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Antifungal activities of extracts produced by liquid fermentations of Chilean *Stereum* species against *Botrytis cinerea* (grey mould agent)

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ABSTRACT

Extracts obtained from liquid mycelial fermentations of Chilean species of the *Stereum* genus showed antifungal activity against *Botrytis cinerea*. Thirty-six strains were evaluated *in vivo* and *in vitro* assays, 25 belonging to *Stereum hirsutum* (Sh) and 11 to *Stereum rameale* (Sr). Two types of extracts were obtained: EtOAc-extract (liquid phase) and MeOH-extract (mycelial phase). Plate diffusion assay showed that EtOAc-extracts were more active than MeOH-extracts. *S. hirsutum* included 4 strains with the highest antifungal activity (Sh134-11, Sh144-11, Sh152-11, Sh155-11) and *S. rameale* highlighted with two strains (Sr25-11, Sr27-11). Effects on the mycelial growth of *B. cinerea* showed that EtOAc-extracts produced by *S. hirsutum* (Sh134-11, Sh152-11) inhibited from 1000 μg mL^{-1}, reaching 67 and 49%, respectively. At 2000 μg mL^{-1}, these strains inhibited nearly 80% of mycelial growth. EtOAc-extract of Sh134-11 was more effective to control the sporogenesis, inhibiting in 100% the sporulation at 500 μg mL^{-1}. Assays showed that Sh134-11 and Sh152-11 exhibited a minimal fungicidal concentration (MFC) of 50 and 100 μg mL^{-1} respectively and minimal inhibitory concentration (MIC) at 20 μg mL^{-1}. EtOAc-extracts of Sr25-11 and Sr27-11 showed MFC value at 100 μg mL^{-1} and MIC at 20 and 50 μg mL^{-1}, respectively. Strawberries treated with 1000 μg $mL^{-1}$of Sh134-11 and Sr25-11 reached 82 and 72% of decay inhibition, respectively. Treatments with 2000 μg mL^{-1} showed a decay inhibition of 90% approximately. *In vivo* tests are in accordance with the results obtained *in vitro* assays, confirming the efficacy of Sh134-11 and Sr25-11 to control *B. cinerea*. Differences in antifungal activities observed in the different strains suggested that the ability to produce bioactive compounds is not homogenously distributed among *S. hirsutum* and *S. rameale*. Our study would suggest that submerged fermentations of Chilean *S. hirsutum* strain Sh134-11 produce extracts, which could be used as possible biofungicides and an alternative to synthetic fungicides.

1. Introduction

A number of fungi from several genera cause diseases and pests on agricultural produce. Among these are representatives of *Alternaria, Aspergillus, Botrytis Colletotrichium, Fusarium, Penicillium, Mucor* and *Rhizopus* (Bautista-Baños et al., 2013). However, grey mould caused by *Botrytis cinerea* Pers ex. Fr is regarded as the most important since it has a wide host range. Grey mold caused by *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a ubiquitous fungal pathogen; it is a necrotrophic, haploid, heterothallic ascomycete that infects more than 200 crop species reducing the yield and quality of the production. *B. cinerea*

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attacks fruits, vegetables, ornamental and forest trees in humid and temperate geographical zones across the world. The losses inflicted to fruits by *B. cinerea* after harvest are considerable if the disease is not properly managed (Williamson et al., 2007). Grey mould is one of the most common fruit diseases occurring during storage and shipment of fruits, resulting in important economic losses to producers and exporters. Grey mould control in Chile is carried out using cultural and agrochemical disease management methods. For example, the chemical control programs in table grapes are based on the use of fungicides of various groups (dicarboximides, anilinopyrimidines, phenylpyrroles, carboxamides and, mainly, hydroxyanilides), with four to six fungicide applications each season (Díaz and Latorre, 2013). However, the use of these synthetic chemicals to control postharvest deterioration has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution, effects in food and other side effects on humans and, the development of resistance to commonly used fungicides within populations of postharvest pathogens have become a significant problem (Kast-Hutcheson et al., 2001).

This fact has stimulated the search for alternative control strategies. At present, there are three emerging non-conventional technologies for the control of postharvest fungal diseases: application of antagonist microorganisms, sanitizing products and natural products with antimicrobial properties (Drobya et al., 2009).

Research in Chile using natural products to control the fungal infections in storage is scarce and poorly documented. Some studies have been focused mainly to plant extracts and essential oils (Céspedes et al., 2014).

In the previous research was found that submerged fermentations of Chilean fungi are producers of new and active molecules (Aqueveque et al., 2002, 2006). Recently, we detected that some Chilean species of genus *Stereum* produced extracts, which exhibited a strong inhibitory activity against grey mould. Thus, in this study, we report the *in vitro* assays to control the mycelial growth and spore germination of *B. cinerea*, *in vivo* test of selected extracts, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of extracts.

2. Materials and methods

2.1. Fungal material

Fruiting bodies of *Stereum hirsutum* (Sh) and *Stereum rameale* (Sr) were collected from the native and exotic forests in different zones of Bio-Bio Region, Chile. The identification was done on the basis of specialized literature and macro- and micro-morphological features, such as size and color of the fruit bodies, presence of clamps, size and shape of spores, type and diameter of hyphae and septa, and size and color of colonies (Eriksson and Ryvarden, 1976; Garrido, 1985). A voucher specimen of the fungi is deposited in the herbarium of the Laboratory of Applied Microbiology and Mycology, Department of Agroindustries, Faculty of Agricultural Engineering, University of Concepción.

2.2. Obtaining pure mycelial cultures

Mycelial cultures were produced from spore prints, which were then grown in yeast malt glucose medium (YMG) composed of yeast extract 0.4%, malt extract 1%, glucose 0.4%, and agar 1.5%, with pH 5.5. The strains were deposited in the culture collection of the Laboratory of Microbiology and Mycology Applied, Department of Agroindustries, University of Concepción, Chile and maintained at 4 °C on agar slants with YMG medium.

2.3. Submerged fermentation

Small sections of 5-mm diameter plug (15–10) of each isolate were cut under sterile conditions and transferred into 1-L Erlenmeyer flask containing 500 mL of liquid YMG medium. The flasks were incubated at 20–22 °C in an orbital agitator with constant agitation at 120 rpm. The cultures were stopped when abundant mycelia were observed, the glucose source was emptied, and the pH was approximately 7 (Rojas et al., 2006).

2.4. Obtaining total extracts

The liquid cultures (1 L) were filtered to separate the broth and mycelium. The mycelium was extracted with methanol and the broth culture was extracted with ethyl acetate (1:1), obtaining the MeOH-extract and the EtOAc-extract, respectively. Both total extracts were concentrated to dryness in a rotating evaporator, weighted and stored at 4 °C (Kettering et al., 2005).

2.5. Isolation of B. cinerea and inoculum preparation

B. cinerea was obtained from naturally infected strawberries, transferred and grown on PDA medium for 6–7 days and incubated at 22 °C. Four replicates of the pure culture were deposited in the collection and maintained at 4 °C on agar slants with PDA medium. To prepare the initial inoculum, spores were harvested using a sterile detergent solution (0.03% Tween 20). Spores were counted using a Neubauer counting chamber and a microscope (40x), and the concentration was adjusted to obtain 10^5 –10^6 spores mL^{-1}. Fresh suspension of spores was used for each set of treatment conditions (Lichter et al., 2003).

2.6. In vitro antifungal assays

2.6.1. Plate diffusion assay

All extracts were dissolved in ethanol and paper filter discs (Whatman No.3; 6 mm diameter) were impregnated with concentrations of 100 μg of the total extract or Rovral® 50% WP (Iprodione-Dicarboximides) purchased from Bayer CropsSciencie-Chile as a positive control, plus ethanol (negative control) and then deposited on Petri dishes (diameter 9 cm) with 20 mL of the fungal inoculum (10^5–10^6 spore mL^{-1}). The plates were incubated at 22 °C and the inhibition halo around the discs was measured (mm) after 24–48 h. Extracts that showed the highest activity against *B. cinerea* were considered potentially active and selected to evaluate the inhibition of mycelial growth (MGI), inhibition of sporogenesis and determine MIC and MFC values with the serial dilution assays (Jasso de Rodríguez et al., 2011).

2.6.2. Mycelial growth inhibition (MGI)

The inhibition of the mycelia growth of the extracts was determined by measuring the radial growth on PDA plates containing EtOAc-extracts at the concentration range of 10, 100, 200, 500, 1000 and 2000 μg mL^{-1}. The diluted samples were deposited and uniformly spread on the agar surface of Petri dishes. The same aliquots of ethanol and Rovral® 50% WP were added in place of extracts in untreated plates (Sayago et al., 2012). Mycelial discs of 0.5 cm in diameter, cut out from the periphery of 4–5 day-old cultures of *B. cinerea*, were placed upside down on the center of Petri dishes. This was done for each different extract and concentration. Treated and untreated plates were incubated at 22 °C in darkness. Each extract was assayed in triplicate. Mycelial growth inhibition (MGI) was calculated at the 5th-6th day after incubation as follows: MGI = [(DC −DO)/DC] X 100, where, DC is the average of colonies diameter in untreated plates, DO is the average of colonies diameter