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Exploiting antifungal metabolites of the fungus *Purpureocillium lilacinum* for effective control of *Botrytis cinerea* in chickpea plants

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Abstract Endophytic fungi have garnered interest as biocontrol agents and plant growth promoters. In this study, we investigated the biocontrol potential of *Purpureocillium lilacinum* against *Botrytis cinerea* in chickpea seeds and plants. Molecular analysis confirmed the strain identity and its potential as a biocontrol agent was supported by inhibitory effects on *B. cinerea* growth and sporulation. In this sense, high-performance liquid chromatography tentatively identified three bioactive compounds in the *P. lilacinum* extract, suggesting mechanisms of antifungal activity. Notably, the extract mitigated the negative impact of *B. cinerea* on germination rates and seedling growth and demonstrated plant growth-promoting effects by enhancing germination rates and seedling growth of chickpea either in the absence or in the presence of the pathogen. Furthermore, the extract improved root length and number of roots in mature plants, indicating potential benefits for nutrient uptake and stress tolerance. Additionally, the extract exhibited antioxidant activity and enhanced nutrient mobilization, further supporting its role in plant health and productivity. Overall, this study highlights the multifaceted potential of the extract of *P. lilacinum* as a sustainable biocontrol agent and plant growth promoter.

Keywords Biocontrol · *Botrytis* gray mold · *Cicer arietinum* · Fungal endophyte · *Purpureocillium lilacinum*

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Introduction

Chickpea (*Cicer arietinum* L.) is a vital legume crop grown in over 57 countries, covering more than 15 million ha and yielding 15.9 million tons of grain in 2021 (FAO 2023). While its global production share might be modest, chickpeas are crucial in Mediterranean countries, particularly Spain, which is Europe's leading producer (Merga and Haji 2019). Chickpeas thrive in the Mediterranean's rainfed lands, showing remarkable adaptability to arid climates and low-fertility soils that make them a sustainable, low-input crop that enhances agricultural biodiversity and soil health (Poveda 2021). Nutritionally, chickpeas are a rich source of energy, proteins (18–29%), lipids (4–7%), and starch (50–60%) (Boukid 2021), that provide a neutral, well-appreciated taste.

Despite their adaptability, chickpea productivity faces challenges from water scarcity linked to climate change (Laranjeira et al. 2022), genetic erosion (Summo et al. 2019), and especially from pests and diseases, particularly those caused by *Ascochyta rabiei*, *Botrytis cinerea,* and *Fusarium oxysporum* (Manjunatha et al. 2022). Botrytis gray mold, caused by *B. cinerea*, is particularly problematic, causing up to 50% yield losses, especially in humid regions (Anuradha et al. 2011). In southwestern Spain, this disease is prevalent in winter sowings, particularly affecting cultivars like Pedrosillano in Extremadura (del Moral et al. 1992).

The lack of *Botrytis*-resistant chickpea varieties (Vijayabharathi et al. 2018) leads to heavy fungicide use, which is not always cost-effective and can incur annual costs of around €1 billion (Dean et al. 2012). Fungicides also face issues like pathogen resistance and pose health and environmental risks (Hahn 2014). Biological control offers a sustainable alternative, with fungal endophytes gaining interest. These symbiotic fungi, which live in plant tissues without causing disease, may produce bioactive metabolites that can protect the host from biotic and abiotic stresses (Nisa and Kamili 2019). Among them, *Purpureocillium lilacinum* has shown biocontrol potential by producing compounds like volatile organic compounds, extracellular enzymes, toxins, and secondary metabolites with antagonistic activity against various pests and pathogens (Wang et al. 2016; Chen and Hu 2022), including *B. cinerea* (Liu et al. 2020). Additionally, *P. lilacinum* may promote host plant growth (Baron et al. 2020), making it a promising biocontrol agent for crops like cotton, tomatoes, eggplants, maize, beans, and soybeans (Dahlin et al. 2019; Baron et al. 2020).

The efficacy of *P. lilacinum* can be influenced by environmental factors, host plant species, and specific pathogens. Direct application of bioactive compounds from endophyte filtrates, instead of the living organisms, may mitigate these issues. Filtrates, solutions containing bioactive compounds produced in vitro, can reproduce the fungus's effects in vitro and in planta (García-Latorre et al. 2022). These compounds can be extracted using different solvents, obtaining various bioactive substances. Endophyte filtrates or their extracts offer several advantages: they are generally non-toxic, environmentally safe, easy to produce, cost-effective, and reduce reliance on chemical pesticides (Farhat et al. 2019). Given that *P. lilacinum* filtrates have already shown to control pathogens like *Penicillium digitatum* in orange fruit (Elsherbiny et al. 2021) and that this fungus has shown biocontrol activity against *B. cinerea* (Liu et al. 2020), our study hypothesizes that *P. lilacinum* filtrates or extracts can control *B. cinerea* and protect chickpea plants. The main goal was to assess the antifungal activity of *P. lilacinum* filtrates/extracts against *B. cinerea* in vitro and their protective effects on chickpea seeds and seedlings. We also evaluated other traits of the endophyte, such as phytohormone production, antioxidant activity, and nutrient solubilization, to understand the mechanisms involved in plant protection.

Materials and methods

Fungal and plant material

The endophytic fungus used was isolated from healthy leaves of *Trifolium subterraneum* growing in dehesas from Extremadura, southwestern Spain, as previously described by Lledó et al. (2016). This isolate (lab code E496) was selected for its frequent isolation from the plant host and its promising bioactivity in preliminary studies (unpublished data). It was identified as *Purpureocillium lilacinum* through sequencing of the internal transcribed spacer (ITS) region, the large subunit (LSU) of the nuclear ribosomal RNA (rRNA) gene complex, and a partial fragment of the beta-tubulin (*tub2*) gene, comparing the

results with sequences in the GenBank database using BLAST (https://www.ncbi.nlm.nih.gov/). Molecular procedures included DNA extraction from colonies grown 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) using the Fungal gDNA Miniprep Kit EZ-10 Spin Column protocol (NBS Biologicals, Cambridgeshire, UK). Loci amplification followed the methods of Vilgalys and Hester (1990) and White et al. (1990). PCR products were purified and sequenced using Sanger Cycle Sequencing at Microsynth Seqlab GmbH (Göttingen, Germany), and consensus sequences were obtained with Geneious® 7.1.9 (http://www.geneious.com) (Kearse et al. 2012).

The pathogen used was *B. cinerea* (CECT 20518), provided by the University of Extremadura's Food Quality and Microbiology research group. This fungus was grown on potato dextrose agar (PDA) at 23 °C in the dark. After seven days, the mycelia plates were exposed to direct sunlight for 14–21 days to induce sporulation. Spores were recovered by flooding the plates with sterile distilled water, and their concentration was calculated using a Neubauer chamber. Commercial seeds of *Cicer arietinum* (cv. Pedrosillano) were used for greenhouse experiments.

Filtrate and extract obtention

Two 5 mm agar discs from a 7-day-old colony of the endophyte were placed in three 500 ml Erlenmeyer flasks containing 250 ml of yeast malt broth (YMB) with yeast extract (6 g l^{-1}), malt extract (10 g l^{-1}), and D-glucose (6 g l^{-1}), adjusted to pH 6.3. The flasks were incubated in a thermoshaker (Orbital Shaker Incubator COMECTA 1102) at 23 °C and 140 rpm. Two days after glucose depletion, the culture was filtered using sterile paper discs (0.2 µm) to separate the mycelium from the liquid filtrate containing secondary metabolites. Part of this filtrate was extracted following Halecker et al. (2014): the filtrate was mixed with an equal amount of ethyl acetate and shaken for 2 min. The mixture was poured into a separatory funnel, phases were separated, and sodium sulfate was added to the organic phase to remove water residues. This was filtered (0.16 mm) and evaporated using a rotary evaporator (Hei-Vap ML/G1) to remove ethyl acetate. The residue was resuspended in methanol for in vitro tests and in water for *in planta* assays.

Effect of fungal filtrate on pathogen mycelial growth in vitro

The inhibitory effect of the filtrates on *B. cinerea* mycelial growth was evaluated in vitro. Petri dishes were prepared by adding 2 ml of fungal filtrate to 18 ml of sterilized PDA medium, mixing uniformly just before solidification when the medium was at a temperature of around 40 °C. Control dishes had 2 ml of sterilized YM medium. A 5 mm mycelium plug of the pathogen was placed in the center of each dish (Santamaría et al. 2004). Samples were incubated at 23 °C for 72 h, and colony length was measured every 12 h in quadruplicate.

Minimum inhibitory concentration in vitro of filtrates and extracts

The Minimum Inhibitory Concentration (MIC) of the filtrate against *B. cinerea* was determined using a serial dilution assay in 96-well plates, following Halecker et al. (2014). Each well received 280 µl of a spore solution (6.7×10^5 spores ml^{-1}) and 20 µl of undiluted filtrate in the first row, followed by 50% serial dilutions. Controls included 1.5 mg ml^{-1} cycloheximide (positive) and 20 µl of sterilized YM medium (negative). Plates were incubated at 23 °C and 600 rpm for 48 h. The same procedure was used for extracts, with 20 µl (300 µg ml^{-1}) of the sample in the first row, and methanol as the negative control. Tests were performed in triplicate.

In planta experiments

Two greenhouse experiments evaluated the efficacy of the extracts to protect chickpea plants against *B. cinerea*. One experiment applied the fungal extract to seeds (mycopriming assay) and the other to seedlings (post-emergence assay). Mycopriming is a type of seed priming where the seed is induced to germinate in a solution containing the fungus extract in order to increase its defensive system to improve plant tolerance against biotic or abiotic stresses. Prior to both experiments, chickpea seeds were surface disinfected with 2% sodium hypochlorite and washed with sterile distilled water.

In the mycopriming assay, disinfected seeds were immersed in fungal extract (3 mg ml^{-1} in sterilized distilled water) for 6 h. Controls used sterile distilled

water. 60 seeds per treatment were sown in plastic pots (7×7×6 cm) containing a substrate and perlite mixture (pH 7.0, EC 1.5 dS m^{-1}, organic matter 60%, N 1.29%, P_2O_5 0.58%, K_2O 1.25%). Half the pots were inoculated with 100 ml l^{-1} soil solution containing the pathogen (2×10^4 spores ml^{-1}), and the other half received pathogen-free substrate. Pots were placed in a greenhouse and irrigated every 2–3 days to field capacity. After 15 days, with a daily monitoring of germination, five plants per treatment were harvested, and shoot and root elongation, number of roots, and pathogen presence were recorded. Every set of 60 seeds was considered a repetition. This assay, repeated thrice, was conducted from February 18, 2020, to March 4, 2020.

In the post-emergence test, another 60 disinfected seeds per treatment were sown in pots with the same substrate mixture and placed under the same conditions. After one month, half the pots received 5 ml of pathogen solution (2×10^4 spores ml^{-1}) per plant. Twelve hours later, each plant received 1 ml of the extract (3 mg ml^{-1}) by spraying. Controls used sterilized distilled water. Disease severity was measured weekly for one month, based on visual symptoms (yellowing, drying, rotten leaves, black spots). Severity levels ranged from 0 (healthy) to 5 (death) based on the percentage of the affected part. The area under the disease progress curve (AUDPC) was calculated per pot by summing the areas of corresponding trapeziums considering as a unit each period between two consecutive measurements. After the final measurement, five plants per treatment were analyzed in the laboratory. Root and shoot lengths, number of roots, and dry weight (after oven drying at 60 °C) were recorded. This assay was conducted in triplicate from February 18, 2020, to April 30, 2020.

Evaluation of traits related to protection in *P. lilacinum*

The potential of *P. lilacinum* to produce substances related directly or indirectly to plant protection was evaluated through three traits: (1) production of phytohormone-like substances, (2) antioxidant activity and synthesis of phenolic compounds, and (3) nutrient mobilization, including phosphate solubilization, siderophore production, and ammonia production.

Estimation of phytohormone-like substances production

It was assessed by quantifying auxin-like (IAA) and gibberellin-like substances in the fungal filtrate. IAA was quantified using a colorimetric test (Devi et al. 2016). Fungal filtrate (1 ml) was mixed with Salkowski reagent (2 ml), shaken, incubated in the dark for 30 min, and absorbance was measured at 530 nm. IAA concentration was determined using a standard curve of pure indole-3-acetic acid (Sigma Aldrich). Additionally, the endophyte was cultivated in YMB medium supplemented with L-tryptophan (5 mM). Gibberellin-like substances were estimated as gibberellic acid equivalents (GAE) using the colorimetric method of Holbrook et al. (1961). Fifteen ml of fungal filtrate were mixed with zinc acetate (21.9%) and potassium ferrocyanide (10.6%), centrifuged, and the supernatant was treated with HCl (30%) and incubated. Absorbance was measured at 254 nm, and results were compared to a gibberellic acid (GA3) calibration curve. Samples were analyzed in triplicate, and results were expressed in mg ml^{-1} of fungal filtrate.

Determination of the antioxidant activity

It was determined using a DPPH radical scavenging assay. Fungal extracts at different concentrations (3 mg ml^{-1}) were mixed with methanolic DPPH solution (0.1 mM), incubated in the dark for 30 min, and absorbance was measured at 517 nm. Controls used methanol instead of extract. The radical scavenging activity was estimated through the absorbance, computed as a percent reduction compared to the control. All samples were analyzed in triplicate.

Determination of total polyphenol content (TPC)

It was determined using the Folin–Ciocalteau method (Yadav et al. 2014). A mixture of 1 ml of fungal extract (1 mg ml^{-1}), 500 µl of 50% aqueous Folin–Ciocalteau reagent, 1.5 ml of 20% aqueous Na_2CO_3, and 2 ml of distilled water was prepared. After incubation for 30 min at room temperature in the dark, absorbance was measured at 765 nm. Gallic acid was used to generate a standard curve (0.01–0.05 mg ml^{-1}). TPC results were expressed as

mg of gallic acid equivalents (GAE) per ml of extract. All samples were analyzed in triplicate.

Phosphate solubilization

It was evaluated by growing the endophyte on National Botanical Research Institute's Phosphate (NBRIP) growth medium (Nautiyal 1999). Phosphate solubilization was detected by clear halos around colonies after seven days of incubation at 27 °C. The solubilization index (SI) was calculated as the ratio of the halo diameter to the colony diameter. Three replicates were performed.

Siderophore production

It was detected using the chrome azurol S (CAS) assay described by Pérez-Miranda et al. (2007). The CAS agar plates were inoculated with the endophyte and incubated at 27 °C for seven days. A color change from blue to orange around colonies indicated siderophore production. The diameter of the color change zone was measured to quantify siderophore production. The protocol was repeated with a non-deferrated medium to confirm that siderophore production was not induced under normal conditions. A blank using a plug of uninoculated PDA medium was also included, and three replicates were performed.

Ammonia production

It was assessed by inoculating the endophyte in peptone water and incubating at 28 °C for 72 h. Nessler's reagent (0.5 ml) was added to the culture, and a yellow to brown coloration indicated ammonia production. A faint yellow color indicated minimum ammonia production, while a deep yellow to brown color indicated maximum ammonia production (Singh et al. 2014). Each assessment was performed in triplicate.

Chemical analysis of bioactive compounds by HPLC

High-performance liquid chromatography (HPLC) was used to identify and quantify bioactive metabolites in the fungal extract. The analysis was performed using an Agilent 6520 Accurate Mass Q-TOF LC/MS and a timsTOF Pro 2 system. The fungal extract was injected into a C18 column, and a gradient elution with a mixture of acetonitrile and water (both containing 0.1% formic acid) was used. The specific leucinostatins were targeted based on their retention times and mass spectra compared with standard compounds.

Statistical analysis

The effect of the fungal filtrate and extract on the mycelial growth of *B. cinerea* was analyzed by means of a split-plot ANOVA, a mixed-design model that compares the means of two factors, one being a repeated-measures factor, or within-subjects factor (a random effects factor: 'day of measurement'), and the other one being a between-subjects factor (a fixed effects factor: 'treatment'). Their interaction was also included in the model. For the rest of experiments, the protective effect of the endophyte towards the pathogen on the different parameters was evaluated using a two-way ANOVA, including the pathogen inoculation, the filtrate/extract treatment, and their interaction in the model. Significant differences between treatments were analyzed using Fisher's LSD test at $P \leq 0.05$. The Shapiro–Wilk and Levene's tests were used to verify the assumptions of normal distributions and homoscedasticity, respectively. Statistical analyses were performed using Statistix v. 8.10 package (Analytical Software, USA).

Results

Identification

The endophyte E496 was identified as *Purpureocillium lilacinum* based on a BLAST search of NCBI's GenBank nucleotide database using ITS, LSU, and *tub2* sequences (GenBank accession numbers: OR756259, OR743742, and OK161079, respectively). It showed 100.0% and 99.8% nucleotide similarity with *P. lilacinum* CBS 226.73B in ITS and LSU, respectively, and 99.7% similarity with *P. lilacinum* CBS 248.33 in *tub2*.

Effect of filtrate and extract of *P. lilacinum* on pathogen growth in vitro

The endophyte completely inhibited the pathogen in vitro at least during the experimental time, as no pathogen growth was observed after 72 h when the filtrate was added to the culture media, in comparison with controls (14.19 ± 0.34 mm colony length; ± SE; n = 4). In the MIC assays, while the filtrate did not inhibit *B. cinerea* sporulation in serial dilutions, the extract did at a minimum concentration of 150 µg ml^{-1} (*vs*. 2.34 µg ml^{-1} for the cycloheximide control).

Effect of mycopriming with *P. lilacinum* extract on *B. cinerea* control in chickpeas

The germination rate of chickpeas was significantly affected by the endophytic extract and the presence or absence of *B. cinerea* from the fourth to the fifteenth day after sowing (Table 1). Without *B. cinerea*, seeds treated with the extract of *P. lilacinum* had consistently higher germination rates compared to the water-treated control group, particularly from day 12 onwards. While control seeds showed about 82% germination, extract-treated seeds reached almost 100% in the last days of measurement. When *B. cinerea* was inoculated, the germination rate of non-treated seeds dropped significantly (up to 24%), but extract-treated seeds' rates matched those of the control seeds (Fig. 1). Regarding plant growth parameters, pathogen inoculation significantly decreased the number of formed roots in seedlings (Table 2). However, pre-treatment with the endophytic extract improved the root formation in pathogen-affected seedlings. Without pathogen inoculation, the extract significantly enhanced root length and the number of roots (Table 2).

Effect of post-emergence application of *P. lilacinum* extract on *B. cinerea* control in chickpeas under greenhouse conditions

Inoculation with *B. cinerea* in untreated plants led to severe disease symptoms (estimated through AUDPC) and significant decreases in growth parameters (shoot and root length, shoot, root, and total dry weight) (Table 3). However, treatment with the endophytic extract reduced disease severity by 43% and restored or enhanced growth parameters compared to non-infected plants. In non-inoculated plants, the extract enhanced root length, suggesting a plant growth promotion effect (Table 3).

Biocontrol traits of *P. lilacinum*

The biocontrol potential of *P. lilacinum* involves producing phytohormone-like substances, antioxidants, and mobilizing essential nutrients. *Purpureocillium lilacinum* produced 4.43 ± 0.09 µg ml^{-1} (n = 3) indole-acetic acid (IAA) and 451.85 ± 1.50 µg ml^{-1} gibberellins (GA3). Even when amending the culture medium with tryptophan (IAA +) IAA production was increased seven-fold (34.45 ± 0.57 µg ml^{-1}), indicating effective synthesis of this plant growth hormone. On the other hand, the fungus produced 74.94 ± 3.87 mg GAE per g extract in total polyphenol content and exhibited 25.29 ± 2.09% DPPH

Table 1 Summary of the ANOVAs for the effect of the extract of *Purpureocillium lilacinum,* the presence of *Botrytis cinerea,* and its interaction on the germination rate of the seeds of *Cicer arietinum* under greenhouse conditions

	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Endophyte (E)	45.06***	61.59***	32.86***	80.02***	48.62***	191.48***
Pathogen (P)	129.52***	125.69***	152.97***	300.93***	85.09***	210.35***
E×P	6.90*	5.02	7.37*	7.50*	1.27	4.69
	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Endophyte (E)	97.12***	81.06***	689.52***	77.10***	187.38***	193.85***
Pathogen (P)	92.49***	93.67***	689.52***	73.24***	178.22***	184.26***
EP	1.71	2.48	122.54***	9.00*	8.26*	7.76*

The F-values, and the levels of significances (*P ≤ 0.05; ***P ≤ 0.001) are shown for each factor. All F-values are on 1 and 8 df

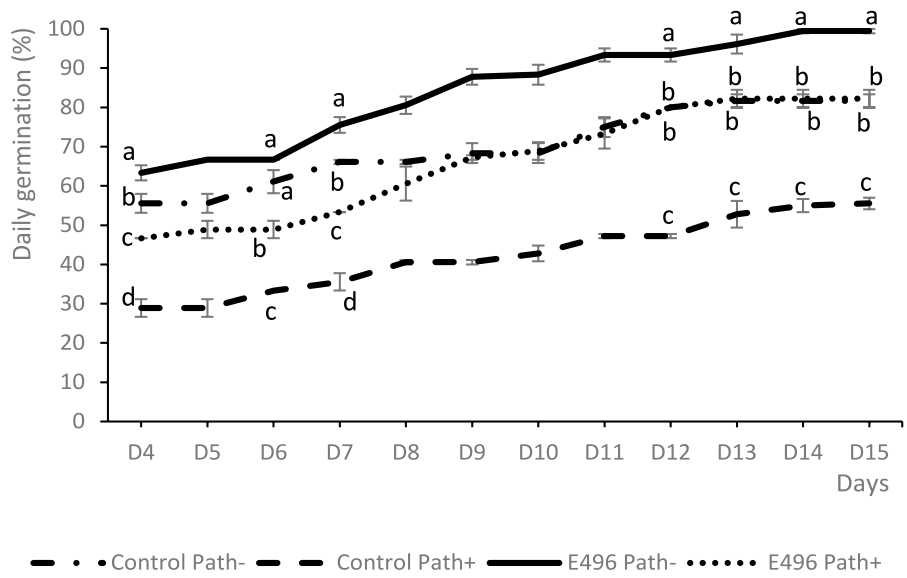

Fig. 1 Effect of endophytic extracts (E496) on the germination percentage of *Cicer arietinum* seeds inoculated (Path+) and not inoculated (Path−) with *Botrytis cinerea* from four to 15 days (D4 to D15) after sowing (12 measurements). Values are expressed as mean ± SE (n = 3). In each day of measurement, different letters (if any) show significant differences between treatments according to the LSD (least significant difference) test of the endophyte × pathogen interaction at α = 0.05

Table 2 Chickpeas growth parameters (shoot length, root length and number of roots) as affected by the *Purpureocillium lilacinum* extract treatment (with extract of E496 and without extract, control) of the seeds, the inoculation with *Botrytis cinerea* (Path+: inoculation with the pathogen; Path−: without the pathogen), and their interaction under greenhouse conditions

Endophyte	Pathogen	Shoot length (cm)	Root length (cm)	Number of roots
E496	Path−	35.88 ± 0.72	15.68 ± 0.90 a	15.00 ± 0.58 a
	Path+	35.43 ± 1.25	8.65 ± 1.14 b	13.50 ± 0.29 b
Control	Path−	38.00 ± 1.08	8.14 ± 0.57 b	13.00 ± 0.58 b
	Path+	35.88 ± 0.72	7.63 ± 0.52 b	11.00 ± 0.58 c
Endophyte (E)		2.71	46.11***	0.24
Pathogen (P)		3.17	35.28***	19.64***
EP		1.30	26.22***	29.33***

Values are expressed as mean ± SE (n = 5). A summary of ANOVAs is also given at the bottom of the table. The F-values, and the levels of significances (*** P ≤ 0.001) are shown for each factor and parameter. All F-values are on 1 and 16 df

For each parameter, different letters (if any) indicate significant differences according to LSD (least significant difference) test of the endophyte × pathogen interaction at α = 0.05

scavenging activity at 3 mg ml^{-1}. The endophyte solubilized phosphate and synthesized ammonia but did not produce siderophores.

Tentative identification of metabolites by HPLC

HPLC analysis identified potential metabolites responsible for plant protection effects. Compounds tentatively identified include brevianamide F, sphingofungin B, and paecilaminol, known for their antibiotic activity (Table 4). Mass spectrometry data supporting these identifications are shown in Supplementary Figures S1–S3. A specific search for leucinostatins showed peaks between 11.09 and 11.21 min, with m/z [M + H] + of 1218.84 and 1204.83 Da, assigned to leucinostatins A, B, and H. A peak at 11.29–11.52 min with m/z [M + H] + of 1148.80 Da was assigned to leucinostatin C (Supplementary Figures S4–S5). Nevertheless, further research is needed to confirm these metabolites and fully characterize the compounds produced by this isolate.

Table 3 Effect of the post-emergence application of the extract of *Purpureocillium lilacinum* (with the extract: E496; and without: control), the inoculation with the pathogen *Botrytis cinerea* (with the pathogen: Path+; and without: Path−), and their interaction on disease severity (estimated though the area under the disease progress curve; AUDPC) and different growth traits (shoot and root length, shoot, root and total dry weight) in chickpea plants under greenhouse conditions

Endophyte	Pathogen	Shoot length (cm)	Root length (cm)	AUDPC
E496	Path−	34.75 ± 0.60	21.51 ± 0.21 a	0.00 ± 0.00 c
	Path+	23.84 ± 0.53	20.35 ± 0.34 b	46.90 ± 2.37 b
Control	Path−	26.12 ± 0.31	19.12 ± 0.40 c	0.00 ± 0.00 c
	Path+	13.70 ± 0.67	13.00 ± 0.34 d	82.00 ± 6.77 a
Endophyte (E)		297.50***	219.98***	23.94***
Pathogen (P)		459.61***	122.89***	322.88***
EP		1.93	57.05***	23.94***
Endophyte	Pathogen	Shoot dry matter weight (g)	Root dry matter weight (g)	Total dry matter weight (g)
E496	Path−	393.24 ± 29.11 b	249.00 ± 2.59	642.24 ± 27.70 a
	Path+	477.50 ± 2.56 a	203.90 ± 2.95	681.40 ± 3.77 a
Control	Path−	406.64 ± 14.26 b	245.50 ± 2.92	652.14 ± 12.26 a
	Path+	265.82 ± 5.89 c	195.08 ± 1.88	460.90 ± 6.90 b
Endophyte (E)		36.00***	5.53*	45.30***
Pathogen (P)		2.93	332.18***	23.62***
EP		46.39***	1.03	54.22***

Values are expressed as mean ± SE (n = 5). A summary of ANOVAs is also given at the bottom of the table. The F-values, and the levels of significance (*P ≤ 0.05; ***P ≤ 0.001) are shown for each factor and parameter. All F-values are on 1 and 16 df

For each parameter, different letters (if any) indicate significant differences according to LSD (least significant difference) test of the endophyte × pathogen interaction at α = 0.05

Table 4 Peak assignment for methanolic extracts of *Purpureocillium lilacinum*

Code	Purposed formula	Rt (min)[a]	Obs. m/z[b] (Da)	Proposed compound	Activity	References
Cpd11	$C_{16}H_{17}N_3O_2$	13.592	283.131	Brevianamide F	Antibacterial	Zhang et al. (2007); Cai et al., (2012)
Cpd13	$C_{20}H_{39}NO_6$	18.493	389.2763	Sphingofungin B	Antimicrobial	VanMiddlesworth et al. (1992)
Cpd24	$C_{20}H_{43}NO$	24.239	313.3335	Paecilaminol	Anticancer, antinematode[1], Antimicrobial[2]	Chen y Hu (2022); Ui et al. (2006)

[a]Rt: retention time; [b]Obs m/z: observed mass/charge relationship

Discussion

Our study focused on the biocontrol potential of the fungal endophyte E496, identified as *P. lilacinum* through ITS, LSU, and *tub2* sequence analysis. This species is already known for its biocontrol efficacy against fungal pathogens like *Verticillium dahliae* and *Penicillium digitatum* (Elsherbiny et al. 2021). In our case, the complete inhibition of *B. cinerea* mycelial growth by the *P. lilacinum* filtrate and the significant reduction in sporulation by its extract underscore the potential of this endophyte as a biocontrol agent against this pathogen. Notably, the inhibition of sporulation is particularly critical as it is a key reproductive process for disease spread. Disrupting sporulation represents a valuable trait for reducing pathogen outbreaks in crops (Fedele et al. 2019). Such inhibitory effects are likely due to specific antimicrobial metabolites produced by *P. lilacinum*, consistent with previous findings on its antifungal activity (Chen and Hu 2022).

A preliminary exploration of the metabolites in the *P. lilacinum* extract via HPLC led to the tentative identification of several compounds, i.e., brevianamide F, sphingofungin B, paecilaminol, and leucinostatins A, B, C, and H, all recognized for their biocontrol activities (Tanvir et al. 2019). Brevianamide F has shown antimicrobial properties, particularly against bacteria, which might extend to fungal pathogens like *B. cinerea*. While sphingofungin B is effective against a broad spectrum of microorganisms, including bacteria and fungi (Boddy 2016), paecilaminol is known for its anticancer, nematicidal, and antimicrobial activities (Ui et al. 2006; Chen and Hu 2022). Finally, leucinostatins are noted for their antimicrobial effects against yeasts, filamentous fungi, and gram-positive bacteria (Chen and Hu 2022), and as entomopathogens (Momose et al. 2019). Specifically, leucinostatins A and B have shown antagonistic effects against the oomycete *Phytophthora* (Wang et al. 2016), while leucinostatins H and K have demonstrated antibiotic and cytotoxic properties (Radios et al. 1987). Nevertheless, further research is needed to confidently confirm their identity in the *P. lilacinum* extract and to explore their specific roles in the biocontrol and growth-promotion mechanisms.

Another aspect to consider in our study was the differences in biocontrol efficacy between filtrates and extracts. Filtrates include both fungal metabolites and components of the growth medium (YM broth in this study), which may contain substances that could interfere with metabolite activity. Extracts, by contrast, are typically more concentrated and purer, where many of the potentially interfering substances have been removed during the extraction process. These differences in biocontrol efficacy between filtrates and extracts have been observed in other studies as well (Chen et al. 2019). In our serial dilution assays, *P. lilacinum* exhibited the greatest antagonistic potential against *B. cinerea* when applied as extracts, suggesting that more purified products might be more effective for practical biocontrol applications.

Beyond its antifungal activity, our isolate of *P. lilacinum* also demonstrated significant plant growth-promoting properties. This dual functionality is particularly desirable for biocontrol agents, as enhancing plant health and vigor can improve overall crop resilience (El-Saadony et al. 2022). The growth-promoting effects of *P. lilacinum* were particularly pronounced in the presence of *B. cinerea*. This suggests that the endophyte not only directly antagonizes the pathogen but also indirectly supports the plant's defense mechanisms by improving growth and fitness (Zhang et al. 2022). In this regard, *P. lilacinum* extract produced a substantial increment on the germination rate of *C. arietinum* seeds, consistent with findings from studies on other crops, like tomato (Cavello et al. 2015). This fact could be explained by the production by *P. lilacinum* of phytohormone-like substances, including indoleacetic acid (IAA) and gibberellins (GA3), which are known to facilitate seed germination (Miransari and Smith 2014). The effectiveness of mycopriming in the improvement of seed germination has also been demonstrated for other endophytes and crops, such as *Alternaria leptinellae* in tomato (García-Latorre et al. 2024a) or *Pseudopithomyces chartarum* in *Lupinus luteus* (García-Latorre et al. 2024b). Notably, the extracts mitigated the negative effects of *B. cinerea* on germination, restoring results comparable to non-infected plants. Similar protective effects observed in studies applying mycopriming with other endophytes have been attributed to the upregulation of genes involved in antioxidant production and jasmonic acid pathways (Batool et al. 2022). In our study, although the presence of antioxidants in the endophytic extract was observed, which may support such an explanation, the induction of the jasmonic acid production in seeds or seedlings by *P. lilacinum* after mycopriming should be further proved.

The endophyte's extracts also enhanced seedling growth parameters, especially root length and root number, when applied as mycopriming or directly on seedlings. Again, the production by the endophyte of indoleacetic acid and gibberellin-like substances, known phytohormones able to stimulate plant growth (Egamberdieva et al. 2017), specifically shoot and root length (Fonseca et al. 2017), may explain such a growth promotion. Moreover, the endophyte's ability found in the present study to mobilize essential nutrients, such as phosphate and ammonia, may support its role in improving plant nutrient uptake and utilization. This capability may contribute to the overall nutrient status of the host plant, promoting growth and development (Adeleke et al. 2022). Such nutrient mobilization could also explain the observed improvements in growth parameters.

The application of the extract produced also a protective effect on the number of roots in the presence

of *B. cinerea*, highlighting its potential for enhancing seedling resilience. Healthy root development is crucial for nutrient uptake and overall plant stability, as longer roots can explore a larger soil volume, accessing more nutrients and water (Barber and Silberbush 1984). The increased number of roots provides better anchorage and facilitates greater nutrient uptake, further supporting plant growth (Wu et al. 2016). The production by the endophyte of metabolites with antifungal properties may explain such a protection. But the antioxidants contained in the endophytic extract could have also contributed to the protection by effectively neutralizing the reactive oxygen species produced after a pathogenic attack, preventing and mitigating oxidative damage to plant cells (Alfiky and Weisskopf 2021). Further research should be necessary to understand more deeply the mechanisms underlying those protective effects.

In conclusion, our study highlights the potential of *P. lilacinum* as a biocontrol agent and plant growth promoter. Its ability to inhibit pathogenic fungi, promote plant growth, and enhance seed germination and seedling development underscores its value in sustainable agriculture. By offering an environmentally friendly alternative to chemical pesticides, *P. lilacinum* represents a promising tool for improving crop resilience and productivity. However, further research is necessary to ensure the safety of *P. lilacinum*-derived products, particularly concerning mycotoxin production or other undesirable metabolites. Comprehensive studies on secondary metabolites in different growth media are essential to ensure their safe application in agriculture.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Research involving humans and animal participants This study did not include human participants and/or animals.

Informed consent Informed consent was not applicable to this study.

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