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Antagonism of tomato endophytic fungi against grey mold disease

Alireza Pourmad
University of Tehran
Leila Ebrahimi
le_ebrahimi@ut.ac.ir
University of Tehran
Khalil-Berdi Fotouhifar
University of Tehran

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Abstract

Endophytic fungi are a group of microorganisms living inside plant tissues without any immediate and visible symptoms, and in most cases have benefits for their host. In this research, 50 samples of healthy tomato plants were collected from Isfahan and Tehran provinces of Iran. Totally, 89 strains of endophytic fungi were isolated from root, stem, and leaf tissues. Based on morphological characteristics, the isolates were belonged to 12 different genera including *Fusarium*, *Alternaria*, *Cladosporium*, *Acremonium*, *Arthrinium*, *Aspergillus*, *Penicillium*, *Moniliophthora*, *Rhizoctonia*, *Nigrospora*, *Trichoderma* and *Colletotrichum*. The biocontrol effects of the isolates were investigated against *Botrytis cinerea in vitro*. Based on the results of dual culture and volatile organic compounds (VOC) production, six isolates were selected with the most inhibitory effect on *B. cinerea* that were belonging to the genus *Fusarium*. Among them, isolate 41R1 with 65.8 and 65% inhibitory rate on mycelia growth of the isolates B1 and B2, respectively, showed the highest value in the dual culture test. In the VOC test, isolate 28S1 had the most inhibitory effect on mycelia growth of isolates B1 and B2, with the values of 66.6 and 62.6%, respectively. Six selected isolates reduced the tomato grey mold disease severity about 35 to 58% in greenhouse. Isolate 28S1 increased the plant dry weight, but without significant effect on other plant growth indices. Isolates 41R1, 28S1, and 16S3 with the highest inhibition, were identified based on the partial sequence of *tef-1α* gene, which belonged to *Fusarium falciforme*, *Fusarium falciforme* and *Fusarium solani* species, respectively.

Introduction

Tomato (*Solanum lycopersicum* L.) is the most broadly cultured vegetable globally which is susceptible and affected by over 200 pathogens during cultivation and postharvest all over the world (Singh, 2017). This plant is susceptible to pathogenic fungi causing losses in crop yields up to 78% (Chavan & Borkar, 2020), in which *Botrytis cinerea* Pers., causing grey mold disease, is a highly damaging necrotrophic plant pathogen, mainly affecting fresh fruits and vegetables. This fungus can invade different plant parts such as stems, leaves, flowers, fruits, and seeds before and after harvesting, leading to significant crop losses (Hua et al., 2018). This pathogen enters the host through pruning cuts, leaves, and flowers in greenhouse (Droby et al., 2007).

Many fungicides are used to control *B. cinerea*, but this management is not method considered sustainable due to their harmful effects on human health and the environment. Also, their frequent use increases the risk of developing resistance in pathogenic fungi, which is an important limiting factor in chemical control of *B. cinerea* (Rodrı´guez et al., 2014; Lu et al., 2016). Therefore, finding non-chemical techniques like biological control as a viable and promising alternative method has been considered. But, the number of biological fungicides available at the market shelves effective against *B. cinerea* is limited (Haidar et al., 2016). Consequently, there is a necessity to explore the novel microorganisms or their metabolites with the ability to combat *B. cinerea*. This quest for new control measures against the phytopathogens has prompted the utilization of endophytes as biocontrol agents. Endophytic fungi are organisms that can settle down inside the plants without any disease symptoms. They hold significant

influence on host plants by enhancing plant tolerance to both biotic and abiotic stresses (Jia et al., 2016, Aleahmad & Ebrahimi, 2023). Endophytic fungi have been isolated from various plant species, with some showing efficacy in suppressing *B. cinerea* (Bolívar-Anillo et al., 2020). The main modes of action of microorganisms against *B. cinerea* involve interference with pathogen growth, induction of host plant resistance, competition for space and nutrients, production of lytic enzymes, antibiotics, and volatile organic compounds (VOCs) (Haidar et al., 2016). Volatile organic compounds are low molecular weight compounds which vaporize at normal atmospheric temperatures and pressure (Toffano et al., 2017), mostly including alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids, and esters (Piechulla & Degenhardt, 2014). Over 300 different VOCs have been identified in fungi (Morath et al., 2012), which are recognized for their ability to inhibit fungal spore germination (Tyc et al., 2017). For example, Vidal et al. (2020) reported that three endophytic fungi isolated from growing plants Central Chilean Andean Precordillera, inhibited the *B. cinerea* mycelia growth through the production of VOCs. Also, Ebrahimi et al. (2023) have reported that the VOCs produced by endophytic fungi including *Chaetomium globosum* Kunze. 2S1, *C. globosum* 3L2, *Fusarium acuminatum* Ellis & Everh. GO2L1, *F. fujikuroi* Nirenberg. 37F6, and *F. incarnatum* (Desm.) Sacc. 25S3 could inhibit the mycelia growth of *B. cinerea* isolates (B1 and B2) by more than 80%.

In several studies, the endophytic fungal communities of tomato plants have been investigated (e.g., Cao et al., 2004; kim et al., 2007; Larran et al., 2011; Mahmoud & Narisawa, 2013; Demers et al., 2015; Imazaki & Kadota, 2015; Poli et al., 2016; Xia et al., 2019; Dastogeer et al., 2020; Dong et al., 2021). To understand the distribution of the cultivable fungal community in tomato plant tissues, Dastogeer et al. (2020) sampled plants from seven different tomato cultivars in Japan and identified fungi across 29 distinct operational taxonomic units with 97% identity. The isolates were divided based on the ITS region sequences, and the majority of them belonged to Ascomycota (99.3%). The dominant genera include *Fusarium* Link 1809 (45.1%), *Alternaria* Nees. (12.8%), *Gibberella* Sacc. (12.0%), and *Dipodascus* Lagerh. (6.8%). Some *Fusarium*, *Alternaria*, and *Penicillium* Link. isolates were present in all cultivars, while a few were unique to a specific cultivar. In another study, Xia et al. (2019) isolated and identified the following endophytic fungi from tomato plants in USA: *Alternaria* sp., *Aspergillus fumigatus* Fresen., *A. nidulans*, *C. globosum*, *Coniothyrium aleuritis* Teng., *Fusarium* sp., *F. chlamydosporum* Wollenw. & Reinking., *F. oxysporum* Schltdl., *F. proliferatum* (Matsush.) Nirenberg., *Neocosmospora solani* Mart., *Hypoxylon* sp., *Leptosphaerulina chartarum* Cec., *Meyerozyma guilliermondii* Wick., *Nigrospora* sp., *Penicillium helicum* Raper & Fennell., *P. ochrochloron* Biourge., *P. simplicissimum* (Oudem.) Thom., *Periconia macrospinosa* Lefebvre & Aar.G. Johnson., *Pleosporales* sp., *Rhinocladiella* sp., *Trichoderma atroviride* P. Karst., and *T. spirale* Bissett.

Some endophytic fungi have been used to protect tomato crops from fungal pathogens (Ebrahimi et al., 2023). Ebrahimi et al., (2023) found that the isolates of endophytic *C. globosum* 2S1, *F. acuminatum* GO2L1, and *F. fujikuroi* 37F6 decreased the grey mold disease severity by over 80%, on endophytically colonized tomato plants. In another study, endophytic *F. oxysporum* Fo47, treated to root, had significantly controlled Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* (Aimé et al., 2013). Moreover, Qin et al. (2017) noted 20% decrease in Verticillium wilt on tomato plants colonized by

Piriformospora indica Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken. Rodriguez et al. (2006) found that *F. oxysporum* suppressed the growth of *Sclerotinia sclerotiorum* (Lib.) de Bary. in infected tomato plants by releasing the antifungal metabolite cyclosporine.

Endophytic fungi can have a significant role in enhancing the plant growth. They can improve plant growth directly through the release of bioactive compounds such as phytohormones and through the mobilization of phosphorus and nitrogen (Qin et al., 2017), or indirectly by releasing enzymes that are able to break down pathogen cells and prevent damage to the plant (Nisa et al., 2015, Aleahmad & Ebrahimi, 2023). Endophytic isolates of *F. fujikuroi*, *Sphaceloma manihoticola* Bitanc. & Jenkins., *Cladosporium* sp., *Penicillium* sp., and *A. fumigatus* enhanced tomato plants growth by releasing hormones (Schulz et al., 2002; Khan et al., 2012), While *F. equiseti* (Corda) Sacc. synthesized the antifungal compound (Horinouchi et al., 2007). It has also been reported that endophytic fungi can play a protective role against insects (Resquín-Romero et al., 2016), pathogens (Kim et al., 2007), and nematodes (Varkey et al., 2018). Therefore, they act as multiple plant defenders or biological control agents.

Considering the important and effective role that endophytic fungi can play in plants; this study was conducted to identify the endophytic fungi of tomato plant and evaluate their antagonistic effect against the tomato grey mold disease caused by *B. cinerea* in Iran. In this study, endophytic fungi were isolated from the roots, stems and leaves of the healthy tomato plants and their ability to control the grey mold disease was investigated *in vitro* and under greenhouse conditions. The results indicate that endophytic fungi have biological control potential.

Materials and methods

Sampling and endophytic fungi isolation

Healthy and symptomless root, stem, and leaf samples of tomato were collected from 50 plants in farms and greenhouses in Isfahan and Tehran provinces of Iran during October-December 2021. They were placed in paper bags and kept at 4°C and were cultured up to 48 h after sampling. The method described by Strobel & Daisy (2003) was used for surface sterilization of the plant samples. Initially, the plant materials were washed thoroughly in running tap water for 10 min prior to disinfestation. Then, plant samples underwent surface disinfestation using 70% (v/v) ethanol for 45 s, 2% (v/v) sodium hypochlorite for 30 s, and finally 70% (v/v) ethanol for 15 s. Subsequently, the samples were rinsed with sterile distilled water and the external tissue of the roots and stems were removed using a sterile scalpel. As surface sterilization control, 100 µL of water from the last wash was inoculated in a PDA plates. Disinfested plant materials were cut into small pieces (1 × 1 cm) and then placed on water agar (WA) culture medium. Petri dishes were kept at 25°C for one to four weeks. Grown fungi were transferred on PDA by moving hyphal tips and then incubated at 25°C. Then the isolates were stored on a PDA slant at 4°C. Identified fungi were deposited in the Fungal Culture Collection of the University of Tehran.

Endophytic fungi morphological and molecular identification

Morphological characteristics such as colony colors on different cultures media, growth rate, conidia and conidiophores (size, color, shape, and ornamentation), conidiogenous cells, mode of sporulation, and authentic mycological monographs and literatures were used to identify the recovered endophytic fungi (Leslie & Summerll, 2006; Watanabe, 2010; Houbraken, 2011; Varga, 2011; Woudenberg, 2013). Each fungal isolate was sub-cultured separately on PDA; carnation leaf agar (CLA); oatmeal agar (OA); potato carrot agar (PCA); synthetic nutrient agar (SNA) for inducing sporulation. Microscopic slide mounts were prepared in lacto-phenol or lacto-phenol cotton blue solutions after seven and/or 21 days (depending on the fungal genus). Measurement (n = 50) and microphotographs of fungal characteristics were taken from microscopic slide mounts using an Olympus BH2 light microscope (Erma, Tokyo).

Fresh mycelia of fungal isolates grown on PDA were used for DNA extraction based on quick and simplified protocol by Cenis (1992). The extracted DNA was diluted in 50 µL of distilled water and stored at -20°C for future use. Molecular identification of the fungal isolates was carried out based on translation elongation factor-1 alpha (*tef-1α*) sequences which was amplified using the EF1/EF2 primer pairs (O'Donnell et al., 1998). The reaction mixture and PCR conditions for the amplification of *tef-1α* was the same as those were used by O'Donnell et al. (1998). PCR products were purified and directly sequenced in one direction with EF1 primer by Noor Genetics Company, Iran.

The *tef-1α* sequences were compared with the most similar sequences using the NCBI BLAST search tool, to confirm the taxonomic status of the intended fungal isolates. Finally, the sequences were submitted to GenBank (NCBI) database. For phylogenetic analyses, the newly obtained and edited *tef-1α* sequences were aligned with the homologous sequences of the respective genes of the related species obtained from NCBI (Table 1) using Clustal W (Thompson et al. 1994). Maximum likelihood (ML) (Felsenstein, 1981) analysis was performed with MEGA 10.2 (Kumar et al., 2018). Models NEI + G was recommended by MEGA as the optimal nucleotide substitution models for *tef-1α* data. Characters were treated as un-weighted and unordered with gaps treated as missing data. Confidence of all clades was assessed by ML bootstrap analysis (Felsenstein, 1985) with 1,000 replicates.

Table 1
Sequences obtained from the gene bank for use in phylogenetic analyses.

Species	Isolate	Host	Location	Accession number
Fusarium sambucinum	FRC R4712	Soil	USA	MW233148
	MRC 514	Soil	USA	MH582302
Fusarium venenatum	FRC R8978	Soil	USA	MW233205
Fusarium culmorum	NL19-93013	Soil	Netherland	MZ921905
Fusarium brachygibbosum	NFCCI:5206	Soil	India	ON032449
	HBSY3	Tomato	China	OQ572486
Fusarium tanahbumbuense	COUFAL0457	*Nopalea cochenillifera*	Brazil	OP763394
	CBS 131009	*Triticum* sp.	Iran	MN170506
Fusarium caatingaense	CBS 976.97	*Juniper chinensis*	USA	MN170449
Fusarium solani	N-13-2	Potato	China	MT749763
	GR_FS147	*asparagus root*	Spain	MT305235
	16S3	**Tomato**	**Iran**	**xxxxx**
Fusarium falciforme	**28S1**	**Tomato**	**Iran**	**xxxxx**
	41R1	**Tomato**	**Iran**	**xxxxx**
	OK20FF1	Cotton	USA	OM973262
	ATS82	*Homo sapiens*	India	KY556711
	FSSC_3 + 4_SF5	Environmental	India	MG272423
Fusarium tonkinense	JW 234010	soil	Netherland	MZ921928
	NRRL 32755	Turtle head lesion	USA	DQ247073
Fusarium nygamai	JZB3110218	*Prunus avium*	China	OP039349
Fusarium redolens	NRRL 25600	*Macrochloa tenacissima*	Tunisia	MT409453
	177PT19AB	*Solanum tuberosum*	*Russia*	ON292388
Sarocladium kiliense	R3PS(A)	*Solanum tuberosum*	Algeria	MK752489

The surveyed isolates in the current study are indicated in boldface.

Biocontrol experiments in vitro

Dual culture

The dual culture method, adapted from Dennis and Webster (1971), was used to assess the antagonistic activity of 89 endophytic strains against two isolates (B1 and B2) of the pathogenic *B. cinerea* that were obtained from the Fungal Collection of the University of Tehran. Mycelial discs (5 mm diameter) of the endophytic fungi were placed 1 cm from the edge of a nine cm Petri dishes containing PDA. Then a five mm diameter disc of *B. cinerea* (B1 and B2) were placed opposite to the endophyte and incubated in the dark at 20°C for seven days. Three replicates were used for each treatment in a completely randomized design. Simultaneously, three replicates of the control containing only the pathogen were considered. When the pathogen colonized the whole Petri dish, the growth inhibition was calculated using the formula n = (a–b)/a × 100, where n is the % growth inhibition; a is the colony area of control; and b is the colony area of treated *B. cinerea* (Etebarian et al., 2005).

Volatile organic compounds

The effect of the endophytic fungi VOCs on mycelia growth of *B. cinerea* was examined using the Sandwiched Petri plates assay, provided by Lillbro (2005). Endophytic isolates were grown in Petri plates containing PDA and *B. cinerea* was cultured in another Petri plate, and then the Petri dishes containing *B. cinerea* was placed on top of the endophytic isolate petri dishes in a upside down position. This system was completely sealed with parafilm to avoid the leak of the produced VOCs, and then the system was incubated at 20°C. Three replicates were prepared for each treatment. The colony diameter of *B. cinerea* was measured after one week. The impact of VOCs on *B. cinerea* mycelia growth was assessed by comparing the radial growth rate of the *B. cinerea* with control, which consisted of *B. cinerea* inoculated under the same conditions but without the presence of endophytic isolates. The above-mentioned formula was used to evaluate the growth inhibition percentage.

Greenhouse experiments

The disinfected tomato seeds (cultivar 8320, Seminis Company, USA) were sown on seedling trays filled with sterile perlite and coco peat. After 25 days, two seedlings were transplanted into individual 1000 mL pots filled with a mixture of coco peat, perlite and field soil (3:1:2 ratio) sterilized twice in an autoclave at 121°C and 115 psi for 1 h. Then to investigate the biocontrol ability of endophytic isolates, the conidial suspensions of six selected endophytic isolates based on the in vitro assays were prepared (10^8 conidia/m L) and sprayed on tomato plants with 5–6 true leaves. After 48 h, the conidial suspension (10^5 conidia mL^{-1}) of each *B. cinerea* isolate (B1 and B2) was sprayed on tomato plants. One treatment received an inoculation of conidial suspensions of each endophytic isolate, while two other treatments were inoculated with *B. cinerea* conidial suspensions and sterile distilled water as positive and negative controls, respectively. All the pots were individually covered with the plastic bags for maintaining a relative humidity of 100% for 48 h. Then, the pots were kept in a greenhouse with the humidity of > 80%

at 17–20°C. Three pots (each containing two seedlings) were considered for each treatment. The inoculated plants were examined every day to evaluate the disease the appearance of symptoms.

After two weeks, the disease severity was estimated by measuring the area of the shoots, leaves, and disease symptoms on the inoculated plants, and the infection percentage was calculated. Finally, the disease inhibition rate of all antagonists was calculated by comparing with the control. Also, fresh and dry weights (g), and aerial parts height (mm) of the plants were measured in all treatments.

The colonization ability of endophytic isolates on tomato plants was investigated by re-isolation of the endophytic fungi using the above-mentioned method for endophytes isolation. The presence of inoculated endophytes was detected by colonizing the different parts of plant. Isolated endophytes were identified based on the morphological features using the microscopic slide mounts.

Statistical analysis

In all experiments, a completely randomized design was used. The analysis of variance was performed using SAS 9.1. All obtained data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test for determining mean differences (Steel and Torrie, 1980).

Results

Endophytic fungi

A total of 89 endophytic fungal isolates were obtained (46 isolates from roots, 33 isolates from stems, and 10 isolates from leaves) (Fig. 1), and identified morphologically at the genus level. These isolates belonged to 12 different fungal genera including 20 *Fusarium* isolates, 15 *Alternaria* isolates, 5 *Cladosporium* Link isolates, 8 *Acremonium* Link isolates, 1 *Arthrinium* Kunze isolate, 5 *Aspergillus* P. Micheli ex Haller isolates, 4 *Penicillium* isolates, 1 *Moniliophthora* H.C. Evans, Stalpers, Samson & Benny isolate, 9 *Rhizoctonia* DC isolates, 2 *Nigrospora* Zimm. isolates, 2 *Trichoderma* Pers. isolates, 1 *Colletotrichum* Corda isolate, and 16 mycelia sterilia isolates (Fig. 2).

Molecular identification

The isolates with the highest inhibitory effect (above 50%) against *B. cinerea* in dual culture and greenhouse conditions, were identified based on the morphological features and *tef-1α* genomic region (Fig. 3), and based on the results, 28S1 and 41R1 isolates belonged to *Fusarium falciforme* (Carrión) Summerb. & Schroers, and 16S3 belonged to *Fusarium solani* (Mart.) Sacc., respectively.

Dual culture test

In this study, 89 endophytic strains were assessed for their antagonism against *B. cinerea* (B1 and B2 isolates) using dual culture assay (Table 2). Based on the primary test, 14 isolates were selected based on the highest inhibition against *B. cinerea* growth (Fig. 4a, b), and dual culture test was repeated for

these isolates. Finally, based on the results, six isolates including 41R1 and 28S1 (*F. falciforme*), 16S3 (*F. solani*), 9R1 (*Fusarium* sp.), 2R1 (*Fusarium* sp.), and 36S1 (*Fusarium* sp.), which had the greatest inhibitory effect (above 40%) against the pathogen (B1 and B2), were selected for further experiments.

Table 2
Antifungal activity of endophytic fungi of tomato plant based on the dual culture assay

Endophytic fungus	Antifungal activity	Endophytic fungus	Antifungal activity
Acremonium spp. 13S1	+*	*Fusarium* spp. 2R2	+
15S1	-	32R1	-
17S1	-	35R3	-
18S1	-	36S1	+
19R1	-	40R1	±
25S1	-	4R1	-
4S1	+	5R1	+
8S1	-	6R1	+
Alternaria spp. 11S1	-	6R2	-
16S2	-	7R1	-
27R1	+	9R1	+
28L1	-	*Moniliophthora* spp. 14S1	+
30S1	+	Myceila sterilia 10S1	-
31L1	-	12R1	-
34L1	+	15R1	+
34S1	+	18R1	-
35L1	+	1R1	±
39S1	-	20S1	-
3S1	+	21R1	-
40L1	-	22R1	-
42L1	-	25R2	-
42S1	-	26R1	-
5S1	+	35R1	-
33S1	+	36R1	+
6S1	-	3R1	-
12S1	+	8R1	-

*– Not active; ± slightly active; + active.

Endophytic fungus	Antifungal activity	Endophytic fungus	Antifungal activity
21S1	-	9S1	-
36L1	-	*Nigrospora* spp. 37R1	+
44L1	-	45R1	-
44S1	-	*Penicillium* spp. 14R1	+
Cladosporium spp. 10S2	-	24R2	-
34S2	-	35R2	+
37L1	-	48R1	-
3S2	-	*Rhizoctonia* spp. 22R2	-
41R2	+	23R1	-
43R1	-	24R1	-
Fusarium falciforme 28S1	+	29R1	-
41R1	+	38L1	-
Fusarium solani 16S3	+	41S1	-
Fusarium spp. 10R1	+	44R1	-
13R1	+	46R1	±
16S1	+	47R1	+
25R1	+	*Trichoderma* spp. 39R1	-
25S2	+	50R1	+
2R1	+		
*– Not active; ± slightly active; + active.			

Volatile organic compound test

The effect of VOCs produced by endophytic fungi on inhibiting the pathogen mycelia growth was investigated (Fig. 5a, b), and isolate 28S1 (*F. falciforme*) had the highest growth inhibition against B1 and B2 isolates (66.5 and 62.6%, respectively). The isolates 2R1 (*Fusarium* sp.), 9R1 (*Fusarium* sp.), 36S1 (*Fusarium* sp.), and 41R1 (*F. falciforme*) in terms of the growth inhibition of B1 (from 26.7 to 35.9%) and B2 (from 20.9 to 28.6%) had no significant difference, but isolate 16S3 (*F. solani*) had the lowest growth inhibition against the B1 and B2 (18.9 and 16.8%, respectively).

Additionally, when *B. cinerea* was exposed to VOCs from certain endophytic fungi, it developed white mycelia compared to the control that had grey mycelia (Fig. 6). On the other hand, this grey color is due

to the presence of conidia, so it can be assumed that the VOCs produced by the endophytic isolates have affected the *B. cinerea* sporulation.

Biocontrol assays in greenhouse

As mentioned before, six isolates with the highest growth inhibition against *B. cinerea in vitro*, were selected and evaluated in greenhouse, resulting in a reduction of grey mold symptoms on tomato plants compared to the control (Fig. 7, 8). Specifically, isolates 28S1, and 41R1 of *F. falciforme*, and isolate 16S3 of *F. solani* showed the highest antagonistic effect against B1 isolate, with inhibitory rate of 58.3%, 58.3%, and 55%, respectively (Fig. 12). Similarly, these isolates also showed significant antagonistic effects against the B2 isolate as well, with inhibitory rate of 55.8%, 57.5%, and 54.1%, respectively (Fig. 14). Additionally, isolates 9R1 (*Fusarium* sp.), 2R1 (*Fusarium* sp.), and 36S1 (*Fusarium* sp.) also reduced grey mold symptoms (B1 and B2 isolates) by approximately 35–40% (Figs. 7 and 8).

The plant development in greenhouse

A significant difference was observed between the treatments of the pathogen isolates with the antagonist fungi and the infected control treatment in both B1 and B2 isolates of pathogen. The antagonistic isolates prevented the reduction of fresh weight, dry weight, and plant height in these plants compared to the infected control plants treated by the pathogen (Table 3). In treatments inoculated with the antagonists without the presence of the pathogen, the plant height index was not significantly different from the control, but the dry weight index of the treatment inoculated with isolate 28S1 (*F. falciforme*) was significantly different from the control and caused an increase in the dry weight of the plant. In the fresh weight index, the treatment inoculated with isolate 16S3 (*F. solani*) was significantly different from the control and caused a decrease in the fresh weight of the plant (Table 3).

Table 3
Height (mm) of aerial parts, fresh and dry weights (g) of whole parts of tomato plants in different treatments of antagonistic isolates and *Botrytis cinerea*, 14 days after pathogen inoculation under greenhouse conditions.

Treatments	Height (mm)		Fresh weight (g)		Dry weight (g)	
	B1	B2	B1	B2	B1	B2
Pathogen + 41R1	141.66 ± 11.69 cdef	131.66 ± 7.52 de	3.23 ± 0.08 b	7.08 ± 0.07 e	1.41 ± 0.07 fg	1.45 ± 0.04 efg
Pathogen + 2R1	138.33 ± 16.02 def	153.33 ± 5.16 ab	6.20 ± 0.08 b	6.06 ± 0.08 g	1.63 ± 0.05 bcd	1.31 ± 0.07 b
Pathogen + 16S3	143.33 ± 8.16 bcde	165 ± 8.36 bc	6.81 ± 0.07 f	7.96 ± 0.08 c	1.33 ± 0.05 gb	1.35 ± 0.03 gh
Pathogen + 28S1	153.33 ± 5.16 abc	123.33 ± 5.16 cd	7.33 ± 0.08 d	6.7 ± 0.08 f	1.58 ± 0.07 bcde	1.42 ± 0.04 fg
Pathogen + 36S1	130 ± 8.94 f	135 ± 10.48 d	7.18 ± 0.07 e	7.11 ± 0.09 e	1.38 ± 0.07 g	1.50 ± 0.03 def
Pathogen + 9R1	133.30 ± 8.16 ef	136.66 ± 5.16 ed	6.66 ± 0.05 g	6.73 ± 0.05 f	1.56 ± 0.08 cde	1.41 ± 0.07 fgh
41R1	155 ± 5.47 ab	155 ± 5.47 ab	7.75 ± 0.15 c	7.75 ± 0.15 c	1.56 ± 0.10 cde	1.56 ± 0.10 cde
2R1	158.33 ± 7.52 a	158.33 ± 7.52 a	7.15 ± 0.12 e	7.15 ± 0.12 e	1.58 ± 0.13 bcde	1.58 ± 0.13 bcde
16S3	150 ± 8.94 abcd	150 ± 8.94 abcd	8.43 ± 0.21 a	8.43 ± 0.21 a	1.53 ± 0.05 ef	1.53 ± 0.05 ef
28S1	155 ± 8.36 ab	155 ± 8.36 ab	8.1 ± 0.2 bc	8.1 ± 0.2 bc	1.85 ± 0.08 a	1.85 ± 0.08 a
36S1	155 ± 5.47 ab	155 ± 5.47 ab	8.1 ± 0.14 b	8.1 ± 0.14 b	1.51 ± 0.07 def	1.51 ± 0.07 def
9R1	155 ± 13.78 ab	155 ± 13.78 ab	7.2 ± 0.12 de	7.2 ± 0.12 de	1.65 ± 0.10 bc	1.65 ± 0.10 bc
Pathogen	100 ± 8.94 g	108.33 ± 7.52 f	3.6 ± 0.06 i	3.41 ± 0.09 h	0.39 ± 0.01 h	0.35 ± 0.3 i
Control	156.66 ± 8.16 a	156.66 ± 8.16 a	8.51 ± 0.07 a	8.51 ± 0.07 a	1.68 ± 0.14 b	1.68 ± 0.14 b

Data are means of six replications (three pots containing two plants). Means in a column followed with the same letters are not significantly different at $p \leq 0.01$ according to Duncan's multiple range test. B1, B2: *Botrytis cinerea* isolates; 2R1: *Fusarium* sp.; 36S1: *Fusarium* sp.; 9R1: *Fusarium* sp.; 16S3: *Fusarium solani*; 28S1: *Fusarium falciforme*; 41R1: *Fusarium falciforme.*

Endophytic colonization of tomato by fungal isolates

In order to investigate the colonization ability of endophytic isolates, fungi obtained from different plant parts (root, stem, and leaf) which were inoculated with conidial suspensions of the isolates (*Fusarium* sp.) 36S1, (*Fusarium* sp.) 2R1, (*Fusarium* sp.) 9R1, (*F. solani*) 16S3, (*F. falciforme*) 28S1, and 41R1, and obtained isolates were compared with the fungi isolated from un-inoculated control plants. Based on the results, the inoculated fungi were isolated along with some other fungi. So, all six isolates exhibited a systemic colonization in plant.

Discussion

Excessive use of chemical fungicides to control the plant diseases has led to harmful effects such as damage to the health of the environment, humans and animals, as well as emergence of resistance in pathogens. Given the high cost of chemical control and the need for healthy food products and sustainable agriculture, biological control agents have gained significant attention and can be used as a alternative to chemical methods (Lewis and Lumsden, 2001). The exploration of endophytic microorganisms as biological control agents is a result of the search for new alternatives to control pathogens.

An increasing number of endophytes are being explored as potential of new biocontrol agents as they inhabit the same ecological niche in host as pathogens and can be present in roots, stems, leaves, fruits, and seeds (Santoyo et al. 2016). Endophytes are fungi or bacteria that invade living plant tissues during their life cycle, causing inconspicuous and asymptomatic contamination within plant tissues (Wilson, 1995). Endophytic fungi improve plant growth, nutrient absorption and storage, produce useful secondary metabolites, and increase plant resistance and tolerance against pathogens and herbivores during their interaction with the host plant (Mishra et al., 2014). Biological inhibition of endophytic fungi can be effective through different mechanisms such as competition, antibiosis, parasitism, induction of resistance, and improvement of plant growth (Pal and Gardener, 2006).

In the present study, endophytic fungi were isolated from roots, stems, and leaves of the different tomato cultivars in Iran. All of them were identified morphologically at the genus level, and among them, isolates with a significant antagonistic effect against *B. cinerea* (B1 and B2) were selected as biocontrol agents. *Rhizoctonia*, *Alternaria*, and *Cladosporium* were found in all parts of the tomato plant (root, stem, and leaf). *Aspergillus* species were obtained from leaves and stems, while *Fusarium* and *Acremonium* species were found in roots and stems. *Penicillium*, *Nigrospora*, *Trichoderma*, and *Colletotrichum* species were only recovered from roots, and *Arthrinium* and *Moniliophthora* species were isolated from the stems exclusively. Among the endophytic fungi isolated from tomato plants, *Fusarium* and *Alternaria* were the most abundant genera, as it reported by Dastogeer et al. (2020), that the most dominant identified endophytic fungi were the isolates of the *Fusarium*, *Alternaria*, *Aspergillus*, and *Penicillium*. El-Hasan et al. (2022) have found that out of the 357 endophytic isolates from the roots of potato, tomato, bell pepper, and nightshade, 112 isolates (31.4%) were belonged to *Fusarium* species. Similarly, in the previous studies (Cannon & Simmons, 2002; Xia et al., 2019), frequent isolation of *Alternaria*, *Aspergillus*, *Chaetomium*, *Fusarium*, and *Penicillium* from tomato plant tissues has been reported.

Kusari et al. (2013) identified the endophyte species *Paecilomyces lilacinus* (Thom) Samson from the apical buds of *Cannabis sativa* L., and the fungus completely prevented the growth of *B. cinerea* in the dual culture tests. Noumeur et al. (2015) obtained 12 endophytic fungal isolates from the roots of *Artemisia absinthium* L.. These fungi exhibited an inhibition index ranging from 33 to 50% against *B. cienerea in vitro*.

In the present study, six endophytic isolates *F. falciforme* (41R1 and 28S1), *F. solani* (16S3), and *Fusarium* spp. (9R1, 2R1, and 36S1), obtained from tomato plants, exhibited 39.9 to 65.8% inhibition against *B. cinerea* (B1 and B2) in the dual culture tests. In a most similar study, *Trichoderma harzianum* Rifai T1 showed 60 and 71.54% inhibition against *B. cinerea* (B1 and B2) in dual culture tests, respectively (Jalali et al., 2021).

Moreover, endophytic fungi produce VOCs, which induce the plant self-defense against pathogens (Tyc et al., 2017). In a study by El-Hasan et al. (2022), VOCs produced by three endophytic fungal isolates (KB1S1-4, KA2S1-42, and KB2S2-15) from members of Solanaceae family inhibited *Phytophthora infestans* (Mont.) de Bary growth by 48.9%. Zhang et al. (2014) isolated *Fusarium oxysporum* CanR-46 from *Brassica napus* L. that was produced VOCs, and showed inhibitory effect on *B. cinerea* by 91%. In the current study, the VOCs from *F. falciforme* 28S1, showed the highest inhibitory rate against pathogenic growth, resulting in 66 and 63% inhibition of mycelia growth for *B. cinerea* B1 and B2 isolates, respectively. Isolates of *Fusarium* spp. (2R1 9R1, and 36S1), *F. falciforme* (41R1), and *F. solani* (16S3) also caused 17 to 35% reduction in mycelial growth of *B. cinerea* (B1 and B2). Also, Jalali et al. (2021) have reported in the VOCs test, that *T. harzianum* T1 inhibit *B. cinerea* (B1 and B2) mycelia growth up to 95.98 and 100%, respectively.

Kavroulakis et al. (2007) isolated the endophytes *F. solani* and *F. equiseti* from healthy roots of tomato, with the ability to colonize the tomato root tissues and protect them from *F. oxysporum* f. sp. *radicis-lycopersici*. Also, endophytic *F. solani* was able to effectively suppress Fusarium root and crown rot disease of tomato plants by inducing resistance through ethylene and jasmonic acid signaling pathways. Jalali et al., (2021) demonstrated that isolates *T. harzianum* T1 effectively controlled the grey mold of tomato plants caused by *B. cinerea* isolates B1 and B2 in greenhouse, with the average inhibition rates of over 60%. In this study, six endophytic strains were examined for their antagonism against tomato grey mold disease under greenhouse conditions. The isolates *F. falciforme* (28S1 and *F* 41R1), and *F. solani* (16S3) showed a reduction in symptoms and lesions of *B. cinerea* (B1 and B2) by 54.2 to 58.3% compared to control. Additionally, three other *Fusarium* isolates, (9R1, 36S1, and 2R1), demonstrated a 35–40% reduction in symptoms of *B. cinerea* (B1 and B2) compared to control.

Some endophytic fungi, have various positive effects on plant growth and then they are considered as plant growth-promoting fungi (PGPF) (Waqas et al. 2015). The plant growth-promoting endophytes (PGPE) reside in plant tissues, facilitating nutrient exchange and enzyme activity due to their close linkage inside the plant (Hassan, 2017), and promote hormone production in plant (Lin and Xu, 2013). For example, *A. fumigatus* and *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, promoted plant

growth through gibberellin production (Bilal et al., 2018). Andrade-Linares et al. (2011) have found that tomato plants colonized by dark septate endophytes obtained from tomato roots, showed increased shoot biomass in the early stages of growth. In the present study, *F. falciforme* isolate 28S1 increased the dry weight of tomato plants compared to control.

Barra-Bucarei et al. (2019) have reported that the *Beauveria bassiana* (Bals.-Criv.) Vuill. isolates colonized tomato and chili pepper tissues, All have exhibited a systemic mode of action, meaning that the fungus inoculated to the roots was subsequently isolated from the leaves. The endophytes inoculated to the leaves were isolated from all parts of the tomato plants and the systemic colonization of these fungi was determined. The results provide evidence of the antagonism potential of *Fusarium* endophytic isolates to control the *B. cinerea* in tomato plants, as it already shown in the research of Ebrahimi et al. (2023), which demonstrated the endophytes' systematic colonization ability in both tomato and melon plants by recovering all five endophytic isolates from different parts of both host plants after two weeks of inoculation.

Conclusion

The current study is the first report of biological inhibition of grey mold disease by tomato endophytic fungi and also the first record of *F. falciforme* as a tomato endophytic fungus in Iran. It is necessary to accomplish additional studies to achieve the effective and sustainable use of these biocontrol agents to management of plant pathogens in agricultural ecosystems. Generally, we can expect to discover the biological control agents within the fungi that are symbiotic with a plant to combat the plants pathogenic agents. However, extensive biochemical and molecular studies are required for commercial production and use of endophytes under field conditions to control pathogens.

Declarations

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Data availability

All data supporting the findings of this study are available within the paper. Raw data are available upon request to the corresponding author.

Author contributions

LE, and KBF contributed to the research design. Experimental design, material preparation, and data collection were performed by AP, and LE. Statistical analysis of the data were interpreted by AP. The original manuscript draft was written by AP. The manuscript was reviewed and edited by LE, and KBF.

The authors declare that they have no conflicting interests.

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Figures

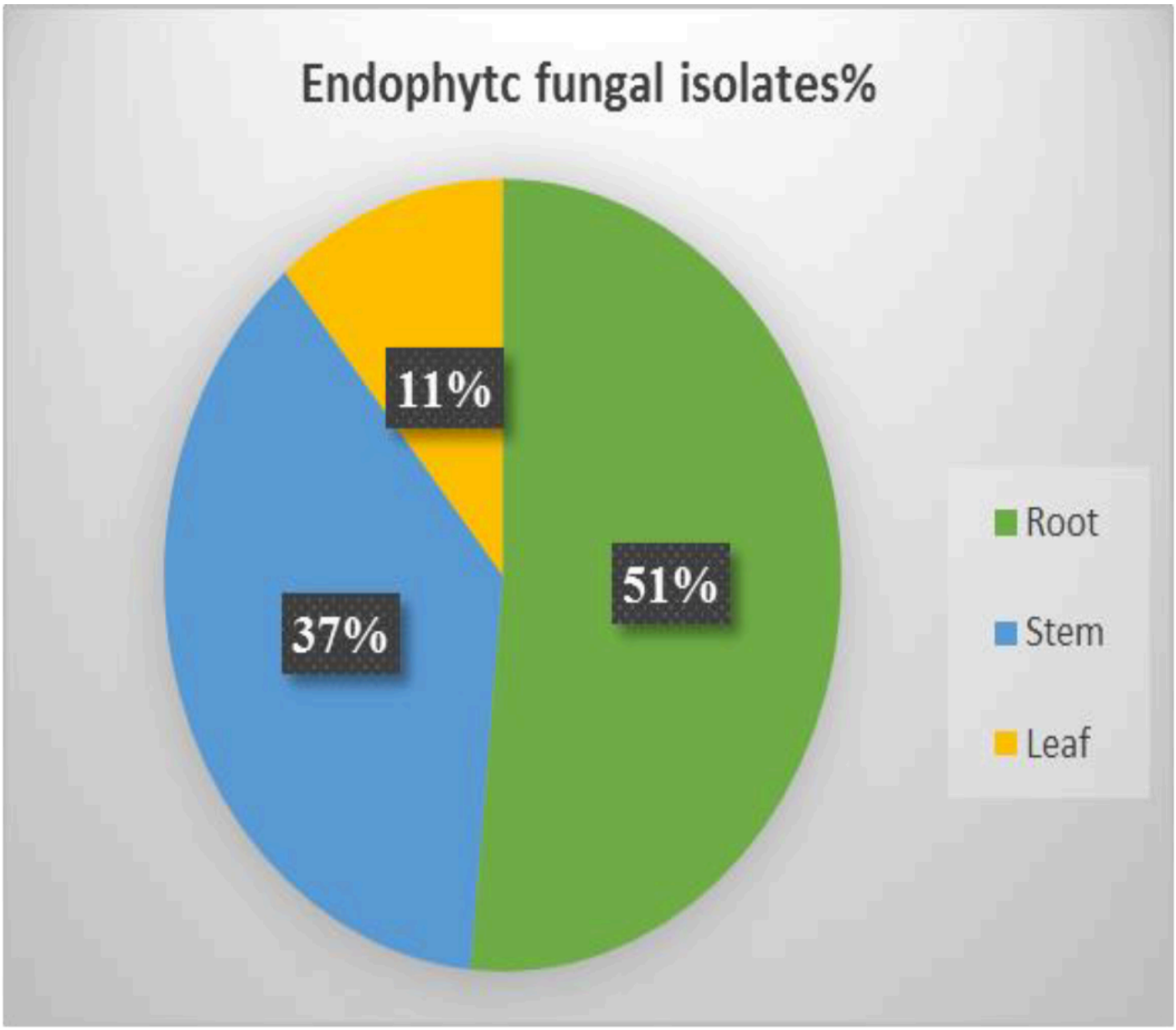


Figure 1

Endophytic fungal isolates were obtained from root, stem and leaf samples of tomato plants collected from Isfahan, and Tehran provinces of Iran.

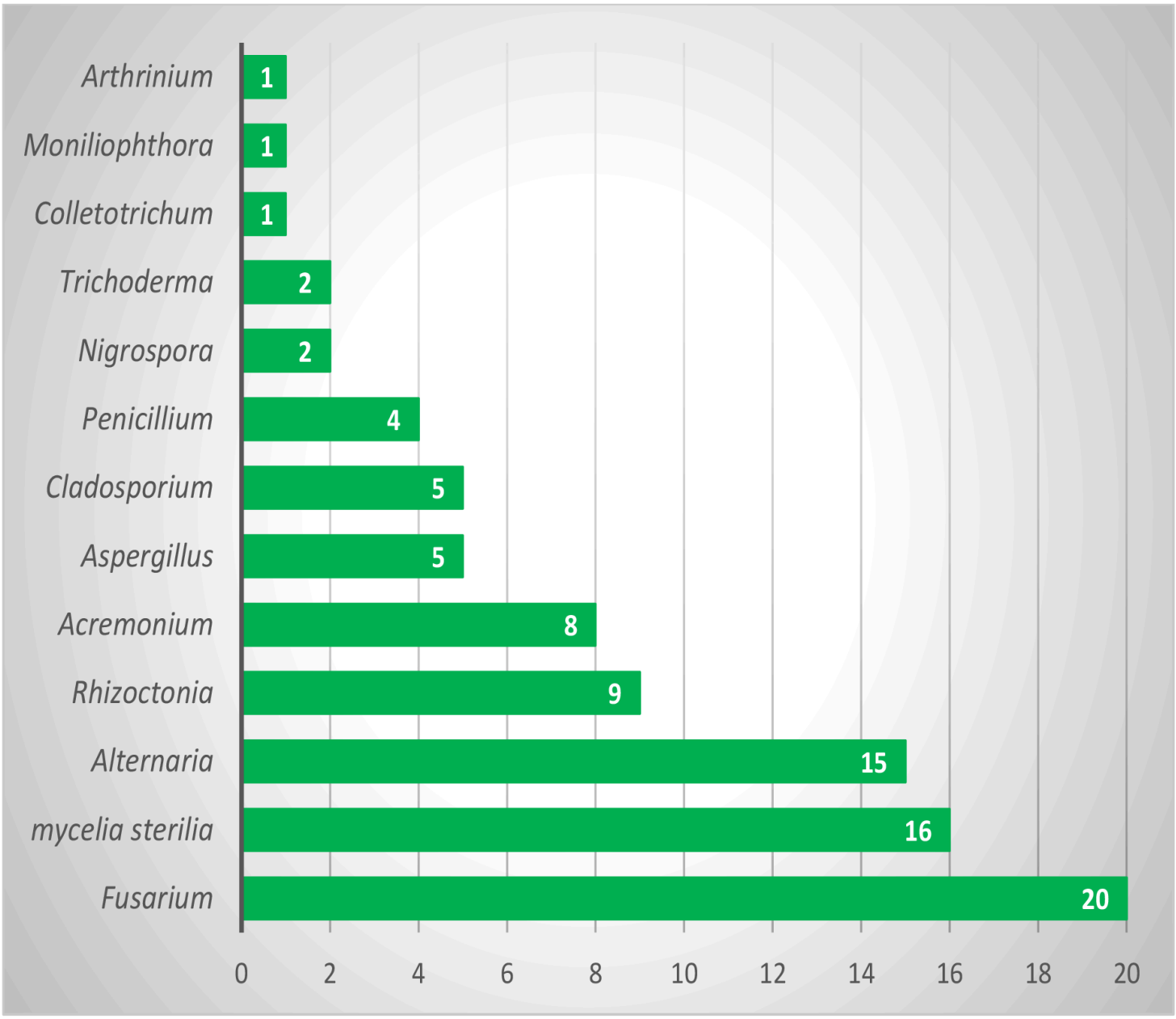


Figure 2

Total isolates of each identified endophytic genus.

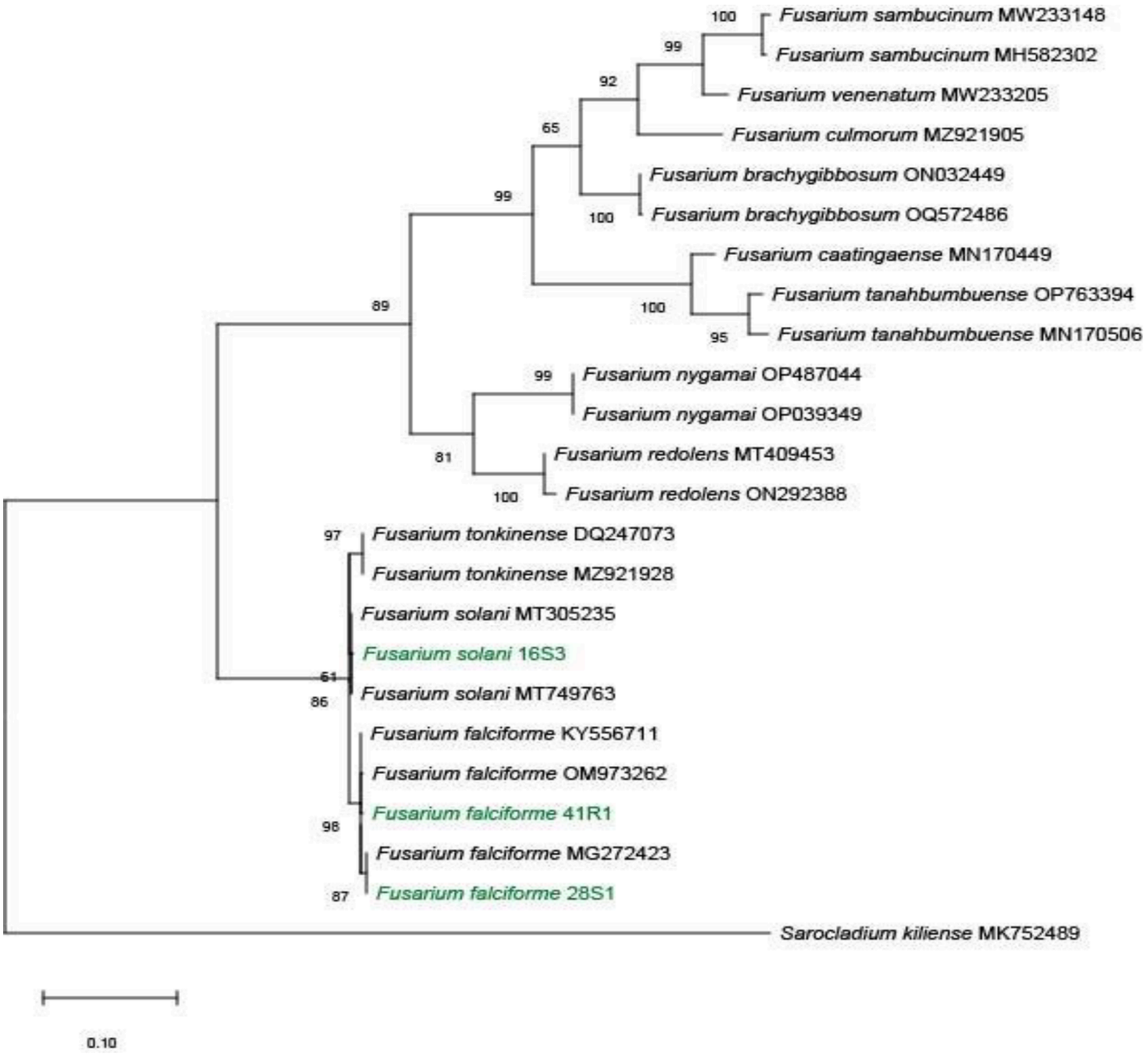


Figure 3

Maximum Likelihood (ML) phylogenetic tree based on aligned sequences of *tef-1α*gene of 24 isolates generated in MEGA 10.2 under NEI + G model. The tree was rooted to *Sarocladium kiliense* (MK752489). Bootstrap values (1,000 replicates) indicated at the nodes. The scale bar indicates nucleotide substitution in ML analysis, values ≥50% are shown above/below the branches. The surveyed isolates in the current study are highlighted.

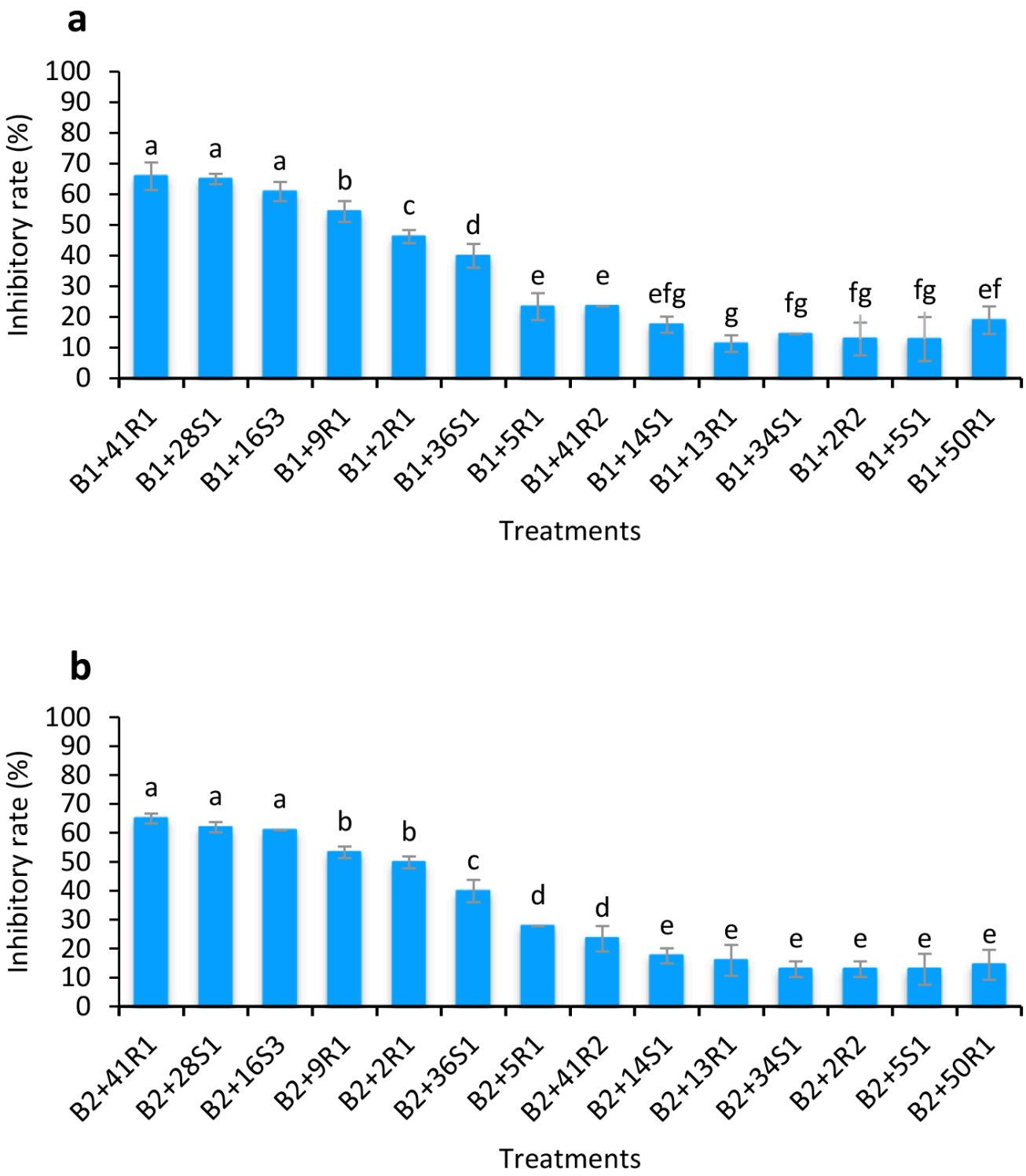


Figure 4

Effects of endophyte isolates (*Fusarium falciforme* 28S1, *Fusarium falciforme*41R1, *Fusarium solani* 16S3, *Fusarium* sp. 36R1, *Fusarium*sp. 9R1, *Fusarium* sp. 2R1) on *Botrytis cinerea* isolates B1 (**a**), and B2 (**b**) mycelia growth in dual culture test after 7 days at 20 °C. Data are the means ± SE of three replicates. Values of histograms with common letters are not significantly different at P≤0.01 according to Duncan's multiple range test.

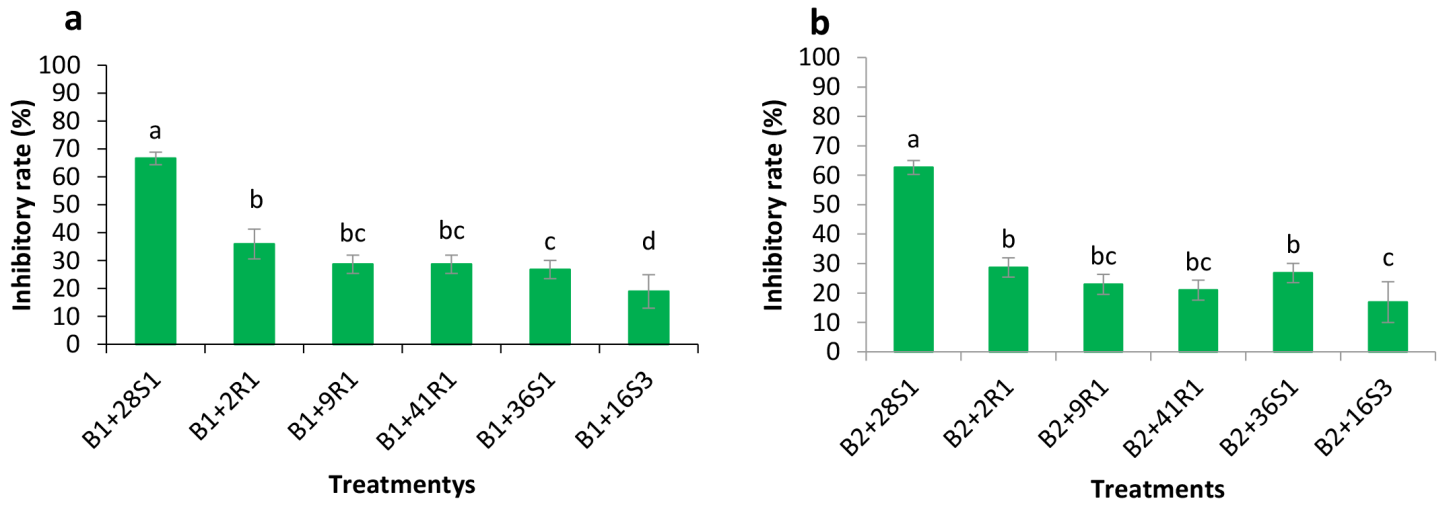


Figure 5

Effects of volatile organic compounds of endophyte isolates (*Fusarium falciforme*28S1, *Fusarium falciforme* 41R1, *Fusarium solani* 16S3, *Fusarium*sp. 36R1, *Fusarium* sp. 9R1, *Fusarium* sp. 2R1) on *Botrytis cinerea* isolates B1 (**a**), and B2 (**b**) mycelia growth after 7 days at 20 °C. Data are the means ± SE of three replicates. Values of histograms with common letters are not significantly different at P≤0.01 according to Duncan's multiple range test.

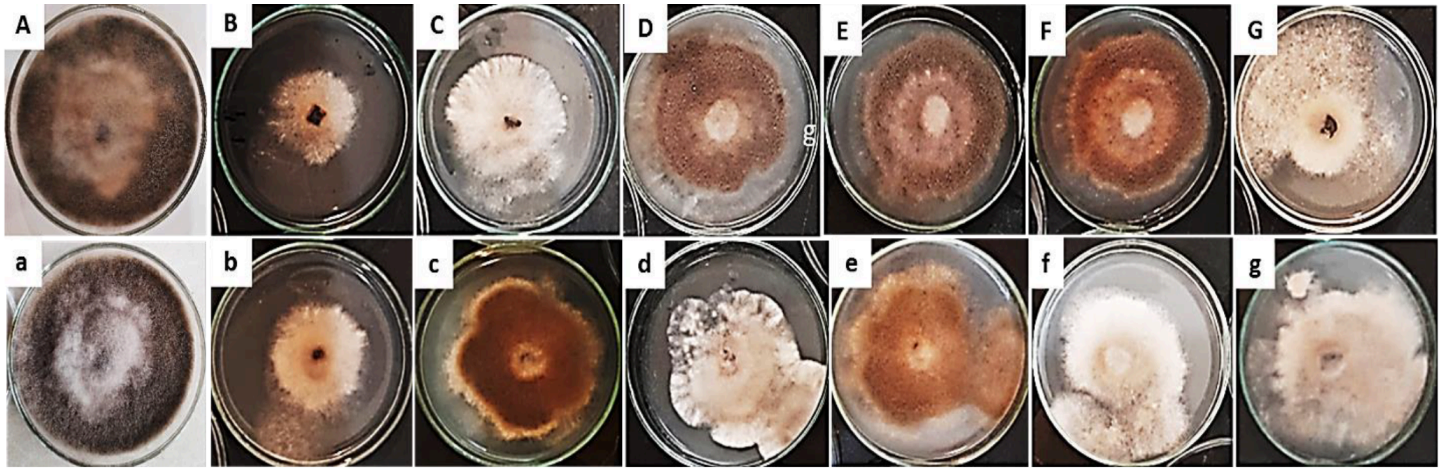


Figure 6

Effects of volatile organic compounds of endophyte isolates (**B, b**) *Fusarium falciforme* 28S1, (**C, c**) *Fusarium* sp. 2R1, (**D, d**) *Fusarium*sp. 9R1, (**E, e**) *Fusarium falciforme* 41R1, (**F, f**) *Fusarium*sp. 36R1, (**G, g**) *Fusarium solani* 16S3 on *Botrytis cinerea*isolates B1 (**A**), and B2 (**a**) mycelia growth after 7 days at 20 °C.

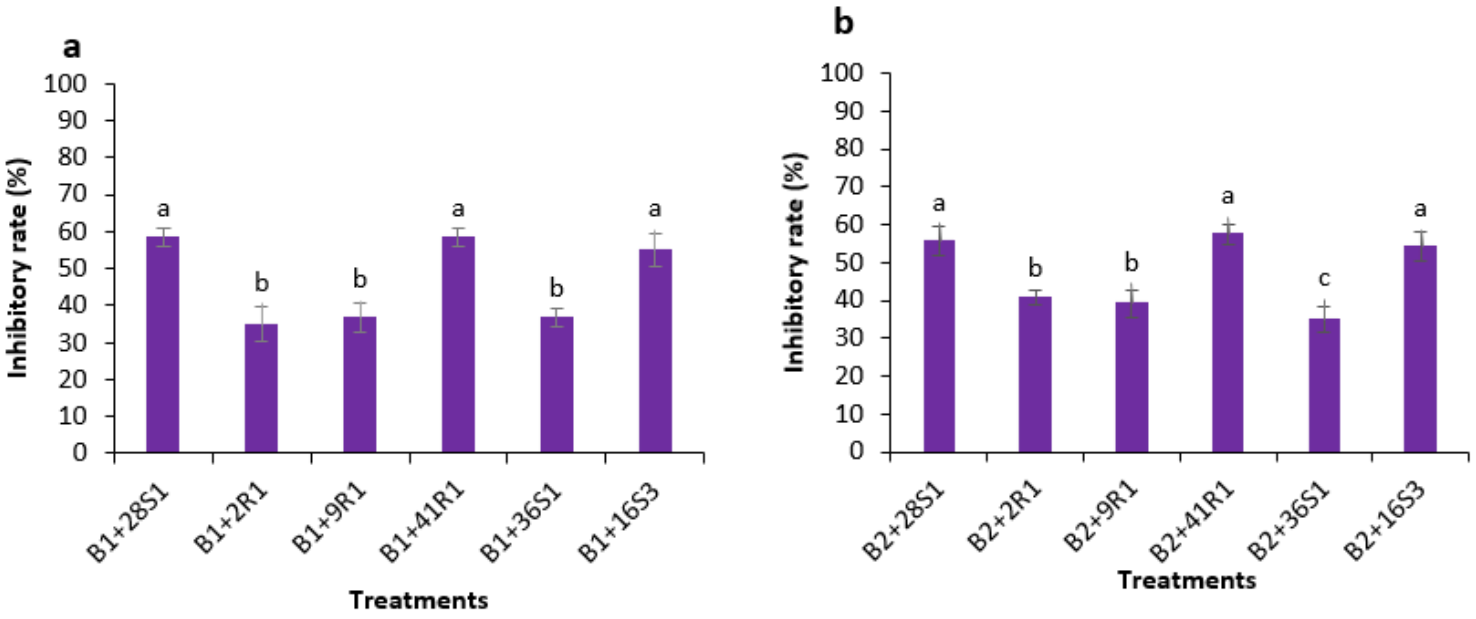


Figure 7

Antagonistic effect of endophytic fungi (*Fusarium falciforme*28S1, *Fusarium falciforme* 41R1, *Fusarium solani* 16S3, *Fusarium*sp. 36R1, *Fusarium* sp. 9R1, *Fusarium* sp. 2R1) against grey mold (*Botrytis cinerea* isolates B1 (**a**) and B2 (**b**)) diseases on tomato plants under greenhouse conditions. Data are the means ± SE of three replicates. Values of histograms with common letters are not significantly different at P≤0.01 according to Duncan's multiple range test.

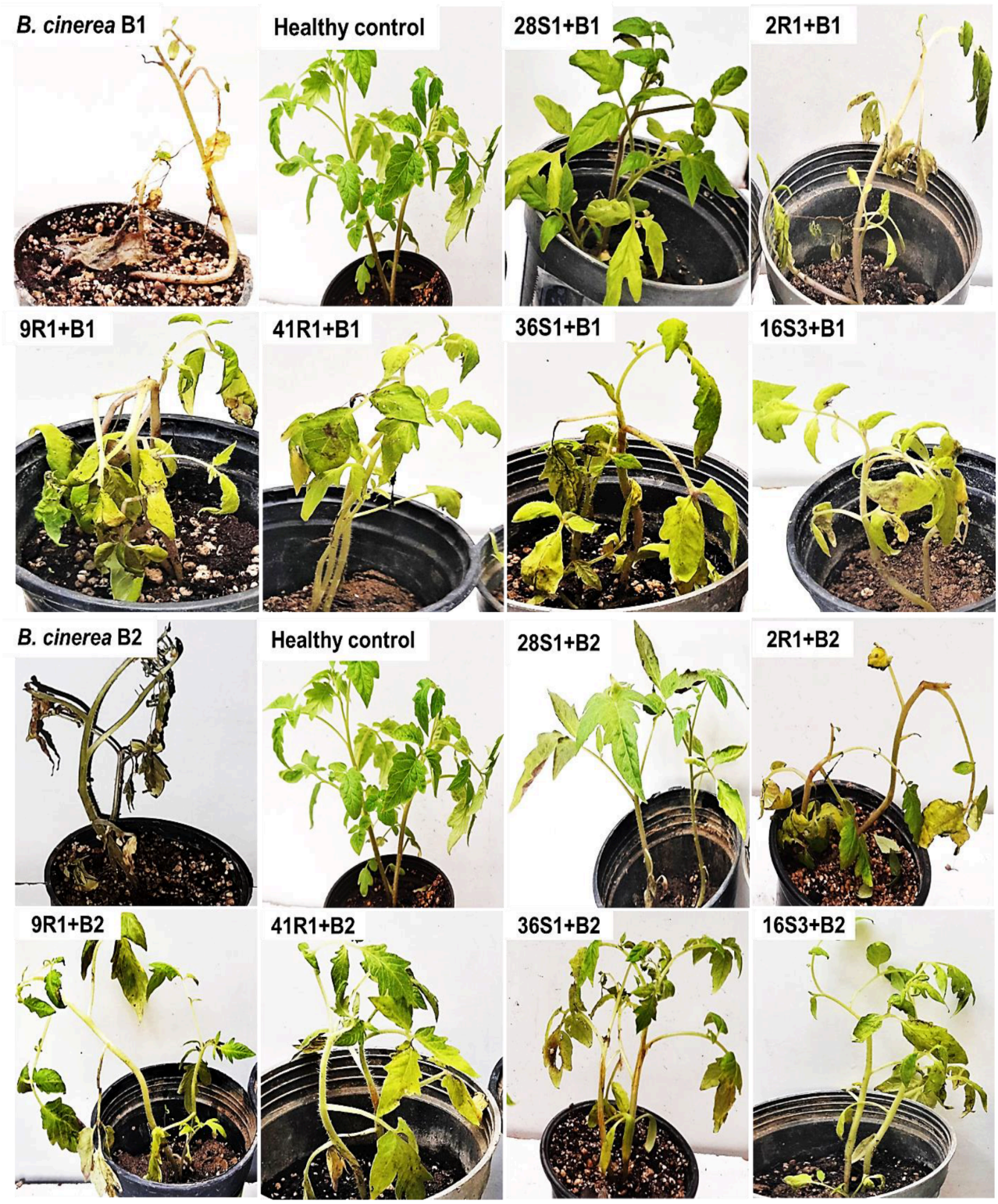


Figure 8

Antagonistic effect of endophytic fungi (*Fusarium falciforme* 28S1, *Fusarium falciforme* 41R1, *Fusarium solani* 16S3, *Fusarium* sp. 36R1, *Fusarium*sp. 9R1, *Fusarium* sp. 2R1) against grey mold (*Botrytis cinerea*isolates B1 and B2) diseases on tomato plants under greenhouse conditions.