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Potential of aqueous extracts of basidiomycetes to control root-knot nematodes on lettuce

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ABSTRACT

The root-knot nematode (*Meloidogyne incognita*) is one of the main pests of lettuce due to the crop's high susceptibility, unavailability of registered nematicides and lack of resistant cultivars. The aim of this study was to evaluate the potential of aqueous extracts of ten basidiomycete fungi for root-knot nematode control (*in vitro* and *in vivo)* on lettuce. The aqueous extracts of these fungi were initially evaluated *in vitro* in relation to their nematostatic and nematicidal activity. All extracts inhibited the hatching of second-stage juveniles of nematodes. The extracts that provided the highest mortality index (*Pleurotus ostreatus*, *P. citrinopileatus*, *P. pulmonarius* and *Boletus* sp.) were applied in pots containing autoclaved and infested soil with root-knot nematode. After 24 h, one lettuce seedling (cv. Regina) per pot was transplanted using soil treated with distilled water as control. After 50 days, we observed that soil treated with fungal extracts reduced, approximately, 70% of nematode reproduction. Plants treated with extracts obtained higher fresh mass and extracts of *Boletus* sp. and *P. pulmonarius* reduced damages to roots, being considered as potential bio-controllers of this nematode.

Keywords: *Lactuca sativa*, *Meloidogyne incognita*, *Pleurotus* spp., *Boletus* sp., biocontrol.

RESUMO

Potencial de extratos aquosos de basidiomicetos no controle de nematoides das galhas em alface

Os nematoides das galhas (*Meloidogyne incognita*) estão entre as principais pragas da alface em função da elevada suscetibilidade da cultura, indisponibilidade de nematicidas e carência de cultivares resistentes. Foi objetivo deste trabalho, avaliar o potencial de extratos aquosos de dez diferentes basidiomicetos no controle de nematoides das galhas *in vitro* e *in vivo* na cultura da alface. Inicialmente os extratos foram avaliados *in vitro* quanto à atividade nematicida e nematostática. Todos os extratos inibiram a eclosão de juvenis de segundo estádio do nematoide. Os extratos que proporcionaram maiores índices de mortalidade (*Pleurotus ostreatus*, *P. citrinopileatus*, *P. pulmonarius* e *Boletus* sp.), foram aplicados em vasos com solo autoclavado e infestado com nematoides das galhas. Após 24 h, transplantou-se uma muda de alface cv. Regina por vaso, usando solo tratado com água destilada como testemunha. Decorridos 50 dias, verificou-se que o tratamento do solo com os extratos fúngicos reduziu, em média, 70% da reprodução do nematoide. As plantas tratadas com extratos obtiveram maior massa fresca e os extratos de *Boletus* sp. e *P. pulmonarius* reduziram os danos às raízes, sendo considerados potenciais biocontroladores desse nematoide.

Palavras-chave: *Lactuca sativa*, *Meloidogyne incognita*, *Pleurotus* spp., *Boletus* sp., biocontrole.

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Lettuce (*Lactuca sativa*) is the most consumed leafy vegetable in Brazil (ABCSEM, 2016) and is of great economic importance (Lima *et al*., 2008), mainly for family farming, since this crop is grown in small areas and contributes for generating direct jobs. Due to the high perishability of this vegetable, lettuce is grown in small areas near consumption centers in consecutive plantations all over the year (Henz & Suinaga, 2009).

The intensive use of the soil, under monoculture system, promotes one of the main phytosanitary problems of lettuce crop, as for example the nematodes of genus *Meloidogyne* (Wilcken *et al*., 2004). High infestation of root-knot nematodes reduces quantity and quality of the harvested product (Santos, 1995) and, considering the soil texture, it can cause host death (Prakob *et al*., 2009).

In Brazil, *M. incognita* and *M. javanica* are the species of greatest incidence on lettuce-producing regions (Pinheiro *et al*., 2013). Although the management of these species is essential, it presents several difficulties, since there are no registered chemicals for nematode control for the crop (AGROFIT, 2017). The majority of commercial cultivars is susceptible and, crop rotation, besides presenting resistance of farmers due to the low availability of productive area, must be careful, since most of the cultivated plant species can be infected by these pathogens (Dias *et al*., 2007).

Thus, evaluating alternative strategies, such as biological control, in order to make production in areas

infested with these pathogens viable, is essential. In this context, the fungi are among the main biological agents used in the management of phytoparasitic nematodes (Li *et al*., 2007), including *Meloidogyne* species (Swe, 2011; Degenkolb & Vilcinskas, 2016a,b). Besides acting as natural enemies capturing and parasiting nematodes (Goswami *et al*., 2006; Haseeb & Kumar, 2006; Swe *et al*., 2011), these organisms are able to produce several antagonistic substances (Tranier *et al*., 2014; Degenkolb & Vilcinskas; 2016a). Several nematicidal-action substances were isolated from basidiomycetes, including different fatty acids produced by species of *Pleurotus* (Li *et al.,* 2007); the efficiency of these fungi was previously demonstrated, reducing galls on tomato plants (Putzke *et al.*, 2007).

Some studies about formulations using vegetative mycelium of basidiomycetes have been highlighted. However, activity, concentration and diversity of compounds in fungi are generally higher in fructification bodies compared to the ones observed in mycelium (Tidke & Raí, 2006).

Given the above, we aimed to evaluate the *in vitro* potential of different mushroom extracts on *M. incognita* biocontrol, select the best isolates for in vivo test and evaluate the efficiency of water extracts previously selected, for *M. incognita* biocontrol on lettuce crop, under greenhouse conditions.

MATERIAL AND METHODS

Obtaining aqueous extracts

The mushrooms *Pleurotus ostreatus*, *P. ostreatoroseus*, *P. citrinopileatus*, *P. sajor-caju* and *P. pulmonarius* were grown in Laboratório de Micologia do Departamento de Microbiologia e Parasitologia do Instituto de Biologia of Universidade Federal de Pelotas. These fungi belong to the collection of the laboratory and were grown following severely pasteurized substrate technique (Bernardi, 2007). Other mushrooms used were *Amanita muscaria*, *Boletus* sp., *Lactarius deliciosus*, *Russula amethystina* and *Suillus* sp.. These were collected from May to August, 2009 at UFPel Campus and Embrapa Clima Temperado, in Pelotas. These mushrooms were identified in the laboratory, observing fruiting body morphological traits. After harvest, basidioms were washed and dehydrated (35°C for 15 days) in the laboratory.

In order to prepare aqueous extracts, the authors followed adapted cold extraction methodology (Fiori-Tutida *et al*., 2007). Dry mushrooms were ground in proportion of 50 g of mushroom/L distilled water. The mixture was kept under refrigeration at 4ºC for 24 hours and, then, filtered through cotton, followed by centrifugation at 5,000 RPM, at 4°C, for one hour. The supernatant was filtered through Whatman # 1 filter, 0.45 and 0.25 µm cellulose acetate membranes, respectively. Finally, the last step was carried out under aseptic conditions in a laminar flow chamber. Extracts were tested within a maximum period of 24 hours after preparation.

Inoculum of *M. incognita*

A pure population of *Meloidogyne incognita* was multiplied and kept in tomato crop cv. Santa Cruz in greenhouse (Carneiro & Almeida, 2001). Eggs and second-stage juveniles (J2) of *M. incognita* were obtained according to Hussey & Barker technique (1973). Second-stage juveniles (J2), used in *in vitro* tests, were extracted from roots infected with nematodes by a modified Baermann funnel technique (Christie & Perry, 1951).

In vitro tests

The nematicidal activity of extracts was obtained in an assay conducted on Elisa plates, four replications per treatment, in a randomized complete block design, considering each replication represented by a plate cavity containing 25 J2 of *M. incognita*. The mortality was tested through the modified methodology described by Ludwig *et al*. (2013). Each Elisa plate cavity was covered with a 20 µL aliquot of distilled water containing J2 of nematode. Then, 80 µL of aqueous extracts of each basidiomycete was added, except for the control, in which just distilled water was used. Afterwards, the plates were sealed with plastic film and kept in a BOD incubator at 25°C in the dark. After 24-hour incubation, in each plate cavity, 10 µL of NaOH 1N at 1% was added adapting the methodology proposed by Chen *et al*. (2000), in which the number of dead and alive juveniles was evaluated. We considered dead the J2 whose body remained completely distended for one minute after adding NaOH, during evaluations under stereoscopic microscope.

Data on the percentage of dead J2 were transformed to arcsine using comparisons of treatment means by Scott-Knott test at 5% probability using SASM- AGRI program (Canteri *et al*., 2001).

To verify the effect of extracts on J2 nematode outbreaks, an Elisa assay was installed, similarly to the mortality assay previously described. In this case, a 20 µL aliquot of distilled water containing approximately 25 eggs was put in each plate cavity, adding J2 nematodes, adding 80 µL of aqueous extracts of mushroom and distilled water on the control. Then, the plates were covered with plastic film and kept in a BOD incubator in the dark for 12 days. After incubation, the number of hatching juveniles and remaining eggs was evaluated, to determine hatching percentage of J2 of *M. incognita.* The percentage was obtained using the formula:

hatching (%) = (number of juveniles)/ (number of juveniles+remaining eggs) x 100.

Then, treatment averages were compared among each other by Scott Knott group test at 5% probability using SASM-AGRI program (Canteri *et al*., 2001).

Effect of fungi extracts on control of *M. incognita* in lettuce crop

According to the obtained results in *in vitro* tests, mushrooms whose extracts caused higher percentage of juvenile mortality of *M. incognita* were selected.

To simulate the use of mushroom extracts in treatment of pre-cultivation soil on lettuce, autoclaved soil (121ºC for 2 hours) was kept in a greenhouse for five days, homogenized, evaluated in relation to field capacity and fractioned into portions of two kilos. Each soil fraction was kept in a plastic bag, with

100 mL water, 18 mL of mushroom extract (10% p/v) and 1.5 mL of eggs suspension containing 5,000 eggs + J2 of *M. incognita* obtained from infested tomato roots, according to Hussey & Barker (1973). Afterwards, soil was homogenized, transferred to pots and covered with plastic film.

For control treatments, pots with autoclaved soil treated with distilled water and with or without nematode infestation were used. One lettuce seedling, cultivar 'Regina', was transferred to the center of each pot, 24 h after treatment application. Subsequently all pots were kept in a greenhouse at 25±3ºC. The experiment consisted of six replications (pots) per treatment distributed in a completely randomized design.

Fifty days after inoculation, lettuce plants were removed from the soil, and shoot fresh mass determined using a semi-analytical scale; roots were carefully washed and root fresh mass, number of galls and damage level were evaluated according to the scale proposed by Zech (1971), being scored from 0 to 10, according to visual inferences in relation to number of galls and severity symptoms caused by nematodes in the plant. Grade 0 corresponded to the plant with no infection and 10 to the dead plant. Right after, the roots of each plant were chopped and grinded in a blender with 0.5% hypochlorite solution for extraction of *M. incognita* (Hussey & Barker, 1973). Total number of eggs and J2 in roots were determined and the reproduction factor of *M. incognita* per plant (final population / initial population), related to each replication was calculated (Oostenbrink, 1966), estimating the control percentage in each treatment. Treatment averages were compared among each other using Duncan test at 5% probability using SASM- AGRI program (Canteri *et al*., 2001).

RESULTS AND DISCUSSION

All evaluated fungal extracts showed some nematostatic and nematicidal activity against eggs and J2 of *M. incognita*, respectively, in relation to control (Table 1), in the *in vitro* tests.

The nematicidal effect of fungi on nematode presented mortality indexes ranging from 90.7 to 100% on different treatments. However, the extracts of *P. ostreatus*, *Boletus sp.*, *P. pulmonarius* and *P. citrinopileatus* provided the highest percentage of dead nematodes (Table 1). Thus, these four isolates were selected to evaluate the potential for biological control of *M. incognita* in lettuce crop, in greenhouse.

The extract potential for controlling nematodes was proved in *in vivo* tests, in which suppressed the reproduction of *M. incognita* and provided the highest values of fresh mass of lettuce plants. Damage severity in lettuce was significantly reduced only with extracts of *Boletus* sp. and *P. pulmonarius*. Nevertheless, the number of eggs and reproduction factor of *M. incognita* were significantly lower in comparison to the control in all treatments (Table 2), showing the efficiency of mushroom extracts, evaluated in this study, on root-knot nematode control.

Nematode control using mushroom extracts achieved an average reduction of nematode reproduction of 62%, compared to the control group, which may have favored the development of plants expressed by an increase of shoot fresh mass (Table 3), being this yield statistically similar to the plants grown without nematodes (Table 3).

Although the aqueous extracts did not affect root fresh mass (Table 3), soil treatment using *P. pulmonarius* and *Boletus sp.* extracts, resulted in lower levels of damaged roots of lettuce (Table 2). Thus, these treatments evidenced the main objective of the use of alternative methods with biological control agents which is damage reduction since the yield of plants grown in a soil treated with extracts was similar to those ones observed in plants grown without nematodes (Table 3).

The use of basidiomycetes may contribute to the phytoparasitc nematodes control a great deal. In this sense, basidiomycetes may affect motility and capacity of penetration of active forms into plants, the attraction of the juvenile by the host, interfering in their hatching or causing the death of these phytoparasites (Kulkarni & Sangita, 2000; Hong *et al*., 2007). Although toxic to nematodes, most fungal compounds present in the basidiocarp have a selective effect, since they preserve non-target species, showing the possibility to develop products which are safer to the environment (Li *et al*., 2007).

The potential of several mushrooms of the genus *Pleurotus* was also showed, controlling *M. javanica, in vitro,* in studies demonstrating the presence of specialized structures in vegetative mycelium which secrete substances able to immobilize nematodes (Heydari

Table 1. Effect of aqueous extract from ten basidiomycete fungi on hatching and mortality (%) of J2 *Meloidogyne incognita*. Pelotas, Embrapa Clima Temperado, 2018.

Treatment	Mortality[1]	Hatching	Hatching inhibition
Pleurotus ostreatus	100.00a	1.96b**	98.40b
Boletus sp.	98.96a	4.98b	95.02b
Pleurotus pulmonarius	98.00a	4.59b	95.41b
Pleurotus citrinopileatus	97.88a	3.09b	96.91b
Amanita muscaria	96.58b	2.95b	97.05b
Russula amethystina	95.69b	5.85b	94.15b
Lactarius deliciosus	95.39b	7.12b	92.88b
Suillus sp.	94.36b	3.00b	97.00b
Pleurotus sajor-caju	94.22b	4.04b	95.96b
Pleurotus ostreatoroseus	90.66b	5.62b	94.38b
Control (distilled water)	4.95c	54.66a	45.34a
CV (%)	8.82	35.51	25.30

Averages followed by same letters in the column did not differ from each other, Scott-Knott test at 5%. [1]Original values transformed into $\arcsin\sqrt{x/100}$.

et al., 2006). Besides, studies on nematode *Panagrellu s*sp. demonstrated that *P. ostreatus* provides different enzymes which may contribute directly to biological control of phytonematoids (Genier *et al*., 2015). Although specialized structures against nematodes are reported in vegetative stage, basidioms or mushrooms are known for the diversity of secondary metabolites with a broad spectrum of biological activities exploited in traditional medicines (Ganeshpurkar & Jain, 2010).

Hatching inhibition potential or nematicidal effect on J2 of *M. incognita* was confirmed in the *in vivo* evaluation, in which the extract of *P. ostreatus* reduced reproduction factor of *M. incognita* in lettuce by more than 70%. The efficiency of *Pleurotus spp.* to control nematodes was also demonstrated in other studies, emphasizing the importance of further studies. *Pleurotus sajor-caju* was used to control mycophagous nematodes which affect the production of champignon *(Agaricus bisporus)*, in which the application of residue extract of *P. sajor-caju* in the substrate reduced population of *Aphelenchoides composticola* in 90% (Sharma, 1994). In another study, the application of substrate colonized by *P. ostreatus* and *P. ostreatoroseus* in soil, provided 70% reduction in the number of galls caused by *M. javanica* in tomato (Putzke *et al*., 2007).

The effect of *Pleurotus* spp. was also described in controlling *M. incognita* in soybean, reporting reduction of number of galls and promotion of plant growth, as well as an increase in number of rhizobium nodules (Okorie *et al.*, 2011), showing, similarly to this study, that the treatment with mushroom can suppress *M. incognita.*

In addition to the reduction of reproduction factor of nematodes, fungal treatments resulted in reduction of *M. incognita* damage up to two levels on Zech scale. Marino & Silva (2013), evaluating isolates of *P. ostreatus* containing coconut powder to control *M. incognita* on lettuce, verified reduction of number of galls and egg masses up to 90%. Similarly, other authors tested different substrates for soil biofumigation in biological control of *M. incognita* on tomato crop and showed that residue of *P. ostreatus* reduced in 70% nematode population density (El-Sherbiny & Awd-Allah, 2014), as observed in this study.

Moreover, fungal potential was verified in experiments with violets, in which application of substrate containing *P. ostreatus* mycelium suppressed *M. javanica* (Abbasi *et al*., 2014). Besides, application of 500 g of substrate containing *P. ostreatus* for each cubic meter of soil was able to reduce *M. incognita* and *Heterodera goldeni* population on rice plants, above 80%, surpassing treatments with *Bacillus thuringiensis* and obtaining almost the same result using nematicide Oxamyl (Awd-Allah & El-Sherbiny, 2015).

Although several studies demonstrate the efficiency of basidiomycetes in the control of plant parasitic nematodes, formulations obtained from vegetative phase of fungi are frequently used. However, activity, concentration and diversity of compounds in fungi are generally higher in fructification bodies compared with the ones observed in mycelium (Tidke & Raí, 2006).

The selected mushrooms for *in vivo* evaluation, such as *Pleurotus* spp., are edible; so they are cultivated and commercially exploited (Ramos *et al*., 2011). That is why, evaluating basidioms as an alternative for pest control, considering both efficiency and possibility to use the residue, generated by the edible mushroom industry, is so important.

Extracts of basidiomycete fungi may represent a promising management strategy in relation to existing difficulties for phytoparasitic

Table 2. Effect of aqueous extract of dried mushrooms on level of damage, control and reproduction of *Meloidogyne incognita* on lettuce cv. Regina cultivar under greenhouse conditions. Pelotas, Embrapa Clima Temperado, 2018.

Treatment	Damages	Number of eggs[1]	FR[2]	Control[4] (%)
Control[3]	5.0 a	155000 a	31.00 a	-
Boletus sp.	3.0 c	83444 b	16.68 b	46.20
P. pulmonarius	2.5 c	58778 b	11.75 b	62.09
P. citrinopiliatus	4.5 ab	51556 b	10.31 b	66.74
P. ostreatus	4.0 abc	39996 b	7.99 b	74.22
CV (%)	33.70	73.6	73.6	

Averages followed by same letters in the column did not differ from each other, Duncan test at 5%. [1]Original values transformed into arcsin $\sqrt{x/100}$; [2]FR= reproduction factor (FR= final population / initial population); [3]control (soil without treatment and infested with *M. incognita*); [4]control in relation to control group. Damage grades varying from 0 (no infection) to 10 (dead plants).

Table 3. Effect of aqueous extracts of dried mushrooms on shoot fresh mass (MFPA) and root fresh mass (MFR) of lettuce plants cv. Regina in soil infested with *M. incognita*. Pelotas, Embrapa Clima Temperado, 2017.

Treatment	MFPA[1] (g)	MFR[2](g)
Boletus sp.	74.06 a	22.95 a
P. pulmonarius	67.11 a	17.78 a
P. citrinopiliatus	66.17 a	18.00 a
P. ostreatus	62.88 a	23.10 a
Non-infested control	64.14 a	12.79 a
Infested control	39.53 b	16.22 a
CV (%)	20.97	50.37

Averages followed by same letters in the column did not differ from each other, Duncan test at 5%; [1]Shoot fresh mass; [2]Root fresh mass.

nematodes management in lettuce crop. However, the importance of its use in integrated management approach must be considered, combining other control techniques in order to suppress this pest and also provide an increase in production and quality of lettuce.

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