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Potential of *Epicoccum purpurascens* Strain 5615 AUMC as a Biocontrol Agent of *Pythium irregulare* Root Rot in Three Leguminous Plants

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***Epicoccum purpurascens* stain 5615 AUMC was investigated for its biocontrol activity against root rot disease caused by *Pythium irregulare*. *E. purpurascens* greenhouse pathogenicity tests using three leguminous plants indicated that the fungus was nonpathogenic under the test conditions. The germination rate of the three species of legume seeds treated with a *E. purpurascens* homogenate increased significantly compared with the seeds infested with *P. irregulare*. No root rot symptoms were observed on seeds treated with *E. purpurascens*, and seedlings appeared more vigorous when compared with the non-treated control. A significant increase in seedling growth parameters (seedling length and fresh and dry weights) was observed in seedlings treated with *E. purpurascens* compared to pathogen-treated seedlings. Pre-treating the seeds with the bioagent fungus was more efficient for protecting seeds against the root rot disease caused by *P. irregulare* than waiting for disease dispersal before intervention. To determine whether *E. purpurascens* produced known anti-fungal compounds, an acetone extract of the fungus was analyzed by gas chromatography mass spectrometry. The extract revealed a high percentage of the cinnamic acid derivative (trimethylsiloxy) cinnamic acid methyl ester. The *E. purpurascens* isolate grew more rapidly than the *P. irregulare* pathogen in a dual culture on potato dextrose agar nutrient medium, although the two fungi grew similarly when cultured separately. This result may indicate antagonism via antibiosis or competition.**

KEYWORDS : Biocontrol, Pathogenicity, *Pythium irregulare*, Root rot

Legumes are a primary source of protein in human diets and animal feed worldwide but diseases caused by fungi and viruses limit their yield and quality [1]. The seeds of the annual legumes *Vicia faba*, *Vigna unguiculata*, and *Lupinus termis* are vital for the daily human nutrition of Egyptians, as they are protein-rich seeds. Cultivated *V. faba* is used as a human food and as an animal feed either green or dried, fresh or canned. It is a common breakfast food in the Middle East, Mediterranean region, China, and Ethiopia and contains a wide variation in protein content among the different varieties (20~41%) [2, 3]. According to Zohary and Hopf [4], both *V. unguiculata* and *L. termis* are appreciated food crops rich in protein content (22.5~53.7% in dry seeds), and they are cultivated in some Mediterranean countries, particularly Egypt. In Egypt, root rot disease of leguminous plants induced by *Pythium* spp. is considered important, especially due to its prevalence, particularly in the new reclaimed land of the desert [5]. It is estimated that *Pythium* diseases are responsible for billion dollar losses around the world [6].

Several reports on the pathogenicity of soil and waterborne *Pythium* spp. indicate that it causes root rot in many economically important agronomic and vegetable plants in the early seedling stage [7-9]. Within 6~12 hr after planting in *Pythium*-infested soil, nearly all cotton seeds are heavily colonized and rotted [10]. Several species of *Pythium* (*P. dissotocum*, *P. acanthicum*, *P. torulosum*, and *P. rostratum*) reduce root system length, while others (*P. ultimum*, *P. irregulare*, and *P. sylvaticum*) cause pre- or post-emergence damping-off, necrosis, and stunting of root and shoot growth in *Medicago sativa* plants in greenhouse pathogenicity tests [11, 12]. Four species of *Pythium* caused significant root rot and reduced growth of mature pepper plants in Florida [13]. *Pythium* species such as *P. aphanidermatum*, *P. debaryanum*, *P. myriotylum*, and *P. ultimum* cause damping-off diseases such as root rot, seedling blight, and stem rot of many plants, including agronomic and vegetable crops [14]. *P. irregulare* has been reported on all major continents except Antarctica and identified on over 200 host species including cereals [15, 16]. This pathogenic fungal species cause root rot in seedlings and older plants [17].

Biological control of plant disease is currently receiving increased research effort to enhance the sustainability of agricultural production systems and to reduce the use of chemical pesticides [18]. Studies have been conducted on the biological control of root rot diseases caused by the cosmopolitan soil-borne *Pythium* spp. growing extensively worldwide but the full potential for biocontrol of these pathogens has not been explored [19]. Several reports indicate the use of fungal species as promising and successful biocontrol agents against root rot diseases of agronomic and vegetable crops caused by pathogenic *Pythium* spp. Treating seeds with *Trichoderma* formulations enhances

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plant biomass under greenhouse and field conditions [20].

Biological control agents and plant pathogenic fungi compete for nutrients as a mode of biocontrol [21-24]. The release of diffusible inhibitors (antibiosis) can affect the hyphae of the host before contact with the antagonist occurs [20, 25].

The goal of this investigation was to assess the potential of *Epicoccum purpurascens* strain 5615 AUMC to manage *P. irregulare* root rot diseases in three legumes. Further, the mode of action of this potential biocontrol agent was partially explored.

Materials and Methods

Preparation of the biocontrol agent homogenate for pathogenicity tests. *E. purpurascens* was isolated from several desert soil samples, which were close to human graves in Assiut Governorate, Egypt. The isolate was deposited at the Assiut University Mycological Center (5615 AUMC). The bioagent species was cultured on Sabouraud agar in Petri dishes at 25°C for 1 wk. Thereafter, mycelial growth (1.5 g biomass) from 7-day-old colonies was harvested by gently scraping the surface with a spatula and crushed in 100 mL of sterilized distilled water under aseptic conditions using a blender. The homogenate, containing all the mycelial fragments of the bioagent, was stored at 5°C for up to 7 days before being used in greenhouse (pot experiment) and laboratory (*in vitro* bioassay) pathogenicity tests.

Seed types. Three types of legume plant seeds (*V. faba*, *V. unguiculata*, and *L. termis* were purchased and collected from El-Quisarria regional markets in Assiut city, Assiut Governorate, Egypt. They were used in the greenhouse and laboratory pathogenicity tests, which were aimed mainly at studying the germination ability of these seeds under different treatments. Additionally, possible root rot infection of these legumes by *P. irregulare* and the potential role for *E. purpurascens* to protect these seeds from infection was investigated.

***E. purpurascens* greenhouse experiment (pot experiment).** The *E. purpurascens* isolate was tested for pathogenicity in the greenhouse using three different annual legumes, *V. faba, V. unguiculata*, and *L. termis*. The previously prepared fungal mycelia water suspension (100 mL) was mixed with 6 kg of sterilized (to avoid interference with any other microorganisms) normal clay field soil in pots. Ten seeds from a single legume species were used in each pot and nine pots were treated with *E. purpurascens*. An additional nine pots (control) were prepared in the same manner but the sterilized soil was mixed with sterile distilled water (100 mL). The pots measured 25 cm in height and ranged from 26 (at the top) to 21 (at bottom) cm in width. Seeds of the three tested plants were cultivated in the pots and watered regularly. Three design replicates (observation) were planted for each seed species and treatment. After 3 wk of cultivation, the seedlings were examined for any symptoms or signs of infection by *E. purpurascens* and compared with the control pots.

Acetone extract preparation of the biocontrol agent for gas chromatography mass spectrometry (GC/MS) analysis. One-wk-old mycelial growth of *E. purpurascens* on Sabouraud agar was collected and crushed in acetone (1.5 g of the fungal biomass in 100 mL acetone at ambient temperature) using a mortar. The acetone extract suspension was centrifuged for 10 min at 600 ×g, and the supernatant was used for GC/MS analysis at the analytical chemistry unit, Chemistry Department, Faculty of Science, Assiut University.

Isolation of the *P. irregulare* pathogen. *P. irregulare* was isolated from field soil at a site where the three species of leguminous plants grew in the Assiut area, Assiut Governorate. The *P. irregulare* isolate was deposited in our laboratory of aquatic fungi, Botany Department, Faculty of Science, Assiut University and given the name PILAF85.

Pathogen purification. Potato dextrose agar (PDA) medium [26] was used to purify and subculture the *P. irregulare*. This medium was also used to conduct the dual culture experiment.

Dual cultures. Agar discs (1 cm) were excised from the margins of actively growing 1-wk-old cultures of the *E. purpurascens* bioagent fungus and placed at the opposite side of the *P. irregulare* pathogen in Petri dishes, which were incubated at 25 ± 1°C. The growth rates of the fungi (the bioagent and the pathogen) in dual cultures were monitored after 12 days of incubation to assess competition. Petri dishes without antagonistic fungi were used as a control.

Preparation of the *P. irregulare* pathogen inoculum. *P. irregulare* PDA mycelial discs were cut using a cork borer (1 cm diameter) from the actively growing margins of the pathogen colonies, and two discs were introduced into 12-cm Petri dishes containing 20 mL of sterilized distilled water and five germinating sesame seeds. Thereafter, the Petri dishes were incubated at 24°C for 7 days for pathogen sporogenesis and zoospore release. Aliquots of encysted zoospores of specific volumes (1.5 L stock) were harvested under aseptic conditions by filtration using hardened, sterilized filter paper (Whatman No. 1) in which the pathogen mats were adsorbed on the filter paper. The encysted zoospores (1×10^3/mL) were kept in a refrigera-

tor and used for the *in vitro* assays of the three types of seeds in the laboratory experiment (Petri dish technique). Zoospore taxis and encystment are of vital importance to the pathogenicity of *Phytophthora* and *Pythium* spp.

***In vitro* assays (laboratory experiment).** The three types of seeds (*V. faba*, *V. unguiculata*, and *L. termis*) to be tested for pathogenicity were surface sterilized with 70% ethanol for 3 min, followed by 1% sodium hypochlorite for 1 min, and rinsed with sterile distilled water five times prior to soaking in the different treatments. Thirty seeds of each legume species were soaked for 10 hr in 250 mL Erlenmeyer conical flasks under aseptic conditions using 150 mL aliquots of the four separate treatments prior to transplanting. These four treatments were: (i) sterilized distilled water for non-inoculated control samples (receiving neither pathogen nor biocontrol amendments), (ii) a suspension of *P. irregulare* encysted zoospores, (iii) an *E. purpurascens* water homogenate, which was the biocontrol agent, and (iv) equal volumes (mixture) of *P. irregulare* and *E. purpurascens*. Treated seeds (10 seeds/Petri dish) of each seed type and each treatment were distributed into sterilized Petri dishes (12 cm in diameter), which contained sterile moist Whatman No. 1 filter paper laid in the bottom. Three Petri dishes were used for each seed species and each treatment. Thereafter, the Petri dishes were covered with lids and incubated at 15~20°C in the dark for 2 wk. The seeds were periodically inspected, and germinating seeds were counted in each Petri dish.

Estimation of root discoloration (root rot severity) and seedling length. Seedlings were examined visually for symptoms of *Pythium* root rot at the end of the laboratory experiment. Root discoloration (root rot severity) was estimated and expressed as the percentage of the root system that had changed color and showed browning symptoms. The number of seedlings with *Pythium* symptoms was scored. Root discoloration (root rot) was selected as a criterion for disease assessment. Germinating seeds (seedlings) were spread over a clean surface and the length of the primary root and the main axis of seedlings stem were measured to the nearest millimeter using a ruler.

Disease assessment. Root rot severity of the three types of seedlings was assessed using a rating scale (disease index) of 0~4 [27, 28] where, 0 = no disease symptoms, 1 = 1~25%, 2 = 26~50%, 3 = 51~75%, and 4 = 76~100% root rot.

Determination of seedling fresh and dry weights. Seedlings were weighed directly at the end of the experiment to determine fresh weight. The dry weights of seedlings were determined by drying the plant material in an oven at 60°C for 24 hr prior to weighing.

Statistical analysis. The laboratory *in vitro* assays were repeated twice, and the two tests showed no significant differences for the different treatments. The data obtained during *in vitro* assays are reported as mean value of three replicates for the two combined tests. Data were subjected to a one-way analysis of variance, and means were compared using the PCSTAT computer program (PC STAT, Nashville, TN, USA). Correlation analysis was used to determine the differences between the estimated parameters for the pathogen treatment and for those of the remaining treatments. The means were compared, and significant differences were identified with the least significant difference test at $p < 0.05$ was considered statistically significant.

Results

Greenhouse *E. purpurascens* pathogenicity test (pot experiment). The *E. purpurascens* isolate was tested for pathogenicity using three different leguminous plants (*V. faba, V. unguiculata* and *L. termis*). Results of the pathogenicity test for our fungal isolate in the greenhouse (pot experiment) showed that *E. purpurascens* did not cause any symptoms or signs of infection on seedlings of the tested plants. The results confirmed that *E. purpurascens* is a nonpathogenic fungal isolate. Notably, treating the soil with *E. purpurascens* before cultivation enhanced plant growth, particularly in the case of broad bean compared with control plants. Soil treated with the *E. purpurascens* homogenate seemed healthy and vigorous compared with untreated control plants.

These preliminary data encouraged us to analyze the characteristics of this isolate and to search for possible biocontrol activity in protecting these three important leguminous plants against root rot disease caused by *P. irregulare*.

GC/MS analysis. A GC/MS analysis was conducted to gather information about the possible chemical composition of the bioagent extract. GC/MS was helpful for understanding the antagonistic nature of *E. purpurascens*. The GC/MS results presented in Table 1 revealed that the potential biocontrol fungus had D-arabinitol as a major component, which represented approximately 29% of the total acetone extract. The most prominent finding in this

Table 1. Percentages of the two major components of the *Epicoccum purpurascens* acetone extract and their molecular weights as indicated by gas chromatography mass spectrometry analysis

Components	Molecular weight	% of total extract
D-Arabinitol	152	29.294
p-(Trimethylsiloxy)cinnamic acid methyl ester	250	15.175

analysis was the presence of P-(trimethylsiloxy) cinnamic acid methyl ester (approximately 15%) in the fungal acetone extract. This compound has direct antifungal activity against several fungal species.

Fig. 1. Dual culture showing competition for space and nutrients between the *Pythium irregulare* pathogen (white restricted growth) and the *Epicoccum purpurascens* potential bioagent fungus (reddish wide growth) on potato dextrose agar medium after 12 days of incubation.

Dual culture test. Neither inhibition zones nor overgrowth between the tested bioagent *E. purpurascens* and the pathogen *P. irregulare* were formed in dual cultures. The potential biocontrol agent (*E. purpurascens*) showed prominent competition for nutrients and space compared with the pathogen (*P. irregulare*) in dual cultures (Fig. 1) on PDA medium. Both the bioagent fungus and the pathogen showed more or less equal growth zones on the PDA medium when they were grown for 12 days on separate Petri dishes.

***In vitro* assays (laboratory experiment): seed germination ability, root rot diseases, and length and weight of seedlings of the three species of legumes in the different treatments.** As indicated in Table 2 and Figs. 2 and 3, soaking the three types of seeds in a suspension of encysted zoospores of the pathogenic fungal species *P. irregulare* for 10 hr resulted in the germination of nearly equal numbers of the three types of seeds, representing 50.5~53.0% of the total number of soaked seeds. This result indicated that seeds dressed in the *P. irregulare* pathogenic inoculum had significantly lower germination ability compared to that of the control treatments. Some of the growing legume seedlings suffered from apparent

Table 2. Effects of soaking of three legume seeds (A, B, C) at different treatments (control, pathogen, biocontrol agent and mixture of the pathogen and the biocontrol agent) for 10 hr on seed germination, % of root discoloration, length of seedlings, and fresh and dry weights of seedlings

Treatments	Seeds type	Number of germinating seeds	% of germinating seeds	% of root discoloration	Seedlings length (cm)	Fresh weight (g/30 seedlings)	Dry weight (g/30 seedlings)
Control	A	29[b]	96.67	0	2.97	31.33	15.29
	B	30[a]	100.00	0	6.03[a]	12.06[a]	2.90[a]
	C	25[a]	83.33	0	4.20	8.74[a]	2.92[a]
Pythium irregulare (Pathogen)	A	16[b]	53.33	18.75	2.20	31.30	14.41
	B	16[b]	53.33	62.50	2.47[b]	9.40[b]	2.59[b]
	C	15[b]	50.00	46.67	4.07	6.55[b]	2.71[b]
Epicoccum purpurascens (Biocontrol agent)	A	30[a]	100.00	0	3.73[a]	38.31[a,b]	17.60[a,b]
	B	30[a]	100.00	0	4.47[a,b]	13.02[a]	3.20[a,b]
	C	25[a]	83.33	0	5.27[a,b]	9.36[a]	3.05[a,b]
P. irregulare and *E. purpurascens*	A	25[a]	83.33	0	2.67	33.90[a,b]	16.76[a,b]
	B	28[a]	93.33	21.43	3.97[a,b]	11.22	2.81
	C	23[a]	76.67	0	4.20	8.81[a]	2.73[b]
LSD ($p = 0.05$) compared with pathogen treatment	A	5			1.07	1.75	1.06
	B	7			1.34	1.76	0.28
	C	5			0.48	1.67	0.11
LSD ($p = 0.05$) compared with control treatment	A	4			0.90	1.67	1.43
	B	6			1.41	1.73	0.25
	C	4			0.51	1.38	0.09

A = *Vicia faba*, B = *Vigna unguiculata*, C = *Lupinus termis.* The root rot disease index was a 0~4 scale where: 0 = no disease symptoms, 1 = 1~25% of seedlings, 2 = 26~50%, 3 = 51~75%, and 4 = 76~100%.

LSD: least significant difference.

[a]Significant difference compared with the pathogen.

[b]Significant difference compared with the control.

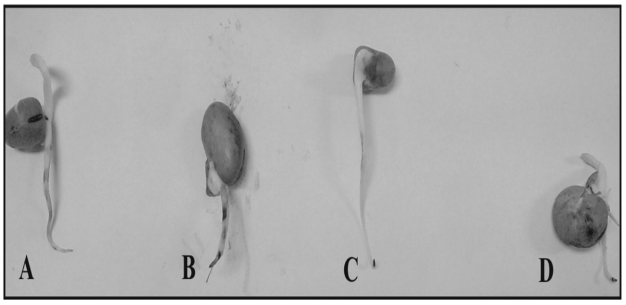

Fig. 2. *Vicia faba* seedlings after a 2 wk incubation with various treatments. A, Control seedling; B, Root rot disease symptoms appeared after soaking the seeds in the *Pythium irregulare* inoculum pathogen; C, Healthy and vigorous seedling after soaking the seeds in the homogenate of the *Epicoccum purpurascens* bioagent fungus; D, Seedling showing recovery from root rot disease after soaking in a mixture of the pathogen and the bioagent fungus.

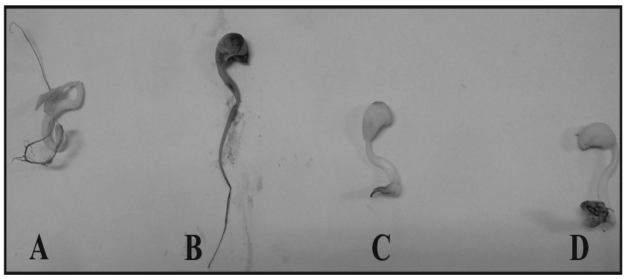

Fig. 3. *Vigna unguiculata* seedlings after a 2 wk incubation in various treatments. A, Control seedling; B, Root rot disease symptoms appeared after soaking the seeds in the *Pythium irregulare* inoculum pathogen; C, Healthy seedling after soaking the seed in the homogenate of the *Epicoccum purpurascens* bioagent fungus; D, Seedling showing recovery from root rot disease after soaking in a mixture of the pathogen and the bioagent fungus.

root rot symptoms (Figs. 2 and 3), and these seedlings showed variability in their pathogen susceptibility. The percentage of root rot (Table 2) in surviving seedlings (of the total number germinating seeds) due to the pathogen inoculation was highest for *V. unguiculata* and *L. termis* (62.50 and 46.67%, respectively), whereas root rot in *V. faba* seedlings was the lowest (18.75%) among the three plant species. Root rot was not observed in the germinating seedlings of the three control plant species. The results, also indicated that soaking seeds of *V. faba* and *V. unguiculata* in the pathogen suspension significantly reduced the length of germinating seedlings as compared with the control treatment, but it resulted in a non-significant reduction in the length of *L. termis* seedlings (Table 2). The seedling fresh and dry weights of the three species soaked in the *P. irregulare* pathogen inoculum decreased compared with the seedling weights of the controls, and this difference was significant in the case of *V. unguiculata* and *L. termis* (Table 2).

The data shown in Table 2, Figs. 2 and 3 show the promising role of *E. purpurascens* as a bioagent fungus, as dressing seeds of the three legume species in the fungal homogenate resulted in significant increments in the number of germinating seeds of the three plant species when compared with the pathogen treatments. All *V. faba* and *V. unguiculata* seeds soaked in the potential biocontrol agent homogenate germinated completely. A visual inspection of the germinating seedlings of the three legume species treated with the bioagent fungal homogenate showed no signs or symptoms of root rot disease (Table 2). Furthermore, these seedlings were healthy and vigorous compared with those in the control treatment (Figs. 2 and 3). The improvement in the seedlings due to the protective role of the bioagent fungus was reflected by the length measurements of the germinating seedlings as well as by the fresh and dry weights (Table 2) of the three leguminous plants, as all of these indicators tended to be higher than those recorded for the pathogen treatments.

Dressing the seeds in equal volumes of the bioagent fungal extract and the pathogen inoculum suspension increased the percentage of germinating seeds of the three plant species to values nearly close to seeds treated in the bioagent fungus homogenate alone (Table 2). A mixture of the potential bioagent fungus and the pathogen significantly reduced the percentage of germinating seeds to two-fold compared to the effect of the pathogen alone. The seedlings of *V. faba* and *L. termis* did not suffer from any symptoms of root rot diseases after mixing the bioagent fungus with the pathogen inoculum, indicating that our tested bioagent fungus could successfully compete with the pathogen infection. However, only about 21% of the *V. unguiculata* germinating seedlings had rotted roots (Table 2). Combining the pathogen and the biocontrol fungus protected the three seed species and the germinating seeds showed either no or only a lower percentage of root rot symptoms than controls (Figs. 2 and 3). Furthermore, disease severity sharply decreased compared with the pathogen-treated seeds. The length as well as the fresh and dry weights of the seedlings of the three plant species tended to increase compared to seedlings attacked by the pathogen and fluctuated between values near to that of the bioagent homogenate and the control treatments (Table 2).

Discussion

Our test results of the *E. purpurascens* isolate on the three leguminous plants in the greenhouse indicated that this isolate was not pathogenic. This *E. purpurascens* isolate is not only non-pathogenic but it promoted vigorous growth

in the treated legume seedlings relative to untreated controls. Similar treatments of tomato seeds with *Trichoderma* formulations enhance plant biomass under greenhouse and field conditions [20]. However, we tested our *E. purpurascens* isolate for its pathogenicity because some isolates of this fungal species have been reported as a causal organism of leaf spot disease in oats [29].

The collective results of the *E. purpurascens* pathogenicity test in the greenhouse and the seedling growth results of the three legumes were helpful to identify a potential role for the biocontrol of root rot disease in these legumes caused by *P. irregulare*.

The GC/MS analysis of the fungal extract was conducted to explore the mode of action of this potential biocontrol agent. The two active major components were D-arabinitol and P-(trimethylsiloxy) cinnamic acid methyl ester, which represented approximately 29 and 15% of the total components of the extract, respectively. This result agreed with previous results of an *E. purpurascens* pathogenicity test. Cinnamic acid and/or its derivative acid have demonstrated antifungal activity against several phytopathogenic fungi. Tawata *et al*. [30] reported that cinnamic acid and its derivatives have antifungal activity against the fungus *Pythium* sp. Furthermore, Brown *et al*. [31] detected six antifungal compounds in *E. purpurascens* cultures grown in two selective media. Four of these compounds, epicorazines A and B and two unknown compounds (X and Y) (designated in the epicorarine fraction) were produced simultaneously at an early stage in the growth of *E. purpurascens* in a sucrose plus casamino acid medium but were not detected in a glucose plus NH_4HPO_4 medium. Flavipin was detected in both media but was preferentially produced in the glucose plus NH_4HPO_4 medium. A third unidentified antifungal compound was detected in both media at a later growth stage. Recently, Park *et al*. [32] reported that cinnamic acid has antifungal activity against the growth of *Phytophthora capsici* in a PDA medium, and that the acid was very effective for controlling root rot in red pepper.

Also, Walker *et al*. [33] reported that trans-cinnamic acid has moderate antifungal activity against *Rhizoctonia solani* and *Fusarium oxysporum*. Said *et al*. [34] found that cinnamic acid at 200 μg/mL reduced *Neurospora crassa* growth by approximately 94% and that the branching pattern of the hyphae was altered. Moreover, Cheng *et al*. [35] evaluated the antifungal activities of a cinnamic acid derivative (cinnamaldehyde) against the white-rot fungus *Lenzites betulina* and brown-rot fungus *Laetiporus sulphureus* and found that this derivative exhibited strong activity against the fungi tested. Recently, Nesci and Etcheverry [36] evaluated the effects of the natural phytochemical trans-cinnamic acid (CA) at concentrations of 1~20 mM on sclerotial production by *Aspergillus flavus* and *A. parasiticus*. They indicated that high concentrations of CA significantly reduced sclerotial production in the *Aspergillus* strains. In another study on the same fungal species, the inhibitory effect of CA on growth and aflatoxin B_1 production in *A. flavus* and *A. parasiticus* was demonstrated at high doses [37]. Moreover, Wu *et al*. [38] found that the hyphal growth of *Fusarium oxysporum* f. sp. *niveum*, the causal agent of watermelon fusarium wilt, is strongly inhibited by CA. At the highest concentration of CA, the biomass in the liquid culture decreased by 63.3%, while colony diameter, conidial germination on plates, and conidial production in liquid culture were inhibited completely. Similarly, Zhang *et al*. [39] indicated that an E-CA methyl ester (1.36%) showed significant antifungal activity against some plant pathogenic fungi.

The GC/MS analysis also detected D-arabinitol as a major component in the extract. The presence of a high percentage of arabinitol in the extract may be a good criterion, as it could allow the bioagent to endure drastic conditions during pathogen interaction. In this regard, Burg and Ferraris [40] reported that some yeast and fungi produce and/or accumulate different polyols such as erythritol, ribitol, arabinitol, xylitol, sorbitol, mannitol, and galacticol.

When the potential biocontrol agent and the pathogen were combined in dual culture, no inhibition zone or overgrowth were found on the growth medium. However, *E. purpurascens* appeared more competitive in the growth medium and space than the *P. irregulare* pathogenic isolate, although they grew similarly in separate cultures. This result may be attributed to antagonism via antibiosis or competition for food and space between the biocontrol agent and the pathogen. Coincident with this finding, Brown *et al*. [31] reported that *E. purpurascens* inhibits colony growth of *Phytophthora* spp. and *Pythium* spp. in culture more than other species tested.

Soaking the legume seeds in encysted *P. irregulare* zoospores reduced the germination ability of the three legume seeds to nearly half compared to the control treatments. The length of the germinating seedlings also decreased significantly in the case of *V. faba* and *V. unguiculata* relative to those in the control treatments, and the seedling length decreased slightly in the case of *L. termis*. Under these conditions, the fresh and dry weights of the seedlings of the three legumes decreased compared with the controls with a significant difference in the case of *V. unguiculata* and *L. termis*. Root rot severity was apparent on a great proportion of the growing *V. unguiculata* and *L. termis* seedlings (63~47%, respectively, of the total geminating seedlings), whereas root rot severity in *V. faba* seedlings was minimal (18.75%) among the three legume species. However, root rot severity was not observed in the germinating control seedlings of the three plant species. The retardation effect of *P. irregulare* on the germination of the three seed species, the frequent root rot of the surviving seedlings, and the concomitant reduction in

seedling length have also been reported by several authors for pathogenic *Pythium* spp. Nearly all planted cotton seeds planted in *Pythium*-infested soil were heavily colonized and rotted within 6~12 hr after planting [10]. Furthermore, Larkin *et al*. [11] found that *P. ultimum*, *P. irregulare*, and *P. sylvaticum* cause severe pre-emergence damping-off, and stunting of root and shoot growth in alfalfa seedlings grown for greenhouse pathogenicity tests. Several other species, including *P. dissotocum*, *P. acanthicum*, *P. torulosum*, and *P. rostratum* had reduced root system length in infected plants compared with non-infected plants.

Dressing the tested legume seeds in the potential biocontrol agent homogenate yielded a significant increase in germination ability of the three species compared with soaking the seeds in the pathogen inoculum. Moreover, the seedlings of the three legumes flourished compared with the control treatments and showed absolutely no signs or symptoms of root rot disease. As expected, the improvement in the seedlings exposed to the bioagent fungus treatment was reflected in the length measurements and the fresh and dry weights of the seedlings of the three legumes, which tended to be higher than those of the pathogen treatments.

Dressing the seeds in the potential biocontrol agent showed that the fungus was protective against the root rot disease caused by *P. irregulare*. Melgarejo *et al*. [41, 42] found that *Epicoccum nigrum* conidia biocontrol is promising, as it acts as an antagonist to several air-borne pathogens, including *Monilinia laxa. E. purpurascens* has been used to protect against a broad spectrum of phytopathogenic diseases, including *Sclerotinia sclerotiorum*, a pathogen of many economically important crops such as oilseed rape/canola and alfalfa [43].

Some other antagonistic fungi have also been safely used to protect against phytopathogenic diseases caused by *Pythium* spp. The biocontrol fungus agent *Clonostachys rosea* f. *catenulata* shows antagonistic properties against a number of phytopathogenic fungi and reduces damping-off caused by *P. ultimum* on ornamental bedding plants [44] and by *P. aphanidermatum* on cucumber [45]. Similarly, Jayaraj *et al*. [20] found that treating seeds with *Trichoderma* formulations reduces the incidence of damping-off disease in tomato by up to 74% and enhances plant biomass under greenhouse and field conditions.

Drenching legume seeds in a mixture of the potential bioagent fungus and pathogen-encysted zoospores increased the number of germinating seeds of each species to levels similar to those occurring in seeds dressed in the biocontrol agent homogenate alone. Additionally, this combination significantly alleviated the germination ability of legume seeds to two-fold compared with individually treating seeds with the *P. irregulare* pathogenic isolate. No rotted root symptoms were observed in germinating seedlings treated with the potential bioagent fungus and the pathogen, with only a few exceptions in the case of *V. unguiculata.* Moreover, legume seedling length and their fresh and dry weights tended to be enhanced relative to those recorded for the pathogen treatments. According to these results, we suggest that our tested isolate is efficient for controlling root rot disease caused by *P. irregulare*. Some previous work agreed with our study results, although they did not investigate the problems of root rot diseases of three important legume plants caused by *P. irregulare*. In this respect, Nelson *et al*. [46] reported that the biological control activity of *Trichoderma koningii* and *T. harzianum* against *Pythiura* seed rot and pre-emergence damping-off of pea increased by adding various compounds to seed treatments. The biological control activity of *T. koningii* increased up to 48%, while *T. harzianum* activity increased up to 44% by incorporating specific compounds into the seed treatments. Moreover, several antagonistic microorganisms such as *Pseudomonas*, *Gliocladium*, and *Trichoderma* spp. have the potential to control damping-off and root rot caused by *P. aphanidermatum*, *P. ultimum*, and *P. irregulare* in a variety of crops [20, 45, 47-49]. In comparative studies to control damping-off diseases of some plants caused by *Pythium* spp., fungal bioagents showed more or less similar results. Lynch *et al*. [50] studied the damping-off of lettuce (*Lactuca sativa*) caused by *P. ultimum* in pots containing a non-sterile potting mix in a glasshouse. Fifty *P. ultimum* sporangia/g compost reduced the plant stand to 15% and shoot dry weight to 18%, but this reduction was totally prevented by applying 2×10^5 *T. harzianum* viable propagules/g potting mix. *Gliocladium virens* also alleviated damping-off. Furthermore, Yamaji *et al*. [51] observed that *Picea glehnii* seedlings are affected by damping-off caused by *P. vexans* in nurseries. *Penicillium frequentans* tended to increase the average percentage of surviving *P. glehnii* seedlings when inoculated together with *P. vexans*, but the increase was not significant.

However, some mechanisms have been proposed to understand the suppressive role of antagonistic fungal species on phytopathogenic *Pythium* spp. Of these, Ahmad and Baker [52] observed that germination of *P. ultimum* sporangia was reduced in the presence of *Trichoderma*-treated seeds as compared with germination in the presence of untreated seeds. Pea seeds treated with *T. harzianum* release significantly lower net amounts of ethanol (a sporangium germination stimulant) and acetaldehyde during germination than do untreated seeds [53].

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