



Biocontrol and plant growth promotion activities of endophytic and rhizospheric fungi from almond trees (*Prunus dulcis*) indigenous in the northeast of Iran

Rezvani V¹, Taheri P^{2*}, Pourianfar HR^{3*}, Drakhshan A¹

¹ Department of Plant Protection, Academic Center for Education, Culture and Research (ACECR)- Khorasan Razavi, Kashmar Higher Education Institute, Kashmar, Iran

² Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

³ Industrial Fungi Biotechnology Research Department, Research Institute for Industrial Biotechnology, Academic Center for Education, Culture and Research, Khorasan Razavi Province Branch, P.O.Box: 91775-1376, Mashhad-Iran

Rezvani V, Taheri P, Pourianfar HR, Drakhshan A 2020 – Biocontrol and plant growth promotion activities of endophytic and rhizospheric fungi from almond trees indigenous to northeastern Iran. Current Research in Environmental & Applied Mycology (Journal of Fungal Biology) 10(1), 50–62, Doi 10.5943/cream/10/1/6

Abstract

Verticillium dahliae and *Phytophthora drechsleri* are important soil borne pathogens which cause severe damages on almond (*Prunus dulcis*, Rosaceae) trees. Endophytic and rhizospheric fungi may be promising biocontrol agents, which could be applied against these destructive phytopathogens. However, scarce information is presently available regarding the endophytic and rhizospheric fungal communities of trees, especially almond. Thus, the present study for the first time was focused on investigating several endophytic and rhizospheric fungi from almond trees with the aim of evaluating their plant growth promotion and biocontrol potential against *V. dahliae* and *P. drechsleri*. Fifty-nine isolates were successfully obtained of which 26 isolates were selected based on colony morphologies. Antagonistic reactions between the isolates and the pathogens were tested using the dual culture method. The growth enhancement potential of the endophytes and rhizospheric fungi was also investigated for lentil and wheat seeds. The findings revealed that the rhizospheric isolates showed higher biological activities as compared to endophytic isolates ($p \leq 0.05$). The KoA1 isolate (identified as *Aspergillus awamori*) obtained from rhizosphere significantly prevented the *P. drechsleri* and *V. dahliae* growth. In addition, the KoA9 isolate (identified as *Paraconiothyrium flavescens*) obtained from rhizosphere significantly increased root and shoot growth of wheat seeds. The data presented revealed the potential of rhizospheric fungi of almond trees on increasing the growth of monocots, as well as their effect as biocontrol agents against fungal pathogens *in vitro*, which needs future *in vivo* investigations.

Key words – Biological control – Endophyte – Rhizosphere – *Phytophthora drechsleri* – *Verticillium dahliae*

Introduction

Almond (*Prunus dulcis*, formerly known as *Amigdalus communis*) is one of the commercially important trees in the family of *Rosaceae*, originated from wild species of central Asia and eastern south mountains (Azizi & Yazdani 2010). According to FAO, the top three countries producing

almond include USA (2 million tons/year), Spain (0.2 million tons/year) and Iran (0.15 million tons/year). According to the statistics of the Ministry of Agriculture of Iran, the area under cultivation of gardens in 2017 was about 2.85 million hectares, of which this amount of almonds is 21.7% of the total area of dry fruit gardens. From the production of 756,000 tons of dried fruits per year, almond production accounts for 19.5 percent of the total dry fruit production. Almond trees are susceptible to a large variety of destructive diseases, such as leaf scorch, witches broom and trunk diseases (Soltaninejad et al. 2017, Arzanlou & Dokhanchi 2012). Among the fungal pathogens, *Verticillium* wilt and *Phytophthora* crown rot pathogens are important almond pathogens with no precise statistics of their damage on almonds. *Verticillium dahliae* is a destructive soil borne pathogen, which causes vascular wilt disease. This disease influences many diverse plant taxa, including almond (Fotoohiyan et al. 2015). The *Verticillium* wilt is most severe on 2 to 6 years old trees. The first observation of *Verticillium* wilt on almond, also known as ‘black heart’, was reported in California (Stapleton 1997). *Verticillium dahliae* Kleb., (initially isolated from Dahlia with the greatest economic effect, is among the most widespread plant pathogens in the world (Klosterman et al. 2009, EFSA PLH Panel 2014). On almond trees, worldwide records show that there are several *Phytophthora* species with symptoms ranging from root and crown rot to pruning wound cankers and lethal cankers (Pérez-Sierra et al. 2010). In Iran, *P. cryptogea* and *Phytophthora* sp. were reported to be involved in almond tree decline, causing yellowing of the leaves and gumming and cracking of the bark near the ground level (Fatemi 1980). The economic damages caused by pathogens and high commercial value of almond trees have encouraged studies to improve their productivity.

One of the methods to control plant pathogens with minimal impact on the environment is biocontrol (Larran et al. 2016). The use of antagonistic endophytic and rhizospheric fungi as biocontrol agents is considered as an attractive option for management of some plant diseases. The rhizosphere is the narrow zone around the root, which is a hot-spot for microbial activity and interactions (Raaijmakers 2015, Wang et al. 2017). In the rhizosphere, complex microbial community members interact with each other and influence the outcome of pathogen infection, host health and growth (Raaijmakers et al. 2009). In addition, rhizospheric fungi are also essential for plant fitness and indirectly affect the composition and functioning of natural plant communities, similar to fungal endophytes (Rudgers et al. 2012, Jogaiah et al. 2013, Philippot et al. 2013). Understanding the composition, dynamics, and activity of the endophytic and rhizosphere fungal community is therefore critical for the development of new strategies to promote plant growth and health in both agro-ecosystems and natural ecosystems (Raaijmakers et al. 2009). Investigations conducted on endophytic fungi have also exhibited that these fungi can play effective roles as biocontrol agents (Gond et al. 2010). Previous studies have also reported the biocontrol ability of endophytic and rhizospheric fungi against *Verticillium* wilt and *Phytophthora* crown root pathogens (Fotoohiyan et al. 2017, De Silva et al. 2018, Saberi-Riseh & Fathi 2018).

To our knowledge, no information is available regarding the potential of almond tree for hosting rhizospheric and endophytic fungi with biological activity. Thus, the present study aimed to characterise rhizospheric and endophytic fungi from roots and shoots of almond trees located in the northeastern region of Iran followed by evaluating their *in vitro* inhibitory effects against *V. dahliae* and *P. drechsleri*. Moreover, effect of the rhizospheric and endophytic fungi on the growth of wheat and lentil seeds was investigated. Inhibitory effects of the selected rhizospheric and endophytic fungi were further verified by evaluating the antifungal activity of volatile compounds produced by the rhizospheric and endophytic fungi.

Materials and methods

The pathogens and isolation of endophytic and rhizospheric fungi

The pathogens used in this study, including *Verticillium dahliae* (isolate VdR65) and *Phytophthora drechsleri* (isolate 17.22.05), were obtained from culture collection of Department of

Plant Protection, Faculty of Agriculture, Shiraz University, and Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad in Iran, respectively.

To obtain the isolates of endophytic and rhizospheric fungi from almond trees, sampling was done during summer and autumn of 2017 from some areas of almond cultivation in Khorasan Razavi province, as shown in Table 1. Sampling was carried out mainly from the branches, roots, and rhizosphere of the healthy trees and the collected specimens were transferred to the laboratory and stored at 4°C until used for fungal isolation.

For isolating endophytic fungi from the branch, almond branches were first washed by running tap water for 10 min and subsequently by sterile distilled water. Then they were surface sterilized by 70% ethanol for 1 min, 5% sodium hypochlorite for 1 min and 70% ethanol for 30 sec, and were finally rinsed 3 times with sterile distilled water (Refaei et al. 2011). Finally, the disinfected parts were transferred onto water agar 2% (Merk KGaA) media. The inoculated petri dishes were transferred to the incubator with absolute dark conditions and 25°C temperature. For preparing the pure colony, the hyphal tips or single spores were transferred to the new PDA medium 7 to 14 days after growth of the fungus. (Strobel & Daisy 2003).

For isolating the fungal endophytes from almond roots, the method described by the Hodkinson et al. (2019) was used with some changes. The roots were subjected to a 10 min wash by running tap water and a subsequent wash by sterile distilled water. They were then surface sterilized by 70% ethanol for 3 min and 5% sodium hypochlorite for 5 min, and were finally washed 3 times by sterile distilled water. The disinfected parts were transferred onto water agar 2% (Merk KGaA). Inoculated petri dishes were transferred to the incubator with dark conditions and temperature of 25°C. Seven to 14 days after the growth of fungi, the hyphal tips were cultured on PDA to prepare pure fungal colonies (Strobel & Daisy 2003).

The method described by Warcup (1950) was used for isolating rhizospheric fungi. The soil or rhizosphere samples (25g) were suspended in 225 mL of sterilized distilled water (1:10 dilution), and subsequently 10 mL of this suspension was added into 990 mL of sterilized distilled water. Petri dishes containing the Sabouraud Agar medium (Himedia) plus chloramphenicol (100mg L⁻¹) and Bengal Rose (50mg L⁻¹) were inoculated with 1 mL of the 1:1000 diluted soil suspension. In order to grow the colony, the plates were kept at room temperature (≈28°C) for 72h. The individual colonies were transferred separately to the same medium containing 50 mg L⁻¹ of chloramphenicol and grown for 72h. The isolates were identified after growth on Czapek Agar (Merk KGaA) and PDA media.

Screening assays

Dual culture assay

Mycelial disks (5 mm in diameter) of *V. dahliae* (VdR65) and *P. drechsleri* (17.22.05) were placed on one side of each petri dish containing PDA, while mycelial disks of the endophytic fungi were placed on the opposite side of the plate. Control plates were inoculated with a 5 mm diameter disk of endophytic and rhizospheric fungi and a disk of PDA medium was placed on the opposite side of the plate. Five to seven days after the incubation at 25±1°C, the overgrowth of colonies of the tested fungi was determined by the antagonist (Küçük & Kivanç 2004). The following equation was used to calculate the percent inhibition of mycelial growth of pathogenic fungi (Aderogba et al. 2013):

$$R (\%) = (R_1 - R_2) / R_1 \times 100\%$$

Where, R is the percentage of inhibiting radial hyphal growth, R₁ is the hyphal growth of the control, and R₂ is the hyphal growth in the Petri dish inoculated with the endophytic fungal isolates.

Effect of endophytic fungi on seed germination

In order to disinfect seeds for cultivation, we first placed wheat seeds (Roshan cultivar) in 6% sodium hypochlorite for 7 min and after 3 times washing in sterilized distilled water. The seeds were placed on sterile filter paper for drying under the laminar hood. After drying, the seeds were placed on PDA medium for germination. For disinfection of lentil (Canadian cultivar), the seeds were disinfected for 5 min in 5% sodium hypochlorite and after 3 times washing in sterile distilled water, the other steps were performed as mentioned above. For germination, the seeds were kept in an incubator (25°C). After 2 days, the germinated seeds were placed on the petri dishes which the endophytic fungi were grown. The seeds were cultivated on a PDA medium in which the fungus was not cultivated as control. When the growth of the seeds in the control filled the petri dish, shoot and root growth were measured.

Morphological identification of the fungal isolates

Based on the type of fungal isolates, the traits used for morphological identification of the fungal isolates were the structure and color of the mycelium, the presence or absence of conidia, morphology of conidia and conidiophores (size, color, form, decoration, etc.) and the nature of the conidiophore and conidiogenous cell features (Klich 2002, Kirk et al. 2013, Verkley et al. 2014).

Ability of the antagonists to produce volatile compounds

The PDA plates were inoculated in the center with a 0.5 cm diameter mycelial disc containing antagonists. The pathogens, including *P. drechsleri* and *V. dahliae*, were individually cultivated in each plate. The lids were removed and two plates containing each *P. drechsleri* and *V. dahliae* and one fungal endophyte were inverted and placed on top of the other plate. The plates were then sealed with parafilm and incubated at 25°C. The same experimental setup was followed for preparing controls, with the exception that antagonist culture was replaced with a PDA disc. In this assay, three replications were used for each treatment. Calculation and statistical analyses related to the inhibitory effect of each antagonist against the pathogens were performed in this study (Lahlali & Hijri 2010). The percentage of radial growth inhibition was measured by using the equation mentioned before.

Statistical analysis

All of the experiments were subjected to an ANOVA analysis by SPSS (Version 22). Means comparison of the treatments was performed by Duncan's test at $p \leq 0.05$. Significant differences were presented by various letters on the figures.

Results

Pre-screening analysis

Totally, 58 isolates of endophytic and rhizospheric fungi were obtained from healthy branches, roots, and rhizosphere of almond trees (Table 1). All the isolates were cultivated on WA (2% w/v) and purified on PDA, leading to purified mycelial cultures. Finally, 26 morphologically distinct groups of isolates were classified based on differences in color, growth rate, and colony type and one isolate from each group was selected for investigating its effect on the plant growth and antagonistic activity against the pathogens.

Inhibitory effects of the endophytic and rhizospheric fungi

Our finding revealed that four isolates obtained from rhizosphere and branch of almond trees, including KoA1, KoA11, KoA8 and KaBsh5 had significant differences in their inhibitory effect on vegetative growth of *P. drechsleri* compared with their controls ($p \leq 0.05$) (Table 1). Among these isolates, KoA1 with 40% inhibitory effect proved to be the most potent isolate against *P. drechsleri*, followed by KoA11 (33%), KoA8 (20%) and KaBsh5 (20%). However the rest of isolates including endophytic and rhizospheric isolates had no significant differences with their

controls. The KoA1 isolate was selected as the most antagonistic isolate against *P. drechsleri* for further identification (Fig. 1, Table 2).

Table 1 The endophytic and rhizospheric fungal isolates obtained from almond trees in the northeast of Iran.

No.	Code of isolate	Plant tissue	Geographic location (North East Iran)	N0.	Code of isolate	Plant tissue	Geographic location (North East Iran)
1	KBR	Root	Kashmar	30	JA1	Rhizosphere	Sabzevar (Village of Jovein)
2	KA2	Root	Kashmar	31	JA2	Rhizosphere	Sabzevar (Village of Jovein)
3	KA1	Root	Kashmar	32	JA3	Rhizosphere	Sabzevar (Village of Jovein)
4	JoRB1	Root	Sabzevar (Village of Jovein)	33	JA4	Rhizosphere	Sabzevar (Village of Jovein)
5	KoAR	Root	Kuhsorkh District	34	JA5	Rhizosphere	Sabzevar (Village of Jovein)
6	KoshB21	Branch	Kuhsorkh District	35	JA6	Rhizosphere	Sabzevar (Village of Jovein)
7	KoshB31	Branch	Kuhsorkh District	36	JA7	Rhizosphere	Sabzevar (Village of Jovein)
8	KoshB32	Branch	Kuhsorkh District	37	KoA12	Rhizosphere	Kuhsorkh District
9	KoshB6	Branch	Kuhsorkh District	38	KoA4	Rhizosphere	Kuhsorkh District
10	KoshB43	Branch	Kuhsorkh District	39	KoA13	Rhizosphere	Kuhsorkh District
11	TBsh3a	Branch	Kashmar (Village of Tak Mar)	40	KoA11	Rhizosphere	Kuhsorkh District
12	GshBa"32	Branch	Kashmar (Village of Jaboz)	41	KoA10	Rhizosphere	Kuhsorkh District
13	KaBsh5	Branch	Kashmar (Village of kahe)	42	KoA9	Rhizosphere	Kuhsorkh District
14	ABsh3	Branch	Kashmar (Village of Argha)	43	KoA7	Rhizosphere	Kuhsorkh District
15	ABsh	Branch	Kashmar (Village of Argha)	44	KoA8	Rhizosphere	Kuhsorkh District
16	ABsh11	Branch	Kashmar (Village of Argha)	45	KoA1	Rhizosphere	Kuhsorkh District
17	GshBa1	Branch	Kashmar (Village of Jaboz)	46	KoA2	Rhizosphere	Kuhsorkh District
18	KBsha	Branch	Kashmar (Village of kahe)	47	KoA5	Rhizosphere	Kuhsorkh District
19	GshBa'2	Branch	Kashmar (Village of Jaboz)	48	KoA51	Rhizosphere	Kuhsorkh District
20	TBsh3b	Branch	Kashmar (Village of Tak Mar)	49	KoA6	Rhizosphere	Kuhsorkh District
21	TBsh2	Branch	Kashmar (Village of Tak Mar)	50	KaBshb1	Branch	Kashmar (Village of kahe)
22	TBsh1	Branch	Kashmar (Village of Tak Mar)	51	GshBa"1	Branch	Kashmar (Village of Jaboz)
23	JoshB1	Branch	Sabzevar (Village of Jovein)	52	Ashb1	Branch	Kashmar (Village of Akbarabad)
24	ABsh1	Branch	Kashmar (Village of Argha)	53	KaBsha1	Branch	Kashmar (Village of kahe)
25	ABsh22	Branch	Kashmar (Village of Argha)	54	KaBsh3	Branch	Kashmar (Village of kahe)
26	Asha	Branch	Kashmar (Village of Akbarabad)	55	KhBshb1	Branch	Khalilabad County
27	Ashb	Branch	Kashmar (Village of Akbarabad)	56	KhBsh1	Branch	Khalilabad County
28	KBsha	Branch	Kashmar	57	KhBsha1	Branch	Khalilabad County
29	KBsh	Branch	Kashmar	58	KhBsha4	Branch	Khalilabad County

The results showed that the rhizospheric isolate KoA1 had a significant difference in its inhibitory effect on vegetative growth of *V. dahliae* compared with control ($p \leq 0.05$). This isolate showed 28.5% inhibitory effect against *V. dahliae*. Other endophytic and rhizospheric isolates did not have inhibitory effect against *V. dahliae* (Table 2).

Effects of endophytic and rhizospheric fungi on plant growth

Our findings showed that one isolate considerably promoted the growth of root and stem of wheat ($p \leq 0.05$), while 25 isolates didn't have a significant effect on wheat seed germination. The isolate KoA9 (obtained from rhizosphere) had the highest effect on growth of wheat root and stem (Fig. 2, Table 3). This isolate were selected for further identification. However, none of the 26 selected isolates significantly influenced the growth of root or stem of lentil ($p \geq 0.05$).

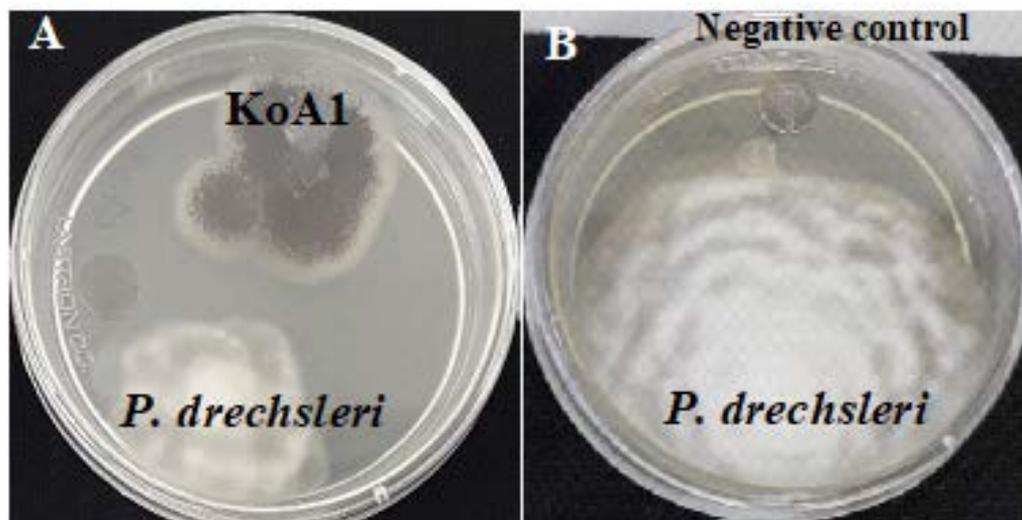


Fig. 1 – *Aspergillus awamori* (isolate KoA1) showed the most antagonistic activity against *Phytophthora drechsleri* using dual culture. Photos have been taken seven days after fungal culture in PDA. A Dual culture of *A. awamori* and *P. drechsleri*. B the negative control.

Table 2 Inhibitory effects of endophytic and rhizospheric isolates against *Phytophthora drechsleri* and *Verticillium dahliae* (n=3; mean±SD).

No.	Isolates	mean radial growth(mm)		No.	Isolates	mean radial growth(mm)	
		<i>V. dahliae</i>	<i>P. drechsleri</i>			<i>V. dahliae</i>	<i>P. drechsleri</i>
1	KaBsh5 (B)	1.50±0.10	1.20 ±0.17^{fg}	15	KoA12 (Rh)	1.43±0.05	1.50±0.10
2	ABsh3 (B)	1.60±0.05	1.70±0.05	16	KoA10 (Rh)	1.76±0.11	1.80±0.17
3	ABsh11 (B)	1.70±0.05	1.53±0.35	17	KoA9 (Rh)	1.43±0.05	1.63±0.20
4	GshBá2 (B)	1.40±0.25	1.60±0.15	18	KoA11 (Rh)	1.60 ±0.32	1.00±0.20^{gh}
5	KaBshb1 (B)	1.40±0.10	1.70±0.11	19	KoA4 (Rh)	1.66±0.05	1.70±0.20
6	KhBshb1 (B)	1.40±0.10	1.60±0.10	20	KoA1 (Rh)	1.00 ±0.05^g	0.90 ±0.05^h
7	GshBa'1 (B)	1.40±0.10	1.40±0.05	21	JA4 (Rh)	1.46±0.20	1.53±0.35
8	GshBa'32 (B)	1.30±0.05	1.60±0.15	22	JA1 (Rh)	1.63±0.25	1.50±0.10
9	GshBa1 (B)	1.80±0.05	1.46±0.10	23	KoA7 (Rh)	1.50±0.20	1.70±0.05
10	KBsha (B)	1.43±0.05	1.46±0.05	24	KoA8 (Rh)	1.50±0.05	1.20 ±0.00^{fg}
11	KoshB43 (B)	1.83±0.05	1.76±0.05	25	KBR (R)	1.20 ±0.00	1.70±0.05
12	TBsh3a (B)	1.20±0.05	1.20 ±0.05	26	JoRB1 (R)	1.56±0.05	1.60±0.10
13	JA7 (Rh)	1.70±0.20	1.40±0.11		Negative	1.40± 0.11 ^{a-f}	1.50± 0.05 ^{a-e}
14	JA6 (Rh)	1.60±0.10	1.43±0.05				

Note: Means with the same letter did not have significant difference according to Duncan's multiple range test at $p < 0.05$. Isolates have become bold with significant effect. B: Branch, Rh: Rhizosphere, R: Root.

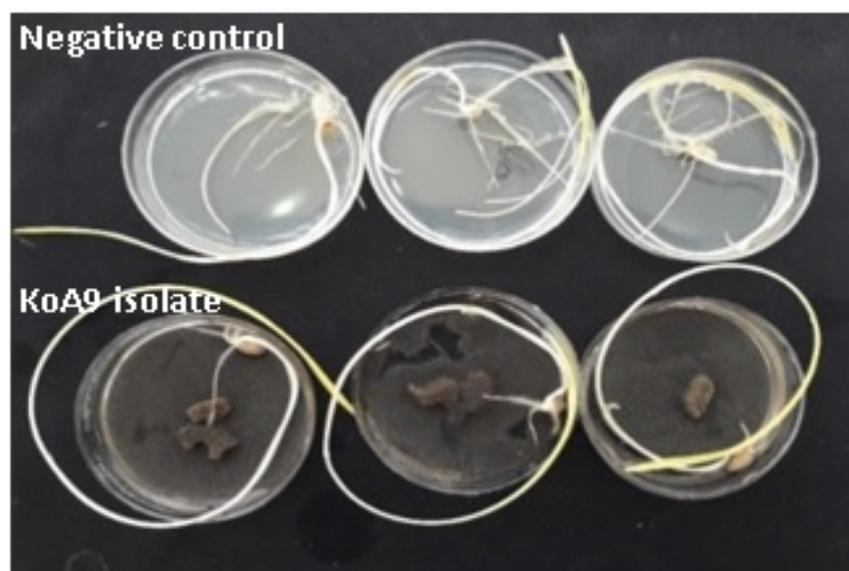


Fig. 2 – Effect of rhizospheric isolate KoA9 on wheat seed growth on PDA medium. First row: Negative control plates. Second row: Plates inoculated with the rhizospheric fungus *Paraconiothyrium flavescens* isolate KoA9.

Table 3 Effect of endophytic and rhizospheric fungi on stem and root growth of wheat and lentil (n=3; mean±SD).

No.	Endophyte code	Growth characteristics (Length, mm)				Treated plant
		SGC	RGC	SGT	RGT	
1	KoA9 (Rh)	17.45±0.08	12.81±1.69	23.1± 1.42*	20.7± 0.40*	Wheat
		4.43±1.17	3.90±0.10	4.00± 0.75	6.26± 0.76	Lentil
2	KoA10 (Rh)	17.45±0.08	12.81±1.69	11.73± 1.86	3.73± 1.32	Wheat
3	ABsh3 (B)	17.45±0.08	12.81±1.69	19.90±3.95	8.30± 1.60	Wheat
4	KhBshb1 (B)	4.43±1.17	3.90±0.10	7.73± 0.24	1.76± 0.23	Lentil
5	KBR (R)	4.43±1.17	3.90±0.10	7.30± 0.35	3.73± 0.33	Lentil

Note: Significant differences were determined using comparison between rhizosphere and endophyte-treated seeds and negative controls using Duncan ($p \leq 0.05$). Significant differences are denoted by asterisk (*0.05). Isolates have become bold with significant effect. SGC: Stem growth rate in control plate. RGC: Root growth rate in control plate. SGT: Stem Growth in Treated plant. RGT: Root Growth in Treated plant. B: Branch. Rh: Rhizosphere. R: Root.

Morphological identification of the fungal isolates

Based on the antagonistic or plant growth enhancement properties, two rhizospheric isolates including KoA9 and KoA1 were selected for morphological characterization using descriptions in the identification keys (Klich 2002, Kirk et al. 2013, Verkley et al. 2014). The morphological analysis revealed that KoA9 belonged to *Paraconiothyrium flavescens* (Verkley et al. 2014) and KoA1 isolate belonged to *Aspergillus awamori* (Klich 2002).

Paraconiothyrium flavescens (Gruyter, Noordel. & Boerema) Verkley & Gruyter, 75: 25 (2012)

Fig. 3A-E

This isolate was obtained from almond rhizospheric soil. Based on morphological characters, the isolate KoA9 was identified as *P. flavescens* (Verkley et al. 2014). The colony on the PDA medium is initially cream-colored and with time and the production of pycnidia, it turns brown. In this species, colony growth on the PDA medium after 14 days at 25°C and darkness averaged 2.5 cm. Brown pycnidia form on the OA medium after seven days. *P. flavescens* is taxonomically

classified as *Ascomycota*, *Dothideomycetes*, *Pleosporales*, and *Didymosphaeriaceae*. Pycnidia were globose with a rather indistinct, non-papillate ostiole, mostly 20-140 µm diam., glabrous or covered by hyphae, solitary or confluent. Conidial exudate were not observed. Conidia were ellipsoidal with 2 very large polar guttules, 4-7 × 2-3.5 µm. Colonies had slow growth, 1.5 cm after 7 days (2.5 cm after 14 days), regular, without any aerial mycelium (Verkley et al. 2014) (Fig. 3, Table 4).

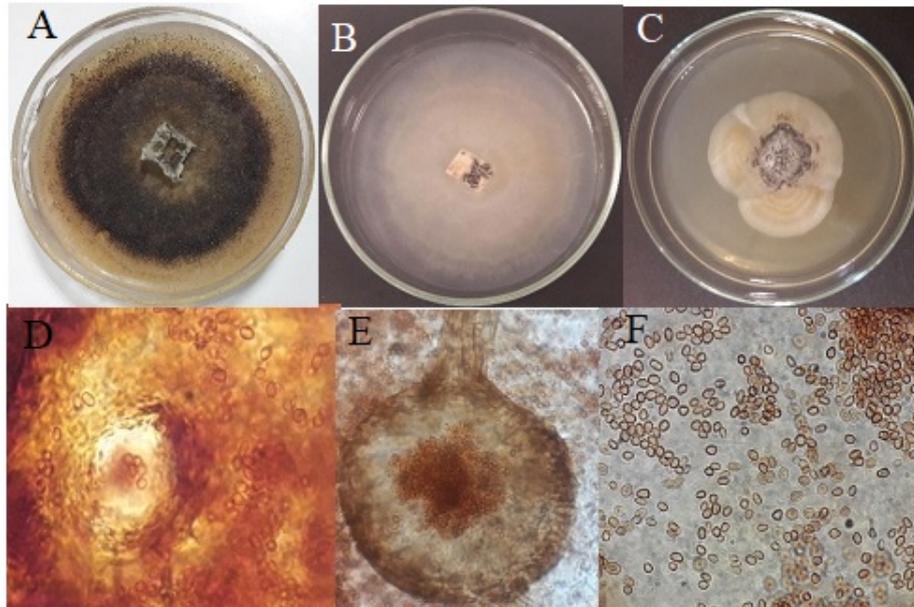


Fig. 3 – *Paraconiothyrium flavescens*. A Colony performance on PDA medium after two weeks. B Colony performance on MEA3% medium after 10 days. C Colony performance on OA medium after 10 days. D Cross section of conidiomata. E Pycnidia .F Conidia.

Table 4 Comparison of the *Paraconiothyrium flavescens* isolate with the original description.

Character	Isolate koA9	Verkley et al. 2014
Conidiogenous cells	phialidic, discrete	phialidic, discrete
Conidial septa	0	0
Conidial size	4-7 × 2-3.5 µm	4-7 × 2-2.5 µm
Conidium wall surface	smooth	smooth
Growth rate on OA	1.5 mm (7 d)	15 mm (7 d)
(colony diam after 10 d)	25 mm (14 d)	25 mm (14 d)

Aspergillus awamori Nakaz, Rep. Gov. Res. Inst. Formosa: 1 (1907)

Fig. 4A–D

This isolate was obtained from almond rhizosphere soil. Based on morphological characters, isolate KoA1 was identified as *A. awamori* (Santos et al. 2013, Cui et al. 2015). The colonies appear on the PDA medium in black. The colonies on the PDA medium are fast-growing and dispersed, filling approximately half an eight-cm plate in one week. This species was not able to form Sclerotia and ascocarp on CZ medium after seven days. It also lacked surface wrinkles on the CZ medium and wrinkles on the back of the colony and was unable to produce secretory droplets. A prominent feature of this species is the black color on the CZ medium due to the presence of black conidiophores (Schuster et al. 2002).

The genus *Aspergillus* is taxonomically classified as *Ascomycota*, *Eurotiomycetes*, *Eurotiales*, and *Trichocomaceae* (Kirk et al. 2013). In order to identify the morphological characteristics of microscopic and macroscopic specimens of *Aspergillus* genus, the CZ, CYA, CY20S and MEA media (Merck) were used (Klich 2002). Colonies on CZ, CYA, CY20S and MEA attain a diam of 8–12 mm in 7 days at 25°C; growing rapidly on CYA at 37°C, with a diam

of 12 mm, substrate mycelium was thin and hyaline, conidial heads white to black brown, loosely globose, 70–125 μm in diameter. Conidiophores usually arose from the foot cell of basal mycelium; stipes straight, mostly 1000–1350 \times 10.2–13 μm , hyaline to pale brown, not constricted below the vesicles; vesicles hemispherical to elongate, 46–50 μm in diameter, black brown, fertile over the upper half to two-thirds. *A. awamori*, metulae variable in size, 12–26 \times 3.8–4.7 μm ; phialides 8.1–9.3 \times 2–3 μm . Conidia globose to subglobose, 3.4–4 μm in diameter, rough, grey brown, parallel in chains (Fig. 4, Table 5).

Inhibitory effects of volatile compounds produced by the rhizospheric fungi

Capability of the antagonistic fungi to produce volatile compounds was evaluated in PDA medium. The visual and quantitative observations clearly indicated that the antagonist isolate-derived volatile substances caused different degrees of inhibition against the growth of *V. dahliae* and *P. drechsleri*. Among the endophytic and rhizospheric isolates, the KoA1 isolate, which showed the most effect on inhibition of *V. dahliae* and *P. drechsleri*, was selected to evaluate the effect of volatile compounds. *P. drechsleri* growth was moderately suppressed by KoA1 volatiles. The inhibition rate was 33%, while no inhibition was found against *V. dahliae* in this assay (Fig. 5).

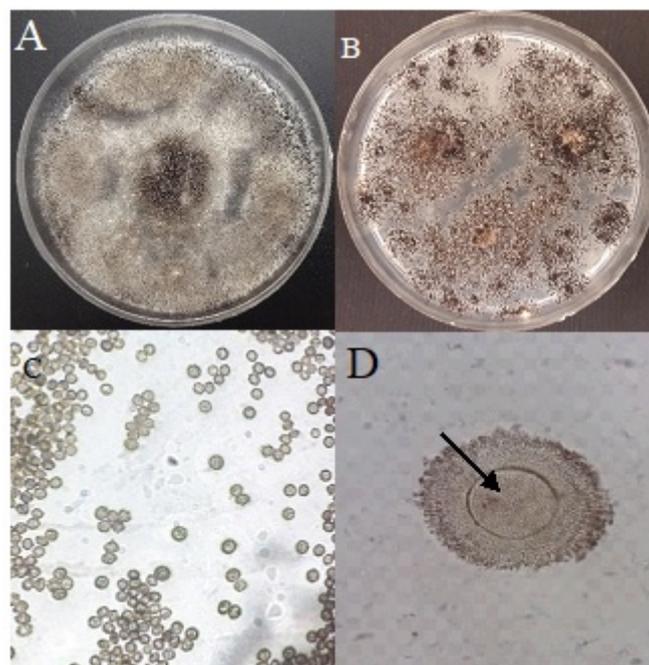


Fig. 4 – *Aspergillus awamori* isolate KoA1. A Colony morphology on PDA medium after 10 days. B Colony on CZ medium after 7 days. C, D Conidia. E conidiophores and Vesicle. Arrows indicate vesicle.

Table 5 Comparison of the *Aspergillus awamori* isolate with the original description.

Character		Isolate KoA1	Cui et al. 2015
Conidiophores	Length (μm)	1000–1350	960–1630
	Width (μm)	10.2–13	10.2–13.4
Conidial heads	Diameter(μm)	70–125	72–127
Vesicles hemispherical	Diameter(μm)	46–50	43–56
Conidia	Diameter(μm)	3.4–4	3.6–4.8
Metulae	Length (μm)	12–26	12–26
	Width (μm)	3.8–4.7	3.8–4.7
Phialides	Length (μm)	8.1–9.3	8.2–9.4
	Width (μm)	2–3	2.5–3

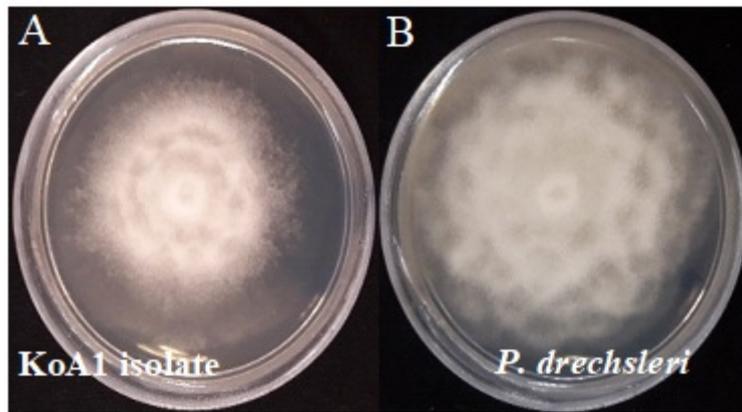


Fig. 5 – The most effective antagonistic isolate against *Phytophthora drechsleri* in the volatile compounds assay on PDA plates. Photos are taken seven days after challenging the antagonist with the pathogen. A, inhibitory effect of volatiles produced by *Aspergillus awamori* (isolate KoA1) on the growth of *P. drechsleri*; B, Control plate containing only *P. drechsleri*.

Discussion

The present study is the first report on characterization of endophytic and rhizospheric fungi isolated from almond trees and evaluating their biocontrol effect and plant growth promoting activities. Totally, 58 endophytic and rhizospheric fungi were successfully isolated from almond trees, which are indigenous to Iran. Two isolates (obtained from rhizosphere) were identified and described based on morphological criteria possessed distinguishable plant growth enhancement or antagonistic activities.

In this research, we investigated the growth enhancer role of rhizospheric and endophytic fungi isolated from almond trees. The best isolate in terms of increasing wheat stem and root growth was KoA9 (*P. flavescens*) but this isolate failed to enhance the growth of lentil. Several studies have provided *in vivo* evidence on the positive effect of endophytic and rhizospheric fungi on the growth of different plants (Rabiey & Shaw 2015, Nassimi & Taheri 2016). Endophytic fungi identified in the present study may help further investigations to address how the isolate KoA9 have promoted the growth of wheat seedlings.

In this study among 26 selected isolates, which obtained from the rhizosphere, one isolate designated as KoA1 (*A. awamori*) showed the highest antagonistic activity with 40% and 28.5% inhibitory against *P. drechsleri* and *V. dahliae* are a destructive pathogen infecting almond trees. Using biological control agents to control plant pathogens has the potential to reduce pesticide use in agriculture. One potential source of biological control agents may be endophytic fungi, because they are already adapted to grow in/on the host species (Kari Dolatabad et al. 2017). Given the economic importance and damage to the almond trees annually, it is important to find endophytic or rhizosphere fungi with biocontrol potential against destructive phytopathogens. To our knowledge, some studies exist on the biocontrol of *Phytophthora* spp. and Verticillium wilt using rhizosphere fungi. Concerning the biocontrol of *P. drechsleri*, most reports have been on the use of *Trichoderma* spp. as a biological control agent (Moayedi & Mostowfizadeh-Ghalamfarsa 2011, Ciancio et al. 2019). In the case of *V. dahliae* biocontrol, a few studies using rhizospheric fungi have been performed (Zheng et al. 2010). All previous studies have used the biocontrol capability of rhizospheric fungi from different plants, and no reports of the use of *A. awamori* in the biocontrol of the two pathogens *P. drechsleri* and *V. dahliae* are available, so far. To our knowledge, this is the first report of the rhizospheric fungus *A. awamori* with the potential of biocontrol of almond pathogens from Iran and in the world. However, further studies are needed to investigate the environmental effects of *A. awamori* and its biocontrol capability *in vivo*.

The results obtained for the production of volatile substances showed that the antagonistic isolate KoA1 (*A. awamori*) produced volatile substances acting against *P. drechsleri*. Fungi produce various mixtures of gas-phase, carbon-based compounds called volatile organic

compounds (VOCs) that due to their small size can diffuse through the atmosphere and soil. In agriculture, the interest in fungal VOCs is for their potential as biological control agents to control fungi (Morath et al. 2012). Considering the biocontrol potential of rhizospheric fungi and their VOCs against different phytopathogens (Moayedi & Mostowfizadeh-Ghalamfarsa 2011, Morath et al. 2012), we investigated the inhibitory effect of volatiles produced by *A. awamori* (isolate KoA1), which showed significant inhibitory effect against *P. drechsleri* *in vitro*. Similarly, Moayedi & Mostowfizadeh-Ghalamfarsa 2011 investigated the antifungal activity of volatile compounds of *Trichoderma* spp. (isolated from the rhizosphere of sugar beet) against *P. drechsleri*, which showed less inhibition than the Rhizosphere fungus KoA1 (*Aspergillus awamori*) used in the present study. To our knowledge, there is no other report on the inhibitory effect of VOCs produced by rhizospheric *Aspergillus* spp. isolates from almond trees against *P. drechsleri*. Considering the biocontrol potential of *A. awamori* volatile compounds against *P. drechsleri*, this study could help future research to evaluate the type of volatile compounds and their antifungal activity in the garden and greenhouse conditions against *P. drechsleri* on almond.

Conclusion

Two rhizospheric fungi were successfully isolated and identified from almond trees locally growing in Northeastern Iran, which exhibited significant antagonistic activities against *P. drechsleri* and *V. dahliae* or showed plant growth promotion activities. Present work, to our knowledge, is the first report of rhizospheric fungi including *Aspergillus awamori* (isolate KoA1) and *Paraconiothyrium flavescens* (isolate KoA9) from almond trees. The KoA1 isolate showed antagonistic effect against *P. drechsleri* in the volatile compound assays. Further *in vivo* studies are ongoing to gain insight into how these antagonistic fungi can be used for biocontrol of fungal pathogens in the almond gardens.

Acknowledgments

This research was part of the master thesis project of V Rezvani in ACECR-Khorasan Razavi, Kashmar Higher Education Institute, Kashmar, Iran. This study was partially funded by Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran and Industrial Fungi Biotechnology Research Department, ACECR- Khorasan Razavi Branch, Iran.

Declaration of interest

There is no conflict of interest to declare.

References

- Aderogba MA, Ndhkala AR, Rengasamy KRR, Staden JV. 2013 – Antimicrobial and selected *in vitro* enzyme inhibitory effects of leaf extracts, flavonols and indole alkaloids isolated from *croton menyanthes*. *Molecules* 18, 12633–12644.
- Arzanlou M, Dokhanchi H. 2012 – *Calosphaeria* canker of almond caused by *Calosphaeria pulchella* in Iran. *Archives of Phytopathology and Plant Protection* 46, 215–226.
- Azizi J, Yazdani S. 2010 – Analysis of Competition Power of Iranian Almond Export. *Journal of Agricultural Science* 2: No 1.
- Ciancio A, Pieterse CMJ, Mercado-Blanco J. 2019 – Editorial: Harnessing Useful Rhizosphere Microorganisms for Pathogen and Pest Biocontrol – Second Edition. *Frontiers in Microbiology* 10.
- Cui R, Fan C, Sun X. 2015 – Isolation and characterization of *Aspergillus awamori* BS05, a root-knot-nematode-trapping fungus. *Biocontrol Science and Technology* 25, 1233–1240.
- De Silva NI, Brooks S, Lumyong S, Hyde KD. 2018 – Use of endophytes as biocontrol agents. *Fungal biology reviews* 33, 133–148.
- EFSA PLH Panel. 2014 – EFSA Panel on Plant Health – Scientific Opinion on the pest categorisation of *Verticillium dahliae* Kleb. *European Food Safety Authority Journal*. 12, 54.

- Fatemi J. 1980 – The role of *Phytophthora* species in almond decline in Iran. *Journal of Phytopathology* 99, 97–100.
- Fotoohiyan Z, Rezaee S, Shahidi Bonjar GhH, Mohammadi AH, Moradi M. 2017 – Biocontrol potential of *Trichoderma harzianum* in controlling wilt disease of pistachio caused by *Verticillium dahliae*. *Journal of Plant Protection Research* 57, 185–193.
- Fotoohiyan Z, Rezaee S, Shahidi Bonjar GhH, Mohammadi AH, Moradi M. 2015 – Induction of Systemic Resistance by *Trichoderma harzianum* Isolates in Pistachio Plants infected with *Verticillium dahliae*. *Journal of Nuts* 6, 95–111.
- Gond S, Verma VC, Mishra A, Kumar A. 2010 – Role of fungal endophytes in plant protection. In book: *Management of Fungal Plant Pathogens*, Chapter: Role of fungal endophytes in plant protection, Publisher: CAB International, UK, Editors: Arya A, Perrello AE, 183–200.
- Hodkinson TR, Doohan FM, Saunders MJ. 2019 – *Endophytes for a Growing World*. Cambridge University Press.
- Jogaiah S, Abdelrahman M, Tran LSP, Shin-Ichi I. 2013 – Characterization of rhizosphere fungi that mediate resistance in tomato against bacterial wilt disease. *Journal of Experimental Botany* 64, 3829–3842.
- Kari Dolatabad H, Javan-Nikkhah M, Shier WT. 2017 – Evaluation of antifungal, phosphate solubilisation, and siderophore and chitinase release activities of endophytic fungi from *Pistacia vera*. *Mycological Progress* 16, 777–790.
- Kirk PM, Cannon PF, Minter DW, Stappers JA. 2013 – *Index Fungorum*. [http:// www. Indexfungorum.org](http://www.indexfungorum.org).
- Klich MA. 2002 – Identification of common *Aspergillus* species. CBS, Utrecht, Netherlands, Pp.116.
- Klosterman S, Atallah Z, Vallad G, Subbarao KV. 2009 – Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology* 47, 39–62.
- Küçük Ç, Kivanç M. 2004 – *In Vitro* Antifungal Activity of Strains of *Trichoderma harzianum*. *Turkish Journal of Biology* 28, 111–115.
- Lahlali R, Hijri M. 2010 – Screening, identification and evaluation of potential biocontrol fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. *FEMS Microbiology Letters* 311, 152–159.
- Larran S, Simón MR, Moreno MV, Siurana MS, Perelló A. 2016 – Endophytes from wheat as biocontrol agents against tan spot disease. *Biological Control* 92, 17–23.
- Moayedi G, Mstowfizadeh–Ghalamfarsa R. 2011 – Antagonistic activities of *Trichoderma* spp. on *Phytophthora* root rot of sugar beet. *Iran Agricultural Research* 29.
- Morath SU, Hung R, Bennett JW. 2012 – Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal biology reviews* 26, 73–83.
- Nassimi Z, Taheri P. 2016 – Endophytic fungus *Piriformospora indica* induced systemic resistance against rice sheath blight via affecting hydrogen peroxide and antioxidants. *Biocontrol science and technology* 2, 252–267.
- Pérez-Sierra A, León M, Álvarez LA, Alaniz S et al. 2010 – Outbreak of a new *Phytophthora* sp. associated with severe decline of almond trees in eastern Spain. *Plant Diseases* 94, 534–541.
- Philippot L, Raaijmakers JM, Lemanceau P, Van Der Putten WH. 2013 – Going back to the roots: the microbial ecology of the rhizosphere. *Nature Review Microbiology* 11, 789–799.
- Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moëne Locozy Y. 2009 – The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341–361.
- Raaijmakers JM. 2015 – “The Minimal Rhizosphere Microbiome,” in *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*, ed. B. Lugtenberg (Cham: Springer International Publishing), 411–417.
- Rabiey M, Shaw MW. 2015 – *Piriformospora indica* reduces fusarium head blight disease severity and mycotoxin DON contamination in wheat under UK weather conditions. *British Society for Plant Pathology* 65, 940–952.

- Refaei J, Jones EBG, Sakayaroj J, Santhanam J. 2011 – Endophytic fungi from *Rafflesia cantley*: species diversity and antimicrobial activity. *Mycosphere* 4, 429–447.
- Rudgers JA, Miller TEX, Ziegler SM, Craven KD. 2012 – There are many ways to be a mutualist: endophytic fungus reduces plant survival but increases population growth. *Ecology* 93, 565–574.
- Saberi-Riseh R, Fathi F. 2018 – The Management of Pistachio Gummosis by Biocontrol Strategies. *Pistachio and Health Journal* 1, 44–51.
- Schuster E, Dunn-Coleman N, Frisvad JC, Van Dijck PWM. 2002 – On the safety of *Aspergillus niger*- a review. *Applied Microbiology and Biotechnology* 59, 426–435.
- Soltaninejad N, Mohammadi H, Massumi H. 2017 – Isolation, identification and pathogenicity of *Botryosphaeriaceae* and *Phaeoacremonium* species associated with decline of *Prunus* species in Iran. *Journal of Plant Pathology* 99, 571–581.
- Santos BAQ, Ntwampe SKO, Doughari JH. 2013 – Continuous Biotechnological Treatment of Cyanide Contaminated Waters by Using a Cyanide Resistant Species of *Aspergillus awamori*. In Tech Online Publishers.
- Stapleton JJ. 1997 – Verticillium wilt of almond in California. *Bulletin OEPP/EPPO Bulletin* 27, 489–492.
- Strobel G, Daisy B. 2003 – Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* 4, 491–502.
- Verkley GJM, Dukik1 K, Renfurm R, Göker M, Stielow JB. 2014 – Novel genera and species of coniothyrium-like fungi in *Montagnulaceae* (*Ascomycota*). *Persoonia* 32, 5–51.
- Wang ZT, Li T, Wen XX, Liu Y et al. 2017 – Fungal communities in rhizosphere soil under conservation tillage shift in response to plant growth. *Frontiers in Microbiology* 8, 1301.
- Warcup JH. 1950 – The soil plate method for isolation of fungi from soil. *Nature* 166, 117–118.
- Zheng Y, Xue QY, Xu LL, Xu Q et al. 2010 – A screening strategy of fungal biocontrol agents towards *Verticillium* wilt of cotton. *Biological Control* 56, 209–216.