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Antifungal activity of β-carbolines on *Penicillium digitatum* and *Botrytis cinerea*

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ABSTRACT

β-carbolines (βCs) are alkaloids widely distributed in nature that have demonstrated antimicrobial properties. Here, we tested *in vitro* six βCs against *Penicillium digitatum* and *Botrytis cinerea*, causal agents of postharvest diseases on fruit and vegetables. Full aromatic βCs (harmine, harmol, norharmane and harmane) exhibited a marked inhibitory effect on conidia germination at concentrations between 0.5 and 1 mM, while dihydro-βCs (harmalina and harmalol) only caused germination delay. Harmol showed the highest inhibitory effect on both fungal pathogens. After 24 h of exposure to 1 mM harmol, conidia revealed a severe cellular damage, exhibiting disorganized cytoplasm and thickened cell wall. Harmol antimicrobial effect was fungicidal on *B. cinerea*, while it was fungistatic on *P. digitatum*. Conidia membrane permeabilization was detected in treatments with harmol at sub-inhibitory and inhibitory concentrations, for both pathogens. In addition, residual infectivity of *P. digitatum* on lemons and *B. cinerea* on blueberries was significantly reduced after exposure to this alkaloid. It also inhibited mycelial growth, preventing sporulation at the highest concentration tested. These results indicate that harmol might be a promising candidate as a new antifungal molecule to control causal agents of fruit diseases.

1. Introduction

Several fungi cause postharvest diseases on fruit and vegetables producing important economic losses (Marquenie et al., 2003). Among them, *Penicillium digitatum*, green mold causal agent, is the most common postharvest pathogen of citrus fruit. Synthetic fungicides such as imazalil and thiabendazole are widely used to control this pathogen (Palou et al., 2002). *Botrytis cinerea* Pers.: Fr. (teleomorph: *Botryotinia fuckeliana*), another economically important phytopathogen, is the causal agent of gray mold in over 200 plant species worldwide, such as grapes, stone-fruit, berries, and vegetables (Cantu et al., 2009; Elad and Evensen, 1995). Benzimidazoles and dicarboximides have been the most widely used fungicides to control the disease caused by this pathogen (Garber et al., 1997).

The continuous use of commercial fungicides has resulted in the appearance of resistant fungal strains (Latorre et al., 1994). The identification of unexplored chemical structures as potential antifungal compounds is an important strategy to control these pathogens (Gellerman et al., 2009). Some natural products isolated from plants exert antifungal activity and could be good alternatives to commercial fungicides (Grayer and Kokubun, 2001). β-carbolines (βCs) comprise a class of natural and synthetic alkaloids that are widely distributed in plants, foodstuffs, marine creatures, insects, mammalians, and humans, among others. βCs are a large group of heterocyclic compounds with a 9H-pyrido[3,4-*b*]indole structural unit that were first isolated from *Peganum harmala* (Zygophyllaceae, Syrian Rue) (Cao et al., 2007). These compounds are of great interest due to their antitumoral, antiviral, antimicrobial and antiparasitic activities (Alomar et al., 2013; Cao et al., 2007). For instance, these alkaloids have demonstrated activity against a wide variety of microorganisms. The βC norharmane exhibited inhibitory effect on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Metarhizium anisopliae* and *Aspergillus nomius* (Chouvenc et al., 2008; Volk and Furkert, 2006; Xing et al., 2012); harmaline

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was active against *Candida albicans* and *Staphylococcus aureus* (Schmeller and Wink, 1998; Xing et al., 2012); harmol against *Trypanosoma cruzi* (Rivas et al., 1999); and norharmane, harmane and harmine against *Toxoplasma gondii* (Alomar et al., 2013).

As a first step towards finding new antifungal molecules to control green and gray molds, the aim of this work was to evaluate the effect of six βCs (harmine, harmol, norharmane, harmane, harmaline and harmalol) on *P. digitatum* and *B. cinerea*.

2. Materials and methods

2.1. Chemicals and stock solutions

Chemical structures of full aromatic βCs (harmine, harmol, norharmane, and harmane) and dihydro-βCs (harmalina and harmalol) are shown in Fig. 1. Drugs were of the highest purity available (>98%, Sigma-Aldrich Co., St. Louis, MO). βC stock solutions (~50 mM) were prepared by dissolving each alkaloid in dimethyl sulfoxide (DMSO, Sigma-Aldrich Co., St. Louis, MO). Solution concentrations were determined by UV–vis spectrophotometry with appropriate dilutions prepared in distilled water at pH 5, using epsilon values previously reported (Alomar et al., 2014; Gonzalez et al., 2009).

2.2. Fungal isolates, growth conditions and conidial suspension preparation

Fungal isolates used in this work were *P. digitatum* PD-A, obtained from naturally infected citrus fruit from Tucumán-Argentina (Cerioni et al., 2009), *B. cinerea* B01, isolated from naturally infected blueberries, and *B. cinerea* B05.10, provided by the Instituto César Milstein – Fundación Cassará. B01 identification was carried out according to keys previously established (Pitt and Hocking, 1997), and confirmed by molecular methods using specific primers $C729^{+}$ and $C729^{-}$ (Rigotti et al., 2002) and genomic DNA extracted by alkaline lysis (Moller et al., 1992). A single band of 0.7 kb that is specific to *B. cinerea* was amplified. B05.10 was used as reference strain. PD-A and B01 isolates were deposited with the codes ICFC 842/15 and ICFC 841/15, respectively, in the ICFC (IIB-INTECH collection of Fungal Cultures, Laboratory of Mycology and Mushroom cultivation, IIB-INTECH; Chascomús, Argentina; WDCM data base reference: 826).

Fungal cells were grown on potato dextrose agar (PDA) plates at 22 ± 1 °C, in the dark for 7–10 d. *B. cinerea* was induced to sporulate by placing a sterile wood stick on the growing colony and incubating for further 7 d.

To obtain conidial suspensions, fungal material from the culture surfaces was resuspended in sterile distilled water containing 0.5% Tween 80 (Sigma-Aldrich Co., St. Louis, MO), thoroughly vortexed and filtered through several layers of sterile cheesecloth. The concentration was adjusted to 10^6 conidia ml^{-1} by counting in a Neubauer chamber and diluting in potato dextrose broth (PDB) (pH 5).

2.3. Conidia germination and viability

Aliquots of βCs dilutions, to final alkaloid concentrations of 0.1, 0.25, 0.5 and 1 mM, were added to wells in microtiter plates containing conidial suspensions in PDB. Controls containing 2% DMSO without βC were included. Plates were incubated at 22 ± 1 °C in the dark and germination was evaluated at different incubation times by observation with an invert light microscope (Olympus IX51 equipped with an Olympus digital camera, QColor5 (Q-imaging)). The percentage of germination was estimated by counting the number of germinated conidia from 300 conidia. Conidia were considered germinated when the germ tube length was equal to or greater than conidial diameter. For each condition, four replicates were performed and the assay was done three times.

To determine whether harmol effect is fungicidal or fungistatic, conidial viability was evaluated after treatments. Conidial suspensions were incubated with different harmol concentrations during 24 h. The alkaloid was then removed by centrifugation at 10,000 rpm for 10 min, and the supernatant was replaced with the same volume of sterile distilled water. Suspensions were serially diluted and spread on PDA medium. Cell survival was quantified as colony forming units (CFU) ml^{-1} after 4 d of incubation at 22 ± 1 °C. Three replicates were performed for each condition, and the trial was repeated three times.

2.4. Conidial membrane integrity

Conidial membrane integrity was studied by the uptake of SYTOX Green. The binding of this fluorescent dye to nucleic acids results in a >500-fold enhancement in its emission, so it has been used to assess the integrity of biological membranes (Roth et al., 1997). The technique was applied as follows: conidial suspensions were prepared in PDB amended with 0.5 μM SYTOX Green. One hundred-microliter aliquots of the suspensions were mixed in 96-

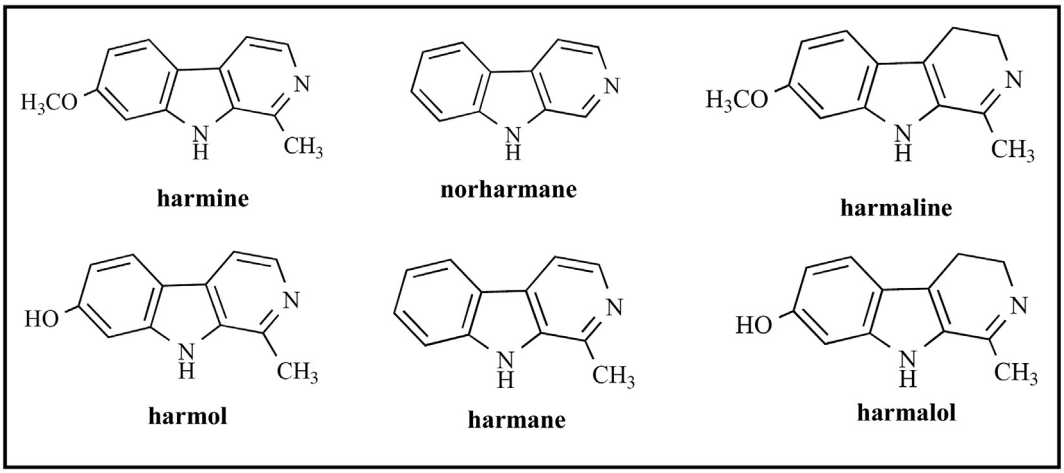

Fig. 1. Structure of the six βCs studied: harmine, harmol, norharmane, harmane, harmaline and harmalol.

well microplates with harmol in order to reach 0.25 mM (subinhibitory), 0.5 mM and 1.0 mM (inhibitory) final concentrations. Microplates were incubated statically for 24 h at 22 ± 1 °C, and conidia were visualized under a fluorescence microscope Olympus IX51 equipped with an Olympus digital camera, QColor5 (Q-imaging). The fluorescence emission was examined and photographed using a filter set of 450–490 nm for excitation and of 515–565 nm for emission. Three replicates were performed for each condition, and the assay was done three times.

2.5. Conidia residual infectivity

Residual infectivity of harmol treated conidia was evaluated on lemons (cv. Genova) for PD-A and blueberries (cv. Snowchaser) for B01. Lemon infections were carried out by wounding peel with a steel rod previously immersed in conidial suspensions prepared in distilled water (controls) or treated with harmol during 24 h. The steel rod was 1-mm-wide and 2-mm-long, and penetrated both flavedo and albedo lemon tissues. Lemons were placed in plastic trays into bags, and stored at 20 °C and 95% relative humidity for 14 d. Blueberries were harvested at a stage between petal fall and early green fruit, being this fruit the most vulnerable to *Botrytis*. Fruit were immersed in untreated (controls) or harmol-treated conidial suspensions during 10 min in an orbital shaker at room temperature. After that, blueberries were air dried and placed in humid chambers, which were maintained at 20 °C for 14 d.

Green and gray mold incidence was expressed as percentage of decayed fruit per total evaluated fruit after 14 d of incubation. For each condition, two replicates of 15 lemons or 25 berries were performed. The assays were repeated twice.

2.6. Mycelial growth

Harmol activity against fungal pathogens mycelium was assayed by spectrophotometry. Conidial suspensions were added into 96-well microtiter plates (polystyrene; Orange Scientific, USA) and incubated under static conditions at 22 ± 1 °C in the dark. After 24 h of incubation, mycelial growth was quantified by determination of OD_{420nm} (SpectraMax Plus384 Absorbance Microplate Reader, US). Then, harmol was added to wells at final concentrations of 0.1, 0.5 and 1 mM. Controls containing DMSO without harmol were included. Mycelial growth was again determined after incubation for further 24 h. Mycelial growth was expressed as the difference between final and initial OD_{420nm}. Three replicates were performed for each condition, and the assay was done three times.

2.7. Statistical analysis

In all assays, data were subjected to analysis of variance followed by Tukey's test with Infostat 2013 version for Windows. Differences of p value ≤ 0.05 were considered significant.

3. Results

3.1. Inhibition of conidia germination by βCs

The biological effect of full aromatic βCs on conidia germination was evaluated after 8, 12, 24 and 48 h of incubation (Fig. 2). All tested compounds exhibited toxicity against fungi in a dose-dependent manner. Harmane completely inhibited conidia germination of both phytopathogens at a concentration of 1 mM. Norharmane inhibited B01 and PD-A germination at 1 mM and 0.5 mM, respectively. 0.5 mM harmine inhibited B01germination, while 1 mM only produced a delay on PD-A germination. Harmol inhibited germination of both microorganisms at 0.5 mM.

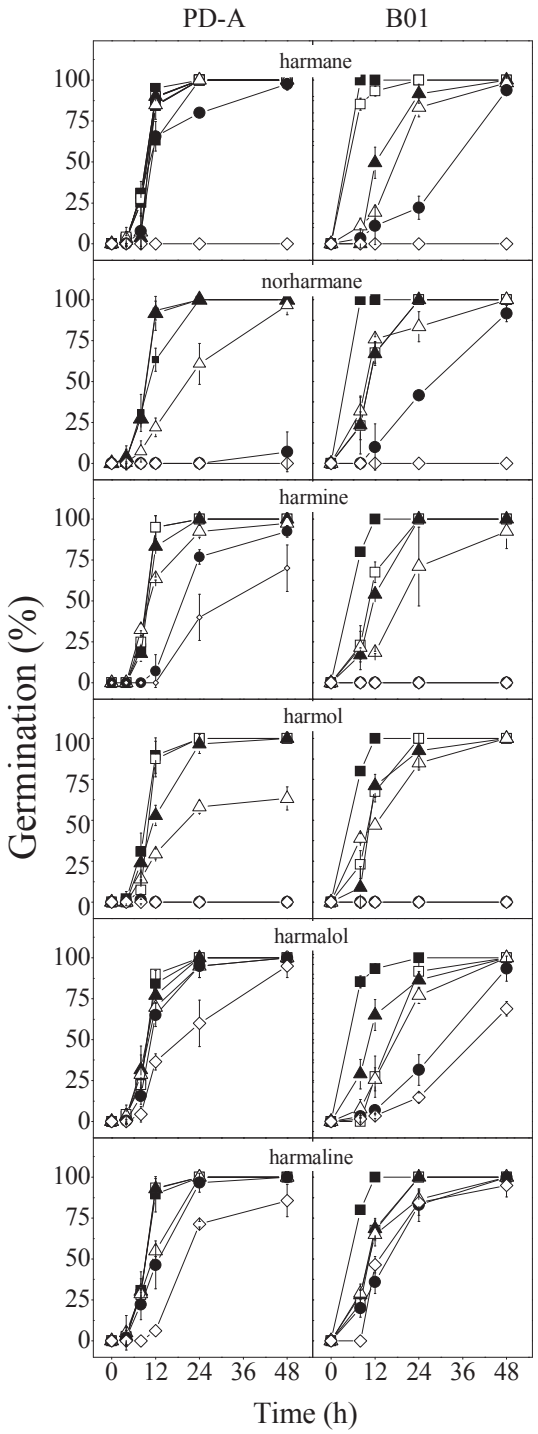

Fig. 2. Effect of βCs on conidia germination. Germination of PD-A and B01 conidia were determined at the indicated incubation times after exposure to different βCs concentrations. ■: control; □: control with DMSO; ▲: 0.1 mM; Δ: 0.25 mM; ●: 0.5 mM; or ◊: 1 mM. Three independent experiments were performed including four replicates for each condition. Results are expressed as the mean percentage of germinated conidia ±SD.

Noteworthy, conidia of both pathogens remained ungerminated after 7 d of incubation in the presence of harmol (data not shown). On the other hand, dihydro-βCs (harmaline and harmalol) caused a weaker effect than full aromatic βCs, since they inhibited around 30% of germination at 1 mM. All βCs were assayed against the *B. cinerea* reference strain B05.10, affecting the germination

similarly to B01 (data not shown). Among assayed alkaloids, harmol was the most active against *P. digitatum* and *B. cinerea*, thus, this compound was selected for further experiments.

In order to determine viability of ungerminated conidia, CFU ml^{-1} counts were carried out at different times of exposure to harmol. B01 conidia treated with 0.5 mM harmol for 24 h showed a viable count reduction from 10^6 to 10^3 CFU ml^{-1}, while treatment with 1 mM completely inhibited colony formation. By contrast, exposure of PD-A conidia to 0.5 mM and 1 mM harmol only reduced viable counts around 2 orders of magnitude in respect to the control. Similar results to those of 24 h were obtained for both pathogens after 48 h of incubation.

3.2. Conidia morphological changes produced by harmol

To analyze whether this compound produced any morphological changes in conidia, PD-A and B01 suspensions were exposed to harmol during 24 h (Fig. 3). In 0.25 mM harmol treatments, around 50% of conidia were able to germinate, but their germination tubes were markedly shorter in respect to controls (PD-A in panels a and c; B01 in panels b and d). Higher harmol concentrations completely inhibited the germination process (panels e to h). Noteworthy, B01 conidia treated with 1 mM harmol exhibited disorganized cytoplasm and thicker cell wall compared to conidia treated with 0.5 mM (inserts in panels f and h).

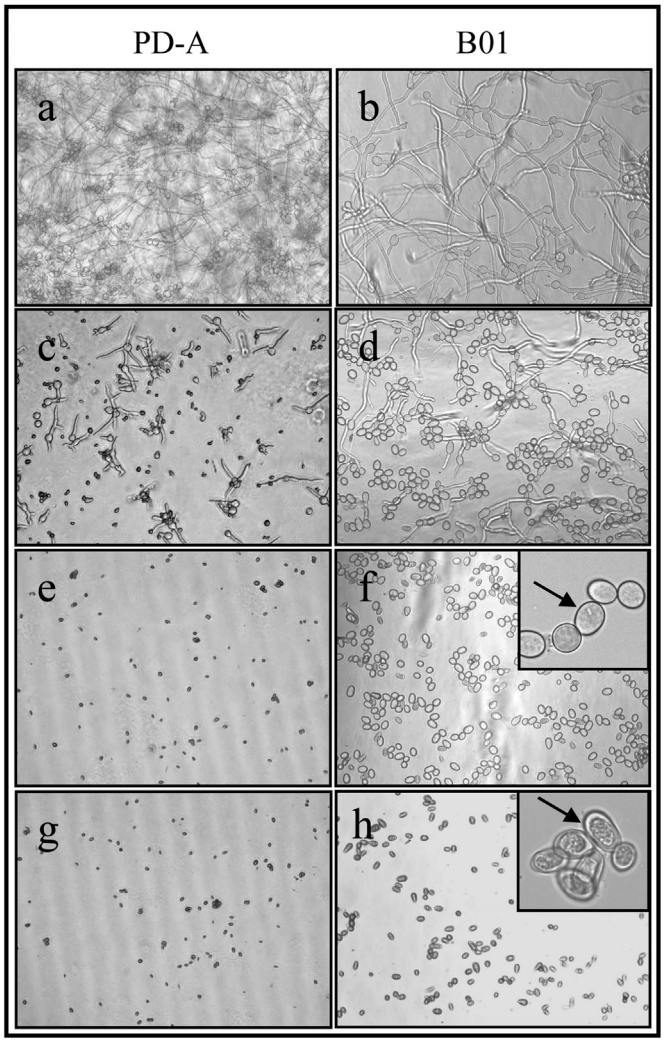

Fig. 3. Effect of harmol on fungal morphology. PD-A and B01 conidia were incubated with the indicated harmol concentrations during 24 h. Photographs in panels and inserts were taken using light microscopy at 40 and 100× magnifications, respectively. Panels show controls with DMSO (**a** and **b**), and treatments with different harmol concentrations: 0.25 mM (**c** and **d**), 0.5 mM (**e** and **f**), and 1 mM (**g** and **h**). Images are representative of three independent experiments.

3.3. Membrane permeabilization by harmol

The integrity of the conidia membrane after exposure to harmol was evaluated with the Sytox Green dye. Fluorescence was not detected in control conidia, while a 24 h exposure to 1.0 mM harmol generated membrane permeabilization in 100% conidia of both strains (Fig. 4). Treatments with 0.5 mM harmol also permeabilized conidia membranes (data not shown). After exposure to 0.25 mM harmol, most of the ungerminated PD-A conidia were fluorescent, while mycelium was not permeable to the dye. In the case of B01, ungerminated conidia exhibited a limited uptake of the fluorescent dye.

3.4. Decreased conidia infectivity after exposure to harmol

As an indicator of conidia residual infectivity, green and gray molds incidence on fruit was evaluated 14 d post inoculation (Fig. 5). In the case of PD-A, treatments with 0.5 mM and 1 mM harmol reduced green mold incidence on lemons to 55% and 17%, respectively, compared with controls. For B01, treatment with 0.5 mM harmol reduced gray mold incidence on blueberries in around 70%. As it was expected, after treatment with 1 mM harmol (lethal condition), no infected blueberries were detected.

3.5. Mycelial growth inhibition by harmol

The exposure of fungal mycelia to both 0.5 and 1 mM harmol during 24 h produced a growth inhibition of 60% for PD-A and 45% for B01 (Fig. 6A). It is important to note that after 7 d of incubation, both pathogens grown in the presence of harmol were unable to sporulate (Fig. 6B).

4. Discussion

Although the βCs antimicrobial activity against bacteria and yeasts has been well documented (Arshad et al., 2008; Cao et al., 2007; Mayad et al., 2013), the present work is the first report on this activity against phytopathogenic fungi. *P. digitatum* and *B. cinerea* germination was inhibited mainly by exposure to harmol and other βCs with planar molecular structure (full aromatic derivatives), whereas dihydro-βCs (harmaline and harmalol) only delayed germination. These two groups differ in their chemical and physicochemical properties: (i) full aromatic βCs are more acidic than the dihydro-derivatives (pK_a values of ~7–8 (Gonzalez et al., 2009) and ~9–10 (Alomar et al., 2014), respectively), allowing deprotonation of the former under neutral pH; (ii) full aromatic βCs are essentially planar and interact with biomolecules (i.e., DNA) by intercalation (Duportail and Lami, 1975), with binding constants 100 to 1000 times higher than those of the corresponding dihydro-derivatives (Taira et al., 1997); (iii) among the four full aromatic βCs investigated, harmol shows the highest polarity. The above mentioned facts and the differences observed in our results are indicative of the relevance of deprotonation, DNA-intercalative capacity, and polarity, in providing antifungal action to these molecules. Previous works reported that molecules with planar structure, such as harmine and harmane, exhibited higher antifungal activity against *Aspergillus niger*, compared with non planar molecules, as harmaline and harmalol (Nenaah, 2010).

Harmol, the most active compound against PD-A and B01,

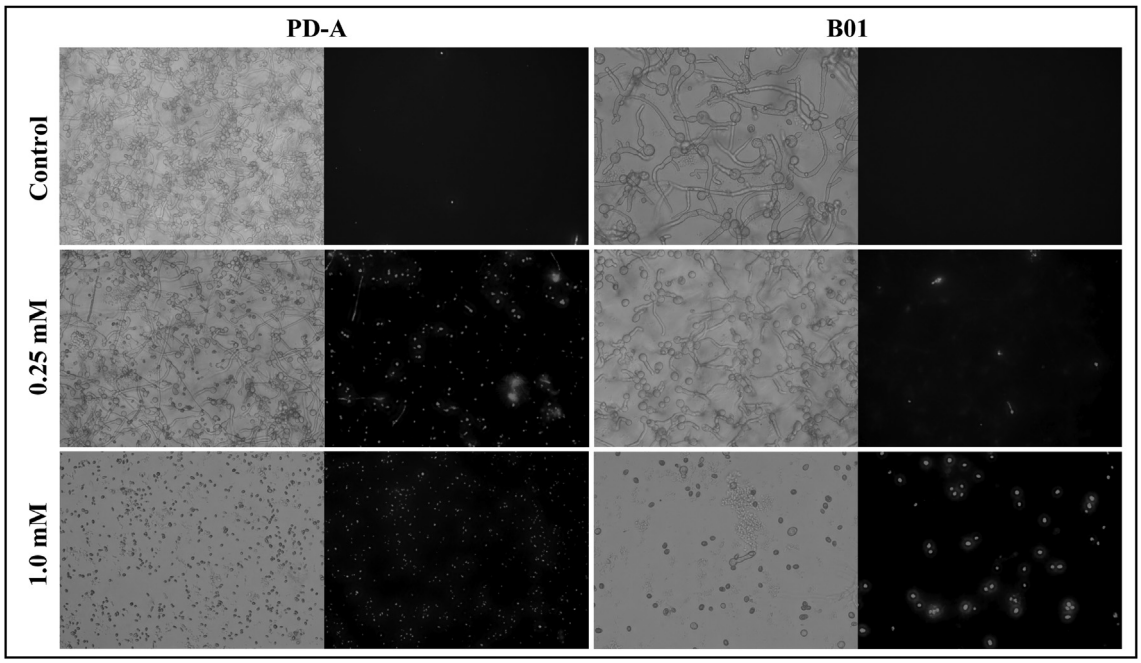

Fig. 4. Effect of harmol on membrane integrity. Conidial suspensions prepared in PDB containing 0.5 μM SYTOX Green were exposed to the indicated harmol concentrations. Photographs were taken using an invert microscope after 24 h of incubation. For each strain, the left panels show the bright field, while the right panels show the fluorescence images of the same samples (Magnification 40×). Photographs are representative of three independent experiments.

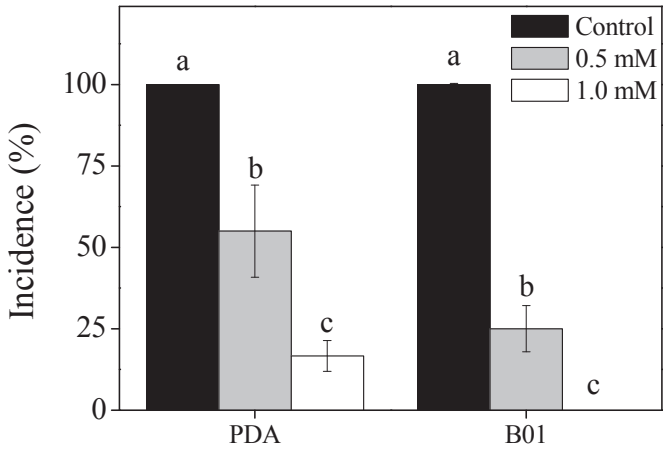

Fig. 5. Green and gray molds incidence on lemons and blueberries. Fruit were inoculated with control or harmol treated conidia and incubated during 14 d. Values are the mean of the incidence percentage ±SD of two independent assays. For each pathogen, different letters indicate significant differences among treatments according to Tukey's test with a p value ≤ 0.05.

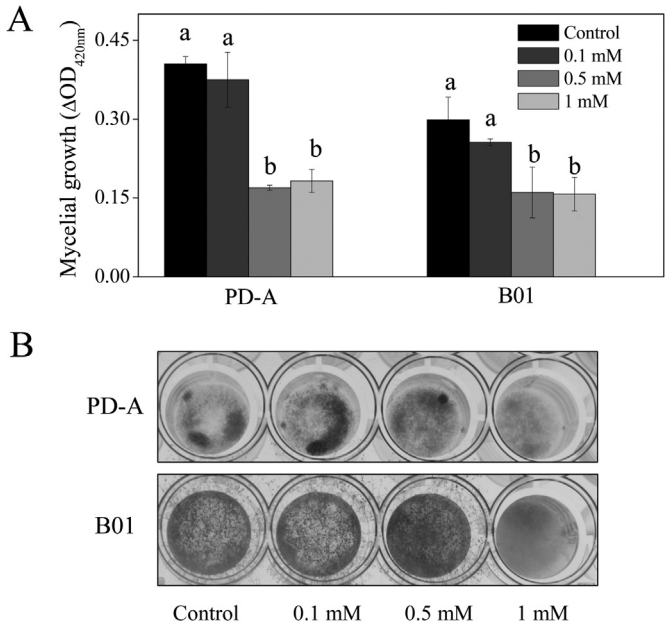

Fig. 6. Effect of harmol on mycelial growth and sporulation. Mycelia grown during 24 h were exposed to the indicated harmol concentrations. Three replicates were performed for each condition, and the assay was done three times. **A**: Differences between final and initial OD_{420nm} ±SD. For each pathogen, different letters indicate significant differences among treatments according to Tukey's test with a p value ≤ 0.05. **B**: Representative images of mycelial growth after 7 d of incubation.

exhibited differential effects when comparing conidia viability of both microorganisms. CFU ml^{-1} counts revealed a fungistatic action against PD-A and a fungicidal effect on B01. In blueberries, the reduction of gray mold incidence was consistent with the lethality confirmed by the viability assay. In respect to PD-A, green mold incidence was significantly reduced after exposure to harmol compared to controls, despite a large proportion of conidia remained viable. This effect can be attributable to a reduction on *P. digitatum* virulence due to the treatment. However, it cannot be discarded that the amount conidia was insufficient to develop disease on fruit (Baert et al., 2008; Vilanova et al., 2012).

The sporulation inhibition achieved by harmol becomes relevant considering that conidia act as an airborne inoculum which determines subsequent disease development (Elad and Evensen, 1995). During postharvest fruit management, the reduction of conidial production by the application of anti-sporulant compounds represents a desirable effect, minimizing cosmetic defects of the unsightly contamination of adjacent fruit (Eckert and Brown, 1986) and protecting fruit from future infection (Njombolwana

et al., 2013).

It has been reported that the mode of action of βCs as antimicrobial agents is related to a broad spectrum of causes, such as arrest of a specific phase of cells cycle, inhibition of certain signal transduction enzymes (Di Giorgio et al., 2004), non-specific membrane damage (Lala et al., 2004), inhibition of respiratory chain (Rivas et al., 1999), and induction of programmed cell death (Rosenkranz and Wink, 2007). Here, harmol caused membrane disruption in conidia of both phytopathogens, maybe as a first step in its antimicrobial action. Namely, this alkaloid might produce an initial structural disruption and alteration of the membrane, before generating intracellular damage. Several authors have already related antimicrobial action of many agents with membrane permeabilization (Badosa et al., 2009; Cerioni et al., 2010; Makovitzki et al., 2006; Muñoz et al., 2006). Our results represent the first report on membrane permeabilization induced by the βC harmol, although further studies should be done to elucidate the mechanism of action of this compound.

5. Conclusions

We report for the first time that full aromatic βCs have significant antimicrobial properties against *P. digitatum* and *B. cinerea*, being harmol the most effective. Exposure to this compound inhibited germination, decreased conidia residual infectivity, permeabilized conidial membrane, and produced intracellular damage. After treatment with this alkaloid, mycelial growth was partially inhibited and sporulation was suppressed. These results indicate that harmol might be a promising candidate as a new antifungal compound to control causal agents of fruit diseases.

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