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Volatile organic compounds from *Hypoxylon anthochroum* endophytic strains as postharvest mycofumigation alternative for cherry tomatoes

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Sabinene (PubChem CID: 3387-41-5)
4-Carene (PubChem CID: 530422)
Limonene (PubChem CID: 22311)
γ-Terpinene (PubChem CID: 7461)
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ABSTRACT

The antifungal activity and chemical composition of the volatile organic compounds (VOCs) produced by four *Hypoxylon anthochroum* endophytic strains were analyzed. The bioactivity of the VOCs synthesized at different periods of incubation on rice medium was assessed, both *in vivo* and *in vitro,* against the phytopathogen *Fusarium oxysporum*. The *in vivo* effect was evaluated on cherry tomatoes, while the mechanism of action was determined *in vitro* analyzing the phytopathogen's growth, respiration and cell membrane permeability. In general, the VOCs from all strains and incubation periods significantly inhibited the growth of *F. oxysporum* on cherry tomatoes with percentages over 50%. They significantly inhibited the pathogen growth and respiration, and altered the cell membrane permeability and hyphal morphology. The chemical composition was analyzed after solid phase microextraction. In total, 36 VOCs were identified in the four strains, mainly sesquiterpenes and monoterpenes. Among the monoterpenes, eucalyptol had the highest fiber affinity (> 60% area) in three of the four strains studied; thus, it could be considered as a chemical marker for *H. antochroum*. Chemical markers are important for the identification and differentiation of species. The *H. anthochroum* strains are potential mycofumigation agents against postharvest diseases caused by *F. oxysporum*.

1. Introduction

Phytopathogens have a great impact on the food system, causing significant damages in both the quality and quantity of crops. To avoid or control the losses due to phytopathogenic microorganisms, synthetic agrochemicals have been used in agroforestry systems notably improving crop yields and quality (Raza et al., 2016; Waller et al., 2005; Cramer, 2000; Cremlyn, 1991). However, excessive use has led to an increase in resistant pathogens and has also contributed to pollution and affected human and animal health. Moreover, it is known that some synthetic pesticides contaminate groundwater and bioaccumulate in food chains, hence affecting a great number of organisms (Cramer, 2000; Cremlyn, 1991).

Current trends look for an efficient crop production to control fungal pathogens via unconventional methods that are not harmful to the crop itself, the environment or humans and animals. An alternative is the use of biological control methods (Raza et al., 2016; Butt et al., 2001). Biocontrol agents are nonpathogenic live organisms with antagonistic potential against phytopathogenic microorganisms such as fungi, oomycetes, bacteria, viruses and insects, which cause numerous diseases of economic importance. These agents are able to suppress the development of the pathogens through one or more action mechanism aiming to reduce the incidence and severity of the diseases (Narayanasamy, 2013; Stinson et al., 2003).

The biological control is an integral systematic procedure that to be effective, three aspects must be considered: control agent, pathogen and

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environmental conditions (Raza et al., 2016).

Agricultural products are generally delivered to supermarkets and stored for a period before reaching the consumers. Postharvest safety measures are thus important to prevent foodborne diseases and maintain human welfare. In this regard, biocontrol is an effective safety measure to reduce postharvest losses in an eco-friendly manner (Qin et al., 2017; Schalchli et al., 2016; Spadaro and Droby, 2016; Di Francesco et al., 2015; Singh et al., 2012; Huang et al., 2011; Mercier and Jiménez, 2004).

Fusarium oxysporum is an important vascular wilt pathogen on many crops worldwide (Leslie and Summerell, 2006). Tomato fruits are susceptible towards infections caused mainly by fungi and bacteria. Tomato fruits rots are often caused by *F. oxysporum* and other *Fusarium* species (Abu Bakar et al., 2013). Rots caused by *F. oxysporum* can be controlled using fungicides, physical and cultural control measures, and biological control agents, such as non-pathogenic *F. oxysporum* strains that could antagonize the pathogenic strains (Ajilogba and Babalola., 2013). *F. oxysporum* particularly damages the quality of postharvest fruits, mainly tomatoes (Ascencio-Álvarez et al., 2008; Leslie and Summerell, 2006; Fravel et al., 2003).

One kind of potential biocontrol agents are endophytic fungi, which produce a great number of chemically diverse secondary metabolites with varied effects on different biological systems (Holighaus and Rohlfs, 2016; Hung et al., 2015; Strobel, 2006; Waller et al., 2005; Mercier and Jiménez, 2004). Volatile organic compounds (VOCs) are among these metabolites; they are low molecular weight compounds, under 300 Da, which evaporate at normal atmospheric pressure and temperature (Vespermann et al., 2007). The mixtures of VOCs produced by endophytic fungi are mainly composed by alcohols, aldehydes, esters, aromatic and aliphatic hydrocarbons, terpenoids, nitrogen and sulfur compounds, etc. (Hung et al., 2015; Strobel, 2011).

Endophytic fungi are used for micofumigation, a process that involves the application of their VOCs' mixtures to control other microorganisms in closed areas (Strobel, 2011; Park et al., 2010; Suwannarach et al., 2013 Mercier and Jiménez, 2004; Strobel et al., 2001). Mycofumigation is an alternative to synthetic toxic pesticides or when plant pathogenic fungi are resistant to them (Butt et al., 2001). Studies have demonstrated that VOCs have great inhibitory effect and are capable of killing test organisms. They also show a synergistic effect on the inhibition of target organisms (Strobel et al., 2001).

The genus *Nodulisporium* and one of its teleomorph, *Hypoxylon*, produce antimicrobial VOCs. Eucalyptol, or 1,8-cineol, is the compound most frequently found in *Hypoxylon* species (Sánchez-Fernández et al., 2016; Ulloa-Benítez et al., 2016; Shaw et al., 2015; Nigg et al., 2014; Suwannarach et al., 2013; Riyaz-Ul-Hassan et al., 2013; Mends et al., 2012; Tomsheck et al., 2010). The endophytic fungus *Nodulisporium* sp. CF016 was evaluated *in vitro* and its volatiles were found to inhibit, or even kill, a wide range of phytopathogens: *F. oxysporum, Pythium ultimum*, *Rhizoctonia solani*, *Phytophthora capsici*, *Sclerotinia sclerotiorum*, *Colletotrichum coccodes*, *Magnaporthe oryzae*, *Alternaria panax*, *Botrytis cinerea* and *Penicillium expansum*; it also worked as an effective mycofumigation agent against postharvest diseases caused by *B. cinerea* and *P. expansum* in apples (Park et al., 2010). The antagonistic effect and the production of volatile and non-volatile compounds of *H. anthochroum* (anamorph: *Nodulisporium* sp. GS4dII21a) isolated from *Gliricidia sepium*, inhibits the growth, and even kills, some plant pathogenic oomycetes and fungi, e.g., *F. oxysporum* (Sánchez-Fernández et al., 2016). Similarly, the endophyte *H. anthochroum* strain Blaci from healthy *Bursera lancifolia* leaves (Blaeg1) (Ulloa-Benítez et al., 2016) whose teleomorph is also *Nodulisporium* sp., biosynthesizes volatile compounds in PDA that possess a significant inhibitory *in vitro* effect against weeds and plant pathogenic microorganisms. Thus, *H. anthochroum* may be a successful mycofumigation agent for postharvest diseases control.

Accordingly, the purpose of this study was to evaluate the potential of VOCs biosynthesized by the *H. anthochroum* strains Blaeg1, Gseg1, Haeg2 and Smeg4 (identified their phenotypic and molecular characteristics) as postharvest mycofumigation agents against the phytopathogen fungus *F. oxysporum*.

2. Materials and methods

2.1. Fungal material

Fungal endophytes included four *H. anthochroum* strains (teleomorph of *Nodulisporium* sp.), two of which were previously identified as *H. anthochroum* Blaci strain (Blaeg1) (Ulloa-Benítez et al., 2016) and *H. anthochroum* GS4d2II1 strain (*Nodulisporium* sp. GS4d2II1) (Gseg1) (Sánchez-Fernández et al., 2016), based on their macro- and micromorphological characteristics and the phylogenetic analysis of the ITS1-5.8S-ITS2 region. The other strains, Haeg2 and Smeg4, are reported for the first time in the present investigation. The four endophytes were isolated from healthy leaves of different host plants: Blaeg1 from *Bursera lancifolia* (Burseraceae), Gseg1 from *Gliricidia sepium* (Fabaceae), Haeg2 from *Hippocratea acapulcensis* (Celastraceae), and Smeg4 from *Sapium macrocarpum* (Euphorbiaceae). Plants were collected at the Reserva de la Biosfera Sierra de Huautla (REBIOSH), located in Quilamula at Tlalquitenango (18°30′4.1″N - 98°51′52″W and 18°32′12.2″N - 99°02′05″W; 1080–1230 masl), Morelos, Mexico.

The four purified endophytic strains are maintained in water agar (0.2%) at 4 °C and in 30% glycerol-potato-dextrose broth (GPDB) at −80 °C at the Laboratorio de Micología C006, Instituto de Biología, UNAM, and in PDA slants at the Instituto de Química UNAM. Dried PDA and oatmeal agar (OA) cultures are deposited in the Fungal Collection of the Herbario Nacional de México (MEXU), UNAM, under the acquisition numbers MEXU 27838 (Blaeg1), 27,541 (Gseg1), 29,004 (Haeg2) and 29,005 (Smeg4).

2.2. Isolation and molecular identification of the strains Haeg2 and Smeg4

Healthy leaves from *H. acapulcensis* and *S. macrocarpum* were respectively used for the Haeg2 and Smeg4 isolation, which was carried out as reported by Sánchez-Fernández et al. (2016).

The taxonomic identification of the fungal strains Haeg2 and Smeg4 was performed based on their phenotypic (macro- and micromorphology) and genotypic (sequencing of the ITS1-5.8S-ITS2 region) characteristics following the same conditions described by Ulloa-Benítez et al. (2016) a detailed description can be found in the supplementary material.

2.3. In vivo effect of the VOCs' mixture from H. anthochroum strains on cherry tomatoes infected with F. oxysporum

The *in vivo* effect of the VOCs' mixtures from the selected strains was evaluated in cherry tomatoes (*Solanum lycopersicum* var. *cerasiforme*), inoculated with the phytopathogenic fungus *F. oxysporum*.

2.3.1. Culture medium optimization for VOCs production

A preliminary evaluation was carried out to determine the effect of the VOCs synthesized by each of the four strains grown on potato dextrose broth (PDB, 200 g of potato infusion and 20 g glucose, in 1 L of distilled water), PDA (200 g of potato infusion, 20 g glucose, and 20 g agar in 1 L of distilled water) and rice medium (RM, 300 g of rice and 300 mL of water), on the growth of *F. oxysporum* in cherry tomatoes. Bioassays were then carried out in sterile 300 mL flasks that contained one cherry tomato with six equidistant, 2 mm deep, wounds inoculated with 20 μL of 10^7 conidia/mL of *F. oxysporum*, and a 30 mL vial with a 5-day culture of the endophyte grown in PDB, PDA or RM. The inside-vial was covered with a thin fabric (pellon; cotton paper constituted by sheets of natural nonwoven fibers, similar to interfacing fabric) to allow for VOCs to saturate the 300 mL flasks while simultaneously avoiding the spread of conidia of the endophytic strains. The flasks were