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Proceeding Paper

Influence of Pine Volatiles on Growth of an Ophiostomatoid Fungi Associated with Pine wilt Disease in *Pinus pinaster* †

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Abstract: Phytopathogenic ophiostomatoid fungi play an important role in pine wilt disease (PWD), caused by the pinewood nematode (PWN), since they begin proliferating once the pine hosts decay, and can serve as a food source for the PWN. In a recent study, the ophiostomatoid fungi associated with naturally infected *Pinus pinaster* were profiled and cultured. To understand the influence of volatiles commonly emitted by pines on fungal growth, the present work aimed at analyzing the influence of α-pinene, β-pinene and *trans*-β-caryophyllene on a *Leptographium* isolate. The volatiles promoted fungal growth in the first 24 h, but lost their effect after 48 or 72 h, probably due to compound volatilization. After 5 days, fungal growth was comparable to that of control cultures, except for α-pinene that appeared to slightly inhibit fungal growth. Profiling the influence of volatile organic compounds on the PWD complex can contribute to a better understanding of the chemical communication occurring between its different intervenients.

Keywords: Ophiostomatales; *Pinus pinaster*; α-pinene; β-pinene; *trans*-β-caryophyllene

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1. Introduction

Ophiostomatoid fungi are a non-taxonomical group of pathogenic fungi vectored by conifer bark beetles. This interaction between fungi and insect can be considered mutualist since the fungi benefit from a faster and wider spread while the insect's survival is improved because the development of some ophiostomatoid fungi damages the host conifer's defenses and provides the insect with essential nutrients [1].

In the pine wilt disease (PWD) complex, these opportunistic fungi are able to take advantage of a pine tree weakened after the uncontrollable growth of the pinewood nematode population (PWN, *Bursaphelenchus xylophilus*) through feeding on its internal structures [2]. In this condition, the tree's mycoflora shifts from an abundant and diverse community to a more limited community of opportunistic pathogenic species, where the Ophiostomatales are favored [3].

Even though some works report on the interactions of the very different organisms that integrate PWD complex, there is still a lack of information on the specific mechanisms of communication between them. In a preliminary effort to pinpoint volatile triggers for the interplay between the intervenients in PWD, this work reports on the influence of volatiles characteristically emitted by pines, α-pinene, β-pinene and *trans*-β-caryophyllene on in vitro growth of a *Leptographium* isolate obtained from wood of a naturally infected maritime pine with strong symptoms of PWD. This study aimed to understand if these volatiles are stimulants for fungal growth and if they can play a role in the complex infection cycle of PWD.

2. Material and Methods

2.1. Chemicals

The volatiles used were analytical standards of the hydrocarbon monoterpenes (−)-*β*-pinene (≥97%), (+)-*β*-pinene (≥98.5%), *α*-pinene (98%) and sesquiterpene *trans*-*β*-caryophyllene (≥80%) acquired from Sigma-Aldrich (St. Louis, MO, USA). Volatiles were diluted in HPLC-grade methanol, acquired from Fisher Chemicals (Fisher Chemicals, NH, USA).

2.2. Maintance of Leptographium

The mycoflora associated with PWD was previously isolated from wood sections of *Pinus pinaster* displaying strong symptoms of the disease [3]. Briefly, infected wood was collected from 5 maritime pines located in Seia (40°15′57.0″ N, 7°42′47.6″ W). Under aseptic conditions, wood pieces were sectioned, surface sterilized (70% ethanol (*v*/*v*) for 15 s), washed with sterile distilled water, and dried in sterilized filter paper. Sections were then carefully transferred to plates with 2% (*w*/*v*) malt extract agar (MEA, Difco, NJ, USA) supplemented with 200 mg/L of cycloheximide and 100 mg/L streptomycin and incubated at 25 °C in the dark. Hyphal tips of emerging colonies were transferred to fresh MEA plates. Representatives from pure cultures were selected for further identification and characterization, and deposited in the culture collection of INIAV institute (Micoteca da Estação Agronómica Nacional (MEAN). Established fungi were transferred to potato dextrose agar (PDA, Difco, NJ, USA) medium and maintained by weekly subculture.

2.3. Bioassays with the Volatiles

The fungal isolate selected was identified through morphological observations and DNA sequencing [3], as a *Leptographium* sp. To analyze the influence of pine volatiles on fungal growth, a petri dish-based technique was devised using PDA as growth medium (Figure 1). Succinctly, a plug of stock culture of *Leptographium* (grown for 7 days on PDA medium) was set in the middle of the petri dish (with 10 mL of PDA) and a disk immersed in a volatile solution was set 5 mm from the side of the dish. The disk was 5 mm in diameter and was obtained from a cellulose Whatman filter paper (Maidstone, UK). Disks were immersed in 2 μL/mL of compound in methanol, for 24 h, before being set in the PDA medium as described above. The Petri dishes were then sealed and kept in darkness, at 25 °C, for 5 days and growth was followed daily by measuring the radius of the fungal culture growing in the section with (section B) and without the disk (A) for comparison (Figure 1). Control experiments were performed with disks immersed for 24 h in pure methanol.

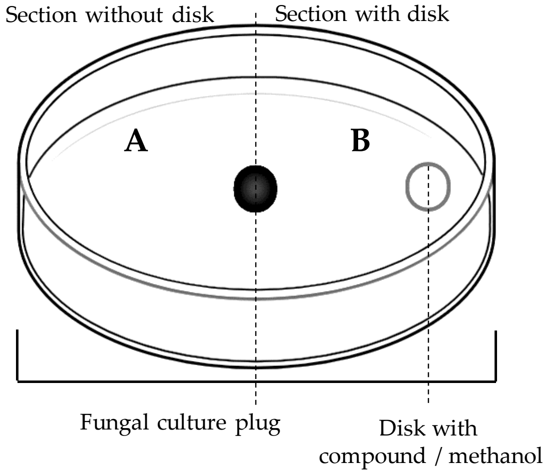

Figure 1. Scheme of the experimental setup used for bioassays with the *Leptographium* isolate.

2.4. Data Treatment and Statistical Analisys

Data of daily fungal radial growth in section A and B of the petri dish was collected for each volatile used. To determine preferential fungal growth in response to the presence of the volatile, the following formula for an Interference Index was applied,

$$\text{Interference Index (II) for compound in section A (or B)} = \text{Radial growth in experimental section } A_{compound} \text{ (or } B_{compound}\text{)/Radial growth in control section } A_{methanol} \text{ (or } B_{methanol}\text{)}.$$

For IIs above 1 ± standard error of controls, growth was considered stimulated by the compound in the respective section, for IIs below 1 ± standard error of controls, growth was considered inhibited, and for IIs above 1 − standard error of controls and below 1 + standard error of controls, growth was considered not influenced by the compound.

Statistical analysis was performed with SPSS version 29 statistics software. Statistical significance was determined with one-way ANOVA, and individual means were compared using the Tukey's post hoc test with $p < 0.05$, the Shapiro–Wilk test ensured data normality, and the Browns–Forsythe test was used for homoscedasticity. Results are presented as the average and standard error of 6 replicates.

3. Results and Discussion

Fungal growth was analyzed in the section in direct contact with the disk immersed in the compound to determine its direct influence and in the section opposite the section with the disk to assess an indirect influence of the compound, i.e., the activity of an environment saturated in the volatile. The assessment of fungal growth in the presence of methanol revealed that no significant differences were found between these two conditions, except after 5 days of growth, where fungi growth in the section with the disk was lower (Figure 2). This may be due to the presence of the cellulose disk, that was reached by the fungal colony at this time-point, leading to a change in growth rate probably because of the difference in the contact surface.

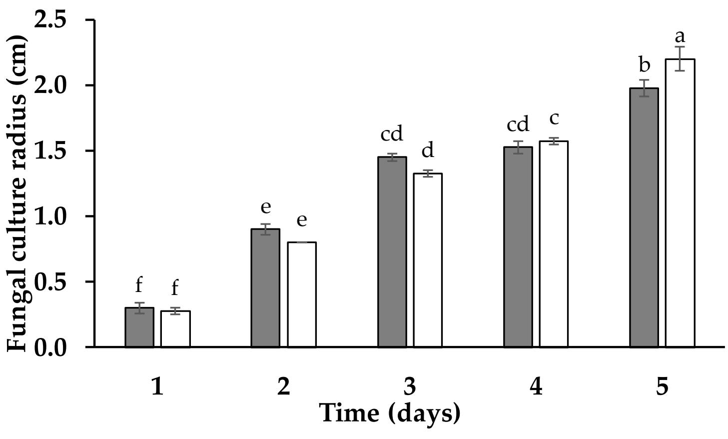

Figure 2. The radial growth of the *Leptographium* isolate on potato dextrose agar (25 °C) in the section without (white columns) and with (gray columns) a cellulose disk imbibed in pure methanol (control). Data are presented as the mean ± standard error of six independent biological replicates. Different letters indicate statistically significant differences ($p < 0.05$).

For the compounds tested, the IIs determined reflected the degree of difference between the result obtained for the compound and its respective control. After 24 h of contact with any of the tested volatiles, fungal growth was increased (Figure 2), but in the following time-points it reduced to values similar to those of the control. This may be due to the rapid volatilization of the compound at room temperature and escape through the gaps of the petri dish. Between the enantiomers of β-pinene, slightly higher values were obtained for the (−) isomer than for the (+) isomer, suggesting a preference of the *Leptographium* isolate for that compound (Figure 2a). Additionally, both isomers induced a higher growth in the section with the disk than in the section without, in the following 2nd and 3rd days (Figure 2b). The monoterpene *α*-pinene showed the highest promotion in fungal growth after 24 h but lost its effect in the following days (Figure 2c).

In the 5th day, fungal growth in the section with the disk seemed to be inhibited, suggesting that after reaching the disk imbibed with *α*-pinene (direct contact), the *Ophistoma* isolate reduced its growth (possible inhibition through contact). This was not observed in the section without the disk. The sesquiterpene *trans*-*β*-caryophyllene induced a growth pattern in *Leptographium* similar to that of the other compounds, with a stimulation of growth after 24 h and a reduction to control values in the following days (Figure 3d).

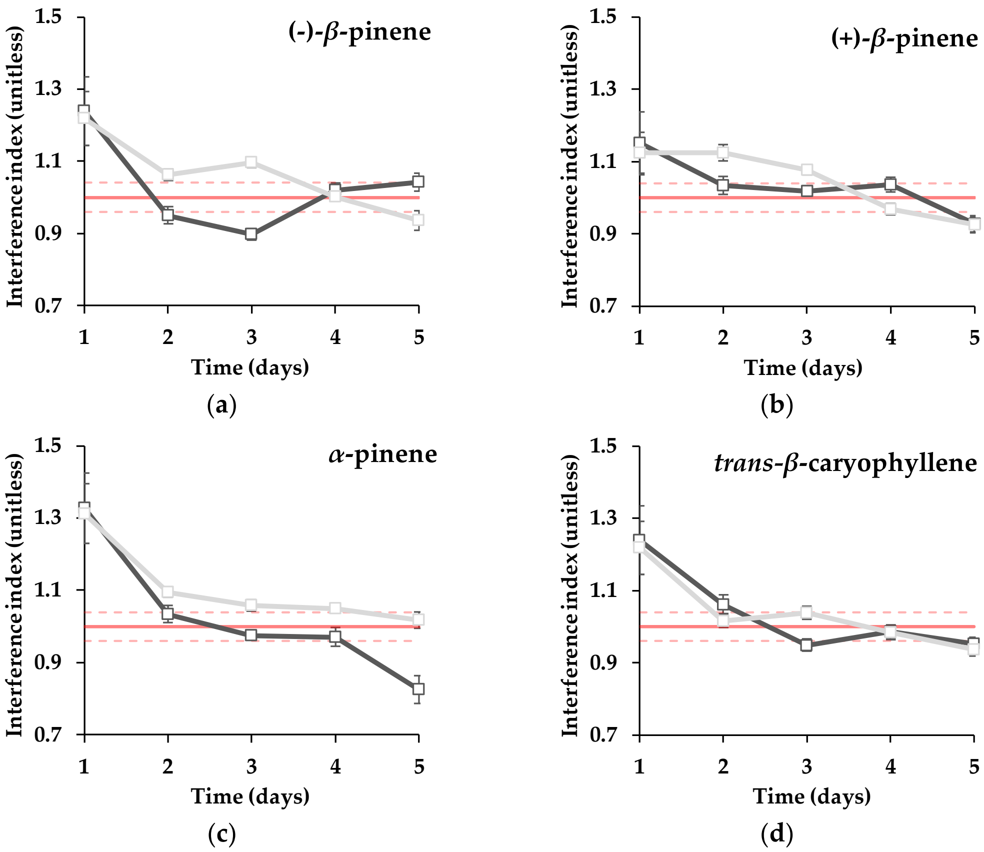

Figure 3. Interference indexes (ratio of radial growth of the *Leptographium* isolate in contact with the compound over that of the control) for fungi growing in the section without (white line) and with (gray line) a cellulose disk imbibed in the volatile compound. Data are presented as mean ± standard error of six independent biological replicates. Different letters indicate statistically significant differences ($p < 0.05$). The red line represents a condition of no influence (experimental = control) with respective standard error based on variation of control values (interrupted red lines).

4. Conclusions

Analyzing the influence of volatiles characteristic of susceptible pines on the growth of fungi associated to PWD is an important contribute for understanding the relationships and communication between the different intervenients in PWD. The *Leptographium* isolate tested appeared to be more influenced by *α*-pinene than by the other compounds. This monoterpene is a very important volatile emitted by the pine and is an attractant for many pine bark beetles. Further research will assess combinations of volatiles simulating the emissions of the pine in its natural environment.

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Conflicts of Interest: The authors declare no conflict of interest.

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