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DISEASE CONTROL

Evaluation of two nematophagous fungi for the control of false root-knot nematode *Nacobbus aberrans* in pepper crops

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Abstract

Nacobbus aberrans is an endophytic parasitic nematode that induces root galls, negatively affecting plant growth and development, and decreasing the production of economically important crops. Currently, low environmental impact alternatives are being developed for the control of nematodes, such as biological control agents, to reduce the use of soil disinfectants. Nematophagous fungi are microorganisms that can suppress nematode reproduction due to different mechanisms, such as parasitism of eggs, toxin production and stimulation of plant growth. The aim of this study was to investigate the effect of inoculation with nematophagous fungi on *N. aberrans* in greenhouse-grown pepper (*Capsicum anuumm* L.) plants and to analyze the response of the infected plants. Two fungal isolates, *Purpureocillium lilacimum* and *Pleurotus ostreatus* were tested. The plants were inoculated with these fungi at the time of transplantation in the presence and absence of *N. aberrans.* The reproduction factor of the nematode was 23.17; 5.90 and 36.4 for *P. lilacinum, P. ostreatus* and control, respectively. Both in plants parasitized and not parasitized by nematodes, the fungi increased the content of soluble proteins and photosynthetic pigments. Additionally, a favorable impact on growth parameters was also observed. This beneficial effect was also verified by a lower accumulation of proline and sugars, metabolites used by plants as osmoregulators in stress situations, and a low accumulation of malondialdehyde, a metabolite resulting from oxidative stress. These results show that both fungi are suitable for use in the biocontrol of *N. aberrans* and as growth promoters in plants.

Keywords Biocontrol · *Capsicum anuumm* L. · *Pleurotus ostreatus* · *Purpureocillium lilacinum*

Introduction

Annual crop losses in global agricultural production due to infection by plant parasitic nematodes are estimated to be close to 11%, with annual economic losses around 80 billion dollars (EPPO 2019). In Argentina *Nacobbus spp.* and *Meloidogyne spp.* are the most economically important root-knot nematodes stands out (Jones et al. 2013). *Nacobbus aberrans* is a sedentary endoparasite that induces root galls on its hosts. These external symptoms on the roots resemble those produced by species of the genus *Meloidogyne* (the root-knot nematode), which is why it is commonly referred to as the false root-knot nematode (Lax et al. 2013). The species is indigenous to America and is present in Argentina, Bolivia, Chile, Ecuador, Mexico, Peru and the USA (EPPO 2019). It is polyphagous, and sedentary females cause exomorphological alterations, while juveniles cause lacerations and necrosis in the roots due to repeated entrances and exits to its (Manzanilla-Lopez et al. 2002) affecting cell integrity and the uptake of water and nutrients.

In intensive crops, the control of nematodes has traditionally been based on soil fumigants (such as Methyl Bromide), often complemented by crop rotation and resistant cultivars. However, as the use of fumigants was reduced due to their prohibition established by international agreements, the appearance of this pest increased, along with the indiscriminate use of chemical nematicides. Therefore, there is an urgent need to find innovative, environmentally friendly and effective management strategies for the control of nematode populations to achieve more sustainable horticulture. One of

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the new technologies is the use of biological products, which entails incorporating organisms selected for their functions in various biological processes into the productive system (Puente et al. 2010). These bio-inputs are frequently applied to sites that receive other inputs, such as phytosanitary products, fertilizers and soil amendments, and may interact to control a particular pest (Abd-Elgawad and Askary 2018). Some microorganisms have nematicidal potential, such as mycorrhizal fungi, rhizobacteria and nematophagous fungi (Askary and Martinelli 2015). The latter can attack, kill and digest nematodes. Although they belong to different ecological groups (Sagués et al. 2011), four main categories can be distinguished based on the mechanism of action: trappers, endoparasites, parasites and toxin producers.

For this work, a parasitic fungus, *Purpureocillum lilacinum* (Luangsa-Ard et al. 2011) and a toxin producer fungus, *Pleurotus ostreatus* have been characterized as potential biocontrol agents for nematodes. *P. lilacinum* hyphae have been shown to penetrate all nematode stages after appressorium formation. This structure releases chitinases and lipase enzymes that degrade the cuticle of the different states of the nematode, thus achieve penetration to the host (Mukhtar et al. 2013). Fungi belonging to the genus *Pleurotus* are powerful biological agents that can thrive on agricultural residues (substrates) and produce fruiting bodies that are safe for human consumption (Golovko et al. 2022). The majority of *Pleurotus* species demonstrate nematicidal activity by colonizing nematodes. Also, *P. ostreatus* fungus specifically produces toxins such as trans-2 decenedioic acid (Kwok et al. 1992). Upon contact with nematodes, these toxins immobilize the nematodes, which are then colonized and digested entirely, thus serving as a source of nitrogen (Genier et al. 2015). Additionally, these fungi produce various biomolecules, such as lectins, hydrolytic enzymes and organic acids, which have substantial biological activities (Papaspyridi et al. 2011). Due to their eco-friendly nature, ease of reproduction, fast-growing and harmless to the environment, which makes them feasible to be studied as a potential bio-input in the control of nematodes in horticultural crops. The aim of this study was to investigate the effect of inoculation with nematophagous fungi on *N. aberrans* in greenhouse-grown pepper (*Capsicum anuumm* L.) plants and to analyze the response of the infected plants.

Materials and methods

Nematode inoculum for the experiments

For this study, an isolate of *N. aberrans* derived from the locality of Los Hornos, La Plata (Buenos Aires province, Argentina) was employed. This particular isolate has been recognized for its ability to propagate in pepper plants (Lax et al. 2011). Eggs of *N. aberrans* were collected from indigenous cultivation cultures displaying symptomatic manifestations (galls) of nematode infection. Sedentary females were classified as *N. aberrans* with the aid of a magnifying glass, while juveniles were identified using a microscope. The egg masses were employed for the inoculation of tomatoes "platense" landrace (*Solanum lycopersicum* L.), a confirmed susceptible host, in order to amplify the nematode population for subsequent experimentation. The Coolen (1979) flotation technique was employed for inoculum preparation. In this approach, diseased roots were subjected to a 30-s maceration using a mixer along with a solution comprising water and low-concentration sodium hypochlorite (5%). This process served to dissociate the eggs from the root material. The resulting mixture was rinsed with water, and the ensuing suspension underwent a series of centrifugation and flotation procedures. Nematode eggs were quantified using an optical microscope and promptly employed for inoculation on the same day to ensure their vitality.

Fungi inoculum for the experiments

P. lilacinum strain LPSC # 876 and *P. ostreatus* strain were obtained from the culture collections of the Instituto Spegazzini, UNLP, La Plata, Argentina, and the Laboratorio de Micología y Cultivo de Hongos Comestibles y Medicinales, IIB-INTECh (CONICET), Chascomús, Argentina, respectively. To propagate the inoculum, both strains were cultured separately on Petri dishes containing a 1.5% potato glucose agar (PGA) medium with a pH of 6. The dishes were then incubated in the dark at 28–30 °C for 7 days until complete colonization of the culture medium was achieved. Subsequently, several 6-mm diameter mycelial plugs from agar cultures were used to inoculate glass bottles filled with an autoclaved substrate composed of oat seeds and Salicaceae wood chips in a 1:1 ratio. The cultures were incubated under solid-state fermentation conditions in a culture chamber at 28 °C in the dark (Inalbón et al. 2015).

Greenhouse experiment

The experiment was conducted in a greenhouse of the Instituto de Fisiología Vegetal located in La Plata, Argentina, between December and March. Paco f1 pepper seeds were disinfected with NaOCl (10%) for 5 min and then sown in 72-cell plastic plug trays filled with a substrate mix of perlite-vermiculite (1:1), which had been previously autoclaved. After 40 days, seedlings were transplanted into 5 kg pots filled with a substrate mix of tindalized soil and sand (1:1). During transplantation, 100 cm^3 of the substrate with mycelium were added to the pots to inoculate the plants with the different fungi and half of the pots were inoculated with 5000 *N. aberrans* eggs.

The control treatments were supplemented with fungi-free autoclaved substrate (100 cm^3) to ensure consistent test conditions across all treatments.

The treatments were: two nematophagous fungi *P. ostreatus* (PO), *P. lilacinum* (PL) and plants without fungi (NI); each of the three treatments was further divided into 2 equivalent parts, with *N. aberrans* (N1) and without *N. aberrans* (N0). Once the experiment was finished, at 90 days after transplant, the height, diameter of the final stem and dry weight were measured. Then a sampling of the aerial part and roots was carried out to determine the following parameters:

- Number of nematode eggs in roots, extracted by Hussey and Barker method (1973), separated and purified by the Coolen method (1979).
- Nematode Reproductive Factor (RF), was determined according to Oostenbrink (1966). The RF was calculated using the expression RF = Fp·Pi − 1, where: Fp = final population (number of eggs and larvae extracted from roots) and Pi = number of inoculated eggs. Plants with RF = 0 were considered immune; with RF < 1 were considered resistant, and those with RF > 1 were considered susceptible.
- Soluble protein content: determined from 100 mg of leaves (fresh weight). The absorbance was measured at a wavelength of 595 nm using a spectrophotometer UV-160 (Shimadzu, Kyoto, Japan) and protein concentration was calculated using a standard curve prepared with different concentrations of bovine serum albumin (BSA, SiFMa Chemical Co) according to the method described by Bradford (1976).
- Chlorophyll and carotenoids content: determined from a 0.5 cm diameter leaf disk. N,N-Dimethylformamide was used as the extraction solvent and the absorbance of the solution was measured at 647, 664 and 480 nm using a spectrophotometer spectrophotometer UV-160 (Shimadzu, Kyoto, Japan). Pigment content was calculated according to Wellburn's (1994) method.
- Proline content: measured from 100 mg of leaves and roots (fresh weight). The absorbance was measured at a wavelength of 520 nm using a spectrophotometer UV-160 (Shimadzu, Kyoto, Japan), and the proline content per unit of fresh weight (FW) was calculated as follows: µmol proline.g^{-1} FW = [(µg proline/ml x ml toluene)/115.5 µg/µmol]/[(g FW)/5], according to the method described by Bates et al. (1973).
- Total sugar content: measured from 500 mg of fresh material using the Somogy method (Cronin and Smith 1979). For total sugar determination, acid hydrolysis was conducted with 0.1 N HCl in a water bath at 100 °C, and the resulting reaction was obtained using cupric reagent. The absorbance was measured at a wavelength of 520 nm using a spectrophotometer UV-160 (Shimadzu, Kyoto, Japan).
- Malondialdehyde content (MDA): measured from 200 mg of leaves and roots using a spectrophotometer UV-160 (Shimadzu, Kyoto, Japan) as an indicator of peroxidation of cell membranes lipids using the method described by Heath and Packer (1968).
- Persistence of fungi in the soil: Following completion of the experiment, soil sampling was carried out to determine the persistence of fungi. Samples of 100 g were collected from each pot, from which 1 g of soil was taken and placed in 10 ml Falcon tubes. The samples were washed ten times with distilled water and the washed soil was then placed in Petri dishes with filter paper and placed in an oven for 24 h. Once dry, five soil particles were placed in Petri dishes with potato glucose agar (PGA) culture medium with 1 ml of antibiotic solution (1 g chloramphenicol + 0.5 g streptomycin in 200 ml of distilled water). The plates were incubated in dark conditions at 28 °C for 7 days to observe colony formation.
- Experimental design.

The trial was conducted using a completely randomized design, in a 3 × 2 factorial scheme, of three fungal conditions (NI, PO, PL) and two soil infection conditions (substrate N0, N1). Ten replicates per treatment were carried out, with each replicate representing a plant. The obtained results were analyzed using analysis of variance (ANOVA) and the mean values were compared by LSD at a significance level of 0.05 using the SISVAR 2018 statistical software.

Results

The inoculation of nematophagous fungi resulted in a significant decrease in root infection by nematodes, unlike the non-inoculated treatment with such fungi. This was evidenced by the quantification of a lower number of nematode eggs, resulting in a decrease in Reproduction Factor (RF) compared to NI treatment (Table 1).

Table 1 Number of total eggs, number of eggs/grams of root and Oostenbrick Reproduction Factor of the control treatments (NI) and the treatments inoculated with *Pleurotus ostreatus* (PO) and *Purpureocillium lilacinum* (PL) in the presence of *Nacobbus aberrans*

Treatment	Total eggs[a]	Eggs.gr^{-1} [a]	RF[a]
NI	182,000 a	2476.91 a	36.4 a
PO	29,500 c	481.95 c	5.90 c
PL	115,833 b	1730.04 b	23.17 b

[a]Different letters indicate significant differences ($p < 0.05$) between nematophagous fungi

In the absence of nematodes, the treatments with fungal inoculants exhibited greater plant heights and higher dry weights compared to the control (NI N0). However, these treatments resulted in smaller stem diameter. In the presence of nematodes, plants inoculated with PL exhibited increased height and dry weight compared to the control treatment (Table 2).

In the presence of the nematode, there was a significant decrease in the chlorophyll and carotene content. However, the inoculation with PO and PL increased the content of these pigments in plants, as compared to the treatments without inoculation (Table 3). A similar trend was observed with the content of soluble proteins in leaves, where the control and PO treatments obtained the highest contents ($p < 0.05$). Among the treatments inoculated with *N. aberrans,* it was observed that the protein content was higher in those where the nematophagous fungi were inoculated, with the PO treatment showing the highest increase. In the absence of the nematode, no significant differences were observed between the treatments in roots. However, in the presence of *N. aberrans,* the PO and PL treatments showed a higher protein content than the control without inoculation (Table 3).

Table 2 Height, diameter of the stem and dry weight of pepper plants in the absence (N0) and presence (N1) of *Nacobbus aberrans* and without inoculation (NI) and inoculated with *Pleurotu ostreatus* (PO) and *Purpureocillium lilacinum* (PL)

N. aberrans	Treatment	Height (cm)[a]	Diameter of the stem (cm)[a]	Dry weight (gr)[a]
N0	NI	65.17 Ba	8.11 Aa	18.40 Ba
	PO	74.70 Aa	7.58 Ba	19.40 ABa
	PL	74.40 Aa	7.67 Bb	20.20 Aa
N1	NI	67.00 Ba	8.36 Aa	13.40 Bb
	PO	67.36 Bb	6.79 Bb	15.30 ABb
	PL	73.50 Aa	8.23 Aa	16.40 Ab

[a]Different lower case letters indicate significant differences between the presence and absence of *N. aberrans*, different upper-case letters indicate significant differences between fungi for the same infection treatment with *N. aberrans* ($p < 0.05$)

Damage caused by nematodes results in plant damage similar to that caused by water deficit due to the damage inflicted on the roots. This stress is evidenced by the accumulation of osmolytes such as proline and total and reducing sugars, which is necessary to sustain water absorption. In the presence of nematodes, the NI treatment accumulated a significantly higher amount of proline than the treatments inoculated with nematophagous fungi, both in leaves and in roots. Moreover, proline accumulation was higher in the leaves than in the roots (Figs. 1a and b).

The total sugar content in leaves was significantly higher in the NI treatment compared to the other treatments in the presence of the nematode (Fig. 2a). In the absence of the nematode, PO exhibited a higher accumulation of total sugars. In turn, in the presence of the nematode, the sugar content in the treatments was higher than in its absence (Figs. 2a and b). The content of reducing sugars in the leaves was lower ($p < 0.05$) in the treatments inoculated with nematophagous fungi compared to the NI treatment in the presence of *N. aberrans* (Fig. 2b). No significant differences were observed in the roots, both in total and reducing sugars (data not shown).

The MDA content in leaves was higher in the presence of the nematode compared to the NI treatments. In the absence of the nematode, the content was higher in the plants treated with PO and PL compared to NI ($p < 0.05$) (Fig. 3). Although in the presence of the nematode, the trend was the opposite, with the NI treatment having a higher MDA content than the PO and PL treatments ($p < 0.05$) (Fig. 3). There was no clear trend observed in the roots, and therefore, the data is not presented.

Although PO was not recovered from the washed and analyzed soil particles, PL LPSC# 876 was detected in 30% of the particles from the soil sampled from the treatments. No phenotype similar to the two fungi studied was detected in the fungal colonies developed in the agarized medium when the seeded particles corresponded to the treatments without fungal inoculation (data not shown).

Table 3 Content of chlorophyll, carotenes, leaf and root proteins of pepper plants in the absence (N0) and presence (N1) of *Nacobbus aberrans* and without inoculation (NI) and inoculated with *Pleurotus ostreatus* (PO) and *Purpureocillium lilacinum* (PL)

N. aberrans	Treatments	Chlorophyll ($\mu g.cm^{-2}$)[a]	Carotenes ($\mu g.cm^{-2}$)[a]	Leaf proteins ($\mu g.mg^{-1}$ PF)[a]	Root proteins ($\mu g.mg^{-1}$ PF)[a]
N0	NI	54.30 Aa	8.66 Aa	7.96 Aa	0.95 Aa
	PO	55.11 Aa	7.32 Aba	6.99 Aa	0.76 Ab
	PL	29.56 Ba	6.97 Aa	5.64 Ba	0.73 Ab
N1	NI	15.32 Bb	4.40 Bb	3.78 Bb	0.87 Ba
	PO	31.16 Ab	6.01 Ab	8.13 Aa	1.36 Aa
	PL	27.51 Ab	5.17 ABb	4.17 Bb	1.15 Aa

[a]Different lower case letters indicate significant differences between the presence and absence of *N. aberrans*, different upper case letters indicate significant differences between fungi for the same infection treatment with *N. aberrans* ($p < 0.05$)

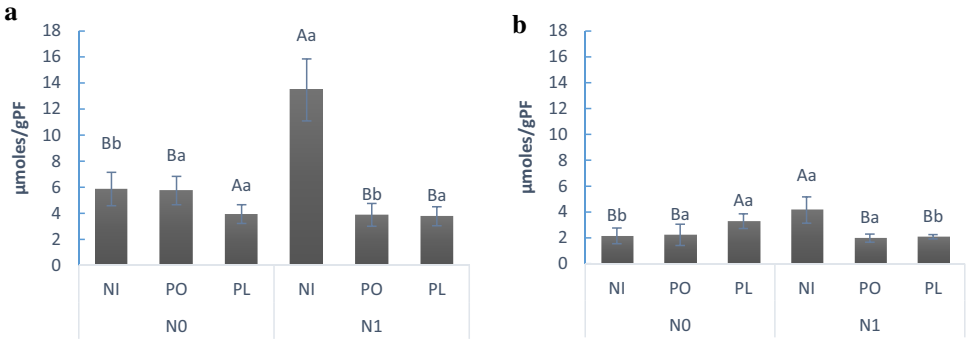

Fig. 1 Proline content in leaves **a** and roots **b** of pepper plants not inoculated (NI) and inoculated with nematophagous fungi: *Pleurotus ostreatus* (PO) and *Purpureocillium lilacinum* (PL); in the absence (N0) and presence (N1) of *Nacobbus aberrans* in the soil. Different lower case letters indicate significant differences between the presence and absence of *N. aberrans,* and different upper case letters indicate significant differences between fungi for the same infection treatment with *N. aberrans* ($p < 0.05$)

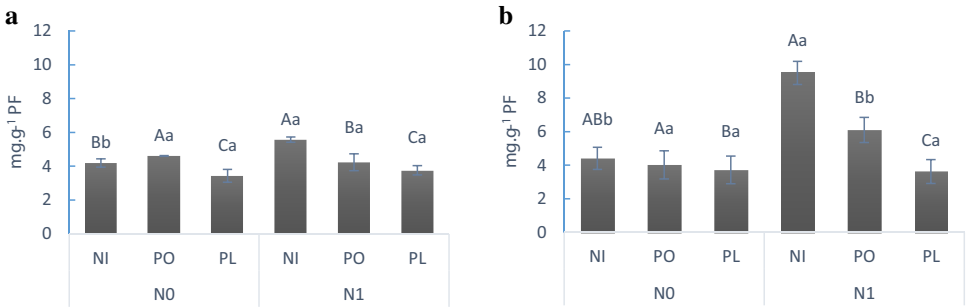

Fig. 2 Total sugars content **a**, reducing sugars content **b**, in leaves of pepper plants not inoculated (NI) and inoculated with the nematophagous fungi: *Pleurotus ostreatus* (PO) and *Purpureocillium lilacinum* (PL); in the absence (N0) and presence (N1) of *Nacobbus aberrans* in the soil. Different lower case letters indicate significant differences between the presence and absence of *N. aberrans*, and different upper case letters indicate significant differences between fungi for the same infection treatment with *N. aberrans* ($p < 0.05$)

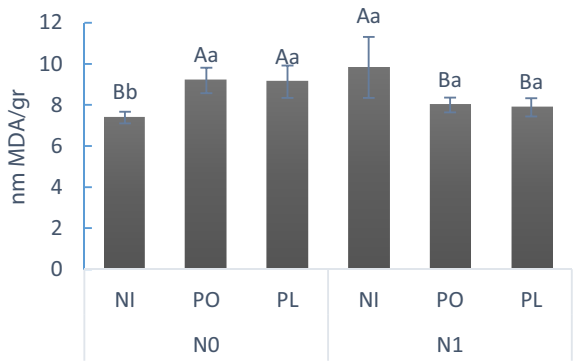

Fig. 3 MDA content in leaves of pepper plants not inoculated (NI) and inoculated with nematophagous fungi: *Pleurotus ostreatus* (PO) and *Purpureocillium lilacinum* (PL); in the absence (N0) and presence (N1) of *Nacobbus aberrans* in the soil. Different lower case letters indicate significant differences between the presence and absence of *N. aberrans*, and different upper case letters indicate significant differences between fungi for the same infection treatment with *N. aberrans* ($p < 0.05$)

Discussion

In this study, the inoculation with the fungi at transplant significantly reduced the infection in the roots by the nematode. Our results are consistent with previous studies reporting a significant reduction in the reproduction of *Meloidogyne incognita, M. hapla* and *M. javanica* in different horticultural crops such as lettuce and tomato, by inoculating with *P. ostreatus* and *P. lilacinum* (Khun-In et al. 2015; Kiewnick and Sikora 2006a,b; Wille et al. 2019). While in vivo investigations involving *N. aberrans* remain scarce, in vitro examinations utilizing *P. lilacinum* # 876 have unveiled its efficacy in parasitizing eggs (Gortari and Hours 2019). These researchers have successfully documented visual evidence of this strain parasitizing *N. aberrans* eggs. Similarly, Genier et al. (2015) have documented the paralysis of *Panagrellus* sp. by *P. ostreatus*, followed by a subsequent process of colonization and digestion.

Plants exposed to nematodes can wilt, exhibit reduced growth, loss of yield and even die (Cristobal et al. 2001). In this experiment, although there were no significant differences in stem height and diameter, PL stood out as having the highest height compared to controls. Concerning dry weight, a discernible decrease was noted in plants afflicted by nematode infestation. Conversely, plants subjected to PL inoculation exhibited elevated dry weight in comparison to the alternative treatments, irrespective of the presence or absence of *N. aberrans*. Okorie et al. (2011) observed higher fresh and dry weight of soybean sprouts inoculated with *P. ostreatus* in the presence of *M. incognita*, demonstrating a growth-promoting effect. Both fungi also increased the chlorophyll and soluble protein content of the plants in the presence of the nematode. Roy et al. (2015) observed that fertilizing pepper plants with residual *P. ostreatus* substrate increased levels of chlorophyll, carotenes and proteins in the plant and fruits, in addition to promoting height growth. In a study with carrots inoculated with *P. lilacinum* and *Aspergillus niger* and exposed to *M. javanica* and *P. carotovorum pv. carotovorum* and *X. campestris pv. carotae,* inoculation with *P. lilacinum* alone not only reduced the nematode population but also increased dry weight and chlorophyll content (Nesha and Siddiqui 2017). *P. lilacinum* strain 251 was evaluated by Kiewnick and Sikora (2006a, b) to control the root-knot nematode in tomato. These authors inoculated the fungus in aqueous suspension before sowing in the substrate, achieving a reduction in the number of egg masses by 74% and the final population of nematodes in the roots by 71% compared to the inoculated control. In this trial, the inoculation was carried out at the transplant and with a substrate colonized with the mycelium of the fungus, achieving a significant reduction of the nematode reflected in the Reproduction Factor and efficient protection in the plant. In both cases of inoculation, a single application was sufficient for a significant reduction of the nematode population, regardless of the moment, demonstrating the efficiency of this fungus for biocontrol. Furthermore, the presence of *P. lilacinum* in the soil subsequent to the experiment was validated via soil particle cultivation. This fungus, commonly encountered in soil ecosystems, displays vigorous growth upon organic substrates linked with solid soil particles, thus maintaining its attachment to them (Gams 1992). The diminished proportion of fungal retrieval in soil specimens can be ascribed to the washing procedure utilized, which targets the actively proliferating fungal phases, in contrast to the dilution methodology that tends to overestimate spore quantities (Cabello and Arambarri 2002).

Rumbos et al. (2008) that evaluated the persistence of *P. lilacinum* in silty loam soils in the presence of *Meloidogyne spp*. reported also a decrease in the persistence of the fungus with the addition of sand and increasing with the addition of organic matter. In both cases, although the density and time varied, it was possible to observe the persistence of *P. lilacinum.* The persistence of microorganisms in the soil depends on various conditions, both biotic and abiotic. These conditions include the type of soil, its humidity and conditions such as salinity, competition in the rhizosphere with other microorganisms, as the management practices habitually carried out by producers, such as the use of agrochemicals. Moreover, the absence of PO in the soil particle cultivation within our investigation suggests its limited establishment and diminished competitiveness within the soil environment. This observed pattern parallels the conduct of other white rot fungi, which preferentially colonize lignocellulosic debris and nematodes (Hestbjerg et al. 2003; Mostafa et al. 2019).

In conclusion, the study demonstrates the efficacy of inoculating PO and PL cultures into a solid fermentation substrate for the management of *N. aberrans*. This approach employs two distinct control strategies: the reduction of nematode populations, leading to a decline in the reproduction factor, and the stimulation of plant growth in pepper plants impacted by this biotic stress. These findings hold significant relevance within the context of the growing need to boost productivity while minimizing dependence on agrochemicals.

The utilization of beneficial microorganisms and by-products derived from edible mushroom cultivation, encompassing harvest residues and crop remnants, aligns with the principles of the Circular Economy. This practice not only holds the potential for generating valuable biofertilizers for horticultural producers but also contributes to the cultivation of sustainable practices.

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Authors' contributions Performed the experiments, analyzed the data and wrote the paper: VFB Supervised the work, review and editing: MFR and MCNS Funding acquisition: MFR and MCNS All authors conceived and designed the experiment, contributed to the measurements, processing of the material and participated in the critical review of the manuscript.

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Declarations

Conflict of interest No potential conflict of interest was reported by the authors.

Ethical approval There were no human and/or animal participants in the research.

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