acceleration voltage of 10 kV.

Effect of the lipopeptide extract on membrane permeability and integrity

Extracellular conductivity

G. persicaria spores were inoculated into a PDB medium at 27 °C and 150 rpm for 48 h. The resulting mycelium was harvested via centrifugation. Subsequently, 1 g of the harvested mycelium was resuspended in 10 mL of distilled water containing various concentrations of the lipopeptide extract

(0, 36, and 1200 μg/mL). The conductivity of the suspension at 25 °C was measured at specific intervals (0, 1, 2, 4, and 6 h)[13,49].

Trypan blue staining

A 100 μL aliquot of the *G. persicaria* spore suspension (1×10^7 sporangiospores/mL) was added to tubes containing 900 μL of PDB with different concentrations of the lipopeptide extract (0, 300, 750, and 1200 μg/mL). The tubes were incubated at 27 °C for 24 h; followed by centrifugation at 10 000 rpm for 5 min at 25 °C. The resulting pellet was resuspended in 100 μL of PBS and stained with 100 μL of 0.4% (w/v) trypan blue. The stained mixture was incubated for 3 min at 25 °C, after which 10 μL aliquots were taken and placed in a Neubauer chamber to observe spore staining under an Imager A2 microscope (Zeiss, Germany)[18,43].

Identification of lipopeptide fractions

Thin-layer chromatography (TLC)

Samples of the lyophilized extract (15 mg) were dissolved in 1 mL of methanol and filtered using Whatman syringe filters with a nylon membrane of 0.22 μm pore size. Aliquots of 5 μL of the filtered methanolic extract were applied as 8 mm bands on TLC plates using a semi-automated application system (CAMAG™ TLC Linomat 5). Similarly, fengycin, iturin, and surfactin standards (30 μL), prepared at a concentration of 1 mg/mL in methanol, were spotted onto the same TLC plate. The samples and standards were eluted with a chloroform/methanol/water (65:25:4, v/v/v) mobile phase inside a dual-channel chamber. The chamber was saturated manually with the mobile phase for 10 min, and the migration distance was set to 60 mm. Subsequently, the TLC plate was dried using a stream of cold air for 2 min and visualized under ultraviolet light (254 and 366 nm) in a CAMAG™ UV Cabinet 4 system. The presence of lipopeptides was confirmed by comparing the retention factors (Rf values) of the lipopeptide fractions with those of the analytical standards.

$$Rf = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent}}$$

Mass spectrometry (MS)

Each of the lipopeptide fractions separated by TLC was excised from the aluminum plate. The fractions were recovered by scraping the sorbent and mixing it with 100 μL of methanol. The filtered methanolic extract (using Whatman syringe filters with a nylon membrane of 0.22 μm pore size) from each fraction was introduced via direct infusion into a mass spectrometer (Waters Xevo TQ-S), controlled by Mass Lyx 4.1 software. Positive-mode electrospray ionization (ESI+) was employed under the following conditions: a capillary voltage of 3.5 kV, a cone voltage of 28 V, a source temperature of 150 °C, a desolvation temperature of 400 °C, and a desolvation gas flow of 650 L/h.

Antifungal activity of the lipopeptide fractions

Aliquots of 10 μL of the filtered methanolic extract, corresponding to each lipopeptide fraction, were applied to five equidistant spots on the surface of the PDA medium in Petri dishes. The plates were previously inoculated with 100 μL of a *G. persicaria* spore suspension (1×10^7 sporangiospores/mL). Afterward, the plates were incubated at 27 °C for 20 h, and antifungal activity was evaluated by observing inhibition zones in fungal growth.

Data analysis

Each experiment was performed in triplicate. Data processing was conducted using Microsoft Excel 2019 and the results were expressed as the mean ± standard deviation (SD). For the effect of the lipopeptide extract on mycelial growth, data were analyzed using one-way analysis of variance (ANOVA). Prior to analysis, inhibition percentages were transformed to stabilize variances and improve normality. Fisher's least significant difference (LSD) test was subsequently applied to identify pairwise differences between treatments. One-way ANOVA was performed independently for each time interval to compare the effect of the lipopeptide extract on extracellular conductivity. The data met the assumptions of normality and homogeneity, and no transformation was required. To identify which treatments differed from control, Dunnett's test was applied. The statistical analysis was conducted using SPSS 17.0 software.

Results

Effect of the lipopeptide extract on mycelial growth

The lipopeptide extract of *B. amyloliquefaciens* B17 inhibited the mycelial growth of *G. persicaria* at all concentrations tested (Fig. 1A). The inhibition of *G. persicaria* mycelial growth increased progressively as the extract concentration rose. Inhibition ranged from 22.97% at 15 μg/mL to 98.09% at 150 μg/mL. Fisher's LSD test revealed significant differences in mycelial growth inhibition between most concentrations pairs ($p < 0.05$), except for comparisons between 90, 120, and 150 μg/mL, where no statistically significant differences were observed (Fig. 1B).

Effect of the lipopeptide extract on sporangia maturation

Among the plates treated with different concentrations of the lipopeptide extract, those equal to or greater than 36 μg/mL showed no visible sporangial development. Therefore, the concentration of 36 μg/mL was selected, as it not only prevented sporangial maturation but also achieved 50.6 ± 0.65% inhibition of mycelial growth. Although higher concentrations could have been selected, further increases in concentration led to greater inhibition of mycelial growth, which reduced the surface area available for cutting 1 cm mycelial discs required for scanning electron microscopy. At this concentration, the lipopeptide extract not only

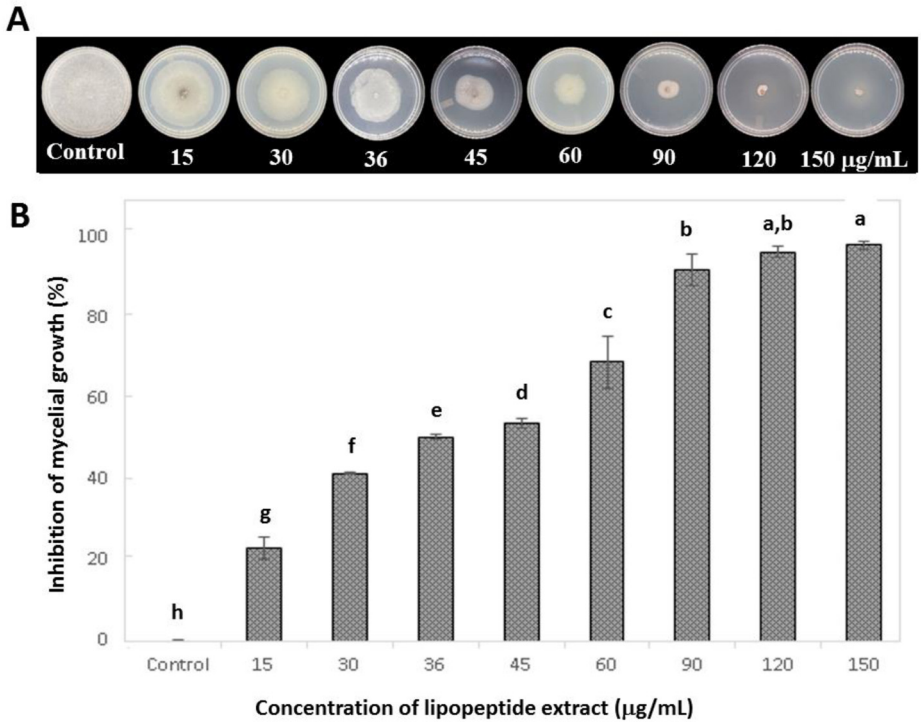

Figure 1 Antifungal activity of *B. amyloliquefaciens* B17 lipopeptide extract against *Gilbertella persicaria*. (A) Mycelial growth of *G. persicaria* on PDA medium after 48 h of incubation at 27 °C. (B) The histogram illustrates the inhibition of mycelial growth, where different letters above the bars indicate statistically significant differences between treatments according to Fisher's LSD test ($p < 0.05$).

inhibited mycelial growth but also negatively affected sporangial maturation. This was evidenced by the formation of hollow globose sporangia on the treated plates (Fig. 2C), suggesting a delay in their maturation. In contrast, sporangia of *G. persicaria* on the control plates reached full maturity after 48 h of incubation (Fig. 2A), characterized by the opening of the sporangial wall into two halves, allowing the release of sporangiospores (Fig. 2B).

Effect of the lipopeptide extract on spore germination

G. persicaria spores germinated at a low concentration of lipopeptide extract (75 µg/mL) in the culture medium; however, the resulting hyphae showed abnormal bulges (Fig. 3B). Increasing the extract concentration to 300 µg/mL led to a lower spore germination rate, and germinated spores exhibited multiple germ tubes (white arrows: Figs. 3C and D). At a concentration of 750 µg/mL, only a few spores germinated, and the germ tubes displayed deformities (Figs. 3E and F). In contrast, spores germinated on PDA medium without extract (control), showing normal germination, with germ tubes elongating into hyphae free of bulges or deformations (Fig. 3A).

Effect of the extract on membrane permeability and integrity

The electrical conductivity of samples treated with the lipopeptide extract (36 and 1200 µg/mL) was significantly higher ($p < 0.05$) than that of the control at all evaluated time intervals (see Supplementary Material 1). This increase in electrical conductivity indicated a rise in cell membrane permeability, suggesting that the lipopeptide extract adversely affects the cell membrane integrity of *G. persicaria*.

Trypan blue staining was used to verify the effect of the lipopeptide extract on membrane permeability and integrity. In this analysis, spores with intact membranes excluded the dye (control), whereas the spores treated with the lipopeptide extract acquired a blue-violet coloration, indicating membrane damage. This effect became more evident as the extract concentration increased (Fig. 4).

Identification of lipopeptide fractions

Thin-layer chromatography (TLC) revealed three distinct lipopeptide fractions characterized by retention factors (Rf) of 0.03, 0.34, and 0.89. By comparing these retention factors with those of established standards such as fengycin (Rf = 0.04), iturin A (Rf = 0.25), and surfactin (Rf = 0.9), fraction 1 was identified as belonging to the fengycin family, while fraction 3 was associated with the surfactin family. Nevertheless, this approach failed to definitively classify fraction 2 within any established lipopeptide family (Fig. 5A).

To overcome this limitation, TLC technique was combined with mass spectrometry (ESI-MS), enabling the identification of the molecular characteristics of the three fractions. In fraction 1, protonated molecular ions ($[M+H]^+$) with m/z values of 1449.14, 1463.16, 1477.14, 1491.13, and 1505.15

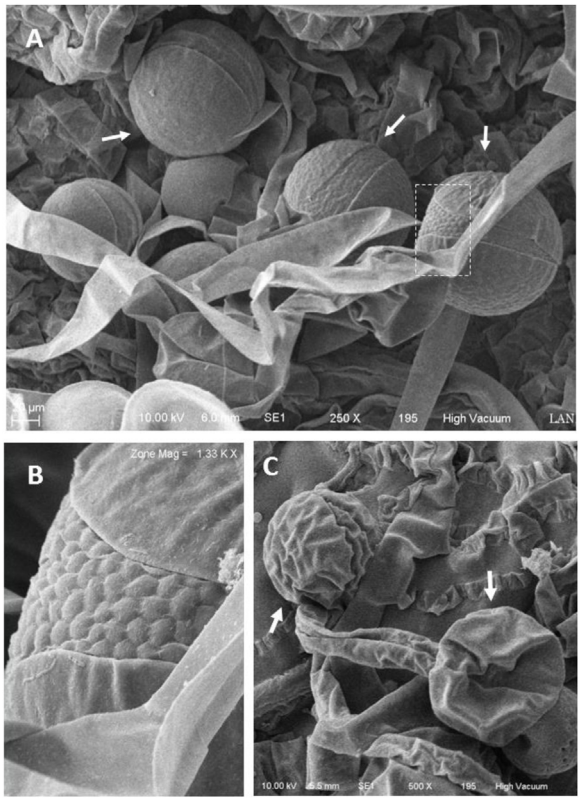

Figure 2 SEM micrographs showing the effect of lipopeptide extract on the maturation of sporangia of *Gilbertella persicaria* after 48 h at 27 °C. (A and B) Mature sporangia of *G. persicaria* grown on PDA medium without the addition of lipopeptide extract. (B) A mature sporangium of *G. persicaria* (control) showing the sporangiospores and the sporangial wall divided into two halves. (C) Immature sporangia of *G. persicaria* grown on PDA medium supplemented with 36 μg/mL of lipopeptide extract.

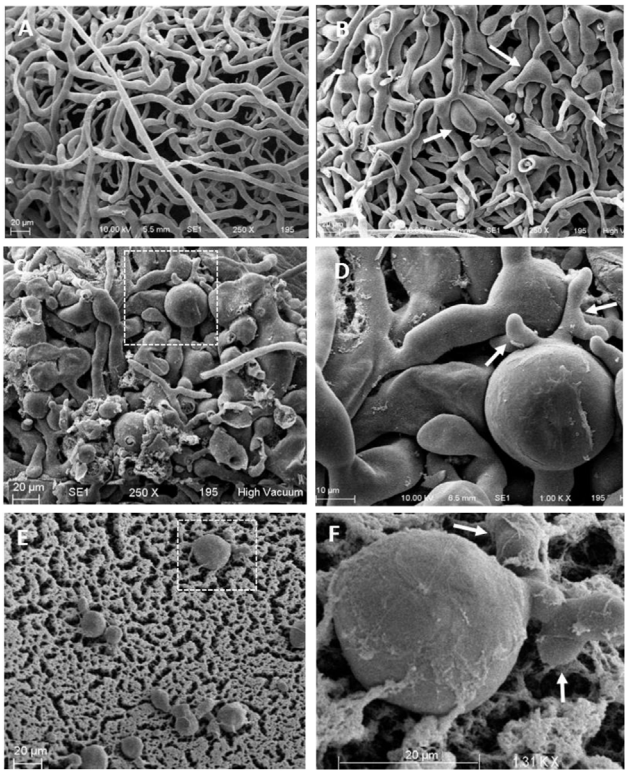

Figure 3 SEM micrographs showing the effect of lipopeptide extract on the germination of sporangiospores of *Gilbertella persicaria* after 48 h at 27 °C. (A) Sporangiospores of *G. persicaria* germinated on PDA medium without the addition of the lipopeptide extract. (B–F) Sporangiospores of *G. persicaria* germinated on PDA medium supplemented with different concentrations of the lipopeptide extract: 75 μg/mL (B), 300 μg/mL (C and D), and 750 μg/mL (E and F). Square = magnification zone. White arrows indicate abnormal swellings in the hyphae or malformations in the germ tubes.

corresponded to homologs of fengycin isoforms A and B. Conversely, in fraction 2, ionic species with *m*/*z* values of 1031.08, 1045.10, 1053.05, 1067.07, and 1081.10 were detected, indicating the presence of homologs of bacillomycin D. Finally, in fraction 3, peaks observed in the mass spectrum at *m*/*z* 1008.23, 1022.23, and 1036.23, differing only by 14 Da, were attributed to variations in the fatty acid chain length. This indicated the presence of surfactin homologs C13, C14, and C15. Additionally, the peaks with *m*/*z* values of 1044.20 and 1058.21 corresponded to sodium adducts, associated with surfactins C14 and C15 (Table 1).

Antifungal activity of the lipopeptide fractions

The antifungal activity of the three lipopeptide fractions was assessed by measuring inhibition zones formed against *G. persicaria*. The results indicated that only fractions 1 and 2 were effective, with average inhibition zone diameters of 11.3 ± 2.6 mm and 8.9 ± 1.7 mm, respectively. In contrast, fraction 3 did not demonstrate antifungal activity, as it failed to produce inhibition zones (Fig. 5B).

Discussion

Lipopeptides have received considerable attention due to their structural diversity and multifunctional properties. They exhibit broad-spectrum antimicrobial and antifungal activity, while maintaining low toxicity and reduced risk of resistance development[54].

In agricultural contexts, lipopeptides produced by strains of the genus *Bacillus* represents a promising and sustainable strategy for crop protection. These compounds reduce dependence on synthetic fungicides and help mitigate environmental, health, and economic impacts. Notably, lipopeptides from *Bacillus* spp. exhibit potent antifungal activity against plant pathogenic fungi, primarily through disruption of the fungal cell membrane, leading to structural damage and impaired development[19,44,52].

In this context, the effect of a lipopeptide extract from *B. amyloliquefaciens* B17 on *G. persicaria* was studied, observing inhibition in mycelial growth and spore germination, as well as malformations in germ tubes and abnormal swellings in the hyphae. Similar swellings have been observed in fungi such as *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, and *Mucor* sp., due to the antifungal

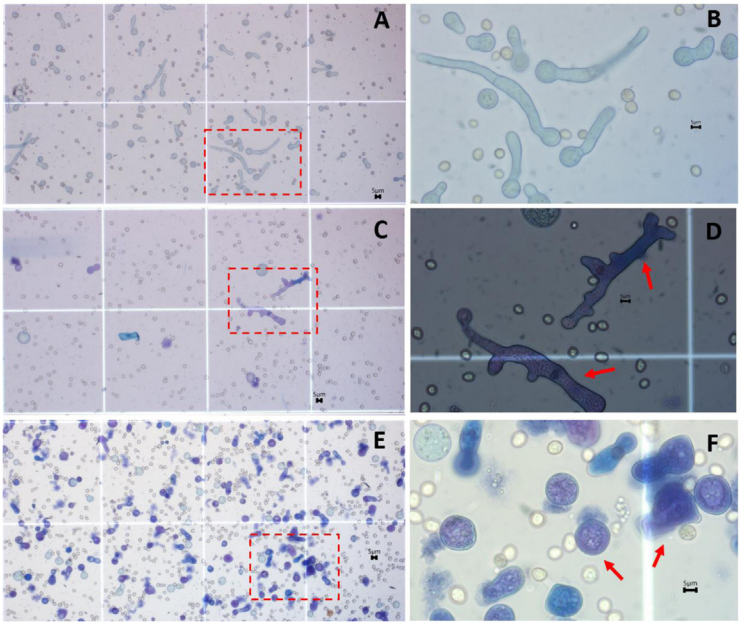

Figure 4 Staining of sporangiospores of *Gilbertella persicaria* with trypan blue. (A and B) Sporangiospores of *G. persicaria* germinated (22 °C, 24 h) in PDB medium without the lipopeptide extract. (C and D) Sporangiospores of *G. persicaria* exposed to 750 μg/mL of extract. (E and F) Sporangiospores of *G. persicaria* exposed to 1200 μg/mL of extract. Spores with damaged membranes do not exclude trypan blue and stain blue-violet (red arrow). Rectangle = magnification zone.

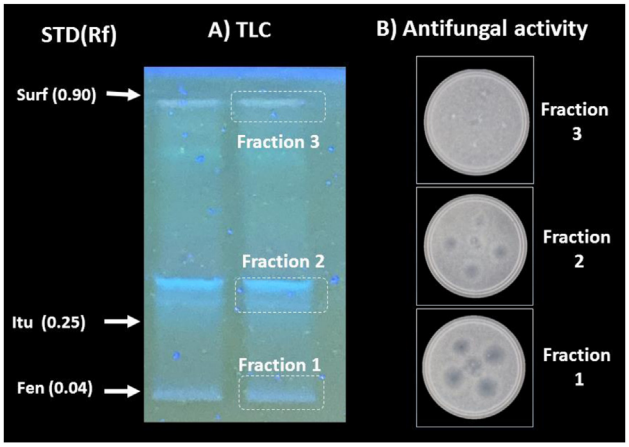

Figure 5 Separation and antifungal activity of lipopeptide fractions against *Gilbertella persicaria*. (A) Lipopeptide extract, fractionated by TLC (in duplicate). The retention factor (Rf) values of the standards fengycin (Fen), iturin A (Itu), and surfactin (Surf) are shown on the left. (B) Antifungal activity detected in fractions 1 and 2 against *G. persicaria*.

activity of lipopeptides, mainly fengycin and iturin[32,45,49,53]. These alterations could be explained by the fact that the hyphal tip is the site of the greatest addition of membrane and cell wall material in fungi. In this area of cellular expansion, there is a secretion of proteins and extracellular enzymes[21,24]. These extracellular enzymes are found within secretory vesicles, whose main function is to direct growth and cell morphogenesis[4]. The secretory vesicles are fused to the plasma membrane, therefore, any alteration to the membrane produced by the action of lipopeptides could result in abnormal fungal growth due to the abnormal release of these enzymes[5,8].

Several authors suggest that lipopeptides have a strong affinity for sterols found in fungal membranes. This affinity promotes the formation of ion-conducting pores, thereby altering the structure and permeability of fungal membranes. Consequently, measuring electrical conductivity becomes a useful technique to evaluate this permeability, as damage to the membrane leads to the release of intracellular electrolytes. This release results in increased conductivity in the surrounding medium[3,19,44].

Likewise, trypan blue staining was used as a confirmatory test of the effect of the lipopeptide extract on membrane integrity. This simple yet effective assay is based on the principle that cells with intact membranes remain impermeable to the dye, whereas those with membrane damage allow the passage of trypan blue and stain blue-violet. These changes in membrane permeability can also hinder spore germination[3,18].

On the other hand, lipopeptides are produced as mixtures of structural analogs, resulting in a wide range of biological activities within each species. This occurs because each lipopeptide family can consist of a mixture of isoforms and homologs. The classification into isoforms is based on the number of carbon atoms present in the peptide portion, whereas the subclassification of isoforms into homologs takes into account the number of carbons in the fatty acid chain. For instance, the surfactin family includes more than 30 variants, each with specific compositions in its peptide and lipid portions. Similarly, the iturin family comprises variants such as iturin A, C, mycosubtilin, bacillomycin D, F and L; and the fengycin family is subdivided into fengycin A, B and S[9,19,22,42].

Table 1 Identification of the lipopeptides present in the different fractions of the *Bacillus amyloliquefaciens* B17 extract via mass spectrometry (ESI-MS).

Fraction of extract	Monoisotopic mass	Lipopeptide molecule	Molecular formula	References
Fraction 1	1449.14	$[M+H]^+$ Fengycin A C15	$C_{71}H_{108}N_{12}O_{20}$	15,17,27,30,48
	1463.16	$[M+H]^+$ Fengycin A C16	$C_{72}H_{110}N_{12}O_{20}$	15,17,27,30,48
	1477.14	$[M+H]^+$ Fengycin A C17	$C_{73}H_{112}N_{12}O_{20}$	15,17,27,30,48
	1491.13	$[M+H]^+$ Fengycin B C16	$C_{74}H_{114}N_{12}O_{20}$	15,27,30,42,48
	1505.15	$[M+H]^+$ Fengycin B C17	$C_{75}H_{116}N_{12}O_{20}$	15,27,30,42,48
Fraction 2	1031.08	$[M+H]^+$ Bacillomycin D C14	$C_{48}H_{74}N_{10}O_{15}$	27,31,35
	1045.10	$[M+H]^+$ Bacillomycin D C15	$C_{49}H_{76}N_{10}O_{15}$	27,31,45
	1053.05	$[M+Na]^+$ Bacillomycin D C14	$C_{48}H_{74}N_{10}O_{15}$	2,34
	1067.07	$[M+Na]^+$ Bacillomycin D C15	$C_{49}H_{76}N_{10}O_{15}$	2,34
	1081.10	$[M+Na]^+$ Bacillomycin D C16	$C_{50}H_{78}N_{10}O_{15}$	2,34
Fraction 3	1008.23	$[M+H]^+$ Surfactin C13	$C_{51}H_{89}N_7O_{13}$	10,17,27,31,45
	1022.23	$[M+H]^+$ Surfactin C14	$C_{52}H_{91}N_7O_{13}$	17,31,45
	1036.23	$[M+H]^+$ Surfactin C15	$C_{53}H_{93}N_7O_{13}$	17,31,42,45
	1044.20	$[M+Na]^+$ Surfactin C14	$C_{52}H_{91}N_7O_{13}$	17,34,42
	1058.21	$[M+Na]^+$ Surfactin C15	$C_{53}H_{93}N_7O_{13}$	17,34,42

In the case of the *B. amyloliquefaciens* B17 extract, the analysis using TLC allowed the visualization of three fractions of lipopeptides. Of these, two were identified at the family level with the help of commercial standards: fengycin (fraction 1) and surfactin (fraction 3). Fraction 2 could not initially be identified with this method due to the limitations of available standards. However, with the combined use of TLC and mass spectrometry (ESI-MS), the lipopeptides present in all three fractions were identified[3,42].

Specifically, in the extract of *B. amyloliquefaciens* B17, a total of 11 homologs were identified: three of fengycin A (C15, C16, and C17), two of fengycin B (C16 and C17), three of bacillomycin D (C14, C15, and C16) and three of surfactin (C13, C14, and C15). These results slightly contrast with those of Ley-López et al.[29], who reported a total of 16 homologs (four of fengycin A, three of fengycin B, four of bacillomycin D and five of surfactin) in an extract of lipopeptides with oomyceticidal activity against zoospores of *Phytophthora capsici*, in which the same strain of *B. amyloliquefaciens* was used[28]. The variation in the number of homologs produced under different cultivation conditions could be related to the differences in fermentation times and temperatures, as well as to the presence or absence of specific inducers during the production of both extracts.

Since the structural classification of lipopeptides is strongly related to their biological activity, the evaluation of the antifungal activity of the three lipopeptide fractions was carried out separately, observing that at the evaluated dose, only fractions 1 (fengycin A and B) and 2 (bacillomycin D) generated inhibition halos in the growth of *G. persicaria*. These results coincide with those reported by various authors, who mentioned that compounds of the iturin and fengycin families are responsible for the antifungal activity and that, particularly fengycin, depending on the dose, is the compound that exhibits the greatest effect against filamentous fungi; while the compounds of the surfactin family are typically not reported as antifungal, but can have a synergistic effect when combined with other lipopeptides, mainly from the iturin family[1,3,19,26,35,50].

The results obtained in this study demonstrated the antifungal effects of the lipopeptide extract produced by *B. amyloliquefaciens* B17 on *G. persicaria* HP15. These findings provide a solid basis for future studies focused on evaluating the impact of this lipopeptide extract against other plant pathogenic fungi, with the aim of expanding the evidence on

its antifungal efficacy and its potential use in the control of various plant pathogenic fungi.

Conclusion

The lipopeptide extract from *B. amyloliquefaciens* B17 had adverse effects on mycelial growth, sporangia maturation, and spore germination of *G. persicaria* HP15. These inhibitory effects were concentration-dependent and mainly attributed to the combined action of compounds identified within the extract, such as fengycin A, fengycin B, bacillomycin D, and surfactin. To reinforce its potential as a promising biocontrol method against *G. persicaria*, future studies should assess its efficacy against additional *Gilbertella* strains. Such expanded evaluations would support its development as a sustainable alternative to conventional chemical fungicides.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.ram.2025.11.006.

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