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MALDI mass spectrometry-based identification of antifungal molecules from endophytic *Bacillus* strains with biocontrol potential of *Lasiodiplodia theobromae*, a grapevine trunk pathogen in Peru

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ABSTRACT

Lasiodiplodia theobromae, a grapevine trunk pathogen, is becoming a significant threat to vineyards worldwide. In Peru, it is responsible for Botryosphaeria dieback in many grapevine-growing areas and it has spread rapidly due to its high transmissibility; hence, control measures are urgent. It is known that some endophytic bacteria are strong inhibitors of phytopathogens because they produce a wide range of antimicrobial molecules. However, studies of antimicrobial features from endophytic bacteria are limited to traditional confrontation methods. In this study, a MALDI mass spectrometry-based approach was performed to identify and characterize the antifungal molecules from *Bacillus velezensis* M1 and *Bacillus amyloliquefaciens* M2 grapevine endophytic strains. Solid medium antagonism assays were performed confronting *B. velezensis* M1 - *L. theobromae* and *B. amyloliquefaciens* M2 - *L. theobromae* for antifungal lipopeptides identification. By a MALDI TOF MS it was possible identify mass spectra for fengycin, iturin and surfactin protoned isoforms. Masses spectrums for mycobacillin and mycosubtilin were also identified. Using MALDI Imaging MS we were able to visualize and relate lipopeptides mass spectra of fengycin (1463.9 *m/z*) and mycobacillin (1529.6 *m/z*) in the interaction zone during confrontations. The presence of lipopeptides-synthesis genes was confirmed by PCR. Liquid medium antagonism assays were performed for a proteomic analysis during the confrontation of *B. velezensis* M1 - *L. theobromae*. Different peptide sequences corresponding to many antifungal proteins and enzymes were identified by MALDI TOF MS/MS. Oxalate decarboxylase bacisubin and flagellin, reported as antifungal proteins, were identified at 99 % identity through peptide mapping. MALDI mass spectrometry-based identification of antifungal molecules would allow the early selection of endophytic bacteria with antifungal features. This omics tool could lead to measures for prevention of grapevine diseases and other economically important crops in Peru.

1. Introduction

Grapevine Trunk Diseases (GTDs) are currently considered the most destructive and significant threats to the global grape industry (Claverie et al., 2020). GTDs are caused by a taxonomically unrelated fungal complex categorized mainly in three groups: Esca disease, Eutypa dieback and Botryosphaeria dieback. Each group consist of a large list of pathogens that grow inside the wood, causing vascular system necrosis, decay and death of grapevine, which results in significant economic losses for major grape-producing countries, affecting production, processing and export (Fontaine et al., 2016; Armijo et al., 2016; Kenfaoui et al., 2022).

Peru has been affected by the increasing propagation of phytopathogens in many vineyards installed for more than a decade. Studies carried out in recent years have detected the presence of GTDs in Piura, northwestern Peru (Rodríguez-Gálvez et al., 2015; Javier-Alva et al., 2023). In particular, *Lasiodiplodia theobromae* (responsible for Botryosphaeria dieback), one of the most commonly reported fungi as trunk

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pathogen (Mehl et al., 2017), has highly spread throughout all the grapes-producing areas due to high incidence and pathogenicity, not only in grapevine but, also in other economically important crops in Piura (Rodríguez-Gálvez et al., 2017, 2021).

Management and complete eradication of GTDs is not possible, so control measures are mainly focused on prevention (Gramaje et al., 2018). Traditional agrochemical management are the measures applied in vineyards for the treatment of fungal diseases (Leroux, 2007). However, the excessive use of chemical products (35 % of all pesticides are used in viticulture) brings with it negative consequences on human health and environment (Compant et al., 2013). In this era, when the global organic food requirement is increasing, innovative strategies and efforts are necessary to produce chemicals-free grapes that do not have negative consequences for the health of consumers or the ecosystem (Lester, 2006; Mditshwa et al., 2017).

The evaluation and use of new bioactive ingredients and biological control agents have become the main priority for viticulture in the last decade (Blundell et al., 2021). Endophytic bacteria are promising candidates for biological control agents (Berg, 2009; Lugtenberg and Kamilova, 2009). A long list of culturable and non-culturable endophytic bacteria such as *Bacillus* and *Pseudomonas* have been described as antifungal agents because their exceptional properties in different host plants including grapevine (Compant et al., 2011; Niem et al., 2020; Cobos et al., 2022). Moreover, many endophytic *Bacillus* strains have strong antimicrobial properties and 4–5 % of its genome is devoted to antifungal compounds synthesis as proteins and cyclic lipopeptides (Cai et al., 2017; Jiao et al., 2021). However, many insights remain to be discovered regarding endophytes and plants synergy at the molecular level.

Some researchers have joined efforts to reveal the potential of endophytic bacteria and their bioactive compounds through dual culture studies. These methods have made it possible to evaluate the inhibition of different grapevine fungal pathogens (including *Lasiodiplodia*) and the results have been promising (Alfonzo et al., 2012; Piccolo et al., 2016; Rezgui et al., 2016; Chukeatirote et al., 2018; Bustamante et al., 2022). However, the traditional dual culture methods are only based on the simple observation of mycelial growth inhibition and spore germination (Kerr, 1999; Shehata et al., 2016). Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI TOF MS)-based proteomics has turned out to be a fast, cost-effective and accurate method for the identification and characterization of bioactive molecules from microorganisms endophytes (Toubal et al., 2017; López et al., 2018; LaMontagne et al., 2021; Munakata et al., 2022).

In this study, we focused on the potential of grapevine endophytic bacteria for the inhibition of *L. theobromae* by dual confronting in solid and liquid culture medium. Thus, through MALDI TOF MS, MALDI Imaging MS and MALDI TOF MS/MS-based proteomics approaches for the visualization and identification of antifungal molecules during the dual confrontations, our efforts aim to understand how the molecular characterization of two endophytic *Bacillus* strains could assist in the management grapevine diseases in Peru based on biocontrol.

2. Material and methods

2.1. Microorganisms isolation, identification and selection

2.1.1. Sampling

Field sampling between April and December 2018 was carried out in the vineyards of Chapairá, department of Piura (5°05′57.8"S 80°35′03.8"W), a main table grapes-producing areas with 1200 hectares for export. Seven-years-old grapevine (cv. Red Globe) plants with Botryosphaeria dieback-symptoms and asymptomatic (healthy plants), were pruned in their aerial parts to obtain branches samples of approximately 15 cm in length. Samples were aseptically placed in hermetic bags and immediately placed in a cooler box at 4°C and transported to the lab.

2.1.2. Isolation

Fungal isolation from Botryosphaeria dieback-symptomatic grapevines was done as described by Bruez et al. (2014). Morphological and microscopic characteristics were previously used for identification of *L. theobromae* (Phillips et al., 2013). Endophytic bacteria isolation from internal tissues of asymptomatic grapevines branches was based on the protocol described by Rashid et al. (2012). Both types of microorganisms were purified and stored in a refrigerator at -20°C for subsequent molecular analysis.

2.1.3. DNA extraction, PCR and identification

L. theobromae and endophytic bacteria were used for DNA extraction using CTAB protocol described by Karthikeyan et al. (2010) and Worden (2009) respectively. DNA concentration and purity were quantified using a spectrophotometer BioPhotometer UV/Vis (Eppendorf, Germany). PCR was carried out using a *Taq* DNA Polymerase Recombinant Kit (Invitrogen™) according to the manufacturer's instructions. ITS (ITS1/ITS4 primers) and 16S rRNA (27F/1492R primers) regions amplification were used for *L. theobromae* and endophytic bacteria identification, respectively. The amplification was carried out in a thermocycler Labcycler Gradient (SensoQuest, Germany), and the temperature conditions were as mentioned by Stefani et al. (2015). The PCR products were sequenced in both directions using Sanger technology (Macrogen, USA). For *L. theobromae* isolates confirmation and endophytic bacteria identification, the sequences were blasted against the non-redundant nucleotide database of the NCBI.

2.1.4. Selection of isolates

Preliminary selection of endophytic bacteria with antifungal features was carried out by simple antagonism tests (Johnson and Curl, 1972). Three bacilliform strains named M1, M2 and A1 were chosen from ten endophytic bacteria previously isolated (asymptomatic branches) because they showed greater inhibition of *L. theobromae* mycelial growth.

2.2. Antifungal molecules identification by MALDI TOF and MALDI Imaging MS

2.2.1. Solid medium antagonism assays

To evaluate the antifungal biomolecules effect from M1, M2 and A1 strains on *L. theobromae* mycelial growth, we confronted both microorganisms in solid culture medium following Moreira et al. (2014) protocol slightly modified. Five PDA plugs (ø 4 mm) containing 5-day-old *L. theobromae* mycelium were plated on a Petri dish (90 mm) containing PDA medium. Thereafter, sterile filter paper discs (ø 4 mm) were placed 1 cm in front of mycelium plugs and 10 µl bacterial suspension (1×10^5 CFU/ml) were placed on these paper discs (see Fig. 3). Treatments included: 1) M1, M2 and A1 strains against fungal pathogen and 2) control dishes with only bacteria. The dishes were incubated at 28 ± 2°C for 72 h. All assays were performed in triplicate.

2.2.2. Lipopeptides analysis by MALDI TOF MS

The dishes with *L. theobromae* and endophytic bacteria confronting were evaluated following Sajitha et al. (2016) method modified. The interaction zone was superficially washed with 200 µl extraction buffer [solution A (0.1 % TFA in water HPLC grade) and solution B (0.1 % TFA in acetonitrile)]. The washing suspension was vortexed and centrifuged at 2500 rcf for 5 min. The supernatant with lipopeptides was recovered and mixed with 10 mg/ml CHCA matrix solution in 1:1 (v/v). One microliter of each mixture was spotted on an opti-TOF 384 plate (AB SCIEX System). Lipopeptides analysis was carried out on a MALDI TOF/TOF MS 5800 (AB SCIEX System, USA) proteomic analyzer in positive reflector ion mode, 349 nm Nd:YAG laser intensity at a speed of 600 µm/s, 750 shots/spectrum and analyzed to 100 - 1800 (m/z) mass

range. Mass standards kit (AB SCIEX System) was used for calibration. The masses (*m/z*) were processed in SPECLUST software and identified with METLIN database.

2.2.3. MALDI Imaging MS analysis

MALDI Imaging MS was used to visualize the antifungal lipopeptides spatial distribution in fungal-bacterial solid medium antagonism following Yang et al. (2012) methods. Two square centimeters of the interaction zone (including bacterial control without confronting) were cut and placed on an Opti-TOF/LC MALDI Plate Inserts (AB SCIEX System) avoiding the formation of bubbles. With a sieve (53 μm) Universal MALDI matrix (Sigma-Aldrich, USA) was applied to saturate and cover the samples. The dehydration step was carried out in an oven at 37°C for 24 h. The plate was inserted into a MALDI TOF/TOF MS 5800 proteomic analyzer in positive reflector ion mode, 349 nm Nd:YAG laser intensity at a speed of 600 μm/s, 10 shots/spectrum and analyzed to 100 - 1800 (*m/z*) mass range. The analysis was performed using TissueView™ v. 1.1 software for building images.

2.2.4. PCR of lipopeptides biosynthesis genes

Specific primers for fengycin synthetase (*fenD*), surfactin synthetase subunit 1 (*srfAA*), bacyllomicin L synthetase B (*bmyB*), iturin A synthetase C (*ituC*) and bacylisin biosynthesis protein (*bacA*) were used for PCR confirmation of lipopeptides biosynthesis genes from M1, M2 and A1 strains. PCR was carried out using *Taq* DNA Polymerase Recombinant Kit (Invitrogen™). The amplification cycles and primers sequence were like those proposed by Mora et al. (2011). Amplification products were verified by 1.5 % agarose gel electrophoresis and sequenced in both directions using Sanger technology (Macrogen, USA).

2.3. Proteomics analysis

2.3.1. Liquid medium antagonism assays

Two PDA plugs (ø 4 mm) containing 5-day-old *L. theobromae* mycelium were placed in 100 ml flasks containing 80 ml of Malt Extract Broth (Fernandes et al., 2014). Thereafter, 10 μl of bacterial suspension (1×10^5 CFU/ml) was added to flasks. Treatments included: 1) Only M1 strain against fungal pathogen; 2) only M1 strain without fungal pathogen; and 3) a control of fungal pathogen. The flasks were placed in a Shaker-Incubator ES-20 (Biosan) at 100 RPM and 28 ± 2°C for 72 h. All assays were performed in triplicate.

2.3.2. Extracellular proteins extraction

The cultures from flasks were individually collected by filtration using several layers of sterile gauze, then centrifuged to maximum speed and the supernatants were recovered. The extraction of extracellular proteins from supernatants was performed using Pasquali et al. (2010) protocol with certain modifications. Briefly, 8 ml supernatant was treated with 16 ml precipitation buffer (20 % TCA and 0.1 % DTT in acetone) and incubated at -20°C for 12 h. The proteins were then centrifuged at 22,000 rcf for 60 min at 4°C and the pellet was twice washed with 1 ml of 0.1 % DTT in acetone. Protein pellet was resuspended in 250 μl of rehydration buffer (8.0 M Urea; 20 mM DTT and 4 % CHAPS).

2.3.3. SDS-PAGE and proteins digestion

The proteins were separated by SDS-PAGE on a 12 % gel (Laemmli, 1970). Molecular weight marker (Blue Wide Range, Cleaver Scientific) was used as a reference. Electrophoresis was carried out at 90 V - 120 A for 3 h. Then, the proteins were fixed and stained with Coomasie R-250 (Merck, USA) in acid-alcohol solution (Steinberg, 2009). The bands were collected, dehydrated and later digested with trypsin according to Shevchenko et al. (2006). The peptides were resuspended in 0.1 % TFA in water HPLC grade.

2.3.4. MALDI TOF MS/MS analysis

The peptide samples were mixed 1:1 (v/v) with 10 mg/ml CHCA matrix solution. One microliter of each mixture was spotted on an Opti-TOF 384 plate. Peptides analysis was carried out on a MALDI TOF/TOF MS 5800 proteomic analyzer as previously mentioned, with 800 - 3500 (*m/z*) mass range. Tandem mass spectrometry (MS/MS) data were acquired by Protein Pilot™ v. 4.0 software using Paragon algorithm for peptides sequence mapping.

3. Results

3.1. Microorganisms identification

By Nucleotide BLAST it was possible to identify *L. theobromae* (E2M isolate) with 100 % identity. Endophytic bacteria were identified as *Bacillus velezensis* strain M1, *Bacillus amyloliquefaciens* strain M2 and *Burkholderia cenocepacia* strain A1, each with 100 % identity (Table S1). In addition, endophytic M1, M2 and A1 strains showed higher inhibition (in decreasing order) of *L. theobromae* mycelial growth in the simple antagonism tests (Fig. 1).

3.2. Antifungal lipopeptides

3.2.1. Detection and visualization of antifungal lipopeptides

Different lipopeptides from *B. velezensis* M1 and *B. amyloliquefaciens* M2 that inhibited the mycelial growth of *L. theobromae* were identified by MALDI TOF MS. Mass spectra obtained from solid medium assays showed peaks (*m/z*) for fengycin and iturin isoforms. Protonated isoforms [m+H^+] detected of fengycin were 1449.8 (Fgy-C_{15}), 1463.8 (Fgy-C_{16}) and 1477.8 (Fgy-C_{17}) as well as its protonated isoforms [m+K^+] were 1487.7 (Fgy-C_{15}), 1501.7 (Fgy-C_{16}) and 1515.8 (Fgy-C_{16}) (Fig. 2a). It was also possible to obtain peaks (*m/z*) for protonated isoforms [m+H^+] of iturin 1043.5 (Itu-C_{14}) and 1057.5 (Itu-C_{15}) as well as their protonated isoforms [m+K^+] 1081.4 (Itu-C_{14}) and 1095.5 (Itu-C_{15}) (Fig. 2b). Protonated forms [m+H^+] peaks of mycobacillin 1529.8 and mycosubtilin 1071.5 were also detected (Fig. 2a and b). Finally, surfactin protonated isoforms [m+K^+] 1046.6 (SrfA-C_{13}), 1060.6 (SrfA-C_{14}) and 1074.7 (SrfA-C_{15}) were only found in *B. velezensis* M1 (Fig. S1).

Using MALDI Imaging MS was possible to correspond the masses detected by MALDI TOF MS. Image analysis allowed us to visualize *in vitro* spatial distribution of bacterial lipopeptides responsible for the inhibition of *L. theobromae*. The interaction between *B. velezensis* M1 - *L. theobromae* allowed us detect signals for mycobacillin 1529.6 *m/z* and fengycin 1463.9 *m/z*, while for control dishes (only *B. velezensis* M1) the signal of both lipopeptides were decreased. On the other hand, the interaction between *B. amyloliquefaciens* M2 - *L. theobromae* allowed detection of an intense signal for mycobacillin 1529.6 *m/z* while the signal was decreased for the control (Fig. 3). Lipopeptides signals observed for both M1 and M2 were not visible for A1 strain.

3.2.2. PCR confirmation of lipopeptides biosynthesis genes

PCR analysis was carried out to confirm lipopeptide biosynthesis genes in M1 and M2 strains. The fengycin (269 bp), surfactin (201 bp), bacyllomicin (370 bp), iturin (423 bp) and bacylisin (498 bp) genes amplified to the expected size for *B. velezensis* M1 and *B. amyloliquefaciens* M2 (except iturin) (Fig. 4). There was no amplification for *B. cenocepacia* A1 strain. Finally, the sequences were confirmed by homology with BLAST.

3.3. Antifungal proteins identification

3.3.1. Peptide sequence by MALDI TOF MS/MS

MALDI TOF MS/MS proteomic analysis of *B. velezensis* M1 - *L. theobromae* confrontation allowed obtaining peptide sequences from UniProt and Protein BLAST databases. Different antifungal proteins and non-ribosomal peptide biosynthesis enzymes were identified (Table 1),

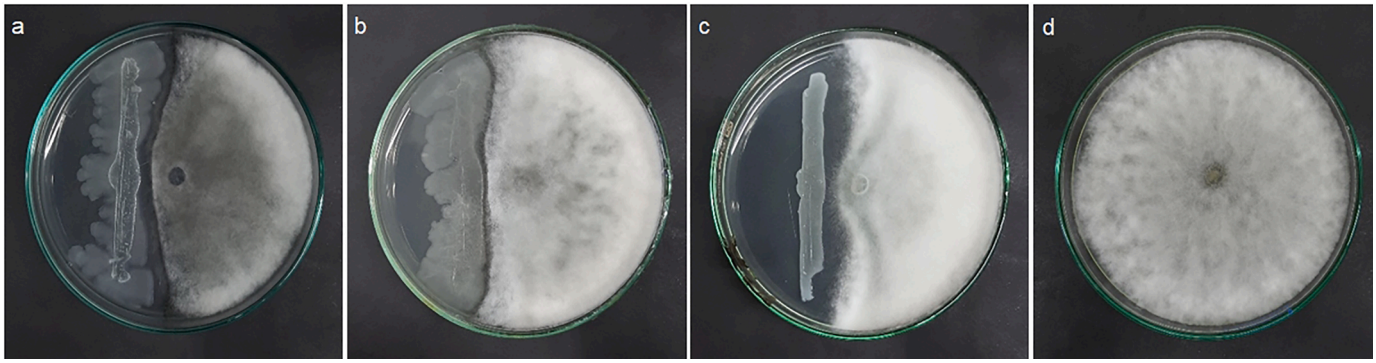

Fig. 1. Three days-old dual culture showing interactions between a) *B. velezensis* M1 - *L. theobromae*, b) *B. amyloliquefaciens* M2 - *L. theobromae*, c) *B. cenocepacia* - *L. theobromae*; d) *L. theobromae* control. Microorganisms were isolated from grapevine branches tissues with Botryosphaeria dieback-symptoms (*L. theobromae*) and asymptomatic (endophytic bacteria).

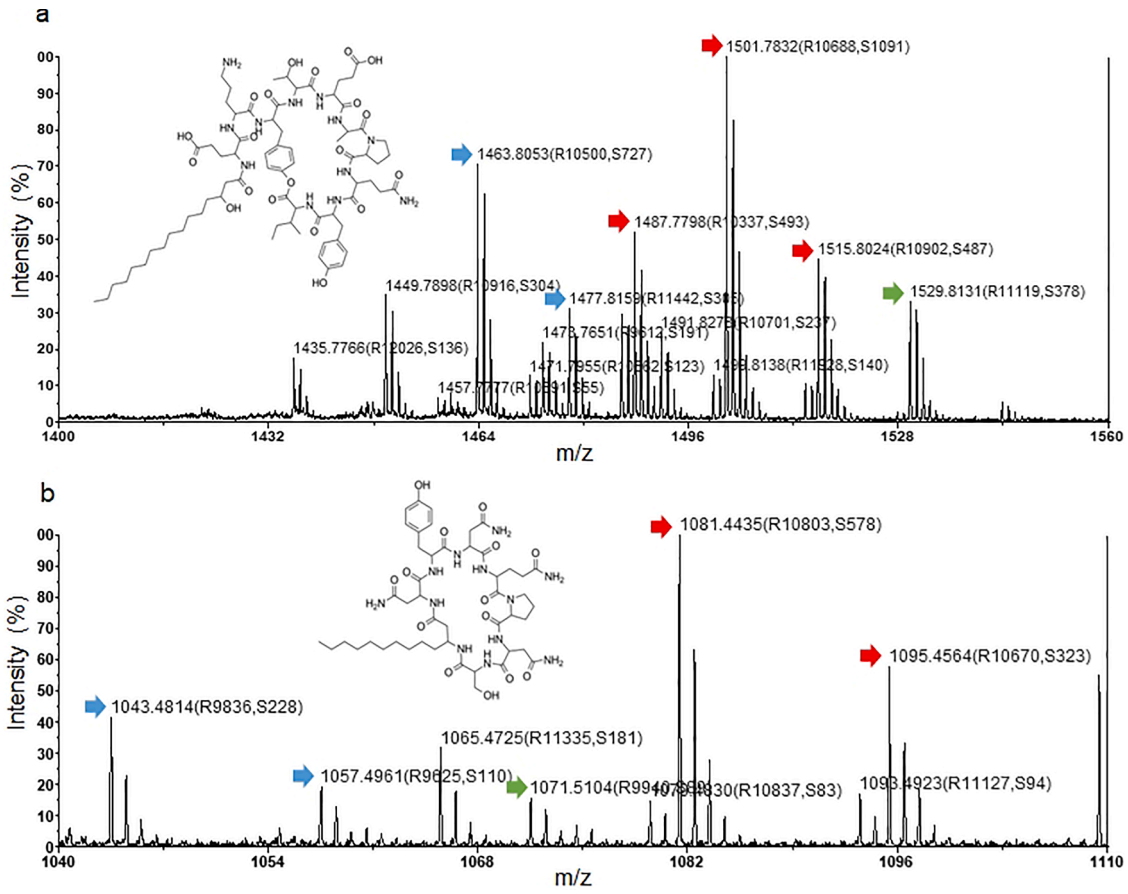

Fig. 2. MALDI TOF MS mass spectra of bacterial lipopeptides from dual culture. Masses ranges 1400–1560 (*m/z*) for fengycin and 1040–1110 (*m/z*) for iturin were proposed. a) Fengycin peaks with different intensity found in *B. velezensis* M1 - *L theobromae* and *B. amyloliquefaciens* M2 - *L. theobromae* confrontations. The fengycin isoforms protonated with H^+ adducts [m+H^+] were 1449.8 *m/z* Fgy-C_{15}, 1463.8 *m/z* Fgy-C_{16} and 1477.8 *m/z* Fgy-C_{17} (blue arrows), while 1487.7, 1501.8 and 1515.8 correspond to the same isoforms but protonated with K^+ adducts [m+K^+] (red arrows). b) Iturin peaks with different intensity found in *B. velezensis* M1 - *L. theobromae* confrontation. The iturin isoforms protonated with H^+ adducts [m+H^+] were 1043.5 *m/z* Itu-C_{14} and 1057.5 m/z Itu-C_{15} (blue arrows), while 1081.4 and 1095.5 correspond to the same isoforms but protonated with K^+ adducts [m+K^+] (red arrows). In addition, 1529.8 m/z and 1071.5 *m/z* peaks of mycobacillin and mycosubtilin respectively (green arrows) were found in M1 and M2 endophytic strain.

as well as another set of proteins and enzymes with different biological functions (Table S2).

Interestingly, four peptide sequences for an oxalate decarboxylase bacisubin protein and three sequences for a flagellin protein were identified. Fig. 5a shows mass spectra 2309.06, 1572.76 and 1513.79 *m/z* belonging to oxalate decarboxylase. The MS/MS fragments of the precursor ion 2309.06 *m/z* of a twenty amino acid peptide (LKDDIVEGPNGEVPYPFTYR), are shown in Fig. 5b. Similarly, mass spectra 1323.65, 2273.00 and 2695.24 *m/z* and their MS/MS fragments for flagellin protein were analyzed (Fig. S2).

4. Discussion

L. theobromae has been recognized as the predominant pathogen

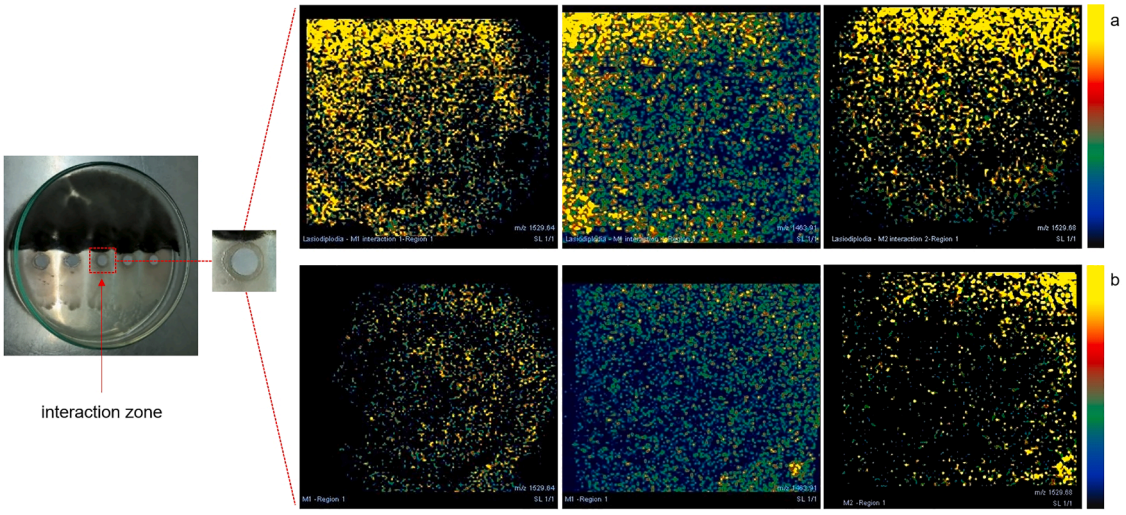

Fig. 3. MALDI Imaging MS from solid medium assays of *B. velezensis* M1 and *B. amyloliquefaciens* M2 against *L. theobromae*. The confrontations show the expression and spatial distribution of antifungal lipopeptides in interaction zones. Antifungal lipopeptide signals are interpreted on the color scale as relative intensity. a) Spatial distribution of mycobacillin 1529.6 m/z [higher signal of yellow dots] present in *B. velezensis* M1 - *L. theobromae* interaction (left); spatial distribution of fengycin 1463.9 m/z [higher signal of yellow dots] present in *B. velezensis* M1 - *L. theobromae* interaction (center); spatial distribution of mycobacillin 1529.6 m/z [higher signal of yellow dots] present in *B. amyloliquefaciens* M2 - *L. theobromae* interaction (right). b) Spatial distribution of same lipopeptides for M1 and M2 strains but without interaction with the pathogen (yellow signal decreased).

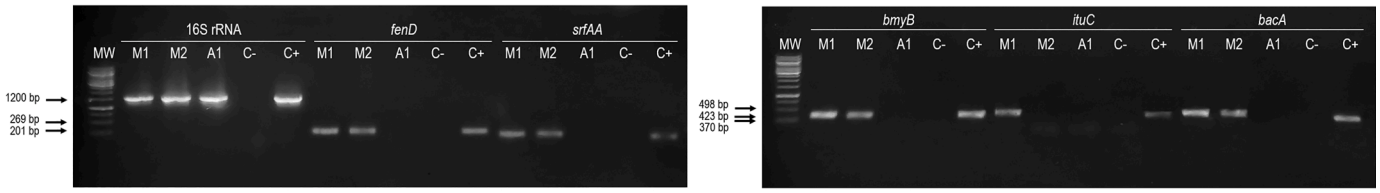

Fig. 4. Lipopeptides genes were confirmed by PCR. The *B. velezensis* M1 exhibited all lipopeptides *fenD, srfAA, bmyB, ituC* and *bacA* followed by *B. amyloliquefaciens* M2 (except ituC) and none for *B. cenocepacia* A1. MW (molecular weight marker), C+ (positive control) and C- (negative control).

associated with canker and dieback of grapevine worldwide (Úrbez--Torres, 2011). Therefore, many efforts have led to the search for microorganisms from different sources (soil, rhizosphere, compost, plants, etc.) to be used as biocontrol alternatives (Compant et al., 2013; Sajitha et al., 2014; Kamil et al., 2018; Chukeatirote et al., 2018; Bustamante et al., 2022).

Dual confrontation studies have permitted evaluation of the inhibition of *L. theobromae* mainly by different bacterial strains and their biomolecules (Alfonzo et al., 2009; Sajitha et al., 2014; Che et al., 2015; Piccolo et al., 2016; Seethapathy et al., 2016; Borges et al., 2018; Russi et al., 2020). However, MALDI MS-based proteomics has proved to be a higher resolution method for the selection of antagonistic microorganisms based on the identification and characterization of their bioactive molecules (Toubal et al., 2017; López et al., 2018; LaMontagne et al., 2021; Munakata et al., 2022).

A characterization study of *B. subtilis* B1 antifungal lipopeptides by MALDI TOF MS revealed the potential of this bacterium isolated from compost to inhibit *L. theobromae*, responsible for economic losses in the timber industry in India (Sajitha et al., 2016). However, our current investigation was mainly focused on the potential of the grapevine endophytic strains *B. velezensis* M1 and *B. amyloliquefaciens* M2 to inhibit *L. theobromae* through an in-depth characterization of their antifungal molecules.

Lipopeptides are a class of secondary metabolites synthesized by a multienzyme complex called non-ribosomal peptide synthetases. The metabolites can be classified into three families: fengycin, surfactin and iturins (Guo et al., 2014a). These possess antifungal character and have received increasing attention as key compounds in the biocontrol of plant diseases (Ongena and Jacques, 2008; Penha et al., 2020). Lipopeptides have been studied and characterized in most of the Bacillaceae species for the control of plant pathogens (Desmyttere et al., 2019). In this study, several *m/z* of lipopeptides were identified and visualized in dual confrontations by MALDI Imaging MS.

Fengycin is a cyclic lipopeptide synthesized by several *Bacillus* species as an immune response to fungal infections. It has been shown that fengycin has direct effects on cell membrane disruption, thus altering its permeability and triggering necrotic cell death (Tao et al., 2011; Sur et al., 2018). *B. velezensis* M1 and *B. amyloliquefaciens* M2 strains were able to express fengycin isoforms in solid medium confrontations. This was verified by its *m/z* (Fig. 2a). The dual cultures showed inhibition and degradation (darkening) of the mycelium at the interface between the two microorganisms presumably due to the presence of fengycin. Clearly, higher expression of this lipopeptide was detected by Imaging MS during the *B. velezensis* M1-*L. theobromae* interaction (Fig. 3a). These results are in agreement with those reported by Sajitha et al. (2016), who demonstrated the presence of *B. subtilis* B1 fengycin A and fengycin B homologs by MALDI TOF MS. Guo et al. (2014b) characterized the antifungal activity of *B. subtilis* NCD-2 against *Rhizoctonia solani* affecting cotton plants. Using MALDI TOF MS and biocontrol tests at the seedling level, they demonstrated that fengycin-type lipopeptides were the main active compounds restricting colonization by this fungus at the rhizosphere level. Interestingly, we were also able to identify a peptide sequence for fengycin synthetase C enzyme by MS/MS analysis (Table 1).

Surfactin is a lipopeptide synthesized by several *Bacillus* strains (Yeh et al., 2005; Chen et al., 2015). This molecule has four isomers (surfactin

Table 1
Peptides sequence corresponding to different antifungal proteins and enzymes from *B. velezensis* M1 - *L. theobromae* interaction by MALDI MS/MS analysis.

Interac[a]	Prec [m/z]	Peptide sequence	Recognized proteins	Identified by	Score[b]	Acc number[c]	Domains	Recognized species
M1-Lt	2309.06	LKDDIVEGPNGEVPYPFTYR	Oxalate decarboxylase bacisubin protein	P. Pilot	99 %	E5LLI0	Conserved	*B. subtilis*
M1-Lt	1572.76	ELPISENLASVNMR	Oxalate decarboxylase bacisubin protein	P. Pilot	99 %	E5LLI0	Conserved	*B. subtilis*
M1-Lt	1513.79	TIASALVVVEPGAMR	Oxalate decarboxylase bacisubin protein	P. Pilot	98.9 %	E5LLI0	Conserved	*B. subtilis*
M1-Lt	1016.51	VTIVDEQGR	Oxalate decarboxylase bacisubin protein	P. Pilot	93 %	E5LLI0	No conserved	*B. subtilis*
M1-Lt	1323.66	INHNIAALNTSR	Flagellin	P. Pilot	99 %	A0A1D9PP28	No conserved	*B. methylotrophicus*
M1-Lt	2273.00	LEHTINNLGTSSENLTSAESR	Flagellin	P. Pilot	99 %	A0A1D9PP28	Conserved	*B. methylotrophicus*
M1-Lt	2695.25	NAQDGISLIQTSEGALNETHSILQR	Flagellin	P. Pilot	99 %	A0A1D9PP28	Conserved	*B. methylotrophicus*
M1-Lt	2309.11	VRNTDDLKQTLSKDYLDQR	Bacteriocin biosynthesis protein SagD	P. BLAST	89 %	KJD55488	No conserved	*B. amyloliquefaciens*
M1-Lt	1568.68	SATGFKFENSVPER	Bacteriocin biosynthesis protein SagD	P. BLAST	86 %	KJD55488	No conserved	*B. amyloliquefaciens*
M1-Lt	1108.52	IGLQTLGHNR	Surfactin NRP synthetase SrfAA	P. BLAST	100 %	WP077411021	No conserved	*B. velezensis*
M1-Lt	2309.08	RLSDGTFEFIGRADDQVKIR	Surfactin NRP synthetase SrfAB	P. BLAST	100 %	WP103031544	Conserved	*B. subtilis*
M1-Lt	1298.57	AGNAALCAYVAPR	Surfactin NRP synthetase SrfAB	P. BLAST	100 %	QGT58224	No conserved	*B. velezensis*
M1-Lt	1804.70	AGATYVSIDPDYPESR	Surfactin NRP synthetase SrfAB	P. BLAST	75 %	WP159353763	Conserved	*B. velezensis*
M1-Lt	1423.64	GAGPEQIIAIMADR	Surfactin NRP synthetase SrfAB	P. BLAST	71 %	WP013350973	No conserved	*B. amyloliquefaciens*
M1-Lt	2309.08	EAVNGNPDAPALTYSGQTLSYR	Surfactin NRP synthetase SrfAC	P. BLAST	100 %	WP015382745	Conserved	*B. subtilis*
M1-Lt	1177.54	IAPPYVPHGAR	Bacylisin biosynthesis protein BacB	P. BLAST	91 %	SCV38295	No conserved	*B. subtilis*
M1-Lt	1423.65	AGGAYLPIGDDVPR	Fengycin synthetase C	P. BLAST	100 %	AFG19383	No conserved	*B. amyloliquefaciens*
M1-Lt	1108.46	QAWCYGAPGV	Lanthionine synthetase C	P. BLAST	89 %	WP059367795	Conserved	*B. velezensis*
M1-Lt	1323.66	MNRRDAMEER	Polyketide synthase PksN	P. BLAST	100 %	ARW06807	No conserved	*B. atrophaeus*
M1-Lt	1423.64	AGGNLAFEVAQAME	Plipastatin NRP synthetase PpsE	P. BLAST	100 %	WP086343902	No conserved	*B. subtilis*

[a] M1-Lt: *B. velezensis* M1 - *L. theobromae* interaction.
[b] Score of peptides sequence mached by Protein Pilot software or Protein BLAST.
[c] Accession number for databases UniProt or NCBI.

A, B, C, and D) and in recent years has been characterized as a metabolite with antibacterial, antifungal, and even antiviral capacity (Santos et al., 2018). In this study, we were able to identify *m/z* with K^+ adducts (Fig. S1) as well as peptide sequences of the *srf*A, B, and C surfactin synthetase enzymes belonging to *B. velezensis* M1 (Table 1). Surfactin is a potent biosurfactant that plays a crucial role in biofilm development (Bais et al., 2004), and this was observed in dual cultures of *B. velezensis* M1 and *B. amyloliquefaciens* M2 (Fig. 1). Sarwar et al. (2018) evaluated the biocontrol activity of surfactin A isolated from *Bacillus* strains against *Fusarium* spp. causing rice bakanae disease. The purified surfactin A molecules showed strong antifungal activity suppressing up to 84 % of *F. moniliforme*. Similarly, Nigris et al. (2018) evaluated the biocontrol traits of the grapevine endophyte *B. licheniformis* GL174 against *B. cinerea* by reducing mycelial growth *in vitro*. LC-MS/MS analysis showed that GL174 strain produced the cyclic lipopeptides surfactin and lichenysin. This is a proof that surfactin is associated with biofilm production and inhibition of mycelial growth. The inhibitory effect can be explained due to the participation of other lipopeptides of the iturin and fengycin family acting in synergy, which was corroborated by Jiang et al. (2016). Our strain *B. velezensis* M1 was able to simultaneously express different lipopeptide biosynthesis enzymes that inhibited the growth of *L. theobromae*. These enzymes were identified by MS and MS/MS analysis.

Iturin is a cyclic lipopeptide used as an active biological control ingredient that targets fungal plant pathogens (Ongena and Jacques, 2008; Dunlap et al., 2019). The mechanism of action of iturin has been reported to involve the formation of pores in the cell membrane, causing osmotic perturbation (Maget-Dana and Peypoux, 1994). Several mass spectra of iturin were detected in *B. velezensis* M1 and *B. amyloliquefaciens* M2. Iturin A [m+H^+] isoforms (1043.54 and 1057.56 *m/z*) were detected in this study (Fig. 2b). These have also been reported by Sajitha et al. (2016). Dang et al. (2019) increased the production of iturin A from *B. amyloliquefaciens* and evaluated its inhibitory activity against fungal pathogens, achieving 74 % inhibition against *Colletotrichum gloeosporioides*. Calvo et al. (2019) also detected iturin A produced by *B. amyloliquefaciens* BUZ-14 for the control of fungi causing postharvest diseases. Mass spectra for iturin were supported by PCR amplification in this study.

Mycosubtilin, another interesting member of the iturin family, was also detected in the assays (Fig. 2b). Mycosubtilin possesses antifungal activity and negative effects on mycotoxin production. Yu et al. (2021) demonstrated the efficacy of mycosubtilin from *B. subtilis* ATCC6633 on the suppression of mycelial growth and biosynthesis of deoxynivalenol and fumonisins B1, B2 and B3 of *F. graminearum* and *F. verticillioides*. On the other hand, mycobacillin is a cyclic tripeptide that disrupts fungal membranes and disintegrates fungal cell walls by agglutination (Nayak et al., 2017; Caulier et al., 2019). Its effect on isolates of *Aspergillus niger* (Majumdar and Bose, 1958) and *Candida* (Nayak et al., 2017) has been demonstrated. Mass spectra for mycobacillin were detected (Fig. 2a) with an *m/z* of 1529.8 [m+H^+]. Homologs of mycobacillin have also been reported by Sajitha et al. (2016). In this study, it was possible to detect mycobacilllin through MALDI Imaging in the interaction between

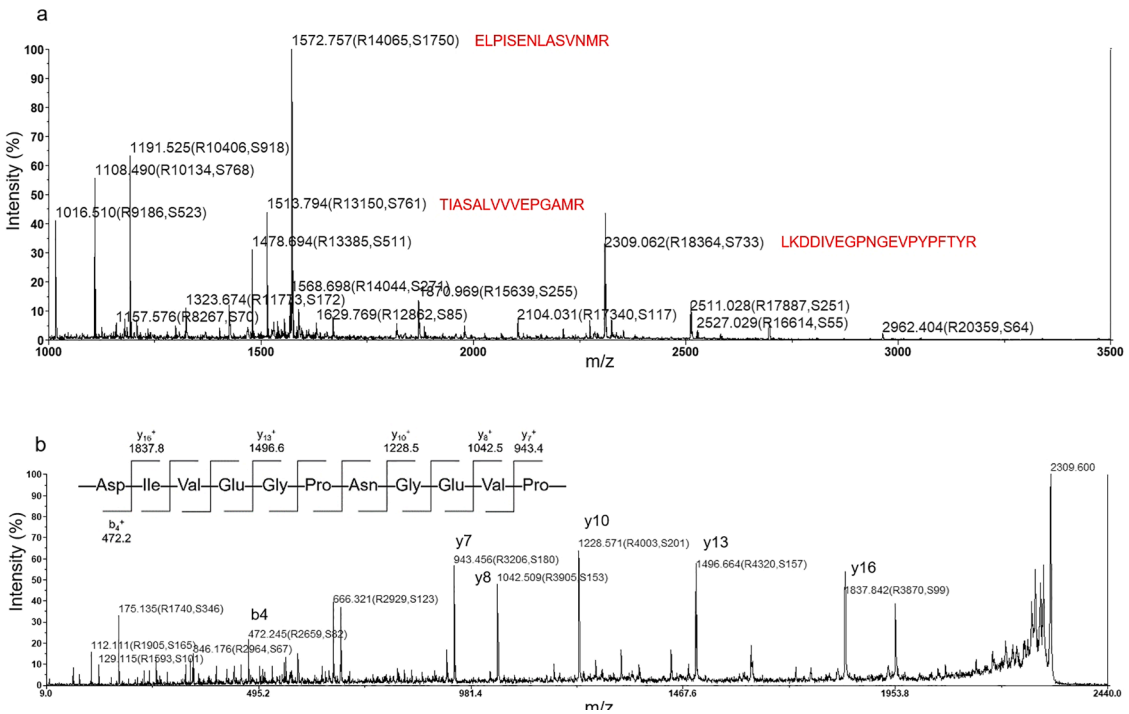

Fig. 5. MALDI TOF MS/MS mass spectra of different peptide sequences from oxalate decarboxylase bacisubin protein. a) the masses of different precursor ions 1513.79, 1572.75 and 2309.06 m/z that correspond to peptide sequences (in red) of bacisubin protein are shown. b) the spectrum exhibits y^{+} fragments for sequence 2309.06 m/z (LKDDIVEGPNGEVPYPFTYR).

B. velezensis M1 - *L. theobromae* (Fig. 3a).

Oxalate decarboxylase is an enzyme with *in vitro* induced production in acidified media (pH 5.0) in some bacteria such as *B. subtilis* (Tanner and Bornemann, 2000). Oxalate decarboxylase acts specifically on oxalic acid which is used as a pathogenicity factor by some phytopathogenic fungi in host infection (Williams et al., 2011; Heller and Witt--Geiges, 2013; Kabbage et al., 2013). Liu et al. (2007) isolated a protein from *B. subtilis* B-916 that exhibited mycelial growth inhibitory activity of six phytopathogens *in vitro*. They also identified a sequence of the protein as bacisubin. Qi et al. (2017) referred to bacisubin as an oxalate decarboxylase gene of *B. subtilis* BS-916 that acts as a modulator of fungal infection. Interestingly, in this study, four peptide sequences corresponding to an oxalate decarboxylase from *B. velezensis* M1 were identified by MALDI MS/MS when confronted against *L. theobromae* (Table 1). Analysis of three peptide sequences of strain *B. velezensis* M1 generated match at 99 % identity with strain *B. subtilis* B-916 (Table 1). This suggests that other endophytic *Bacillus* species are capable of producing antifungal proteins such as bacisubin.

Flagellin is a protein subunit that polymerizes to form the filaments of flagella in *Bacillus* and other eubacteria. Although most flagellins are assembled in the flagellum, flagellin can also be secreted and accumulate in the extracellular environment as a result of leakage and shedding (Komoriya et al., 1999). Flagellins possess antifungal activity by inhibiting mycelial growth. Some studies have reported that flagellins from *Bacillus* strains are able to decrease mycelial growth of pathogens even more than tested chemical fungicides. In these studies, flagellins could be identified by peptide sequence analysis and estimation of their molecular weight (Ren et al., 2013; Zhao et al., 2013; Jiang et al., 2019).

Our study was able to identify flagellin peptide sequences to 99 % identity (Table 1). Our results are consistent with previous reports suggesting that flagellin has antifungal activity. We believe that all the molecules of *B. velezensis* M1 endophyte identified by MALDI acted, somehow, synergistically against *L. theobromae*. It is important to understand how the mechanisms of activation of these antifungal molecules, their succession and their mechanisms of action on phytopathogens are carried out, in order to comprehend the molecular warfare between these microorganisms. On the other hand, these results are encouraging in the search for potential biocontrollers for viticulture in Peru.

Finally, the endophytic strain *B. cenocepacia* A1, isolated in our study, showed very good antifungal characteristics in dual confrontation assays. However, no information could be retrieved from molecular and proteomic analyses because many of the molecule's databases consulted are specific for Bacillaceae. We believe that *B. cenocepacia* A1 could be a good candidate for future research when standardized methodology is available for the purpose of evaluating bacteria from other groups.

5. Conclusion

Grapevine endophytic *B. velezensis* M1 and *B. amyloliquefaciens* M2 strains showed strong antagonism against *L. theobromae* in dual culture assays. MALDI TOF MS-based proteomics and MALDI Imaging MS made it possible to elucidate antifungal lipopeptides such as fengycin, iturin, mycobacillin and others from its molecular masses. By MALDI TOF MS/MS was possible to demonstrate the presence of antifungal proteins from mapping of protein sequences. Oxalate decarboxylase (bacisubin), flagellin and several enzymes of lipopeptides biosynthesis were identified. We believe that these omics technologies would be supportive in evaluating *in vitro* potential biological control agents, specifically on endophytes to ensure that results in field can be promising and above all applicable safely for the prevention of grapevine fungal pathogens and other crops of commercial importance in Peru.

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CRediT authorship contribution statement

Manuel Saucedo-Bazalar: Conceptualization, Methodology, Investigation, Data curation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Pedro Masias:** Methodology, Investigation, Formal analysis. **Estefanía Nouchi-Moromizato:** Methodology, Validation. **César Santos:** Methodology, Validation. **Eric Mialhe:** Conceptualization, Investigation, Formal analysis, Visualization, Resources, Supervision, Writing – review & editing. **Virna Cedeño:** Investigation, Formal analysis, Visualization, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100201.

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