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Effect of environmental factors and food resources on the antagonistic activity of *Acrophialophora jodhpurensis*, biocontrol agent of *Rhizoctonia solani*

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Abstract: *Rhizoctonia solani* is a destructive pathogen on several plant species. The isolates of *R. solani* AG- 2-2 IIIB are important due to their diverse host range and association with bean diseases such as web blight, hypocotyl rot, and damping off. In response to the adverse environmental effects of chemical fungicides, the possibility of resistance in pathogen populations, and unpredictable prices of chemicals, adopting alternative strategies such as the use of biological fungicides is necessary for managing the diseases caused by *R. solani*. Members of the genus *Acrophialophora*, such as *A. jodhpurensis*, are valuable sources of metabolites with diverse applications in agriculture, biotechnology, industry, and medicine. This study investigated the effects of different culture conditions on the biocontrol of *R. solani* AG-2-2 IIIB by *A. jodhpurensis*. The addition of fructose (1%), ammonium chloride (3.5%), and asparagine (0.1%) as the most effective carbon, nitrogen, and amino acid sources, respectively, significantly increased the growth rate and release of volatile and non-volatile metabolites, as well as the antagonistic activity of *A. jodhpurensis* against the pathogen. Microscopic observations showed structural changes in *R. solani* hyphae affected by *A. jodhpurensis*. Seed coating was performed with *A. jodhpurensis* spores containing 1% alginate, molasses, or Arabic gum as stickers. Among the stickers, alginate had the greatest effect in reducing the disease index and increasing growth factors in beans. Therefore, seed coating with the endophytic fungus *A. jodhpurensis* is effective in protecting beans against *R. solani*.

Keywords: Antagonistic fungus, Bean, Biological control, Culture conditions, *Thanatephorus cucumeris*.

INTRODUCTION

The fungus *Rhizoctonia solani* is one of the most important basidiomycetes and a necrotrophic pathogen affecting a wide range of plants, including beans (Palacioğlu et al. 2019, Zhang et al. 2021). This fungal pathogen poses a significant threat to a wide range of hosts, including ornamental plants, field crops, and forest trees (Molla et al. 2020, Goad et al. 2020). Common beans are susceptible to severe diseases caused by *R. solani*, including blights (both web and foliar blights), damping-off, and a variety of rots such as seed, stem, root, crown, collar, and hypocotyl rots (Basbagci & Dolar 2020, Gondle 2018, Xue et al. 2018). Furthermore, infections caused by *R. solani* are significant threats to crop yield, with losses ranging from 20% to 100% in various regions (Costa-Coelho et al. 2014, Akarca 2013, Peña et al. 2013, Palacioğlu et al. 2019). Isolates of *R. solani* AG-2-2, AG-4, and AG-5 can cause a range of bean diseases, including damping-off, root rot, and hypocotyl rot (Eken & Demirci 2004). On the other hand, various taxonomic groups of *R. solani*, such as AG-1-1A, AG-1-1B, AG-2-2, and AG-4, cause web blight (Godoy-Lutz et al. 2003, Yang et al. 2007). Also, AG-1-1E, AG-1-1F, and AG-2-2 WB, can lead to web blight in bean crops (Godoy-Lutz et al. 2008). Researchers have reported environmental conditions, such as rainy weather, moderate to hot temperatures (20-30 °C) as well as high relative humidity (more than 80%) as causes of blight epidemics (Godoy-Lutz et al. 2008, Mora-Umaña et al. 2013).

Plant vulnerability to environmental hazards, fungal resistance to chemical fungicides (Zhang et al. 2016), reduction in their effectiveness (Zhou et al. 2017, Adzmi et al. 2021), and subsequent loss of biodiversity (Ghorbanpour et al. 2018) are all factors contributing to the necessity of exploring sustainable environmental strategies such as biological control for managing fungal diseases (Ab Rahman et al. 2018). In the last two decades, researchers have devoted extensive research on endophytic fungi and other microbial antagonists to control fungal diseases (Ibrahim et al. 2020, Noumeur et al. 2020, Zheng et al. 2022). Fungal endophytes live intracellularly or intercellularly inside plant tissues and do not harm

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host plants (Singh et al. 2021, Yadav et al. 2021). Endophytic fungi represent a valuable source of antifungal metabolites. These metabolites can be influenced by a range of factors including physical and chemical parameters, such as carbon availability, nitrogen levels, amino acid composition, temperature, and pH. These essential components of various metabolic pathways significantly affect the biological processes and play a key role in the synthesis of antifungal metabolites via various pathways. Selecting a precise combination of the culture medium compounds and environmental parameters is a critical step in biological control. Carbon compounds play a vital role inside fungal cells because they constitute about half of the dry weight of fungal cells. Due to their varying carbon structures, organic nitrogen compounds can serve as substrates for certain fungi, facilitating the release of volatile and non-volatile metabolites. In addition, these compounds contain a wide range of vitamins and rare metals that show potential effects on metabolic processes. In addition, endophytic fungi participate in interactions with host plants and are reported in recent studies as key producers of natural compounds (Ludwig-Müller 2015). Therefore, food sources and environmental factors such as pH and temperature have great importance in the synthesis of antifungal metabolites by these beneficial fungi (Gunasekaran & Poorniammal 2008).

Previous studies have reported efficacy of the endophytic *Acrophialophora* spp. as potential biocontrol agents against various fungal pathogens, such as *Pythium aphanidermatum* (Ramzan et al. 2014), *Macrophomina phaseolina* and *Fusarium solani* (Ramzan et al. 2014), *Fusarium udum*, *Pythium debaryanum*, *Phytophthora capsica*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *R. solani*, and *Gaeumannomyces graminis* (Turhan & Grossmann 1989), *Alternaria alternata* (Daroodi et al. 2022), and *Alternaria porri* (Abdel-Hafez et al. 2015). Numerous studies have emphasized the effectiveness of biological control agents in seed treatments for managing fungal diseases in various crops. Rocha et al. (2019) reported that seed coating with endophytic fungi or their metabolites as an effective strategy to increase seed germination rates and protect against pathogens due to its precision, stability, and economic efficiency. In addition, El-Mougy et al. (2007) reported effective management of the diseases caused by *R. solani*, *Fusarium solani*, and *Sclerotium rolfsii* in *Vicia faba* through seed coating with *Trichoderma viride*, *T. harzianum*, and *T. hamatum*. Azadi et al. (2016) have identified significant effects of tomato seed coating with *Beauveria bassiana* in reducing infection of *R. solani* and wheat seed coating with *B. bassiana* and *Metarhizium brunneum* in reducing the disease caused by *Fusarium culmorum* (Jaber 2018).

Several studies have demonstrated the effectiveness of seed coatings with *Chaetomium globosum* in reducing the infection levels of *Fusarium roseum* f. sp. *cerealis* in *Zea mays* (Chang 1968, Kommedahl & Mew 1975), as well as *Fusarium solani* f. sp. *pisi* in *Phaseolus vulgaris*, *Cucurbita pepo*, and *Cicer arietinum* (Hubbard et al. 1982). Vilich et al. (1998) reported the efficacy of seed coating by *Chaetomium globosum* and *C. funicola* in reducing contamination by *Erysiphe graminis* f. sp. *hordei* in *Hordeum vulgare*. According to the studies by Soytong et al. (1992), seed treatments with *Chaetomium trilaterale*, *C. globosum*, and *C. cochliodes* were effective in protecting *Oryza sativa* from *Pyricularia oryzae* (Soytong 1992). Researchers have demonstrated activation of defense responses and induction of resistance in *Solanum lycopersicum* against *Alternaria solani* and *A. alternata* by seed coating with *Pestalotiopsis microspores* (Sujatha et al. 2021) and *A. jodhpurensis* (Daroodi et al. 2022), respectively. In addition, numerous studies revealed the effectiveness of endophytic fungi such as *Neocosmospora haematococca* (syn. *Fusarium haematococcum*) (Prema Sundara Valli & Muthukumar 2018), and *Fusarium* spp. (Nefzi et al. 2019) in stimulating plant growth and protecting crops against stresses. Current research aims to explore the potential of *A. jodhpurensis* as a sustainable substitute for chemical methods in the control of *R. solani* on bean plants. It includes identifying ideal environmental conditions and food sources for growth, analyzing the release of volatile and non-volatile metabolites, and evaluating the biocontrol effect of this endophytic fungus against *R. solani* AG-2-2 IIIB on bean plants under both *in vitro* and *in vivo* conditions. In addition, the research focused on controlling the disease caused by *R. solani* AG-2-2 IIIB and studying the growth factors of bean plants through this endophytic fungus and seed coating.

MATERIALS AND METHODS

Fungal isolates

The fungal strains *R. solani* AG-2-2 IIIB (BRS45) and *A. jodhpurensis* (BAJ50) were obtained from the fungal collection at Ferdowsi University of Mashhad. These strains were isolated from beans exhibiting infection in underground organs and from healthy beans, respectively, in Khorasan Razavi Province during 2021-2022. For short-term storage, the fungal strains were kept on slants containing Potato Dextrose Agar (PDA). For long-term storage, the strains were preserved on sterile filter papers colonized by each fungus.

Effect of various culture media on antagonistic activity of *A. jodhpurensis*

To select the most suitable culture medium, the endophytic fungus was initially grown on various media, such as Potato Dextrose Agar (PDA), 1/2 PDA, 1/4 PDA, Potato Carrot Agar (PCA), Oat Agar (OA), Olive Meal Agar (OMA), Malt Extract Agar (MEA), and Corn Meal Agar (CMA) (Wang et al. 2019). After incubating the Petri dishes in the dark at 28 °C for 10 days, the best culture medium was selected for further investigations.

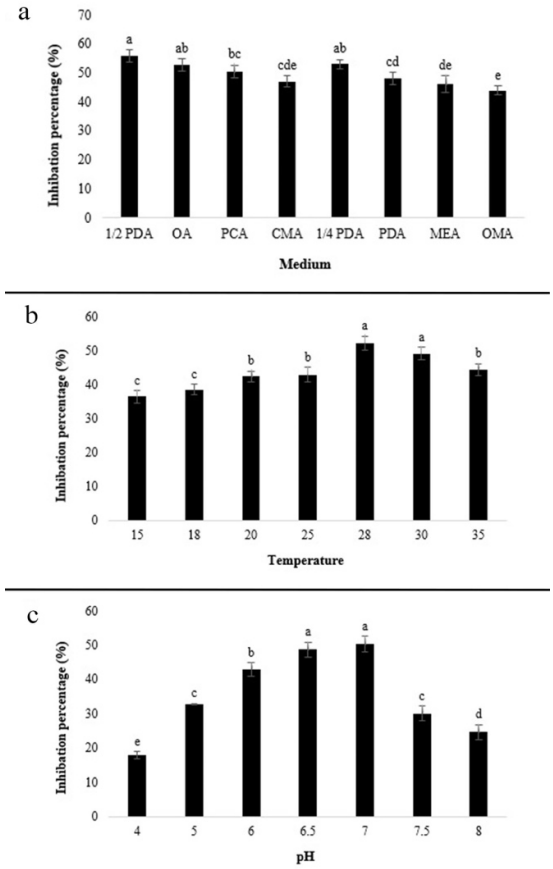

Fig. 1. Effect of culture medium (a), temperature (b), and pH (c) on inhibitory effect of *Acrophialophora jodhpurensis* against *Rhizoctonia solani* AG-2-2 IIIB on 1/2 Potato Dextrose Agar (1/2 PDA). IP: Inhibition percentage.

Effect of various temperatures on antagonistic activity of *A. jodhpurensis*

Understanding the best growth temperature is important in determining the efficacy of endophytic fungi as biological control agents. To discover the ideal temperature, the pathogenic and endophytic fungi were cultured on selected medium (1/2 PDA) and kept in complete darkness at temperatures of 15 °C, 18 °C, 20 °C, 25 °C, 28 °C, 30 °C, and 35 °C for 5 days (until the control Petri dishes only containing *R. solani* AG-2-2 IIIB display full fungal coverage) and then the antagonistic activity of the endophyte fungus was evaluated against *R. solani* AG-2-2 IIIB (Lazarević et al. 2016, Deka & Zha 2018).

Effect of pH on antagonistic activity of *A. jodhpurensis*

In large-scale fermentation processes designed to synthesis bioactive metabolites, the pH of the culture medium is of great importance due to its influence on various physiological functions of the fungus, including growth, proliferation, and nutrient efficiency (Taragano & Pilosof 1999). The formation of morphological structures and the growth of endophytic fungi are affected by the pH level, which is sensitive to hydrogen ion concentration. In addition, studies have shown its key role in metabolic processes and the synthesis of antifungal metabolites by influencing enzymatic reactions. The pH is associated with the permeability of the cell wall and membrane. Therefore, the absorption and release of hydrogen ions in the environment are influenced by this factor. Different pH levels including 4, 5, 6, 6.5, 7, 7.5, and 8 were selected for this research via addition of hydrochloric acid (HCl) and sodium hydroxide (NaOH) to the culture medium 1/2 PDA. After determining the pH levels, 1/2 PDA Petri dishes were kept at 28 °C for 5 days until the control plates showed full occupancy by the hyphae of *R. solani*. Then, the growth and antagonistic activity of *A. jodhpurensis* against *R. solani* were evaluated (Mitrović et al. 2021, Pope & Hill 1991, Deka & Jaha 2018).

Effect of carbon, nitrogen, and amino acid sources on antagonistic activity of *A. jodhpurensis*

Following the determination of the best culture medium, temperature, and pH, carbon compounds such as fructose, lactose, glucose, dextrose, sucrose, and maltose at concentrations of 0.5%, 1%, 1.5%, 2%, 2.5%, and 3% were combined with 1/2 PA. Furthermore, nitrogen sources like ammonium nitrate (NH_4NO_3), sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3), ammonium oxalate ($(NH_4)_2C_2O_4$), ammonium sulfate ($(NH_4)_2SO_4$), and ammonium chloride (NH_4Cl) were examined in a 1/2 PDA medium at concentrations between 1%, 1.5%, 2%, 2.5%, 3%, and 3.5% (Mitrović et al. 2021, Pope & Hill 1991 Deka & Jha 2018). Amino acid sources such as isoleucine, phenylalanine, serine, glycine, arginine, and asparagine at concentrations of 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, and 0.6%, were mixed in a 1/2 PDA medium (Gramisci et al. 2018).

Investigation of antagonistic activity

Initially, both *A. jodhpurensis* and *R. solani* were cultured separately on PDA at 28 °C for seven days. Then, at the best temperature and pH a hyphal plug of the antagonist (8 mm diameter), and a disc of PDA without fungal antagonist as a control, were cultured separately on one side of a Petri dish containing 1/2 PDA medium. After incubating for 48 h, a hyphal plug of the pathogen (8 mm diameter) was cultured in each Petri dish, opposite the antagonist and control discs. When control Petri dishes were filled with *R. solani* AG-2-2 IIIB hyphae (72 h), the treatments were morphologically and microscopically evaluated (BH2, Tokyo, Japan) compared to the control (Sánchez-Fernández et al. 2016). The effectiveness of *A. jodhpurensis* in inhibiting the radial growth of *R. solani* was evaluated according to the method of Asran-Amal et al. (2010). This experiment was conducted under two distinct conditions: one with the addition of nitrogen, carbon, and amino acid sources to the 1/2 PA medium at 3.5%, 1%, and 0.1% concentrations, respectively, and the other without these nutrient supplements in the 1/2 PDA medium.

Effect of volatile and non-volatile metabolites of *A. jodhpurensis*

To investigate the synthesis of volatile antifungal compounds by the endophyte, 5 mm diameter discs from young cultures of *A. jodhpurensis* and *R. solani* were cultured separately in the center of Petri dishes containing 1/2 PDA medium. Then, under sterile conditions, after placing the Petri dishes containing *R. solani* in front of the Petri dishes containing *A. jodhpurensis* and sealing them, they were incubated at 28 °C until the control Petri dishes were occupied with *R. solani*. For the control, a 1/2 PDA plate containing *R. solani* disk was placed in front of a plate without the antagonist (Nishino et al. 2013).

To study the biocontrol effects of non-volatile metabolites, two hyphal plugs from young cultures of *A. jodhpurensis* (1 cm × 1 cm) on PDA were transferred into flasks containing 100 mL of sterile Potato Dextrose Broth (PDB) medium. The flasks were then placed on a rotary shaker at 30 °C and 150 rpm for 10 days (Xiao et al. 2013). Control flasks were maintained without inoculation of *A. jodhpurensis.* Subsequently, the liquid culture was filtered through Whatman filter paper to remove hyphae and then sterilized with a 0.2 µm pore biological membrane filter (Xiao et al. 2013). Subsequently, the supernatant was transferred to 1/2 PDA medium at concentrations of 2.5%, 5%, 10%, and 15% (v/v) (Li et al. 2015). Afterward, hyphal plugs (8 mm diameter) from young cultures of *R. solani* were cultured in the center of 1/2 PDA Petri dishes containing supernatant of the antagonistic fungus. The Petri dishes were kept at 28 °C until the control Petri dishes were filled with the pathogenic fungus. To investigate the effect of non-volatile metabolites, the hyphae from the edges of young cultures of *R. solani* AG-2-2 IIIB (after 72 h) during interaction with *A. jodhpurensis* and also from control cultures without this endophyte and were examined by a light microscope (Olympus, BH2, Tokyo, Japan).

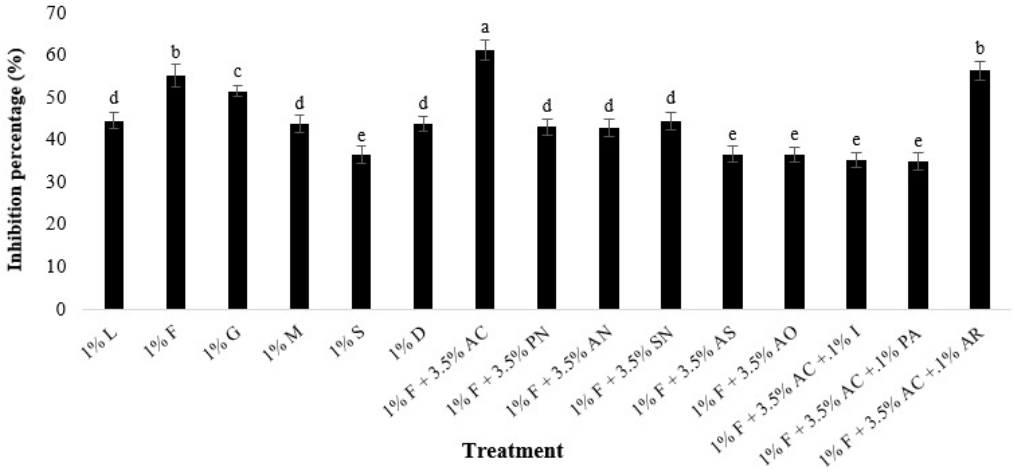

Fig. 2. Effect of carbon, nitrogen and amino acid sources on the inhibitory potential of *Acrophialophora jodhpurensis* against *Rhizoctonia solani* AG-2-2 IIIB on 1/2 Potato Agar (1/2 PA). IP: Inhibition percentage, L: Lactose, F: Fructose, G: Glucose, M: Maltose, S: Sucrose, D: Dextrose, SN: Sodium nitrate, AS: Ammonium sulfate, AC: Ammonium chloride, PN: Potassium nitrate, AN: Ammonium nitrate, AO: Ammonium oxalate, AP: Ammonium phosphate, G: Glycine, I: Isoleucine, AR: Arginine, AS: Asparagine, S: Serine, PA: Phenylalanine.

Effects of *A. jodhpurensis* on formation and germination of *R. solani* sclerotia

Hyphal plugs (8 mm diameter) from young cultures of *R. solani* AG-2-2 IIIB were cultured in the center of each 1/2 PDA medium containing non-growth supernatant at different concentrations of 0%, 2.5%, 5%, 10%, and 15% v/v, then incubated at 28 °C for 14 days. Subsequently, fresh and dry weights of sclerotia were measured after heating in a 70 °C oven for 48 h (Lu et al. 2016). On the other hand, after 30 days, 10 sclerotia were transferred to water agar medium, and germination of these sclerotia was examined under a light microscope (Olympus, BH2, Tokyo, Japan) (Mukherjee et al. 1995).

Inoculation of bean seeds with *A. jodhpurensis* and plant growth conditions

Initially, the seeds of bean cv. Yaghoot (Obtained from Bean Research Station in Khomein, Iran) was surface sterilized with 1% sodium hypochlorite solution for 2 min, followed by three times rinsing with sterile distilled water. The seeds were inoculated with spore suspension of *A. jodhpurensis* at 1×10^7 spores mL^{-1} concentration, incorporating various stickers such as 1% Arabic gum, 1% alginate (Chin et al. 2021), or 1% molasses (Daroodi et al. 2022). Control seeds were solely treated with sterile distilled water containing either 1% molasses, 1% Arabic gum, or 1% alginate solution. After drying the seeds, five of them were transferred to a falcon tube containing 10 mL of sterile distilled water to assess seed colonization. After shaking the falcon tube, measuring the spore concentration, and obtaining a concentration of 1×10^7 spores mL^{-1}, the seeds were transferred to the pots containing sterilized soil, perlite, and sand (2:1:1), and were then kept at greenhouse conditions (30 ± 4 °C with a 16/8-light/dark photoperiod).

Investigating root colonization by *A. jodhpurensis*

To investigate colonization of bean roots by *A. jodhpurensis*, following the protocol of Dingle & Mcgee (2003), the plants were removed from the soil at intervals of 0, 7, and 14 days after inoculation with the endophytic fungus, and were washed with running water. After separating the roots from the aerial parts, they were stained with cotton blue and examined under a light microscope (Olympus BH2, Tokyo, Japan) (Bajaj et al. 2018). After cutting the roots into 1 cm pieces, they were successively disinfected in a 2 % solution of sodium hypochlorite and then in 70 % ethanol for 2 min each, followed by three times immersions in sterile distilled water, and finally air-dried on sterile filter paper. Subsequently, slices of these roots were allocated into PDA Petri dishes containing 0.05% streptomycin. The Petri dishes were subsequently incubated at 25 °C for 10 days, after which fungal colonies were counted to assess root colonization (Dingle & Mcgee 2003).

Preparation of *R. solani* inoculum

For preparing inoculum of *R. solani*, after placing 15 sterile toothpicks (2 cm length) on the surface of PDA culture medium and a block of *R. solani* AG-2-2 IIIB in the center of the same Petri dish, the Petri dishes were incubated at 28 °C for 7 days (Taheri et al. 2007). Finally, the toothpicks colonized by the pathogen were placed on the plant stem at a two cm distance from the soil surface, to inoculate bean plants by the pathogen.

Effect of *A. jodhpurensis* on biocontrol of bean disease caused by *R. solani*

To evaluate the biological control effects of *A. jodhpurensis* on the disease caused by *R. solani* AG-2-2 IIIB *in vivo*, bean seeds were initially inoculated with a spore suspension of the biocontrol agent at 1×10^7 spores mL^{-1} concentration. Fourteen days later (when the plant roots were colonized by the endophyte), the plants were inoculated with *R. solani* AG-2-2 IIIB using the toothpicks colonized by the pathogen. These plants were subsequently kept in a greenhouse with 22 °C and 90% relative humidity. Disease severity progress was evaluated seven days post-pathogen inoculation, with plants removed from their pots and subjected to thorough washing. Disease severity was evaluated based on the scales including 0 = no necrotic lesion, 1 = root necrosis up to 2.5 mm in length, 2 = necrosis 2.5 to 5.0 mm in length, 3 = necrosis longer than 5.0 mm, 4 = lesions covering the crown and shoots, and 5 = seedling damping-off (Wen et al. 2005). Disease index (DI) was subsequently determined via the formula $DI = (1n_1 + 2n_2 + 3n3 + 4n4 + 5n1) \div 5N \times 100$. In this formula, the variables n_1 to n_5 represent the number of plants with disease scores of 1 to 5, respectively, and N denotes the total number of plants under investigation in the experiment (Taheri & Tarighi 2010).

Effect of *A. jodhpurensis* on growth parameters of bean seedlings

Plant growth factors, including fresh weight, dry weight, length of underground organs, and height of aerial organs, were evaluated 7 days after inoculation with *R. solani*. To determine the dry weight, after

separating the aerial parts from the underground parts and placing them in paper envelopes, they were kept in an oven at 70 °C for 48 h, and the dry weight was measured.

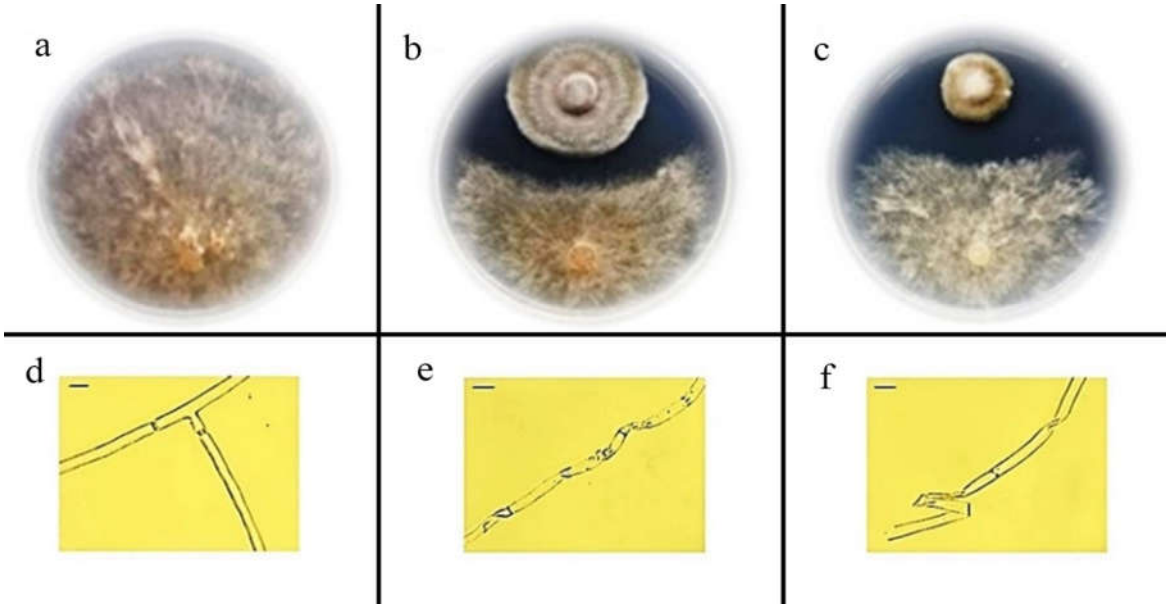

Fig. 3. Effect of *Acrophialophora jodhpurensis* on hyphal growth and structural characteristics of *Rhizoctonia solani* AG-2-2 IIIB in dual culture test on 1/2 Potato Dextrose Agar (1/2 PDA). control (a), normal (b) and optimized conditions (1/2 PDA medium, at a temperature of 28 °C, pH 7, with a combination of fructose at 1%, ammonium chloride at 3.5%, and asparagine at 0.1%) (c), normal morphology of *Rhizoctonia solani* hyphae (control; d), Structural changes and vacuolation in *R. solani* hyphae affected by *Acrophialophora jodhpurensis* (normal; e), Twisting, entanglement, breakage, and disintegration of *R. solani* hyphae affected by *A. jodhpurensis* (optimized; f). Scale bars = 20 µm.

Statistical analysis

In this research, the experiments were conducted in a completely random design. All data were subjected to analysis with Minitab 18 software via one-way analysis of variance (ANOVA). The means were separated by the Fisher test at the level of $P \leq 0.05$. Diagrams were drawn via Excel 2013. Data for each assay include mean values with their associated standard errors based on the results of three independent experiments. All tests were done with three repetitions and three replications for each treatment.

RESULTS

Effect of various culture media on antagonistic activity of *A. jodhpurensis*

Among the various culture media examined, the highest inhibition of *A. jodhpurensis* against *R. solani* AG-2-2 IIIB was recorded in the 1/2 PDA medium (Fig. 1A). Therefore, this medium was selected to be used in the rest of the experiments.

Effect of temperature on antagonistic activity of *A. jodhpurensis*

The maximum growth and antagonistic activity of *A. jodhpurensis* against *R. solani* AG-2-2IIIB were recorded at 28 °C. Therefore, this temperature was chosen as the most favorable temperature for the next tests. Conversely, lower (15 °C) and higher (35 °C) temperatures showed decreased growth and antagonistic effects of the endophyte against the pathogen (Fig. 1B).

Effect of pH on antagonistic activity of *A. jodhpurensis*

Different pH levels showed a prominent effect on the growth behavior and antagonistic potential of *A. jodhpurensis*. The growth rate and antagonistic activity of *A. jodhpurensis* against *R. solani* AG-2-2 IIIB significantly increased with increasing pH levels until pH = 7 and then decreased. The maximum growth and antagonistic activity of *A. jodhpurensis* were recorded at pH=7, which was selected as the best pH for the rest of the experiments (Fig. 1C).

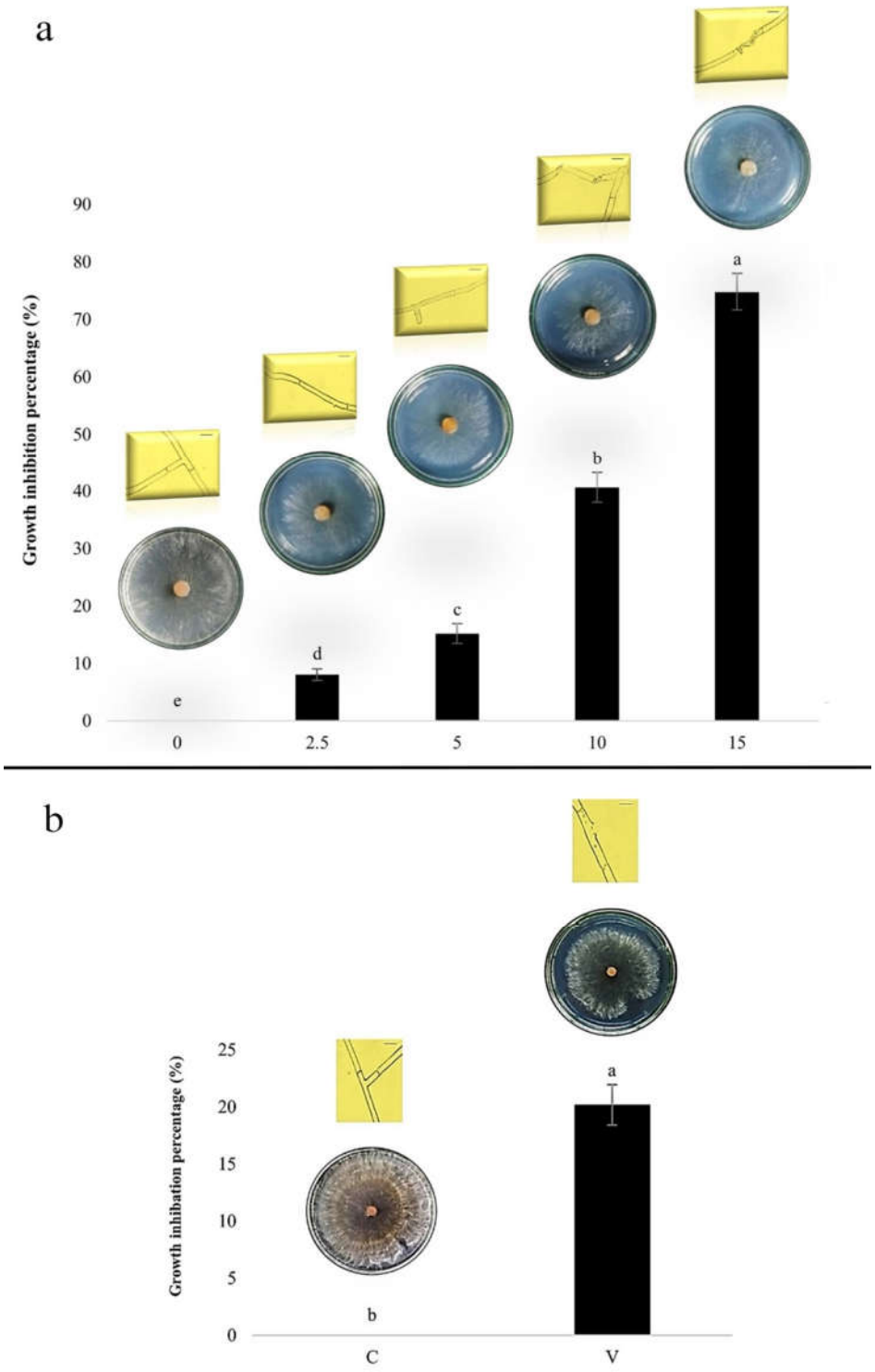

Fig. 4. Effect of *Acrophialophora jodhpurensis* growth-free supernatant (non-volatile metabolites) at concentrations of 0%, 2.5%, 5%, 10%, and 15% (v/v) on the hyphal growth and structural characteristics of *Rhizoctonia solani* AG-2-2 IIIB (a), and volatile metabolites (b) in control and under optimized conditions. C: control, V: volatile metabolites. Scale bars = 20 µm.

Effect of carbon, nitrogen, and amino acid sources on antagonistic activity of *A. jodhpurensis*

The addition of fructose (1%) as a carbon source to 1/2 PA medium maximized the growth, antagonistic activity, and release of volatile and non-volatile metabolites by *A. jodhpurensis*. Conversely, media without this carbon source showed minimal growth and antagonistic activity of *A. jodhpurensis*. Many nitrogen sources were found to be effective in promoting the growth *A. jodhpurensis* and enhancing the antagonistic effect of volatile and non-volatile metabolites against *R. solani* AG-2-2 IIIB. However, the addition of ammonium chloride (3.5%) resulted in the maximum growth and antagonistic activity against the pathogen. Amino acids enhanced the antagonistic effect of *A. jodhpurensis* against *R. solani* AG-2-2 IIIB, and among them, asparagine (0.1%) showed the best effectiveness (Fig. 2). Therefore, concentrations of 1%, 3.5%, and 0.1% for carbon, nitrogen, and amino acids, were selected for the subsequent tests, respectively.

Investigation of antagonistic activity

After culturing *A. jodhpurensis* on one side of the Petri dishes containing 1/2 PDA and *R. solani* on the opposite side, the Petri dishes were kept at 28 °C until the control Petri dishes (Fig. 3A) were fully taken over by the pathogen. After investigating the growth and antagonistic effect and determining an inhibition rate of 28% (Fig. 3B), the Petri dishes were re-examined by adding different food sources in varying amounts to maximize the parameters of growth and antagonistic effect. The inhibitory effect of *A. jodhpurensis* was significantly increased to 55.5% (Fig. 3C) by adding food sources including carbon, nitrogen, and amino acid sources to 1/2 PA medium at concentrations of 1%, 3.5%, and 0.1%. Control Petri dishes of *R. solani* showed hyphae with normal morphology (Fig. 3D). During microscopic examination, the hyphae showed structural changes and vacuolation in 1/2 PDA Petri dishes without the above food sources (Fig. 3E), while the hyphae of *R. solani* exposed to *A. jodhpurensis* in 1/2 PA Petri dishes containing carbon, nitrogen, and amino acid sources showed twisting, entanglement, breakage, and disintegration (Fig. 3F).

Effect of volatile and non-volatile metabolites of the endophyte against *R. solani*

The endophytic fungus *A. jodhpurensis* had various inhibitory effects against *R. solani* AG-2-2 IIIB at different concentrations of non-volatile metabolites. The supernatant without growth of *A. jodhpurensis* at 15% concentration had a more pronounced inhibitory effect against *R. solani* AG-2-2 IIIB compared to the other concentrations (Fig. 4A). In microscopic examinations, the hyphae of *R. solani* without the non-volatile compounds treatment (control) showed normal morphology. However, *R. solani* hyphae in Petri dishes containing non-volatile metabolites at concentrations of 2.5% and 5% underwent morphological changes, such as vacuolation. While at concentrations of 10% and 15%, they showed changes, such as folding and disintegration into small fragments, respectively. These metabolites not only inhibited the development of *R. solani* hyphae, but also caused deformation, twisting, and vacuolation (Fig. 4A). On the other hand, the volatile metabolites of *A. jodhpurensis* also had a significant effect on reducing the hyphal growth of *R. solani* compared to the control. Also, volatile metabolites of this endophytic fungus caused a wide range of morphological changes in *R. solani* hyphae, such as increased hyphal thickness, abnormal branching, vacuolation, folding, breakage, twisting and disintegration of hyphae. In contrast, the hyphae in the control Petri dishes containing *R. solani* had normal morphology (Fig. 4B).

Effects of *A. jodhpurensis* on formation and germination of *R. solani* sclerotia

The impact of *A. jodhpurensis* on the growth, differentiation, and germination of *R. solani* AG-2-2 IIIB sclerotia was explored via the addition of non-growth supernatant of *A. jodhpurensis* at different concentrations (0%, 2.5%, 5%, 10%, and 15% v/v) to 1/2 PDA medium. The control group and the group treated with *A. jodhpurensis* supernatant showed different levels of sclerotia formation. Notably, the control Petri dishes (0% concentration of culture supernatant without growth) showed more sclerotia formation compared to the treatments containing growth-free supernatant of *A. jodhpurensis* (Fig. 5A). Furthermore, in light of these findings, 10% and 15% concentrations of the growth-free supernatant exhibited significant inhibitory effects on germination of *R. solani* AG-2-2 IIIB sclerotia. This is even though 2.5% and 5% concentrations did not show a significant difference compared to the controls (Fig. 5B). In addition, the sclerotia in the control condition showed higher fresh and dry weights compared to the Petri dishes containing supernatant metabolites at different concentrations with no growth. In other terms, the 10% and 15% concentrations of these metabolites showed the highest efficacy in reducing the fresh (Fig. 5C) and dry (Fig. 5D) weights of sclerotia compared to the control Petri dishes.

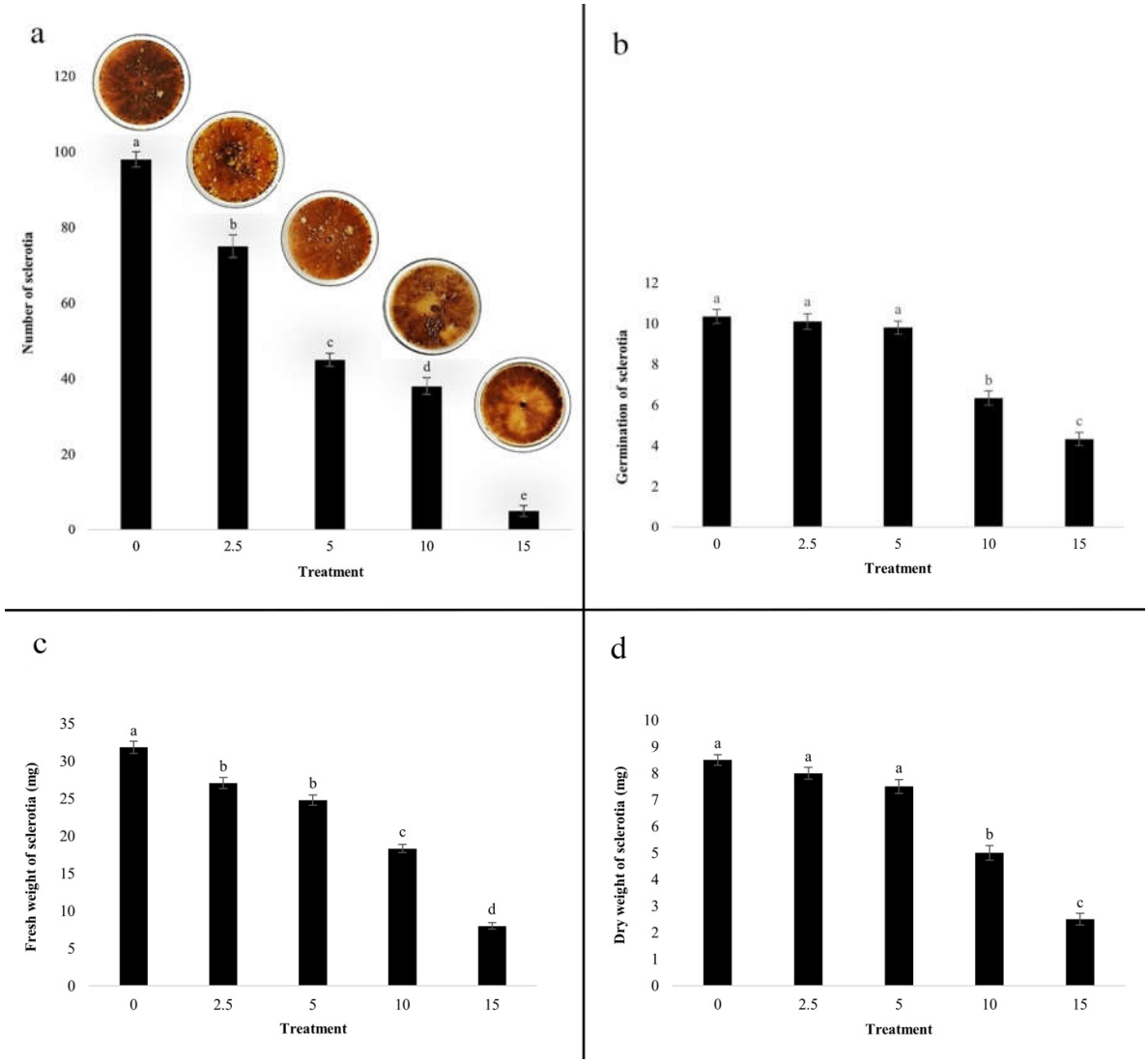

Fig. 5. Effect of growth-free supernatant (non-volatile metabolites) of *Acrophialophora jodhpurensis* at 0%, 2.5%, 5%, 10%, and 15% (v/v) concentrations on formation and germination of the sclerotia of *Rhizoctonia solani* AG-2-2 IIIB. Formation of sclerotia (a), germination of sclerotia (b), fresh weight of sclerotia (c), and dry weight of sclerotia (d).

Detection of *A. jodhpurensis* in bean roots and its *in vivo* effect on the control of *R. solani*

Bean roots were subjected to microscopic analysis to investigate the host tissue colonization by *A. jodhpurensis* at the time intervals of 0, 7 and 14 days after inoculation. Intracellular hyphae of *A. jodhpurensis* were detected within bean roots through microscopic analysis at 14 days post-inoculation (Fig. 6A). Colonization percentage of bean roots by *A. jodhpurensis* was assessed via different seed coating solutions, including spores of *A. jodhpurensis* with 1% alginate, 1% Arabic gum, and 1% molasses as stickers. Seed treatment via *A. jodhpurensis* spores in combination with alginate as a sticker showed a higher colonization percentage than those with Arabic gum and molasses (Fig. 6B). This endophytic fungus had a notable (45%) effect on suppressing the disease caused by *R. solani* AG-2-2 IIIB compared to the plants without *A. jodhpurensis* treatment (Fig. 6C).

Evaluating the impact of *A. jodhpurensis* on growth parameters of bean plants

After coating bean seeds with 1×10^7 spores mL^{-1} concentration of the endophytic fungus, plant growth parameters such as the height of aerial organs and underground parts (Fig. 7A) in plants under treatment showed a significant increase compared to the controls. Additionally, fresh weight (Fig. 7B) and dry

weight of the aerial and underground organs (Fig. 7C) significantly increased in the plants treated with *A. jodhpurensis* compared to the control plants.

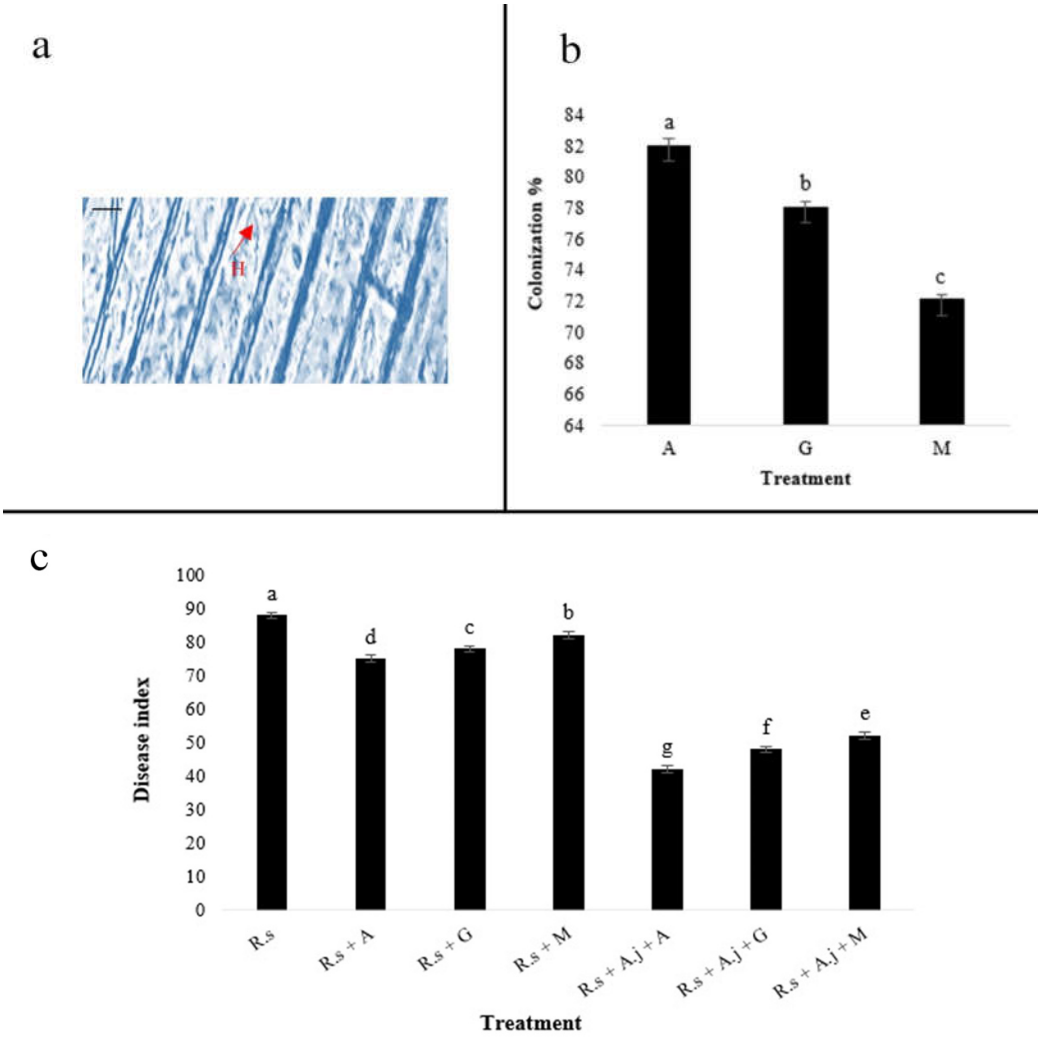

Fig. 6. Detection of *Acrophialophora jodhpurensis* in colonized roots of bean at 14 days post inoculation (a), H: *A. jodhpurensis* hyphae, colonization of bean roots with spore suspension of *A. jodhpurensis* containing 1% alginate (=A), 1% Arabic gum (=G), and 1% molasses (=M) solution as sticker at 14 days post inoculation (b), and effect of bean seed coating with spore suspension of *A. jodhpurensis* and stickers on progress of the disease caused by *Rhizoctonia solani* AG-2-2 IIIB on bean seedlings (c). R.s: inoculation with *R. solani* AG-2-2 IIIB, A.j: inoculation with *A. jodhpurensis*.

DISCUSSION

In this study, the biocontrol effect of the endophytic fungus *A. jodhpurensis* was evaluated against *R. solani* AG-2-2 IIIB pathogenic on bean plants. The growth, antagonistic activity, and release of volatile and non-volatile metabolites of *A. jodhpurensis* were significantly enhanced in 1/2 PDA medium compared to the other media investigated. Daroodi et al. (2024) also reported the highest biocontrol effect of *A. jodhpurensis* in 2% PDA culture medium. Furthermore, Neeta Sharma & Srivastava (2011) introduced oat agar (OA) as the most suitable culture medium for the growth and sporulation of *C. globosum* isolates via evaluating colonies in various culture media, such as PDA, OA, CMA, and MEA.

According to the research by Kok & Peppert (2002), temperature is an important factor in shaping the radial growth, sporulation, and inhibitory effect characteristics of antagonistic fungi. At 28 °C, *A. jodhpurensis* showed the highest growth rate, sporulation, and inhibitory effect, and it also grew

significantly at 30 °C. On the contrary, at lower (15 °C) and higher (35 °C), temperatures this endophytic fungus had the least growth and minimal inhibition effects. The morphology of *A. jodhpurensis* was affected by temperature changes, especially at 35 °C, where colonies showed specific abnormalities. The evidence from this research indicates the disruption of metabolic processes under lower temperatures and the adverse effects of higher temperatures on the structural integrity of endophytic fungal cells. Daroodi et al. (2024) recorded the maximum growth rate and antagonistic characteristics of *A. jodhpurensis* at 30 °C, parallel to the observations of Buston & Basu (1948) for *C. globosum*. Neeta Sharma & Srivastava (2011) reported 28 °C as the ideal temperature for vigorous radial growth of *C. globosum* and also showed considerable growth at 30 °C and minimal growth at 15 °C and 10 °C. The findings of these researchers were in close agreement with our data.

Fungi exhibit a wide range of acidic and alkaline growth requirements from pH 3.0 to above 8.0 (Elzwai et al. 2018). The endophytic *A. jodhpurensis* reached maximum growth and antagonistic activity against *R. solani* AG-2-2 IIIB at pH 7. Different fungi, including *A. jodhpurensis* (Daroodi et al. 2024), *Mucor racemosus*, *Aspergillus tamarii*, and *Aspergillus terreus* showed desirable growth at pH 7, and the results of the present study were consistent with the findings of these researchers. Whereas *Cladosporium* sp., *Cadophora* sp., and *Rhizopus stoloifer* favored alkaline conditions with pH 9.0 (Elzwai et al. 2018). Singh et al. (2014) identified pH 4.1 to 8.6 as the most favorable for the growth and sporulation of *Trichoderma harzianum*, *T. viride*, *T. hamatum*, and *T. asperellum*. Colonies of these fungi showed diameters greater than 7.3 cm at pH 5.1 to 5.6. However, at higher pH levels, the diameter decreased to 6.1 cm. Piwtamai et al. (2013) identified the ideal pH for the growth of *C. globosum* as between 5.5 and 6. These results did not align with our findings. Fogle et al. (2008) identified pH 6.5 as the most favorable pH for maximizing the metabolites and antagonistic activity of *G. pallida*. Additionally, in line with our results, they found neutral pH to be more favorable for the growth and maximization of *C. globosum* mycotoxins.

In this research, fructose (1%), ammonium chloride (3.5%), and asparagine (0.1%) were identified as the best sources of carbon, nitrogen, and amino acids, respectively, for growth stimulation, antagonistic capabilities, and the release of non-volatile and volatile metabolites of *A. jodhpurensis* against *R. solani* AG-2-2 IIIB. Whereas, Daroodi et al. (2024) demonstrated the importance of glucose, sodium nitrate, and asparagine as crucial sources of carbon, nitrogen, and amino acids, respectively, in *A. jodhpurensis* of tomato. Our findings were in agreement with these researchers' results only concerning the addition of asparagine, while the results for carbon and nitrogen sources did not match their findings. Buston & Basu (1948) emphasized the importance of glucose (0.5%) as the preferred carbon source for promoting the growth and sporulation of *C. globosum*. Additionally, researchers identified glucose and maltose as the most suitable carbon sources, and peptone as the optimal nitrogen source to facilitate the growth and sporulation of *C. globosum*. Barros et al. (2010) identified lactose as the preferred carbon source to achieve the highest level of endo-1,4-β-glucanase and xylanase enzymes in *Acrophialophora nainiana*. Piwtamai et al. (2013) introduced cellulose and asparagine as carbon and nitrogen sources, respectively, to stimulate the growth and sporulation of *C. globosum*. The findings of the present study were consistent with these reports only in terms of asparagine as a superior nutrient source.

In this research, volatile metabolites of *A. jodhpurensis* showed significant effects in inhibiting the growth of *R. solani* and causing morphological changes in the pathogen's hyphae during microscopic examination. Previous studies by Bjurman & Kristensson (1992) and Korpi et al. (1998) have documented the release of antifungal volatile organic compounds (VOCs) by fungi belonging to *Chaetomiaceae*, which is in accordance with our findings. In addition, Sharma et al. (1981) reported the release of antifungal volatile organic compounds (VOCs), such as geosmin, 2-phenyl ethanol, and phenylacetaldehyde from *A. nainiana*. Satyanarayana & Johri (1981) reported inhibition of conidia germination and growth of *Humicola lanuginosa* due to the release of volatile compounds by *Chaetomium thermophile*, which is in accordance with the findings of this study. The 15% concentration of non-growth supernatant had the greatest inhibitory effect on *R. solani* AG-2-2 IIIB through the release of non-volatile metabolites in this study. Sharma et al. (1981) demonstrated a delay in the onset of *Pythium* rot in *Cucurbita maxima* by treating the fruits with the non-vegetative supernatant of *A. nainiana* before inoculation with *Pythium aphanidermatum*. Results of the present research were consistent with the findings of these researchers.

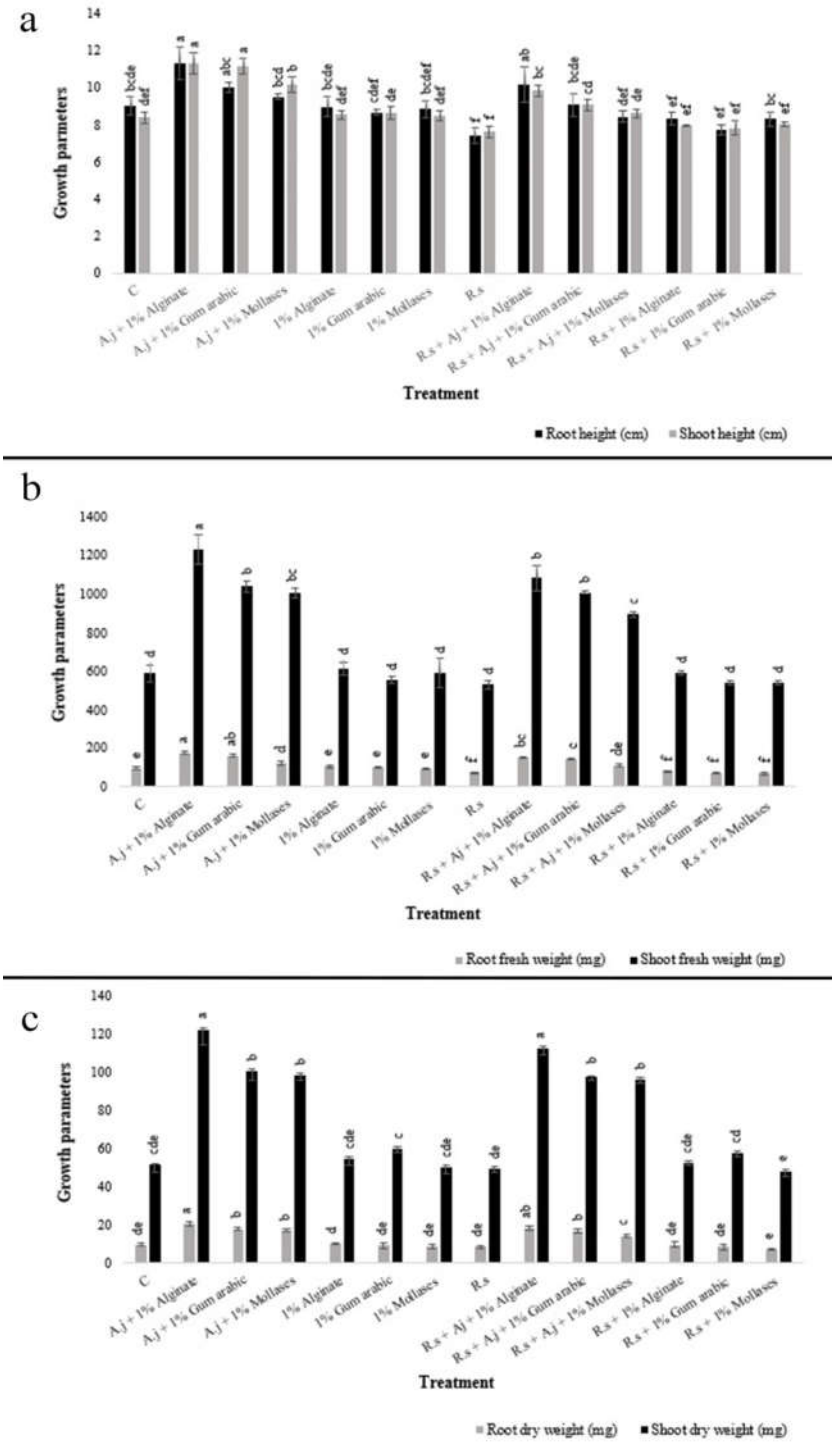

Fig. 7. Effect of bean seeds coating with spores of *Acrophialophora jodhpurensis* and stickers such as 1% alginate, 1% Arabic gum, and 1% molasses on growth factors, including length (a), fresh weight (b), and dry weight (c) of underground and aboveground organs in plants under treated with *Rhizoctonia solani* AG-2-2 IIIB and control plants. Evaluation of statistical analysis with Minitab 18 software, based on analysis of variance and Fisher's comparative test, at a significance level of $P \leq 0.05$. R.s: inoculation with *R. solani* AG-2-2 IIIB, A.j + R.s: inoculation with *A. jodhpurensis* after 14 days of inoculation with *R. solani* AG-2-2 IIIB, A.j: inoculation with *A. jodhpurensis*, C: control.

In this study, covering bean seeds with *A. jodhpurensis* had a significant effect in reducing the disease caused by *R. solani* by 45%. Another report demonstrated coating tomato seeds with *Beauveria bassiana* spores as a method to induce resistance and reduce disease severity caused by *R. solani* (Azadi et al. 2016) and our results were consistent with their findings. Although limited information is available on the antagonistic activities of other *Acrophialophora* species. Examples of other *Acrophialophora* species, such as *A. fusispora*, have shown efficacy against various plant pathogens, including *Fusarium udum* (Upadhyay & Rai 1983), *Macrophomina phaseolina* (Siddiqui & Mahmood 1992), *Rhizoctonia solani* (Demirci et al. 2011), *Fusarium solani*, *Macrophomina phaseolina*, *Pythium aphanidermatum*, *Rhizoctonia solani* and *Sclerotium rolfsii* (Ramzan et al. 2014), and *Alternaria porri* (Abdel-hafez et al. 2015). Furthermore, reports have highlighted the efficacy of *Acrophialophora* species such as *A. levis* against numerous fungus-like organisms and fungi, including *Pythium debaryanum*, *Phytophthora capsica*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Gaeomannomyces graminis*, and *R. solani* (Turhan & Grossmann 1989).

Bean plants under treatment with *A. jodhpurensis* together with sticker compounds, especially 1% alginate, showed a significant increase in growth factors, including fresh weight, dry weight, and the length of both aerial and underground parts, compared to the control plants. Similar to these results, Khan et al. (2012) reported stimulation of growth characteristics in *Piper nigrum* due to seed coating with *C. globosum*, through the secretion of gibberellins and indole acetic acid. Meanwhile, Zhai et al. (2018) obtained similar results in the growth parameters of *Salvia miltiorrhiza* through seed coating with *C. globosum*. Consistent with our findings, researchers have documented increases in plant growth parameters by coating *Solanum lycopersicum* seeds with *Piriformospora indica* (Fakhro et al. 2010), *Trichoderma hamatum* and *Trichoderma atroviride* (Tucci et al. 2011), as well as *Penicillium simplicissimum* (Khan et al. 2015). Colla et al. (2015) showed an increase in plant biomass, yield, and seed quality as a result of coating *Triticum aestivum* seeds with *Glomus mossae*, *G. intraradices*, and *Trichoderma atroviride*. In agreement with our findings, Cardarelli et al. (2019) have also recorded an increase in the yield and quality of *Cynara cardunculus* subsp. *scolymus* due to seed coating with *Trichoderma koningii* and arbuscular mycorrhizal fungi.

CONCLUSION

Different culture parameters, such as nutritional supplements, their concentration, and environmental conditions, as well as the interaction between these physical, chemical, and biological factors, play a fundamental role in influencing the release of bioactive metabolites by endophytic fungi. The results of this research demonstrated significant changes in the growth of hyphae and the antagonistic effect of *A. jodhpurensis* against *R. solani* AG-2-2 IIIB in both laboratory and *in vivo* conditions, under the influence of various culture conditions. Understanding the variables affecting the biological activities of *A. jodhpurensis* to maximize its effectiveness against phytopathogens is of considerable importance. The results of this study provide valuable guidance for researchers, who are seeking to identify the best nutrient sources and examining temperature and pH variables. Based on these results, *A. jodhpurensis* isolate BAJ50 has favorable characteristics for commercial biofungicide formulation suitable for sustainable agricultural practices. Therefore, considering the antimicrobial properties of *A. jodhpurensis*, continuous efforts are required to investigate the effect of other isolates of this endophyte with practical field applications in crop protection. Comprehensive research to explore the efficacy of *A. jodhpurensis* against other fungal pathogens, as well as safety evaluations for humans and animals, is of considerable importance before large-scale applications. In this regard, the formulation of *A. jodhpurensis* or its metabolites and the evaluation of their field applications can be interesting subjects for future research.

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اثر عوامل محیطی و منابع غذایی بر فعالیت آنتاگونیستی *Acrophialophora jodhpurensis* و پوشش‌دهی بذر با آن برای کنترل *Rhizoctonia solani*

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چکیده: قارچ *Rhizoctonia solani* یک بیمارگر مخرب بر روی میزبان های گیاهی مختلف است. جدایه های *R. solani* AG-2-2 IIIB به دلیل طیف میزبانی متنوع و ارتباط با بیماری های لوبیا از قبیل سوختگی شبکه ای، پوسیدگی هیپوکوتیل و مرگ نهال از اهمیت قابل توجهی برخوردار هستند. در پاسخ به اثرات نامطلوب زیست‌محیطی قارچ‌کش‌های شیمیایی، امکان مقاومت در جمعیت‌های بیماری‌زا و قیمت‌های غیرقابل پیش‌بینی مواد شیمیایی، اتخاذ استراتژی‌های جایگزین مانند قارچ‌کش‌های بیولوژیکی برای مدیریت بیماری‌های ناشی از *R. solani* ضروری می باشد. اعضای جنس *Acrophialophora*، همچون *Acrophialophora jodhpurensis*، منابع ارزشمندی از متابولیت ها با کاربردهای متنوع در کشاورزی، بیوتکنولوژی، صنعت و پزشکی هستند. در این مطالعه، تاثیر شرایط مختلف کشت بر کنترل زیستی *R. solani* AG-2-2 IIIB توسط *A. jodhpurensis* بررسی گردید. افزودن فروکتوز (۱٪)، کلرید آمونیوم (۳/۵٪) و آسپاراژین (۰/۱٪) به ترتیب به عنوان مؤثرترین منابع کربن، نیتروژن و آمینو اسید به طور چشمگیری باعث افزایش رشد و ترشح متابولیت های فرار و غیرفرار و همچنین فعالیت آنتاگونیستی *A. jodhpurensis* در برابر بیمارگر شد. مشاهدات میکروسکوپی تغییرات ساختاری را در ریسه *R. solani* تحت تاثیر *A. jodhpurensis* نشان داد. پوشش دهی بذر با اسپورهای *A. jodhpurensis* حاوی ۱٪ آلژینات، ملاس یا صمغ عربی به عنوان ترکیباتی با خاصیت چسبندگی صورت گرفت. در میان این ترکیبات، آلژینات بیشترین تأثیر را در کاهش شاخص بیماری و افزایش فاکتورهای رشدی در لوبیا نشان داد. بنابراین پوشش دهی بذر با قارچ اندوفیت *A. jodhpurensis* در محافظت از لوبیا در برابر *R. solani* تاثیرگذار است.

کلمات کلیدی: شرایط کشت، قارچ آنتاگونیست، کنترل زیستی، لوبیا، *Thanatephorus cucumeris*

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