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ANTAGONIZING IMPACT OF ENDOPHYTIC FUNGAL ISOLATES AGAINST POTATO BLACK SCURF (*RHIZOCTONIA SOLANI*)

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ABSTRACT

Four endophytic fungal isolates, naturally associated with *Solanum tuberosum* L., were evaluated for their antagonistic activity against *Rhizoctonia solani* and their ability to suppress black scurf disease of potato tubers. To identify the potential implication of antifungal compounds in their inhibitory activity, cell-free culture filtrates were produced and tested for their antifungal potential against *R. solani* mycelial growth and for their protective effect against development of on potato tuber slices. Results showed that percent inhibition of *R. solani* mycelial growth, based on *in vitro* antibiosis tests, ranged between 16 to 59%. Hyphal damage and lysis were the most frequent stress responses exhibited by the target pathogen during its *in vitro* interactions with the potato-associated fungi tested. All Cell-free culture filtrates of tested fungi had significantly inhibited the radial growth of *Rhizoctonia solani* Rs20. The cell-free culture filtrates of *Penicillium chrysogenum* and *Aspergillus niger* had decreased pathogen growth by more than 60% over control. These two filtrates (*P. chrysogenum* and *A. niger*) were found to be the most effective in decreasing the decay incidence in potato slices by 36 and 40%, respectively, as compared to pathogen-inoculated and untreated control. Thus, the present study clearly demonstrated that fungal isolates, occurring ubiquitously within potato plants, may be explored as potent biocontrol agents against potato-associated fungal pathogens and as source of bioactive metabolites for *R. solani* suppression.

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INTRODUCTION

Potato production is threatened by many soilborne fungal diseases worldwide, including black scurf caused by *Rhizoctonia solani* J.G. Kühn. Black scurf is responsible for 50% economic losses (Keiser, 2008) and significant reduction in potato quality, especially for export-oriented potato tubers (Daami-Remadi *et al.*, 2008b). *Rhizoctonia solani* is an important fungal pathogen that causes both stem canker and black scurf of potato. The fungus forms dark masses on tuber surface. These resting bodies are called sclerotia and causing malformed cracked tubers (Wood *et al.*, 2013). *R. solani* is a necrotrophic pathogen that kills host in advance of colonization with the production of extracellular enzymes or toxins (Daami-Remadi *et al.*, 2008a).

The high survival rate of sclerotia and a wide host range renders the use of crop rotation an ineffective management practice and highlight the need for other

effective and safer management options. Furthermore, all cultivated potato varieties commonly grown in Tunisia are susceptible to *R. solani* attack (Djébali and Belhassen, 2010). In Tunisia, all registered fungicides applied to potato crops are often inefficient to control Rhizoctonia damping off under field conditions (Tarhouni, 2007).

Due to the increased need to reduce use of chemical fertilizers and pesticides, for sustainable agriculture and environment protection, there was a growing interest in searching for beneficial microorganisms (Figueiredo *et al.*, 2016). Biological control of plant diseases is a promising alternative that could enrich our set of tools to control *Rhizoctonia* induced diseases more effectively and its use is being increasingly promoted. Several bacterial and fungal agents have been tested both *in vitro* and in plant assays for their ability to control *R. solani* (Grosch *et al.*, 2006; Khedher *et al.*, 2021; Mrabet *et al.*, 2013). Nevertheless, most of them are not able to protect crops when applied under field conditions and very few of these discoveries have been developed into commercially available solutions for farmers (Grosch *et al.*, 2005; Parnell *et al.*, 2016). In recent years, endophytic microorganisms (bacteria, or fungi) have received much attention as plant growth promoters as well as biological control agents against many plant pathogens (Aydi Ben Abdallah *et al.*, 2016; Mahdi *et al.*, 2014; Nefzi *et al.*, 2018). This strategy is very promising for the improvement of crop productivity and as an alternative to chemical fungicides and synthetic fertilizers (Wang *et al.*, 2016). Since they colonize plant tissues asymptomatically whilst sharing the same biological niche as pathogens, endophytic fungi may offer a route to more consistent effect in the field (Collinge *et al.*, 2019).

On numerous times, several endophytic fungi such as *Alternaria, Cladosporium*, *Curvularia, Fusarium*, *Phaeoacremonium*, and *Trichoderma* have been isolated mainly from the star anise, tomato, mangrove (Debbab *et al.*, 2013), and were used as biocontrol agents against pathogenic fungi. Fungal endophytes are a group of microorganisms that grow in internal and intercellular plant tissue at some of their life cycle without causing symptoms of disease in host plants (Strobel, 2003). They have a great diversity and potential as an agent of bioactive compound and metabolites that are important in the pharmaceutical industry, agriculture, and environment (Strobel, 2006). Endophytes synthesize bioactive metabolites for the competence with co-occurring endophytes, host and pathogens in order to colonize the host, and as well as for the nutrition (Tan and Zou, 2001). Many novel secondary metabolites from higher fungi have been isolated and reported to provide lead compounds for new drug discovery such as antimicrobial, antioxidant, and anticancer agents, etc. (Palanichamy *et al.*, 2018). Various secondary metabolites can be produced from endophytic fungi including alkaloid compounds, steroids, terpenoids, diterpenes, flavonoids, phenols, aliphatic compounds, and others (Kumar *et al.*, 2014).

In previous studies, we investigated representative isolates of the fungal endophytic communities naturally associated to potato tissues and we have demonstrated the ability of 20 potato-associated isolates belonging to *Aspergillus* and *Penicillium* genera to suppress *Fusarium* dry rot to 50% (Mejdoub-Trabelsi *et al.*, 2016b) and *Fusarium* wilt by 47% (Mejdoub-Trabelsi *et al.*, 2016a) in potato tubers and plant. Hence, this is the first study reporting on possible exploration of these endophytic fungi for their antifungal potential against another potato-associated pathogen i.e., *R. solani*.

MATERIALS AND METHODS

Isolation of Pathogen

R. solani was isolated from potato cv. Spunta tubers showing typical black scurf symptoms from a Tunisian potato-growing area in Tunisian Centre-East (Chott-Mariem, N35°56'20.451''; E10°33'32.028''). The fungus pathogenicity was confirmed on potato plants by Daami-Remadi *et al.* (2008b) study. Isolate was grown on Potato Dextrose Agar (PDA) medium at 25 °C for seven days in the dark and stored at 4 °C until use.

Endophytic fungi used and their growth conditions

The potato-associated fungi used in the present work were originally recovered from visibly disease-free tubers collected from various potato-growing fields in Tunisian Centre-East (Chott-Mariem, N35°56'20.451''; E10°33'32.028''). They were previously selected in a previous work for their effectiveness against *Fusarium* spp. (Mejdoub-Trabelsi *et al.*, 2016a). Four isolates, namely E.13.11 (*Aspergillus niger*; Accession number KU305732), E.25.11 (*A. flavus*; KU305733), E.36.11 (*Penicillium chrysogenum*; KU305735) and E.29.11 (*P. polonicum*; KU305734), were used in the present work. Stock cultures were maintained at -20 °C in a 20% glycerol solution. Tested isolates were grown on PDA at

25 °C for one week before being used in the bioassays.

Screening of the *in vitro* antifungal activity of endophytes

Dual culture trial

The antifungal activity of four fungal isolates (E.13.11, E.25.11, E.36.11, and E.29.11) against *R. solani* was assessed using the dual culture technique in which the pathogen and the antagonist were plated in the same Petri plate containing PDA medium supplemented with streptomycin (300 mg/L). Agar plugs (6 mm in diameter), already colonized by the pathogen or the antagonist and removed from 7-day-old cultures, were placed at 2 cm apart from the edge of the Petri plate and equidistant of 5 cm. Untreated control plate was plated with pathogen plug only. Each individual treatment was replicated four times. The whole experiment was repeated twice. After 5 days of incubation at 25 ◦C, the diameter of pathogen colony (DPC) was measured. The percentage of radial growth inhibition (GI) was calculated according to the following formula (Elkahoui *et al.*, 2015).

$$\text{GI}\ (\%) = 100 - \frac{DPC\ in\ presence\ of\ treatment}{DPC\ in\ control} \times 100$$

From each treatment, an aliquot of mycelium was stained with lactophenol-cotton blue and observed under light microscope (Olympus Model is not given) to assess hyphal interactions at the confrontation zone between the dual cultured fungi and all abnormal morphological alterations occurring in mycelium, in comparison to control.

Test of cell-free culture filtrates

The cell-free culture filtrate of *Aspergillus* spp. and *Penicillium* spp. antifungal activity test against *R. solani* was assessed according to the procedure adopted by Vibha (2010). Each tested antagonistic agent was cultured in Potato Dextrose Broth (PDB) and subjected to continuous stirring at 150 rpm for 15 days at 25 °C. After incubation, mycelial pellets were separated from the supernatant by filtration through Whatman No.1 filter papers and the collected filtrate was further centrifuged thrice for 10 min at 10,000 rpm. Supernatant fluids were collected and sterilized by filtration through a 0.22 µm pore size filter. Filtrates were aseptically injected at the concentration 20% (v/v) into Petri plates containing molten 10 mL PDA medium cooled at 45 °C and amended with streptomycin sulfate (300 mg L-1) (w/v). After solidification of the mixture, three agar plugs of the target pathogen (6 mm in diameter) were placed equidistantly in each Petri plate. In control plates, PDB medium filtrate was added at the same proportion to PDA. The effect of tested treatments on pathogen growth (in treated and untreated control plates) was measured as described above. Each individual treatment was replicated three times and the whole experiment was repeated twice.

Screening of the antifungal activity of cell-free culture filtrates on tuber slices

Cell-free culture filtrates from endophytic fungi tested were evaluated for their protective effect against *R. solani* development on potato tuber slices. Potato cv. Spunta tubers, undamaged and ascertained as visibly free from scurf signs, were surface sterilized with sodium hypochlorite (2 %) and alcohol (70 %), rinsed with sterile distilled water then air-dried at room temperature and cut into 2 cm slices. Bioassay consists of dipping separately potato slices for 30 min into different cell-free culture filtrates from *A. niger* (E.13.11), *A. flavus* (E.25.11), *P. chrysogenum* (E.36.11), *P. polonicum* (E.29.11) and sterile distilled water used as a negative control, 24 h prior the slice inoculation with a 6-mm agar disc colonized by *R. solani*. Tuber slices were sealed in plastic boxes to maintain high humidity and incubated at 25 °C for seven days in the dark. All treatments consisted of three replicates and five potato slices in each replicate, and the experiments were repeated twice. The incidence of decay was assessed by measuring the lesion area on each potato slice as compared to the total potato slice surface area using a visual scale from 0 to 4, where 0 = absence of lesion area, 1 = 1–25% potato slice surface with lesion area, 2 = 26–50% potato slice surface with lesion area, 3 = 51–75% potato slice surface with lesion area, 4 = 76-100% potato slice surface with lesion area. Each treatment was replicated five times and the antagonism assay was repeated twice. The reduction of decay incidence was calculated according to the following formula:

$$\text{Decay severity}\ \% = \frac{\text{decay severity of untreated potato slice} - \text{decay severity of treated potato slice}}{\text{decay severity of untreated potato slice}} \times 100$$

Statistical analyses

Data were subjected to analysis of variance using SPSS software. Mean values were compared using Duncan's Multiple Range test at the 5% ($P < 0.05$) level of significance.

RESULTS

Effect of endophytic fungi against pathogen radial growth

The diameters of *R. solani* colony dual cultured with the four endophytic fungi (E.25.11, E.13.11, E.29.11 and E.36.11) and noted after 7 days of incubation at 25 °C varied significantly (at $P \leq 0.05$). All endophytic agents had significantly decreased pathogen growth by 16.9 to 59.5% compared to control. Data from the dual culture assay given in Figure 1 showed that the highest inhibition was recorded using *P. chrysogenum* (E.36.11) and *A. niger* (E.13.11) by 49.16 and 59.54%, respectively. These inhibition rates revealed the ability of the four endophytic isolates E.13.11, E.25.11, E.36.11 and E.29.11 to inhibit growth through their antifungal metabolites released in the growth medium (Figure 2 b, c, d). In the case of *A. niger* (E.13.11), it invaded the total surface of the pathogen colony (Figure 5e) and its mycelium was able to actively sporulate on it. Dual plate confrontations suggested the presence of antifungal metabolites secreted by these strains characterizing competitive ability on PDA medium exerted by each antagonist.

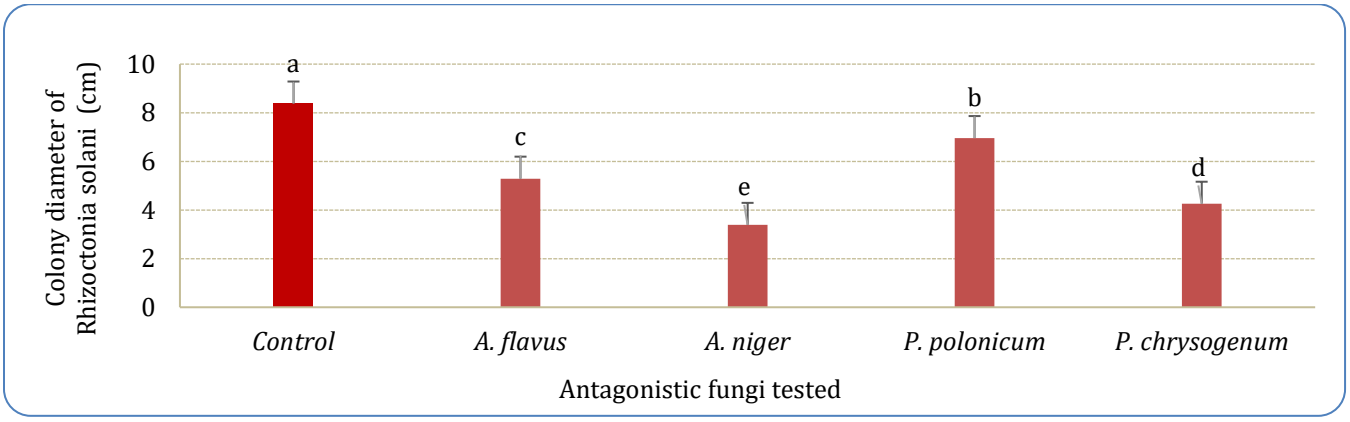

Figure 1. Effect of four endophytic fungi against *Rhizoctonua solani* mycelial growth noted after 5 days of incubation at 25°C as compared to untreated control.
Bars sharing the same letter are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$.

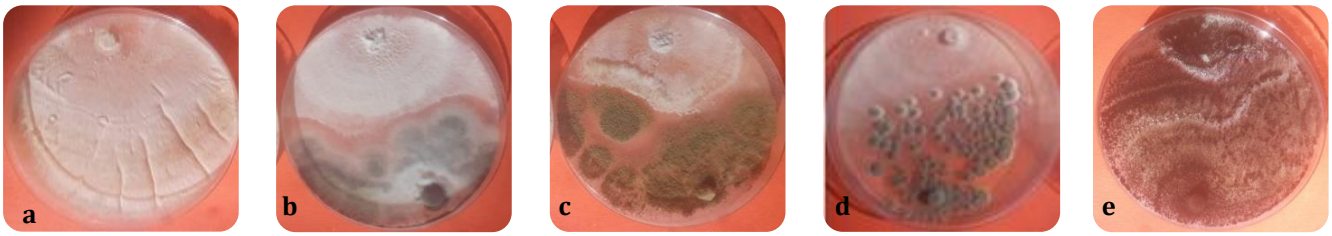

Figure. 2 Inhibitory effects of four isolates of endophytic fungi on the mycelial growth of *Rhizoctonia solani* using dual culture assay on potato dextrose agar (PDA) plate with antagonistic fungi and *R. solani*. (a) *R. solani* alone (control), (b) *R. solani* in the presence of *P. polonicum*, (c) *R. solani* in presence of *A. flavus*, (d) *R. solani* in the presence of *P. chrysogenum*, (e) *R. solani* in the presence of *A. niger*.

Light microscopic studies of hyphal *in vitro* interactions performed at the confrontation zone of *R. solani* with the endophytic fungi revealed varied antagonistic effects. Indeed, five days after inoculation, the antagonists were abundantly multiplied, reduced and lysed *R. solani* mycelia (Figure 3b), and coiled around its hyphae (Figure 3c).

Antifungal potential of cell-free culture filtrates from endophytic fungi

Data given in Figure 4 showed a significant ($P \leq 0.05$) decrease in *R. solani* colony diameter induced by cell-free culture filtrates tested as compared to the

untreated control. The cell-free filtrates from *A. flavus, P. polonicum, A. niger,* and *P. chrysogenum* had decreased the pathogen radial growth by 41.53, 45.56, 60.88, and 64.91%, respectively, relative to the untreated control.

Antagonistic effects of endophytic fungi were further confirmed by the incorporation of their cell-free culture filtrates into PDA medium (Figure 5). The Cell-free culture filtrates of *P. chrysogenum* (E.36.11) and *A. niger* (E.25.11) showed strongly inhibited mycelial growth of *R. solani* with the appearance of browning and disappearance of the fungal mycelium depending on tested agents (Figure 5 d, e).

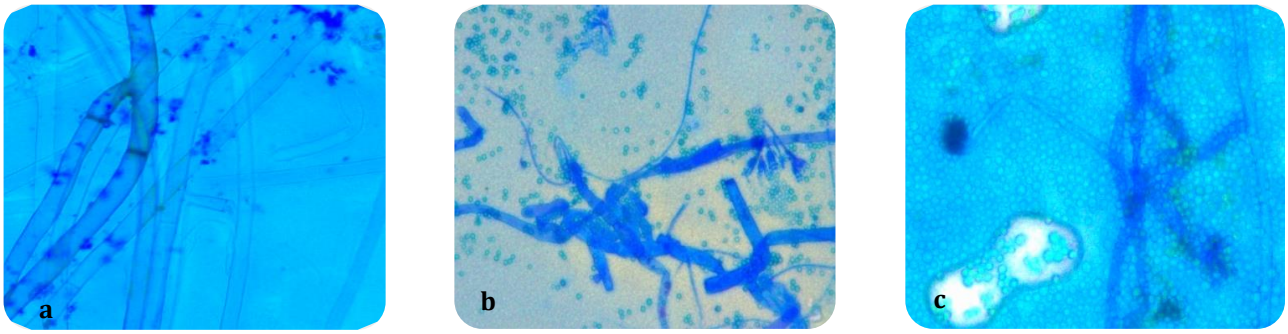

Figure 3. Microscopic observations of *Rhizoctonia solani* hyphae growing on the PDA medium in presence of tested endophytic agents as compared to control. (a) *R. solani* alone (control), (b) *R. solani in* dual culture with *Penicillium chrysogenum* (c) *R. solani* in dual culture with *Aspergillus niger* (Magnification: 40 x).

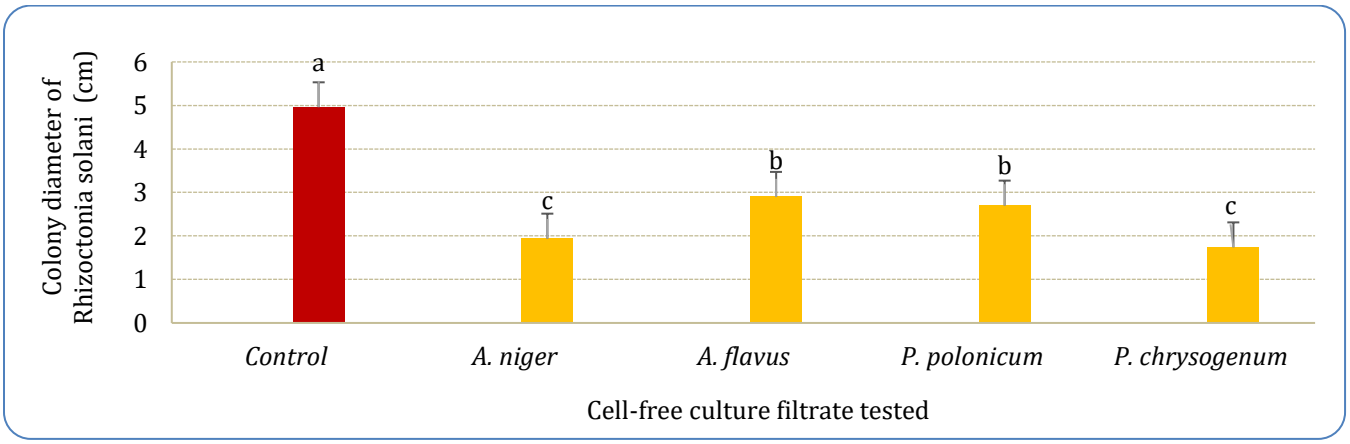

Figure 4. Effect of cell-free culture filtrates from endophytic fungi tested on *Rhizoctonia solani* mycelial growth noted after 5 days of incubation at 25°C as compared to control. Bars sharing the same letter are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$.

Figure 5. Inhibition of *Rhizoctonia solani* mycelial growth on solid medium amended with different cell-free culture filtrates of endophytic fungi observed after 7 days of incubation at 25°C. (**a**): Control Filtrate of Potato Dextrose Broth (PDB); (**b**): Cell-free culture filtrate from E.25.11 (*Aspergillus flavus*); (**c**):Cell-free culture filtrate from E.29.11 (*Penicillium polonicum*); (**d**):E.13.11: Cell-free culture filtrate from *A. niger*; (**e**): Cell-free culture filtrate from E.36.11 (*P chrysogenum*). The filtrates were added at the concentration of 20% (v/v).

Effect of cell-free culture filtrates of tested endophytes

The application of cell-free culture filtrates from endophytic fungi, tested applied 24 h prior to inoculation with the fungus Rs20 showed a significant ($P \leq 0.05$) decrease in the decay severity in tuber slices. Like results *in vitro* assay, cell-free culture filtrates of *A. niger* (E.13.11) and *P. chrysogenum* (E.36.11) decreased decay incidence in potato slices by 40 and 36% respectively, in comparison to the inoculated but untreated control (Figure 6).

The observations from treated potato tuber slices and untreated ones showed differences in decay severity. Potato slices solely inoculated with pathogenic fungus showed profuse mycelial growth that was highly pronounced at 7 days post-inoculation (Figure 7a). It should also be noted that *P. chrysogenum* cell-free culture (E.36.11) had strongly reduced the *R. solani* infection on potato slices (Figure 7e) as compared to the other treatments.

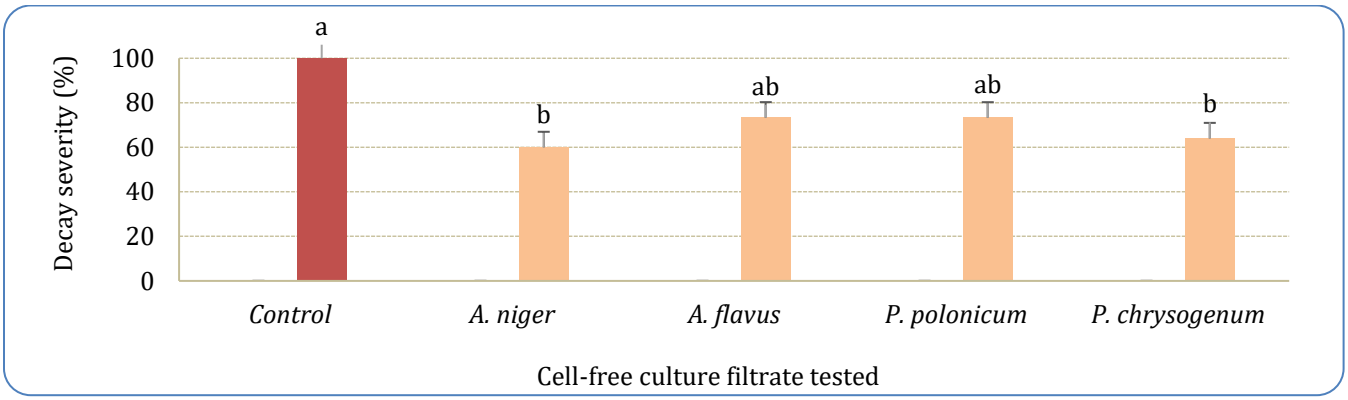

Figure 6. Decay severity induced by *Rhizoctonia solani* on potato tuber slices following their treatments with cell-free culture filtrates from endophytic fungi noted after 5 days of incubation at 25°C as compared to control. Bars sharing the same letter are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$.

Figure 7. Severity of potato decay recorded after 7 days of incubation of tuber slices inoculated with *Rhizoctonia solani* and treated with cell-free culture filtrates from endophytic fungi tested (a) Potato slices treated with LB medium and inoculated with *R. solani* plug; (b) Potato slices treated with cell-free culture filtrate from (*A. flavus*); (c)Potato slices treated with cell-free culture filtrate from (*P. polonicum*); (d) Potato slices treated with cell-free culture filtrate from (*A. niger*); (e) Potato slices treated with cell-free culture filtrate from (*P. chrysogenum*)

DISCUSSION

Recently, an interesting alternative to soilborne disease control that has been developed and gained particular interest is the exploration of endophytic microorganisms (fungi or bacteria) as potential biocontrol agents (BCAs). Many plant-associated microorganisms can synthesize bioactive compounds involved in its defense against plant pathogens (Hyder *et al.*, 2020; Schulz *et al.*, 2002; Shakoor *et al.*, 2015) Their antagonistic potential against some soilborne fungi such as *Verticillium dahliae* and *Rhizoctonia solani* has been previously showed (Berg *et al.*, 2005). In our previous studies, (Mejdoub-Trabelsi *et al.*, 2016a), we have demonstrated the ability of 20 nonpathogenic isolates of potato-associated fungi belonging to *Aspergillus*, *Penicillium*, *Colletotrichum*, and *Trichoderma* genera, to suppress *Fusarium* dry rot disease severity in potato incited by *Fusarium sambucinum* and *F. solani*. These endophytic fungi, were reported to be more adapted to the ecological niche of targeted pathogens and exhibiting, thus, interesting activities in bioprotection of their hosts (Narisawa *et al.*, 2000). The current study is among the few reports on

the use of endophytic fungi for the biocontrol of *Rhizoctonia solani* infecting potato.

Dual interactions in co-cultures showed that *Aspergillus* spp. and *Penicillium* spp. significantly reduced *R. solani* mycelial growth. This seems to suggest that the four endophytic fungi were effective to control *R. solani* by exhibiting diffusion of metabolites into the culture medium.

Inhibition effect expressed by *A. niger* (E.13.11) was more effective in comparison with the rest of antagonists being their percentage of radial growth inhibition (GI) sensibly lower. It was observed that this antagonist exhibited, in addition to its lytic activity, high capacity of colonization of the medium preventing the fast-growing rates of *R. solani* once the isolate began to grow on the plate. Interestingly control mechanisms described for *Rhizoctonia* are usually the competition for nutrients and space induced resistance (Olson and Benson, 2007).

In relation to potato diseases, several studies have found variable efficacies in reducing mycelial growth in plate assays by employing different *Aspergillus* or *Penicillium* species against *Rhizoctonia* solani and other specific soilborne pathogens. In this sense, Khedher *et al.* (2015) assayed the antagonistic effect of strain *Bacillus subtilis* V26, a local isolate recovered from the Tunisian soil, to control potato black scurf caused by this pathogen, and noted a mycelial growth reduction reaching 80% like the results reported in the current investigation. For the rest of the endophytic fungi tested (*A. flavus* E.25.11, *P. polonicum* E.29.11, *P. chrysogenum* E.36.11), it was noted that GI values obtained did not exceed 50%. This could be explained either by the slow growth rate of these species thus that *R. solani* invaded the plate before antagonists could secrete enzymes and/or inhibitory secondary metabolites into the culture medium or due to the nutrient composition used (standard PDA medium) that may not in favor of their production. Previous investigations have also explored the possibility of using potato-associated fungi like *Aspergillus* spp. to control the potato diseases. This antagonism effect was already observed by authors like Aydi *et al.* (2013) and Aydi *et al.* (2014) reporting the ability of several *Aspergillus* species isolated from soil and compost allowing the selection of new biocontrol agents active against *F. sambucinum* and *Pythium aphanidermatum* causing dry rot and leak on potato tubers, respectively. Interestingly, *Penicillium* species are well reported to produce antifungal metabolites. In fact, secondary metabolites produced by *Penicillium* sp. were shown to be effective to inhibit *Botrytis cinerea* and *Alternaria solani* on tomato fruits (Hassine *et al.*, 2013; Marwa *et al.*, 2014).

Concerning the inhibitory effects of culture filtrates of the four endophytic fungi, results revealed that extracellular metabolites present in cell-free culture filtrate of the endophytic fungi tested influence the ability for mycelial growth inhibition in comparison with control. Among the tested cell-free culture filtrates, those of E.13.11 (*A. niger*) and E.36.11 (*P. chrysogenum*) were found to be highly effective against the growth of *R. solani* by inhibiting its radial growth by more than 50% compared to the untreated control. The current findings suggest that the antifungal activity of the cell-free culture filtrates from 15 days old liquid cultures of *Aspergillus* spp. and *Penicillium* spp. be due to their biologically active and stable secondary metabolites that may be antibiotics and/or lytic enzymes presumably associated with a release of diffusible compounds that infiltrated the pathogen hyphae. Similarly, due to their antagonistic properties against fungal pathogens, the genera *Penicillium* and *Aspergillus* have been largely recorded as biocontrol agents. In fact, Larena *et al.* (2003) found that *P. oxalicum*, applied by watering of tomato plants by spore suspension, 7 days prior transplanting, decreased vascular wilts caused by *V. dahliae* and *Fusarium oxysporum* f. sp. *lycopersici* under greenhouse and field conditions. In the same sense, Wang *et al.* (2008) found metabolites with antifungal activity in an endogenous fungus *Penicillium* sp. associated to *Hopeaha inanensis*. In a previous wortk, Vibha (2010) demonstrated the inhibitory effects of metabolites from most ubiquitous *Aspergillus* spp. *(A. ochraceus, A. niger, A. fumigatus, A. flavus,* and *A. terreus*) against *R. solani*. In this sense, assessed in a 4-year field study, non-aflatoxigenic, indigenous *A. flavus* isolates, K49 and CT3, exhibited biocontrol through reducing aflatoxin contamination of corn (Abbas *et al.*, 2006). The present study revealed that extracellular metabolites present in cell-free culture filtrate of E.13.11 (*A. niger*) and E.36.11 (*P. chrysogenum*), were only found to be effective in suppressing *R. solani in vivo* growth. The control of black scurf disease in potato tubers by these two endophytic fungi could be due to its capability to produce hydrolytic enzymes. In fact, Sreevidya *et al.* (2015) showed that *P. citrinum* VFI-51 was able to produce siderophore, indole acetic acid (IAA),

hydrocyanic acid (HCN), lipase, protease and β-1,3glucanase. Siderophores help plants not only to acquire iron but also helps in disease suppression. In the same way, other studies have previously demonstrated the biocontrol efficacy of *Aspergillus* spp., recovered from soil and compost, against infection by *F. sambucinum* in potato tubers Aydi *et al.* (2014).

CONCLUSION

This study presents the first evidence of the potency of endogenous *Aspergillus* and *Penicillium* species naturally associated to potato as potential inhibitors of black scurf disease caused by *Rhizoctonia solani*. These endophytic fungi were found to be potent source of extracellular metabolites. Thus, it can be concluded that naturally occurring potato-associated fungi and their extracellular metabolites can suppress *in vitro* and *in vivo* growth *R. solani* infecting potato tubers. Further chemical studies are needed to more elucidate the efficiency of the secondary metabolites of these four antagonists and to identify major compounds present in their chloroform extracts.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTIONS

BMT and RAB planned and designed the research experiments. BMT performed the experiments and wrote the research article. HJK coordinated the laboratory works. BMT, and FA performed data interpretation and statistical analysis. MDR supervised the results analysis and corrected the manuscript draft. All authors have read and approved the manuscript.