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Research paper

Plant extracts inhibiting mycelial growth of *Fusarium solani f. sp. piperis* and *Phytopythium helicoides*

Extractos de plantas que inhiben el crecimiento micelial de Fusarium solani f. sp. piperis *y* Phytopythium helicoides

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Abstract

Mixtures of compounds with antifungal effect may increase the efficiency of products against fungi. The objective of this work was to evaluate the effect of different formulations based on coffee extracts, neem extracts and coffee pyroligneous extracts on the inhibition of *Fusarium solani* f. sp. *piperis* and *Phytopythium helicoides* mycelial growth. Three formulations with different concentrations of the extracts plus technical glycerin at 2.5; 5; 10; 15; and 20 mL L^{-1}, previously sterilized at 120°C for 20 minutes, were added in PDA (Potato-Dextrose-Agar) culture medium. Discs containing culture medium with mycelium of *F. solani* f. sp. *piperis* or *P. helicoides* were transferred to the center of Petri dishes containing the treatments. After an incubation period of three days for *P. helicoides* and seven days for *F. solani* f. sp. *piperis* the diameter of the colonies were measured. In general, highest concentrations of the extracts resulted on inhibition of mycelial growth up to 100% for both microorganisms.

Keywords: Antifungal activity, *Azadirachta indica*, Coffee extract, Phenolic compounds, Pyroligneous extract.

Resumen

Mezclas de compuestos con efecto antifúngico pueden aumentar la eficacia de productos contra los hongos. El objetivo de este trabajo fue evaluar el efecto de diferentes formulaciones a base de extractos de café, extractos de neem y extractos piroleñosos de café sobre la inhibición de crescimento micelial de Fusarium solani f. sp. piperis *y* Phytopythium helicoides*. Tres formulaciones con diferentes concentraciones de los extractos más glicerina técnica al 2,5; 5; 10; 15; se agregaron 20 mL L^{-1}, previamente esterilizados a 120°C por 20 minutos, en medio de cultivo PDA (Papa-Dextrosa-Agar). Discos que contienen medio de cultivo con micelio de* F. solani f. sp. piperis *o* P. helicoides *se transfirieron al centro de las cajas de Petri que contenían los tratamientos. Después de un período de incubación de tres días para P. helicoides y siete días para F. solani f. sp. piperis se midió el diámetro de las colonias. En general, como resultado las concentraciones más altas de los extratos inhibieron hasta um 100% del crecimiento micelial de ambos microorganismos.*

Palabras Clave: *Actividad antifúngica,* Azadirachta indica, *Extractos de café, Compuestos fenólicos, Extractos piroleñosos.*

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Introduction

Fusarium spp. and *Phytopythium helicoides* (Drechsler) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque are not easy to control and easily disperse microorganisms, which infect several crops of economic importance such as black pepper (*Piper nigrum* L.) and lettuce (*Latuca sativa* L.). Prevention is the best control strategy for these pathogens (Sutton *et al*., 2006; Secundino *et al*., 2018) since the use of chemical control is not efficient in both cases. Therefore, new products with fungicidal and/or fungistatic activity, with less toxicity for humans and safer to the environment have been pursuit.

During the process of thermal decomposition of organic materials, several compounds which can be used in agriculture as environmentally-friendly antimicrobial agents, promoters of seed germination and plant growth and production enhancers, are released (Theapparat *et al*., 2015; Grewal *et al*., 2018; Gmach *et al*., 2020; Ramos *et al*., 2020; Júnior *et al*., 2022; Cândido *et al*., 2023). An example of these compounds is the pyroligneous extract obtained from materials such as, wood of bamboo (*Dendrocalamus asper*), *Eucalyptus camaldulensis*, *E. urograndis, E. grandis, E. urophylla, Enterolobium contorstisiliquum, Leucaena leucocephala, Azadirachta indica, Hevea brasiliensis* (rubberwood), black wattle (*Acacia mearnsii*), pine (*Pinus taeda*), coconut (*Cocus nucifera*), sugarcane (*Saccharum* spp.) and coffee residues, in a process called pyrolysis (Theapparat *et al*., 2015; Grewal *et al*., 2018; Angelo *et al*., 2022; Cândido *et al*., 2023; Fernández-Ferreras *et al*., 2023; Smaniotto *et al*., 2023). Such extracts can be used to control *Oidium leucoconium* in rose, white rot fungus (*Trametes versicolor* and *Rigidoporopsis amylospora*), brown fungus (*Gloeophyllum trabeum*), sapstain fungus (*Botryodiplodia threobromae*), white mold (*Sclerotinia sclerotiorum*), as well as *Aspergillus* spp., *Fusarium* sp. and *Rhizoctonia* sp. occurring in *Schizolobium amazonicum* seeds (Theapparat *et al*., 2015; Macedo *et al*., 2019; Ramos *et al*., 2020; Smaniotto *et al*., 2023).

A number of plant extracts have been reported controlling or inhibiting the mycelial growth of plant pathogenic fungi. Among them, the extracts of *Azadirachta indica* L. (neem), pyroligneous extracts from wood and coffee extracts, which have effect on insects, phytonematodes and fungi. Neem extracts have more than 50 compounds, however, the major constituent is azadirachtin which is found mainly in neem seeds and has nematicidal, fungicidal and insecticidal properties (Li *et al*., 2019; Adusei and Azupio 2022; D'Errico *et al*. 2023).

The antifungal effect of coffee and neem extracts and pyroligneous extracts against *Botrytis cinerea, F. oxysporum*, *Aspergillus* sp., *Alternaria* sp., *Curvularia* sp., *Rhizopus stolonifer* and *Oidium leucoconium* has been reported in the literature (Ramos *et al*., 2020; Silva *et al*., 2023; Kahya *et al*., 2023; Pertile and Frac 2023). Andaló *et al*. (2019) evaluated the effect of exposing the nematode *Heterorhabditis amazonensis* to three formulations composed of neem extract, pyroligneous extract and coffee extract and found that the formulation with 50% neem extract and pyroligneous extract was considered slightly harmful. However, little information is available about the mixture of extracts aiming new and more efficient formulations to control microorganisms. In view of this, the objectives of this work were to evaluate *in vitro* formulations based on coffee and neem extracts and coffee pyroligneous extracts on the inhibition of *F. solani* f. sp. *piperis* and *P. helicoides* mycelial growth.

Materials and methods

The research was performed in the Laboratory of Plant Pathology of the Institute of Agrarian Sciences (ICA) at Federal University of Minas Gerais (UFMG), *Campus* Montes Claros, Minas Gerais.

In this study we used the isolate CML 3828 of *P. helicoides*, from the Fungi Collection of the Plant Pathology Department of the Federal University of Lavras, Lavras, MG, used as causal agent of lettuce root rot in hydroponic systems. The isolate of *F. solani* f. sp. *piperis* was acquired from the Fungi Collection of the Laboratory of Plant Pathology from ICA/UFMG, pathogenic to black pepper. Discs of pure cultures of these isolates were transferred to 9 cm diameter Petri dishes, containing PDA (Potato-Dextrose-Agar) culture media, previously sterilized by autoclaving at 120° C for 20 minutes. Subsequently, the plates were sealed with clear PVC film, and incubated in a BOD-type growth chamber at 25°C and 12 h photoperiod for five days. After this period the isolates were used in the experiments.

Formulations were prepared by diluting different concentrations of neem leaf extracts, pyroligneous coffee extract and technical glycerin in water (**Table 1**). In the first experiment, concentrations of 2.5; 5; 10; 15; and 20 mL L^{-1} of the formulations, previously sterilized at 120° C for 20 minutes, were added to the PDA culture medium in 9 cm diameter Petri dishes, to evaluate the *in vitro* effect of formulations on mycelial growth of *F. solani* f. sp. *piperis*. Subsequently, 4 mm diameter culture discs containing mycelium of the microorganisms were transferred to the center

of Petri dishes. Plates containing only culture with mycelium discs of the microorganisms were considered as control. The plates were incubated at 25°C and 12 h photoperiod. The experimental design was completely randomized in a factorial arrangement 3x5 (3 formulations x 5 concentrations) plus the control, totalizing 16 treatments, with five replications.

The evaluation of *F. solani* f. sp. *piperis* mycelial growth was performed seven days after inoculation, by calculating the mean of two perpendicular colony diameters on each replication with the aid of a millimeter ruler. Then, the percentage inhibition of mycelial growth was determined according Rabuske *et al*. (2023), using the equation: I (%) = [(DC-DT)/DC]x100, where: I = percentage of inhibition; DC = diameter of fungal colony in the control plate, and DT= diameter of the fungal colony in the treatment plate.

In the second experiment, the same methodology and the same concentrations and formulations described in the first experiment were tested (**Table 1**), however in the case of *P. helicoides* the evaluation was performed after three days of incubation.

Generalized linear models (GLM) were constructed using the statistical program R, version 3.2.4 (R Development Core Team, 2021) (Crawley, 2007) for statistical analysis. The analyses evaluated whether the percentage of mycelial growth inhibition (response variable) varies as a function of the formulations and their different concentrations (explanatory variables) for isolates of *F. solani* f. sp. *piperis* and *P. helicoides*. The error distribution used was Quasi-binomial with Logit link function, being the significance of the complete models tested and terms not significant removed from the model (Crawley, 2007). Next, the residual analysis of the minimal models was performed to verify their adequation. ED_{50} (Efficient Dose to inhibit 50% of mycelial growth) was calculated with the Logit link function for each product and isolates. Values of $p<0.05$ were considered statistically significant.

Results and discussion

The values of ED_{50} for *F. solani* f. sp. *piperis* and *P. helicoides* varied according to the formulations tested (**Table 2**). The chemical composition of coffee extract obtained during the roasting process and used in the formulations had the following characteristics: humic acid<1.0 % m/v; fulvic acid = 1.50 % m/v; organic matter = 0.83 % m/v; electrical conductivity = 257.6 uS/cm; total phenols = 99.60 mg C_6 H_5 OH L^{-1}; pH = 5.53; total Nitrogen = 2550 mg L^{-1}; K_2O = 0.70 mg L^{-1}; P_2O_5 = 57.52 mg L^{-1}; Mg = 0.326 mg L^{-1}; Ca = 1.80 mg L^{-1}; Zn = 0.202 mg L; Fe = 0.015 mg L^{-1}; Cu <0.003 mg L^{-1}; TOC = 13350 mg L^{-1}.

Formulation 3 inhibited 100% mycelial growth in both microorganisms at concentrations of 5; 10; 15; and 20 mL L^{-1} (**Figures 1 and 2**). However, the inhibition of mycelial growth of the isolates varied according to the 1 and 2. Formulation 1 did not

Table 1. Percentages of composition and hydrogen ionic potential (pH) of the formulations accessed.

Formulations	Chemical composition	pH
1	65% neem extract, 15% pyroligneous coffee extract, 5% coffee extract, 2% phenolic compounds, 15% technical glycerin*	3.54
2	85% neem extract, 15% technical glycerin	4.99
3	50% neem extract, 50% pyroligneous coffee extract	3.54

*Technical glycerin – obtained from the acid hydrolysis of the phosphoric acid from the biodiesel fabrication process.

Table 2. ED_{50} values for formulations inhibiting mycelial growth of *Fusarium solani* f. sp. *piperis* and *Phytopythium helicoides* isolates after 7 and 3 days of incubation, respectively.

Formulations	Isolates	
	F. solani* f.sp. *piperis	***P. helicoides***
1	6.13 mL L^{-1}	21.73 mL L^{-1}
2	28.49 mL L^{-1}	3.26 mL L^{-1}
3	2.59 mL L^{-1}	1.66 mL L^{-1}

ED_{50} (mL L^{-1}) - concentration which inhibited growth by 50%.

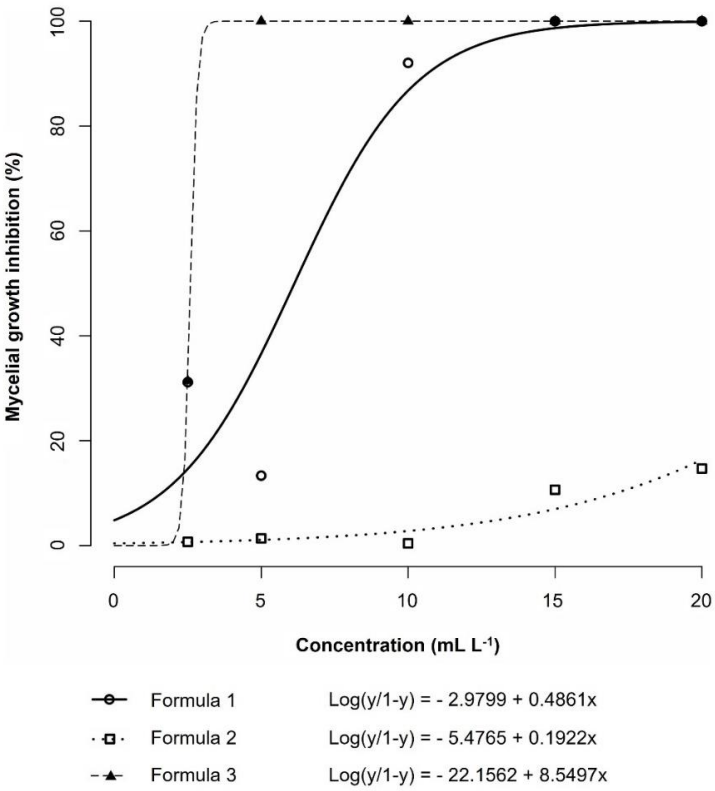

Figure 1. Inhibition of mycelial growth (%) in *Fusarium solani* f. sp. *piperis* seven days after incubation at different concentrations of the tested formulations.

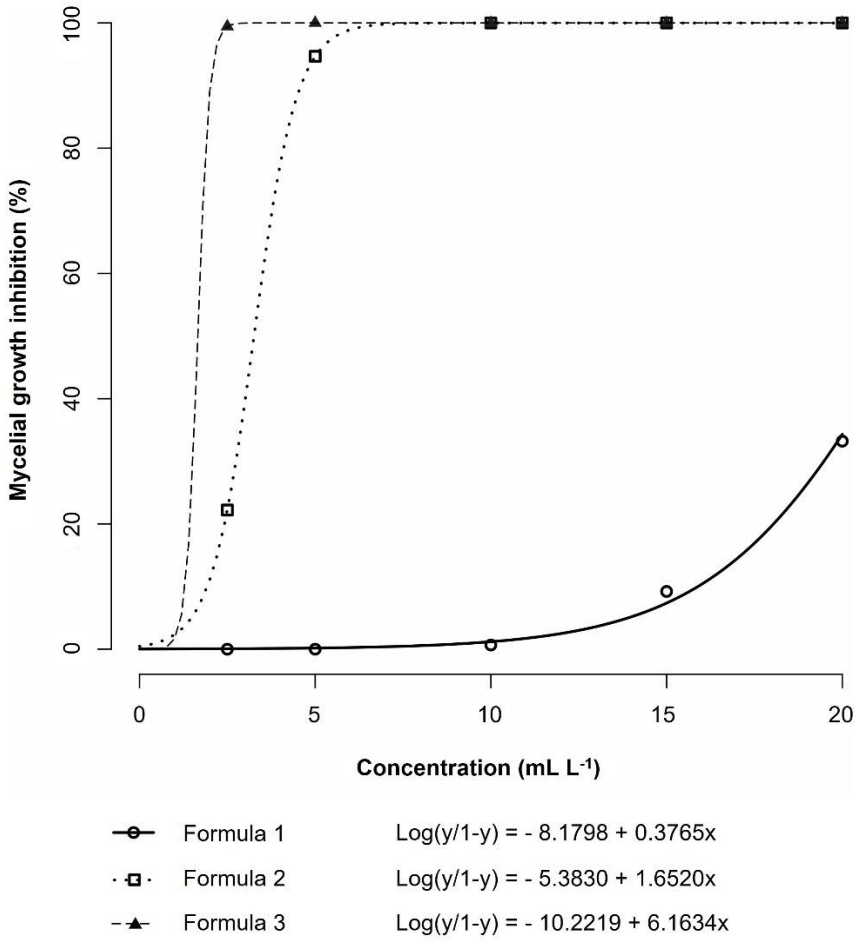

Figure 2. Inhibition of mycelial growth in *Phytopythium helicoides* three days after incubation at different concentrations of the tested formulations.

inhibit *P. helicoides* at concentrations of 2.5 and 5 mL L^{-1}, however, for *F. solani* f. sp. *piperis* these concentrations inhibited mycelial growth in 31.17% and 13.14%, respectively. Formulation 2 completely inhibited the mycelial growth of *P. helicoides* at concentrations of 10; 15; and 20 mL L^{-1}, while for *F. solani* f. sp. *piperis* the inhibition of mycelial growth at these concentrations were of 0.43 %, 10.65% and 14.69%, respectively (**Figures 1 and 2**). According to ED_{50} (**Table 2**) values formulation 3 showed higher inhibitory effect on mycelial growth for both isolates. Then, to inhibit 50% of *F. solani* f. sp. *piperis* mycelial growth it required 2.59 mL L^{-1} of the formulation, while *P. helicoides* was inhibited with only 1.66 mL L^{-1}, being this formulation more efficient when compared with the formulations 1 and 2.

The percentage and the composition of the plant extracts presents in the formulations can explain the inhibition of mycelial growth in the microorganisms accessed. In fact, formulation 3, which contains in their composition 50% of neem and coffee pyroligneous extracts resulted in high rate of mycelial growth inhibition for both isolates. However, when the effect of inhibition for *F. solani* f. sp. *piperis* was tested with formulation 2, which has on its composition 35% more of neem extract when compared to formulation 3 and has 15% of technical glycerin instead of pyroligneous coffee extract, the mycelium growth inhibition rate of *F. solani* f. sp. *piperis* drastically decreased, demonstrating that pyroligneous coffee extract exhibited higher fungicidal effect that the neem extract for this fungus. However, when the percentage of pyroligneous coffee extract was reduced by 15% in the composition of formulation 1, the inhibition of mycelial growth was of 100% for *F. solani* f. sp. *piperis*, only at higher concentrations (15 and 20 mL L^{-1}). This result can be attributed to the inclusion of coffee extract in the formulation, although in lower percentage. Coffee extracts have compounds such as phenols, fulvic and humic acids, copper, zinc and potassium on its chemical composition, which may have fungicidal activity (Chong and Dumas, 2012; Santos *et al*., 2019; Silva *et al*., 2021; Okur *et al*., 2021). Chlorogenic acids are the main components of the phenolic fraction from green c offee beans, with levels of up to 14% dry matter (Farah and Donangelo, 2006). In fact, phenolic compounds and chlorogenic acids have been reported to inhibit mycelial growth and spore germination in *Sclerotinia sclerotiorum*, *F. solani*, *Verticillium dahliae*, *Botrytis cinerea*, *Cercospora sojina, Colletotrichum gloeosporiodes, Trichophyton mentagrophytes*, *T. rubrum* and *Candida parapsilosis* (Martínez *et al*., 2017; Zhang *et al*., 2022; Calheiros *et al*., 2023). According to Calheiros *et al*. (2023), compounds present in spent coffee grounds caused a significant reduction of the ergosterol, chitin, and β-(1,3)-glucan content of *C. parapsilosis*, revealing the synthesis of this membrane and cell wall components as possible targets for these extracts.

Yi *et al*. (2021) observed the application of 800 mL of neem leaf extracts (50g 100mL^{-1}) to 25 kg soil, reduced the occurrence of *F. oxysporum* f. sp. *cubense* Race 4 in Cavendish banana plantlets (*Musa* spp. AAA group cv. Grand Naine) and significantly improved the crop height, stem diameter, root size (root surface area, root diameter, and root volume) and root-shoot ratio, as well as soil physicochemical properties. Rodrigues *et al*. (2019), demonstrated that neem oil at a concentration of 0.3% inhibited the mycelial growth of *Aspergillus carbonarius* strains in more than 95%.

Regarding *P. helicoides* sp. only the formulations 3 and 2 were significantly effective to reduce the mycelial growth after three days of incubation (**Figure 2**). Hence, it was noticed that the absence of pyroligneous coffee extract (formulation 2) did not reduce the mycelial growth of this microorganism, however, differently of the result obtained for *F. solani* f. sp. *piperis*, *P. helicoides* seems more sensitive to neem extracts when compared to pyroligneous coffee extracts. On the other hand, the reaction of substances constituting neem and pyroligneous coffee extracts (formulation 3) could be associated to the potential fungistatic against *P. helicoides* and *F. solani* f. sp. *piperis*. New *in vivo* studies are required to validate the results obtained in the present work.

Conclusion

The highest concentrations of the tested formulations caused reduction of the mycelial growth in *F. solani* f. sp. *piperis* and *P. helicoides*. However, considering the ED_{50}, the formulation 3, which contain 50% of neem and pyroligneous coffee extracts is more efficient to control both microorganisms.

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