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Identification of new antifungal metabolites produced by the yeast *Metschnikowia pulcherrima* involved in the biocontrol of postharvest plant pathogenic fungi

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ABSTRACT

Several strains of the yeast *Metschnikowia pulcherrima* exhibit strong antagonistic activity against postharvest pathogens and may have broad biotechnological potential as biocontrol agents. However, the nature and interplay of the mechanisms contributing to this antifungal activity are still largely unknown. This study characterizes the antifungal compounds present in the exometabolome of two yeast strains that previously showed an efficient inhibition of *Botrytis cinerea* infection. We show that a yeast-fungus co-culture assay is a good system to examine the antagonistic interaction and elucidate the nature of the produced yeast metabolites. As a result, our UPLC-MS/MS analysis identified a total of 35 differentially secreted metabolites, potentially involved in the biocontrol of gray mold. Subsequent *in vitro* analysis and *in vivo* tomato, grape and apple fruit protection assays with such metabolites allowed us to identify several new antifungal compounds, with 3-amino-5-methylhexanoic acid, biphenyl-2,3-diol and sinapaldehyde being the most active (with up to 90–100% reduction in the infection of tomato and apple with *B. cinerea*). In addition, the first two metabolites protected tomatoes against *Alternaria alternata* infection. It was observed that these metabolites negatively affected the cell membrane integrity and mycelial morphology of *B. cinerea* and increased the intracellular level of ROS. Furthermore, other unexpected metabolites with interesting biotechnological applications were identified for the first time as being secreted by yeast cells, such as piperideine and protoemetine (alkaloids), p-coumaroyl quinic acid (phenylpropanoid), β-rhodomycin (antibiotic), hexadecanedioic acid (long chain fatty acid) or taurocholic acid (bile acid). This fact highlights that the antifungal activity of *M. pulcherrima* may result from synergistic action of several active molecules.

1. Introduction

Microbial antagonists are effective biocontrol agents (BCAs) of postharvest diseases and nowadays they are considered a promising alternative to treatments with conventional synthetic chemicals (Dukare et al., 2019; Freimoser et al., 2019). The *Metschnikowia* yeast genus contains species with broad biotechnological potential for application in various industrial processes, although their most studied property is their strong antimicrobial activity (Sipiczki, 2020). In particular, the species *M. pulcherrima* is widely distributed and frequently isolated from fruits and flowers (Sláviková et al., 2007; Vadkertiová et al., 2012). It has been reported that several strains of this species exhibit strong *in vivo* antagonistic activity against fungal postharvest pathogens such as *Alternaria*, *Botrytis* or *Penicillium* spp. among others (Fernandez-San Millan et al., 2021; Gore-Lloyd et al., 2019; Janisiewicz et al., 2001; Oztekin and Karbancioglu-Guler, 2021; Piano et al., 1997; Ruiz-Moyano et al., 2016; Saravanakumar et al., 2008; Sipiczki, 2020; Spadaro et al., 2002; Türkel et al., 2014). Moreover, Hilber-Bodmer et al. (2017) identified *M. pulcherrima* as the overall strongest antagonist among 40 yeasts against a diverse set of 16 filamentous fungi. In fact, several *Metschnikowia*-based products have been commercialized as BCAs against pre and postharvest diseases such as *Botrytis* or *Monilinia* spp. (Noli, Koppert Biological Systems), and as bioprotectans from possible microbial alterations in the winemaking industry (Sipiczki, 2020).

In our previous studies with a collection of 69 yeast strains isolated from Spanish vineyards (Fernandez-San Millan et al., 2021,

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2020), *M. pulcherrima* Mp-22 and Mp-30 strains were identified as excellent antagonists against economically important fungal diseases, such as *Botrytis cinerea* on postharvest tomato and grape fruits or *Fusarium oxysporum* in tomato plants. These strains had several capabilities related to fungal biocontrol activity such as production of cell wall-degrading enzymes, solubilization of nutrients, plant growth promotion, biofilm formation or secretion of iron chelating agents. However, we showed that each one of these capabilities seems to be an important but non-essential aspect of its biocontrol activity. Recent studies also indicate that the biocontrol of yeasts involves multiple modes of action (Liu et al., 2019), including the release of volatile compounds and the production of killer factors (Dukare et al., 2019; Freimoser et al., 2019; Muccilli and Restuccia, 2015; Mukherjee et al., 2020). In this context, although iron immobilization by pulcherrimin seems to be a robust mechanism in the antimicrobial antagonism of *M. pulcherrima*, there is a considerable debate among researchers about the involvement of non-iron-related mechanisms as major antagonistic factors. Hence, the nature and interaction of the compounds and mechanisms contributing to *M. pulcherrima* antifungal activity are still largely unknown and need to be studied and elucidated in much more detail (Gore-Lloyd et al., 2019).

Microorganisms coexist in most environments and interact by responding to each other, eliciting the production of signaling and defense molecules (Hardoim et al., 2015; Köhl et al., 2019). Accordingly, the effect of a specific yeast strain on its surrounding environment and the interaction with other microorganisms will be unique and characterized by the metabolites it secretes (exometabolome). So far, little is known about the production of such antagonistic metabolites by yeasts. Elucidating the nature of the produced active compounds and their mechanisms of action against pathogens will help understanding the role of BCAs in disease control (Jangir et al., 2021). In fact, even though BCAs have many advantages, their use is still minimal due to their limited shelf life, narrow activity spectrum, inconsistency, quality issues, stringent registration procedures and environmental restrictions (Babalola, 2010; Koskey et al., 2021; Raymaekers et al., 2020). Hence, the success of new antifungal strategies will require in the future cutting-edge technologies to identify novel biocontrol compounds. In this context, untargeted metabolomic techniques may help to find new bioactive molecules and ascertain the importance of gene expression and stimuli in the synthesis of already discovered metabolites (Alawiye and Babalola, 2021). In addition, the use of antimicrobial metabolites in a commercial postharvest protection product instead of the living microorganism is easier to register and apply.

With its great resolution and excellent mass accuracy, ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) is the perfect tool to analyze the exometabolome generated during yeast-fungus interactions (also named the interactome). Recently, metabolomic analyses have been applied to study the mechanisms by which different agents exert their antimicrobial effects against bacteria and fungi (Arora et al., 2020; Bo et al., 2014; Cao et al., 2016, 2013; Mousavi et al., 2016; Shahid et al., 2021). However, to date, no studies have employed a metabolomic approach to examine the antagonistic interaction of a yeast species on phytopathogenic fungi in a co-culture system. In fact, co-culture of different microorganisms is a valuable approach for activating biosynthetic pathways to increase chemical diversity and triggering the production of new antibiotics or antifungal compounds (Bertrand et al., 2013a,b; Meilin et al., 2019; Netzker et al., 2018; Oppong-Danquah et al., 2018, 2020; Vallet et al., 2017; Yu et al., 2016). For example, a recent co-culture assay between the fungus *B. cinerea* and several fungi led to the discovery of novel antifungal substances (Serrano et al., 2017).

The main objective of this metabolomic study is the characterization of bioactive yeast metabolites against a fungal phytopathogen. A comprehensive metabolomics survey has been undertaken to identify changes in the exometabolome to shed light on the mechanisms and metabolites responsible for the antifungal strategies employed by the *M. pulcherrima* Mp-22 and Mp-30 strains against *B. cinerea*. As a result, the co-culture approach used in conjunction with subsequent *in vitro* analysis and *in vivo* fruit protection assays, allowed us to identify several new antifungal compounds and other unexpected metabolites with interesting agronomic and biotechnological applications.

2. Materials and methods

2.1. Yeast strains and culture conditions

Native *Metschnikowia pulcherrima* Mp-22 and Mp-30 strains (Fernandez-San Millan et al., 2021) isolated from *Vitis vinifera* (cv. Grenache) by the Lev2050 company (https://lev2050.com/) were stored at − 80 ºC in Cryoinstant vials (VWR, Barcelona, Spain). Identification was performed as described (Oztekin and Karbancioglu-Guler, 2021) by PCR amplification and sequencing of the D1/D2 domain of the 26 S rDNA using the primers NL1 (5′ – GCATATCAATAAGCGGAGGAAAAG – 3′) and NL4 (5′ – GGTCCGTGTTTCAAGACGG – 3′). For solid culture, yeasts were plated onto YMA medium (Condalab, Spain). Two different media were used for liquid culture: (i) YNBS (Yeast Nitrogen Base, Condalab) with additional (g L^{-1}) glucose 20.0 and ammonium sulfate 5.0 at pH 6.8; (ii) YPD broth (Condalab). Flasks or tubes were incubated on a rotary shaker at 150 rpm and 28 ºC for 2 d. The cell concentration to be used in subsequent assays was determined by culture of serial dilutions on YMA plates or with an automated TC20 cell counter (Bio-Rad, Hercules, USA).

2.2. Fungal strains and culture conditions

Alternaria alternata (strain 20560), *Botrytis cinerea* (strain 20754) and *Penicillium expansum* (strain 20906) were obtained from the CECT (Paterna, Spain). For liquid culture, YNBS or PDB (Condalab) were used and for solid culture, all fungi were grown on PDA medium (Condalab).

The conidial suspensions were prepared and conserved as previously described (Fernandez-San Millan et al., 2021; Janisiewicz, 1992). The conidia concentration was determined by culture of serial dilutions and with a hemocytometer.

2.3. Measurement of the antifungal activity of culture filtrates from M. pulcherrima against B. cinerea

The effect of the yeast culture filtrates (CFs) on the mycelial growth of *B. cinerea* was assessed according to a previous method (Spadaro et al., 2002) with some modifications. Briefly, 100 µL (10^8 cells mL^{-1}) of the antagonistic yeast strains were transferred to 50 mL-flasks containing 10 mL of YNBS media. The flasks were placed on a rotary shaker (150 rpm at 28 ºC) for 12 h. Filtrates of *M. pulcherrima* Mp-22 and Mp-30 (10^6 cells mL^{-1}) cultures were obtained by filtration through a 0.22 µm cellulose membrane filter and diluted with YNBS medium at 1:2 and 2:1 (filtrate:YNBS) ratios. Non-diluted CFs or YNBS medium alone were used as controls. The final concentration of all medium components was adjusted to be equal in all treatments.

The antifungal activity of the culture filtrates was tested in glass tubes containing 3 mL of the different media. Thirty µL of a *B. cinerea* suspension (10^3 conidia mL^{-1}) were inoculated in each tube and tubes were incubated on a rotary shaker at 150 rpm and 28 ºC. Mycelium diameter was measured in triplicate after three days.

2.4. Metabolomic analysis of extracellular components on the liquid medium

2.4.1. Metabolite secretion in the co-culture of B. cinerea and yeast for metabolomic analysis

One hundred µL of a *B. cinerea* suspension (10^3 conidia mL^{-1}) were inoculated into 50-mL flasks containing 10 mL of YNBS medium and incubated on a rotary shaker at 150 rpm and 28 ºC for 48 h (the fungal

mycelium had a diameter of approximately 0.5 cm). Then, 100 µL of a 24-hour grown pre-inoculum of *M. pulcherrima* Mp-22 or Mp-30 cells (10^6 cell mL^{-1} YNBS) were added to each flask for fungal co-culture for 12 additional hours. Monocultures of *B. cinerea* and yeasts were used as controls. Cell-free CFs for metabolomic analysis were obtained after centrifugation at 2000 *g* and subsequent 0.22 µm cellulose filtration. Each condition was replicated three times. CFs from all treatments and controls were freeze-dried for conservation prior to metabolomic analysis.

2.4.2. Metabolomic analysis

Five mL of freeze-dried CFs were resuspended in 1 mL of extraction buffer (H_2O:MeOH:formic acid (90:10:0.01)) and were randomly injected into the instrument. The untargeted metabolomics was performed using a Kinetex 2.6 µm EVO C18 UPLC column (Phenomenex Inc.) in the ACQUITY UPLC I-Class System and a SYNAPT G2-S high-definition mass Spectrometer MS/MS detector (Waters®). In total, three biological replicates per sample were analyzed, and each of the samples was injected twice into the instrument. MassLynx 4.2 (Waters®) was used for data acquisition on the SYNAPT G2-S and the DataBridge package (Waters®) was used to transform the file extension to CDF. A peak-picking algorithm was run in R using XCMS library. MetaboAnalyst 5.0 and MarVis-suite 2.0 were used for data analysis and interpretation (Kaever et al., 2015; Pang et al., 2021).

A total of 16,684 cations (ESI+) and 2705 anions (ESI-) signals were detected by UPLC-MS/MS, and data were Pareto scaling prior to multivariate analysis in MetaboAnalyst 5.0. The confidence level for metabolite identification was established based on Schrimpe-Rutledge et al. (2016). Validated identification was performed with reference standard (level 1), putative identification MS/MS online spectrum match (level 2) or tentative identification *m/z* online database match (level 3). For level 1, we used an internal library with more than 200 secondary metabolites. We used the Kyoto Encyclopedia of Genes and Genomes (Kegg) (https://www.genome.jp/kegg/) for *m/z* tentative identification with an accurate mass error of 1 mDA and Massbank (https://massbank.eu/MassBank/Search) and Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage) for online MS/MS spectra.

2.5. In vitro antifungal activity assay of metabolites

2.5.1. Preparation of metabolites

Metabolites commercially available were purchased and prepared at 100 mM stock solution following the manufacturer's advice. The pH of each metabolite was adjusted accordingly between 5.5 and 7 (it was verified that *B. cinerea* growth was unaffected in this pH range).

2.5.2. B. cinerea spore germination plate assay

The effect of metabolites on conidia germination was performed using the Oxford cup diffusion inhibition assay method (Cabañas et al., 2020; Khawaja et al., 2018). Aliquots (100 µL) of a suspension of 10^4 conidia mL^{-1} of *B. cinerea* were spread out on PDA plates. A 5 mm-diameter Oxford cup was made with a cork borer and 100 µL of metabolites (100 mM) were deposited inside. As controls, corresponding metabolite solvents were assayed. After incubation at 28 ºC for 4 d, a transparent halo zone across the Oxford cup was caused by the antifungal effect of the corresponding metabolite. The diameter of the inhibition halo zone was measured. Three replicates for each metabolite were made.

2.6. Efficacy of metabolites to control postharvest fruit spoilage: wound protection assays

2.6.1. Measurement of biocontrol activity of metabolites against B. cinerea in tomato, apple and grape

The efficacy of commercially available metabolites as *B. cinerea* biocontrol agents was evaluated on three economically relevant species highly susceptible to the gray mold infection that represent different cultivation systems: tomato as a vegetable, apple as a fruit tree and grape for wine production. For cherry tomato fruit (*Solanum lycopersicum* var. cerasiforme) we used a previously described method for wounding and infection (Fernandez-San Millan et al., 2021; Magoye et al., 2020). Three wounds (2 mm deep × 1 mm wide approximately) were made per fruit and each wound was inoculated with 5 µL (100 mM) of the metabolite or 5 µL of corresponding solvent in negative control tomatoes (MES 10 mM, ethanol, DMSO:H_2O 1:20 or chloroform). After 15 min for complete air drying, each wound was inoculated with 5 µL of a *B. cinerea* conidial suspension (10^4 conidia mL^{-1}). Tomatoes were set in plastic supports inside plastic trays with water-soaked paper to maintain a high relative humidity environment. After 7 d storage in a dark chamber at 25 °C, the disease incidence (DI) was measured as described (Parafati et al., 2015): DI % (percentage of infected wounds) = (number of infected wounds / number of total wounds) x 100). After 14 d of infection, the disease severity (DS) was measured using a 0-to-4 scale: 0 =no symptoms; 1 = 1–25% of the surface with fungal damage; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%. Then, the DS percentage was calculated (Parafati et al., 2015): DS %= [$\sum$ (number of infected fruits in each scale x disease scale) / (total fruits analyzed x highest disease scale)] x 100. This experiment was repeated twice.

A *B. cinerea* biocontrol assay with selected metabolites was performed with white grapes (*Vitis vinifera*) as described (Fernandez-San Millan et al., 2021). The process was identical to the one applied to the tomatoes and the experiment was repeated twice.

For testing selected metabolites in apples (*Malus domestica* cv. Golden Delicious), four wounds were made per fruit and three apples per treatment were tested. DI at 7 d post-inoculation was recorded as described above. The DS index was calculated as DS %= (D_Y/D_0 x 100), where D_y is the diameter of the rot and D_0 is the maximum rot diameter on the metabolites-treated apples (Fernandez-San Millan et al., 2021; Magoye et al., 2020). This experiment was repeated twice.

2.6.2. Assessment of the antifungal spectrum of metabolites

The metabolites that positively controlled *B. cinerea* were subsequently tested for biocontrol efficacy against *A. alternata* and *P. expansum* on cherry tomatoes. The procedures for infection and testing were performed as described before. *A. alternata* and for *P. expansum* conidia were used at 10^4 and 10^5 conidia mL^{-1}, respectively. The experiment was repeated twice.

2.7. Characterization of the antagonistic activity of metabolites against fungi

2.7.1. Quantification of the oxalic acid produced by B. cinerea

The effect of metabolites on oxalic acid production was performed as previously described (Wang et al., 2021). Briefly, 200 µL of a *B. cinerea* suspension (10^3 conidia mL^{-1}) were inoculated into 50-mL flasks containing 10 mL of PDB medium with selected metabolites at 100 µM. Flasks amended with the corresponding amount of solvent (MES 10 mM for 3-amino-5-methylhexanoic acid, or chloroform for biphenyl-2,3-diol and sinapaldehyde) were used as controls. After incubation at 150 rpm and 28 ºC for 6 d, the cultures were centrifuged at 2000 *g* for 10 min, and the oxalic acid content in the corresponding supernatants was measured at 510 nm (Synergy HI microplate reader, Biotek). The oxalic acid concentration was calculated according to an oxalic acid standard curve (Sigma-Aldrich) ranging from 10 to 75 mg L^{-1}.

2.7.2. Cell membrane permeability test

The cell membrane permeability test was performed as previously described (Wang et al., 2021). Flasks with 25 mL of PDB medium were inoculated with 25 µL of a *B. cinerea* suspension (10^5 conidia mL^{-1}) and incubated on a rotary shaker at 150 rpm and 28 ºC. After 72 h, mycelia cultures were amended with 1 mM of the selected metabolites. Flasks amended with the corresponding amount of solvent were used as controls. After incubation at 150 rpm and 28 ºC for an additional 2 h, mycelia were collected and rinsed three times with double-distilled

water. Then, 1.5 g of mycelium per sample was suspended in 10 mL of double-distilled water. The electrical conductivity was measured at 0, 1, 2, 3 and 4 h with a SensION Portable conductivity-meter (Hach, Loveland, CO, USA) to assess the leaching of cell contents through mycelial cell membranes. At the end of the experiment, each sample was boiled for 10 min to quantify the final conductivity. The relative conductivity was calculated as follows: (conductivity at each time / final conductivity) × 100%. Three repeats per metabolite treatment were performed.

2.7.3. Quantification of cell membrane integrity and intracellular level of reactive oxygen species (ROS)

The effect of selected metabolites on *B. cinerea* cell membrane integrity and the production of intracellular ROS was evaluated as described (Zhang et al., 2015) by using 10 μg mL^{-1} propidium iodide (PI; Sigma-Aldrich) and 5 μM 2, 7-dichlorodihydrofluorescein diacetate (DCHF-DA; Sigma-Aldrich), respectively. Briefly, spores of *B. cinerea* (10^5 conidia mL^{-1}) were cultured in PDB medium containing 1 mM of the selected metabolites for 2 h at 23 ℃. Then, spores were stained for 30 min with the different dyes and washed twice with phosphate-buffered saline (PBS) to eliminate the residual dye. The spores were observed under a Leica MC170 HD fluorescence microscope (excitation 488 nm and emission 525 nm). Percentage of fluorescent spores was calculated by counting them in six different fields for each fluorochrome. Means and standard error were calculated. Percentages represent ROS level and spores that lost membrane integrity.

2.8. Statistical analysis

The data are presented as the mean and standard error. To normalize the distribution before performing the statistical analysis of DI and DS percentages, data were transformed into arcsine square-root values as described (Fernandez-San Millan et al., 2021). T-tests or one-way ANOVA with Tukey's pairwise comparison ($p < 0.05$) were used to evaluate the statistical significance of differences among the treatments. SPSS.24 software was used to perform all calculations.

3. Results

3.1. M. pulcherrima produces extracellular antifungal compounds with antagonistic activity of B. cinerea growth

The ability of *M. pulcherrima* strains to produce extracellular antifungal compounds was evaluated. Cell-free aseptic culture filtrates (CFs) of the Mp-22 and Mp-30 strains in the exponential growth phase were added at different concentrations to the culture medium for *B. cinerea* growth. Mycelium development was measured by the proliferation in liquid culture after 2 d. The results showed an inhibitory effect of CFs that was dose-dependent (Fig. 1A). Growth inhibition was greater from the Mp-30 strain CF than the Mp-22 strain CF. Mycelial growth inhibition was observed with low concentrations of the CFs (29% in Mp-22% and 79% in Mp-30). Fungal growth was totally suppressed with high concentrations of the yeast CFs.

3.2. Identification of yeast metabolites with antifungal activity

3.2.1. Establishment of a yeast-fungus co-culture protocol for UPLC-MS/MS analysis of culture filtrates

A protocol was developed to identify metabolites present in the yeast CF and to monitor changes in the exometabolome under antagonistic co-culture conditions with *B. cinerea*. It was observed that the yeast growth was in the exponential phase from 12 h-culture on, and it was similar in the presence or absence of the fungus (Fig. 1B and Fig. S1A). However, the presence of the yeast stopped the growth of the fungus (Fig. S1B).

3.2.2. UPLC-MS/MS metabolomic analysis of culture filtrates

To find the metabolites that *M. pulcherrima* strains secrete into the medium in the presence of *B. cinerea*, we performed an untargeted metabolomic analysis of yeast CFs in the presence (co-culture) and the absence of the fungal pathogen. The Principal Component Analysis (PCA) showed that the secreted metabolite profile of Mp-22 significantly differed from the *B. cinerea* profile, the latter being much closer to the control medium (Fig. 2A) and indicating poor secretion of metabolites from *B. cinerea*. Interestingly, the PCA also showed that the loading plots of Mp-22 co-cultured with *B. cinerea* (Mp-22 + *B.c.*) displayed a different profile of metabolites compared to the Mp-22 monoculture, which showed a greater distance to the control medium in the first principal component (PC1) than Mp-22 alone. PC1 represents the maximum percentage of variability in the data, meaning that the Mp-22 + *B.c.* profile had the most significant impact on the analysis. A similar outcome was observed in the PCA of Mp-30. The loading plots of Mp-30 co-cultured with *B. cinerea* (Mp-30 + *B.c.*) had the greatest impact in the analysis compared to the loading plots of *B. cinerea* and Mp-30 monoculture (Fig. 2B).

To identify secreted metabolites with a potential antifungal activity against *B. cinerea*, we performed a heatmap analysis to find signals with greater accumulation in Mp-22 and Mp-30 co-cultured with *B. cinerea* relative to the other three conditions (Fig. 2C, D). Before clustering, we performed a statistical analysis and filtered all the signals with a significance of $p < 0.05$ (Kruskal-Wallis) to ensure variability among the

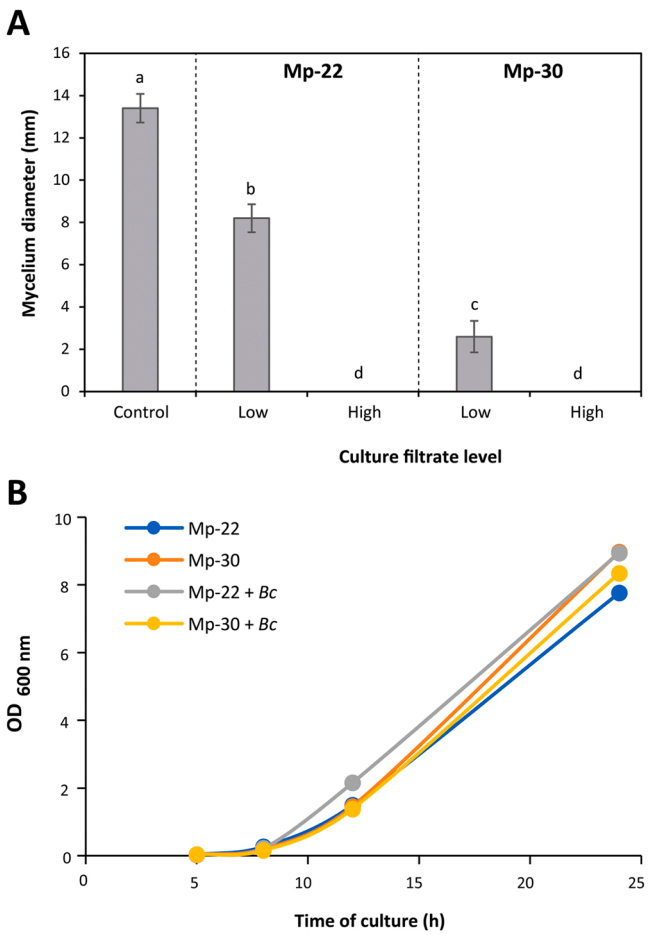

Fig. 1. (A) Antagonistic activity of *M. pulcherrima* Mp-22 and Mp-30 culture filtrates on mycelium growth of *B. cinerea* in liquid medium. Control: without yeast culture filtrate. Low: culture filtrate/YNBS medium at a 1:2 ratio. High: culture filtrate/YNBS medium at a 2:1 ratio. Data are presented as the means ± SE (n = 5). Values with the same letter do not differ significantly ($p < 0.05$) according to the Tukey's multiple range test. **(B)** Time-course of yeast growth in the absence/presence of the fungus. Mp-22 and Mp-30: *M. pulcherrima* strains. Mp-22 + *B.c.* and Mp-30 + *B.c.*: yeast-fungus co-culture.

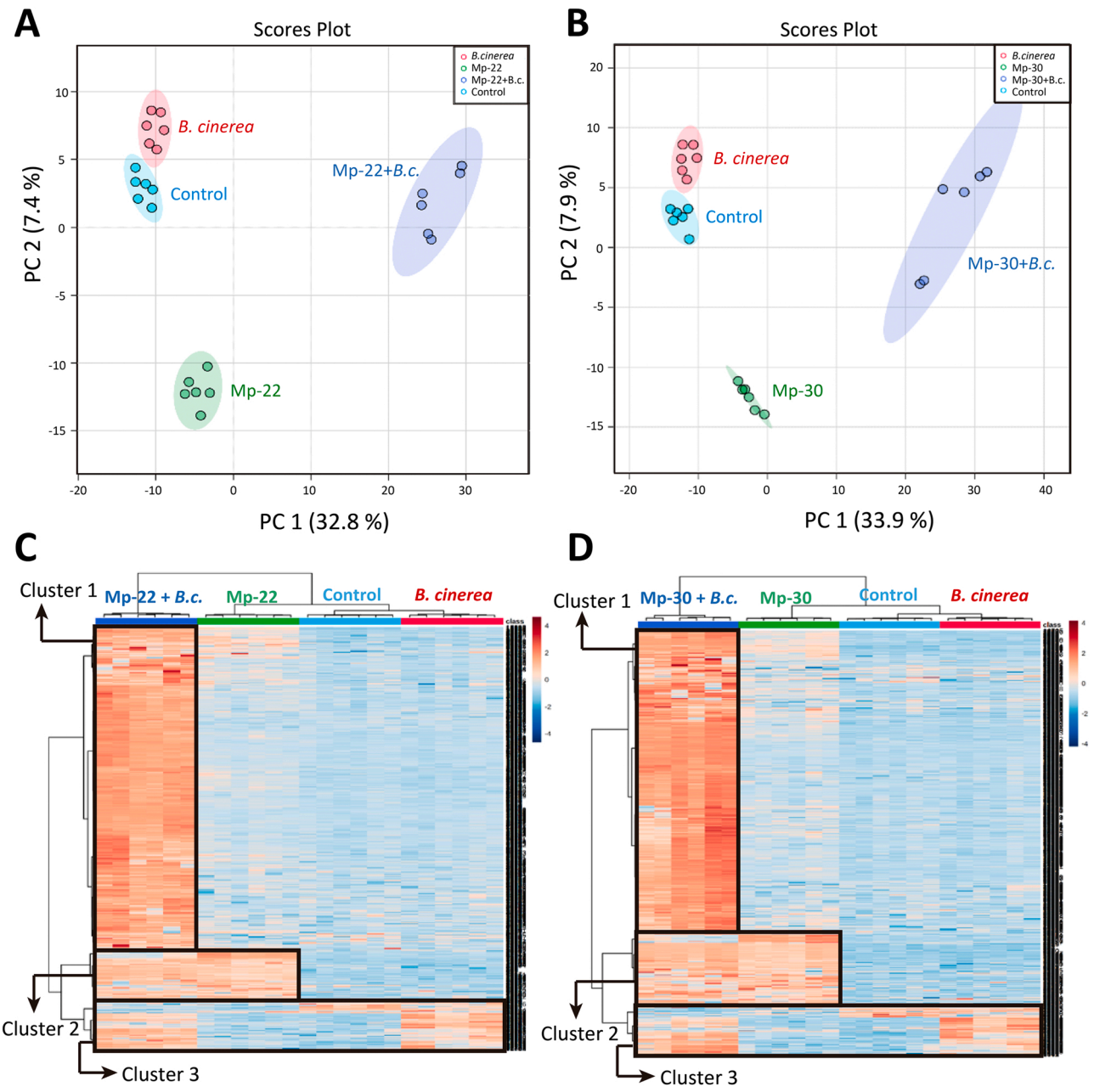

Fig. 2. Principal component analysis (PCA) representing UPLC-MS/MS data of the culture filtrates. Non-targeted metabolomic profiling of *M. pulcherrima* Mp-22 **(A)** and Mp-30 **(B)** in the presence or the absence of *B. cinerea*. Heatmap analysis of culture filtrates generated from ESI+ and ESI- signals with p-value< 0.01 of Mp-22 **(C)** and Mp-30 **(D)**. The relative amounts of the metabolites were determined in all the samples by normalizing the chromatographic pick area of each compound with the dry weight of the corresponding sample. Control: culture medium. *B. cinerea*: fungal culture. Mp-22 and Mp-30: *M. pulcherrima strains*. Mp-22 + *B.c.* and Mp-30 + *B.c.*: yeast-fungus co-culture. The heatmap analysis shows the behavior of differentially accumulated compounds within the samples. Cluster 1 includes signals with higher accumulation levels in the yeast-fungus co-culture alone. Cluster 2 includes signals with higher accumulation levels in individual *M. pulcherrima* cultures and yeast-fungus co-culture. Cluster 3 includes signals with higher accumulation levels in individual fungal cultures. Three biological replicates per sample were analyzed, and each of the samples was injected twice into the instrument.

samples. Subsequently, the combined heatmap analysis of ESI+ and ESI- features displayed a cluster of signals produced by *M. pulcherrima* only in the presence of *B. cinerea* (cluster 1). Moreover, constitutive metabolites from *M. pulcherrima* and *B. cinerea* were also visible in the analysis (cluster 2 and 3). To establish a tentative identity of the secreted metabolites in cluster 1, we annotated a total of 35 metabolites based on internal library matching, online fragmentation spectra or accurate masses (identification levels 1–3 in Table 1).

Among the most representative functional categories, we found phenylpropanoids, alkaloids, 2-oxocarboxylic acid-derivatives and amino acid-related compounds (Table 1), indicating that these pathways may have a relevant function in the yeast-fungus interaction.

3.3. *In vitro antifungal activity assessment of metabolites*

An *in vitro* assay was conducted with 18 metabolites commercially available as a first approach to evaluate their biocontrol potential against the fungal pathogen *B. cinerea*. Among the 18 metabolites tested, nine had an obvious inhibitory effect on *B. cinerea* spore germination and mycelial growth according to the Oxford cup method assay (Fig. 3 and Fig. S2). In particular, the inhibitory activity of biphenyl-2,3-diol was the highest followed by dethiobiotin.

Table 1
List of compounds identified by MS/MS data.

Compound	*m/z*	ESI	Yeast Strain	Functional category	Identification level	Antifungal effects *in vitro* / *in vivo*
5-Hydroxy-1-(4-hydroxyphenyl)− 3-decanone	265.177	+	Mp-22	Microbial metabolism	3	–
5,10-Methenyltetrahydrofolate	456.165	+	Mp-30	Microbial metabolism	3	–
6-Aminocaproic acid	132.102	+	Mp-22 / Mp-30	Microbial metabolism	2	No
(S)− 3-Amino-5-methylhexanoic acid	146.118	+	Mp-22 / Mp-30	Microbial metabolism	2	*In vitro* and *in vivo*
Biphenyl-2,3-diol	187.076	+	Mp-22 /Mp-30	Degradation of aromatic compounds	3	*In vitro* and *in vivo*
Catechol	110.036	–	Mp-22	Degradation of aromatic compounds	2	*In vitro*
2,3-Dihydroxybenzoate	153.018	–	Mp-30	Phenylpropanoid pathway	1	*In vitro*
p-Coumaroyl quinic acid	337.090	–	Mp-30	Phenylpropanoid pathway	3	No
Sinapaldehyde	209.078	+	Mp-30	Phenylpropanoid pathway	2	*In vitro* and *in vivo*
5-Methyltetrahydrofolate	460.197	+	Mp-22 / Mp-30	Carbon metabolism / Aa synthesis	3	*In vitro*
2'''-N-Acetyl-6'''-deamino-6'''-hydroxyneomycin C	658.313	+	Mp-22	Antibiotic synthesis	3	–
8,8a-Deoxyoleandolide	371.240	–	Mp-22 / Mp-30	Antibiotic synthesis	3	*In vitro*
β-Rhodomycin	544.219	+	Mp-22 / Mp-30	Antibiotic synthesis	3	–
Dethiobiotin	215.140	+	Mp-30	Biotin metabolism	3	*In vitro*
Hexadecanedioic acid	285.205	–	Mp-30	Fatty acid metabolism	3	*In vitro*
16-Hydroxytabersonine	353.180	+	Mp-22 / Mp-30	Alkaloid biosynthesis	3	–
Deacetylisoipecoside	524.210	+	Mp-22 / Mp-30	Alkaloid biosynthesis	3	–
Piperideine	84.080	+	Mp-30	Alkaloid biosynthesis	3	–
Protoemetine	316.187	–	Mp-30	Alkaloid biosynthesis	3	–
2-Isopropylmaleate	157.049	–	Mp-30	2-Oxocarboxylic acid metabolism / Aa synthesis and degradation	3	No
2-(2'-Methylthio) ethylmalic acid	207.029	–	Mp-22 / Mp-30	2-Oxocarboxylic acid metabolism / Aa synthesis and degradation	3	–
3-Methyl-2-oxopentanoic acid	129.054	–	Mp-30	2-Oxocarboxylic acid metabolism / Aa synthesis and degradation	2	No
S-(Hydroxyphenylacetothiohydroximoyl)-L-cysteine	271.075	+	Mp-22 / Mp-30	2-Oxocarboxylic acid metabolism / Aa synthesis and degradation	3	–
4-Trimethylammoniobutanoate	146.118	+	Mp-22 / Mp-30	Aa synthesis and degradation	2	–
Glutamic acid	148.061	+	Mp-22 / Mp-30	2-Oxocarboxylic acid metabolism / Aa synthesis and degradation	1	No
Homocitrate	205.034	–	Mp-22	2-Oxocarboxylic acid metabolism / Aa synthesis and degradation	3	–
L-Arginine phosphate	255.084	+	Mp-22 / Mp-30	Aa synthesis and degradation	3	–
Val-Leu	231.170	+	Mp-30	Aa synthesis and degradation	2	No
Pantetheine	277.121	–	Mp-30	Taurine and hypotaurine metabolism / Pantothenate and CoA biosynthesis	3	No
Taurocholic acid	530.277	–	Mp-22 / Mp-30	Taurine and hypotaurine metabolism	2	No
2-C-Methyl-D-erythritol 4-phosphate	215.033	–	Mp-22 / Mp-30	Isoprenoid backbone biosynthesis	3	–
Cellohexaose	989.321	–	Mp-30	Starch and sucrose metabolism	2	No
2-Deoxyglucose 6-phosphate	243.030	–	Mp-22	Amino sugar and nucleotide sugar metabolism	2	–
Adenine	136.063	+	Mp-30	Purine metabolism	2	–
Cytidine 5'-triphosphate	481.981	–	Mp-30	Pyrimidine metabolism	2	–

Identification levels represent: level 1, validated identification with a reference standard; level 2, putative identification by exact mass and online fragmentation spectrum match; level 3, tentative identification by *m/z* online database match. -: metabolite not tested. No: no *in vitro / in vivo* antifungal effects. Aa: Amino acid.

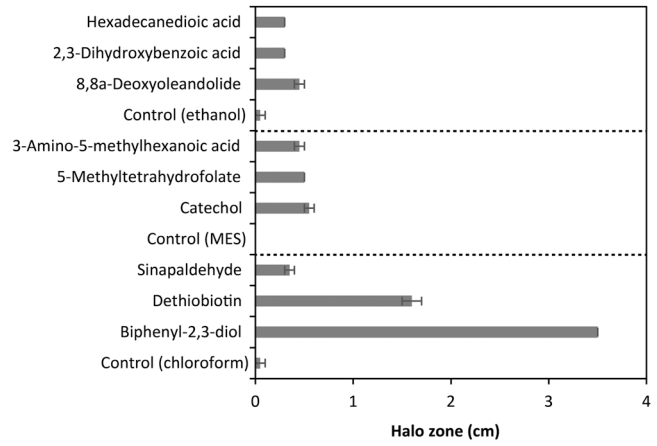

Fig. 3. *In vitro* plate assay of the antagonistic activity of bioactive metabolites against *B. cinerea* spore germination. Data (halo zone) are presented as the means ± SE (n = 6). Groups of metabolites dissolved with the same solvent (ethanol, MES or chloroform) are separated by dashed lines. Control: solvent without metabolite.

3.4. *In vivo* antifungal activity assessment of metabolites: efficacy to control postharvest fruit spoilage

The 18 metabolites were tested for the study of *in vivo* biocontrol aptitudes against *B. cinerea* as one of the main tomato postharvest pathogens. Fungal spores were inoculated into wounded fruits and after 14 d, *B. cinerea*-inoculated tomato controls without metabolite treatment reached DI and DS values above 95% (Fig. 4A-C). Only the presence of three metabolites (out of 18 tested) in the fruit wounds was associated with reductions in DI and DS values against *B. cinerea* infection and damage: 3-amino-5-methylhexanoic acid, biphenyl-2,3-diol and sinapaldehyde (Fig. 4A-C, F; Fig. S3A). The DI was significantly reduced (below 10%) in relation to control tomatoes in treatments of 100 mM of 3-amino-5-methylhexanoic acid and biphenyl-2,3-diol. At this concentration, the DS for these two metabolites was close to zero and increased with decreasing metabolite concentrations. In the sinapaldehyde treatment (Fig. 4C), although the DI was high, the DS was reduced around 60% relative to control tomatoes.

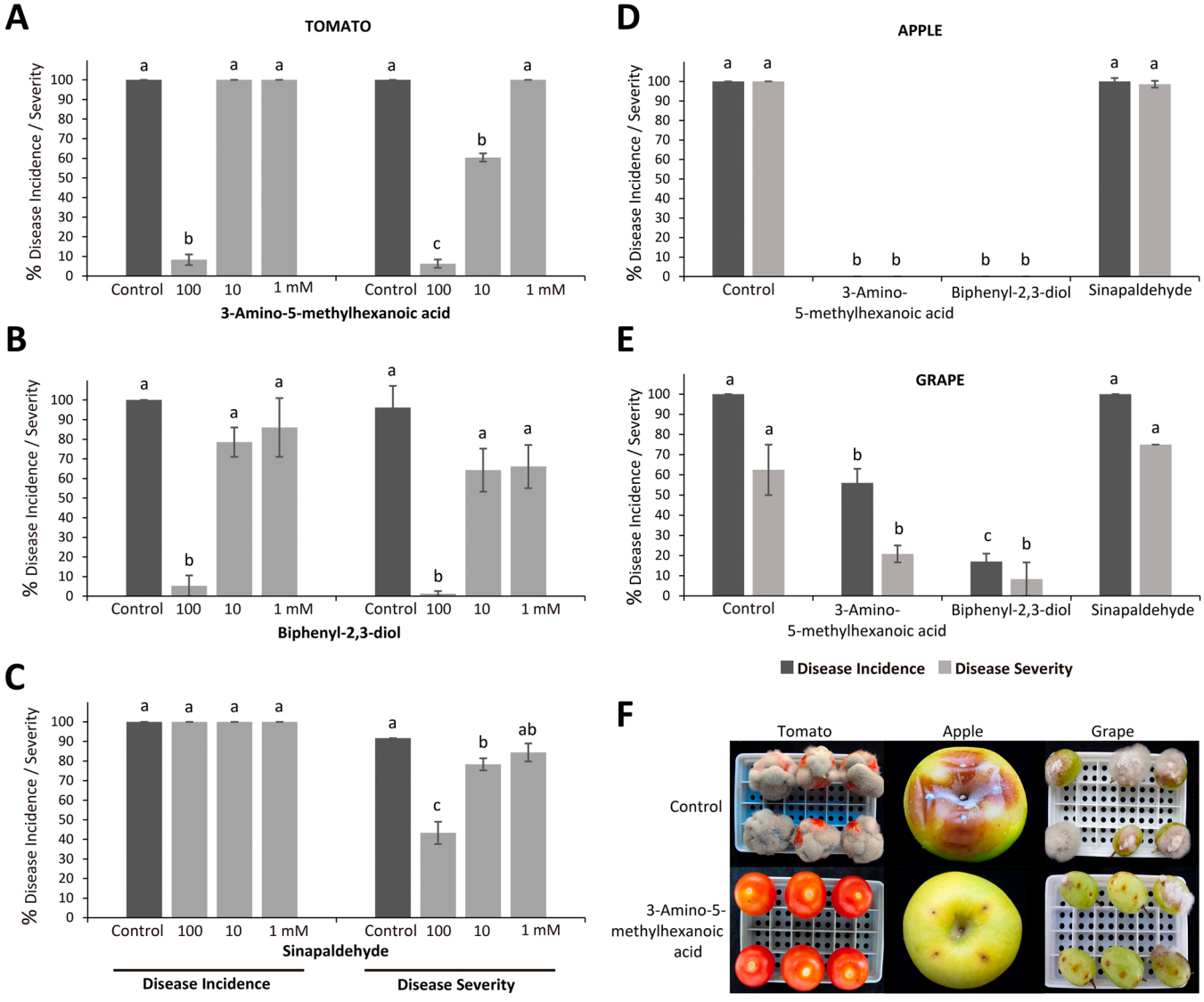

Fig. 4. Biocontrol of postharvest gray mold in different fruits. Disease incidence and disease severity of cherry tomatoes inoculated with *B. cinerea* and treated with 3-amino-5-methylhexanoic acid **(A)**, biphenyl-2,3-diol **(B)** or sinapaldehyde **(C)** at different concentrations (100 – 10–1 mM). **(D)** Disease incidence and disease severity of apples **(D)** and grapes **(E)** treated with the selected metabolites (100 mM). **(F)** Disease proliferation in experimentally wounded fruits: control fruits inoculated with spores of *B. cinerea* (up) and fruits treated with 3-amino-5-methylhexanoic acid (100 mM) and then inoculated with the fungus (down). Data are presented as the means ± SE (n = 36 for tomato and grape, and n = 12 for apple). Values with the same letter do not differ significantly ($p < 0.05$) according to the Tukey's multiple range test. Control: infected fruits without any treatment.

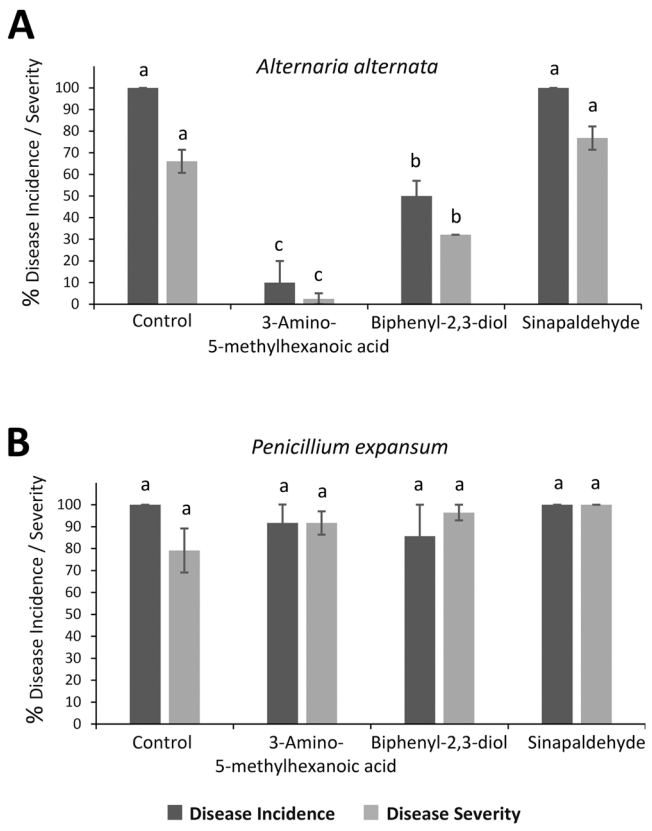

Fig. 5. Biocontrol of other postharvest fungal diseases. Disease incidence and disease severity of cherry tomatoes inoculated with *A. alternata* **(A)** or *P. expansum* **(B)** treated with the selected metabolites (100 mM). Data are presented as the means ± SE (n = 36). Values with the same letter do not differ significantly ($p < 0.05$) according to the Tukey's multiple range test. Control: infected fruits without any treatment.

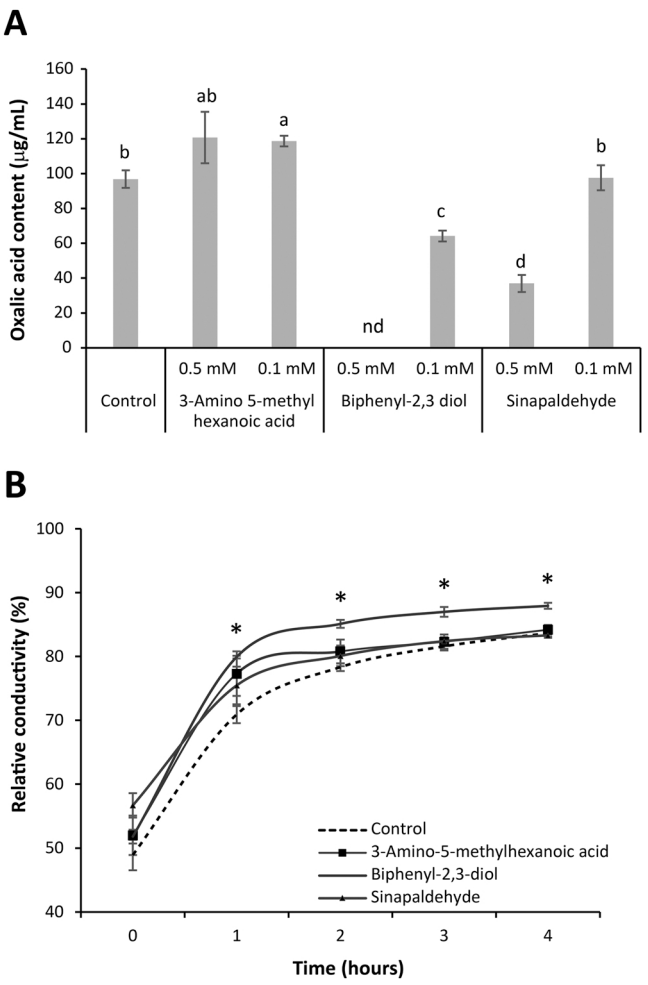

Fig. 6. Effect of different concentrations of the selected metabolites on oxalic acid content **(A)** and cell membrane permeability **(B)** of *B. cinerea* cultures. Data are presented as the means ± SE (n = 6). Values with the same letter do not differ significantly ($p < 0.05$) according to the Tukey's multiple range test. Asterisk indicates statistical significance compared with the control, as determined by Student's t-test ($p < 0.05$). Control: fungal culture without any metabolite. nd, not detected.

The favorable results against *B. cinerea* in tomato fruits prompted us to study the biocontrol effect of the three selected metabolites on other fruits that are highly susceptible to gray mold infection. Consequently, the same assay was repeated with apples and white grapes. Total protection of apples inoculated with *B. cinerea* spores was observed after 14 d with two (3-amino-5-methylhexanoic acid and biphenyl-2,3-diol) out of the three metabolites tested (Fig. 4D, F; Fig. S3A). Both metabolites also offered a protection higher than 80% in infected grapes (Fig. 4E, F; Fig. S3A). However, sinapaldehyde did not show any level of protection for other fruits except tomato (Fig. 4C-E; Fig. S3A).

The three selected metabolites that inhibited *B. cinerea* were subsequently assayed for protection on cherry tomatoes against two additional postharvest fungal pathogens: *A. alternata* and *P. expansum*. In the case of *A. alternata,* two of the metabolites, 3-amino-5-methylhexanoic acid and biphenyl-2,3-diol, led to respective decreases in the DI of 90% and 50% and the DS of 95% and 70% (Fig. 5A, Fig. S3B). However, none of the metabolites could protect tomato fruits from *P. expansum* (Fig. 5B, Fig. S3C).

3.5. Characterization of the antagonistic activity of metabolites against B. cinerea

The relative abundance of the three antagonistic metabolites in the CFs is represented by Box plots (Fig. S4A, B). Secretion of 3-amino-5-methylhexanoic acid and biphenyl-2,3-diol was enhanced in both Mp-22 and Mp-30 co-cultures with *B. cinerea*. However, the presence of sinapaldehyde only increased significantly in the Mp-30 co-culture. While the secretion of biphenyl-2,3-diol into the extracellular medium only occurred in the presence of *B. cinerea*, the other two metabolites already appeared at a basal level in yeast control monocultures. The chemical structures of the three metabolites are completely different (Fig. S4C) and belong to separate functional categories (Table 1): microbial metabolism, degradation of aromatic compounds and phenylpropanoids.

3.5.1. Effect of metabolites on oxalic acid content and cell membrane permeability

As shown in Fig. 6A, the oxalic acid content present in the culture medium significantly decreased in a dose-response way after treatment with biphenyl-2,3-diol or sinapaldehyde. Interestingly, oxalic acid slightly increased in the presence of 3-amino-5-methylhexanoic acid.

To study changes in the cell membrane of *B. cinerea*, the relative conductivities of mycelial suspensions treated with the three antifungal metabolites were measured. The results showed that the relative conductivity significantly increased in the presence of biphenyl-2,3-diol, indicating that this metabolite could damage the fungal cell membranes and lead to an increase in electrolyte leakage from the mycelium (Fig. 6B).

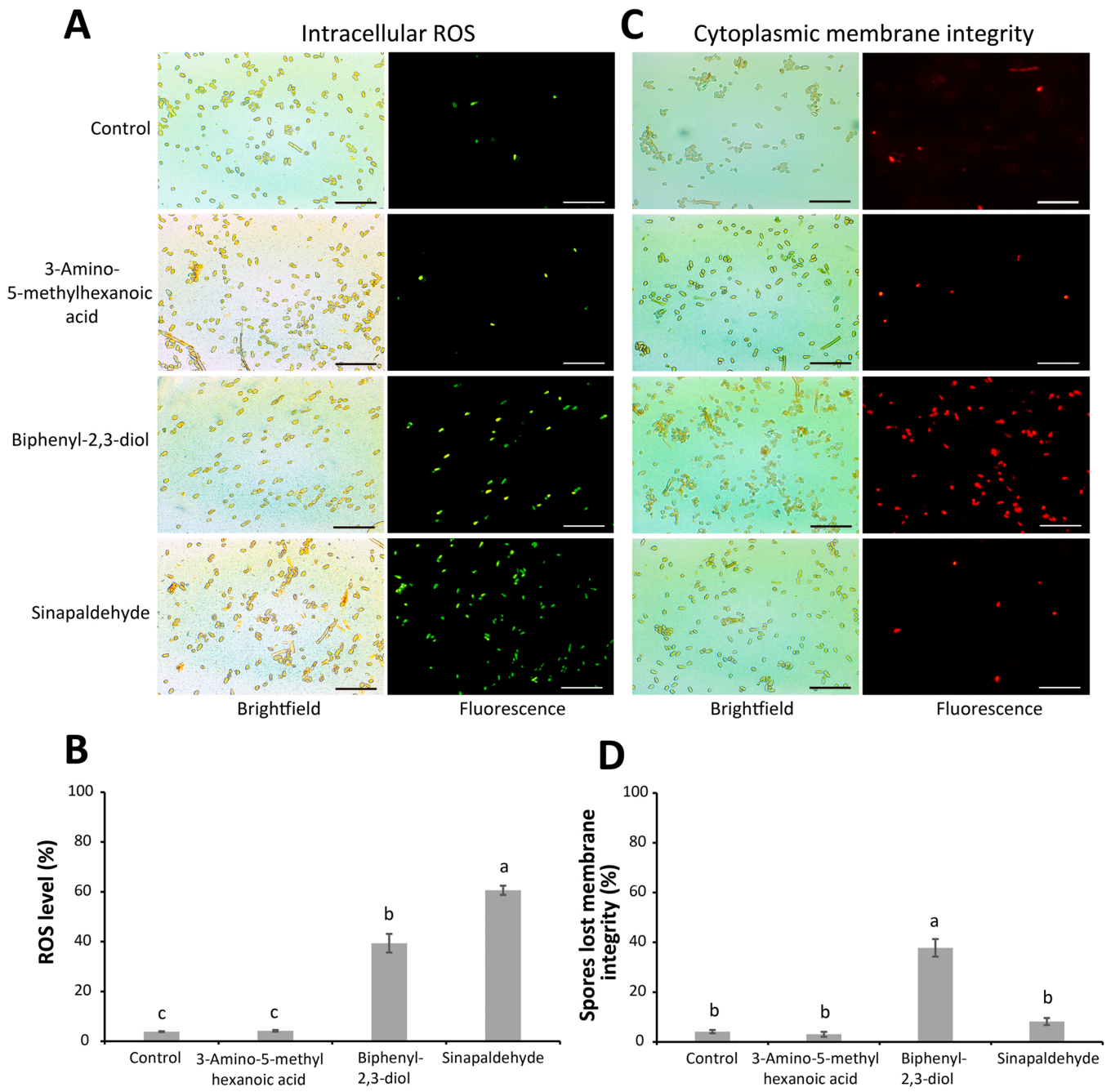

Fig. 7. Loss of cytoplasmic membrane integrity and accumulation of intracellular reactive oxygen species (ROS) in *B. cinerea* spores after incubation with the selected metabolites (100 mM). **(A)** Spores with ROS accumulation showed green fluorescence. **(B)** Percentage of *B. cinerea* spores exhibiting visible ROS accumulation after treatment. **(C)** Spores with damaged cytoplasmic membranes showed red fluorescence. **(D)** Percentage of *B. cinerea* spores with loss of cytoplasmic membrane integrity after treating with the corresponding metabolite. Bars represent 100 μm. Data are presented as the means ± SE (n = 6). Values with the same letter do not differ significantly ($p < 0.05$) according to the Tukey's multiple range test. Control: fungal culture without any metabolite.

3.5.2. Effect of metabolites on the intracellular level of ROS, cell membrane integrity and mycelial morphology

As illustrated by Fig. 7A, antifungal metabolite treatment significantly induced the intracellular ROS accumulation in *B. cinerea* spores, as determined by the use of the fluorescent dye DCHF-DA. The percentages of fluorescent spores with DCHF-DA reached 39% and 59% following 100 mM treatment with biphenyl-2,3 diol and sinapaldehyde, respectively (Fig. 7B). By contrast, no effect was observed when the spores were treated with 3-amino-5-methylhexanoic acid.

The fluorescent dye PI was chosen to determine the cell membrane integrity of *B. cinerea* after exposure to the three antifungal metabolites. PI can enter damaged membranes and combine with nucleic acids to produce fluorescence. As shown in Fig. 7C, few fluorescent spores (less than 5%) were observed in the control but they significantly increased (40%) after the treatment with biphenyl-2,3-diol (Fig. 7D). No differences with respect to the control spores were observed after the treatment with 3-amino-5-methylhexanoic acid or sinapaldehyde. These results indicate that only biphenyl-2,3 diol was able to damage the cell membranes of *B. cinerea* spores.

Changes in the mycelial morphology of *B. cinerea* after the 2 h treatment with biphenyl-2,3-diol were observed under an optical microscope (Fig. S5). The width of *B. cinerea* hyphae decreased compared

to the control, particularly in the tips of the mycelium. After the metabolite treatment, hyphal plasma contracted while the control was natural with the hyphal plasma evenly distributed. No changes were observed in mycelia treated with the other two metabolites.

4. Discussion

Nowadays, the side effects of chemical-based methods to manage plant pathogens and the growing concern for the environment demand alternative approaches (Agler et al., 2020). Biomolecules of microbial origin are potential candidates for the new generation of antimicrobial and pesticidal agents (Kumar and Khurana, 2021). Within this context, the identification of mechanisms and metabolites responsible for the antifungal strategies employed by the *M. pulcherrima* Mp-22 and Mp-30 strains against the necrotrophic pathogen *B. cinerea* is of great interest. By using untargeted metabolomics, we have discovered novel candidate molecules with antagonistic activity against fungal postharvest phytopathogens.

As a preliminary study, this work has identified a concentration-dependent growth inhibition of *B. cinerea* by *M. pulcherrima* culture filtrates, confirming that both of the yeast strains secrete inhibitor compounds against gray mold growth. It is well known that microorganisms and plants secrete a wide range of molecules into the extracellular space where they play crucial roles in stress responses, development and signaling (Delaunois et al., 2014). Although proteins are the most studied group, a cocktail of nonproteinic molecules such as small peptides, nucleic acids or secondary metabolites are also secreted. However, how all these secreted molecules interact to regulate communication mechanisms between organisms in both pathogenic and beneficial interactions, remains unclear (Uhse and Djamei, 2018; Vincent et al., 2020). To date, this knowledge has been generally derived from genomic analysis of genes putatively encoding enzymes involved in biocontrol activity or the biosynthesis of secondary metabolites rather than from direct investigations of the metabolites synthesized during the interaction (Demissie et al., 2020, 2018; Dubey et al., 2014; Karlsson et al., 2015; Ocampo-Suarez et al., 2017; Sipiczki et al., 2020; Sui et al., 2020).

To address the gap in our understanding of the mechanisms underlying the biocontrol activity of Mp-22 and Mp-30 strains against *B. cinerea*, an experimental confrontation assay comprising yeast and fungus co-culture in liquid medium was set up to compare the secreted metabolites (exometabolome) between interacting species and monoculture controls. Among various culture-based approaches exploited thus far to investigate this chemical diversity, microbial co-culture in the same confined environment has attracted tremendous attention in the literature. It allows the microorganisms to initiate direct chemical interactions and such stimuli may induce the synthesis of compounds not previously observed in monocultures (Jones and Wang, 2018; Shi et al., 2021). As a result, the characterization of the metabolites produced in a microbial consortium is more relevant if metabolites are detected and isolated in a co-culture system (Vallet et al., 2017). Other than the well-studied *S. cerevisiae* and medically relevant yeasts, little is known about the metabolism of other wild yeast species. Thus, the UPLC-MS/MS analysis identified a total of 35 differentially secreted metabolites potentially involved in growth inhibition of the gray mold. These metabolites significantly increased in Mp-22 and Mp-30 culture filtrates inoculated with *B. cinerea* compared to the control yeast conditions. Among these metabolites, 15 were common for both yeast strains. The metabolites belong to several functional categories such as antibiotics, the phenylpropanoid pathway, 2-oxocarboxylic acid metabolism, or alkaloid biosynthesis, among others. We were able to analyze the antifungal capabilities of 18 of these metabolites *in vitro* and *in vivo*. Antifungal activity was observed with nine metabolites in the *in vitro* plate experiments and three of them also showed *in vivo* postharvest fruit protection.

4.1. In vivo bioactive antifungal metabolites produced by M. pulcherrima

One of the best protective metabolites discussed here was 3-amino-5-methylhexanoic acid. This molecule is known as β-homoleucine and belongs to a class of unusual non-proteinogenic amino acids known as β-amino acids. They are involved in the primary and secondary metabolism of all kingdoms (von Nussbaum and Spiteller, 2004), being rare substances in nature in comparison to proteinogenic α-L-amino acids (Kudo et al., 2014). Whereas a variety of natural β-amino acids have been found as secondary substructures of diverse metabolites (*i.e.* alkaloids, peptides or depsipeptides), their free counterparts, such as 3-amino-5-methylhexanoic acid, seem to be less common (von Nussbaum and Spiteller, 2004). Indeed, non-proteinogenic amino acids are of interest for numerous medical applications: discovery of new antibiotics, improvement of protein and peptide pharmaceuticals, or studies of toxic cyanobacteria, among others (Fichtner et al., 2017; Kudo et al., 2014; Wani et al., 1971). In this context, our results show that 3-amino-5-methylhexanoic acid is secreted by both *M. pulcherrima* strains. The increased levels of the metabolite in the presence of *B. cinerea* indicates that it must have an important function in the interactions between the yeast and fungus. Secretion of 3-amino-5-methylhexanoic acid was only reported for sclerotia (*Sclerotinia sclerotiorum*) exudates stimulated with *Bacillus* sp. (Sarajlic, 2018), which led support to the idea of a role for this metabolite in microbial interactions. In our *in vivo* infection model, 3-amino-5-methylhexanoic acid demonstrated high levels of tomato, apple and grape protection against *B. cinerea* and *A. alternata* infection. Little is known about such antimicrobial capacity. Homoleucines are unusual amino acids whose formation is attributed to the low specificity of enzymes involved in the synthesis of isoleucine, leucine and valine. They can act as antagonists of ordinary aliphatic amino acids at the cellular level, but their biological importance and the reason for their accumulation in some plants, fungi or bacteria still remain unclear (Zahradníčkovμ et al., 2008). Clearly, the high antagonistic efficacy of this metabolite and the lack of precedents in the literature make further research necessary to investigate its commercial potential.

Biphenyl-2,3-diol is the other metabolite that showed very high antifungal activity. It was also produced by both *M. pulcherrima* strains. This metabolite displayed a fungal control behavior similar to 3-amino-5-methylhexanoic acid in the *in vivo* biocontrol experiments and it was the strongest inhibitor of *in vitro* mycelial growth. Biphenyl-2,3-diol is a biphenyl derivative member of catechols, which are toxic to many microorganisms. They can react with membranes and biomolecules (such proteins or DNA), ultimately leading to irreversible damage as a consequence of their chemical properties (Schweigert et al., 2001). On the other hand, biphenyl derivatives have demonstrated a broad spectrum of biological activities in humans for medical treatments. In fact, biphenyl-containing compounds have found applications in various areas, including pesticides (revised in Ali et al., 2018 and Jain et al., 2017). In the field of food technology, biphenyl has been used in some countries for the preservation of citrus fruit through impregnation of the packaging material (Lück and Jager, 1997). In addition, biphenyls are members of the phytoalexin family that are formed *de novo* in plants in response to infection by bacterial and fungal pathogens. For example, eight natural biphenyl-type phytoalexins exhibiting antifungal effects were isolated from *Sorbus pohuashanensis* leaves infected by *Alternaria tenuissi* (Song et al., 2021). An *in vivo* activity assay by these authors confirmed the biocontrol effect of the biphenyls on tobacco leaves, and there was also significant activity against other fungal phytopathogens. Similar to our findings for *B. cinerea*, observations of mycelial morphology revealed that the natural biphenyls triggered adverse effects on the growth of *A. tenuissi* hyphae (Song et al., 2021). Clearly, biphenyl-2,3-diol is a very interesting *M. pulcherrima* metabolite with strong antifungal activity *in vivo*. There is no precedent for its commercial use and its possible value to agronomic production needs further investigation.

Sinapaldehyde is the third compound that showed significant *in vitro* and *in vivo* protection against *B. cinerea,* although it was only found in culture filtrates from the Mp-30 strain. Sinapaldehyde is a member of cinnamaldehydes, which are very active phenolic compounds mainly found in plants alongside other phenolic acids. However, some phenolic acids are also of microbial origin (Mandal et al., 2010). Phenolic acids are known to play multifunctional roles in rhizospheric plant-microbe interactions and can act as agents in plant defense (Mandal et al., 2010). In particular, evaluation of antimicrobial compounds from the plant *Ixora megalophylla* confirmed the presence of sinapaldehyde and evidenced its antimicrobial activity against some human oral pathogens (Panyo et al., 2016). Phenolic acids are produced in plants *via* shikimic acid through the phenylpropanoid pathway. In this pathway, phenylalanine ammonia lyases are crucial enzymes because they catalyze the formation of *trans*-cinnamate, which is the first committed step in the biosynthesis of several classes of plant phenylpropanoids. Although phenylalanine ammonia lyases are also found in fungi or prokaryotes (Moffitt et al., 2007), little is known about this pathway and the production of phenolic compounds in microorganisms. Therefore, as we have proven the ability of the *M. pulcherrima* Mp-30 strain to produce phenolic compounds, the door is open to study this metabolic pathway and the related metabolites.

In order to elucidate the mechanisms by which the three active metabolites inhibited mycelial growth in *B. cinerea*, we analyzed the integrity of the cytoplasmic membrane, oxalic acid production and intracellular ROS generation of *B. cinerea* after metabolite treatment. These methods have been used before to investigate the mode of action of cinnamic acid to control fruit decay after *B. cinerea* infection (Zhang et al., 2015). Previous studies also have reported that oxalic acid is critical to the pathogenicity of *B. cinerea* and the development of sclerotia (Wang et al., 2021). Biphenyl-2,3-diol significantly impaired fungal membrane integrity, generated an increase in ROS and was able to inhibit the secretion of oxalic acid into the medium by the fungal mycelium. Accordingly, earlier work has indicated that catechols may cause uncoupling of NADH formation, leading to the production of ROS such as hydrogen peroxide (Held et al., 1998; Suske et al., 1997). In our experiments, sinapaldehyde also enhanced intracellular ROS levels and reduced the secretion of oxalic acid by *B. cinerea*, but the membrane integrity was not affected. However, no significant changes in any measured parameter were detected after 3-amino-5-methylhexanoic acid treatment. These results suggest different modes of action of these metabolites. Biphenyl-2,3-diol and sinapaldehyde are stress agents leading to increase ROS levels that can cause oxidative damage to different cell components resulting in cytoplasmic membrane damage and decreased viability of the fungal pathogen. By contrast, 3-amino-5--methylhexanoic acid seems to exert its fungal inhibition by alternative means.

4.2. Other metabolites produced by M. pulcherrima with in vitro antifungal activity

Along with sinapaldehyde, two additional metabolites from the phenylpropanoid pathway (p-coumaroyl quinic acid -PCQA- and 2,3-dihydroxybenzoate −2,3-DHB-) were found to be secreted at higher levels into the extracellular medium in the presence of *B. cinerea*, confirming the importance that this pathway might have in yeast-fungal interactions. The fact that these three metabolites are only secreted by one of the two yeast strains tested indicates that the production of secondary metabolites by the phenylpropanoid pathway might be a strain-dependent adaptative strategy.

It has been described that 2,3-DHB is an important phenolic compound in bacteria, it being the immediate precursor of catecholate siderophores such as enterobactin, chromobactin or viobactin (Batista et al., 2019). It is known to be secreted by bacteria under iron-limiting conditions and to have metal-chelating capacity (Wienhausen et al., 2017). In response to iron deficiency in their environment, many microorganisms produce and export compounds called siderophores, which are able to chelate Fe^{3+} to provide iron for metabolic processes. For example, the metal-binding 2,3-DHB was also detected in the exometabolome of the bacterium *Dinoroseobacter shibae* (Wienhausen et al., 2017). *M. pulcherrima* is also known for its capacity to deplete iron as a way to inhibit fungal and bacterial growth. Although the pigment pulcherrimin secreted by *M. pulcherrima* seems to be the best described iron immobilizing agent (Arnaouteli et al., 2019; Sipiczki, 2020), yeast cells might produce other siderophores and we can speculate that 2,3-DHB might be one of them. However, reports on the antifungal activity of 2,3-DHB is limited in the literature. Benny et al. (2010) purified 2,3-DHB from fruits of *Flacourtia inermis* Roxb and demonstrated its antifungal efficiency against some human opportunistic fungi such as *Aspergillus* and *Chrysosporium*, and suggested that 2,3-DHB can be used as a powerful medical antifungal agent. In the present study we also confirmed 2,3-DHB antifungal activity against *B. cinerea*, although only under *in vitro* conditions.

PCQA is the other metabolite found in this phenylpropanoid pathway. It is a type of chlorogenic acid, which are metabolites widely distributed and studied in many kinds of seeds and fruits (Mullen et al., 2011; Tajik et al., 2017), although not previously mentioned from microorganisms. Although no antifungal activity was detected under our experimental conditions, several studies claim the antioxidant and antimicrobial activity of chlorogenic acids against human pathogens (Tajik et al., 2017). In addition, Chen et al. (2019) described PCQA as one of the components of *Helianthus tuberosus* leaves, whose extracts are active against the oomycete pathogen *Phytophthora capsici* Leonian.

Other metabolites with increased secretion by one or both yeast strains and with *in vitro* antifungal activity were 5-methyltetrahydrofolate, 8,8a-deoxyoleandolide, catechol, dethiobiotin and hexadecanedioic acid. The antifungal activity of some of them has been reported before. For example, the capacity to produce catechol is naturally present in several microbial species. A plethora of investigations have documented its broad-spectrum against bacteria and phytopathogenic fungi; for example against *Botryosphaeria dothidea* (Li et al., 2020), *F. oxysporum* and *Penicillium italicum* (Kocaçalişkan et al., 2006). The polyketide 8,8a-deoxyoleandolide is secreted by both yeast strains. Its production was reported in *Streptomyces* and it is an intermediate in the biosynthesis of antibiotics such as oleomycin (Parisi et al., 2020). In the current context, both yeast strains secreted the antibiotic β-rhodomycin in the co-culture with *B. cinerea*. Secretion of this antibiotic has been described in *Streptomyces* sp. (Vanek et al., 1977) but never by a yeast. In addition, the strain Mp-22 also showed enhanced secretion of an intermediate in the biosynthesis of neomycin, kanamycin and gentamycin (2’’’-N-Acetyl-6’’’-deamino-6’’’-hydroxyneomycin C). Therefore, it seems that the presence of *B. cinerea* in the co-culture conditions stimulated the metabolic pathways for production of antibiotics by the yeast.

After biphenyl-2,3- diol, dethiobiotin was the second metabolite that showed a more marked *in vitro* inhibition of mycelium growth. It is the last intermediate in the biosynthesis of vitamin B7 (biotin), a vitamin required as a cofactor for many carboxylation reactions (Wu et al., 2005). Biotin can be produced by some yeasts (Wu et al., 2005), and it has also been detected in the exometabolome of the bacterium *Phaeobacter inhibens* (Wienhausen et al., 2017). Although exogenous supply of dethiobiotin has been demonstrated to cause growth inhibition in several biotin requiring fungi and bacteria, the significance of released dethiobiotin on microbial communities is still open and needs further studies (Wienhausen et al., 2017).

Hexadecanedioic acid is a naturally occurring long chain dicarboxylic acid (LCDA). A possible explanation of its antifungal effect observed *in vitro* stems from evidence for these lipophilic weak acids crossing the plasma membrane by passive diffusion and causing cellular toxicity (Cabral et al., 2001; Legras et al., 2010). Another aspect worth noting is the current focus of the biotechnology industry on sustainable production of LCDAs, which are used in the manufacture of a number of

products including polyamides, polyesters, perfumes, plasticizers, lubricants, and adhesives (Huf et al., 2011). Some yeasts such as *Candida tropicalis* have been studied for their ability to produce LCDAs when cultured on alkanes or fatty acids as the carbon source (Craft et al., 2003). From an industrial point of view, it would be interesting to explore the ability of the Mp-30 strain to naturally produce this LCDA (even on a minimal medium) when grown in co-culture with *B. cinerea*.

Screening of new BCAs often starts by using *in vitro* assays to detect antagonists that secrete antimicrobial metabolites in the artificial environment. However, this usually leads to an overestimation of the importance of this mode of action relative to other mechanisms that cannot be detected with *in vitro* assays, thus generating a biased result and excluding other modes of action (Köhl, 2019). In fact, our experimental data showed that the *in vitro* antifungal effects cannot always be extrapolated to *in vivo* protection. Therefore, conducting *in vitro* and *in vivo* studies in parallel is a more rigorous approach. According to our results and other reports in the literature (Crowley et al., 2013; Le Lay et al., 2016), antifungal activity can result from a large panel of active molecules of various chemical families that may have antifungal power on their own or that need to act synergistically. This fact would partially explain the discrepancies between the *in vitro* and *in vivo* antifungal results.

4.3. Other metabolites identified with putative significance for fungal antagonism and/or microbial interaction

The observed increase in alkaloids or their precursors in culture filtrates in the presence of *B. cinerea* is another point of great interest. Alkaloids are one of the most diverse groups of secondary metabolites. They are produced by a large variety of organisms including animals, plants, fungi and bacteria and have a wide range of pharmacological and antimicrobial activities in humans (Goyal, 2013). In addition, alkaloids have antifungal properties against plant phytopathogens (Ma et al., 2000; Singh et al., 2002; Zhou et al., 2003). The metabolite deacetylisopecoside is a precursor of protoemetine, an isoquinoline alkaloid that is also precursor of other known alkaloids such as emetine or cephaeline, with high value in the pharmaceutical industry. Among known natural fungal products, isoquinoline alkaloids are rare, although some studies suggest that fungi are capable of producing them (Baccile et al., 2016). Another alkaloid that has been reported as being produced naturally by the plant *Catharanthus roseus* is 16-hydroxytabersonine (Sun et al., 2018). This chemical is an intermediate in the formation of vindoline, a precursor needed for the synthesis of the pharmaceutically valuable alkaloids vinblastine and vincristine, which are widely used to treat cancers (Nobili et al., 2009). *C. roseus* is still the exclusive source for the industrial production of these compounds. Lastly, piperideine represents the precursor of another family of alkaloids with demonstrated efficacy against phytopathogens such as *Pythium ultimum* for controlling damping off in cucumber (Li et al., 2012). In this work we could not perform antifungal bioassays with four alkaloids and/or precursors found in culture filtrates due to commercial unavailability, so their putative roles as antifungal secondary metabolites against *B. cinerea* is only a hypothesis, and they could play important functions in other aspects of yeast metabolism. However, their discovery in the *M. pulcherrima* exometabolome represents a valuable starting point to investigate alkaloid pathways in yeasts and possible new applications.

Another remarkable point is that the 2-oxocarboxylic acid (2-OCA) pathway was clearly enhanced, with several metabolites significantly secreted, including amino acids. The 2-OCAs are important intermediates in central carbon metabolism, both in anabolism and catabolism. Since they form the link between the metabolism of carbohydrates, fats and amino acids (Stottmeister et al., 2005), it is not surprising to find this pathway enhanced. However, it is difficult to elucidate a particular role for these metabolites in the co-culture assay. In this context, the significant accumulation of the Val-Leu dipeptide in the culture filtrate of Mp-30 strain is striking. In a recent publication, Luzarowski et al. (2021) made a global mapping of protein–metabolite interactions in *Saccharomyces cerevisiae*, revealing that of the 74 small molecules co-eluting with proteins, 36 were proteogenic dipeptides, one of them being Val-Leu. They concluded that the role of dipeptides is very important as metabolic regulators at the interface of protein degradation and central carbon metabolism. Here, we demonstrated that yeast Mp-30 secretes the dipeptide Val-Leu in response to the presence of *B. cinerea*. However, when applied externally in *in vitro* or *in vivo* biocontrol assays, no antagonism at all was observed, pointing towards a more intricate metabolic function.

Interestingly, taurocholic acid, described as a mammalian bile acid, was present in the culture filtrates of both strains as well as its precursor pantetheine in the Mp-30 exometabolome. To date, *M. pulcherrima* strains Mp-22 and 30 are the first example of a taurocholic acid-producing yeast. Kim et al. (2010) reported as a rare finding a marine bacterium that produced this acid. Recently, Cheema et al. (2021) also observed this metabolite in a *Streptomyces* culture, although rather than a true secondary metabolite, they attributed its presence to the bioconversion of medium components. In our study, secretion of taurocholic acid was observed as a direct result of the presence of *B. cinerea* in the culture medium, confirming that it likely acts as a secondary metabolite. From the pharmaceutical point of view, taurocholic acid is used as cholagogue in the production of vaccines and as a vehicle to assist with drug delivery. It is currently manufactured commercially by purification from cattle bile. Exploring an alternative yeast source would result in great biotechnological interest.

Finally, the presence and increased accumulation of 2-C-methyl-D-erythritol-4-phosphate (MEP) in the culture filtrates of both yeast strains was surprising. It is generally accepted that the mevalonic acid (MVA) pathway in yeast and most other eukaryotes and the MEP pathway in most plant plastids and bacteria are responsible for the synthesis of isoprenoids (Maury et al., 2005). The formation of isoprenoids by yeasts is limited to trace concentrations by a small number of non-*Saccharomyces* species, with *M. pulcherrima* being among them (Carrau et al., 2005), and it has been shown that isoprenoids of five fungal species, including the yeast *Rhodotorula glutinis*, are synthesized exclusively *via* the MVA pathway (reviewed by Lange et al., 2000). In fact, in the entirely sequenced genomes of *Schizosaccharomyces pombe* and *S. cerevisiae*, homologues for all genes of the MVA pathway are found, with no evidence for the occurrence of MEP precursor enzyme genes (Lange et al., 2000). Although these studies support the notion that MEP pathway would not be functional in yeasts (Disch and Rohmer, 1988), the confirmed presence and accumulation of MEP in co-culture with *B. cinerea* may have a significance that deserves further investigation.

5. Conclusions

While efforts are ongoing to identify the mechanistic components underlying fungal antagonism, this is the first report to describe a metabolomic approach to understanding the biocontrol effects of yeast against a fungal plant pathogen. In this study, we characterized the nature of antifungal compounds present in the exometabolome of yeast strains that previously showed an efficient inhibition of *B. cinerea* infection. *In vitro* and *in vivo* methods were used in parallel to look for antifungal metabolites present in bioactive culture filtrates, yielding several new biocontrol metabolites with potential agronomic applications in the future. Formulating antimicrobial metabolites into a post-harvest protection product without the living microorganism being present could result in a more convenient product. Furthermore, this is the first time that yeast cells have been shown capable of synthesizing and secreting several metabolites including a number of alkaloids, antibiotics, long chain fatty acids and even bile acids, with a potential biotechnological use. The development of this comprehensive study brings insights into the complex regulatory processes that prompt yeast to secrete metabolites during a direct confrontation with a fungal counterpart and highlights the generation of a large panel of active

molecules that probably act synergistically in antifungal activity.

CRediT authorship contribution statement

Alicia Fernandez-San Millan: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Jordi Gamir:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Luis Larraya:** Formal analysis, Methodology. **Immaculada Farran:** Methodology, Funding acquisition. **Jon Veramendi:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Funding acquisition.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2022.111995.

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