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

Gabriela M. Olmedo [a], Luciana Cerioni [a], M. Micaela González [b], Franco M. Cabrerizo [b], Viviana A. Rapisarda [a], Sabrina I. Volentini [a, *]

[a] Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Química Biológica "Dr. Bernabé Bloj", Facultad de Bioquímica, Química y Farmacia, UNT. Chacabuco 461, CP T4000ILI, San Miguel de Tucumán, Tucumán, Argentina
[b] IIB-INTECH (sede Chascomús), UNSAM-CONICET, Intendente Marino Km 8,2, CC 164, CP 7130, Chascomús, Buenos Aires, Argentina

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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

* Corresponding author.
E-mail address: sabrina@fbqf.unt.edu.ar (S.I. Volentini).

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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-

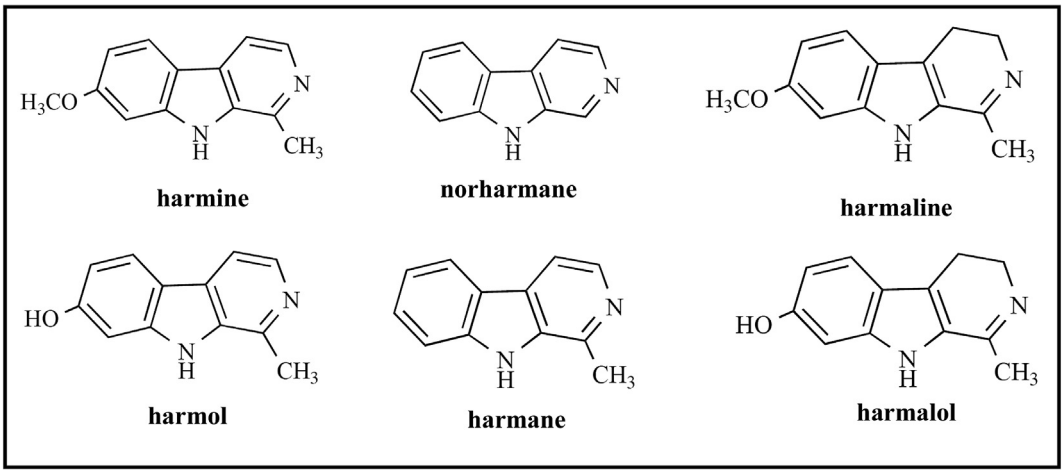

Fig. 1. Structure of the six βCs studied: harmine, harmol, norharmane, harmane, harmaline and harmalol.