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Research Article

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Efficacy of ergosterol peroxide obtained from the endophytic fungus *Acrophialophora jodhpurensis* against *Rhizoctonia solani*

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Abstract

Aim: To investigate antifungal activity of the extract and major metabolite of the endophytic fungus *Acrophialophora jodhpurensis* (belonging to Chaetomiaceae) against crown and root rot caused by *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*), as an important pathogen of tomato.

Methods and Results: The endophytic fungus *A. jodhpurensis*, has high inhibitory effect against *R. solani* AG4-HG II *in vitro* and *in vivo*. The media conditions were optimized for production of the endophyte's metabolites. The highest amounts of secondary metabolites were produced at pH 7, 30°C temperature, and in the presence of 0.5% glucose, 0.033% sodium nitrate, and 1 gl^{-1} asparagine as the best carbon, nitrogen, and amino acid sources, respectively. The mycelia were extracted by methanol and the obtained extract was submitted to various chromatography techniques. Phytochemical analysis via thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) spectroscopy showed that ergosterol peroxide was the major component in the extract of this endophyte. Antifungal activities of the methanolic extract and ergosterol peroxide in the culture media were studied against *R. solani*. Minimum inhibitory concentrations of the extract and ergosterol peroxide against the pathogen were 600 and 150 μg ml^{-1}, respectively. Ergosterol peroxide revealed destructive effects on the pathogen structures in microscopic analyses and induced sclerotia production. Histochemical analyses revealed that it induced apoptosis in the mycelia of *R. solani* via superoxide production and cell death. Application of ergosterol peroxide in the leaf disc assay reduced the disease severity in tomato leaves.

Conclusions: Antifungal metabolites produced by *A. jodhpurensis*, such as ergosterol peroxide, are capable of controlling destructive *Rhizoctonia* diseases on tomato.

Impact Statement

Ergosterol peroxide could be used as a bioactive compound and replaced with hazardous chemical fungicides for protecting tomato against *Rhizoctonia solani*.

Keywords: *Acrophialophora jodhpurensis*; beneficial fungi; cell death; ergosterol peroxide; root and crown rot; tomato

Introduction

The genus *Acrophialophora* Edward was first described by Edward (1959) with the species *Acrophialophora nainiana*. Later, Samson and Mahmood reintroduced this genus in 1970 as a thermotolerant fungus with the species *A. fusispora*, *A. levis*, and *A. nainiana*. The genus *Acrophialophora* belongs to Ascomycota and is a member of the Chaetomiaceae family (Zhang et al. 2017). Twenty-eight species have been reported for this genus, so far . The Cheatomiaceae family is an excellent source of metabolites with diverse biotechnological and industrial applications (Ibrahim et al. 2021). Also, the species belonging to the Chaetomiaceae family, such as *Chaetomium globosum* (Wang et al. 2012, Li et al. 2014, Kumar et al. 2020a, Darshan et al. 2021), *C. cupreum* (Sibounnavong et al. 2012), *C. brasiliense* (Khumkomkhet et al. 2009), *A. levis* (Agrawal et al. 2021), and *A. jodhpurensis* (Daroodi et al. 2022, Daroodi and Taheri 2023) have considerable potential for production of bioactive metabolites with medicinal and agricultural applications (Hyde et al. 2020, Ibrahim et al. 2021).

To our knowledge, there is no phytochemical investigation on the metabolites produced by *A. jodhpurensis*. However, studies have reported phenolic acids, phenylpropanoids, sesquiterpenes, tannins, lignans, and flavonoids for another species of this genus, *A. levis* (Agrawal et al. 2021). Nevertheless, various compounds have been reported from the *Chaetomium* genus, which formerly *A. jodhpurensis* belonged to *Chaetomium*. There are some examples about identification of the metabolites produced by *Chaetomium* species, such as alkaloids of *C. globosum* (An et al. 2020, Ji et al. 2021), polyketide of *C. seminudum*, *Chaetomium* sp. (Li et al. 2018), *C. mollipilium* (Teigo et al. 2012), and *C. indicum* (Lu et al. 2013), pyranones of *C. globosum* (Fatima et al. 2016), orsellides of *Chaetomium* sp. (Xu et al. 2018), xanthones of *C.*

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globosum (Hani and Eman 2015), steroids of *C. elatum* (Thohinung et al. 2010), and *C. globosum* (Thohinung et al. 2010, Ji et al. 2021).

Ergosterol peroxide (5α, 8α-epidioxyergosta-6,22-dien-3β-ol), a steroidal specialized metabolite with 28 carbons, is produced by fungi, lichens, sponges, and halotolerant algae. Wieland and Prelog (1947) isolated this compound from *Aspergillus fumigatus* for the first time. The ergosterol peroxide (EP) has been isolated from mushrooms and fungal endophytes, including *Hygrophoropsis aurantiaca* (Nowak et al. 2016); *Phoma* sp. (Wu et al. 2018); *Fomitopsis* sp., *Daldinia concentrica*, *Ganoderma* sp., *Phellinus igniarius*, and *Trametes gibbosa* (Trung et al. 2018); *Leccinum scabrum*, *Armillaria mellea*, *Laccaria* spp., *Suillus variegatus*, *Xerocomus badius*, *Clitocybe gibba*, *Coprinus micaceus*, *Fomitopsis betulina*, *Trametes betulina*, and *Macrolepiota procera* (Nowak et al. 2022); *Xylaria striata* (Zhang et al. 2023); and *Alternaria alternata* (Khazaal et al. 2023).

The EP has many biological effects, such as antimicrobial, cytotoxic, immunosuppressive, anti-inflammatory, antiobesity, antimelanoma, antiprotozoal, and anticancer activities (Jeong and Park 2020, Dembitsky et al. 2021, Xia et al. 2022). This compound has antimicrobial activity against bacteria, fungi, viruses, and protozoa (Merdivan and Lindequist 2017, Suwannarach et al. 2020). The EP has shown antifungal activity against plant pathogens, such as *Septoria* sp., *Rhizoctonia* sp., *Fusarium* sp., *Verticillium* sp., *Pyricularia oryzae* (You et al. 2009), and *R. solani* (You et al. 2019).

To the best of our knowledge, the metabolites produced by *A. jodhpurensis* have not been studied, so far. Therefore, the objectives of present study were to (i) determine the best conditions for secondary metabolites production by *A. jodhpurensis*, (ii) isolate and identify major metabolites of *A. jodhpurensis*, (iii) to examine their antifungal activity against *R. solani in vitro* and *in vivo* conditions on tomato leaves, and (iv) to examine their effects on superoxide ($o_2^{\cdot -}$) production and cell death in the hyphal cells of *R. solani*, as a destructive phytopathogen.

Materials and methods

Source of endophytic and pathogenic fungi

Fungal isolates used in the present study, including *A. jodhpurensis* and *R. solani* AG4-HG II (belong to the Chaetomiaceae and Ceratobasidiaceae families, respectively) were previously isolated from healthy (Daroodi et al. 2022) and infected (Pourmahdi and Taheri 2014) tomato plants in Khorasan province, located in the northeast of Iran and maintained in the culture collection of Ferdowsi University of Mashhad, Iran. The fungal isolates were grown on petri dishes containing potato dextrose agar (PDA) medium and PDA slants and maintained at 28°C, until used.

Optimum conditions for antifungal activity of *A. jodhpurensis*

In our previous study, the endophytic fungus *A. jodhpurensis* reduced mycelial growth of *R. solani* AG4-HG II in dual culture test on PDA medium (Daroodi et al. 2021). Therefore, we investigated effect of various culture media on the biological activity of this beneficial fungus against *R. solani* AG4-HG II using dual culture method on 2% PDA (2 g in 100 ml distilled water), 3% PDA (3 g in 100 ml distilled water), and corn meal agar (CMA) media and compared to PDA medium (3.9 g in 100 ml distilled water).

Effect of carbon, nitrogen, and amino acid sources, temperature, and pH on antifungal activity of *A. jodhpurensis* was studied using dual culture test. The 2% PDA medium, which showed the best results in dual culture test, was selected as the basal medium in this assay. The effects of different temperatures (15°C, 17°C, 20°C, 24°C, 28°C, 30°C, 35°C, and 37°C) and pH 4, 5, 6, 7, 7.4, 8, and 9 were investigated on the antifungal activity of *A. jodhpurensis* in 2% PDA medium (Lazarević et al. 2016).

One % concentrations of starch, dextrose, fructose, sucrose, and also 1% and 0.5% concentrations of glucose were used as the sources of carbon (Piwtamai et al. 2013). Half-strength potato agar medium (2% PA medium) with 0.5% concentration of glucose, which was the best carbon source, was selected as the basal medium for investigating influence of different nitrogen sources on the antifungal activity of *A. jodhpurensis*. Nitrogen sources used were sodium nitrate, ammonium sulfate, ammonium chloride, uric acid, and ammonium phosphate with 0.033% concentration in each case (Piwtamai et al. 2013). Also, various amino acids, including glycine, arginine, leucine, asparagine, serine, and phenylalanine, were used at 1 gl^{-1} concentration in half-strength PA medium containing 0.5% concentration of glucose and were incubated at 28°C (Oso 1975).

The biological activity of *A. jodhpurensis* was evaluated against the phytopathogen *R. solani* AG4-HG II (crown and root rot) using dual culture method. For this purpose, mycelial disks (9 mm diameter) of the endophytic fungus taken from 7-d-old culture were placed on one side of the petri dishes containing various media. For control, a 9 mm disc of PDA without the endophytic fungus was placed on one side of the petri dishes. After 2 d, mycelial disks of *R. solani* AG4-HG II (9 mm diameter) taken from 7-d-old culture were placed on the opposite side of the petri dishes. The vegetative growth of *R. solani* AG4-HG II was examined macroscopically (Sanchez-Fernandez et al. 2016). The growth inhibition percentage (GI %) of pathogen was determined before full development of *R. solani* AG4-HG II mycelia in control petri dishes according to the method of Asran-Amal et al. (2010) using the following equation:

$$L = ((C - T)/C) \times 100,$$

where L = GI %, C = radial growth in the control, and T = radial growth in the treatment. The assays were performed in three repetitions and triplicate in each experiment.

Preparation of the fermentation broth of *A. jodhpurensis*

Two mycelial disks (1 × 1 cm) of *A. jodhpurensis*, taken from 7-d-old culture of *A. jodhpurensis* on PDA, were transferred into 1000 ml Erlenmeyer flasks containing 250 ml half-strength potato glucose broth medium [PG medium: extract of potato (100 g of potato boiled for 30 min, then filtered using a muslin cloth), 5 g glucose in 1 l distilled water], including 0.033% sodium nitrate with pH7 and autoclaved at 121°C for 15 min. The fermentation was incubated at 30°C in a shaker at 150 rpm for 14 d. The mycelia were harvested through two layers of muslin cloth, followed by three times washing in sterile distilled water. Then, the mycelia dried in laminar flow.

Extraction

The dried mycelia (62.15 g) were powdered by an electric mill and extracted with 5 l of methanol (MeOH) by percolation for 2 d at room temperature. The extract was dried using a rotary evaporator at 40°C to afford 5.53 g MeOH residue.

Evaluating antifungal activity and minimum inhibitory concentration of MeOH extract against *R. solani* AG4-HG II *in vitro*

Antifungal activity of MeOH extract was tested on half-strength potato glucose agar (PGA) medium. Briefly, the extract was dissolved in DMSO (dimethyl sulfoxide) and diluted with half-strength PGA media to get the desired concentrations of 300 and 600 μg ml^{-1}. A mycelial plug of the pathogen (9 mm diameter) was inoculated at the center of each culture medium. The culture medium treated with DMSO without any extract was used as a negative control. The petri dishes were kept at 28°C until full development of *R. solani* AG4-HG II in control petri dishes. Mycelial GI % was determined as described by Kumar et al. (2020b). Also, the fungistatic or fungicidal effect of MeOH extract was determined (Plodpai et al. 2013). The assays were performed in three repetitions and triplicate in each experiment.

Preparative isolation

The dried MeOH extract was separated by an open column chromatography (CC) on silica gel (30 × 3 cm, 230–400 mesh). Solvent system was optimized by thin-layer chromatography (TLC). The MeOH extract was fractionated by a gradient of petroleum ether (PE)–ethyl acetate (EtOAc) (100:0–0:100) and EtOAc–MeOH (100:0–0:100), and finally, the column was washed with water. A crystal (10.2 mg) was obtained from the column using PE and EtOAc as solvents. The purified compound was investigated by TLC [EtOAc: PE (2:3)] using vanillin–sulfuric acid as a reagent. The compound appeared as black spots with RF 0.6.

Structural elucidation of the EP

Structure of the EP was elucidated by proton (^{1}H-NMR, 300 MHz) and carbon (^{13}C-NMR, 75 MHz) nuclear magnetic resonance (NMR) spectroscopy. For this purpose, the compound was dissolved in $CDCl_3$ (deuterated chloroform). The NMR data were analyzed by MestreLab Nova 14.2.

Evaluation of antifungal activity and minimum inhibitory concentration of EP

For evaluating the antifungal activity and minimum inhibitory concentration (MIC) of EP, the effect of this compound on the growth of *R. solani* AG4-HG II was investigated using half-strength PGA media containing various concentrations of EP, including 8, 10, 20, 50, 100, and 150 μg ml^{-1} as described before (Kumar et al. 2020b). Also, the fungistatic or fungicidal effect of EP was determined (Plodpai et al. 2013).

Effect of the EP on mycelial structure of the pathogen

To observe the effect of EP on mycelial structure of the pathogen, *R. solani* AG4-HG II was cultured on half-strength PGA containing various concentrations of EP, including 0, 8, 10, 20, 50, and 100 μg ml^{-1} (pathogen had no growth at 150 μg ml^{-1} concentration). Then, the pathogen hyphae were stained using aniline blue) and were investigated using a light microscope (BH2, Tokyo, Japan) before full development of *R. solani* AG4-HG II mycelia in the control petri dishes.

Effects of the EP on production and germination of *R. solani* AG4-HG II sclerotia

Effect of the EP on production of *R. solani* AG4-HG II sclerotia was investigated by addition of EP at 0, 8, 10, 20, 50, and 100 μg ml^{-1} concentrations to half-strength PGA medium (pathogen had no growth at 150 μg ml^{-1} concentration). Then, a mycelial plug (9 mm diameter) of the pathogen taken from 7-d-old culture was transferred to the center of each petri dish and maintained at 28°C for 14 d. After this period, fresh and dry weights of sclerotia were investigated. To determine dry weight, fresh sclerotia were transferred into an oven at 70°C temperature until a stable weight was reached (Lu et al. 2016). The assays were performed with three repetitions and three repetitions for each treatment in each assay.

In order to determine effect of the EP on sclerotia germination, *R. solani* AG4-HG II was cultured on half-strength PGA media containing the EP (0, 8, 10, 20, 50, and 100 μg ml^{-1}), as explained before and petri dishes were maintained at 28°C for 30 d. After this period, 10 sclerotia were placed onto a water agar medium. Then, the vegetative growth of sclerotia was investigated by the light microscope (Olympus, BH2, Tokyo, Japan) (Mukherjee et al. 1999). Sclerotia germination was examined (as a percentage) using the following equation (Li et al. 2015):

$$\text{Total germination rate } (\%) = \left(\text{Germinated sclerotia/Total sclerotia}\right) \times 100.$$

The assays were performed with three repetitions and three repetitions for each treatment in each assay.

Effect of the EP on superoxide production in the hyphae of *R. solani* AG4-HG II

For investigating superoxide ($O_2^{\cdot-}$) production in mycelia cells of the pathogen, a 9 mm diameter plug of the *R. solani* AG4-HG II was transferred onto a coverslip surface. Then, the coverslips were placed inside sterile petri dishes containing moist filter paper. After 3 d, the hyphae were treated with 0, 10, 20, 50, 100, and 150 μg ml^{-1} concentrations of the EP. The petri dishes were sealed with parafilm and maintained at 28°C in the dark for 24 h. After this period, 500 μl of the nitroblue tetrazolium (NBT) (2.5 mM) solution in HEPES buffer (25 mM, pH 7.6) was added to corner of the coverslips. Then, the hyphae were incubated under laboratory conditions (25°C) in the dark for 30 min (Semighini and Harris 2010). Images were taken at different concentrations using a light microscope (Olympus, BH2, Tokyo, Japan), and Image J software (http://rsb.info.nih.gov/ij/index.html) was used for analyzing the staining intensities.

Effect of the EP on cell death in the hyphae of *R. solani* AG4-HG II

The cell death in the hyphal cells of *R. solani* AG4-HG II was investigated using Evans blue solution (1%) in phosphate-buffered saline [PBS: 10 × stock solution: 27 mM potassium chloride (KCl), 1.37 M sodium chloride (NaCl), 18 mM potas-

sium dihydrogen phosphate (KH2PO4), 100 mM sodium dihydrogen phosphate (NaH2PO4), pH 7.2]. For this purpose, a 9 mm diameter plug of the pathogen was transferred onto a coverslip surface. The coverslips were placed inside sterile petri dishes containing moist filter paper. The hyphae were treated with 0, 8, 10, 20, 50, 100, and 150 μg ml^{-1} concentrations of EP after the pathogen growth. After 24 h, 500 μl of Evans blue solution (1%) in 1 × PBS (pH 7.2) was added to the corner of coverslips. Staining was carried out at room temperature for 5 min, followed by washing three times in the phosphate buffer (pH 7.0) (Semighini and Harris 2010). The samples were investigated using a fluorescence microscope (Olympus BX51, Japan), and Image J software (http://rsb.info.nih.gov/ij/index.html) was used for analyzing the staining intensities.

Biocontrol effect of the EP against *R. solani* AG4-HG II on detached tomato leaves

Effect of the EP was evaluated on the disease severity of *R. solani* AG4-HG II in detached tomato leaves. For this purpose, the tomato seeds (*Solanum lycopersicum* cv. Mobil) were surface disinfected with sodium hypochlorite (1%) for 2 min, followed by three times washing in sterile distilled water. In order to prepare the pots, a mixture of farm soil, sand, and leaf mold (2:1:1) was sterilized at 121°C for 45 min. Then, the disinfected seeds were sown in 12 × 10 cm pots (two tomato seeds per pot). The pots were maintained in greenhouse at 30°C ± 4°C, with 16/8 h light/dark photoperiod and 50% relative humidity for 4 weeks. After this period, leaf discs (2 cm diameter) were prepared from the oldest leaves of tomato plants. The leaf discs were treated using the EP at 8, 10, 20, 50, 100, and 150 μg ml^{-1} concentrations for 1 h. For this purpose, the EP was dissolved in dimethyl sulfoxide (DMSO) and diluted with sterile distilled water to get concentrations of 8, 10, 20, 50, 100, and 150 μg ml^{-1}. Sterile distilled water containing DMSO was used as a control. Then, each leaf disc was transferred onto a glass slide surface inside a sterile petri dish containing moist filter paper. Then, the center of each leaf disc was inoculated by a plug of the pathogen (9 mm diameter). In control, the leaf discs were inoculated by plugs of PGA medium without the pathogen. The petri dishes were maintained at room temperature and 12/12 h light/dark photoperiod for 5 d. After this time, the disease severity was investigated using five grades based on the leaf area infected, and the disease index (DI) was determined using the method of Taheri and Tarighi (2010). The assays were performed with three repetitions and three replicates for each treatment in each assay.

Statistical analysis

All experiments were performed with three repetitions and replications. The statistical analyses were carried out using Minitab software (version 17). First, the data were normalized in each experiment. Then, the mean of replications was calculated in each repetition. The means of all data obtained for different treatments at different time points were analyzed using one-way analysis of variance (ANOVA). The means were separated by the Fisher test at the level of $P \leq .05$. The presented data for each assay were means (± SE) of three experiments. Excel 2013 was used for drawing all diagrams. The data of NMR were analyzed using MestreLab Nova 14.2. Also, structure of the metabolite was drawn by ChemDraw Ultera V12.0.

Results

Effect of culture conditions on antifungal activity of *A. jodhpurensis* against *R. solani* AG4-HG II

The effects of carbon, nitrogen, and amino acid sources, temperature, and pH on the antimicrobial activity of *A. jodhpurensis* against *R. solani* AG4-HG II were studied using the dual culture method. The results showed higher antagonistic activity of *A. jodhpurensis* against the pathogen in 2% PDA (Fig. 1a), at 30°C temperature (Fig. 1b) and pH 7 (Fig. 1c). Also, 0.5% concentration of glucose (Fig. 1d), 0.033% sodium nitrate, and 1 gl^{-1} asparagine (Fig. 1e) were selected as the best sources of carbon, nitrogen, and amino acid, respectively.

Antifungal activity and MIC of the MeOH extract against *R. solani* AG4-HG II

The MeOH extract reduced *R. solani* AG4-HG II growth. The GI % of the pathogen using the MeOH extract at concentrations of 300 and 600 μg ml^{-1} were 51.33% and 100%, respectively (Fig. 2a). Also, the MeOH extract showed fungistatic effect against *R. solani* AG4-HG II at 600 μg ml^{-1} concentration. The TLC profile of the MeOH extract is shown in Fig. 2b, which shows many spots. Therefore, this extract contained various metabolites.

Compound identification

The EP (10.2 mg) was obtained as a crystal from the chromatographic column using PE and EtOAc as solvents. The crystal was white (Fig. 2c) and appeared as black spots on the TLC plate using vanillin–sulfuric acid as a reagent (Fig. 2d). According to ^{13}C-NMR and deptsp-135 data, the scaffold contained 7 CH_2 groups, 17 CH and CH_3 groups, and 4 C. Based on ^{1}H-NMR data, there are 6 CH_3 groups, 4 aromatic CH, and 7 aliphatic CH moieties (Fig. 2e). Analysis of the ^{1}H and ^{13}C NMR spectra and comparing them with the literature indicated that the compound was EP (Table 1, Supplementary Fig. 1).

Evaluating antifungal activity and MIC of the EP against *R. solani* AG4-HG II

The EP reduced *R. solani* AG4-HG II growth. The GI % of the pathogen using the EP at concentrations of 8, 10, 20, 50, 100, and 150 μg ml^{-1} were 0%, 3.55%, 8%, 38.66%, 71.55%, and 100%, respectively (Fig. 3a). Also, the EP showed fungistatic effect against *R. solani* AG4-HG II at 150 μg ml^{-1} concentration.

Effect of EP on the hyphae structure of *R. solani* AG4-HG II

Light microscopy was used to characterize the effect of the EP on hyphae structure of the pathogen at concentrations of 8, 10, 20, 50, and 100 μg ml^{-1} before full development of *R. solani* AG4-HG II mycelia in control petri dishes (after 3 d). The results revealed that hyphae formation altered in the presence of the EP. Cytoplasm lysis and deformation of the mycelia were observed in the pathogen treated with the EP compared to the controls (Fig. 3b).

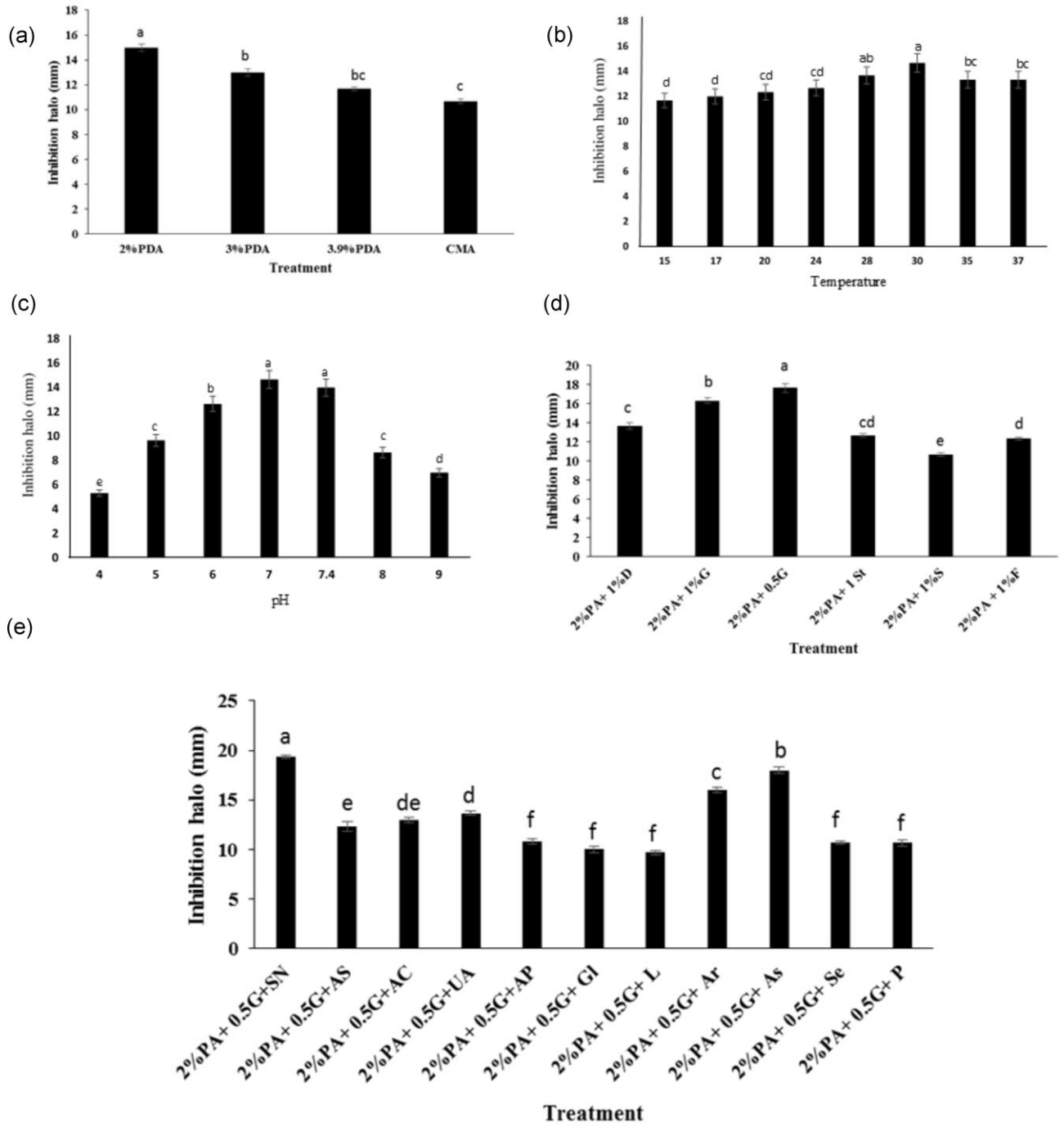

Figure 1. Determining optimum conditions for antagonistic activity of *A. jodhpurensis* against *R. solani* AG4-HG II in dual culture. Effect of medium (a), temperature (b), pH (c), carbon (d), and nitrogen and amino acid (e). PDA: potato dextrose agar, CMA: corn meal agar, D: dextrose, G: glucose, St: starch, S: sucrose, F: fructose, SN: sodium nitrate, AS: ammonium sulfate, AC: ammonium chloride, UA: uric acid, AP: ammonium phosphate, GL: glycine, L: leucine, Ar: arginine, As: asparagine, Se: serine, and P: phenylalanine. Error bars correspond to standard error of three experiments. The assays were performed in three repetitions and triplicate in each assay.

Effects of the EP on production and germination of *R. solani* AG4-HG II sclerotia

Effect of the EP on the production and germination of *R. solani* AG4-HG II sclerotia was investigated on half-strength PGA media containing EP at 8, 10, 20, 50, and 100 μg ml^{-1} concentrations. The sclerotia production of the pathogen changed in the presence of EP compared to the control. Production of sclerotia in plates containing PGA and EP was higher than that of the control (Fig. 4a).

Also, fresh (Fig. 4b) and dry (Fig. 4c) weights of the sclerotia produced in the control were fewer than those of the EP treatments. Maximum sclerotia production was observed by the pathogen cultured in a medium containing 150 μg ml^{-1} concentration of EP (the highest concentration used in the experiment). However, germination of sclerotia decreased in the presence of EP compared to the control (Fig. 4d).

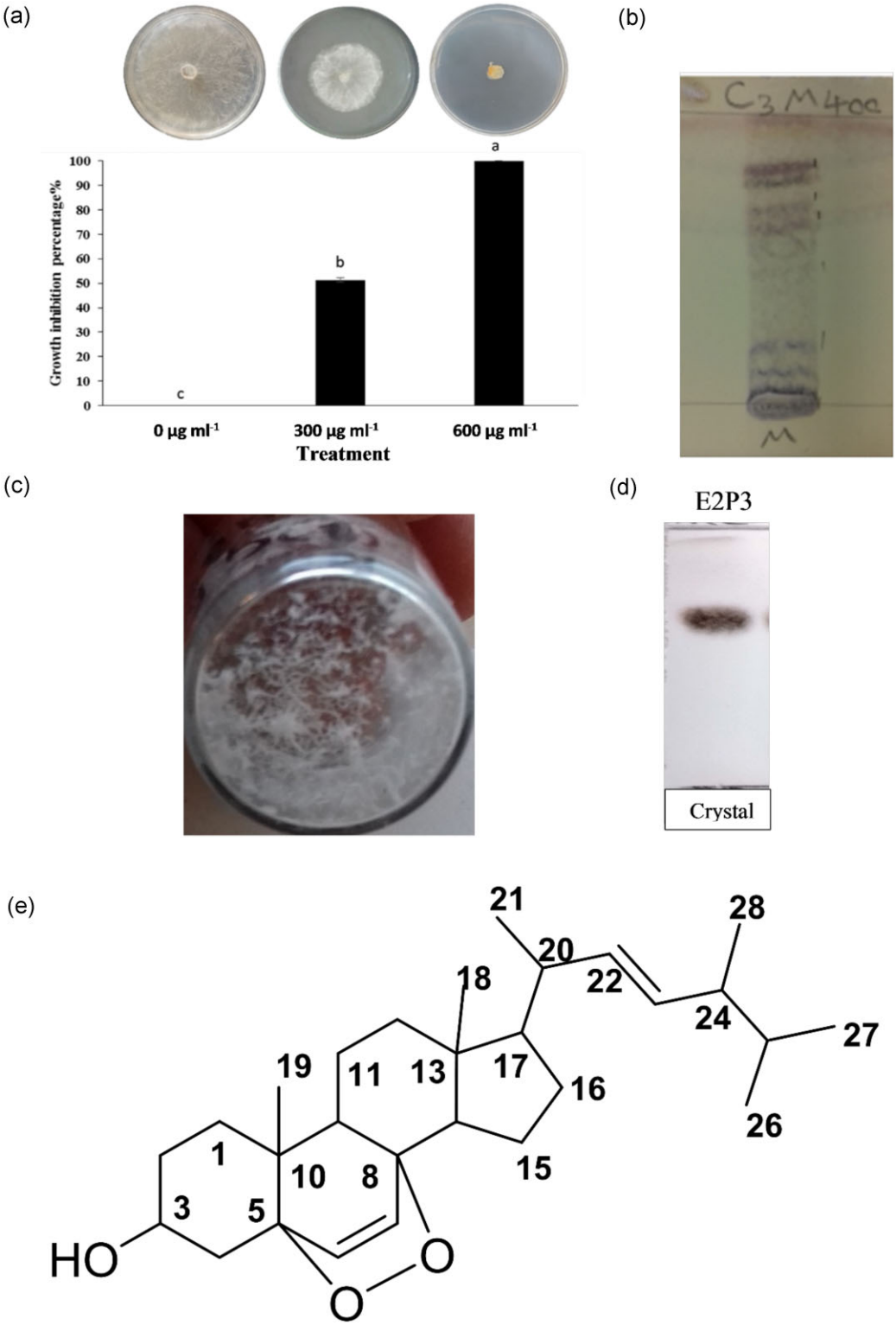

Figure 2. Effect of MeOH extract from the endophytic fungus *A. jodhpurensis* on mycelial growth of *R. solani* AG4-HG II in concentrations of 0, 300, and 600 µg ml^{-1} (a), the TLC of MeOH extract using chloroform: C and methanol: M solvents (b), the structure of EP isolated from *A. jodhpurensis* (c), TLC of EP using ethyl acetate: E and petroleum ether: P solvents (d), and the chemical structure of EP (e). Error bars correspond to the standard error of three experiments and the chemical structure of ergosterol peroxide was drew by ChemDraw Ultera V12.0 software.

The EP induced superoxide production in the mycelia cells of *R. solani* AG4-HG II

Detection of superoxide ($O_2^{\cdot-}$) in the mycelia cells of pathogen was done using NBT staining at 24 h after the treatment with different concentrations of EP (0, 8, 10, 20, 50, 100, and 150 µg ml^{-1}). The reaction of NBT with $O_2^{\cdot-}$ produced a dark blue insoluble formazan compound, which was observed by an Olympus microscope (BH2, Tokyo, Japan). Production of $O_2^{\cdot-}$ in the hyphal cells enhanced with increasing concentration of the EP (Fig. 5a).

Table 1. ^{1}H and ^{13}C NMR spectroscopic data for EP ($CDCl_3$; 300 MHz for ^{1}H and 75 MHz for ^{13}C NMR; and δ in ppm).

Position	δ_C, type	δ_H, (J in Hz)
1	34.7, CH_2	
2	30.1, CH_2	
3	66.5, CH	3.97, m
4	37.0, C	
5	82.2, C	
6	135.4, CH	6.24, d (14.0)
7	130.8, CH	6.51, d (14.1)
8	79.4, C	
9	51.1, CH	
10	37.0, C	
11	23.4, CH_2	
12	39.3, CH_2	
13	44.6, C	
14	51.7, CH	
15	20.6, CH_2	
16	28.7, CH_2	
17	56.2, CH	
18	12.9, CH_3	0.81, s
19	18.2, CH_3	0.87, s
20	39.8, CH	
21	20.9, CH_3	1.00, d (10.8)
22	135.2, CH	5.15, dd (19.8, 12.95)
23	132.3, CH	5.19, dd (19.8, 11.7)
24	42.8, CH	
25	33.1, CH	
26	19.7, CH_3	0.83, d (8.4)
27	20.0, CH_3	0.80, d (11.2)
28	17.6, CH_3	0.91, d (11.3)

Effect of EP in inducing cell death in the hyphal cells of *R. solani* AG4-HG II

Dead cells in the hyphae of *R. solani* AG4-HG II were investigated using Evans blue staining at 24 h after treatment with different concentrations of EP (0, 8, 10, 20, 50, 100, and 150 μg ml^{-1}). Evans blue penetration into nonviable cells was visualized using a fluorescence microscope at 470 to 540 nm. Cell death in the fungal hyphae enhanced with increasing concentration of the EP. Maximum cell death was observed in the fungal hyphae treated with 150 μg ml^{-1} concentration of EP (Fig. 5b).

Effect of EP on biocontrol of tomato disease caused by *R. solani* AG4-HG II

The leaf discs treated with various concentrations of EP (0, 8, 10, 20, 50, 100, and 150 μg ml^{-1}) showed less disease severity than the discs only inoculated with the pathogen (without the EP treatment) (Fig. 6a). Maximum reduction of the disease index (>80%) was observed on the leaf discs treated with 150 μg ml^{-1} concentration of EP (Fig. 6b).

Discussion

In our previous study, the fungal endophyte *A. jodhpurensis* showed antifungal activity against *R. solani* AG4-HG II *in vitro* (44%) and *in vivo* (40% in leaf disc bioassay and 33% on tomato seedlings) (Daroodi et al. 2021). In this research, the best fermentation conditions were optimized for the antifungal activity of *A. jodhpurensis* against *R. solani* AG4-HG II in dual culture. The data revealed that the maximum inhibitory effects of *A. jodhpurensis* against the pathogen were at 2% PDA, temperature of 30°C, and pH 7. Also, 0.5% concentration of glucose, 0.033% concentration of sodium nitrate, and 1 gl^{-1} asparagine were selected as the best sources of carbon, nitrogen, and amino acid, respectively. Similarly, many studies have been carried out on the best fermentation conditions for growth, sporulation, and antagonistic activity in the Chaetomiaceae family. For example, Buston and Basu (1948) studied some factors affecting the growth and sporulation of *C. globosum*. They selected glucose (0.5%) as the source of carbon and the influence of nitrogen compounds was investigated in the presence of glucose. Piwtamai et al. (2013) reported that the optimal pH range for *C. globosum* growth was 5.5 to 6.0. Also, cellulose and asparagine (as carbon and nitrogen sources, respectively) supported growth and sporulation of *C. globosum*, respectively. Barros et al. (2010) investigated effect of different carbon sources, such as steam-pretreated sugarcane bagasse, wheat bran, or lactose on production of enzymes by *A. nainiana*. The highest production of endo-1–4-β-glucanase and xylanase was observed using lactose as a carbon source.

In the current study, the MeOH extract was obtained from the mycelia of *A. jodhpurensis* and showed antifungal activity against *R. solani* AG4-HG II *in vitro*. The MIC value of the MeOH extract was 600 μg ml^{-1}. Similar to our findings, the EtOAc extract of *A. levis* showed strong antioxidant activity against *Staphylococcus aureus* with the MIC value of 4 μg ml^{-1} (Agrawal et al. 2021). In addition, EP was purified from the MeOH extract. This is the first study reporting isolation of EP from the endophytic fungus *A. jodhpurensis*. The EP reduced the vegetative growth of *R. solani* AG4-HG II with the MIC of 150 μg ml^{-1}. Similar to our findings, many researchers reported antimicrobial activity of this compound against phytopathogens, such as *Mycobacterium tuberculosis* (Duarte et al. 2007), *Septoria* sp., *Rhizoctonia* sp., *Fusarium* sp., *Verticillium* sp., and *Py. oryzae* (You et al. 2009), *Helninthosporiun maydwas* (Zhang et al. 2023), *Listeria monocytogenes*, *Clostridium perfringens*, *Streptococcus faecalis*, *Staph. aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli*, *Klebsiella pneumoniae*, *As. niger*, and *Candida albicans* (Khazaal et al. 2023). Also, You et al. (2019) investigated antimicrobial activity of the EP, extracted from bamboo vermicompost, against *R. solani* AG1-IB at various concentrations on top of each plug of the pathogen in PDA medium. Their results showed that concentrations of 150, 300, 600, and 900 μg of the EP could inhibit 13%, 22%, 34%, and 53% vegetative growth of pathogen, respectively. Also, they suggested that the EP was released in vermicompost by microbes during vermicomposting (You et al. 2019). Furthermore, phytosterols such as sitosterol, stigmasterol, and ergosterol from *Bulbine natalensis* (a plant in southern Africa) exhibited antifungal activities against *F. verticilloides*, *Penicillium digitatum* and *As. flavus* (Mbambo et al. 2012).

In this research, the EP showed fungistatic activity against *R. solani* AG4-HG II. Similarly, Zhang et al. (2023) isolated ergosterol and EP from the fungus *X. striata* and the ergosterol showed excellent fungistatic activities against *Valsa mali*, *Sclerotinia sclerotiorum*, *F. graminearum*, and *H. maydwas*.

Also, effects of the EP on hyphal structure and sclerotia production of *R. solani* AG4-HG II were studied. The data revealed that the EP could increase sclerotia production of pathogen. However, the presence of EP resulted in a reduction in germination of sclerotia compared to the control. The structures of *R. solani* AG4-HG II displayed cytoplasm lysis and deformation of hyphae in the presence of EP. To the best of our knowledge, there are no data about effect of the EP on hyphal structures and spore production of phytopathogens,

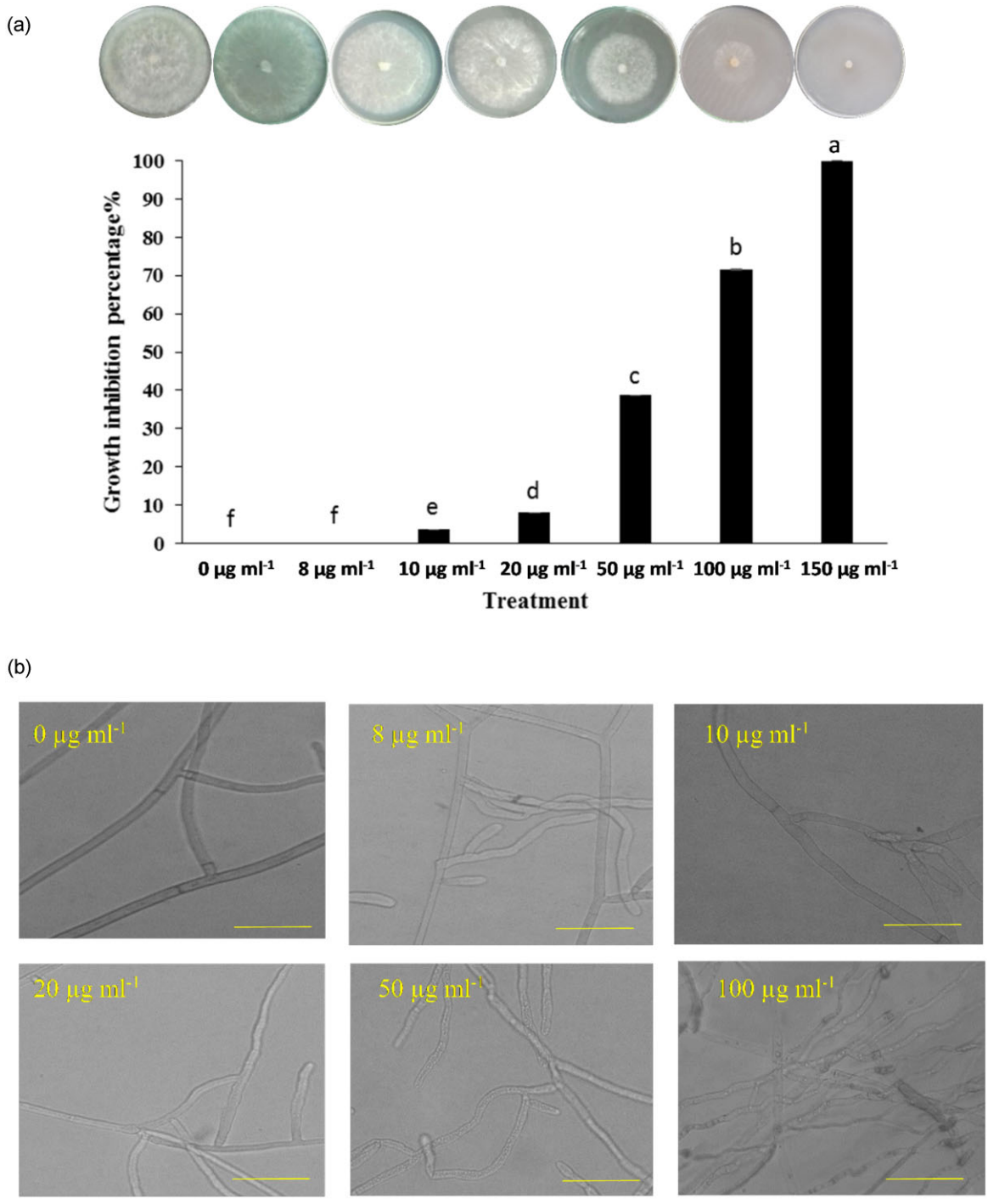

Figure 3. Effect of EP extracted from the endophytic fungus *A. jodhpurensis* on mycelial growth and morphology of hyphae of *R. solani* AG4-HG II. The effect of EP on growth of *R. solani* AG4-HG II in concentrations of 0, 8, 10, 20, 50, 100, and 150 µg ml^{-1} (a) and effect of EP on morphology of hyphae of *R. solani* AG4-HG II in concentrations of 0, 8, 10, 20, 50, and 100 µg ml^{-1} (b). Error bars correspond to standard error of three experiments.

so far. However, many researchers reported altering in hyphal structures of pathogens using other compounds. For example, eugenol, isolated from clove plant (*Syzygium aromaticum*), altered the ultrastructure and morphology of *R. solani* mycelia. The scanning electron microscopy (SEM) observations showed that the mycelia became dehydrated and shrank, and surface unevenness, roughness, and depression were observed. The transmission electron microscopy (TEM) results showed separating the plasma membrane from cell wall, vacuolating the cytoplasm, decreasing cytoplasmic density, thickening, and expanding cell wall. Also, reduction or dissolution of organelles was observed (Zhao et al. 2021). Farnesol, as a quorum-sensing molecule, altered the morphology of *R. solani* mycelia. Alterations in the cytoplasm of mycelia were observed using optical microscopic and alterations in the nuclear membrane, nucleus, mitochondria, septum, parenthesome, septal pore, cytoplasm, cell membrane, and cell wall were observed using TEM analyses. Farnesol also promoted sclerotia formation in *R. solani* (Nassimi et al. 2019). In addition, morphological changes of *Py. oryzae* structures were

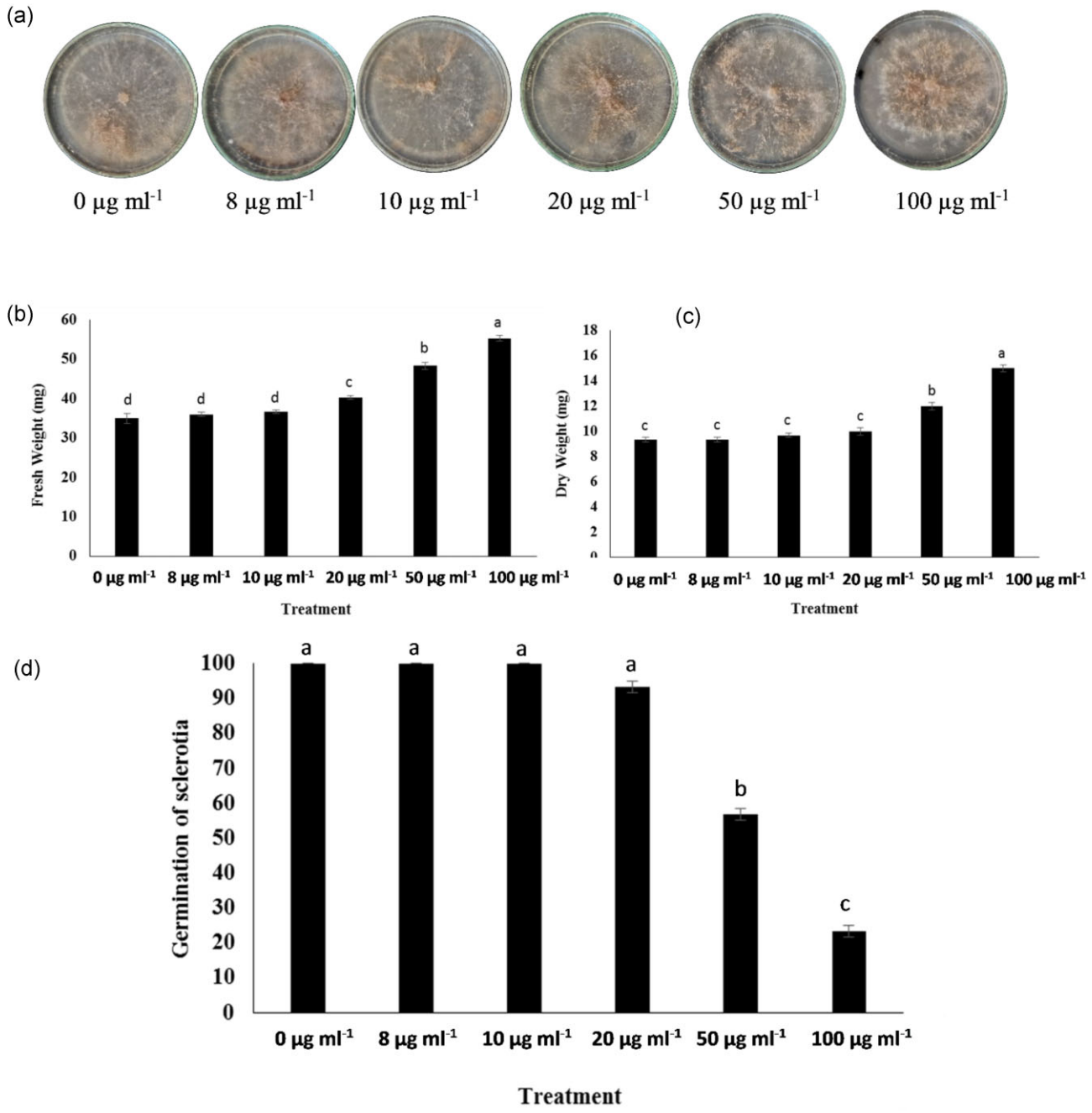

Figure 4. Effect of EP on production and germination of sclerotium of *R. solani* AG4-HG II in concentrations of 0, 8, 10, 20, 50, and 100 µg ml^{-1}. The effect of EP on production of *R. solani* AG4-HG II sclerotia (a), fresh weight of sclerotia (b), dry weight of sclerotia (c), and germination of sclerotia (d). Error bars correspond to standard error of three experiments.

observed at minimum concentration of EP (7.8 µg ml^{-1}) (You et al. 2009).

Apoptosis is a form of programmed cell death, which is involved in biology and physiology of various multicellular organisms (Baehrecke 2002). In this study, apoptotic cells in *R. solani* AG4-HG II were investigated using two staining methods, which include NBT and Evans blue staining to detect $O_2^{\cdot-}$ and cell death, respectively. Detection of $O_2^{\cdot-}$ was performed using NBT (a pale-yellow compound). A reaction between NBT and $O_2^{\cdot-}$ would produce a blue to purple formazan precipitate (Berridge et al. 2005). In Evans blue staining to detect cell death, dead hyphal cells with lysed plasma membrane are unable to exclude the stain Evans blue, while normal hyphae exclude Evans blue and are in their natural color. The data showed that EP induced the production of $O_2^{\cdot-}$ and cell death in the hyphal cells of *R. solani* AG4-HG II. In agreement with our findings, EP from marine fungus *Phoma sp.* induced generation of reactive oxygen species (ROS) and apoptosis in human lung cancer cells (Wu et al. 2018). The EP has exhibited high cytotoxic activity against gastric cancer cells and induces apoptosis via enhancement of ROS levels (Lin et al. 2022). Also, induction of apoptosis and ROS production were observed using EP isolated from the fungus *Pleurotus ferulae* (Yang et al. 2020).

EP decreased the disease severity of *R. solani* AG4-HG II on detached leaf discs of tomato compared to the controls

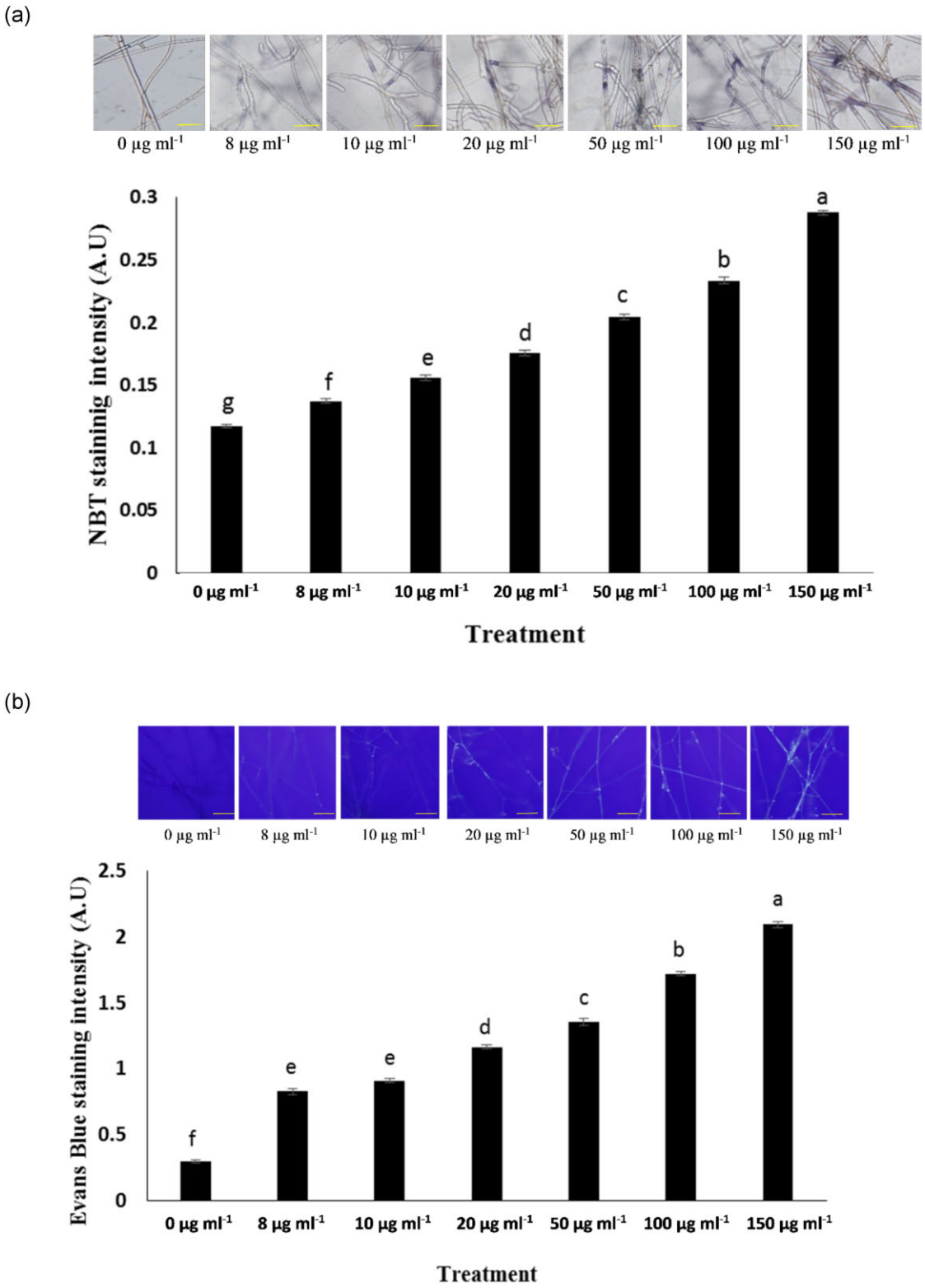

Figure 5. Effect of ergosterol peroxide on production of $O_2^{\cdot-}$ and cell death in the hyphal cells of *R. solani* AG4-HG II in concentrations of 0, 8, 10, 20, 50, 100, and 150 µg ml^{-1}. Accumulation of formazan after NBT staining for detecting $O_2^{\cdot-}$ and the staining intensities of O_2^{-} using Image J software (a), and fluorescence of dead cells in *R. solani* AG4-HG II and intensities of fluorescence in different treatments using Image J software (b). Scale bar = 20 µm. Error bars correspond to standard error of three experiments.

(without EP treatment). The maximum concentration used in the experiment (150 µg ml^{-1}) was superior to the other concentrations in decreasing the disease index on tomato leaves. To our knowledge, there are no data about effect of EP on disease index of *R. solani* on plants. However, other natural compounds obtained from different beneficial microbes have been used to decrease the disease index of *R. solani*. For example, spraying of rice plants with SPM5C-1 (an antifungal aliphatic compound obtained from *Streptomyces* sp. was effective against *R. solani* at 500 µg ml^{-1} concentration and

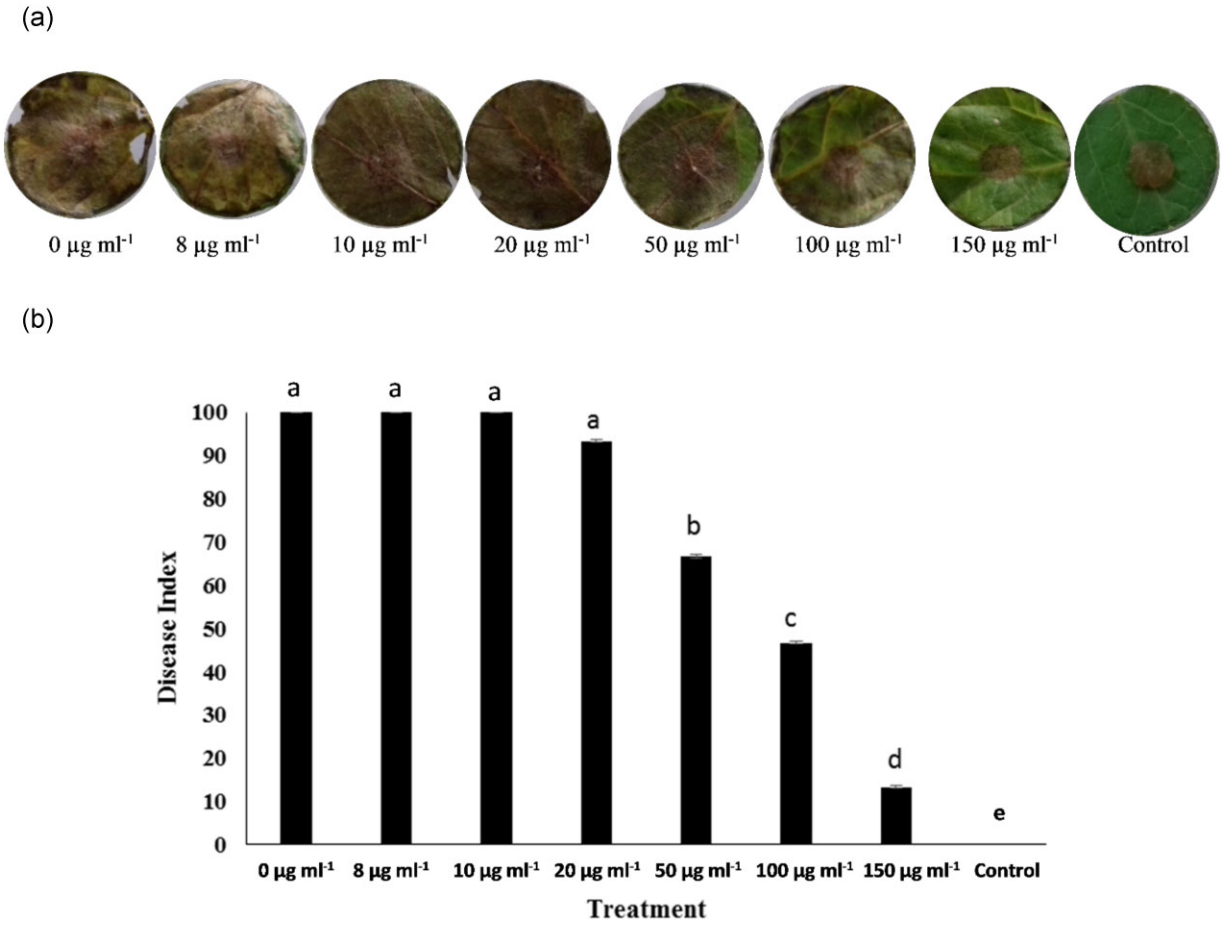

Figure 6. Effect of EP on disease symptoms (a) and disease index (b) of *R. solani* AG4-HG II on the leaf discs.

significantly decreased sheath blight development by 82.3% compared to the control (Prabavathy et al. 2006). Also, a phenazine-like compound significantly reduced lesions caused by *R. solani* AG1-IA at 5 µg ml^{-1} concentration compared to carbendazim and control after 5 d on detached rice leaves (Karmegham et al. 2020).

Conclusion

Overall, this research showed that the EP, obtained from the endophytic fungus *A. jodhpurensis*, is capable of decreasing *R. solani* AG4-HG II growth and protects tomato plants against this destructive pathogen. Therefore, the EP can be used in environmentally safe disease management strategies to control plant diseases caused by *R. solani*. More studies seem to be necessary to investigate effects of the EP against various phytopathogens. Finally, formulation of this natural compound and its application in greenhouse and field conditions might be interesting subjects for future research.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest: The authors have no conflict of interest to declare.

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Author contributions

Zoha Daroodi (Methodology, Software, Visualization, Writing – original draft, Writing – review & editing), Parissa Taheri (Conceptualization, Data curation, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing), Saeed Tarighi (Conceptualization, Investigation, Supervision, Validation, Writing – review & editing), Mehrdad Iranshahi (Methodology, Project administration, Resources, Validation, Writing – review & editing), and Maryam Akaberi (Conceptualization, Investigation, Method-

ology, Project administration, Resources, Writing – original draft, Writing – review & editing).

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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