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ORIGINAL PAPER

Evaluation of the Extract of *Pseudopithomyces chartarum* to be used as Biocontrol Agent Against *Phytophthora cinnamomi* in *Lupinus luteus*

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Abstract
Endophytic fungi offer promising opportunities for a sustainable disease management in agricultural systems, often related to their secondary metabolites. In this study, the potential of the metabolites produced in vitro by *Pseudopithomyces chartarum* to protect the forage crop *Lupinus luteus* against the soil-borne pathogen *Phytophthora cinnamomi* was evaluated in different in vitro and *in planta* tests. The fungal extracts containing the metabolites were applied by two different procedures: to seeds by immersion (mycopriming) and to seedlings by spraying (post-emergence). The antagonism between the endophyte fitrates/extracts and the pathogen was studied by dual culture assays and the minimal inhibitory concentration. Other filtrate/extract traits, such as its phytohormones production and nutrient mobilization, were also analyzed. The metabolites contained in the extract were tentatively identified by mass spectrometry. The endophyte's filtrates and extracts exhibited significant inhibition on the *P. cinnamomi* growth in vitro. Additionally, seed mycopriming and post-emergence extract application positively influenced seed germination, reduced disease severity, and enhanced several growth parameters in treated seedlings. Auxins and gibberellins production, and phosphate solubilization activity were observed in the endophyte, traits potentially contributing to its biocontrol efficacy. The bioactive compounds ciclo(L-Phe-L-Pro), dihydroabikoviromycin, medelamine A, herniarin, and Piptamine, all with antimicrobial properties, were tentatively identified in the extracts, but further research is needed to confirm this identity and to evaluate the extract efficacy under field conditions. This study highlights the potential of certain endophytic fungi as sustainable and environmentally friendly alternatives for a disease management in agriculture.

Keywords Fungal endophytes · Secondary metabolites · Plant protection · *Pseudopithomyces chartarum* · *Lupinus luteus* · *Phytophthora cinnamomi*

1 Introduction

Yellow lupine (*Lupinus luteus* L.), particularly the cultivar 'Tremosilla', is traditionally grown in dehesas, a characteristic agroforestry system in Spain, due to its adaptability to the semiarid Mediterranean climate and acidic soils (Stoate et al. 2000), typical conditions of this ecosystem (Olea and Miguel-Ayanz 2006). Dehesas, artificially maintained by farmers for extensive livestock rearing, are also very valuable for their high biodiversity and provision of ecosystem services. They are considered the best management option for balancing economic activity and conservation under such harsh conditions (Plieninger et al. 2004). However, natural forage resources are often insufficient to meet the nutritional requirements of animals (Vázquez de Aldana et al. 2006), so the cultivation of forage crops is often necessary. Within these crops, the use of legumes is considered very appropriate due to their known ability to improve soil structure and atmospheric nitrogen fixation. In this regard, yellow lupine is also an adequate source of protein and dietary fiber, along with balanced levels of lysine and sulfur amino acids (Llobat and Marín-García 2022). Furthermore,

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the cultivar 'Tremosilla' is valued for its richness in antioxidants and its low levels of antinutritional factors such as trypsin inhibitors or saponins compared to other lupine species (Ferchichi et al. 2021; Villa et al. 2020).

However, the cultivation of *L. luteus* in dehesas also presents several limitations, including its general susceptibility to pathogens such as *Phytophthora cinnamomi*, which can cause root rot, wilting, and crop death (Serrano et al. 2010). The relationship between *L. luteus* and *P. cinnamomi* is particularly important in the context of dehesas ecosystems, as this pathogen can cause damage not only to lupine plants, but also to woody species as a primary root pathogen, particularly affecting holm oaks (Mora-Sala et al. 2018), the main component of the tree layer in dehesas. *Phytophthora cinnamomi* has been identified as a major biotic driver of the severe decline and mortality experienced by evergreen oaks in southern Europe (Domínguez-Begines et al. 2020). The susceptibility of young holm and cork oak seedlings to *P. cinnamomi* has already been demonstrated, potentially limiting the natural regeneration processes of trees in dehesas (Rodríguez-Molina et al. 2002). Therefore, the control of *P. cinnamomi* in all the components of this ecosystem is crucial for its preservation. The relationship between the diversity of soilborne microbial communities and the holm oak decline syndrome due to *P. cinnamomi* is not yet fully understood (Gómez et al., 2019). However, it is known that the action of *P. cinnamomi* may be influenced by various abiotic and biotic factors, such as soil moisture content, edaphic factors, changes in traditional land use, and the presence of other microorganisms and insects in dehesas (Cernadas et al. 2018). Recent research suggests that sustainable control measures based on the management of soil microbiota diversity and composition could play a key role in the implementation of integrated disease management strategies in dehesas (Ruiz-Gómez and Miguel-Rojas 2021).

One of these potential strategies could be based on the use of fungal endophytes, organisms living inside their plant hosts without causing any disease symptoms, which have already demonstrated their potential as biocontrol agents against *P. cinnamomi* (Martins et al. 2021; Tellenbach et al. 2013; Wang et al. 2016). However, the efficacy of the biocontrol exerted by the endophyte can be strongly influenced by its compatibility with the host and by the particular environmental conditions (Arnold et al. 2003). For this reason, the results obtained from the use of endophytic fungi as biocontrol agents are often inconsistent and highly variable depending on the particular conditions of the area of application. In many cases, the potential biocontrol activity of fungal endophytes is due to their ability to produce bioactive compounds, including antimicrobial and antifungal compounds, which can inhibit the growth of plant pathogens (Khan et al. 2020; Segaran and Sathiavelu 2019; Sharma et al. 2022). The extracts of endophytic fungi have been reported to exhibit antimicrobial, antimalarial, and cytotoxic activities (Omomowo et al. 2023). In this sense, the direct application of fungal metabolites, previously produced in vitro, to control pathogens may overcome the limitations of the direct use of the living organism as they may not be as influenced by the environmental conditions. Therefore, more robust and consistent biocontrol results could be expected after their application. This is supported by several studies, where the use of filtrates or extracts produced by fungal endophytes has already been shown to be effective in the biocontrol of Phytophthora (Lawrence et al. 2021), even against *P. cinnamomi* in *Lupinus luteus* plants (García-Latorre et al. 2022).

Therefore, with the hypothesis that the endophytic metabolites with biocontrol properties can be produced in vitro and that these metabolites can be effective in the biocontrol of *P. cinnamomi*, the main goal of this study was to evaluate the potential of a fungal endophyte with biocontrol properties, previously isolated from dehesas and identified as *Pseudopithomyces chartarum*, to protect *Lupinus luteus* plants against the pathogen through its filtrates and extracts obtained in vitro. Several traits of the endophyte filtrate or extract, related directly or indirectly with the biocontrol or with the improvement of plant defense responses, such as its antagonistic activity against the pathogen or its capacity to produce phytohormones or to have nutrient solubilization activity, were also evaluated in order to better understand the mechanisms involved in plant protection. For the same reason, a tentative identification of the metabolites contained in the fungal extract was also performed.

2 Materials and Methods

2.1 Fungal and Plant Material

For this study, a specific endophytic fungus (laboratory code E498), previously isolated from healthy *Hordeum murinum* stems collected in the *dehesas* of Extremadura (Spain), was used. Its isolation followed the procedure described in Lledó et al. (2016). The isolate E498 was selected based on parameters such as the frequency of its isolation from the original plant hosts and the bioactivity observed in previous experiments (data not shown). The fungus, identified as *Pseudopithomyces chartarum* (Berk. & Curtis) Li, Ariy. & Hyde, was characterized by sequence data derived from the internal transcribed spacer (ITS) region and the large subunit (LSU) of the nuclear ribosomal RNA (rRNA) gene complex. Comparison of these with sequences in the GenBank database was performed using a BLAST search (www.NCBI.nlm.nih.gov) and phylogenetic trees (Fig.

S1). The isolate was assigned Genbank accession numbers OK161078 (for the ITS region) and OR999601 (for its LSU region). Molecular procedures included DNA extraction and purification from colonies growing on yeast malt (YM) agar (malt extract 10 g L^{-1}, yeast extract 4 g L^{-1}, D-glucose 4 g L^{-1}, agar 20 g L^{-1}, pH 6.3 before autoclaving) by using the Fungal gDNA Miniprep Kit EZ-10 Spin Column protocol (NBS Biologicals, Cambridgeshire, UK). Amplification of loci (ITS and LSU) followed the methods described in White et al. (1990) and Vilgalys and Hester (1990). PCR products were subsequently purified and sequenced by Sanger Cycle Sequencing method at Microsynth Seqlab GmbH (Göttingen, Germany). Consensus sequences were obtained using Geneious® 7.1.9 (http://www.geneious.com) (Kearse et al. 2012).

The pathogen used in this study, a single morphologically identified *P. cinnamomi strain* (*Ps-1683*), isolated from roots of a *Quercus ilex* tree in Valverde de Mérida, SW Spain (38° 55′ N, 6° 11′ W), had been shown to be highly virulent to seedlings of *Quercus ilex* and *Castanea sativa* (Camisón et al. 2019). The strain was cultured on potato dextrose agar (PDA; 39 g L^{-1}) at a temperature of 23 °C to promote mycelial growth. Spores were obtained by transferring 5 mm plugs from 7-day-old cultures on PDA to V8-agar plates, after their incubation at 22 °C for 5 days. Zoospores were induced by treating the plates with distilled water and non-sterile soil extract, and their concentration was determined using a hematocytometer. Commercial seeds of *Lupinus luteus* L. (cv. Tremosilla) were used for the greenhouse experiments.

2.2 Culture Conditions and Obtention of Filtrates and Extracts from*Pseudopithomyces chartarum*

In order to obtain the filtrates and extracts of *P. chartarum* for the experiments, the endophyte was incubated in triplicate in 500 mL Erlenmeyer flasks containing 250 mL of yeast malt broth (YMB; in g L^{-1}: yeast extract: 6; malt extract: 10; D-glucose: 6, at pH 6.3). The cultures were maintained at 23 °C and 140 rpm in a thermoshaker (Orbital Shaker Incubator COMECTA 1102). Two days after complete glucose consumption in the medium, fungal culture was filtered using sterile paper discs (0.2 µm) to separate the mycelium from the liquid filtrate containing secondary metabolite (Stadler et al. 2001). Crude extracts were then obtained from a portion of these filtrates using ethyl acetate (1:1) according to the method described by Halecker et al. (2014). The organic phase was evaporated on a rotary evaporator (Hei-Vap ML/G1) to eliminate the ethyl acetate. The solid residue was then resuspended in methanol for in vitro tests and in water for *in planta* assays.

2.3 Inhibition of Pathogen Mycelial Growth*in vitro* by Fungal Filtrate

The inhibitory effect of the fungal filtrate of *P. chartarum* on the mycelial growth of *P. cinnamomi* was first evaluated in vitro. Petri dishes were prepared by adding 2 mL of the filtrate to 18 mL of sterilized PDA medium, ensuring uniform mixing before agar solidification. Control dishes were similarly prepared using 2 mL of sterilized distilled water. An actively growing mycelial plug of the pathogen (ø = 5 mm) was placed in the center of each Petri dish (Santamaría et al. 2004). Dishes were then incubated at 23 °C, and colony length measurements were taken every 12 h for 72 h. Four colony length measurements were taken for each dish, one for each of the four radii that defined the quadrants. All samples were tested in triplicate.

2.4 Minimum Inhibitory Concentration (MIC)*in vitro* of the Crude Extract

The Minimum Inhibitory Concentration (MIC) of the crude extract of *P. chartarum* against *P. cinnamomi* was determined using a serial dilution assay in 96-well plates. First, 130 µL of a freshly prepared spore solution (6.7×10^5 spores mL^{-1}) of the pathogen was added to each well. Then, 20 µL of 300 µg mL^{-1} of the extract were added in the first row, following for a 50% serial dilution for the following rows. A positive control of 20 µL of 1.5 mg mL^{-1} cycloheximide, and a negative control of 20 µL of sterilized methanol were included. Plates were then kept at 23 °C and 600 rpm for 48 h before evaluating the results. All samples were tested in triplicate.

2.5 *In planta* experiments

The efficacy of crude extract in protecting *L. luteus* plants against *P. cinnamomi* was evaluated in two greenhouse experiments. In the first experiment, lupine seeds were treated with the fungal extract prior to germination (mycopriming test). In the second experiment, seedlings were treated with the fungal extract one month after sowing (post-emergence test). Before the experiments, the lupine seeds were disinfected in a solution of 2% sodium hypochlorite and then washed 3 times with sterile distilled water (Zabalgogeazcoa et al. 2006).

For the mycopriming test, sterilized seeds were soaked in the fungal extract of *P. chartarum* (3 mg mL^{-1}) for 6 h to ensure metabolite uptake. A negative control of sterile distilled water, the solvent used for the extract, was included. Subsequently, 30 seeds per treatment were sown individually in independent pots ($7 \times 7 \times 6$ cm^3) containing a mixture of substrate and perlite with the following composition:

pH 7.00 ± 0.50; EC 1.50 ± 0.10 dS m^{-1}; organic matter 60.0 ± 2.0%; N 1.29 ± 0.08%; P_2O_5 0.58 ± 0.05%; K_2O 1.25 ± 0.10%. Half of the containers were inoculated with a 100 mL L^{-1} soil solution containing 2×10^4 spores of *P. cinnamomi*, while the other half received pathogen-free substrate mixture. The containers were kept in the greenhouse for 15 days, watered to field capacity each 2–3 days and no receiving additional fertilizers. Each treatment was kept physically separated from each other to ensure no cross-contamination. During such a period, daily germination was monitored. After those 15 days, five randomly selected plants from each treatment were harvested, and various growth parameters, such as aerial and root elongation, number of roots, and dry weight of root and herbage, were measured ($n = 5$). To ensure the validity of the results, this assay was conducted in triplicate simultaneously. This meant that, for the evaluation of the influence of the fungal extract on the germination rate, three sets of 30 seeds were sown independently and each set was considered a repetition ($n = 3$). This experiment was conducted from September 26, 2019, to October 10, 2019.

In the post-emergence test, five independent pots ($7 \times 7 \times 6$ cm^3) per treatment were sown with three sterilized seeds each and irrigated until field capacity each 2–3 days. One month after sowing, the pathogen inoculation (100 mL L^{-1} of soil containing 2×10^4 spores of *P. cinnamomi* mL^{-1}) was performed to half of the pots, followed 12 h later by spraying a dose of 3 mg mL^{-1} extract, at a rate of 1 mL per pot. A control treatment using sterile distilled water was also included. Disease severity was evaluated weekly after the inoculation of the pathogen for a month, by adapting the scale used by Serrano et al. (2010). Thus, the process consisted in visually observing symptoms such as yellowing, drying, rotten leaves, blackish spots, lower vigor and assigning severity levels from 0 (healthy plant) to 4 (death):: 0 - Healthy plant; 1 - Slight signs and symptoms of disease (affecting less than 50% of the plant); 2 - Symptoms of disease (affecting more than 50% of the plant); 3 - Senescence or obvious signs of decay; 4 - Death. The area under the disease progress curve (AUDPC) for each pot was calculated based on the severity values. After the final measurement, the five plants per treatment were collected for laboratory analysis. In those plants, the root number and length were recorded, and samples were then dried in an oven, and the weight of herbage and root dry matter determined. Again, to ensure the validity of the results, the assay was conducted in triplicate simultaneously. This experiment was conducted from September 26, 2019, to November 28, 2019. The climatic conditions (relative humidity and temperatures) during the experiments are shown in Fig. 1.

2.6 Evaluation of Other Traits of *Pseudopithomyces chartarum* Directly or Indirectly Related to Plant Protection

The assessment of the potential of *P. chartarum* to produce substances directly or indirectly related with plant protection was based on two traits: (i) production of phytohormone-like substances, and (ii) nutrient mobilization, including phosphate solubilization, siderophore production, and ammonia synthesis.

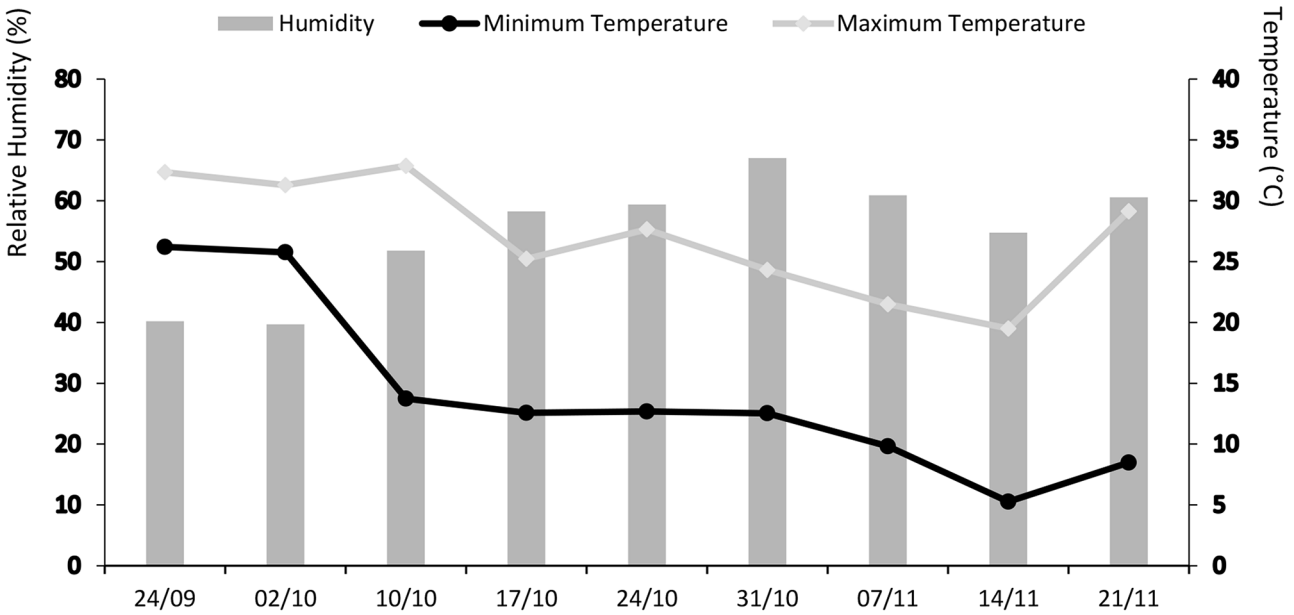

Fig. 1 Climatic conditions regarding temperatures and relative humidity in the greenhouse during the *in planta* assays

*Estimation of phytohormone-like substances production: t*o determine the production of phytohormone-like substances, we evaluated the presence of both auxin-like and gibberellin-like substances in the fungal filtrate. The auxin-like substances were determined by quantifying indoleacetic acid (IAA) through a colorimetric test (Devi et al. 2016). Thus, 1 mL of the fungal filtrate was mixed with 2 mL of Salkowski reagent and vigorously shaken for 1 min in a vortex and kept in the dark for 30 min. The absorbance was then measured at 530 nm using a spectrophotometer (JP Selecta UV 3100). The concentration of IAA was determined using the regression equation from a standard curve of pure indole-3-acetic acid (Sigma Aldrich). Additionally, the endophyte was cultivated in YMB medium supplemented with 5mM of L-tryptophan, to assess its effect as the main precursor of IAA (Gordon and Weber 1951). The estimation of the concentration of gibberellin-like substances, expressed as gibberellic acid equivalents (GAE), was assessed through the colorimetric method described by Holbrook et al. (1961). In summary, 15 mL of the fungal filtrate were mixed with 2 mL of zinc acetate (21.9%) and 2 mL of potassium ferrocyanide (10.6%), sequentially with a two-minute interval. The mixture was then centrifuged at 2000 rpm for 15 min. To the supernatant, 5 mL of HCl (30%) were added, followed by incubation at 20 °C for 75 min. The absorbance was measured at 254 nm and the results were compared to a gibberellic acid (GA3, Sigma Aldrich) calibration curve. All samples were analyzed in triplicate, and the results were expressed as milligrams of compound (i.e., IAA or GAE, respectively) per milliliter of fungal filtrate.

*Phosphate solubilization capacity: a*n actively growing plug of *P. chartarum* was placed in a Petri dish containing the National Botanical Research Institute's phosphate (NBRIP) growth medium amended with 1.5% agar (Nautiyal 1999; Nieva et al. 2019). The plates were incubated at 27 ºC for 7 days, after which they were examined for a clear halo around the colony. The diameter of this clear zone was measured, and the solubilization capacity of the endophyte was estimated using the following formula: Solubilization Index (%) = (Colony diameter + Clear zone diameter)/(Colony diameter). Three replicates were performed.

Siderophore Production it was estimated using a modified Chrome Azurol S (CAS) universal assay described by Pérez-Miranda et al. (2007). An actively growing plug of *P. chartarum* was placed in the center of Petri dishes containing 20 mL of Minimal Medium 9 (MM9) and incubated in a growth chamber at 27 ºC for 7 days. After the incubation period, a modified CAS solution was prepared by dissolving 60.5 mg of CAS, 72.9 mg of HDTMA, 30.24 g of PIPES, FeCl3·6H2O in 10 mL (10 mM) of HCl and agarose (0.9%, w/v). The solution was autoclaved and then poured onto each Petri dish with the 7-day old fungal colony in MM9 medium. After 15 min, a color change might be observed in the CAS medium surrounding siderophore producing microorganisms. The size of the halo, if any, was measured using the same formula as used for the phosphate solubilization. Additionally, the protocol was repeated with a non-deferrated medium to confirm that siderophore production was not induced under normal conditions. For both assays, a blank was introduced using a plug of uninoculated PDA medium, and three replicates were performed.

Qualitative Assessment of ammonia Production ammonia production capacity was assessed qualitatively by observing a color change in the solution after the addition of 1 mL of Nessler's reagent to a culture of the fungus grown in peptone water at 28 ºC for 72 h. The degree of ammonia production was determined based on the observed color change in the solution. A faint yellow color may indicate minimum ammonia production, while a deep yellow to brown color may indicate maximum ammonia production (Singh et al. 2014). Each assessment was performed in triplicate.

2.7 Tentative Identification of Metabolites Contained in the Fungal Extract

To tentatively identify metabolites in the fungal extract, mass spectrometry was conducted using an Agilent 6520 Accurate Mass Q-TOf LC/MS system (Agilent, Santa Clara, CA, USA) with an electrospray ionization interface in positive ion mode. The operating parameters were as follows: capillary voltage, 3500 V; fragmenter, 100 V; nebulizer pressure, 35 psig; drying gas temperature, 300 °C; acquisition range 150–800 m/z. Nitrogen was employed as the drying gas at a flow rate of 12.0 L min^{-1}.

The system also featured a diode array detector operating in the range of 280 to 350 nm with a 2 nm step. Samples were eluted on an Agilent Zorbax eEclipse Plus C18 Rapid Resolution column (4.6 × 100 mm, 3.5 µm) maintained at 30 °C. The mobile phase consisted of 0.1% formic acid in ultrapure water (obtained from Millipore Integral-5 purification system) (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A gradient elution was applied as follows: 0–10% B (0 min), 10–100% B (30 min), 100% B isocratic mode (10 min), and for column re-conditioning, 100–10% B (1 min) and 10% B (7 min). Both formic acid and acetonitrile were of LC/MS grade. The flow rate was set at 0.30 mL min^{-1}, and the injection volume was 1 µL.

Table 1 Effect of *Pseudopithomyces chartarum* (E498) filtrates and extracts on mycelial growth (in solid media) and on the MIC (Minimum Inhibitory concentration) of *Phytophthora cinnamomi*. A summary of the one-way ANOVA for the growth of the pathogen in solid media is shown at the bottom indicating the degree of freedom (df), *F* value and the level of significance (*** $p \leq 0.001$)

	Solid media after 72 h (mm)[1]	MIC (µg of extract mL^{-1})
E498	0 ± 0 b	150.00 ± 0
Negative control[2]	1.98 ± 0.02 a	0.00 ± 0.00
Positive control[3]	-	2.34 ± 0
df	1	-
F	6525.26***	-

[1]For the biocontrol potential assessed in solid media, values are expressed as mean ± error standard ($n = 16$). Different letters indicate significant differences according to LSD (least significant difference) test at $\alpha = 0.05$

[2]Negative control: sterilized distilled war for the solid media test and methanol for the MIC test; [3]Positive control: cycloheximide (1.5 mg mL^{-1}), only in the MIC essay

2.8 Statistical Analysis

To assess the effect of the filtrate and the extract of *P. chartarum* on the mycelial growth of *P. cinnamomi* and on the response variables associated to protection in the greenhouse assays using *L. luteus* plants, both inoculated and non-inoculated with the pathogen, two-way ANOVAs were used. Prior to the analysis, the assumptions of normal distribution and homoscedasticity were ensured by Shapiro-Wilk and Levene's tests, respectively. Factors considered in the analysis included the endophyte treatment, the pathogen inoculation, and their interaction. Significant differences between treatments were further analyzed using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$.

3 Results

3.1 Effect of the Filtrate And The Extract of *Pseudopithomyces chartarum* on Pathogen Growth Plant Protection*in Vitro*

The filtrate of *Pseudopithomyces chartarum* (E498) completely inhibited the growth of the pathogen at least during the 72-h duration of the experiment (Table 1). At the same time, the application of extract produced inhibition at a minimum concentration of 150 µg of extract per mL (Table 1).

3.2 Effect of Mycopriming with *Pseudopithomyces chartarum* Extract on the Control of *Phytophthora cinnamomi* in *Lupinus luteus*

According to the ANOVAs (Table 2), across the different days of observation (from day 5 to 15), mycopriming with the endophyte consistently had a significant impact on the germination rate of *Lupinus luteus* seeds, as did the presence of the pathogen (except on day 6), and the interaction between both variables (except on days 9 and 10; Table 2). The evolution of germination rates for lupines seeds (Fig. 2), showed that in the control group without mycopriming or pathogen (Control Pc-), germination rates steadily increased from Day 5 to Day 15, reaching a peak of 56.67% with relatively low variability. Conversely, in the group of seeds not mycoprimed but inoculated with the pathogen (Control Pc+)-), germination rates declined, delaying germination until day 9 and reaching a maximum germination rate of 23.33% by Day 15. Mycopriming with the extract of the endophyte reversed the negative impact caused by the pathogen, with the values of this group (E498 Pc+) being very similar to those of the control group without the pathogen (Fig. 2). Mycopriming not only inhibited the damage caused by the pathogen but also promoted germination, as mycoprimed seeds without the pathogen (E498 Pc-) significantly increased their germination compared to the control group from day 8 onwards, progressively increasing over time and reaching 93.33% by Day 15, which is 64.7% higher than the control group on the final day (Fig. 2).

Table 2 Summary of the ANOVAs for the effect of the mycopriming with the extract of *Pseudopithomyces chartarum* (E498), the presence of *Phytophthora cinnamomi*, and its interaction on the germination rate of the seeds of *Lupinus luteus* under greenhouse conditions. The degree of freedom (*df*), F-values, and levels of significance (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) are shown for each day and factor

	df	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Endophyte (E)	1	1.00	64.00**	15.12*	75.00***	73.50**	64.00**
Pathogen (P)	1	49.00*	9.14	42.25*	289.00***	147.00**	100.00**
E*P	1	49.00*	30.22*	4.9E + 32***	49.00*	1.00	72.89
	df	Day 11	Day 12	Day 13	Day 14	Day 15	
Endophyte (E)	1	90.25***	162.00***	361.00***	361.00***	147.00***	
Pathogen (P)	1	132.25***	121.00**	529.00***	49.00*	49.00*	
E*P	1	74.25**	134.67**	297.00***	89.22*	73.58*	

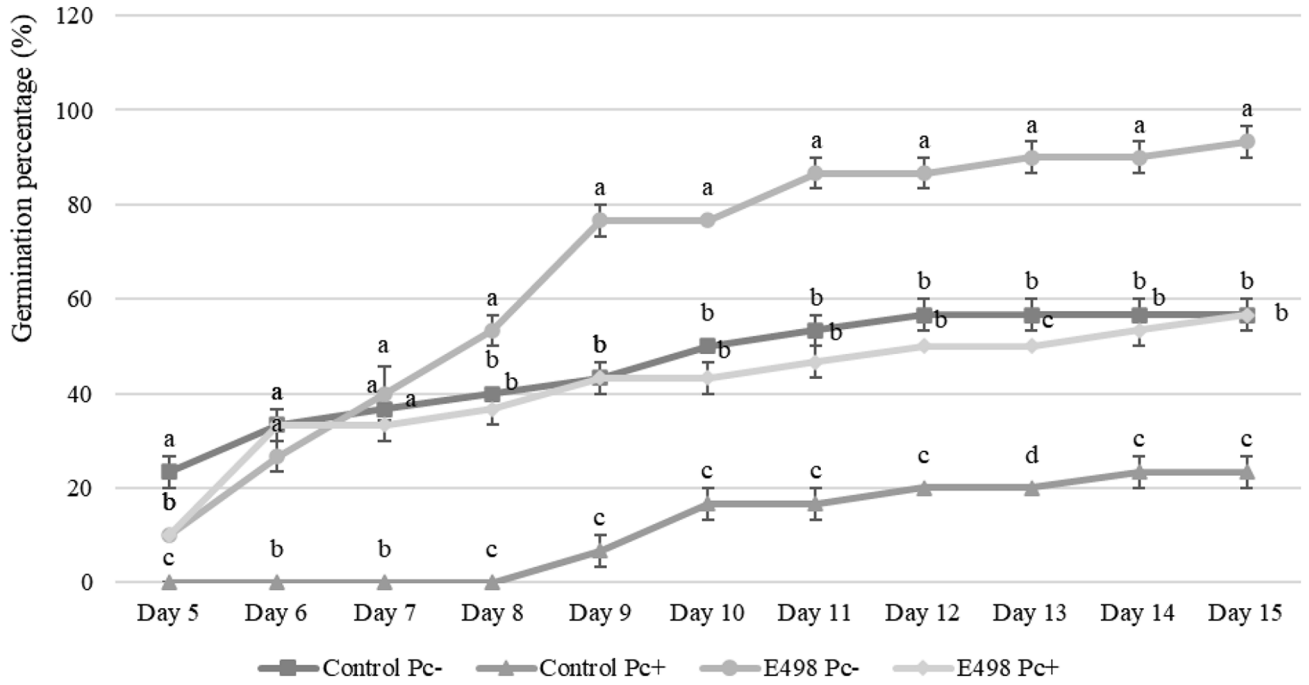

Fig. 2 Effect of the mycopriming with the extract of *Pseudopithomyces chartarum* on the germination rate of *Lupinus luteus* seeds under greenhouse conditions, inoculated or not with *Phytophthora cinnamomi* over 11 measurements (from day 5 to day 15). Results are expressed as the mean ($n=3$) ± standard error (error bars). For each day of observation, different letters indicate significant differences according to the least significant difference (LSD) test ($\alpha=0.05$). Control Pc-: seeds not mycoprimed with the extract and not inoculated with *P. cinnamomi*; Control Pc+: not mycoprimed seeds but inoculated with *P. cinnamomi*; E498 Pc-: mycoprimed seeds but not inoculated with *P. cinnamomi*; E498 Pc+: mycroprimed seeds and inoculated with *P. cinnamomi*

In terms of plant performance traits, inoculation with *P. cinnamomi* (Control Pc+) significantly decreased almost all growth parameters compared to the controls (Control Pc-), especially in shoot length and all dry matter weights (Table 3). However, these negative effects induced by the pathogen were reversed when the seeds were mycoprimed with the endophyte extract (Fig. 3). The values of all growth parameters recorded in plants inoculated with the pathogen were similar to or higher than those observed in the control seedlings (Control Pc-) when seeds were previously mycoprimed with the endophyte extract (treatment E498 Pc+). Similarly, consistent with the germination test, a growth promotion effect of mycopriming was observed in the E498 Pc- group, where *P. cinnamomi* was not inoculated; seedlings presented higher values than control plants in almost all growth traits, except for the shoot DM weight (Table 3).

3.3 Effect of the Post-Emergence Application of *Pseudopithomyces chartarum* Extract on the Control of *Phytophthora cinnamomi* in *Lupinus luteus* Plants Under Greenhouse Conditions

In the post-emergence test, *P. cinnamomi* can be considered as a clear pathogen in the *L. luteus* plants, as its inoculation (group Control Pc+) lead to a notable increase in disease severity (estimated through the AUDPC) and a consistent decrease in almost all the growth traits examined, except for shoot DM weight, compared to the controls (Control Pc-; Table 4). The application of the fungal extract to the seedlings (group E498 Pc+) reduced disease severity caused by the pathogen by threefold and effectively restored or enhanced the different biomass yield traits measured in *Lupinus luteus* plants. Similar to the mycopriming test, a clear growth promotion effect of the endophyte extract was also observed, as its application to not inoculated plants (E498 Pc-) improved growth values in almost all traits compared to the controls. Importantly, the extract application did not produce any toxicity in the seedlings, as evidenced by the absence of damage in the treated plants (AUDPC = 0 in this group of seedlings; Table 4).

3.4 Other Traits of *Pseudopithomyces chartarum* Related Directly or Indirectly with Plant Protection

The analysis revealed the presence of both IAA and GA3 phytohormones in the *P. chartarum* (E498) filtrate (Table 5). The concentration of IAA in the filtrate was more than doubled when the growth media was supplemented with tryptophan (IAA+). In addition, *P. chartarum* (E498) exhibited positive activity in phosphate solubilization, which can

Table 3 Effect of the seed mycopriming with the extract of *Pseudopithomyces chartarum* on different growth traits of *Lupinus luteus* seedlings inoculated or not with *Phytophthora cinnamomi* under greenhouse conditions. Results are expressed as mean ($n=5$) ± standard error. A summary of the ANOVA is also shown (*df*: degree of freedom; *F*: F-value; and the level of significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

Endophyte	Pathogen	Shoot length (cm)	Root length (cm)	Number of roots
E498	Pc-	9.20 ± 0.29 a	8.65 ± 0.61 a	15.75 ± 0.63 a
	Pc+	8.33 ± 0.17 ab	8.63 ± 0.50 a	14.75 ± 0.75 a
Control	Pc-	7.70 ± 0.52 b	6.38 ± 0.06 b	10.75 ± 0.85 b
	Pc+	6.30 ± 0.12 c	5.48 ± 0.89 b	9.00 ± 0.41 b
Source	*df*	*F*	*F*	*F*
Endophyte (E)	1	29.90***	24.70**	178.94***
Pathogen (P)	1	9.54*	0.73	6.15
E*P	1	15.06***	7.28**	22.24***
Endophyte	Pathogen	Shoot dry matter (mg)	Root dry matter (mg)	Total dry matter (mg)
E498	Pc-	83.50 ± 5.27 a	33.00 ± 2.97 a	116.50 ± 5.91 a
	Pc+	78.00 ± 4.81 a	29.00 ± 1.47 ab	107.00 ± 5.12 ab
Control	Pc-	72.00 ± 3.03 a	24.75 ± 2.02 b	96.75 ± 4.42 b
	Pc+	53.00 ± 0.41 b	16.00 ± 0.82 c	69.00 ± 3.67 c
Source	*df*	*F*	*F*	*F*
Endophyte (E)	1	80.34***	43.44**	94.44***
Pathogen (P)	1	9.85*	5.63	11.34*
E*P	1	11.70***	13.49***	17.90***

For each parameter, different letters indicate significant differences according to LSD (least significant difference) test at $\alpha = 0.05$. Pc-: seeds without *P. cinnamomi* inoculation; Pc+: seeds with *P. cinnamomi* inoculation

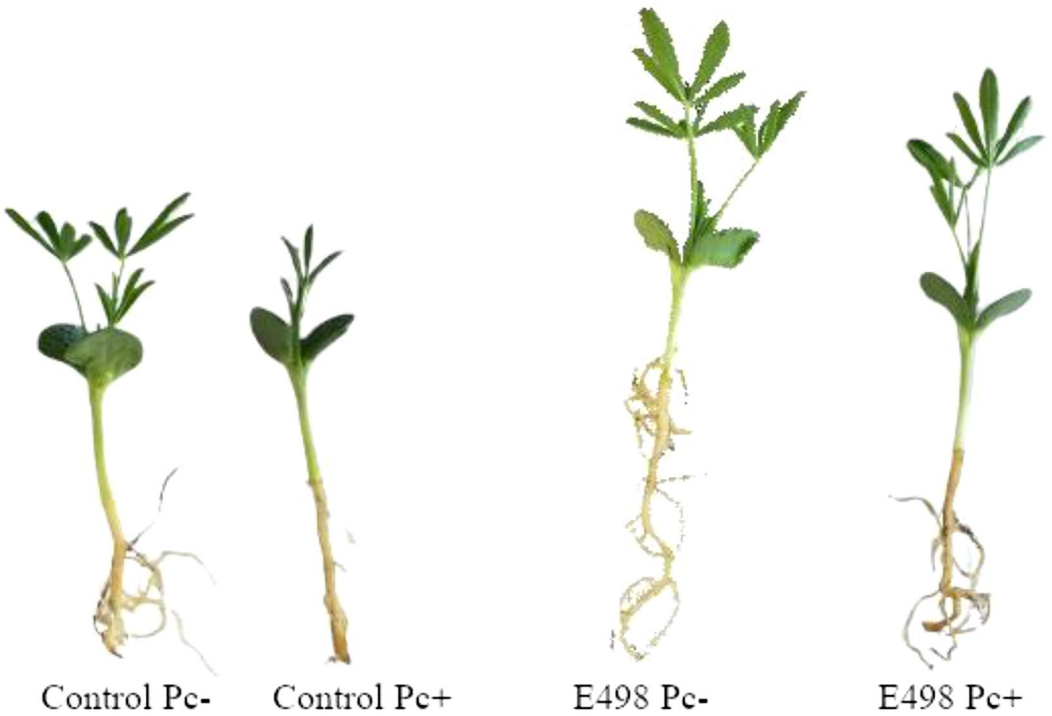

Fig. 3 Appearance of the mycoprimed and not mycoprimed *Lupinus luteus* plants inoculated or not with *Phytophthora cinnamomi* at the end of the experiment. Control Pc-: seedlings not mycoprimed with the extract and not inoculated with *P. cinnamomi*; Control Pc+: not mycoprimed seedlings but inoculated with *P. cinnamomi*; E498 Pc-: mycoprimed seedlings but not inoculated with *P. cinnamomi*; E498 Pc+: mycroprimed seedlings and inoculated with *P. cinnamomi*

Table 4 Effect of the post-emergence application of the extract of *Pseudopithomyces chartarum* on different yield traits of *Lupinus luteus* and on the disease severity, expressed in terms of the area under the disease progress curve (AUDPC), in plants inoculated or not with *Phytophthora cinnamomi* under greenhouse conditions. Results are expressed as mean ($n=5$) ± standard error. A summary of the ANOVA is also shown (*df*: degree of freedom; *F*: F-value; and the level of significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

Endophyte	Pathogen	Root length (cm)	Number of roots	AUDPC
E498	Pc-	25.62 ± 2.03 a	23.6 ± 0.57 a	0.00 ± 0.00 c
	Pc+	21.22 ± 1.23 ab	16.8 ± 0.42 b	57.60 ± 17.70 b
Control	Pc-	19.57 ± 2.66 b	14.2 ± 0.42 c	0.00 ± 0.00 c
	Pc+	14.73 ± 1.41 c	6.2 ± 1.02 d	168.00 ± 0.00 a
Factor	*df*	*F*	*F*	*F*
Endophyte (E)	1	13.17**	246.91***	48.64***
Pathogen (P)	1	11.91*	112.91***	203.13***
E*P	1	6.88**	149.87***	100.14***
Endophyte	Pathogen	Shoot dry matter weight (mg)	Root dry matter weight (mg)	Total dry matter weight (mg)
E498	Pc-	509.72 ± 54.65 b	317.16 ± 33.67 ab	826.90 ± 73.51 b
	Pc+	735.42 ± 24.80 a	354.26 ± 25.08 a	1089.70 ± 44.75 a
Control	Pc-	456.32 ± 51.84 b	271.94 ± 25.99 b	728.30 ± 69.80 b
	Pc+	415.38 ± 42.64 b	242.88 ± 17.56 c	658.30 ± 44.17 c
Factor	*df*	*F*	*F*	*F*
Endophyte (E)	1	17.42**	11.09*	23.44**
Pathogen (P)	1	4.43	0.02	2.08
E*P	1	12.58***	4.39***	12.56***

For each parameter, different letters indicate significant differences according to LSD (least significant difference) test at α = 0.05. Pc+: plants with *P. cinnamomi* inoculation

Table 5 Summary of other traits of *Pseudopithomyces chartarum* (E498) related with plant protection

Phytohormone production		Nutrient mobilization (solid media)	
IAA (μg mL^{-1})	5.34 ± 0.05	Phosphate solubilization	+
IAA+ (μg mL^{-1})	12.85 ± 0.07	Siderophore production	-
GA_3 (μg mL^{-1})	463.01 ± 1.38	Ammonia production	-

IAA, indole-acetic acid; IAA+, indole-acetic acid supplemented with L-tryptophan; GA_3, gibberellic Acid. For the nutrient mobilization: -, no activity; +, positive activity

enhance nutrient availability. However, it did not show either siderophore or ammonia production under the conditions tested (Table 5).

3.5 Tentative Identification of Metabolites by Mass Spectrometry

Table 6 indicates the peak assignments for methanolic extracts of *P. chartarum*. Five compounds were tentatively identified as a first approximation of the metabolites responsible for the results observed in the previous assays.

4 Discussion

The use of endophytic fungi as biocontrol agents has recently gained considerable interest due to their potential to promote plant health and to limit the harmful effects of

Table 6 Peak assignment for methanolic extracts of *Pseudopithomyces chartarum*

#	Proposed formula	Rt (min)	Obs. m/z	Proposed compound	Activity	Reference
Cpd08	$C_{14}H_{16}N_2O_2$	12.95	244.122	Ciclo(L-Phe-L-Pro)	Influence on quorum sensing activity	(Capon et al. 2007; Khan et al. 2021)
Cpd09	$C_{10}H_{13}NO$	14.74	163.100	Dihydroabikoviromycin	Melanin inhibition (reduction of pathogenicity)	(Maruyama et al. 2003)
Cpd11	$C_{14}H_{31}N$	19.26	213.246	Medelamine A	Influence on Heat Shock proteins	(Morino et al. 1995)
Cpd16	$C_{10}H_8O_3$	23.46	176.048	Herniarin	Antioxidant and antimicrobial	(Gebru and Sbhatu 2020; Okezie et al. 2020)
Cpd17	$C_{23}H_{41}N$	24.27	331.324	Piptamine	Antimicrobial	(Schlegel et al. 2000)

Rt: retention time; Obs. m/z: observed mass/charge relationship

pathogens (Kashyap et al. 2023). In this study, we explored the biocontrol potential of an endophytic fungus isolated from the *dehesas* ecosystem against the highly virulent soil-borne pathogen *Phytophthora cinnamomi* to mitigate its negative effects on *Lupinus luteus* plants.

The endophytic strain (E498) used in this study was identified as *Pseudopithomyces chartarum*, a fungus associated with various health and environmental concerns. This fungal species has been recognized as a plant pathogen, causing leaf spot on *Tetrapanax papyrifer* and wheat in different regions (Wu et al. 2023), probably associated to the production of secondary metabolites such as alternariol, alternariol mono-methyl ether, altertoxin I or altertoxine II (Perelló et al. 2017). Additionally, this fungus is also known to produce sporidesmin, a toxin that can cause facial eczema in ruminants (Sidhu et al. 2021). Nevertheless, other authors (Fitzgerald et al. 1998) have also reported the existence of atoxigenic strains of *P. chartarum*. In our case, the strain used was isolated as an endophyte from healthy plants, specifically from the stem of a grass species such as *Hordeum murinum*, suggesting a potential atoxigenic activity in plants. Furthermore, it is important to highlight that in the present study, only filtrates or extracts, and not the living organism, were used. Therefore, although the original strain had the potential to be pathogenic, it does not necessarily mean that the metabolites produced in vitro were also phytotoxic. This presumed atoxigenicity was supported by the fact that the application of the extracts did not cause any symptoms in the plants and no negative effects on their growth were observed. Conversely, a certain promotion of plant growth was observed after the application of the extract. Therefore, the use of metabolites produced by the endophyte for plant protection instead of the living organism, may overcome the aforementioned problems of inconsistent results and potential phytopathogenicity of the fungus. Even if the eventual phytotoxic compound was present in the filtrate/extract, the beneficial metabolite with the biocontrol activity could be isolated, purified and concentrated in order to get a harmless product. Nevertheless, further studies should be performed to confirm the safety of the product, not only on other plant species, but also on other beneficial microorganisms, invertebrates, and vertebrates, before its widespread use.

The in vitro tests revealed a significant inhibitory effect of both *P. chartarum* filtrates and extracts on the growth of *P. cinnamomi*, with complete inhibition of mycelial growth of the pathogen by the filtrate after 72 h. In the case of the serial dilution assay, evaluating the spore germination of the pathogen and its further development, the results were positive when using the extract. The biocontrol activity of *P. chartarum* observed in this study could be primarily attributed to specific metabolites contained in the filtrates/extracts with antimicrobial properties. Mass spectrometry analysis tentatively identified five compounds, including ciclo(L-Phe-L-Pro), dihydroabikoviromycin, medelamine A, herniarin, and Piptamine. Although these results require further confirmation through additional research, these findings may explain the biocontrol potential of our *P. chartarum* isolate, as most of these secondary metabolites have been documented in the scientific literature for their antimicrobial activity. For example, dihydroabikoviromycin reduces the production of melanin in the pathogen (Maruyama et al. 2003), a pigment associated with fungal virulence (Nosanchuk 2015), potentially reducing its pathogenicity. The metabolite herniarin has been shown to have antimicrobial activity (Gebru and Sbhatu 2020; Okezie et al. 2020) through its disruptive effect on the cell wall components of the pathogen (Roca-Couso et al. 2021). Piptamine and other tentatively identified compounds have been described for their antimicrobial properties (Schlegel et al. 2000). However, these aspects are only a preliminary consideration, and further research should be performed in order to deeply understand the potential of the bioactive compounds present in the fungal extracts and their precise role in the plant protection mechanisms observed for *P. chartarum*.

In addition to its antimicrobial activity, the filtrates and extracts of *P. chartarum* showed plant growth promoting activity in *L. luteus* seedlings. This activity might be very positive as it could improve the plant productivity (forage or grain in the case of yellow lupine). Furthermore, this growth promotion reinforce the role of the endophyte as biocontrol agent (BCA), as it contributes to the overall fitness, health and vigor of the host plant, thereby favoring its defensive responses to biotic and abiotic stresses (El-Saadony et al. 2022). *P. chartarum* showed the ability to produce in vitro both indoleacetic acid (IAA) and gibberellins (GA_3), molecules known for their role in stimulating plant growth (Egamberdieva et al. 2017), and activating plant defense mechanisms (Berens et al. 2017). The production of these substances could explain the enhanced growth observed in *L. luteus* seedlings after the application of the extract, consistent with findings from other studies (Fonseca et al. 2017). In particular, the addition of L-tryptophan to the culture medium doubled the content of IAA in the fungal filtrate (IAA+). This highlights the potential of the endophyte to produce this auxin through different metabolic pathways, which may be tryptophan-dependent and independent (Jahn et al. 2021), although the first one seems to be more important.

In the present study two potential procedures for the endophyte extract application were investigated: mycopriming an post-emergence application. Mycopriming, considered preventive, effectively mitigated the negative effect of the pathogen on germination and also improved germination rates of *L. luteus* in the absence of the pathogen. In addition,

mycopriming provided benefits to seedlings, protecting them from pathogen-induced damage and improving plant growth parameters either in the presence or the absence of the pathogen. Mycopriming has already been used successfully in maize using *Beauveria bassiana* and *Trichoderma asperellum* to improve grain yield and to induce defensive responses against the herbivory of *Ostrinia furnacalis* (Batool et al. 2022). In that study, the beneficial effects were attributed to the fact that the mycopriming may have mediated to boost the expression of the responsive gens related with the production of antioxidants and jasmonic acid, which are known to play a role in plant defense responses against fungal diseases (Macioszek et al. 2023). Therefore, a similar mechanism could have acted in our case, although further studies should be performed in order to elucidate the specific mechanisms responsible for the observed plant protection.

Post-emergence application of the endophyte extract to lupine seedlings was proved to be highly effective, significantly reducing the disease severity (estimated through the AUDPC) caused by *P. cinnamomi* by two-thirds, likely due to any of the metabolites with antimicrobial properties tentatively identified by mass spectrometry. The negative effects of the pathogen on root development, as evidenced by reduced root number, length, and subsequent DM weight, were reverted by the application of the extract that not only mitigated pathogen-induced damage, but also enhanced root development above control levels. This could be due to the presence of phytohormones in the extract, auxins and gibberellins, as it is very well-known their involvement in the root development (Tanimoto 2005). In addition, the observed phosphate solubilizing activity of the endophyte may have contributed to the enhanced growth observed in seedlings treated with the extract. All these factors may have favored the fitness and the vigor of the plants to jointly contribute to the plant protection against diseases. Taken together, these results suggest that seed mycopriming could serve as a preventive measure against initial germination problems or seedling damage, while post-emergence application could enhance this protection in the event of disease attack, thereby promoting overall plant health and disease resistance.

5 Conclusions

The filtrates/extracts of the endophyte *Pseudopithomyces chartarum* (E498) produced inhibition of the pathogen growth in vitro, positively influenced seed germination, significatively reduced the disease severity *in planta* and improved several growth parameters in the treated seedlings. Both seed mycopriming and post-emergence application of extracts were equally effective in plant protection, and both strategies could be used jointly to strengthen their beneficial effects. The observation of auxins and gibberellins production, phosphate solubilization activity and the tentative identification of bioactive compounds with biocontrol activity may explain the positive effect of the extract application. Further research should focus on confirming the identity of the bioactive compounds and evaluating the efficacy of the extracts under field conditions. This study contributes to the exploration of sustainable and environmentally friendly alternatives for a disease management in agricultural systems.

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Declarations

Competing Interests The authors declare that they have no conflict of interest.

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