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Endophytic *Trichoderma* strains increase soya bean growth and promote charcoal rot control

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ABSTRACT

Charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goid., is one of the world's most serious diseases because it reduces yield and seed quality. Nowadays, biological control is an environment-friendly option for controlling plant diseases. The goals of this study were to (i) test eight endophytic *Trichoderma* spp. strains as biocontrol agents against *M. phaseolina* and (ii) further investigate two selected strains showing good behaviour against the pathogen. Pathogen-antagonist interactions were studied in dual culture, and the morphological alterations of *M. phaseolina* mycelia in the interaction zone were examined by light microscopy. *Trichoderma* strains were applied to soya bean seeds by a seed coating technique. Their bioprotective effects were assessed by *in vitro* and *in vivo* assays to evaluate radicle length, the germination percentage and the presence of typical charcoal rot symptoms in seedlings. Two *Trichoderma* strains were selected and they were molecularly identified as *T. harzianum* species complex. Their antagonistic ability against *M. phaseolina* was evaluated under different water availability conditions. The mechanisms used by these two endophytic strains against the pathogen were evaluated by cryo-scanning electron microscopy. The results showed that all eight *Trichoderma* strains successfully performed biocontrol activity against *M. phaseolina* by reducing colony growth and causing morphological alterations in the mycelia of *M. phaseolina*. All endophytes improved seed germination and radicle length, and reduced typical symptoms and disease progression on seedlings. Water availability in the medium impacted on fungal growth. At 0.995 α_w, all the fungi grew more and faster. At 0.95 α_w *M. phaseolina* grew more than the *Trichoderma* strains, while the pathogen grew slightly more at 0.98 α_w than the *Trichoderma* strains. However, both selected *Trichoderma* strains grew larger and faster than the pathogen at 0.995 α_w. The mechanisms involved in pathogen control revealed by the light and cryo-scanning microscopy studies included competition for nutrients or space and direct mycoparasitism. All the endophytic *Trichoderma* strains were antagonistic against *M. phaseolina*, however our study allowed us to select two *Trichoderma* strains with good potential to be included for charcoal rot management.

1. Introduction

Fungal pathogens are severe biotic adversities that cause economic yield losses in agriculture and pose a major threat for global food production by affecting important crops like wheat, rice, soya bean, corn and potatoes (Almeida et al., 2019). Soya bean [(*Glycine* max (L.) Merr.)] is the major oilseed produced and traded worldwide for its numerous applications, including human food

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(vegetable oil and protein meal), animal feed and biofuels. Argentina, along with Brazil, the USA and China, are the world's leading soya bean producers (https://www.fas.usda.gov/data/oilseeds-world-markets-and-trade).

Several diseases affect soya bean crops, and reduce yield and seed quality, to result in significant yield losses. Of them, "charcoal rot", caused by *Macrophomina phaseolina*, is one of the most economically important diseases worldwide. The causal agent is a polyphagous soil-borne and seed-borne pathogenic fungus that infects nearly 500 agricultural crops and weed species (Uroos et al., 2022). This pathogen is present under a wide range of climate conditions from arid to tropical (Iqbal and Mukhtar, 2020). *M. phaseolina* is also an opportunistic pathogen whose prevalence can be enhanced by high temperature (28–35 °C), low soil humidity and water stress (Chiesa et al., 2016; Larran, 2016). According to several authors, *M. phaseolina* exhibits a wide range of morphological, pathogenic, physiological and genetic variability that allows it to adapt to a variety of environmental conditions and to spread across North and South America, Asia, Australia, Africa, and parts of Europe (Iqbal and Mukhtar, 2014; Reznikov et al., 2018a, 2018b). In Argentina, charcoal rot is present in all agro-ecological zones, but frequently occurs in the north-eastern, north-western and central-southern regions of Córdoba. Its intensity is high and environmental conditions favour this disease developing. During the 2000/2001 growing season, *M. phaseolina* caused significant yield losses in some Argentinian provinces, where hot and dry weather conditions prevailed for a long period of time (Resnikov et al., 2020).

Soya bean plants are susceptible in almost all the growth stages, and seedling blight, root rot and root and stem rot take place. *M. phaseolina* produces lesions on stems, branches, pods and grains. However, the disease is associated mainly with mature soya bean plants after flowering in growth stages R5 to R7 (Fehr and Caviness, 1977), and patches of wilted plants that may prematurely die appear in the field. Affected tissues look grey due to the development of numerous black microsclerotia, the characteristic sign of this disease. Infected seeds may not be apparent and can also have microsclerotia on seed coats or on seed surfaces and, thus, act as a source of inoculum. When soil is wet, seedling roots can become infected and show lesions in hypocotyl and epicotyls regions, which are reddish brown, and can also be covered by numerous black microsclerotia that darken tissues (Reznikov et al., 2020). The fungus survives as microsclerotia for several years (2–15 years) on plant residue or in soil depending on the environmental conditions and the means by which the pathogen spreads (Ndiaje, 2007). Microsclerotia can germinate and infect root tissue at temperatures between 20 °C and 40 °C (Resnikov et al., 2020).

Different strategies adopted to manage charcoal rot, such as crop rotation, tillage, fungicide seed treatments, soil and irrigation management and those that conserve soil moisture, have been suggested (Mengistu et al., 2007; Ndiaje, 2007). However, charcoal rot management has focused mostly on using fungicides and resistant cultivars. Nevertheless, it is widely known that a large number of negative health and environmental effects have been associated with employing agrochemicals. Additionally, intensive fungicide use causes resistant pathogens to develop and diminishes agricultural sustainability (De Silva et al. 2019). However, host resistance is an essential part of disease control management and may be a feasible method to manage this pathogen. Only the tolerant to moderately tolerant, but not the resistant soya bean genotypes, have been identified against *M. phaseolina* (Mengistu et al., 2007; Chiesa et al., 2016). Therefore, control measures are difficult for several reasons, such as lack of resistant cultivars, opportunistic fungal character, its survival by microsclerotia on soil, seeds and plant debris, the short residuality of fungicidal seeds, and also because of the variability of *M. phaseolina* among populations in distinct agro-ecological regions (Iqbal and Mukhtar, 2014; Zilli et al., 2018).

Nowadays, one of the challenges in the plant pathology area is to ensure the sustainability and quality of food products, and to preserve the environment by searching for alternatives to using chemical products for crop management purposes (Emoghene and Futughe, 2016). In this scenario, researchers focus on a new agricultural concept to deal with healthier food production, and seek a more sustainable and ecological plant disease management approach.

Biological control is considered an alternative strategy and a significant tool as part of integrated disease management, which is why it has steadily grown in recent decades (Köhl et al., 2019). Different alternatives to agrochemicals have been evaluated against *M. phaseolina*. In this way, the antifungal potential of 20 antagonistic plants, such as *Carum copticum*, *Azadirachta indica, Nigella sativa,* among others, has been demonstrated by *in vitro* and *in vivo* assays (Iqbal et al., 2014). Recently, Khan and Javaid (2022a; 2022b) studied the effect of two *Aspergillus* species and quinoa (*Chenopodium quinoa)* dry biomass applied either alone or together in the soil inoculated with *M. phaseolina* on histochemical features of mungbean (*Vigna radiata*) plant. These authors suggested that the two *Aspergillus* species and quinoa dry biomass activated the resistance mechanisms in mungbean plant. Iqbal and Mukhtar (2020) have also demonstrated that seven indigenous *Trichoderma* species were effective biocontrol agents against *M. phaseolina* in *in vivo* and *in vitro* assays. The bacterial endophytes isolated from soya bean tissues were able to antagonise the pathogen in an *in vitro* assay (Senthilkumar et al., 2009). Additionally, the potential of certain fungal endophytes as biological control agents of *M. phaseolina* to cause root rot on different hosts, such as pepper (Mmbaga et al., 2018) and melon (Gonzalez et al., 2020), has been reported. As endophytes are microorganisms that live in healthy plant tissues and are adapted to these internal conditions (White et al., 2019), we considered that their use could be an interesting charcoal rot management alternative for soya bean crops with a minimal impact on the environment and human health. In our country, very few research studies have focused on the biocontrol potential of endophytic *Trichoderma* species against soya bean charcoal rot (Larran, 2016; Larran et al., 2016). For these reasons, the objectives of this work were to (i) test eight endophytic *Trichoderma* spp. strains as biocontrol agents against *M. phaseolina* in *in vitro* and *in vivo* assays and (ii) further investigate two selected strains that showed good behaviour against the pathogen. To obtain more in-depth knowledge of these selected strains, we performed studies to molecularly identify them, study their behaviour according to different water activities under *in vitro* conditions and analyse the mechanisms of action involved in antagonising the pathogen by light microscopy and cryo-scanning electron microscopy.

2. Material and methods

2.1. Fungal strains

The eight *Trichoderma* strains used in this work (Tr1, Tr2, Tr3, Tr4, Tr5, Tr6, Tr7, Tr8) were obtained from the fungal collection of the Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales (FCAyF), Universidad Nacional de La Plata (UNLP), Buenos Aires, Argentina. They were isolated from different healthy soya bean organs and growth stages (GS) according to Fehr and Caviness (1977) as follows: Tr1 and Tr2: isolated from the stem at GS V5; Tr3: isolated from the leaf at GS R4; Tr4: isolated from the pod at GS R6; Tr5: isolated from the stem at GS V1; Tr6: isolated from the stem at GS V7; Tr7: isolated from

the leaf at GS R6 and Tr8: isolated from the pod at GS R6. These endophytic strains were identified according to their cultural characteristics, morphology and features of spores at the genus level.

The *M. phaseolina* strain was obtained from the same aforementioned fungal Centre in Buenos Aires, Argentina. It was previously procured from the Laboratorio Agrícola Río Paraná, Red Nacional de Protección Vegetal (RedNPV) of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina.

2.2. Antagonism in dual cultures

The dual culture technique was applied to screen the antagonistic effect of eight endophytic *Trichoderma* strains against *M. phaseolina* according to the method described by Larran et al. (2016). Mycelial plugs of each fungal colony were taken from the 7- and 4-day cultures (*Trichoderma* spp. and *M. phaseolina*, respectively). They were placed on Petri plates with 2% potato dextrose agar (PDA). A control was performed by placing a mycelial plug of *M. phaseolina.* All the culture plates were incubated at 28 °C ± 2. The experiment was repeated twice and each treatment was replicated 5 times. The colony diameter of the pathogen was measured 3 days after incubation in two directions at right angles to one another. Data were analysed according to a factorial design for two experiments, nine microorganisms (eight *Trichoderma* strains + control) and five replications. To fulfil the ANOVA assumptions, the normality of the data was tested according to the Shapiro-Wilk test (1965). The homogeneity of variances was tested according to the Levene test (1960). Several transformations were tested to normalise and homogenise residual variance and log transformation was selected. Means were compared by the LSD test ($P \leq 0.05$).

Macroscopical and microscopical studies were performed to determine the pathogen-antagonist interaction type and to elucidate the mechanisms of antagonism used by the *Trichoderma* endophytic strains. To evaluate the pathogen-antagonist interaction type, the dual cultures plates were left for 4 weeks under the same conditions as mentioned above and then observed macroscopically. The method proposed by Magan and Lacey (1984) was followed. These authors define interaction types as: (a) mutual intermingling; (b) mutual antagonism on contact or with a 2 mm free space between fungus colonies; (c) mutual antagonism at a distance; (d) dominance upon contact; (e) dominance at a distance. The Index of Dominance (I_D) was also determined by assigning values to each fungus in all the interaction types according to Magan and Lacey (1984) as follows: (a) 1/1; (b) 2/2; (c) 3/3; (d) 4 for the dominant species/0 for the inhibited species; (e) 5 for the dominant species/0 for the inhibited species.

A microscopic study was performed under *in vitro* conditions by a dual microculture technique. All the different pathogen-endophyte combinations were inoculated apart on 1 cm^2 2% PDA plugs placed on microscope slides covered with a coverslip. They were then incubated on Petri plates at 28 °C ± 2 in the dark until both colonies came into contact in accordance with the method followed by Larran et al. (2020). Controls were performed by inoculating each fungus alone. The experiment was designed with four replicates. The pathogen's morphological changes were assessed using the areas of intermingling growth of each pathogen-antagonist combination. Sections were examined using a light microscope.

2.3. Effect of endophytes against M. phaseolina applied via seed coating in an in vitro assay

The antagonistic effects of the eight *Trichoderma* endophytic strains previously studied in the dual cultures against *M. phaseolina* were evaluated by an *in vitro* assay. Endophytes were applied to soya bean seeds *via* seed coating and were sown on colonised Petri plates by *M. phaseolina.* All the endophytes and *M. phaseolina* were multiplied on 2% PDA and incubated at 24 °C for 7 days and 28 °C for 4 days, respectively, when colonies fully covered plates. A sample of 70 g of soya bean seeds, Cv DM 3810 (Thousand kernel weights: 172 g) with a 98% germination percentage, were surface-sterilised using 5% sodium hypochlorite (NaOCl), washed in sterile distilled water and then dried in a laminar air flow chamber. Seed coating was prepared and applied according to the technique described by Cordo et al. (2007) as follows: a suspension of each endophyte was obtained by flooding the 7-day-old colonies with sterile distilled water and then rubbing the culture surface using a sterile glass rod. Suspensions were homogenised with a vortex and filtered through cheesecloth. Tween 20 (0.05%) was added to the suspension as a surfactant. Then the conidial concentration was adjusted to 10^7 conidia/mL by a haemocytometer. A 2 mL aliquot of suspension of each endophyte was added to 18 mL of 0.25% water-agar, which was previously sterilised and placed inside 100 mL beakers. Thirty-five seeds per treatment were immersed in the prepared suspensions with stirring for 20 min and dried to complete dryness and until a fine coating had formed. Five seeds of each treatment were placed on Petri plates on the 2% PDA colonised by *M. phaseolina* and then incubated at 28 °C with a 12 h light/12 h dark photoperiod lasting 7 days. Two controls were performed: (T9) soya bean seeds were coated with 0.25% water-agar and placed on the Petri plates colonised by *M. Phaseolina*; (T10) soya bean seeds were coated with 0.25% water-agar and placed on 2% PDA.

The percentage of germinated soya bean seeds and radicle length were recorded. The effect of endophytes was evaluated by an analysis of variance (ANOVA) for a completely randomised design with five replications. Means were compared by the LSD test ($P \leq 0.05$). Each piece of data was the mean of five measurements. Homocedasticity in variance appeared for treatments. As residuals lacked normality, data were transformed using the log transformation of radicle length and square root + 1 of seed germination.

2.4. Effect of endophytes applied via seed coating against M. phaseolina in a greenhouse assay

The objective of this assay was to evaluate the bioprotective effect of eight endophytic *Trichoderma* strains that had been previously evaluated under *in vitro* conditions (Tr1, Tr2, Tr3, Tr4, Tr5, Tr6, Tr7, Tr8) against *M. phaseolina* applied to soya bean seeds as a seed coating technique to then be sown in inoculated soil with *M. phaseolina.* The germination percentage was evaluated on 15 and 21 days after sowing. Soya bean seedlings were also evaluated 15 and 21 days after sowing for the presence of typical charcoal rot symptoms and grouped as three types: (a) necrosis on cotyledons (brown to dark spots); (b) seedlings that stopped growing in the cotyledon stage (VC, according to Fehr and Caviness, 1977); (c) weak and dead seedlings.

2.4.1. Inoculum preparation and seed coating

M. phaseolina was multiplied on 2% PDA and then incubated at 28 °C for 4 days to prepare an inoculum in accordance with the method proposed by Castellanos et al. (2011). Rice grains (10 g) were washed under tap water, placed on Petri plates with 10 mL of distilled water and autoclaved at 120 °C for 20 min at 1 ATM. Then grains were inoculated with a plug of 1 cm^2 obtained from the margin of the 4-day-old *M. phaseolina* colonies. Plates were incubated for 15 days in the dark at 28 ± 2 °C. The rice grains colonised with the pathogen were left in a laminar air flow chamber to remove excess moisture. Then they were crushed with a mortar, spread on tissue paper and left at 28 ± 2 °C for 24 h. Finally using an electric grinder, grinding was carried out again to obtain a fine

powder. The number of colony-forming units (CFUs) was obtained by plate counting from 1 g of the inoculum prepared by serial dilutions (Waskman, 1927).

2.4.2. Substrate preparation and sowing seeds

The substrate was prepared using soil, sand and perlite at a ratio of 50:35:15. It was then sterilised by tyndallisation (three cycles at 120 °C). Seedling trays of 50 cells (9 × 5 cm each) were filled with the prepared substrate. Soya bean seeds, Cv DM 3810 with a 98% germination percentage, were used in this assay. A 55 g sample of seeds (approx. 300 seeds) was surface-sterilised and coated with eight endophytic *Trichoderma* spp. strains as described in the *in vitro* assay. Substrate inoculation was carried out by placing 0.1 g of inoculum (9000–11000 CFUs) per cell at the planting site before sowing the covered seeds. Controls included the seeds coated with 0.25% water-agar sown in *M. phaseolina*-inoculated soil (T9). The healthy control (T10) included the seeds coated with 0.25% water-agar sown in the non-inoculated soil. The assay remained under controlled greenhouse conditions (25–30 °C and 12–15 °C day/night) with a low irrigation regime. It was repeated 4 times with five units every time.

A first evaluation was made 15 days after sowing by counting the germinated seeds (%) and evaluating the presence of symptoms on cotyledons. Seedlings were removed from cells 21 days after sowing to visually evaluate aerial and underground tissues. Charcoal rot symptoms were recorded, such as reddish-brown sunken lesions on epi- and hypocotyls of seedlings, cotyledons, weak seedlings, basal stem strangulation, root necrosis and seedling death. The effect of endophytes was evaluated by an ANOVA for a randomised design. Means were compared by the LSD test ($P \leq 0.05$).

2.5. Complementary studies of the two selected Trichoderma spp. Strains

Trichoderma strains Tr1 and Tr2, which displayed good behaviour against *M. phaseolina* in our previous assays, were molecularly identified and selected to acquire more in-depth knowledge of the pathogen-antagonist interactions. Complementary studies were performed to evaluate interactions under different water availability conditions by *in vitro* assays and to study the mechanisms of antagonism used under a cryo-scanning electron microscope.

2.5.1. Molecular identification of the selected Trichoderma strains

For the molecular identification of *Trichoderma* strains Tr1 and Tr2, about 100 mg of fungal mycelia were harvested from axenic cultures and DNA was extracted according to Raeder and Broda (1985). The PCR amplification of the internal transcribed spacers (ITS) of the ribosomal DNA (rDNA) was carried out according to Bader et al. (2020). Amplicons were separated by gel electrophoresis in 1.0% w/v agarose supplemented with GelRedTM (Biotium) to then be purified and sequenced using the ITS4 primer with the Macrogen Inc. service.

2.5.2. Effect of water activity on fungal growth

The selected endophytes (Tr1 and Tr2) and the pathogen were subcultured on 2% PDA and incubated at 24 ± 2 °C for 7 days and at 28 ± 2 °C for 4 days, respectively. The antifungal potential of the endophytes against *M. phaseolina* was tested by the dual culture technique. Following the technique proposed by Sempere and Santamarina (2006), PDA was used as a basic medium, and was mixed with glycerol and distilled water to obtain different water activity levels (a_w) (0.90, 0.95, 0.98 and 0.995). The mycelial fungal plugs obtained from margins of colonies were screened on the Petri plates containing the culture media adjusted to different a_w. Controls were performed by placing only disks of the pathogen on all the adjusted media. In all the treatments (control and dual cultures), experiments were performed with five replicates. To keep water activity constant throughout the experiment, the Petri plates with the same a_w were placed inside boxes, which were then filled with beakers containing a glycerol water solution with an identical relative humidity value to the PDA adjusted at each a_w and incubated at 25 °C in the dark. Plates were examined at 24-hour intervals for 7 days. The diameter of colonies was measured in two directions and growth rates (GRs) (mm/day) were calculated by a linear regression of the radius (mm) as opposed to time (days). A multifactorial ANOVA was used to evaluate the influence of water activity on fungi growth, where the factors were species and water activities. Means were compared by the LSD test ($P \leq 0.05$).

In order to analyse the evolution of the fungal species growing in interactions in the different water activities, plates were incubated at 25 °C for 4 weeks and were macroscopically evaluated. The method proposed by Magan and Lacey (1984) was followed to determine the interaction type as mentioned above. Additionally, the I_D of each pathogen-antagonist interaction was determined following the method described by Magan and Lacey (1984).

2.5.3. Cryo-scanning electron microscopy of the endophytes-M. phaseolina interaction

The fungal interactions between Tr1 and Tr2 and *M. phaseolina* were analysed by cryo-scanning electron microscopy to corroborate the mechanisms of antagonism used by the *Trichoderma* strains against the pathogen. A dual microculture technique was applied to inoculate each pathogen-antagonist combination on the margins of the PDA sections adjusted at 0.995 a_w and placed on microscope slides without coverslips. Then, slides were incubated on Petri plates at 25 °C for 3–5 days to be observed according to the technique proposed by Sempere and Santamarina (2011). The controls were the plates with each fungus alone. Four replicates of each pathogen-endophyte combination were performed. Samples were taken from the interaction zone MP-Tr1 and MP-Tr2 and treated by the technique of Sempere and Santamarina (2011) to examine them under a JEOL JSM 5410 scanning electron microscope at the UPV Electronic Microscopic Service (Spain).

3. Results

3.1. Antagonism in dual cultures

The ANOVA results showed that there were differences between experiments and microorganisms, and the experiments $_x$ microorganisms interaction was not significant (Table 1). The means of each experiment were 3.03 and 2.94 (cm). All the tested endophytes significantly reduced the colony diameter of the pathogen compared to the control that had the highest values (Table 2). Tr1, Tr3 and Tr5 were highlighted as the best ones.

According to Magan and Lacey (1984), macroscopic examinations of all the endophytes-pathogen interactions on dual cultures after 4 incubation weeks were classified as "D," dominance on con-

Table 1
ANOVA of *M. phaseolina* growth in dual culture against endophytes.

Source of variation	DF	MS	P Value*
Repetition	4	0.009	
Experiments	1	0.173	≤ 0.001
Endophytes	8	0.465	≤ 0.001
Experiments × microorganisms	8	0.005	0.426
Error	68	0.004	

* Fisher Test ($P \leq 0.05$). DF: Degrees of freedom; MS: Mean squares.

Table 2
Means of *M. phaseolina* growth in dual culture against endophytes.

Treatments	Means (cm)
MP-Tr1	2.88* de
MP-Tr2	2.93 cd
MP-Tr3	2.86 e
MP-Tr4	2.97 b
MP-Tr5	2.84 e
MP-Tr6	2.93 cd
MP-Tr7	2.92 d
MP-Tr8	2.99 b
MP (control)	3.55 a

* The means followed by the same letter are not statistically different. LSD $\leq$ 0.05.

tact. Both colonies grew in the direction of one another. When they came into contact, all the endophytes overgrew the pathogen colony. The I_D value was determined as 4 for each antagonist and as 0 for the pathogen. Fig. 1 shows the interaction model found in all the endophyte-*M. phaseolina* combinations compared to the control after 1, 2 and 3 incubation days (Fig. 1 a, b, c, respectively). A defined black ring and a yellow halo were observed in the initial contact zone of both fungi with Tr1, Tr2, Tr3, Tr4, Tr5 and Tr6. According to these results, all the endophytes reduced the pathogen colony, which suggests that one of the mechanisms followed for the endophytic *Trichoderma* spp. strains was competition for space and nutrients.

3.1.1. Morphological alterations of M. phaseolina in the in vitro assays

The results of the microscopic observations from the interaction of endophytes-pathogen zone growing in dual microcultures showed morphological changes in the hyphae of the pathogen. The light microscopic observations revealed that all eight endophytes grew close to the hyphae of *M. phaseolina* by surrounding and coiling around them, with penetration of the pathogen's hyphae that caused cell wall disruption (plasmolysis) in all the endophyte-pathogen combinations (Fig. 2). The photomicrographs showed that *M. phaseolina* hyphae were surrounded by endophytic *Trichoderma* hyphae (coiling) (Fig. 2 a,b). The microscopic examination of the pathogen-endophyte interaction revealed initial contact between both fungi and a strong attachment of *Trichoderma* hyphae to those of *M. phaseolina* either with or without appressorium formation. Moreover, plasmolysis and swelling of the mycelium, granulation and cytoplasmic vacuolisation were the predominant interference types (Fig. 2 c-f). Additionally, the production of pigmented compounds inside hyphae and in media occurred in most of the endophyte-pathogen interactions (Tr1, Tr2, Tr3, Tr4, Tr5 and Tr6).

According to our results, the endophytic *Trichoderma* strains antagonised *M. phaseolina* with initial contact by coiling around the pathogen, and with a tight attachment between both fungi with appressorium formation, penetration and plasmolysis. Therefore, we demonstrate that direct mycoparasitism is a mechanism involved by the endophytic *Trichoderma* strains to biocontrol *M. phaseolina.*

3.2. Effect of endophytes applied via seed coating against M. phaseolina in an in vitro assay

The ANOVA results showed significant differences between treatments for seed germination and the radicle length of the soya bean seeds covered with endophytes for 7 days in an *in vitro* assay (Table 3). All the endophytes improved seed germination compared to the control on the media colonised by *M. phaseolina* (control, T9). However, Tr8, Tr7 and Tr2 were statistically different compared to control T9. The results obtained for radicle length, showed that all treatments improve the length significantly except Tr4 compared to control T9 (see Table 4).

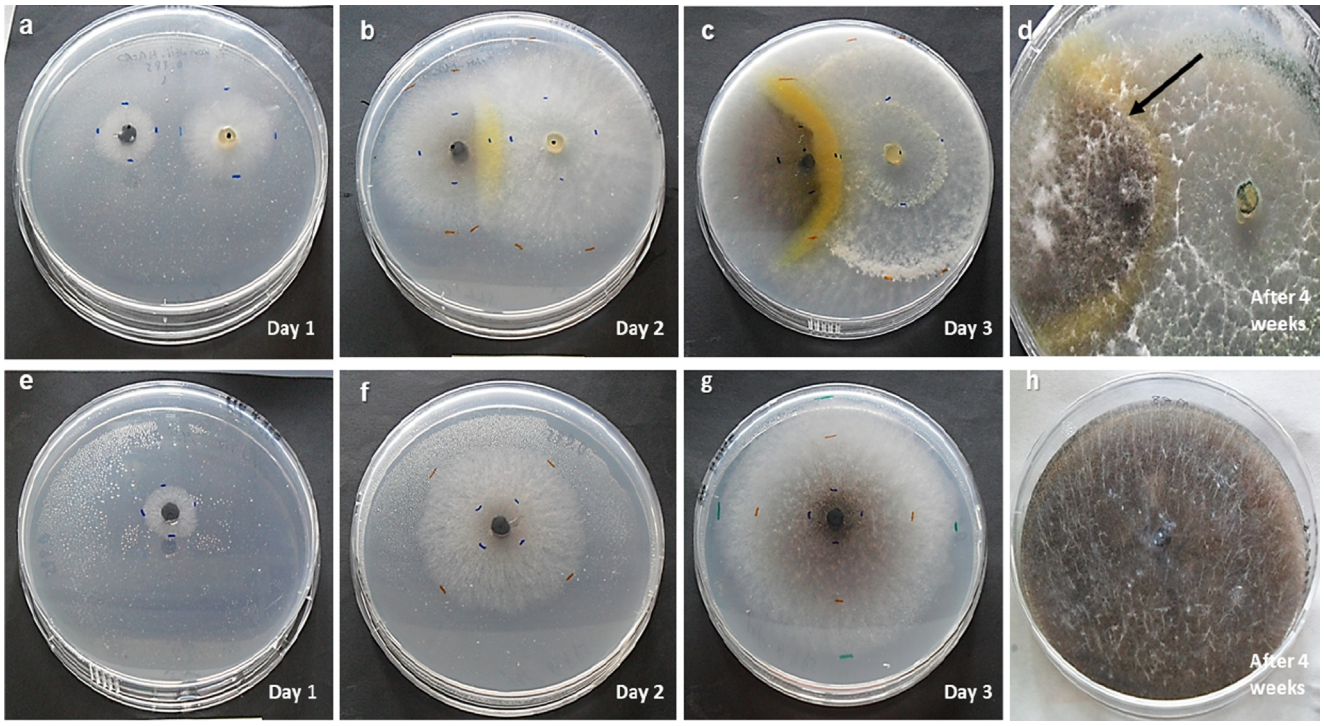

Fig. 1. The interaction model found in all endophyte-*M. phaseolina* combinations in dual culture during the 3-day incubation compared to the control. (a,b,c) Endophyte-*M. phaseolina* interactions at 1, 2 and 3 incubation days compared to the control (*M. phaseolina*) (e,f,g); (d) endophytes-pathogen interaction type "D": dominance on contact after 4 weeks of incubation, according to Magan and Lacey (1984) (enlarged image). Arrow shows overgrowth of endophytic *Trichoderma* covering the *M. phaseolina* colony; (h) *M. phaseolina* after 4 weeks of incubations. (a,b,c) images correspond to Tr4-*M. phaseolina*.

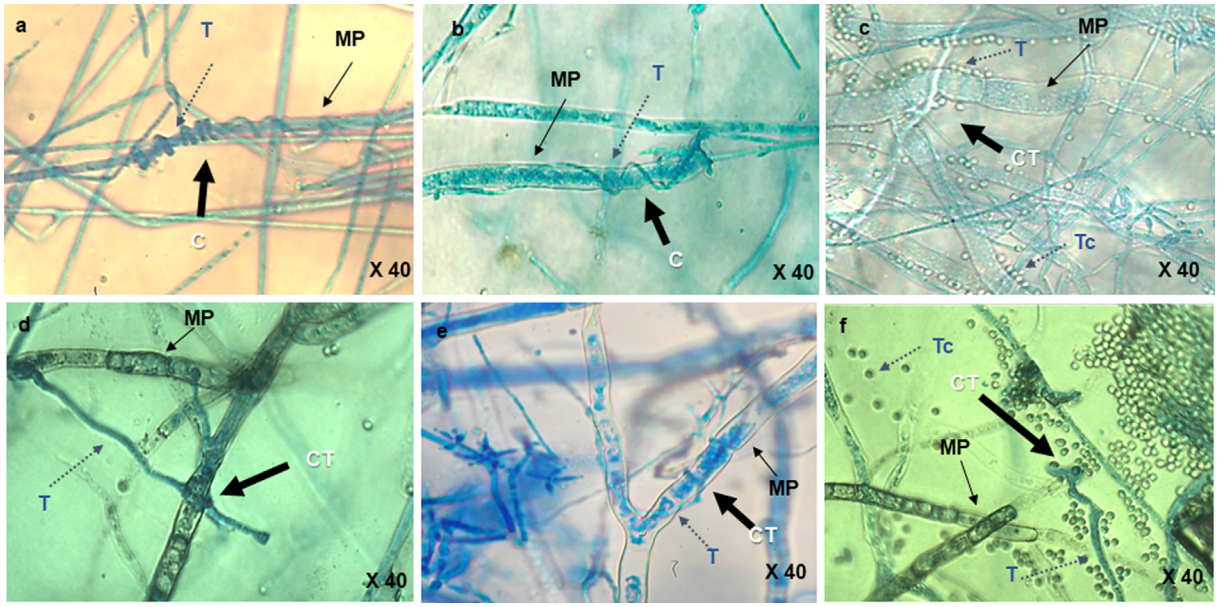

Fig. 2. Microscopic study of *M. phaseolina* in dual culture under a light microscope. (a,b) *Trichoderma* hyphae coiling around *M. phaseolina* hyphae; (c,d,e,f) contact and penetration either with or without formation of appressorium-like structures, cytoplasmatic granules and hyphae integrity completely collapsed (plasmolysis). Arrows indicate T: hyphae of the endophytic *Trichoderma* strains; Tc: conidia of *Trichoderma*; MP: hyphae of *M. phaseolina*; C: coiling; CT: contact and penetration.

Table 3
ANOVA of the effect of endophytes applied as a soya bean seed coating on the germination and radicle length of soya bean against *M. phaseolina.*

	Germination			Radicle length		
Source of variation	DF	MS	P- Value*	DF	MS	P-Value*
Treatments	9	0.954	≤ 0.001	9	1.906	0.032
Repetition	4	0.151		4	1.022	
Error	36	0.179		36	0.716	

* F Test (P ≤ 0.05). DF: Degrees of freedom; MS: Mean squares.

Table 4
Means of the protective effect of endophytes against *M. phaseolina* on the germination and radicle length in *in vitro* assays.

Treatments	Germination (seed numbers)	Radicle length (cm)
Tr1	1.60* cd	0.68* c
Tr2	2.20 bc	0.65 c
Tr3	1.80 bcd	0.61 c
Tr4	1.00 cd	0.22 cd
Tr5	1.40 cd	0.49 ab
Tr6	1.60 cd	0.63 c
Tr7	2.60 bc	1.31b
Tr8	3.60 b	1.76 b
T9**	0.00 d	0.00 d
T10***	5.80 a	2.20 a

* The means followed by the same letter are not statistically different, LSD (P ≤ 0.05); Treatments: Tr1-Tr8: seeds treated with endophytes and sown in inoculated soil with *M. phaseolina*;
** T9: Control: seeds coated with 0.25% water-agar and placed on 2% PDA colonised by *M. phaseolina*;
*** T10: Control: seeds coated with 0.25% water-agar placed on 2% PDA.

3.3. Effect of endophytes applied via seed coating against M. phaseolina in a greenhouse assay

The ANOVA results showed significant differences in the germination percentage between treatments when evaluated 15 days after sowing (Table 5). Not all the *Trichoderma* strains displayed the same behaviour at the different evaluation times (15–21 days after inoculation). Some had a higher growth rate and a better effect after a short time.

After 15 sowing days, the presence of *M. phaseolina* in soil led to a 20 % reduction in the germination percentage of the seeds covered only with water-agar (T9) compared to those covered with water-agar sown in the non-inoculated soil (T10) (Fig. 3). Germination percentages were higher in treatments Tr1 and Tr2. There were also significant differences in the presence of charcoal rot symptoms on cotyledons 21 days after sowing (Table 5). Fig. 3 depicts how 45 % of the emerged seedlings from the control (T9) showed typical charcoal rot symptoms as reddish brown, irregular, isolated and confluent lesions. Several seedlings presented poor root hair and reddish-brown lesions on hypocotyls. However, all the treatments with endophytes significantly reduced disease symptoms, except for Tr1. Tr7 and Tr8 brought about the most marked reduction in the seedlings with symptoms on cotyledons (Fig. 3b).

Significant differences also appeared between treatments for seedlings, which stopped growing in the cotyledon stage 21 days after sowing (Table 5). Treatment Tr2 significantly reduced (by 100%) these symptoms in the cotyledon stage compared to all the other treatments and control T9 (Fig. 3c). There were also significant differences between treatments for the percentage of weak and dead seedlings after 21 days of sowing (Table 5). Some seedlings showed necrosis at the base of stems and reddish coloration. As Fig. 3 illustrates, all the treatments with endophytes lowered the percentage of weak and dead seedlings, except Tr8. Treatments Tr2, Tr3, Tr4 were the most effective in reducing these symptoms.

According to these results, all the endophytes reduced some typical charcoal rot symptoms when they were applied as a seed coating technique compared with T9. A tendency to improve the germination percentage was observed in most treatments, but only

Table 5
ANOVA of the effect of endophytes applied as a seed coating against *M. phaseolina* in a greenhouse assay.

Source of variation		Germination (%)*		Charcoal rot on soybean seedlings					
				Symptoms on cotyledons*		Seedlings stopped growing in the cotyledon stage**		Weak and dead seedlings**	
	DF	MS	P-value***	MS	P-value	MS	P-value	MS	P-value
Treatments	9	0.14	≤ 0.001	0.29	≤ 0.001	0.06	0.021	0.13	≤ 0.001
Repetition	3	0.01		0.01		0.25		0.03	
Error	27	0.03		0.03		0.02		0.02	

* Germination percentage and symptoms on cotyledons were evaluated 15 days after sowing;
** Charcoal rot on soya bean seedlings were evaluated 21 days after sowing;
*** F Test (P ≤ 0.05).

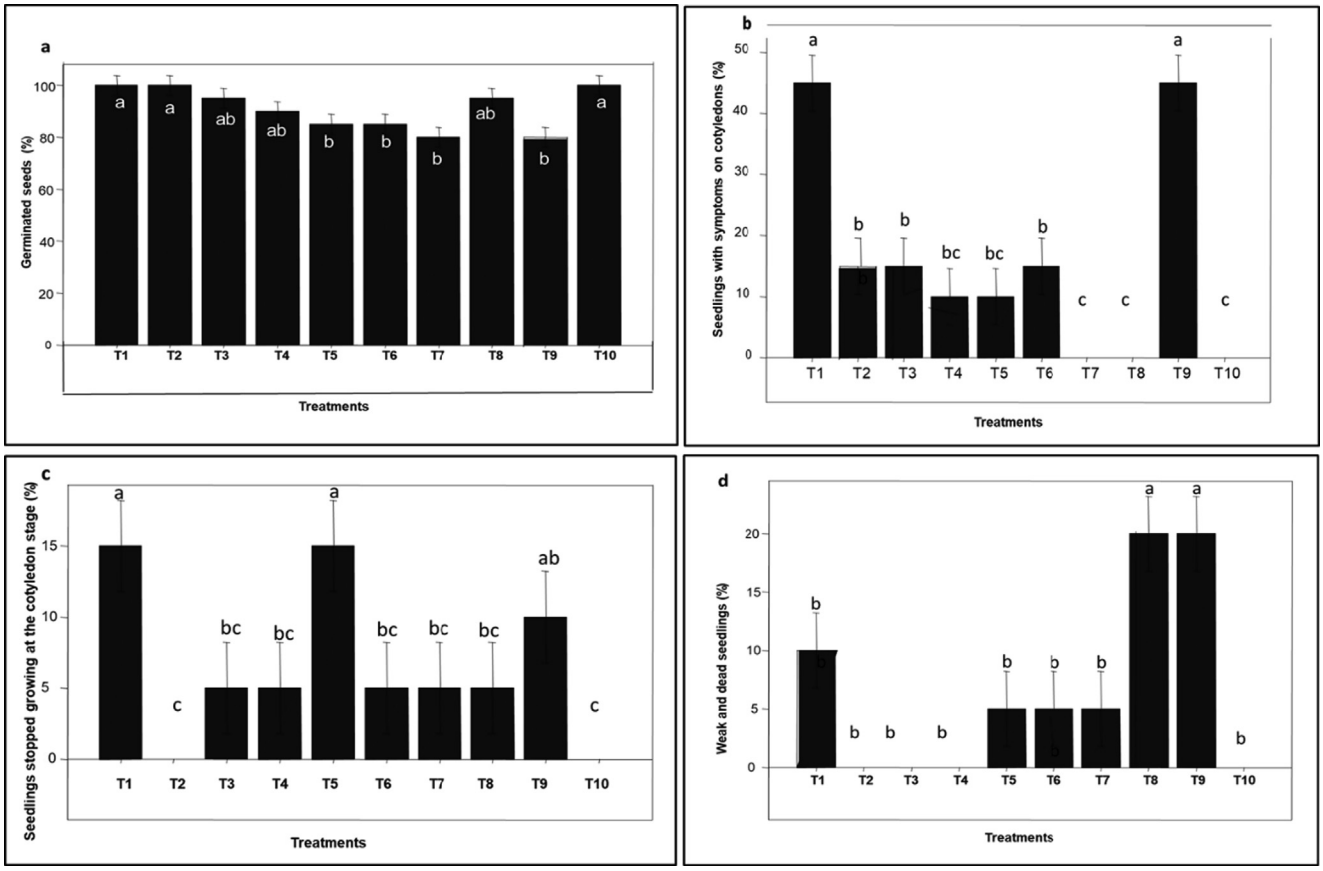

Fig. 3. Effect of endophytes against *M. phaseolina* applied as a seed coating technique in a greenhouse assay, (a) Gemination percentages; (b) seedlings with symptoms on cotyledons (%) evaluated 15 days after sowing; (c) seedlings that stopped growing or died in the cotyledon stage and (d) weak and dead seedlings evaluated 21 days after sowing. Treatments: Tr1-Tr8: seeds treated with endophytes and sown in inoculated soil with *M. phaseolina*; T9: control (seeds coated with 0.25% water-agar and sown in inoculated soil); T10: healthy control (seeds coated with 0.25% water-agar and sown in non-inoculated soil with *M. phaseolina*).

Tr1 and Tr2 were statistically different compared to control T9. Tr7 and Tr8 were the best treatments to reduce symptoms on cotyledons 15 days after sowing (Fig. 3b). However, 21 days after sowing, both treatment increased symptoms of charcoal rot. Tr2 showed the greatest reduction in the percentage of seedlings that stopped growing or died in the cotyledon stage. Considering the mean values for the percentage of seedlings with symptoms on cotyledons 15 and 21 days after sowing, reduced disease progress on seedlings was noted for most treatments (Fig. 3 b,d). In this way, and of all the treatments, treatment Tr2 obtained the highest germination percentage values, displayed good seedling protection behaviour against symptoms on cotyledons, and also diminished disease progress from its emergence to 21 days. According to the obtained results, Tr2 was selected. Tr1 was chosen because together with Tr2 they were the only ones statistically different compared to the control. Furthermore, despite the significant necrosis observed in the cotyledons, seedling death was lower in comparison to other *Trichoderma* strains that caused fewer symptoms (Fig. 3c,d). As a result, we speculate that Tr1 may have a greater effect when the time after inoculation increases. As a result, it may be a good option in the field when germination is delayed compared to greenhouse conditions.

3.4. Complementary studies of the two selected Trichoderma spp. strains

3.4.1. Molecular identification of the selected Trichoderma strains (Tr1 and Tr2)

Strains Tr1 and Tr2 were identified as *Trichoderma harzianum* species complex (Allaga et al., 2021). The nucleotide sequences of the nuclear rDNA region containing the two internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene were deposited in the GenBank database as TH2 and TH4 with accession numbers OM920536 and OM920548, respectively.

3.4.2. Effect of water activity on the in vitro endophytes-pathogens interaction

The growth of endophytes (Tr1 and Tr2) and *M. phaseolina* was strongly affected by water availability in the medium (Fig. 4). The ANOVA results revealed significant differences for α_w and the α_w x species interaction (Table 6; Fig. 5). When radial growth and growth rates were analysed, all the fungi were found to have grown more and had higher growth rates at the highest water activity level (Table 7). As shown, the highest values were obtained at 0.995 α_w and the lowest at 0.95 α_w. The *Trichoderma* strains did not grow at 0.90 α_w. At 0.95 α_w, *M. phaseolina* grew more than the *Trichoderma* strains and at 0.98 α_w, the pathogen grew slightly more than the *Trichoderma* strains. However, it can be highlighted that both *Trichoderma* strains grew larger and faster compared to the pathogen at 0.995 α_w (Figs. 4 and 5, Table 7). Moreover, a yellow halo was observed in the initial contact zone of both fungi, as was a line of concentrated microsclerotia at 0.98 and 0.995 α_w (Fig. 4).

After 4 inoculation weeks, the dual culture endophytes-pathogen results showed that Tr1 and Tr2 inhibited the pathogen colony at 0.995, 0.98 and 0.95 α_w, and overgrew the *M. phaseolina* colony. According to the method proposed by Magan and Lacey (1984), "D" "dominance on contact" was the determined interaction type, except for 0.90 α_w. The calculated I_D assesses the ability of Tr1 and Tr2 to compete and dominate over *M. phaseolina* at several α_w. The values calculated for both Tr1/*M. phaseolina* and Tr2/*M. phaseolina* interactions were: 0.995: 4/0; 0.98: 4/0; 0.95: 4/0. The 0.90 interaction was not considered because Tr1 and Tr2 did not grow with a total of 12/0.

3.4.3. Microscopic alteraations of M. phaseolina in the dual microculture under cryo-scanning electron microscopy

The evaluation of the pathogen's morphological alterations under a cryo-scanning electron microscopy from the interaction zone of the dual microcultures showed initial contact between the *Trichoderma* (Tr1 and Tr2) and *M. phaseolina* strains, with direct penetration of the pathogen's hyphae, as well as plasmolysis (Fig. 6 a,c). The following were noted: a tight attachment between both fungi, veils formed, a woven-like wrapping that enveloped both pathogen microsclerotia and hyphae, and numerous *Trichoderma* conidia that developed in veils (Fig. 6 c,e,f,g). Similar behaviour was observed between both *Trichoderma* strains.

4. Discussion

In the present work, all the evaluated endophytic strains displayed significantly reduced pathogen growth in the dual culture, and also improved seed germination and seedling radicle length in *in vitro* assays when applied *via* seed coatings compared to the control. All these endophytes also reduced some charcoal rot symptoms when they were applied as a seed coating in *in vivo* assays.

Endophytes are widely known as microorganisms that live in plants and colonise their internal tissues as symptomless (White et al., 2019). Interestingly, several studies have demonstrated that some endophytes can benefit their hosts and play an important role in increasing plant growth *via* mechanisms, such as improving water and nutrient absorption, providing protection against pathogens and insects, and increasing stress tolerance (Dalal and Kulkarni, 2014; Larran et al., 2016; Latz et al., 2018; De Silva et al., 2019; White et al., 2019). Several researchers have demonstrated that endophytic microorganisms can play a role as either biocontrol agents or elicitors by mechanisms like inducing resistance and reducing the effect of abiotic stresses (De Silva et al., 2019; Wei et al., 2019; Fontana et al., 2021). Endophytes can present advantages as biological control agents compared to other

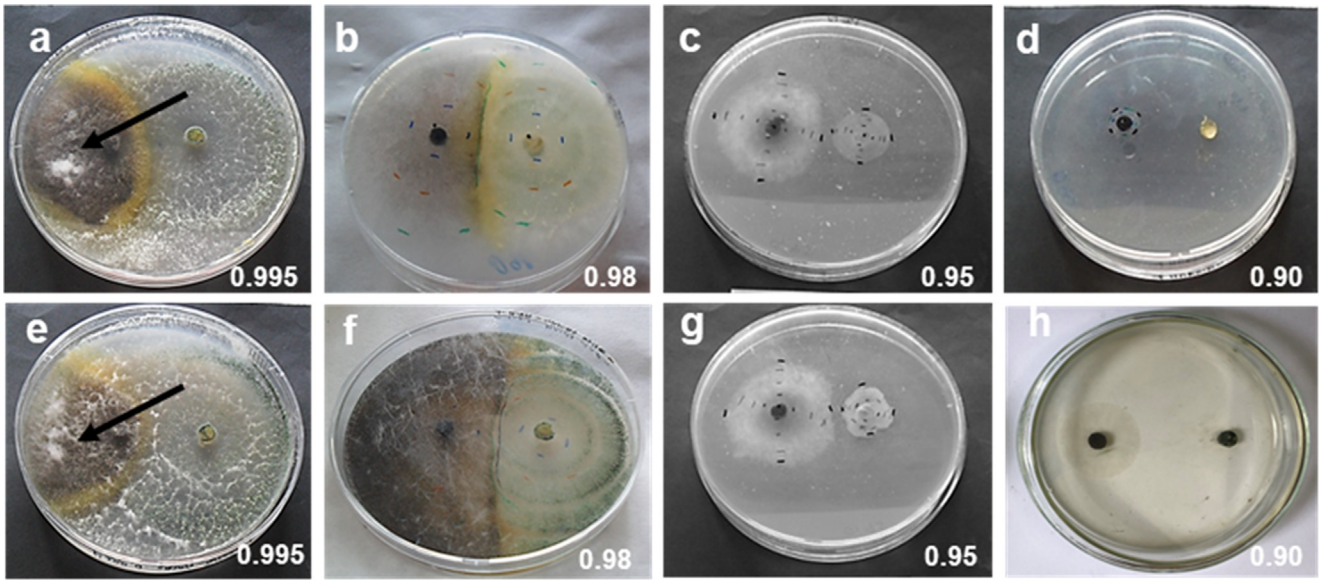

Fig. 4. Interactions between the endophytic *Trichoderma* strains Tr1 and Tr2 and *M. phaseolina* at different water activity levels, (a,b,c,d) Interactions Tr1 (right)-*M. phaseolina* (left) and (e,f,g,h) Tr2 (right)-*M. phaseolina* (left) at 0.995, 0.98, 0.95 and 0.90 α_w. Tr1 and Tr2 did not grow at 0.90 α_w. Arrow indicates overgrowth of endophytic *Trichoderma* covering the *M. phaseolina* colony.

Table 6
ANOVA of the effect of water activity on *M. phaseolina* growth in dual culture with endophytes (Tr1 and Tr2).

Source of variation	DF	Tr1 Mean square	P-value*	DF	Tr2 Mean square	P-value
Water activity	3	24189.63	≤ 0.0001	3	23696.15	≤ 0.0001
Species	1	404.63	0.1054	1	216.58	0.2272
Interaction						
Water activity $_x$ species	3	3682.53	≤ 0.0001	3	4105.56	≤ 0.0001
Error	392	153.66				

* F Test (P ≤ 0.05).

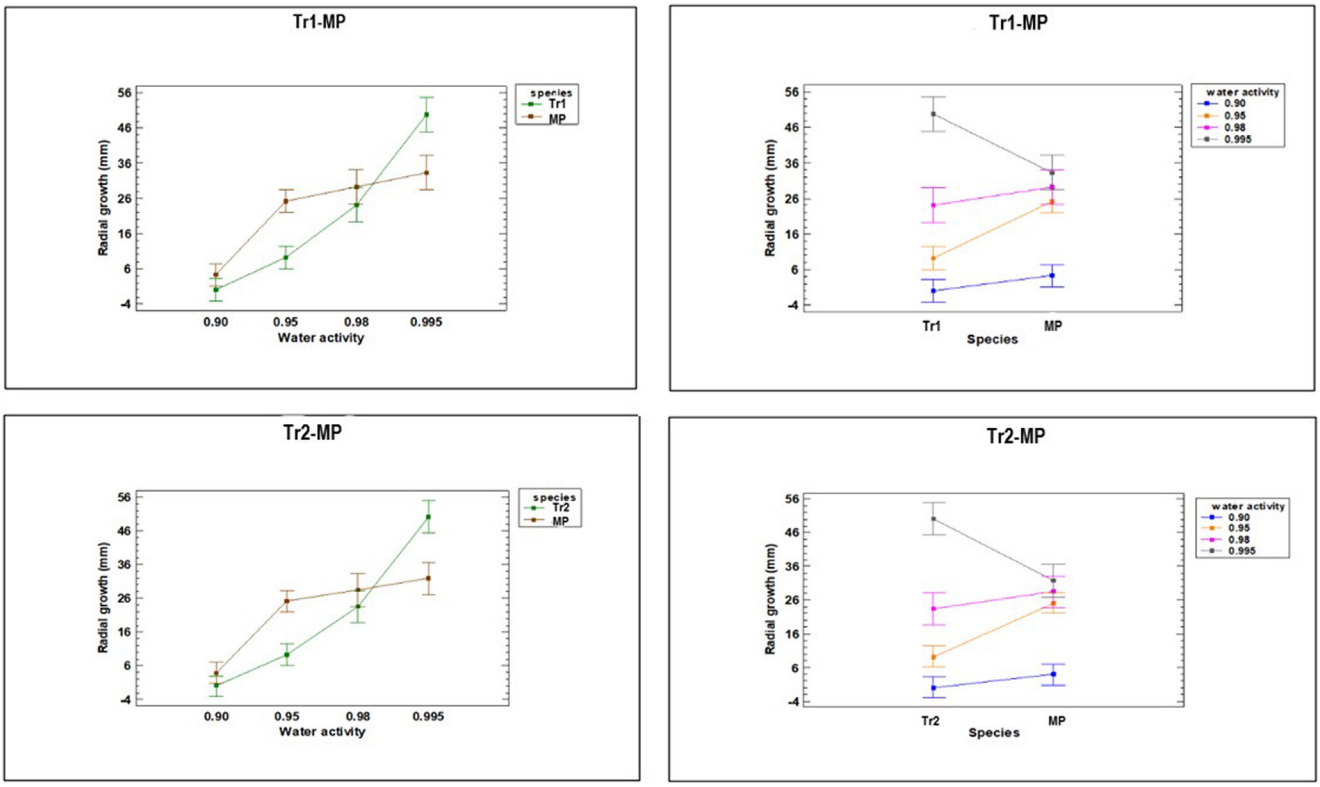

Fig. 5. Interaction intervals (mean radius, species, water activity) between the endophytic *Trichoderma* strains Tr1 and Tr2 and *M. phaseolina* (MP). LSD ≤ 0.05.

biocontrol agents because they internally colonise plant tissues and, thus, remain protected from more adverse environmental conditions because they are adapted to internal conditions. In Argentina, very few studies have been conducted to evaluate the potential of endophytes isolated from crops against phytopathogens that impact soya bean production (Larran et al., 2016; de Almeida et al., 2018).

Previous studies have also provided evidence for the efficacy of different *T. harzianum* isolates collected from soya bean root and crown samples infected by *M. phaseolina* as biological control agents against this pathogen (Khalili et al., 2016). Additionally, researchers have evaluated the biocontrol potential of different *Trichoderma* spp. against *M. phaseolina* and mechanisms of action in *in vitro* assays (Khan and Javaid, 2020; Khan et al., 2021). These authors have demonstrated that *T. pseudokoningii* and *T. viride* were successful biocontrol agents against *M. phaseolina* by means of DNA disintegration and the production of antifungal secondary metabolites. It is also worth noting that distinct *Trichoderma* species have proven effective in reducing fungal growth under different agro-ecological conditions (Singh et al., 2008; Iqbal and Mukhtar, 2020). This increases the significance of testing those species in specific situations. Indeed, knowledge of the mechanisms of action used by the antagonist is essential to adequately select those isolates with a higher potential for controlling targeted phytopathogens. In this sense, studies by Khan and Javaid (2022a; 2022b) revealed that two *Aspergillus* species and quinoa dry mass applied either alone or together to soil previously inoculated with *M. phaseolina* induced defence response in mungbean plants, which would be another mechanism of action involved by biocontrol options. These authors reported normal cell structures from plant sections of mungbean treated with *Aspergillus* species alone or together with quinoa dry mass while cross sections from untreated soil (only inoculated with *M. phaseolina*) showed histochemical changes on sections plants of mungbean in response to *M. phaseolina* as distorted, fragmented, and collapsed cell structures.

According to the results obtained in this work, all the endophytes reduced the pathogen colony in an *in vitro* assay, which suggests that one of the mechanisms was competition for space and nutrients. All the evaluated *Trichoderma* strains caused hyphal alterations in *M. phaseolina*. Our light microscopic observations revealed that all eight endophytes grew close to the *M. phaseolina* hyphae by surrounding and coiling around them, and penetrated the pathogen's hyphae either with or without appressorium-like

Table 7
Effect of water activity on radial growth and growth rates of endophytes-*M. phaseolina* interactions in an *in vitro* assay.

Tr1-*M. phaseolina* interaction	**Mean***	**Lower limit**	**Upper limit**	**GR****
0.90 a_w - Tr1	0 e	0	0	0
0.90 a_w - *M. phaseolina*	4.27 ± 1.48 de	1.35	7.18	1.53 (0.95)***
0.95 a_w - Tr1	9.31 ± 1.48 d	6.40	12.23	2.23 (0.95)
0.95 a_w - *M. phaseolina*	25.16 ± 1.48 bc	22.25	28.08	6.99 (0.99)
0.98 a_w - Tr1	24.07 ± 2.26 bc	19.62	28.52	15.90 (0.99)
0.98 a_w - *M. phaseolina*	29.21 ± 2.26 b	24.76	33.66	18.71 (0.99)
0.995 a_w - Tr1	49.75 ± 2.26 a	45.30	54.20	33.10 (0.99)
0.995 a_w - *M. phaseolina*	33.27 ± 2.26 b	28.82	37.72	22.33 (0.99)
Tr2-*M. phaseolina* interaction				
0.90 a_w - Tr2	0 e	0	0	0
0.90 a_w - *M. phaseolina*	3.90 ± 1.45 de	1.04	6.76	1.42 (0.97)
0.95 a_w Tr2	9.25 ± 1.45 d	6.39	12.11	2.19 (0.96)
0.95 a_w - *M. phaseolina*	25.10 ± 1.45 bc	22.24	27.96	7.18 (0.99)
0.98 a_w - Tr2	23.49 ± 2.22 c	19.12	27.86	15.58 (0.99)
0.98 a_w - *M. phaseolina*	28.48 ± 2.22 bc	24.11	32.84	17.98 (0.99)
0.995 a_w- Tr2	50.16 ± 2.22 a	45.79	54.53	31.68 (0.99)
0.995 a_w - *M. phaseolina*	31.84 ± 2.22 b	27.48	36.21	22.23 (0.99)

* Mean: mean radius ± standard error; the means followed by the same letter in the same column in the same treatment are not statistically different according to the LSD ($P \leq 0.05$).
** GR: calculated growth rates (mm/day-^{1}).
*** R^2: coefficient of determination, all of them significant $P \leq 0.01$.

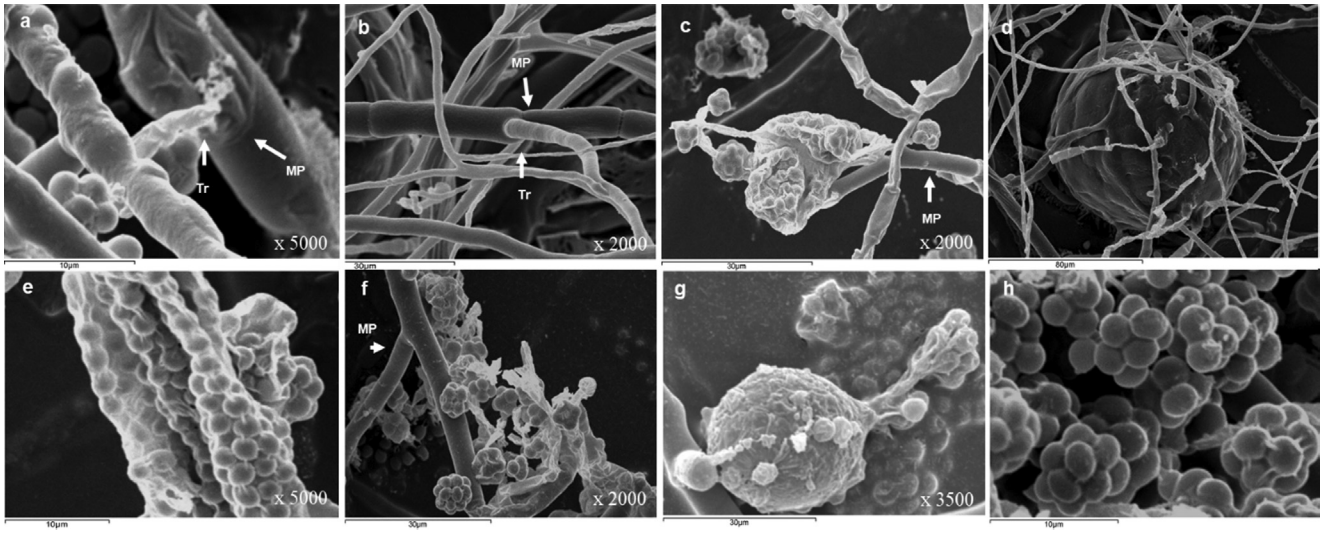

Fig. 6. Cryo-scanning electron micrographs of the interaction between *Trichoderma harzianum* species complex (Tr1 and Tr2) and *M. phaseolina,* (a,b) Direct *T. harzianum* species complex (THSC) penetration in *M. phaseolina* hyphae showing plasmolysis; (c,e) veil formation with conidia developed as woven and showing plasmolysis of *M. phaseolina* hyphae; (f) THSC conidia coming out of the veil; (g) veil formation wrapping the *M. phaseolina* microsclerotia and THSC conidia; (d,h) microesclerotia in the formation stage and *T. harzianum* species complex conidia (controls). Arrows indicate THSC hyphae (Tr) and *M. phaseolina* hyphae (MP).

formation to cause cell wall disruption (plasmolysis). These mechanisms of action observed in the endophytes-*M. phaseolina* interaction have been previously described as a typical mycoparasitism of *Trichoderma* species (Mukhopadhyay and Kumar, 2020. However, one novelty of our work was that veils formed by a woven-like wrapping around *M. phaseolina* microsclerotia or hyphae, and we frequently found numerous *Trichoderma* conidia developing in veils. Interestingly, in dual culture previous studies have screened a *T. harzianum* strain against *Fusarium sudanense,* and we observed a veil-like formation that wrapped the false heads and microconidia of *F. sudanense* under a cryo-scanning electron microscope (Larran et al., 2020). So, we suggest that another mechanism that evolved for endophytes against the pathogen was direct mycoparasitism. Moreover, Khalili et al. (2016) observed the cell lysis of *M. phaseolina* hyphal tissue using non-endophytic *Trichoderma* strains. It is widely known that the species belonging to the *Trichoderma* genus present different mechanisms of action to control pathogens, such as competition for substrate or space, mycoparasitism, antibiosis and DNA disintegration by the production of antifungal secondary metabolites (Khan and Javaid 2020; Mukhopadhyay and Kumar, 2020; Khan et al., 2021). These mechanisms can act independently or together against pathogens and, interestingly, their potential as biological agents can be influenced by diverse factors like *Trichoderma* strains, environmental conditions and targeted pathogens (Mukhopadhyay and Kumar, 2020).

In our work, we also observed yellow pigment production in the pathogen-antagonists interaction zone in dual cultures with Tr1, Tr2, Tr3, Tr4, Tr5 and Tr6. We observed that Tr1 and Tr2 in the dual culture with *M. phaseolina* produced this yellow pigment at 0.995 and 0.98 a_w. Several fungi are known to synthesise pigments as secondary metabolites and, interestingly, many of these fungal pigments play an ecological role, including protection from environ-

mental stress (Dufosse et al., 2014; Rao et al., 2017). It has been previously demonstrated that certain pigments possess antibacterial and antifungal biological properties among other biotechnological applications (da Costa Souza et al., 2016). Different *Trichoderma* species have also been shown as pigment producers (Caro et al., 2017), and numerous studies have reported that pigments or pigment extracts of different fungal genera, such as *Trichoderma*, *Penicillium* and *Aspergillus*, have antimicrobial activity against distinct fungal pathogens (Saravanan and Radhakrishnan, 2016; Kalra et al., 2020). We observed that when the strains of *Trichoderma* and *M. phaseolina* were cultivated individually on 2 % APG, non-yellow pigments formed, as they did in dual cultures. Although the production of fungal pigments and their association with different biological activities are widely known, very few studies are available on their physiological role and the factors that regulate their production. In light of the above, it could be interesting to continue with studies about the production of pigments in the interactions between the *M. phaseolina*-endophytic *Trichoderma* strains herein evaluated to elucidate the chemical structure of the metabolites produced in culture media and their role. We highlight that not all the eight *Trichoderma* strains generated this yellow pigment when screened against the *M. phaseolina* strain. Therefore, we suggest different behaviours of the evaluated strains.

We demonstrated that eight endophytic *Trichoderma* strains antagonised the *M. phaseolina* strain procured from a soya bean field in the Entre Rios province. However, morphological and pathogenic variability among *M. phaseolina* isolates has been demonstrated (Iqbal and Mukhtar, 2014). Therefore, we consider that further studies using different strains of the pathogen are necessary to assess the effectiveness of endophytic *Trichoderma* strains against this pathogen.

Additionally, this work demonstrated that the growth of endophytes (Tr1 and Tr2) and *M. phaseolina* was affected by the water availability of the culture media adjusted for different water activities. This agrees with several reports showing that the water availability of substrate affects fungal growth, particularly mycelial growth, spore germination and production, and also germ tube growth (Magan, 1988; Lupo et al., 2002; Larran et al., 2020). This knowledge contributes to understand how potential biological control agents will behave under natural field conditions, which is a first required step to test their use and effectiveness as alternative integrated disease management tools.

5. Conclusion

All the endophytic *Trichoderma* strains were antagonistic against *M. phaseolina,* however our study allowed us to select two *Trichoderma* strains with good potential to be included for charcoal rot management.

We conclude that soya bean seeds coated with endophytic *Trichoderma* strains can be successful for the bioprotection of soya bean seedlings against *M. phaseolina*. Its use is highly recommended as an environment-friendly and economical tool compared to chemicals. Hence its inclusion as part of integrated strategies for agro-ecological charcoal rot management is suggested.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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