Biocontrol potential of endophytic fungus *Talaromyces trachyspermus* against root rot pathogens of sunflower

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Abstract

Several reports revealed that endophytic fungi have great influence on host plants, as they promote plant growth and minimize disease severity caused by various pathogens. In this study, endophytic fungi isolated from healthy plants were identified as *Aspergillus terreus*, *Curvularia lanata*, *C. hawaiiensis*, *Macrophomina phaseolina*, *Fusarium solani*, *Talaromyces assiutensis* and *T. trachyspermus* were evaluated for antimicrobial activity, using dual-culture plate assay and agar disc diffusion method. They have shown significant activity against root rot pathogens, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *Foxysporum*. *Talaromyces assiutensis* and *T. trachyspermus* were selected for further study, since other fungi are well known plant pathogens or environmental contaminants. They were applied in pots and field plot experiments using sunflower as test plant. Their efficacy was also compared with endophytic *Cephalosporium* sp., and *Chaetomium* sp. *Talaromyces* spp., significantly suppressed root rotting fungi and improved plant biomass. They ameliorated production of plant defense biochemical markers (polyphenolic content and salicylic acid) and antioxidant potential of treated plants. GC-MS profiling of *n*-hexane fraction of *T. trachyspermus* yielded several new compounds from this source. Endophytic fungi associated with healthy plants have great potential to suppress root rotting fungi and stimulate production of plant's defense biochemical markers.

Introduction

Endophytic fungi are the microorganisms that live inside plant tissues but do not cause any disease^{1,2,3}. Recent reports show that they have great influence on host plants as they promote plant growth, minimize disease severity, produce antiherbivore/antimicrobial products and improve defense mechanism^{4,5,6,7}. Endophytic fungi produce bioactive compounds, involved in increasing the adaptability of fungi and their host, utilization of nutritional elements in the form of nitrogen and phosphorus which are beneficial for plants^{8,9}. They improved plant growth via secretion of different hormonal substances and plant's defense biochemical markers¹⁰. *Penicillium* species, which are environment contaminants, are now being reported as endophytes that improve systemic resistance of plants against abiotic and biotic stresses^{6,11,12,13}. The IAA produces endophytic *Penicillium roqueforti* enhanced nutrient uptake by plant and tolerance against stresses¹⁴. Similarly, it has been reported that solubilization of phosphate by *Penicillium oxalicum* resulted in improved maize growth, while, *Gliocladium* sp., an endophytic fungus produces volatile antibiotics identified as Annulene¹⁵.

Endophytic fungi may have several characteristics which enable them to be widely used as biological control agents¹⁶. It is now known that mutualistic fungal endophytes may contribute to a wide range of plant tolerance to various biotic and abiotic stresses including elevating resistance level to drought stress to extreme temperatures and high salinity^{17,18}. An endophytic fungus *Daldinia concentrica* has been reported to suppress plant parasitic nematodes, *Meloidogyne javanica*¹⁹. Whereas endophytic *Trichoderma*²⁰ and endophytic *Penicillium*^{3,6} significantly suppressed root rotting fungi on tomato, sunflower and okra. One of the most important threats facing world agriculture is root rot disease caused by fungi that reduces yield and quality in economic crops^{21,22}. They also attack sunflower plants in most parts of the world including Pakistan²³. Stem and root rot disease of sunflowers caused by *Fusarium* spp., and charcoal rot by *Macrophomina phaseolina* are major limiting factors in sunflower growth^{22,24}. Endophytic fungi produce bioactive compounds and have suppressive effects on pathogenic fungi are emerging as possible alternatives to chemical pesticides^{3,5,6}. This study describes the isolation, identification, molecular characterization and antimicrobial potential of endophytic *Talaromyces* species associated with healthy plants. Biocontrol potential of some endophytic fungi against root rotting fungi was also evaluated using sunflower as a test plant. GC-MS profiling of *n*-hexane fraction of culture filtrate of endophytic *Talaromyces trachyspermus* is also being reported here.

Results

Morphological Identification of endophytic fungi. The endophytic fungi isolated were identified as *Aspergillus terreus*, *Curvularia lanata*, *C. hawaiiensis*, *Macrophomina phaseolina*, *Fusarium solani*, *Talaromyces assiutensis* and *T. trachyspermus* on the basis of morphological characters (Table 1).

Table 1

Growth inhibition of *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum* in dual culture plate assay by the endophytic fungi isolated from different wild and cultivated plants.

Fungi	Host name	<i>M. phaseolina</i>	<i>R. solani</i>	<i>F. solani</i>	<i>F. oxysporum</i>
		Zone of inhibition (mm)			
<i>Chaetomium</i> sp. (KUCC-1359)	<i>Momordica charantia</i> L. (G.H.No. 94618)	29 ^c ± 4.9	15 ^a ± 3.5	25 ^{bc} ± 2.1	31 ^f ± 2.9
<i>Talaromyces assiutensis</i>	<i>Euphorbia hirta</i> L. (G.H.No. 12615)	20 ^{ab} ± 1.6	24 ^c ± 3.8	27 ^{bc} ± 1.4	15 ^a ± 1.8
<i>Talaromyces trachyspermus</i>	<i>Haloxylon stocksii</i> (Boiss.) (G.H.No. 93479)	25 ^{bc} ± 2.4	26 ^c ± 3.1	33 ^d ± 3.1	20 ^{cd} ± 1.8
<i>Curvularia lanata</i>	<i>Allium cepa</i> L. (G.H.No. 95608)	18 ^a ± 3.1	16 ^{ab} ± 1.6	30 ^a ± 1.8	25 ^{ab} ± 2.1
<i>Curvularia hawaiiensis</i>	<i>Euphorbia hirta</i> L.	20 ^{ab} ± 1.6	16 ^{ab} ± 3.1	18 ^a ± 1.9	27 ^e ± 0.81
<i>Fusarium solani</i>	<i>Chenopodium album</i> L. (G.H.No. 89235)	25 ^{bc} ± 2.4	16 ^{ab} ± 3.1	16 ^a ± 2.1	20 ^{cd} ± 1.4
<i>Macrophomina phaseolina</i>	<i>Indigofera hochstetteri</i> Baker (G.H. No. 38211)	17 ^c ± 4.9	19 ^{bc} ± 2.1	18 ^b ± 3.0	27 ^d ± 2.2
<i>Aspergillus terreus</i>	<i>Sida ovata</i> Forssk. (G.H. No. 12278)	15 ^a ± 3.5	20 ^{abc} ± 4.7	15 ^a ± 1.9	18 ^{bc} ± 1.8
<i>Fusarium solani</i>	<i>Corchorus olitorius</i> L. (G.H. No. 50663)	18 ^a ± 4.3	26 ^c ± 1.6	29 ^{cd} ± 3.0	25 ^e ± 2.4
<i>Talaromyces assiutensis</i>	<i>Solanum melongena</i> L. (G.H. No. 82045)	21 ^{ab} ± 2.0	26 ^c ± 3.2	17 ^{cd} ± 2.4	28 ^{cd} ± 0.81
Values in column bearing same superscript letter are not significantly different at p < 0.05 according to Duncan's multiple range test					

Molecular Identification of endophytic fungi. The dendrogram revealed the genetic relatedness among and between fungal isolates. The fungal genera are divided into three clades. The nucleotide sequence of MK894136, MK894137 and MK894138 are similar to each other. The nucleotide sequence of MK894141, MK894142 and MK894139 are closely related with one another and exhibit sequence similarity at nucleotide level. MK894144 and MK894149 exhibit higher heterogeneity than other species and hence placed into distinct groups within a cluster. Neighbour-Joining phylogenetic tree constructed by MEGA software illustrates that all species were placed into respective groups on the bases of nucleotide similarity (Fig. 1).

Antifungal activity of endophytic fungi in dual culture plate Assay. Endophytic fungi have shown significant activity against four test root rot fungi and produced zones of inhibition. Most of them showed more than 15 mm zone against *M. phaseolina* and more than 20 mm against each *R. solani*, *F. oxysporum* and *F. solani* (Table 1).

Antifungal activity of culture filtrates of endophytic fungi. Culture filtrates of endophytic fungi were examined against *F. oxysporum*, *F. solani*, *M. phaseolina* and *R. solani*, caused inhibition of test fungi and produced inhibition zone of 10mm or larger against them at 60 µL/disc (Table 2).

Table 2 In vitro growth inhibition of *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum* by culture filtrates of PGPEF isolated from healthy plants

Fungi	Culture filtrates	M. phaseolina	R. solani	F. solani	F. oxysporum
Zone of inhibition(mm)					
Control		0 ^a ± 0.0	0 ^a ± 0.0	0 ^a ± 0.0	0 ^a ± 0.0
+ve Control	Carbendazim(20µg/disc)	5 ^{bc} ± 0.8	7 ^{bcd} ± 1.8	10 ^{cdef} ± 2.0	9 ^{cdef} ± 2.9
Chaetomium sp.					
	20µl/disc	11 ^{efghi} ± 2.1	10 ^{defg} ± 2.0	9 ^{cdef} ± 2.1	8 ^{bcd} ± 1.8
	40µl/disc	14 ^{hijk} ± 2.2	15 ^{klmn} ± 2.9	15 ^{hijk} ± 2.4	11 ^{efg} ± 1.9
	60µl/disc	19 ^{lmn} ± 4.6	19 ^{lm} ± 3.1	21 ^{mn} ± 2.1	19 ^{ijk} ± 2.3
Talaromyces assiutensis					
	20µl/disc	5 ^{bc} ± 0.8	4 ^b ± 1.4	5 ^{bc} ± 0.8	7 ^{bcd} ± 0.8
	40µl/disc	10 ^{cdef} ± 2.0	13 ^{ghi} ± 3.1	14 ^{hij} ± 2.1	19 ^{ijk} ± 2.2
	60µl/disc	18 ^{lmn} ± 2.1	18 ^{mn} ± 2.1	19 ^{lmn} ± 2.3	23 ^{mn} ± 3.1
T. trachyspermus					
	20µl/disc	8 ^{bcd} ± 1.8	9 ^{cdef} ± 2.0	6 ^{bc} ± 1.8	10 ^{cde} ± 2.0
	40µl/disc	15 ^{ijkl} ± 3.6	17 ^{jkl} ± 2.4	15 ^{hijk} ± 1.6	19 ^{ijk} ± 2.5
	60µl/disc	20 ^{mn} ± 2.5	23 ⁿ ± 2.6	21 ^{mn} ± 2.0	24 ⁿ ± 2.9
Curvularia lanata					
	20µl/disc	4 ^b ± 1.4	0 ^b ± 0	4 ^{cdef} ± 2.3	5 ^{bcd} ± 1.8
	40µl/disc	11 ^{cdef} ± 2.9	8 ^{cdef} ± 2.9	7 ^{efghi} ± 1.8	9 ^{cde} ± 1.9
	60µl/disc	16 ^{ijkl} ± 2.4	10 ^{hij} ± 2.2	11 ^{jkl} ± 2.1	12 ^{ghi} ± 2.2
Curvularia hawaiiensis					
	20µl/disc	8 ^{bcde} ± 1.8	4 ^d ± 1.0	5 ^{bc} ± 0.8	8 ^{bcd} ± 1.8
	40µl/disc	15 ^{ijkl} ± 3.6	7 ^{bcd} ± 1.8	8 ^{bcde} ± 1.8	13 ^{fgh} ± 2.1
	60µl/disc	17 ^{lmn} ± 2.9	12 ^{fghi} ± 1.8	16 ^{ijkl} ±	14 ^{fgh} ± 2.2
Fusarium solani					
	20µl/disc	6 ^{bcd} ± 1.8	5 ^{bc} ± 0.8	7 ^{bcd} ± 1.4	8 ^{bcd} ± 1.8
	40µl/disc	9 ^{cdef} ± 2.9	8 ^{bcde} ± 1.8	10 ^{cdef} ± 1.8	9 ^{def} ± 2.4
	60µl/disc	13 ^{ghij} ± 3.1	11 ^{efgh} ± 1.9	15 ^{hijk} ± 1.6	16 ^{hij} ± 2.5
Macrophomina phaseolina					
	20µl/disc	5 ^{bcde} ± 1.8	0 ^a ± 0	7 ^b ± 2.1	7 ^{bc} ± 0.8
	40µl/disc	9 ^{fghi} ± 1.8	6 ^{bcde} ± 1.6	11 ^{cde} ± 1.4	9 ^{bcde} ± 1.5
	60µl/disc	11 ^{ijkl} ± 2.1	12 ^{ghi} ± 1.9	11 ^{fgh} ± 2.1	18 ^{ghi} ± 1.9
Aspergillus terreus					
	20µl/disc	5 ^{bc} ± 0.8	4 ^b ± 1.4	5 ^{bc} ± 1.9	5 ^{bc} ± 0.8
	40µl/disc	11 ^{efgh} ± 1.9	9 ^{cde} ± 1.9	11 ^{def} ± 0.8	10 ^{cdef} ± 1.7
	60µl/disc	15 ^{ijk} ± 2.2	14 ^{hij} ± 2.1	16 ^{ijk} ± 2.0	14 ^{fgh} ± 2.0

Values in column bearing same superscript letter are not significantly different at p < 0.05 according to Duncan's multiple range test

Table 2 In vitro growth inhibition of <i>Macrophomina phaseolina</i> , <i>Rhizoctonia solani</i> , <i>Fusarium solani</i> and <i>F. oxysporum</i> by culture filtrates of PGPEF isolated from healthy plants					
<i>Fusarium solani</i>	20µl/disc	4 ^b ± 1.4	5 ^{bc} ± 0.8	7 ^{bcd} ± 2.1	4 ^b ± 1.4
	40µl/disc	10 ^d ± 2.0	12 ^{efgh} ± 1.6	9 ^{cdef} ± 0.8	8 ^{bcde} ± 1.8
	60µl/disc	17 ^{klmn} ± 2.9	11 ^{efg} ± 2.0	15 ^{hijk} ± 1.3	12 ^{defg} ± 1.8
<i>T. assiutensis</i>	20µl/disc	10 ^d ^{efg} ± 2.0	6 ^{bc} ± 1.8	7 ^{bcd} ± 0.8	7 ^{bcd} ± 0.8
	40µl/disc	17 ^{klmn} ± 2.9	15 ^m ± 2.1	17 ^{ijkl} ± 1.3	16 ^{hij} ± 1.4
	60µl/disc	21 ⁿ ± 2.1	22 ⁿ ± 2.5	22 ⁿ ± 1.8	20 ^{jk} ± 1.8
Values in column bearing same superscript letter are not significantly different at p < 0.05 according to Duncan's multiple range test					

In vitro **antifungal activity of solvent fractions (n-hexane and chloroform) of culture filtrates of endophytic fungi**. All endophytic fungi were found to produce 15mm or more than 15mm zone against *F. solani* and *M. phaseolina*, while more than 10mm inhibition zone were measured against *F. oxysporum* and *R. solani* at 60µL/disc of *n*-hexane soluble fraction. In case of chloroform soluble fraction of endophytes, more than 10mm inhibition zone was measured against common phytopathogen including *M. phaseolina*, *R. solani*, *F. solani*, and *F. oxysporum* at 60µL/disc (Table 3).

Table 3
In vitro antifungal activity of n-hexane and chloroform soluble fractions of culture filtrates of endophytic fungi.

Endophytic Fungi	Conc. of culture filtrates	Zone of inhibition (mm)							
		<i>M. phaseolina</i>		<i>R. solani</i>		<i>F. solani</i>		<i>F. oxysporum</i>	
		Hexane	Chloroform	Hexane	Chloroform	Hexane	Chloroform	Hexane	Chloroform
	Control	0	0	0	0	0	0	0	0
<i>Chaetomium</i> sp.	+ve control*	5	10	12	9	10	10	9	15
	20µl/disc	12	10	8	7	9	6	12	9
	40µl/disc	17	12	11	9	12	10	16	13
	60µl/disc	19	15	13	11	17	13	20	16
<i>Talaromyces assiutensis</i>									
	20µl/disc	9	7	11	8	9	6	8	5
	40µl/disc	13	9	14	11	12	9	10	9
	60µl/disc	17	14	20	14	15	11	16	13
<i>Talaromyces trachyspermus</i>									
	20µl/disc	8	5	9	7	11	9	6	4
	40µl/disc	12	9	11	10	13	11	11	8
	60µl/disc	15	10	15	12	15	13	13	11

GC-MS profiling of n-hexane fraction of culture filtrates of *Talaromyces trachyspermus*. A total 24 compounds were isolated and identified from *T. trachyspermus* of which 14 compounds were found new from this source when compared at Science finder. New compounds identified are 2-(2-Hydroxy-hex-1-enyl)-3-methyl-5, 6-di-hydropyrazine, Acetic acid, [3-hydroxy-4-(1-oxopropyl) phenyl] ester, l-Alanine, N-(2-fluorobenzoyl)-, heptadecyl ester, 6-Acetamido-2-methylbenzothiazole, 9-[6-Hydroxyhexyl] hypoxanthine, Caffeine, cis-10-Heptadecenoic acid, cis-9-Hexadecenoic acid, Methoxyacetic acid, 2-tetradecyl ester, Cyclopropane-octanoic acid, 1,2-Benzenedicarboxylic acid, diisooctyl ester, 2-[[2-[(2-ethyl-cyclopropyl)-methyl]- cyclopropyl]-methyl], methyl ester-, 3-(1-Phenethyl-but-3-enyloxy)-butyric acid, Methoxyacetic acid, 2-pentadecyl ester, Stigmasta-5-22-di-en-3-ol, acetate, (3β)- (Fig. 2, Supplementary Fig. S-1, S-2, S-3; Supplementary Table S-1).

Effect of endophytic fungi on root rotting fungi and growth of sunflower in pot experiment (2017). No infection of *F.solani* was observed in *Chaetomium* sp., and *T. trachyspermus* treated plants. *Chaetomium* sp., *Cephalosporium* sp. and *T. assiutensis* treated plants showed complete suppression of *F. oxysporum*. *T. trachyspermus* also completely suppressed *M. phaseolina*. *R. solani* was not found in endophytes treated plants as compared to control plants (18.7 %)

(Table 4). All endophytic fungi treated plants produced significantly ($p < 0.05$) greater fresh shoot-weight and shoot-length than control plants. *Cephalosporium* sp., treated plants showed comparatively better results as compared to other endophytic fungi (Table 5).

Table 4
Effects of endophytic fungi on the infection of *Fusarium solani*, *F. oxysporum*, *Rhizoctonia solani* and *Macrophomina phaseolina* of sunflower roots in pots experiments.

Treatments	Infection %							
	<i>F. solani</i>		<i>F. oxysporum</i>		<i>M. phaseolina</i>		<i>R. solani</i>	
	2017	2018	2017	2018	2017	2018	2017	2018
Control	25	31.2	25	18.75	50	62.5	18.7	18.5
Carbendazim	12.5	18.75	12.5	18.75	25	25	6.2	6.25
<i>Cephalosporium</i> sp.	18.7	6.25	18.7	0	18.7	18.75	0	0
<i>Chaetomium</i> sp.	0	0	0	0	12.5	6.25	0	0
<i>Talaromyces assiutensis</i>	12.5	18.75	12.5	0	25	31.2	0	0
<i>T. trachyspermus</i>	0	0	0	12.5	0	0	0	0
*LSD _{0.05}	Treatments = 9.5 ¹ Pathogens = 8.5 ²							
¹ In column, mean values showing differences more than LSD value are significantly different at p < 0.05								
² In row, mean values showing differences more than LSD value are significantly different at p < 0.05								

Table 5
Effects of endophytic fungi on growth of sunflower plants in pots experiment.

Treatments	Shoot length		Shoot wt.		Root length		Root wt.	
	(cm)		(g)		(cm)		(g)	
	2017	2018	2017	2018	2017	2018	2017	2018
Control	29.3	30.8	5.8	5	3.9	4.2	0.4	0.3
Carbendazim	38	36	9	8.3	5.4	5.4	0.6	0.5
<i>Cephalosporium</i> sp.	43.5	45.8	11.1	11.2	7.1	8	1.1	1.4
<i>Chaetomium</i> sp.	38.2	39.5	9.4	8.5	7.3	6.5	0.7	1
<i>Talaromyces assiutensis</i>	34.1	35.7	7	7.2	5.6	5.1	0.6	0.9
<i>T. trachyspermus</i>	41.3	40.6	9.8	8.2	6.8	6.2	0.6	1.0
L.S.D($p < 0.05$)	4.1	6.1	2.4	1.4	1.9	1.4	0.3	0.3
¹ Mean values in the column showing difference greater than LSD value are significantly different at $p < 0.05$								

Phenolic content was found highest in *Cephalosporium* sp., (0.68 mg mL⁻¹) treated plants than other treatments. *Chaetomium* sp., (0.55 mg mL⁻¹), *T. assiutensis* and *T. trachyspermus* (0.53 mg mL⁻¹) showed increased in polyphenolic contents as compared to carbendazim treated plants (0.41mg mL⁻¹) and control plants (0.17 mg mL⁻¹) (Fig. 3). Highest level of salicylic acid content was found in *T. assiutensis* treated plants (0.60mg mL⁻¹) followed by *Cephalosporium* sp., (0.54 mg mL⁻¹), *T. trachyspermus* and *Chaetomium* sp., (0.51 mg mL⁻¹) as compared to carbendazim treated plants (0.33 mg mL⁻¹) and control plants (0.16 mg mL⁻¹) (Fig. 4). Antioxidant activity of endophytic fungi treated plants was also found greater as compared to control plants, where *T. trachyspermus* showed highest activity (Fig. 5).

Effect of endophytic fungi on root rotting fungi and growth of sunflower in pot experiment (2018). Less numbers infection of *F. oxysporum* and *R. solani* was observed in all treatments including control plants, while *F. solani* significantly ($p < 0.05$) suppressed by all test endophytic fungi than control plants. *Macrophomina phaseolina* significantly suppressed in all treatments than control plants, where *M. phaseolina* was not found in *T. trachyspermus* treated plants (Table 4). All isolates of endophytic fungi treated plants produced greater fresh shoot weight and shoot length than control plants (Table 5).

Phenolic content was again found greater in plants treated with endophytic fungi as compared to control or carbendazim treated plants with highest in *Cephalosporium* sp., (0.57 mg mL⁻¹) and lowest in control plants (0.24 mg mL⁻¹) (Fig. 6). Salicylic acid content increased in all endophytic fungi treated plants than control or carbendazim treatments (Fig. 7). Highest level of salicylic acid content was found in *T. assiutensis* (0.62 mg mL⁻¹) and lowest in carbendazim treated plants (0.17 mg mL⁻¹).

Antioxidant activity was found to be increase with time, at 1 minute maximum was found in plants treated with *Cephalosporium* sp. (46.2 %), while at 30 minutes antioxidant status increased in all plants treated with endophytic fungi. Maximum level was found in plants treated with *Chaetomium* sp. (69.8%) as compared to control plants (16.9%) at 30 minutes (Fig. 8).

Effect of endophytic fungi on sunflower growth and suppression of root rotting fungi in field experiment (2017). Significant suppression of root rot fungi was observed in *Chaetomium* sp. and *T. trachyspermus* treated plants at both time intervals i.e. 45 d and 60 d (Table 6). *Cephalosporium* sp. and *T. assiutensis* were found effective against *F. oxysporum*, *R. solani*, and *M. phaseolina*, at both time intervals. All endophytic fungi enhanced plant height and fresh shoot-weight than control plants (Table 7).

Table 6. Effects of endophytic fungi on the infection of Fusarium solani, F. oxysporum, Macrophomina phaseolina and Rhizoctonia solani of sunflower plants in field experiments.

Treatments	Infection %															
	F. solani				F. oxysporum				M. phaseolina				R. solani			
	2017		2018		2017		2018		2017		2018		2017		2018	
	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d
Control	25	31.2	37.5	43.7	18.7	25	12.5	25	43.7	50	61.2	75	18.7	25	12.5	25
Carbendazim	25	25	18.7	25	12.5	12.5	6.2	18.7	25	43.7	37.5	43.7	12.5	18.7	12.5	18.7
Cephalosporium sp.	18.7	25	6.2	12.5	0	12.5	0	0	18.7	25	18.7	25	0	0	0	0
Chaetomium sp.	0	0	0	0	0	0	0	0	18.7	25	25	31.2	0	0	0	0
Talaromyces assiutensis	18.7	25	12.5	25	0	0	0	0	25	25	12.5	18.7	6.2	12.5	12.5	18.7
Talaromyces trachyspermus	0	0	0	0	6.2	12.5	6.2	18.7	0	6.25	0	12.5	0	0	0	0
LSD _{0.05}	Treatments = 4.7 ¹ ; Pathogens ² = 3.8; Time = 2.7 ³															

¹In column, mean values showing differences more than LSD value are significantly different at p < 0.05

²In row, mean values showing differences more than LSD value for pathogens are significantly different at p < 0.05

³In row, mean values showing differences more than LSD value for time are significantly different at p < 0.05

Table 7. Effects of endophytic fungi on growth of sunflower plants under field condition.

Treatments	Shoot length (cm)				Shoot weight (g)				Root length (cm)				Root weight (g)			
	2017		2018		2017		2018		2017		2018		2017		2018	
	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d
Control	57.5	73.7	64.4	74.3	37.3	48.7	36.7	61.4	7.4	8.1	7.7	9.9	7.6	11.5	10.5	12.9
Carbendazim	72.5	104.2	78.6	90.9	61.6	113.2	61.5	115	11.7	11.8	10.6	14.1	12.6	16.5	14.3	18.7
Cephalosporium sp.	98.7	150.1	102.5	129.8	83.6	193	93.3	170	14	20.5	13.8	19.9	14.7	19.1	15.2	23.2
Chaetomium sp.	91.7	112.5	95.5	107.3	66.6	133	77.2	127	11.7	12.3	12	15.8	12.5	15	11.2	19.9
Talaromyces assiutensis	84.7	101	88.7	100.6	56.1	98.6	60.4	113	10	12.32	10.2	12.4	7.8	8.4	10.7	15.8
Talaromyces trachyspermus	92.2	131.5	99.3	112.6	74.7	143.7	77	130.5	12.2	14.7	9.5	16	10.6	14.5	12	20.7
LSD _{0.05}	7.9 ¹	10.3 ¹	8.7 ¹	8.9 ¹	10.6 ¹	27.8 ¹	7.9 ¹	29.5 ¹	2.3 ¹	2.8 ¹	3.2 ¹	4.1 ¹	1.7 ¹	2.3 ¹	2.4 ¹	2.3 ¹

¹In column, mean values showing differences more than LSD values are significantly different at p < 0.05

Concentrations of plant biomarkers like polyphenolic contents and salicylic acid were found significantly higher in endophytic fungi treated plants than control plants at both time intervals (Fig. 9, 10). Similarly, antioxidant status of endophytic fungi treated plants were found better than control plants at both time intervals (Fig. 11).

Effect of endophytic fungi on sunflower growth and suppression of root rotting fungi in field experiment (2018). All four test endophytic fungi, *Cephalosporium* sp., *Chaetomium* sp., *T. assiutensis* and *T. trachyspermus* significantly suppressed all four test pathogenic fungi on sunflower roots at both time intervals (Table 6). Highest activity against *M. phaseolina* was found by *T. trachyspermus*. Endophytic fungi treated plants again produced significantly taller plants with greater fresh shoot weight as compared to control plants (Table 7).

The endophytic fungi treatment again found to improve production of polyphenolic content and salicylic acid with increasing antioxidant levels of plants as compared to control plants at both time intervals (Figs. 12, 13 and 14). All four tests endophytic fungi showed a similar trend in suppressing root rot fungi and stimulating plant defense biochemical markers and improved antioxidant levels of the plant as found in a 2017 experiment.

Principal Component Analysis (PCA). A principal component analysis (PCA) was performed to highlight the high promising isolate for endophytes that promote sunflower growth and increased plant defense biochemical markers in pots and field plot experiments. *Talaromyces trachyspermus* was found more effective in improving plant biomass and enhanced plant defense biochemical markers in general both in pots and field experiments as compared to other isolates. The results are presented in (Figs. 15 and 16).

Discussion

Chemical pesticides are essential agents to minimize losses caused by plant pathogens to agricultural crops. Due to their adverse impact on nature, their applications are being reduced. There is a basic need for an emerging attempt to explore compounds which are nature- friendly and can be substituted for chemically synthesized commercial products. Alternate sources are more essential to synthesize natural products are not yet commercially feasible and are costly²⁵. To search for novel bioactive metabolites, microorganisms which make symbiotic association with plants are gaining special attention²⁶. In this study, endophytic fungi isolated from healthy wild and cultivated plants and were identified by using molecular biological tools, showed potential against plant pathogenic fungi. These findings are an agreement with previous studies regarding the presence of potential fungi in plants as endophytes^{3,6,27,28}. Each plant species is host specific to few or more endophytic fungi and they are considered as hyper diverse groups¹. They are found in most of the known plants and plant-like organisms; liverworts and algae²⁹. The relationship between host plants and their fungal endophyte range from symbiotic to mutualistic or sometimes antagonistic. It is described that diversity of fungal endophytes in ecologies, and there is no reliable estimation of endophytes yet, but environmental factors are one of the complex features³⁰. However, some endophytes are predominant in specific hosts and some are rare³¹. In this study, some endophytic fungi isolated are also known as plant pathogens like *Fusarium* sp., and *Macrophomina phaseolina* that attack on a wide range of economically important plants²². However, *Talaromyces* isolated in this study are the perfect stage of *Penicillium* known to possess biocontrol potential³².

In this study, endophytic fungi showed significant antibacterial and antifungal activity *in vitro*. There are reports that fungal endophytes isolated from various plants have shown significant antimicrobial activity^{3,27}. In this study, *n*-hexane and chloroform soluble portion of culture filtrates of endophytic fungi like *Chaetomium* sp., *T. assiutensis* and *T. trachyspermus* showed strong antimicrobial activity against root rotting fungi. In this study, *n*-hexane soluble portion of culture filtrate of *T. trachyspermus* revealed the presence of several compounds, when subjected to GC-MS analysis, and many of them appeared as new from this source. Secondary metabolites reported from *Talaromyces* are alkaloid³³ terpene³⁴ and polyketide classes^{26,34} identified talaperoxides from an endophytic fungus *Talaromyces flavus* from medicinal plants showed potent cytotoxic activity against cancer cell lines. Presence of these compounds may play a vital role in antimicrobial activity of *T. trachyspermus*.

In this study endophytic fungi showed significant results on sunflowers both in pots and field plot experiments by reducing root rot incidence and improvement of plant biomass. *Talaromyces* spp. and *Cephalosporium* sp. showed the best result in improving the plant growth and suppressing plant pathogenic fungi on sunflowers. Active metabolites produced by fungal endophytes have a capability to act as a biological control agent and enhance plant growth and protect them from phyto-pathogens³. There is a report that *Talaromyces flavus* can suppress root rotting fungi on chickpeas³⁵. *T. trachyspermus* is known to produce hydrolytic enzymes, chitinase, amylase, cellulose, protease and pectinase having antagonistic property along with producing indole acetic acid (IAA), siderophore, and solubilize phosphate which plays a role in plant growth promotion³⁶. *Cephalosporium* spp., are well known for the production of cephalosporin like antibiotics Its role as a potential biological control agent against root rotting fungi is interesting, and needs further investigation.

Endophytic fungi used in our study also improved plant growth, and stimulated the production of plant defense biochemical markers like, polyphenolic content and salicylic acid (SA). Polyphenolic compounds work as antibiotics in plants and act as non-host resistance to filamentous fungi³⁷ which improve antioxidant status of plant^{5,38}. Similarly, salicylic acid (SA) has now been reported to increase plant resistance to abiotic and biotic stress^{39,40}. It has also positive impacts on development of plant growth and regulation of uptake of the several plants based on beneficial elements^{41,42}. SA also decreased oxidative stress by modulating their antioxidant defense systems⁴³. The disease suppression in sunflower may be attributed due to enhance plant defense biochemical markers, besides producing antimicrobial compounds by endophytes, as evident from the production of higher amount of plant defense biochemical markers like, polyphenol content, salicylic acid and improving antioxidant status in plants. It seems that endophytic fungi associated with healthy plants could be an alternative to chemical pesticides in plant root diseases management.

Materials And Methods

Isolation of Endophytic fungi. Healthy plant samples viz., *Allium cepa* L., *Chenopodium album* L., *Corchorus olitorius* L., *Euphorbia hirta* L., *Haloxylon stocksii* (Boiss) Benth. & Hook, *Sida ovata* Forssk., and *Solanum melongena* L., growing in the experimental field of Botany Department, Karachi University and agricultural field of Malir, Karachi were collected (along with root, shoot and leaves) and endophytic fungi were isolated within 24 hours. Permission of Chairman, Department of Botany and Campus Office was obtained for collecting the plants from the campus, whereas, plants collected from agricultural field at Malir with the permission of Owner of the field. Identification of each plant species was confirmed by a taxonomist and herbarium number was assigned (Table 1). **All the experiments were performed in accordance with institutional guidelines and regulations.**

After washing with tap water, plant samples were cut into small pieces (1 cm long), then sterilized with 1% bleach [Ca(OCl)₂] for three minutes, followed by sterilization with 70% alcohol for three minutes and then with sterile water. Sample was crushed in 50 mL sterilized water by using an electric grinder and a

dilution was made up to 1:10⁴. From final dilution 0.1mL was spread over Petri plates containing Potato Dextrose Agar (PDA) amended with streptomycin (0.2 g/ L) and Penicillin (100000 units/L). Fungi isolated after incubation at 28°C for 7 days were initially identified on morphological characters according to^{44,45,46,47,48,49}. Identification of endophytic fungi was confirmed by using molecular biological tools.

Molecular identification of endophytic fungi. Fungal mycelium (1.5-2 cm²) was obtained from fresh culture in sterile Eppendorf tubes and the cell was disrupted with glass beads in liquid nitrogen. The DNA extracted by using Biobasic EZ-10 Spin Column, Fungal Genomic DNA Mini-Preps Kit, (CANADA) according to their manufacturer instructions. DNA quality was assessed by using Agarose gel electrophoresis while the purity of DNA was assessed by spectrophotometer (Shimadzu- UV-1800). The primer sets i.e., ITS1 region (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 region (3'-TCCTCCGCTTATTGATATGC-5') used for amplification of rDNA ITS region. For the amplification of targeted region, each PCR reaction mixture comprised of fungal genomic DNA (100ng), 1.0 µL of ITS1 primer (10pmole), 1.0µL of ITS4 (10 pmole), 12.5µL of 2X PCR Master Mix (Thermo Fisher Scientific, MA, USA) up to final volume 25 µL with nuclease free water. PCR amplification was carried out on ABI 2700 thermal cycler (California, USA) using the following conditions: first hold for 5 minutes at 94 °C for DNA denaturation followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 1 minute, then second hold for 10 min at 72 °C after that hold at 4 °C. Amplified products evaluated by using 2% Agarose gel electrophoresis at 100 V for 35 min. The amplified products were submitted to BGI Genomic Services (Shenzhen, Guangdong, China) for Sanger sequencing of both ITS1 and ITS4. The consensus sequences were retrieved from both sets of primers and the sequences were edited and aligned manually by using BioEdit software (Version 7.2.6). BLAST analysis carried out for the identification of fungal isolates. Phylogenetic relationships of all isolates constructed using neighbor joining (NJ) method with MEGA-X software (PMID: 29722887). Each Sequence submitted to NCBI Genbank.

Antifungal activity of endophytic fungi. Antifungal activity of endophytic fungi was determined against *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium solani* and *Foxysporum* by using Dual culture plate assay. Test isolates were inoculated in Petri dishes having Czapek's dox agar and on its opposite side pathogenic fungi was placed and incubated at 28°C for 5-7 days. Inhibition zone produced, if any was recorded⁵⁰.

Antifungal activity of culture filtrates of endophytic fungi. Test fungi grown at (25-30°C) for 15 days in 250mL flasks containing 100mL Czapek's Dox broth. The broths were filtered over Whatman #1 filter paper in flask and exposed to chloroform vapors under laminar flow hood to kill fungal propagules. Thick sterile discs were impregnated with endophytic fungal culture filtrates at 20, 40 and 60µL/disc and dried. Discs along with control (disc loaded with Czapek's Dox broth) and +ve control (carbendazim at 20µg/disc) were placed at the periphery of plates. Test fungi (5 mm disc) were inoculated in the center. Zone of inhibition, if any was recorded after 5-7 days of incubation at 30°C²⁷.

Fractionation of culture filtrates of endophytic fungi. The culture filtrates (*Chaetomium* sp., *T. assiutensis*, *T. trachyspermus*) were extracted separately 3 times with *n*-hexane in a separating funnel. *n*-hexane insoluble fraction was further extracted with chloroform. Both fractions were dried separately on a rotary vacuum evaporator (Eyela-NE, Japan) and finally gummy mass was obtained⁵¹.

Antifungal activity of solvent fractions of endophytic fungal culture filtrates. Each fraction was re-dissolved in their respective solvent at 1.5 mg/mL and 5mm diameter sterile disc of filter papers were loaded at 20µl (30µg/disc), 40µl (60µg/disc) and 60µl (90µg/disc) and dried. Other details are the same as described in antifungal activity of culture filtrates of endophytic fungi.

Gas Chromatography-Mass Spectrometry (GC-MS). The *n*-hexane soluble portion was oily in nature, which was subjected to GC/MS analysis. GC/MS was conducted on Agilent (6890) Gas Chromatograph linked with Mass Spectrometer: Jeol, JMS: (600H). Operational mode was EI having the ion source at 50eV and their electron energy then maintained at 70 eV. Carrier Gas Volume placed in a range of 1.0-5.0µL. Individual peaks of each compounds were assigned, their mass spectra and retention indices (RI) were matched online with National Institute of Standards and Technology, USA (NIST: Mass Spectrometry Data Center (mainlib) NIST#: 352898 ID#: 113419 DB) and finally compared with Science finder.

Biocontrol potential of endophytic fungi against root rotting fungi. *Talaromyces assiutensis* and *T. trachyspermus* isolated in this study were selected for further study as biocontrol agents against root rotting fungi, since other fungal isolates are well known plant pathogens or environmental contaminants. Efficacy of *Talaromyces* spp., was examined in pots and also in field plots, using sunflower (*Helianthus annuus* L.) as test crop. Efficacy of *Talaromyces* spp., was also compared with endophytic *Chaetomium* sp. (KUCC1359) and *Cephalosporium* sp. (KUCC1358) obtained from the culture collection center at University of Karachi.

Screen house experiments. The experiment was conducted in February, 2017, in clay pots containing sandy loam soil (15 cm diam.) at 1 Kg/pot and aqueous suspensions of endophytes *Talaromyces assiutensis*, *T. trachyspermus*, *Chaetomium* sp., and *Cephalosporium* sp., (107 cfu/mL), grown on potato dextrose broth (pH 5.6) at room temperature were applied onto each pot (25 mL/pot). Soil was naturally infested with 3 to 13 % colonization of *R.solani*, using sorghum seed as bait⁵², 3000 cfu/g of soil of *Fusarium* spp., using soil dilution technique⁵³ and 3-9 sclerotia/g of soil of *M.phaseolina*, by using wet sieving and dilution technique⁵⁴. Aqueous suspension (25 mL) of endophytes (8 × 10⁷ CFU mL⁻¹) grown in potato dextrose broth (PDB) for 15 days at 28°C was drenched in each pot after placing the seeds, then covered with soil (about 1.5 cm). Population of endophytes in suspension was determined by dilution plate method on potato dextrose agar using the formula:

$$\text{CFU per mL} = \text{Number of colonies on plate} \times \text{Dilution factor}$$

Seeds of sunflower (6 seeds per pot) were sown in each pot and randomized with four replicates. Temperature ranged from 25 °C- 35°C. Carbendazim (200 ppm in water) at 25mL in each pot used as +ve control; untreated plants were kept as control. In each pot, four seedlings maintained and excess were removed after germination. Observations were recorded after 45 days, plants were uprooted, washed under the tap water, and then blot dried. Root length and

shoot length were measured and their fresh weight was recorded on an electronic balance. To determine the incidence of infection of fungi on roots, tap roots were cut into (1 cm) pieces, surface sterilized with 1% bleach. The root pieces were transferred onto PDA plates containing antibiotics, streptomycin and Penicillin as described above. Fungi grown on root pieces after 5 days of incubation at 25°C were identified and infection (%) of each fungus was calculated by using the formula⁵⁵.

$$\text{Infection \%} = \frac{\text{Number of plants infected by a fungus}}{\text{Total number of plants}} \times 100$$

The experiment was repeated in 2018 in similar conditions, in order to confirm the efficacy of endophytic fungi.

Field plot experiments. The experiment was also conducted in field plots (2x2 m) of the Department of Botany in 2017 and repeated in 2018. Aqueous suspension of above mentioned endophytes applied in the planting row at 400 mL⁻¹ 2 m. The sandy loam soil (pH 8.0) was infested naturally with root rotting fungi *M.phaseolina* (6-11 sclerotia / g soil), 7-16 % colonization of *R.solani*, on seed of sorghum used as a bait, 3300 cfu/g of soil having *Fusarium* spp. Sunflower seeds (30 per 2m row) were sown and watered after 2-3 days. Complete randomized block design was used in each experiment with 4 replicates. Carbendazim (200 ppm in water) at 400 mL/ 2m row served as +ve control. After 45 and 90 days, 4 plants from each row were uprooted, washed under tap water and data collected from plant growth, suppression of root infecting fungi and plant stress markers were determined.

The experiment was repeated in 2018 in similar conditions to confirm the efficacy of endophytic fungi.

Biochemical parameters. Leaves (1 g) from each plant sample were dried overnight at 70°C in an oven, crushed in 100 mL ethanol (96% v/v) and centrifuged at 504 g for 20 minutes. The supernatant was collected to analyze polyphenol, salicylic acid and antioxidant activity.

Estimation of polyphenols. The Folin-Ciocalteu reagent was used to determine the total phenolic content⁵⁶, aliquots (100µL) were mixed with 2 mL of Na₂CO₃ (2% w/v), kept for 2 minutes at room temperature and then Folin-ciocalteu phenol reagent (100µL of 50%) was added, finally kept in dark for 30 minutes. Absorbance (at 720 nm) was recorded and phenolic content was expressed in mg of Gallic acid equivalents (GAE)/g of dried sample.

Estimation of salicylic acid. Sample aliquot (100 µL) was mixed with the freshly prepared (0.1%) solution of ferric chloride, and volume was made up to (3.0 mL). Absorbance was measured on a spectrophotometer at 540nm. The results were expressed as salicylic acid equivalent (mg of SA per gram of dried extract)⁵⁷.

Determination of antioxidant activity. Antioxidant activity (leaves) was determined by using 2, 2-Di-phenyl-1-picrylhydrazyl (DPPH) as a free radical scavenging activity as described⁵, where aliquot (0.2mL) was added in (0.8 mL) of 100 mM, Tris-HCl buffer (pH 7.4), mixed with 30µM DPPH (1 mL) and vortex finally. Their absorbance was recorded at 517 nm at 01 minutes, and at 30 minutes after keeping in dark, using 1 mL aqueous ethanol with 01 mL of DPPH served as a control. Following formula used to determine their activity:

$$\text{Antioxidant activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Analysis of data. The data was analyzed by using software SPSS, Costat, one-way ANOVA for plant growth and biochemical parameters. Significant value at P < .005 was calculated to compare the means. For fungal infection, two- or three-way ANOVA was used and least significant difference (LSD) was calculated at P < 0.05. and their means were compared at significant level (p<0.05). To highlight the performance of the most effective isolate of endophytic fungi on plant growth, production of resistance markers and antioxidant activity, Principal Component Analysis (PCA) was performed by using software CANOCO Engine Version 5.0⁵⁸.

Declarations

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Author contributions

Hafiza Farhat, Faizah Urooj and Hafiza Asma Shafique collected the plants, isolated and identified the endophytic fungi and conducted experiments on sunflowers. Muhammed Irfan and Saima Majeed helped in the molecular identification of endophytic fungi. H. Farhat and Nida Sohail extracted metabolites from endophytic fungi, and did antifungal activity. GC-MS analysis was conducted by Sidra Fatima Hameedi. Syed Ehteshamul-Haque conceived and designed the study, supervised research work and improved the quality of the final version of manuscript. All authors read and approved the final manuscript.

Conflict of Interest

No conflict of interest declared.

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Figures

Figure 1

Molecular bases of endophytic fungi showing their ancestral relationship and their accession number downloaded from NCBI.

Figure 2

New compounds identified from n-hexane soluble fraction of culture filtrate of *Talaromyces trachyspermus*.

Figure 3

Effect of endophytic fungi as soil drench on polyphenol content of sunflower plants in pots experiment (2017).

Figure 4

Effect of endophytic fungi as soil drench on salicylic acid of sunflower plants in pots experiment (2017).

Figure 5

Effect of endophytic fungi as soil drench on antioxidant activity of sunflower plants in pots experiment (2017).

Figure 6

Effect of endophytic fungi as soil drench on polyphenol content of sunflower plants in pots experiment (2018).

Figure 7

Effect of endophytic fungi as soil drench on salicylic acid of sunflower plants in pots experiment (2018).

Figure 8

Effect of endophytic fungi as soil drench on antioxidant activity of sunflower plants in pots experiment (2018).

Figure 9

Effect of endophytic fungi as soil drench on polyphenol content of sunflower plants in field experiment (2017).

Figure 10

Effect of endophytic fungi as soil drench on salicylic acid of sunflower plants in field experiment (2017).

Figure 11

Effect of endophytic fungi as soil drench on antioxidant activity of sunflower plants in field experiment (2017)

Figure 12

Effect of endophytic fungi as soil drench on polyphenol content of sunflower plants in field experiment (2018).

Figure 13

Effect of endophytic fungi as soil drench on salicylic acid of sunflower plants in field experiment (2018).

Figure 14

Effect of endophytic fungi as soil drench on antioxidant activity of sunflower plants in field experiment (2018).

Figure 15

Bioplots are showing the principal component analysis (PCA1 and PCA2) of plant growth parameters, concentration of plant defense biochemical markers in sunflower and antioxidant activity due to endophytic fungi as compared to control plants. RW= root weigh, RL= root length, SL= shoot length, SW= shoot weight, SA= salicylic acid, PC= polyphenolic content, AO1= antioxidant activity at 1 minute, AO30= antioxidant activity at 30 minutes. Control= control, Carbendz= carbendazim, Chaetomi= Chaetomium sp., Cephalos= Cephalosporium, TAssiu= Talaromyces assiutensis, TTrach= T. trachyspermus

Figure 16

Bioplots are showing the principal component analysis (PCA1 and PCA2) of plant growth parameters, concentration of plant defense biochemical markers in sunflower leaves and antioxidant activity due to PGPEF as compared to control plants. . RW45= root weigh at 45 d, RL45= root length at 45 d, SL45=- shoot length at 45 d, SW45= shoot weight at 45 d, SA45= salicylic acid at 45 d, PC45= polyphenolic content at 45, AO145= antioxidant activity at 1 minute at 45 d, AO3045= antioxidant activity at 30 minutes at 45 d. RW60= root weigh at 60 d, RL60= root length at 60 d, SL60=- shoot length at 60 d, SW60= shoot weight at 60 d, SA60= salicylic acid at 60 d, PC60= polyphenolic content at 60 d, AO160= antioxidant activity at 1 minute at 60 d, AO3060= antioxidant activity at 30 minutes at 60 d. T1= control, T2= carbendazim, T3= Chaetomium sp., T4= Cephalosporium, T5= Talaromyces assiutensis, T6 T. trachyspermus

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