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Antifungal Activity of Oleoresin and Fractions of *Pinus elliottii* Engelm and *Pinus tropicalis* against Phytopathogens

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Abstract

Microorganism resistance to the existing products is yet another difficulty that agriculturalists have to deal with. In this context, the search for new agricultural products that can fight phytopathogens has become increasingly important. Plants have played an important role in this process, because they can serve as a source of new compounds for drug discovery. Plants belonging to the genus *Pinus* produce an oleoresin that protects the plant against herbivores and pathogens. With a view to developing products that can combat fungal pathogens without harming the environment, this work aimed to evaluate the antifungal activity of the oleoresins and fractions of *Pinus elliottii* Engelm and *Pinus tropicalis* against phytopathogens. The methodology based on NCCLS M38-A standards aided antifungal activity assessment. The microdilution method helped to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). The oleoresins of *P. elliottii* and *P. tropicalis* afforded the most significant results—they displayed fungicidal activity against all the tested species. MIC values were promising, especially the MIC of the oleoresin of *P. elliottii* against *S. rolfsii* (1.95 μg·mL^{-1}). The MIC values of the oleoresins of *P. elliottii* and *P. tropicalis* ranged from 1.95 to 1000 μg·mL^{-1} and from 31.25 to 250 μg·mL^{-1}, respectively. Fraction PT2 of *P. tropicalis* furnished the best results among all the assayed fractions: MIC values lay between 125 and 500 μg·mL^{-1}. In conclusion, the oleoresin of *P. tropicalis* is a promising source of new antifungal agents for application in the treatment of phytopathogenic infections.

Keywords

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1. Introduction

Phytopathogenic diseases affect the amount and quality of agricultural produce. For this reason, controlling pathogenic fungi has become a matter of great concern for agriculturalists worldwide [1]. Plant contamination by phytopathogens has increased significantly, especially because these microorganisms have developed resistance to the currently marketed products. The development of microbial resistance, the great environmental impact of modern pesticides [2], and the large amount of pesticides that remain on the leaves and fruits intended for human consumption have motivated the search for new products.

All around the world, consumers have sought to purchase products of organic origin; *i.e.*, products that do not contain chemical residues and that grow in conditions that harm the environment to a minimum. In this context, plant-derived products are an attractive alternative. In particular, Brazil is rich in plants that may contribute to the development of new antifungal agents. Besides that, the genus *Pinus* has been planted in Brazil for over thirty years, especially in the southern region, where these plants have thrived in favorable climate conditions [3]. *Pinus tropicalis* and *Pinus elliottii* Engelm are among the *Pinus* species that are most cultivated in the Brazilian territory. These plants produce a oleoresin that confers protection against the attack of herbivores and pathogens; indeed, the oleoresin can repel, inhibit, or kill these invaders [4].

Traditionally, the oleoresin derived from coniferous *Pinus* can 1) function as an antiseptics or analgesic, 2) act by relieving cough and inflammation, 3) aid treatment of skin burns and wound diseases, and 4) fight pulmonary tuberculosis [5] [6]. The oleoresin consists of a minor fraction called turpentine and a major fraction known as oleoresin or pitch. The latter fraction is composed mainly of diterpenes [7], especially those belonging to the class of pimaranes, abieatanes, and labdanes [8].

Studies have shown that various classes of diterpenes display potential antifungal activity against plant pathogens such as *Colletotrichum gloesporioides* [9], which is the causative agent of anthracnose. Savluchinske-Feio *et al*. [10] have demonstrated that several diterpenes inhibit *Botrytis cinerea* growth, because they elicit morphological changes in the plasma membrane of this fungus.

Pinus tropicalis is a species of the genus *Pinus* that is rich in diterpenoids. It is believed to possess fungicidal activity, particularly against phytopathogenic fungi. Literature reports have stated that the oleoresin of *Pinus tropicalis* has other important biological activities. Leyva *et al*. [11] found that both the oleoresin and turpentine of *P. tropicalis* have insecticidal action against *Aedes aegypti*. Hevia *et al*. [12] observed that the oleoresin of this same plant is also active against *Biomphalaria havanensis*, and that its molusquicidal potential stems from its ovicidal activity.

Pinus elliottii Engelm is also known as common pine or American pine [13]. This species is also the one that is most grown in the south of Brazil, and it has found wide application in the chemical industry. In fact, it serves as raw material to produce adhesives, rubbers, paints, disinfectants, and perfumes. In addition to presenting diverse biological activities, activity against multidrug-resistant bacteria and anaerobic bacteria [14] [15] was included.

On the basis of evidence that plants belonging to the genus *Pinus* exert antifungal activity, and given the fact that the search for new agricultural products to combat these microorganisms has increased, this study aimed to evaluate the *in vitro* antifungal activity of the oleoresins and fractions of *Pinus elliottii* and *Pinus tropicalis* against eight phytopathogens.

2. Materials and Methods

2.1. Plant Material, and Bioguided-Assay Fractionation for *P. tropicalis*

Certified oleoresin of *Pinus elliottii* Engelm (PE; 100.0 mg) and *Pinus tropicalis* (PT; 120.0 mg) was kindly provided by ARESB (Associação dos Resinadores do Brasil). Bioguided-assay fractionation with *P. tropicalis* (PT) was performed, due to the fact that this oleoresin showed to be very effective against a panel of fungal. This plant material was subjected to vacuum chromatography over silica gel 60H (500 g; Merck, art. 7736) using *n*-hexane and increasing amounts of ethyl acetate as eluant (1500 mL each fraction). After solvent evaporation, this procedure afforded five fractions (PT1 - PT6); which were also assayed against fungal.

2.2. Fungal Strains

The phytopathogenic fungi assayed in this study, namely *Macrophomina phaseolina*, *Lasiodiplodia theobromae*,

Colletotrichum gloeosporioides, *Pestalotiopsis* sp., *Sclerotium rolfisii*, *Fusarium solani*, *Fusarium oxysporum*, and *Phytophthora infestans*, were donated by Brazilian Corporation of Agricultural Research.

The fungal isolates were maintained in Laboratory of Research in Applied Microbiology of the University of Franca. The samples were kept in sterile water, at room temperature. The fungi employed here were isolated from infected plants, as depicted in **Table 1**.

2.3. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The Minimum Inhibitory Concentration (MIC) values were determined by the microdilution broth method in 96-well microplates, in triplicate. The samples were dissolved in dimethylsulfoxide—DMSO (Sigma-Aldrich) at 1.0 mg·mL^{-1}, followed by dilution in RPMI (Difco) with MOPS pH 7.2; concentrations ranging from 0.98 to 2000.0 µg·mL^{-1} were achieved. The final DMSO content was 5%.

The inoculum was prepared on the basis of the standards recommended by the NCCLS M-38A [16] with some modifications. First, the fungi were cultivated in tubes containing Potato Dextrose Agar—PDA (Difco) at 35˚C, for seven days, to allow for sporangiospores or conidia to form. The *Fusarium* species were incubated at 35˚C for 72 h and were then kept at 25˚C until the seventh day. After this period, the inoculum was obtained by adding 1 mL of sterile 0.85% saline to the test tube containing the culture, thereby forming a suspension. The mixture was transferred to a sterile tube and allowed to stand for 5 min. After sedimentation, the homogeneous supernatant was transferred to another tube and mixed for 15 s. The density of the suspensions was read in a spectrophotometer at a wavelength of 550 nm; adjustments ensured that the optical density reached values between 0.09 and 0.11 (80% to 82% transmittance), and between 0.15 and 0.17 (68% to 70% transmittance) for *Fusarium*. After adjusting the spectrophotometer parameters, the suspension was diluted in RPMI medium at a 1:50 ratio, which corresponded to twice the required concentration range (from 4×10^5 to 5×10^4).

Amphotericin B was the positive control at concentrations ranging from 0.031 to 16 µg·mL^{-1}. A reference strain of *Aspergillus fumigatus* (ATCC 204305) was included as reference fungi. The following controls were employed: negative (RPMI broth only), positive (RPMI plus inoculum, without addition of antifungal agent), and diluent (DMSO and inoculum).

Aliquots of the MIC wells were transferred to Sabouraud Dextrose Agar—SDA (Difco) plates without the drug. The plates were incubated at 28˚C for seven days, to provide the concentration that was fungicidal —Minimum Fungicidal Concentration (MFC)—defined as the lowest concentration of the compound that did not generate visible microbial growth in the medium.

3. Results and Discussion

The present work evaluated the antifungal activity of *P. tropicalis* and *P. elliottii* against phytopathogenic fungi. According to Holetz *et al.* [17], an extract with MIC lower than 100 µg·mL^{-1}, from 100 to 500 µg·mL^{-1}, from 500 to 1000 µg·mL^{-1}, and over 1000 µg·mL^{-1} constitutes a good, moderate, weak, and inactive antimicrobial agent, respectively. The oleoresin of *P. tropicalis* provided promising results: MIC values ranging from 31.25 to 250 µg·mL^{-1} (**Table 1**), and this oleoresin exerted a fungicidal effect on all the tested fungi. The best result was obtained against *C. gloesporioides* and *F. solani* (MIC = 31.25 and 62.5 µg·mL^{-1}, respectively). As for the fractions, PT2 furnished the highest antifungal activity: MIC values ranged from 125.0 (against *F. oxysporum*, *Pestalotiopsis* sp., and *L. teobromae*) to 500.0 µg·mL^{-1}, attesting that this fraction constituted a moderate antifungal agent. PT2 also had a fungicidal effect on *C. gloeosporiodes*, *S. rolfisii*, *F. oxysporum*, *L. theobromae*, and *M. phaseolina*, which confirmed its good activity. Fractions PT1, PT3, PT4, PT5, and PT6 yielded MIC values that lay between 125.0 and 1000.0 µg·mL^{-1} (**Table 1**), so they could be considered from moderate to inactive agents. PT1 exhibited fungistatic activity against *F. oxysporum*, *P. infestans*, and *M. phaseolina*; as for the other phytopathogenic fungi, it acted as a fungicide. Despite its poor activity, PT3 worked as a fungicide against all the tested fungi. PT4 was fungistatic against *F. oxysporum* and *F. solani*; it served as fungicide against the other fungi. Except for *P. infestans* and *C. gloesporioides*, PT5 acted as fungicide against all the tested fungi. PT6 was fungistatic against *F. oxysporum*, *P. infestans*, and *F. solani*, and it exerted a fungicidal effect on the other tested fungi.

The antifungal activity of the oleoresin of *P. elliottii* varied from 1.95 to 1000 µg·mL^{-1}. This oleoresin was the most active against *S. rolfisii* (MIC = 1.95 µg·mL^{-1}) as well as *F. solani* and *M. phaseolina* (MIC of 62.5 µg·mL^{-1}

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values (µg·mL^{-1}) of the oleoresin of *P. tropicalis* and *P. elliottii* and *P. tropicalis* fractions.

Fungi	Oleoresin						*P. tropicalis* fractions						Oleoresin			
	P. tropicalis		PT1		PT2		PT3		PT4		PT5		PT6		*P. elliottii*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Pestalotiopsis sp.	250	250	1000	1000	125	250	500	500	500	500	1000	1000	1000	1000	1000	1000
C. gloesporioides	31.25	31.25	250	250	250	250	250	250	250	250	250	500	500	500	125	125
S. rolfsii	250	250	1000	1000	500	500	500	500	500	500	250	250	125	125	1.95	1.95
F. oxysporum	125	125	250	500	125	125	500	500	500	1000	250	250	500	1000	500	500
P. infestans	250	250	500	1000	500	1000	1000	1000	1000	1000	1000	2000	1000	2000	1000	1000
L. theobromae	250	250	250	250	125	125	125	125	250	250	500	500	500	500	1000	1000
F. solani	62.5	62.5	1000	1000	500	1000	1000	1000	1000	2000	250	250	250	500	62.5	62.5
M. phaseolina	125	125	500	1000	250	250	500	500	500	500	1000	1000	1000	1000	62.5	62.5

in both cases). This oleoresin displayed moderate activity against *C. gloesporioides* and *F. oxysporum*; it exerted a fungicidal effect on all the other tested fungi. To ensure the accuracy of the technique, amphotericin B was tested against *A. fumigatus* (ATCC 204305), to give a MIC value of 1.0 µg·mL^{-1}, which lay within the expected standard established by NCCLS M38-A [16] (between 0.5 and 2.0 µg·mL^{-1}).

To date, there have been no reports on the antifungal activity of *P. tropicalis* and *P. elliottii*, which has precluded comparisons with the work of other authors. Therefore, here we present some data obtained by other authors for the same pathogens; however, the compounds assayed in such works and the assessment techniques differed from those employed in the present study.

Most literature papers have evaluated the antifungal activity by incorporating the tested extracts in the culture medium, followed by inoculation with the tested fungus. The authors then monitored fungus growth.

Lins *et al*. [18] studied the antifungal potential of a variety of compounds against *L. teobromae*. They conducted their study by immersing fruits in diluted extracts and then inoculating the phytopathogenic the fruit. After the incubation period, they measured phytopathogen growth. These authors described that the garlic extract, potassium phosphate, and sodium chloride exerted antifungal action.

David *et al*. [19] investigated whether incorporation of the extract of *Furcraea giganteia* into Tomato agar at a concentration of 75000.0 µg·mL^{-1} was effective against *Phythophtora* sp. Mojica-Marín *et al*. [20] incorporated the extract of *Larrea tridentata* in PDA culture medium and observed that it was considerably active against *P. capsici*. In our experiment, this fungus proved to be resilient: high concentrations of the extract were necessary to inhibit microorganism growth, and most of the evaluated compounds were only fungistatic against this species.

Domingues *et al*. [21] demonstrated that hexane extracts of *Rutagra veolens*, *Allamanda ridentat*, and *Impatiens walleriana* inhibited 100% mycelial growth of *S. rolfisii*. Faria *et al*. [22] observed that both the aqueous extract and the hydroethanolic extract of *Momordica charantia* had fungitoxic potential against *S. rolfsii*. According to Ramírez-Chávez *et al*. [23], the extract of *Heliopsis longipes* exhibited fungicidal activity against *S. rolfsii* at a concentration of 25 µg·mL^{-1}. As reported by other authors, *S. rofsii* proved to be a very sensitive species: the oleoresin of *P. elliottii* possessed excellent activity against this pathogen. Concerning the other extracts and fractions, they also afforded satisfactory MIC results, which attested to their fungicidal potential against *S. rolfsii*.

According to Rozwalka *et al*. [24] some compounds present in plant extracts such as rosemary (*Rosmarinus officinalis*), basil (*Ocimum basilicum*), burdock (*Articum lappa*, *Articum minus*), calendula (*Calendula officcinalis*), chamomile (*Chamomila recutita*), and lemongrass (*Cymbopo goncitratus*) displayed fungitoxic properties against *C. gloesporioides*. Indeed, the MIC and MFC results confirmed its high sensitivity; only fraction PT5 presented fungistatic activity against this species. Additionally, the oleoresin of *P. tropicalis* was the most effective against this phytopathogen.

Costa *et al*. [25] studied the antifungal potential of the essential oil of *Syzygium aromaticum*, popularly known as clove, against the pathogens *F. solani*, *F. oxysporum*, and *M. phaseolin*. These authors concluded that the oil had fungicidal activity against *F. solani* and *F. oxysporum*, but it was inactive against *M. phaseolina*. Cunico *et al*. [26] reported the antifungal potential of extracts of *Ottonia martiana* against three phytopahogens and found

that the extracts were only active against *F. oxysporum* and *Rhizoctonia* sp. In another study, the extract of *Piper marginarum* exhibited strong antifungal activity against *F. oxysporum* [27]. On the basis of the results above, *F. oxysporum* was more sensitive to the tested compounds than *F. solani*. Our experiments revealed an opposite trend: both the MIC and the MFC results indicated greater sensitivity of *F. solani*.

Seixas *et al*. [28] studied the antifungal activity of the essential oil of Citronella-grass and the isolated compound Citronellal against the pathogen *F. subglutinans*. Because the essential oil led to stronger fungi static effect than the isolated compound Citronellal, the activity of the Citronella-grass oil most likely originated from the synergism of the compounds that constituted the oil. Other authors [29] [30] have also verified the synergistic effects of compounds derived from natural products.

Analysis of the MIC and MFC results obtained in the present work evidenced that the fractions derived from the oleoresin of *P. tropicalis* afforded better MIC values than the corresponding oleoresin. Therefore, the antifungal properties of the oleoresin of this plant are possibly related to the synergism of the compounds present in it.

4. Conclusion

In conclusion, fraction PT3 of the *P. tropicalis* and the oleoresins of *P. elliottii* and *P. tropicalis* exerted a fungicidal effect on all the tested phytopathogens. Hence, the oleoresins of these species constitute a potential source of new antifungal agents to treat infections caused by phytopathogens, mainly *C. gloesporioides* the main causative agent of anthracnose in tropical fruits such as papaya, mango, guava and passion fruit, and *F. solani*, which causes root rot in soybeans. The antifungal activity of the oleoresins probably originates from the synergism of the compounds that constitute the oleoresin.

Acknowledgements

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