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ANTIFUNGAL ACTIVITY OF VOLATILE COMPOUNDS RELEASED FROM MYCELIAL CULTURES OF CHILEAN NATIVE SAPROPHYTIC FUNGI ON PLANT PATHOGENIC FUNGI GROWTH

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"Antifungal activity of volatile compounds released from mycelial cultures of Chilean native saprophytic fungi on plant pathogenic fungi growth"

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Abstract

Fungi produce and release a wide range of volatile organic compounds which can be used in biological control of plant pathogenic fungi. The antifungal activity of Chilean saprophytic fungi *Trichoderma viride*, *Schizophyllum commune* and *Trametes versicolor*, on the plant pathogenic organisms, *Botrytis cinerea*, *Fusarium oxysporum* and *Mucor miehei*, was investigated using two types of inhibition bioassay: (i) bi-compartmented Petri dishes, and (ii) two Erlenmeyer flasks connected by their top parts. The chemical composition of volatile organic compounds (VOCs) released by the saprophytic fungi were investigated using headspace solid-phase microextraction (HS-SPME) and gas chromatography/mass spectrometry (GC-MS) analysis. The antifungal activity of *Schizophyllum commune* on the plant pathogenic fungus *Botrytis cinerea* grown in different substrates was evaluated using the bi-compartmented Petri dish bioassay. Finally, an artificial mixture and individual compounds of *S. commune* volatiles were tested. One strain of *S. commune* showed the highest inhibitory activity against *B. cinerea* and *M. miehei*, 86.0 ± 5.4% and 99.5 ± 0.5% respectively. The volatile profiles were different among the saprophytic fungi strains evaluated. Different classes of volatile compounds produced by the evaluated fungi were found, such as alcohols, esters, ketones and terpenes. The major components in the headspace of mycelial cultures were 6-pentyl-α-pyrone (*T. viride*), ethanol and β-bisabolol (*S. commune*), and a sesquiterpene alcohol (*T. versicolor*). Volatiles released by *S. commune* growing in potato-dextrose-agar (PDA) showed the highest inhibitory activity on the *B. cinerea* growth. The minimum inhibitory concentration (MIC) and median lethal dose (LD50) values of the artificial mixture made from the mixture of volatiles released from *S. commune* (Schi-2) grown in PDA were 0.70 and 0.29 µl per ml of aerial space, respectively. The results of inhibition of *B. cinerea* produced by the artificial mixture confirmed that volatile compounds released from *S. commune* have antifungal activity that could be used in controlling the growth of this plant pathogen. Further bioassays will be carried out for testing the artificial mixture of *S. commune* volatiles on the control of *B. cinerea* on fresh berries and others plant pathogenic organisms.

List of abbreviations

BCAs	Biocontrol Agents
C-Dox	Czapek-Dox
C/N	Carbon/Nitrogen
FVOCs	Fungal Volatile Organic Compounds
GC-MS	Gas Chromatography - Mass Spectrometry
HS-SPME	Headspace Solid Phase Micro-Extraction
LD50	Median Lethal Dose
MA	Malt Agar
MIC	Minimum Inhibitory Concentration
MM	Minimal Media
NIST-MS	National Institute of Standards and Technology - Mass Spectral
PDA	Potato Dextrose Agar
PDMS/DVB	Polydimethylsiloxane/Divinylbenzene
SD	Standard Deviation
SE	Standard Error
VOCs	Volatile Organic Compounds
YSA	Yeast Sucrose Agar
6PAP	6-pentyl-α-pyrone

Chapter 1

General Introduction

Hypothesis and objectives

1.1 Introduction

Fungi produce and release a wide range of volatile organic compounds (VOCs) including hydrocarbons, alcohols, ketones, aldehydes, ethers, esters, terpenes, terpene derivatives, and a variety of sulphur and nitrogen compounds (McAffe and Taylor 1999; Kuske at al. 2005). These compounds have been studied with different purposes, such as food additives (flavor/aroma), indicators of fungal contamination within buildings and stored cereals, and antibiotic agents (Börjesson et al. 1989; Kuske et al. 2005; Mitchell et al. 2010).

The biological activity of VOCs produced by fungi has been tested on several plant pathogenic organisms, such as fungi, bacteria, nematodes and insects (Ghisalberti and Sivasithamparam 1991; Strobel et al. 2001; Grimme et al. 2007). One of the most studied volatile-producing fungus is *Muscodor albus* which has been postulated for controlling post harvest diseases (Mercier and Jimenez 2004), and their activity has been associated to the VOCs emission (Strobel et al. 2001). Other fungi reported as releaser of antifungal volatile metabolites belong to the genus *Trichoderma* sp. This genus is one of the most studied due to the production of enzymes, bioactive non-volatile and volatile compounds and to develop mycoparasitism. The most abundant volatile compound released by this fungus is the lactone 6-pentyl-α-pyrone (6PAP). The antifungal characteristics of this compound were demonstrated on *Rhizoctonia solani* (Worsatit et al. 1994), *Fusarium oxysporum* (Scarselletti and Faull 1994), *Gaeumannomyces graminis* (Dewan and Sivasithamparam 1988) and *Botrytis cinerea* (Pezet et al. 1999). The use of volatile-producing fungus and/or their VOCs as biocontrol agents (BCAs) has been studied for controlling various fungal diseases (Strobel et al. 2004; Mercier and Jimenez 2004).

Both losses caused by plant diseases and the cost associated with its control has led to yield loss in agricultural production systems. Unlike insects and weed plants, diseases are more difficult to detect, identify, and control on time (Agrios 2005). Total crop lost from diseases and pests has been estimated at about 36% or one-third of the potential production of the world, of which 14% of losses are due to diseases despite all types of controls used (Agrios 2005).

Over time, chemical control has been the most effective method for controlling plant diseases. However, the disadvantages associated with it use has led to the search for new alternatives for disease control, such as biological control. The main disadvantages of using fungicides are the increase of genetic resistance to fungicides (e.g. *Botrytis cinerea*), toxin accumulation in foods and environmental contamination (Tripathi and Dubey 2005; Minerdi et al. 2009).

Due to their bioactivity, biodegradability and long-distance mechanism, VOCs constitute attractive candidates for the development of agrochemicals for controlling plant diseases (Parker et al. 1997). An important source of potential bioactive VOCs are world native forests which preserve fungal strains with valuable biological properties that are waiting to be discovered. Among them, the saprophytic fungi are the most important and efficient microorganisms involved in natural process of wood decomposition because they uptake the nutrients from dead organic matter by means of enzymatic process (Tortella et al. 2008). Because of these characteristics, saprophytic fungi isolated from the forest can afford new candidates for studying the antifungal activities associated to the VOCs. Nowadays, the study of antimicrobial activity of saprophytic fungi in Chile has been focused in the production of non-volatile secondary metabolites (Astudillo et al. 2000; Piovano et al. 2005; Aqueveque et al. 2006). However, to date, there are no reports related to the study of production and bioactivity of volatile compounds released from Chilean native fungi.

The aims of this thesis was mainly based on: a) current need for searching new alternatives to control fungal diseases, b) literature reports on the bioactivity of volatiles released from different fungi against plant pathogens, c) the limited information on the bioactivity of saprophytic volatile-producing fungi, and d) the diversity of Chilean native fungi whose volatile compounds have not yet been studied. Volatile-producing fungi were collected from a Chilean template native forest and the plant pathogenic fungi isolated from blueberry (*Vaccinium corymbosum*). Two species of plant pathogenic fungi were selected according to their importance in the agriculture worldwide. First, the pathogen *Botrytis cinerea* Pers. Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel), an airborne plant pathogen with a necrotrophic lifestyle, affecting more than 235 plant species throughout the world including vegetables, ornamentals, fruits and even some field crop. These polyphagous parasites are geographically widespread, having been found from cold to sub-tropical regions. Although there are fungicides for its control, many classes of fungicides have failed due to the fungi genetic plasticity (Collado et al. 2007; Williamson et al. 2007). The genus *Fusarium* is a soilborne, necrotrophic, plant pathogenic fungus with many species that causes serious diseases around the world. *Fusarium oxysporum* causes primarily vascular wilts on many crops and consists of more than 120 *formae specialis* according to the hosts they infect (Agrios 2005). The use of fungicide to control *Fusarium* wilt is effective controlling the pathogen but it also kills beneficial soilborne organisms. Both plant pathogens cause serious economic losses to commercial crops being necessary new control alternatives allow to decrease the use of fungicides and therefore the resistance to fungicides, residues in food, and the environment contamination.

1.2 Hypothesis

Saprophytic fungi have been studied for a variety of purposes including the use of their antibiotic secondary metabolites. Volatile compounds and their biological activity have been mainly studied in endophytic fungi, unlike saprophytic fungi that have been less studied. The use of VOCs released from fungi constitutes an alternative concept for the biological control of fungal pathogens. The antifungal activity of volatile metabolites released from fungi on plant pathogenic fungi shows a potential application in biological control strategies of fungal plant diseases. However, there are relatively little data concerning the use of volatile metabolites released from saprophytic fungi as biocontrol agents. In Chile, there are no reports related to the antifungal activity of volatile metabolites released by native fungi.

Based on these antecedents, the work **hypothesis** is:

There are native saprophytic fungi that grow in template forests able to release volatile compounds with antifungal activity on plant pathogenic fungi growth.

1.3 Objectives

General objective

To evaluate the antifungal activity of volatile organic compounds released from Chilean native saprophytic fungi on plant pathogenic fungi growth.

Specific Objectives

1. To select and identify the most active saprophytic fungus on *Botrytis cinerea* and/or *Fusarium oxysporum* growth based on its volatile antifungal compounds.
2. To identify volatile organic compounds released by the most active saprophytic fungus.
3. To evaluate the effect of the culture media on the bioactivity of the volatile produced by the selected saprophytic strain.
4. To determine the responsible volatile(s) of the bioactivity