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Molecular typing, biodiversity, and biological control of endophytic fungi of *Triticum aestivum* L. against phytopathogenic fungi of wheat

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Abstract

The current study aimed to evaluate the biodiversity and antagonistic potential of 22 endophytic fungal species belonging to 10 endophytic genera, namely *Acremonium, Alternaria, Aspergillus, Fusarium, Gliocladium, Nigrospora, Penicillium, Pochonia, Scopulariopsis*, and *Stachybotrys*, along with 2 morphospecies of mycelia, namely a yellow sterile septate mycelium (YSSM) and a dark sterile septate mycelium (DSSM), living within healthy *Triticum aestivum* L. against nine wheat pathogenic fungi identified as *Alternaria alternata, Alternaria cichorii, Alternaria dianthi, Alternaria sonchi, Aspergillus petrakii, Curvularia penniseti, Fusarium oxysporum, Fusarium semitectum*, and *Fusarium solani* obtained from naturally infected plants. The highest count and frequency in *T. aestivum* were recorded for *Fusarium subglutinans* (n = 81 and Rf = 34.91%) followed by DSSM (n = 32 and Rf = 13.79%). *In vitro* DSSM studies showed the highest antagonistic activity of 56.52% reduction against *A. petrakii*, while *F. subglutinans* inhibited *F. semitectum* by 72.97%. These two endophytic isolates were then selected as fungal bioagents to control root rot disease in wheat caused by *A. petrakii* and *F. semitectum* under greenhouse conditions. Wheat plants growing from seeds inoculated with DSSM were more resistant to root rot disease in soil infected with *A. petrakii*, and they showed reduced root rot severity percentage (15.56%) and disease index (0.27) compared to the values of 40% and 1.13, respectively, for these parameters in untreated plants. Furthermore, treatment of seeds with *F. subglutinans* before planting in soil infected with *F. semitectum* reduced the disease severity percentage and the disease index from 50% and 2.20 to 20% and 0.47, respectively. These endophytic fungi also markedly increased the growth parameters (length, fresh weight, and dry weight of shoots and roots) of wheat plants growing in soils infected with these phytopathogens. The present study showed that wheat is a promising ecological niche for different fungal endophytes that assist their host plants to survive and resist the harmful effects of phytopathogenic fungi, improve wheat growth, and develop fungal strains as biological control solutions against wheat diseases.

Key words: endophyte fungi, biocontrol agent, phytopathogenic fungi, green house, root rot disease

Introduction

Endophytic fungi are a group of fungi with similar characteristics that inhabit different plant organs such as leaves, stems, and roots without causing any damage (Teimoori-Boghsani et al., 2020). In recent decades, there has been much interest in the fungal endophytes of plant regarding 1) entry, colonization, and distribution of endophytic microorganisms, 2) benefits of "plant-endophyte" interactions for sustainable cultivation of medicinal plants, 3) plant growth-promoting mechanisms of endophytes, 4) endophytes inducing systemic resistance in strategic crop plants, 5) seed priming by endophytes and its agricultural applications, 6) the chemical pathways involved in endophytic microorganism-plant relations, 7) endophytic microbial influence on plant stress response, 8) microbial bioformulation-based

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plant bio-stimulants as a reasonable methodology for subsequent generation of sustainable agronomy, 9) genomic insights of plant-endophyte interaction and prospects and impact on plant health, 10) gene expression studies of fungal endophytes in disease management of field crop plants, 11) endophytic fungi, a treasure house of novel nanoparticles for disease management, 12) therapeutic and biotechnological applications of the diverse fungal endophytic flora, 13) biocommercial aspects of microbial endophytes for sustainable agriculture, and 14) legal issues and IPR in microbial research (Comby et al., 2017; El-Gendy et al., 2017a; El-Bondkly et al., 2020a; Ripa et al., 2019; Kumar and Radhakrishnan, 2020). Cereal crops serve as the most important source of calories, carbohydrates, and some proteins for the human population (Jalili et al., 2020). Wheat (*Triticum aestivum*) is one of the strategic essential cereal crops for human consumption worldwide, with yearly production reaching approximately 750 million metric tons (Ikram et al., 2019). Wheat yield is susceptible to fungal diseases such as *Fusarium* head blight (FHB) caused by several *Fusarium* species (Rojas et al., 2020). However, phytopathogenic fungi are not the only microorganisms that inhabit wheat organs. Wheat organs are also colonized by extremely diverse communities of endophytic fungal genera that live inside the host plant without causing any signs of disease (Ikram et al., 2019). These species appear to be ubiquitous as symbionts within all organs and species in the plant kingdom in a close specific relationship that is believed to be important in stimulating plant fitness and tolerance to biotic and abiotic stress (Kumar and Radhakrishnan, 2020). Because endophytic fungi have similar characteristics and essentially share the same biological niches as plant pathogenic fungi, it is important to recognize their associations and behaviors during pathogen attack and disease development, especially as several of these fungi may be used as new sources of biological control agents against phytopathogenic fungi (Larran et al., 2007; Rojas et al., 2020).

The key objectives of the present study were to evaluate the overall composition of the endophytic mycobiome within the inner tissues of wheat plants, including root, stem, leaves, glumes, and grains, to conduct a large-scale screening of phytopathogenic fungi of diseased wheat plants. We also aimed to explore and use fungal endophytes of healthy wheat as biological control agents against the fungal pathogens isolated from *in vitro* diseased wheat plants by using the dual culture technique under greenhouse conditions. To the best of our knowledge, this is the first study to assess the structure of endophytic fungi in wheat microbiome and their antagonistic properties against fungal pathogens of wheat.

Materials and methods

Isolation of endophytic fungi from wheat plants

Healthy wheat samples (*Triticum aestivum* cultivar Gimaza 9) were collected from Al-Qalyubia Governorate located between 30°06′11″ and 30°36′36″ North and 31°03′20″ and 31°35′32″ East, north of Cairo, Egypt, in the growing seasons of 2015 and 2016. To obtain the highest isolation frequency of endophytic fungi from wheat, samples of each organ, namely leaves, roots, stems, glumes, and grains, were acquired at the growth stage GS 11.1 (milky ripe growth stage) as recommended by Larran et al. (2007). The samples were placed in plastic bags and immediately transported to the laboratory for processing. Wheat organs were washed under running tap water and surface sterilized by immersion in 96% ethanol for 1 min and 2% sodium hypochlorite for 3 min, followed by rinsing in sterile deionized water for 3 min. The surface-sterilized materials were dried on a sterile filter paper and cut into 1-cm pieces. Sets of five segments of each organ were then placed evenly in 90 mm Petri dishes. Three isolation media, namely malt yeast extract agar (MYA), potato dextrose agar (PDA), and starch casein agar (SCA), were used in 6 replicas for each medium for each of the five organs. All inoculated plates were incubated at 28°C for 2 weeks in dark and examined periodically. All fungal colonies from the plant tissues that grew on agar were isolated, purified, and maintained on the same isolation medium at 4°C as previously described by El-Gendy and El-Bondkly (2014) and El-Gendy et al. (2014; 2016; 2017a, b; 2018a, b).

Isolation of pathogenic fungi from naturally infected wheat plants

Naturally infected wheat plants that exhibited disease symptoms during the same growing season in the same field (El-Qalyubia Governorate, Egypt) were collected, surface sterilized, and dried as described above. Once the plants were dry, five pieces per tissue were placed on PDA and incubated at 28°C for 7 days, and the

obtained isolates were purified and stored at 4°C for further analysis (Hassanein, 2010; Hassanein et al., 2010, 2016).

Identification of endophytic and phytopathogenic fungi of wheat

All fungal isolates were identified on the basis of their phenotypic characteristics by using international taxonomic keys at the Mycological Center, Faculty of Science, Assiut University (AUMC). The identification of the dark septate sterile mycelium (DSSM) of the selected endophytic strains and *Fusarium* sp. NAZ6 was confirmed based on the phylogenetic analysis of these endophytic strains. Both endophytic strains were grown in 50 ml potato dextrose broth for 5 days at 28°C. Mycelium was harvested, washed with deionized water, and pulverized with liquid nitrogen. The genomic DNA of these fungi was extracted and purified using a QIAGEN DNeasy Tissue Kit following the manufacturer's protocol for gram-positive bacteria and animal tissue. The ribosomal DNA was amplified using puReTaq™ Ready-To-Go™ polymerase chain reaction (PCR) beads (GE Healthcare). The internal transcribed spacer (ITS) of the nuclear rDNA was amplified using ITS1 (5′-TCCGTAG GTGAACCTGCG-3′) and ITS2 (5′-GCTGCGTTCTT CATCGATGC-3′) primers. The PCR reaction and conditions as well as sequencing parameters were according to the method described by El-Bondkly (2012), El-Bondkly and El-Gendy (2012), El-Gendy et al. (2018a), and White et al. (1990). The amplified DNA was sequenced in the Animal Health Institute in Giza, Egypt. Sequence data were edited with Lasergene Software SeqMan (DNAStar Inc.) and analyzed for the identification of the closest homologs of the fungal strains by using BLAST provided in the NCBI (National Center for Biotechnology Information) GenBank database (Altschul et al., 1990). The phylogenetic tree and molecular evolutionary relationships were inferred using the ITS data for each isolate with closest relatives. The ITS1 – 5.8S – ITS2 ribosomal DNA region sequences of the DSSM and *Fusarium* sp. NAZ6 of the selected endophytic strains were deposited in the GenBank database.

In vitro biocontrol characteristics of fungal endophytes of healthy wheat against pathogenic fungi

The inhibitory activity of the endophytic strains of healthy wheat against pathogenic fungi derived from diseased wheat plants was assessed *in vitro* by the dual culture technique (Hassanein, 2010; Hassanein et al., 2010, 2016). Each endophytic isolate was streaked onto one side of a Petri dish containing PDA and incubated in dark at 28°C for 72 h to allow the secretion and diffusion of biocontrol agents from the active antagonistic strains into the medium. Next, agar disks with the 7-day-old mycelium of the fungal pathogen were placed on the reverse side of the streaked plates. Mycelial discs of pathogens inoculated in PDA plate alone served as controls. All inoculated plates were incubated at 28°C for 6 days and monitored periodically for the inhibition (%) of radius of each pathogen during 6 days of incubation using the following formula: percentage of inhibition I(%) = A – B / A × 100, where A – radius of the pathogen in the control plate, B – radius of pathogen in the dual culture plate (Nouari et al., 2013).

In vivo greenhouse studies

On the basis of the *in vitro* studies, the hyper-antagonistic endophytic strains, DSSM, and *F. subglutinans* were selected for greenhouse studies against the hypersensitive pathogenic strains *Aspergillus petrakii* and *Fusarium semitectum*.

Preparation of pathogen inoculate, soil infestation, and pathogenicity tests

The selected pathogenic strains*, A. petraki* and *F. semitectum*, were grown on PDA medium at 28°C for 7 days in dark, and 4 agar discs (8 mm in diameter) of each pathogen were then individually inoculated into 250 ml Erlenmeyer flasks containing a maize grain-based medium (10 g of grains in deionized water, autoclaved at 121°C for 30 min) and incubated at 28°C for 14 days with occasional shaking (Wong et al., 1984). Pathogen inoculates were carefully dispersed throughout sterile soil in 3 sets of plastic pots of 12 cm diameter each and filled with 500 g of sandy soil. The following treatments were performed: the 1st set contained *A. petrakii* inoculum, the 2nd set contained *F. semitectum* inoculum, and the 3rd set contained the same quantity of sterilized non-infested soil that served as a control. Eight wheat seeds (Gimaza 9) obtained from Wheat Research Department, Field Crops Research Institute (FCRI), ARC, Cairo, Egypt, were surface sterilized and sown at 2 cm depth in triplicates pots; the pots were then placed in the greenhouse at 25°C with a 14-h photoperiod and watered with equal amount of water on alternate days.

Table 1. Total count, relative frequency, and specificity of the endophytic fungal genera and species isolated from the wheat plant *T. aestivum*

Genus	Species	Organ					Total species count in plant (CFU)
		roots	stems	leaves	glumes	grains	
Fusarium	*F. acuminatum* NAZ1	14	–	1	–	–	15
	F. graminum NAZ2	1	–	–	–	–	1
	F. nygamai NAZ3	7	–	–	–	–	7
	F. oxysporum NAZ4	37	2	–	–	–	39
	F. semitectum NAZ5	8	–	–	–	–	8
	F. subglutinans NAZ6	11	–	–	–	–	11
	total count	78	2	1	0.0	0.0	81
	total RF [%]	33.62	0.86	0.43	0.0	0.0	34.91
Alternaria	*A. alternata* NAZ7	–	–	7	–	–	7
	A. chlamydospore NAZ8	–	10	11	–	–	21
	total count	0.0	10	18	0.0	0.0	28
	total RF [%]	0.0	4.31	7.76	0.0	0.0	12.07
Aspergillus	*A. aculeatus* NAZ9	2	–	3	–	–	5
	A. fumigatus NAZ10	–	–	3	–	–	3
	A. parasiticus NAZ11	3	4	3	–	–	10
	A. terreus NAZ12	–	–	1	–	–	1
	total count	5	4	10	0.0	0.0	19
	total RF [%]	2.16	1.73	4.32	0.0	0.0	8.19
Penicillium	*P. chrysogenum* NAZ13	–	2	6	–	–	8
	P. oxalicum NAZ14	–	11	10	–	–	21
	total count	0.0	13	16	0.0	0.0	29
	total RF [%]	0.0	5.61	6.90	0.0	0.0	12.50
Acremonium	*A. blochii* NAZ15	1	–	–	–	–	1
	A. roseolum NAZ16	–	5	–	–	–	5
	A. strictum NAZ17	–	–	–	–	1	1
	total count	1	5	0.0	0.0	1	7
	total RF [%]	0.43	2.16	0.0	0.0	0.43	3.02
Gliocladium	*G. roseum* NAZ18	9	–	–	–	–	9
	total RF [%]	3.88	0.0	0.0	0.0	0.0	3.88
Nigrospora	*N. oryzae* NAZ19	–	–	3	2	1	6
	total RF [%]	0.0	0.0	1.29	0.86	0.43	2.59
Pochonia	*P. suclasporia* NAZ20	1	2	–	–	–	3
	total RF [%]	0.43	0.86	0.0	0.0	0.0	1.29
Scopulariopsis	*S. brumptii* NAZ21	–	3	2	1	–	6
	total RF [%]	0.0	1.29	0.86	0.43	0.0	2.59
Stachybotrys	*S. bisbyi* NAZ22	3	–	–	–	–	3
	total RF [%]	1.29	0.0	0.0	0.0	0.0	1.29
Yellow sterile mycelium (YSSM)	total YSSM	9	–	–	–	–	9
	total RF [%]	3.88	0.0	0.0	0.0	0.0	3.88
Dark septate sterile mycelium (DSSM)	total DSSM	14	3	8	2	5	32
	total RF [%]	6.03	1.29	3.45	0.86	2.16	13.79

RF – relative frequency, DSSM – dark septate sterile mycelium isolate, YSSM – yellow septate sterile mycelium isolate, CFU – colony frequency unit

The growing wheat seedlings were observed for 10 days daily after planting, and the percentages of damping-off were evaluated according to Leath et al. (1989) as follows:

$$\text{Percentage of damping-off [\%]} = \frac{\text{no. of ungerminated seeds/pot}}{\text{no. of sown seeds/pot}} \times 100$$

Table 2. Total count (CFU) and species richness of endophytic fungi isolated from different organs of *T. aestivum*

Parameter	Organ species count (CFU) and relative frequency					Total in plant
	roots	stems	leaves	glumes	grains	
Total count (CFU)	120.0	42.0	58.0	5.0	7.0	232.0
CFU percentage [%]	51.72	18.10	25.0	2.16	3.02	100.0
Species richness	14.0	9.0	12.0	3.0	3.0	24.0
Species richness percentage [%]	58.33	37.5	50.0	12.5	12.5	100.0

Table 3. Pathogenic fungi isolated from different diseased wheat plant organs

Pathogenic fungi	Plant organ				
	roots	stems	leaves	glumes	grains
A. alternate	–	√√	√√	√√	√√
A. cichorii	–	√√	√√	–	√√
A. dianthi	–	–	√√	–	–
A. sonchi	–	√√	√√	–	–
A. petrakii (Vörös-Felkai)	–	√√	–	–	√√
C. penniseti	√√	√√	–	–	–
F. oxysporum (Schlechtendal)	√√	–	–	–	√√
F. semitectum (Berkeley)	√√	–	–	–	–
F. solani (Martius) Saccardo	√√	–	–	–	–

One month after planting, the growing wheat plants were taken out from soil, washed, and examined for root rot disease by determining the percentage of disease severity and the severity index by comparing with control plants (Liu et al., 1995; Beccari et al., 2011).

Preparation of endophytic fungal inoculums

The endophytic fungi were grown on PDA medium for 10 days at 28°C until maximum sporulation was obtained. Suspensions of the endophytic antagonist were then prepared by flooding the cultures with sterile deionized water containing 0.02% Tween 80, rubbing their surfaces with a sterile glass rod and then vortexing. The conidial concentration used for *F. subglutinans* NAZ6 and the DSSM isolate was 2×10^6 conidia/ml and 2×10^6 propagules/ml, respectively.

Effect of the selected endophytic fungi on root rot fungal pathogens

Fifty surface-disinfected wheat seeds (Gimaza 9) were coated by immersing in 3 ml of spore suspension of the selected endophytic fungi for 3 h and dried for 2 h in a laminar air flow cabinet. *A. petrakii* and *F. semitectum* maize grain-based inoculants were added and mixed with sterilized (autoclaved) soil individually. Plastic pots (12 cm diameter and filled with 500 g soil) were half-filled with the infested soil and then overlaid with a 2 cm layer of noninfested sterilized soil. The seeds were planted on that layer and further overlaid with a 2 cm layer of noninfested soil (Coombs et al., 2004). The wheat seed treatments were as follows: 1) seeds treated with *A. petrakii* only, 2) seeds treated with *A. petrakii* + DSSM isolate, 3) seeds treated with *F. semitectum* only, 4) seeds treated with *F. semitectum* + *F. subglutinans*, and 5) surface-disinfected noninoculated seeds planted in noninfested soil (−ve control) and surface-sterilized seeds coated with water planted in soil with each pathogen (+ve control). Each plastic pot was planted with 8 coated seeds, and each treatment was performed in three replicates. The pots were placed in a greenhouse at 25°C with a 14-h photoperiod and watered with equal quantity of water on alternate days. After 30 days from planting, wheat plants were removed from the soil and washed carefully, and the severity of

Table 4. *In vitro* antagonistic activity of the endophytic fungal isolates of healthy *T. aestivum* against phytopathogenic fungi of wheat

Endophytic fungi	*Pathogenic fungi																							
	A. alternate			*A. cichorii*			*A. dianthi*			*A. sonchi*			*A. petraki*			*F. oxysporum*			*F. semitectum*			*F. solani*		
	A	B	I [%]	A	B	I [%]	A	B	I [%]	A	B	I [%]	A	B	I [%]	A	B	I [%]	A	B	I [%]	A	B	I [%]
Dark sterile isolate DSSM	3.3	1.6	51.52	3.5	3.5	0.0	3.0	3.0	0.0	2.5	2.5	0.0	2.3	1.0	56.52	3.5	3.5	0.0	3.7	3.7	0.0	3.0	3.0	0.0
F. nygamai NAZ3	3.3	1.1	66.67	3.5	3.5	0.0	3.0	3.0	0.0	2.5	2.5	0.0	2.3	2.3	0.0	3.5	3.5	0.0	3.7	1.1	70.27	3.0	3.0	0.0
F. subglutinans NAZ6	3.3	1.2	63.64	3.5	1.0	71.43	3.0	1.0	66.67	2.5	2.5	0.0	2.3	1.1	52.17	3.5	1.0	71.43	3.7	1.0	72.97	3.0	3.0	0.0
F. oxysporium NAZ4	3.3	3.3	0.0	3.5	0.7	80.00	3.0	3.0	0.0	2.5	2.5	0.0	2.3	2.3	0.0	3.5	1.1	68.57	3.7	1.1	70.27	3.0	1.1	63.33
F. graminum NAZ2	3.3	3.3	0.0	3.5	3.5	0.0	3.0	3.0	0.0	2.5	1.0	60	2.3	2.3	0.0	3.5	3.5	0.0	3.7	3.7	0.0	3.0	3.0	0.0
G. roseum NAZ18	3.3	1.6	51.52	3.5	3.5	0.0	3.0	3.0	0.0	2.5	2.5	0.0	2.3	2.3	0.0	3.5	3.5	0.0	3.7	3.7	0.0	3.0	3.0	0.0
P. chrysogenum NAZ13	3.3	3.3	0.0	3.5	3.5	0.0	3.0	3.0	0.0	2.5	2.5	0.0	2.3	2.3	0.0	3.5	3.5	0.0	3.7	3.7	0.0	3.0	3.0	0.0
P. suclasporia NAZ20	3.3	1.1	66.67	3.5	3.5	0.0	0.0	3.0	3.0	2.5	2.5	0.0	2.3	2.3	0.0	3.5	3.5	0.0	3.7	1.1	70.27	3.0	3.0	0.0
Yellow sterile isolate YSSM	3.3	1.3	60.61	3.5	3.5	0.0	3.0	1.1	63.33	2.5	2.5	0.0	2.3	1.2	47.83	3.5	3.5	0.0	3.7	3.7	0.0	3.0	3.0	0.0
A. alternate NAZ7	3.3	3.3	0.0	3.5	1.0	71.43	0.0	3.0	3.0	2.5	2.5	0.0	2.3	0.8	65.22	3.5	1.0	71.43	3.7	3.7	0.0	3.0	1.0	66.67
A. fumigatus NAZ10	3.3	3.3	0.0	3.5	3.5	0.0	0.0	3.0	3.0	2.5	2.5	0.0	2.3	1.4	39.13	3.5	1.2	65.71	3.7	3.7	0.0	0.0	0.0	0.0
S. brumptii NAZ21	3.3	3.3	0.0	3.5	3.5	0.0	0.0	3.0	3.0	2.5	2.5	0.0	2.3	1.2	47.83	3.5	3.5	0.0	3.7	1.1	70.27	0.0	0.0	0.0

* I [%] – inhibition percentage [%] = A – B / A × 100; A – radius of the pathogen in the control plate, B – radius of the pathogen in the dual culture plate

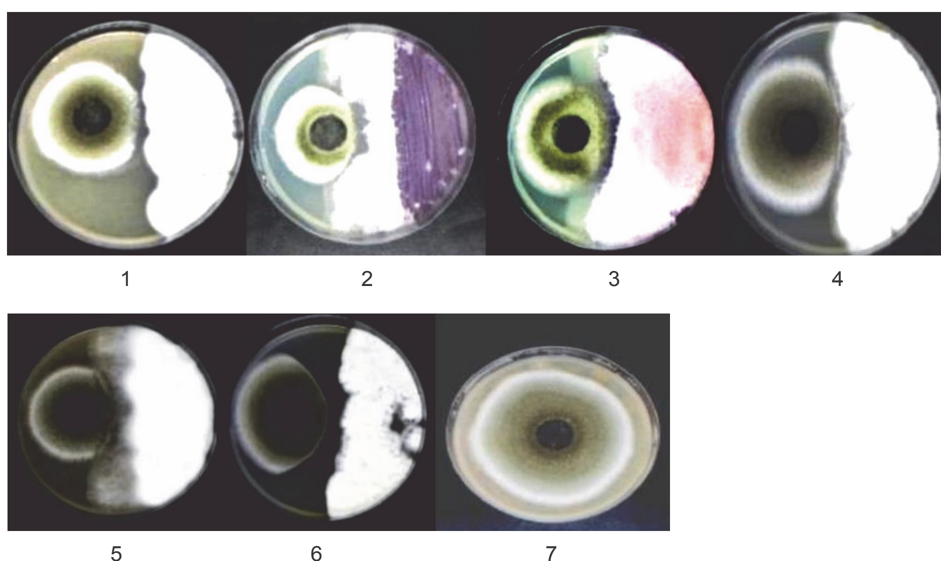

Fig. 1. Antagonistic activity of 1) DSSM isolate, 2) *F. nygamai*, 3) *F. subglutinans*, 4) *G. roseum*, 5) *P. suclasporia*, and 6) YSSM isolate against *A. alternata*, and 7) control

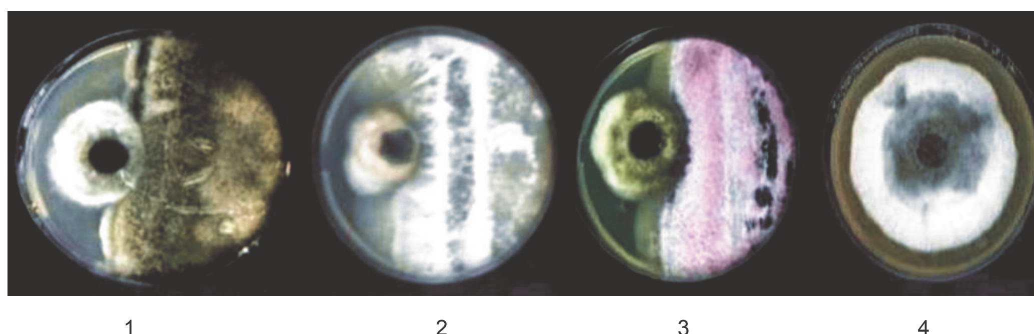

Fig. 2. Antagonistic activity of the endophytic fungi 1) *A. alternata*, 2) *F. oxysporum*, 3) *F. subglutinans* against *A. cicharii*, and 4) control

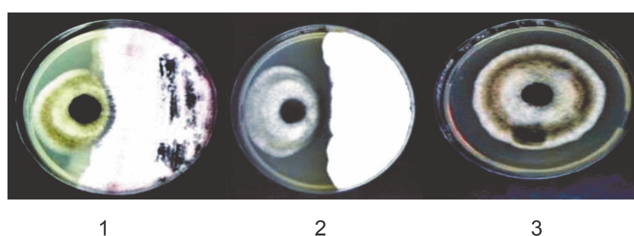

Fig. 3. Antagonistic activity of the endophytic fungi 1) *F. subglutinans*, 2) YSSM isolate against *A. dianthi*, and 3) control

root rot disease was assessed. The mean of the severity of root rot disease (disease severity index [DSI]) for each replicate was estimated using the formula DSI = $(\Sigma d/d_{max} \times n) \times 100$ (Liu et al., 1995), where d is the disease rating of plants, dmax is the highest disease rating, and n is the total number of plants examined for each replicate, [disease rating: 0 – roots without discoloration (no infection), 1 – 1–20%, 2 – 21–40%, 3 – 41–75%, 4 – 75–100% discoloration of root mass, and 5 – totally dead plants].

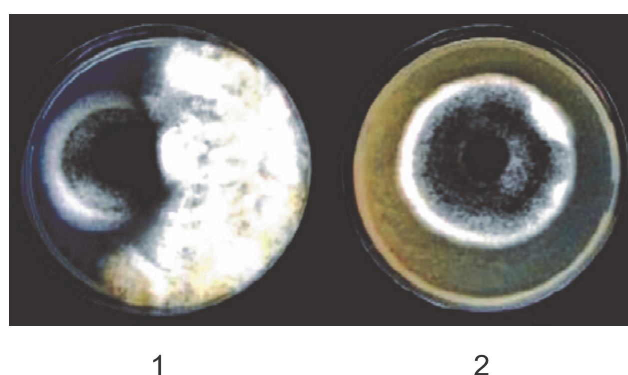

Fig. 4. Antagonistic activity of the endophytic fungi 1) *F. graminum* against *A. sonchi*, and 2) control

Primary root disease symptoms were calculated using the formula of Beccari et al. (2011): disease index (DI)

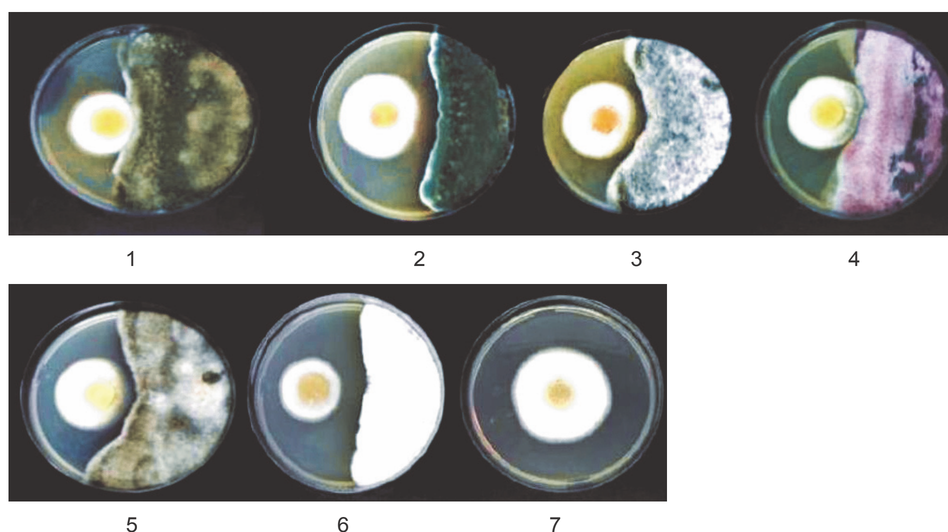

Fig. 5. Antagonistic activity of the endophytic fungi 1) *A. alternata*, 2) *A. fumigatus*, 3) DSSM isolate, 4) *F. subglutinans*, 5) *S. brumptii*, 6) YSSM against *A. petrakii*, and 7) control

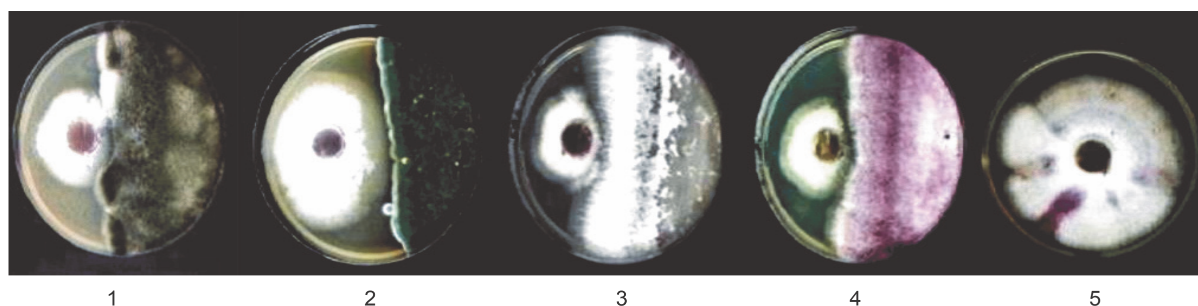

Fig. 6. Antagonistic activity of the endophytic fungi 1) *A. alternata*, 2) *A. fumigatus*, 3) *F. oxysporum*, 4) *F. subglutinans* against *F. oxysporum*, and 5) control

= SE × BI, where SE is symptom extension (cm) and BI is browning index based on a five-point scale: (0 – symptomless; 1 – slightly necrotic; 2 – moderately necrotic; 3 – severely necrotic and 4 – totally necrotic). The shoot and root growth parameters, including length (cm) and fresh and dry weights (g), were also measured.

Statistical analysis

IBM SPSS statistics (V. 24.0, IBM Corp., USA, 2016) were used for data analysis. The results are expressed as mean ± SD for quantitative parametric measures. Analysis of variance (ANOVA) was used for comparing more than 2 groups for parametric results. Multiple comparisons (Post hoc test or least significant difference [LSD]) were performed in a similar manner to examine the possible statistical significance between two groups. The probability of error at 0.05 was considered significant, while that at 0.01 and 0.001 was considered highly significant.

Results and discussion

Isolation and identification of endophytic fungi from T. aestivum

Ten endophytic fungal genera, namely *Acremonium, Alternaria, Aspergillus, Fusarium, Gliocladium, Nigrospora, Penicillium, Pochonia, Scopulariopsis,* and *Stachybotrys*, along with two morphospecies of mycelia species, namely yellow septate sterile mycelium (YSSM) and DSSM, were obtained from the roots, stems, leaves, glumes, and grains of healthy *T. aestivum* plants (Table 1). According to the international phenotypic taxonomic keys, *Fusarium* genus was characterized as *F. acuminatum* NAZ1, *F. graminum* NAZ2, *F. nygamai* NAZ3, *F. oxysporum* NAZ4, *F. semitectum* NAZ5, and *F. subglutinans* NAZ6; *Aspergillus* species was identified as *A. aculeatus* NAZ9, *A. fumigatus* NAZ10, *A. parasiticus* NAZ11, and *A. terreus* NAZ12; *Penicillium* genus in-

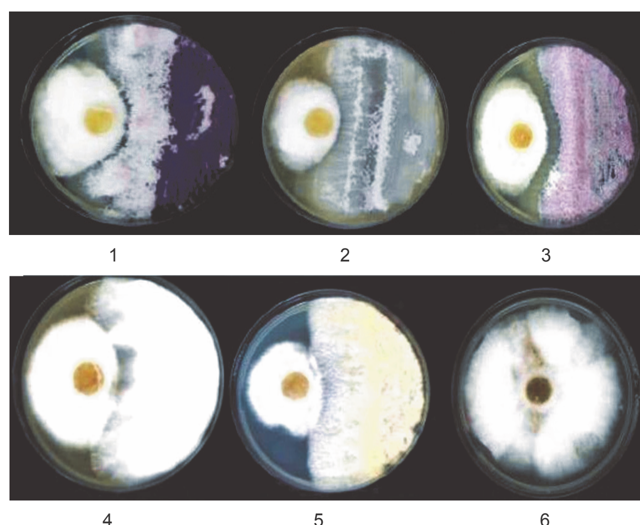

Fig. 7. Antagonistic activity of the endophytic fungi 1) *F. nygamai*, 2) *F. oxysporum*, 3) *F. subglutinans*, 4) *P. suclasporia*, 5) *S. bisbyi* against *F. semitectum*, and 6) control

cluded *P. chrysogenum* NAZ13 and *P. oxalicum* NAZ14; *Acremonium* genus included *A. blochii* NAZ15, *A. roseolum* NAZ16, and *A. strictum* NAZ17 along with one species of *Gliocladium (G. roseum* NAZ18), *Nigrospora* (*N. oryzae* NAZ19), *Pochonia* (*P. suclasporia* NAZ20), *Scopulariopsis* (*S. brumptii* NAZ21), and *Stachybotrys* (*S. bisbyi* NAZ22) genera (Table 1). Our data largely agree with the findings of Comby et al. (2017) who isolated *Alternaria, Acremonium, Cladosporium*, *Phoma*, *Chaetomium*, and *Epicoccum* species as the most frequent fungal endophytes of wheat. Moreover, sixteen endophytic fungi comprising *Aspergillus niger*, *A. flavus*, *A. stellatus*, *Fusarium oxyporum*, *F. incarnatum*, *F. proliferatum, F. equiseti, Penicillium aurantiogriseum*, *P. janthinellum, A. alternata*, *A. tenuissima*, and *Cladosporium cladosporioides* were characterized as dominant wheat endophytic fungi (Ripa et al. 2019; El-Bondkly et al., 2020b).

Tissue specificity, relative frequency, and species richness of the endophytic fungi of T. aestivum

The total count and relative frequency (Rf %) of each fungal species isolated (calculated as the number of one species divided by the total number of isolates and expressed as percentage) are given in Table 1. The highest count and Rf of endophytic fungal species in *T. aestivum* were recorded for *Fusarium* species (n = 81 and *Rf* = 34.91%), followed by DSSM (n = 32 and *Rf* = 13.79%), *Penicillium* (n = 29 and *Rf* = 12.50%), *Alternaria* (n = 28 and *Rf* = 12.07%), and *Aspergillus* (n = 19 and *Rf* = 8.19%).

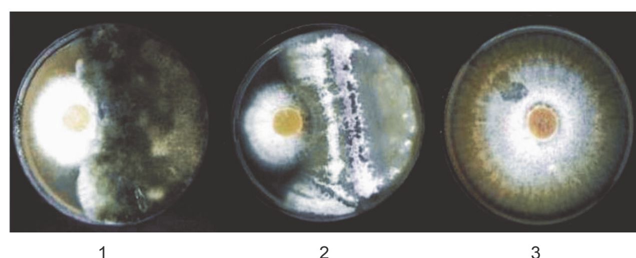

Fig. 8. Antagonistic activity of the endophytic fungi 1) *A. alternata*, 2) *F. oxysporum* against *F. solani*, and 3) control

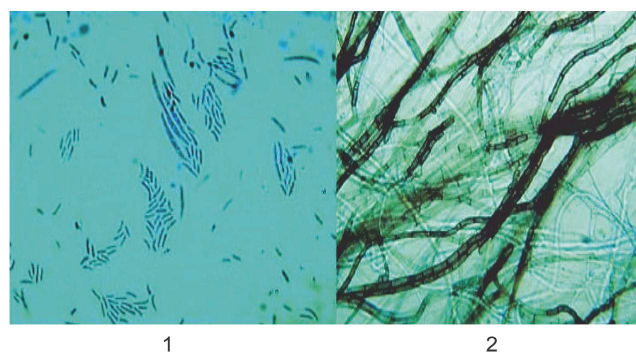

Fig. 9. Photomicrograph of the endophytic fungi 1) *F. subglutinans* and 2) DSSM isolate

An equal count (n = 9) with an equal frequency (3.88%) was found for YSSM and *Gliocladium* species, and the total number of 7, 6, and 3 with *Rf* = 3.02, 2.59, and 1.29% were recorded for *Acremonium*, (*Nigrospora* and *Scopulariopsis*), and (*Pochonia* and *Stachybotrys*) species, respectively. Some endophytic fungi can colonize certain plant tissues more often than others. For instance, all *Gliocladium* (n = 9), *Stachybotrys* (n = 3), YSSM (n = 9), and *Fusarium* species were detected only in roots (n = 78), except for *F. acuminatum* NAZ1 (n = 14 in roots and n = 1 in leaves) and *F. oxysporum* NAZ4 (n = 37 in roots and n = 2 in stems). None of the *Alternaria, Penicillium, Nigrospora*, and *Scopulariopsis* species as well as 50% of *Aspergillus* (*A. fumigatus* NAZ10 and *A. terreus* NAZ12) and 66.66% of *Acremonium* species (*A. roseolum* NAZ16 and *A. strictum* NAZ17) were detected in the roots (Table 1). Moreover, *A. roseolum* NAZ16 was specific only for stem tissue, while *A. alternata* NAZ7, *A. fumigatus* NAZ10, and *A. terreus* NAZ12 showed specificity for leaves. Furthermore, glumes and grains were poor sources of endophytic isolates; only *N. oryzae* NAZ19 (n = 2), *S. brumptii* NAZ21 (n = 1), and DSSM (n = 2) isolates were detected in glumes, while *A. strictum* NAZ17 (n = 1) and *N. oryzae* NAZ19 (n = 1) were detected in grains. *Scopulariopsis*

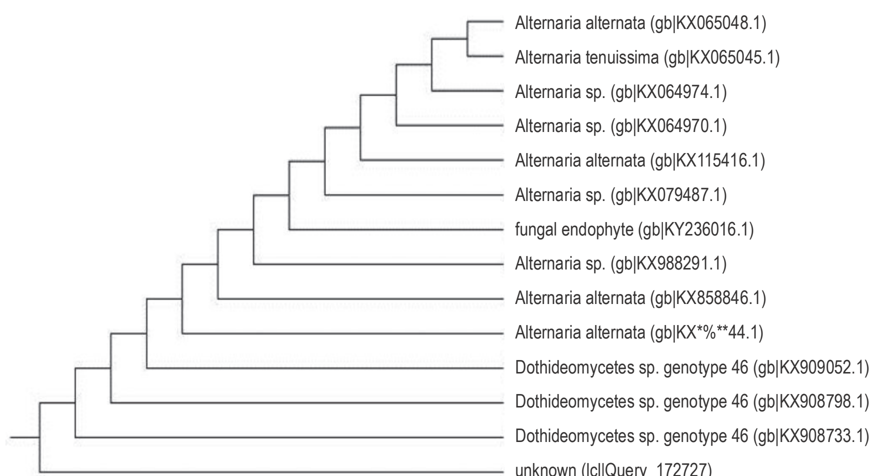

Fig. 10. Phylogenetic tree based on the ITS1 – 5.8S – ITS2 region sequence for the DSSM isolate

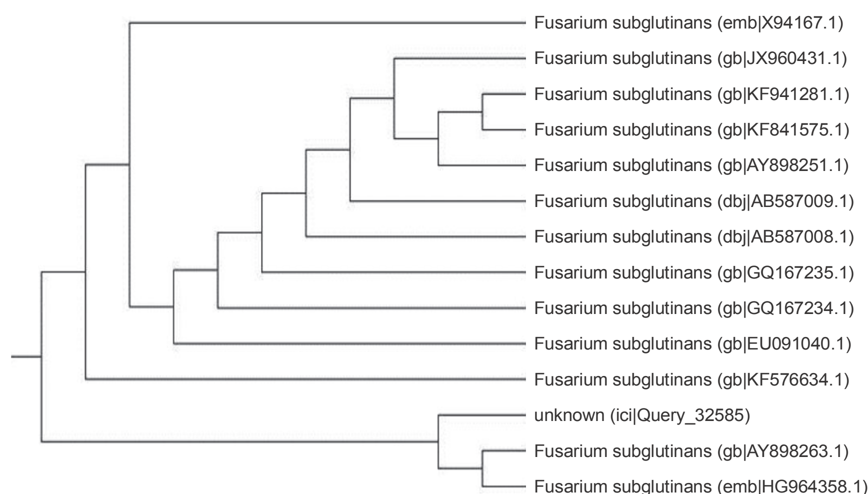

Fig. 11. Phylogenetic tree based on the ITS1 – 5.8S – ITS2 region sequence for *F. subglutinans*

was found only in wheat glumes (Table 1). Hence, the total wheat organ species count (CFU), total relative frequency (%), species richness, and species richness percentage (%) in roots, stems, leaves, glumes, and grains were found to be (120.0, 51.72%, 14.0, and 58.33%), (42.0, 18.10%, 9.0, and 37.5%), (58.0, 25.0%, 12.0, and 50.0%), (5.0, 2.16%, 3.0, and 12.5%), and (7.0, 3.02%, 3.0, and 12.5%), respectively (Table 2). Many previous studies have shown tissue specificity between host plants and their endophytes (El-Bondkly and El-Gendy, 2010; El-Gendy et al., 2014; 2017a), and this aspect may be attributed to their varying contact with sunlight, wind, moisture, aeration, and nutrient availability (Andreote et al., 2014, Ripa et al., 2019). Moreover, El-Gendy et al. (2017a) proposed that organ specificity in some endophytic fungi could be restricted to only one organ of the plant, thus showing the importance of isolating endophytes from each organ of each plant to increase the number of most active organisms. Conversely, Comby et al. (2017) reported that there was not much difference in 21 endophytic filamentous fungi isolated from aerial parts or roots of wheat.

Identification of pathogenic fungi isolated from diseased wheat tissues

Nine pathogenic fungal species belonging to 4 genera (*Alternaria, Aspergillus*, *Curvularia*, and *Fusarium)* that are responsible for several wheat diseases (Smiley et al., 2005; Beccari et al., 2011; Comby et al., 2017; Hartmann et al., 2017) were isolated and identified from the diseased wheat plant tissues during 2015–2016 growing

Table 5. Pathogenicity of the selected root rot pathogens on wheat plants under greenhouse conditions

Treatments	Disease		
	damping-off [%]	root rots severity [%]	disease index
A. petrakii	41.67	40	1.13
F. semitectium	45.83	50	2.20
Control (non-infected)	8.33	–	–

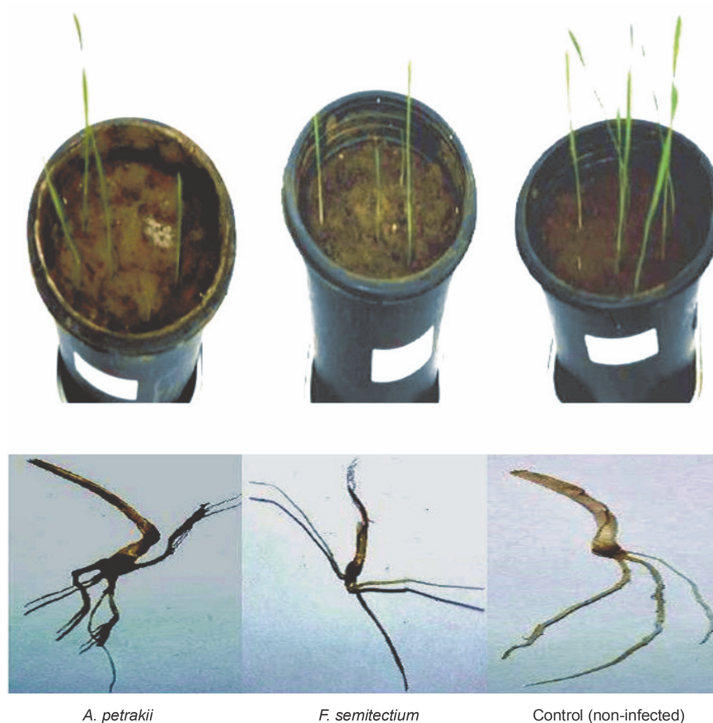

Fig. 12. Damping-off and root rot severity of wheat plants caused by root rot pathogens from left to right: *A. petrakii, F. semitectum* and control

season (Table 3). The highest number of species found was five (*A. alternata, A. cichorii, A. sonchi, A. petrakii*, and *C. penniseti*) in stems, followed by four species in roots (*C. penniseti, F. oxysporum, F. semitectum*, and *F. solani*), four species in leaves (*A. alternata, A. cichorii, A. dianthi*, and *A. sonchi*), four species in grains (*A. alternata, A. cichorii, A. petrakii*, and *F. oxysporum*), and one species in glumes (*A. alternata*) (Table 3). Importantly, this is the first report on the isolation of *A. petrakii* from a wheat plant. Wheat is among the most strategic cereal crops and staple food sources worldwide, but many diseases greatly affect its yield and quality (Rojas et al., 2020). Moreover Panwar et al. (2016) and Rojas et al. (2020) have reported that *Alternaria* species, in particular *A. alternata* and *A. sonchi*, as well as *Fusarium* species such as *F. oxysporum, F. solani*, and *F. semitectum* are the most common phytopathogens of wheat.

Biological control activity of the endophytic fungi of healthy T. aestivum against the phytopathogenic fungi of wheat

To investigate the effective antagonistic microorganisms, the *in vitro* screening test is the first and the most important step in finding biological control agents to manage wheat fungal pathogens (Comby et al., 2017). We conducted in vitro antagonism screening (Table 4 and Figs. 1–8) to evaluate the ability of 12 endophytic fungal species, namely DSSM, *F. graminum* NAZ2, *F. nygamai* NAZ3, *F. oxysporium* NAZ4, *F. subglutinans* NAZ6,

Table 6. Efficiency of the selected endophytic antagonists on the severity of root rot disease and disease index of wheat plants under greenhouse conditions

Pathogens	Treatments	Root rots severity [%]	Disease index
A. petrakii	+ ve control	40	1.13
	+ dark septate sterile mycelium isolate inoculant	15.56	0.27
F. semitectum	+ ve control	50	2.20
	+ dark septate sterile mycelium isolate inoculant	20	0.47

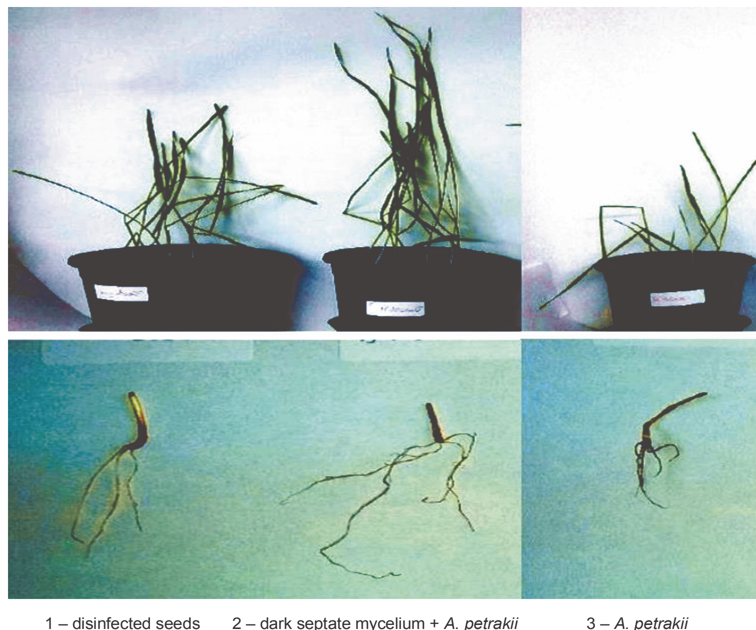

Fig. 13A. Efficiency of the endophytic DSSM isolate on the growth parameters of wheat shoots and roots in soil infested with the root rot pathogen *A. petrakii*; from left to right 1) disinfected seeds; 2) seeds inoculated with DSSM isolate and then planted in soil infested with *A. petrakii*, and 3) seeds planted in soil infested with *A. petrakii* without inoculant

A. alternata NAZ7*, A. fumigatus* NAZ10*, P. chrysogenum* NAZ13*, G. roseum* NAZ18*, P. suclasporia* NAZ20*, S. brumptii* NAZ21, and YSSM, to inhibit the radial growth of nine phytopathogens, including *A. alternata, A. cichorii, A. dianthi, A. sonchi, A. petrakii*, *C. penniseti*, *F. oxysporum, F. semitectum*, and *F. solani*. Overall, all endophytic fungi were inactive against *C. penniseti*. The endophytic fungi *F. nygamai* NAZ3 and *P. suclasporia* NAZ20 were the active fungal inhibitors of the growth of pathogenic *A. alternata* by 66.67%, followed by *F. subglutinans* NAZ6, YSSM isolate, *G. roseum* NAZ18, and DSSM isolate with inhibition percentages of 63.64, 60.61, 51.52, and 51.52%, respectively. The endophytic fungi *F. oxysporum* NAZ4 showed the highest inhibitory activity toward the radical growth of *A. cichorii* by 80%, while the endophytic fungi *F. subglutinans* NAZ6 and *A. alternata* NAZ7 inhibited its growth by 71.43% (Table 4 and Fig. 1 and Fig. 2). Interestingly, only the endophytic fungi *F. subglutinans* NAZ6 and YSSM showed the ability to inhibit *A. dianthi* by 66.67% and 63.33%, respectively; the endophytic fungi *F. subglutinans* NAZ6 and *A. alternata* NAZ7 repressed the growth of pathogenic *F. oxysporum* by 71.43%, and only *F. graminum* NAZ2 could inhibit the growth of *A. sonchi* by 60%. Six endophytic fungi, including *A. alternata* NAZ7, DSSM, *F. subglutinans* NAZ6, YSSM, *S. brumptii* NAZ21, and *A. fumigatus* NAZ10 inhibited the growth of *A. petrakii* by 65.22, 56.52, 52.17, 47.83, 47.83, and 39.13%,

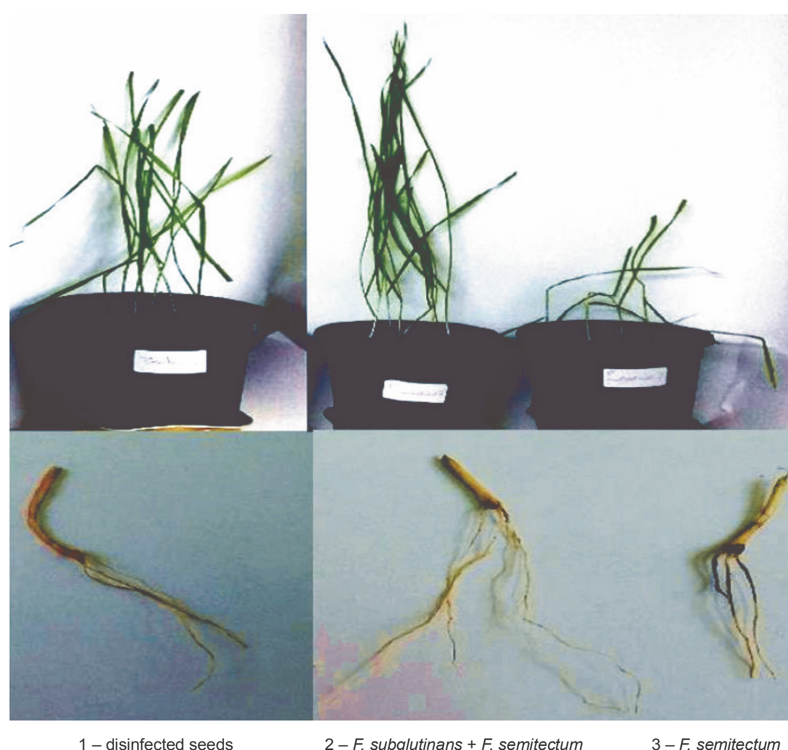

Fig. 13B. Efficiency of the endophytic *F. subglutinans* on the growth parameters of wheat shoots and roots in soil infested with root rot pathogen *F. semitectium*; from left to right: 1) disinfected seeds in sterilized soil; 2) seeds inoculated with *F. subglutinans* and then planted in soil infested with *F. semitectium*, and 3) seeds planted in soil infested with *F. semitectium* without inoculant

respectively (Table 4 and Figs. 3–5). Furthermore, the endophytic fungi *F. oxysporum* and *A. fumigatus* decreased the viability of the pathogenic *F. oxysporum* by 68.57 and 65.71%, respectively, while the growth of *F. semitectum* was repressed by 72.97% by *F. subglutinans* NAZ6 and by 70.27% after treatment with each of *F. nygamai* NAZ3, *F. oxysporum* NAZ4, *P. suclasroria* NAZ20 and *S. Brumptii* NAZ21. *F. solani* growth was repressed by 66.67% and 63.33% by the endophytic antagonistic fungi *A. alternata* NAZ7 and *F. oxysporum* NAZ4 (Table 4 and Figs. 6–8). Due to the highest number and frequency of *Fusarium* species (n = 81 and Rf = 43.9%) and DSSM (n = 32 and Rf =13.79%) in *T. aestivum* mycobiome along with their hyperantagonistic activity against *A. petrakii* and *F. semitectum*, respectively, these endophytic fungi were selected for phylogenetic analysis and *in vivo* studies under greenhouse conditions. Troncoso et al. (2010) and Vergara et al. (2018) also reported that the endophytic fungi *F. subglutinans* and DSSM were the predominant fungi among the endophytic fungal populations in the plant mycobiome with unique biologically active substances that do not cause any signs of diseases; these results are in line with our findings. Similarly, Teimoori-Boghsani et al. (2020) reported that fungal endophytes of wheat proved to be promising biological control agents and offer an alternative safe strategy to wheat disease management. Fungal endophytes adapt particularly well to their host plant, colonize the same ecological niches as those of phytopathogens, and produce a wide range of secondary metabolites necessary for their survival and for the protection of their host against many phytopathogens and insect herbivores.

Phylogenetic analysis of the hyper antagonistic fungi DSSM and F. subglutinans NAZ6

The phenotypic identification of the selected endophytic isolates DSSM and NAZ6 as dark septate mycelium isolate and *F. subglutinans* strains, respectively, (Fig. 9) was confirmed on the basis of the molecular

Table 7. Efficiency of the selected endophytic antagonists on the growth parameters of wheat shoots and roots in soil infested with phytopathogens

Treatments	Wheat shoots parameters											
	length [cm]				fresh weight [g]				dry weight [g]			
	mean	mean different	*P*	significant	mean	mean different	*P*	significant	mean	mean different	*P*	significant
Disinfected seeds (−ve control)	26.967	–	–	–	0.230	–	–	–	0.034	–	–	–
Seeds infected with *A. petrakii*	21.992	4.974	0.01	HS	0.177	0.052	0.005	HS	0.029	0.005	0.044	S
A. petrakii + DSSM	35.396	−13.403	0	HS	0.322	−0.145	0	HS	0.048	−0.018	0	HS
Seeds infected with *F. semitectum*	21.114	5.852	0	HS	0.179	0.050	0.003	HS	0.029	0.005	0.056	NS
F. semitectium + *F. subglutinans*	34.783	−13.669	0	HS	0.296	−0.117	0	HS	0.042	−0.013	0	HS
Wheat roots parameters												
Disinfected seeds (−ve control)	4.514	–	–	–	0.032	–	–	–	0.010	–	–	–
Seeds infected with *A. petrakii*	3.485	1.02	0	HS	0.031	0.001	0.63	NS	0.010	−0.001	0.765	NS
A. petrakii + DSSM	4.683	−1.19	0	HS	0.040	−0.009	0.001	HS	0.015	−0.004	0	HS
Seeds infected with *F. semitectum*	3.200	1.314	0.002	HS	0.025	0.007	0.006	HS	0.009	0.001	0.13	NS
F. semitectum + *F.subglutinans*	4.850	−1.650	0	HS	0.038	−0.013	0	HS	0.012	−0.003	0	HS

S – significant, NS – nonsignificant, HS – highly significant

characterization of both endophytic strains by using the ITS1 – 5.8S – ITS2 ribosomal DNA region sequence. The DNA of each fungus was extracted, purified, and sequenced, and the sequencing data were aligned and analyzed for finding the closest homologs for these fungal strains. Phylogenetic analyses and molecular evolutionary relationships were inferred using the ITS data for each isolate with their closest relatives, and the identity was confirmed and illustrated in Figure 10 and Figure 11. BLAST search for strains yielded 100% identity with that of dark septate endophyte isolate (Fig. 10) and *F. subglutinans* (Fig. 11), respectively; these species were then deposited in NCBI GenBank under accession numbers KY236016.1 and AY898263.1, respectively.

Pathogenicity test with root rot pathogens

Pathogenicity tests of the selected pathogenic fungi *A. petrakii,* and *F. semitectum* on wheat cultivar Gimaza 9 (Table 5) showed that they caused damping-off after 10 days of planting and developed root rot diseases in plants after 30 days of planting in soil infested with these pathogens. *F. semitectum* recorded the highest percentage for all pathogenicity criteria in the diseased wheat plants, including damping-off (45.83%), root rot severity (50%), and disease index (2.20), followed by *A. petrakii*, which caused 41.67% damping-off and 40% root rot severity, with 1.13 disease index (Table 5 and Fig. 12). In line with the results of our work, the surveys conducted by Güney and Güldür (2018) for wilt and root rot diseases caused by *R. solani*, *M. phaseolina*, *F. oxysporum*, and *F. solani* in pepper fields proved that all the tested isolates led to stem and root rot, leaf chlorosis, and bruising when the soil was infested with these pathogens. Furthermore, Smiley et al. (2005) found that all 178 fungal isolates belonging to *Bipolaris sorokiniana*, *F. avenaceum*, *F. culmorum*, *F. pseudograminearum*, and *Microdochium nivale* could cause crown rot disease in wheat under greenhouse conditions ($P < 0.05$), and they decreased the length of the plant and the density of mature heads, while increasing the occurrence and severity of crown rot relative to noninoculated controls. Interestingly, Hartmann et al. (2017) reported that the fungal wheat pathogen *Zymoseptoria tritici* developed host specialization by rearranging large-scale chromosomes in the affected plants.

Efficiency of seed coating treatments with the selected endophytic fungi against root rot pathogens

The effect of coating the seeds with DSSM and *F. subglutinans* on the suppression of root rot disease severity in soil infested with *A. petrakii and F. semitectum*, respectively, is shown in Table 6 and Figure 13A and Figure 13B. The root rot severity percentage and the disease index in the soil infested with *A. petrakii* were repressed from 40% and 1.13 in control treatment to 15.56% and 0.27, respectively, in wheat plants whose seeds were inoculated with DSSM isolate before plating in infested soil (Table 6 and Fig. 13A). Moreover, *F. subglutinans* treatment of seeds before planting in soil infested with *F. semitectum* reduced the disease severity percentage and disease index from 50% and 2.20 in *F. semitectum* control treatment to 20% and 0.47, respectively, in plants that grew from the inoculated seeds (Table 6 and Fig. 13B). Hence, the selected endophytic bioagents can be used as effective protection agents of wheat plants against root rot disease. Both DSSM and *F. subglutinans* are unique sources of bioactive substances such as salicylic acid, jasmonic acid, subglutinol A and B, gibberellin, and gibberellin oxidase that have been reported to regulate defense hormone signaling networks in plants and enhance resistance against pathogens and insect attack (Troncoso et al., 2010; Gong et al., 2012, Ozan, 2019; Teimoori-Boghsani et al., 2020).

Efficiency of the selected endophytic fungi on the growth parameters of wheat

The selected endophytic fungi were evaluated for their effectiveness in promoting the growth of wheat plants following *in vivo* inoculation when compared with control. After one month of planting, wheat plants were harvested and washed, and their growth parameters were evaluated. Results shown in Table 7 and Figure 13 a and b indicate that the growth parameters of both shoots and roots, including length, fresh weight, and dry weight, were increased markedly in plants grown from seeds inoculated with the endophytic fungi DSSM or *F. subglutinans* either in soil infected with *A. petrakii* and *F. semitectium*, respectively, (+ve control) or uninfected soil (−ve control). The enhanced growth parameters increased the absorption of phosphate and other

nutrients, thereby improving the quality of wheat and spikes yield per unit area and subsequently leading to higher yields with uniform grain (Comby et al., 2017; Ripa et al., 2019).

Conclusions

The findings of the present study confirmed that healthy wheat plants are a promising ecological niche for different fungal endophytes occurring in natural conditions. These endophytic fungi have a crucial function to improve plant growth because of their capability to secrete elevated amounts of various growth metabolites that inhibit invasive fungi and to assist their host plants to survive and resist the harmful effects of phytopathogenic fungi. Furthermore, these growth inhibitor metabolites can act as innovative biological control agents that are environmentally friendlier than traditional pesticides. Further studies are necessary to investigate in detail the interaction of these endophytic fungi with the host plant, the metabolite production of both strains, and improvement of the inoculation technology.

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