Identification of L-asparaginase-producing endophytic fungi isolated from medicinal plant species (#38991)

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Identification of L-asparaginase-producing endophytic fungi isolated from medicinal plant species

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In this study, endophytic fungi isolated from medicinal plants were studied for the production of L-asparaginase enzymes. Endophytic fungi from seven medicinal plants including *Matricaria chamomilla, Anthemis triumfetii, Anthemis parthenium, Anthemis altissima var. altissima, Achillea millefolium, Achillea filipendulina, Cichorium intybus* were investigated for production of L-asparaginase. Asparaginase activity was assayed by the nesslerization method. Isolated endophytic fungi were identified by morphological and molecular. Of the 104 species of endophytic fungi isolated from seven species of medicinal plants, 37 isolates were able to produce L-asparaginase. Asparagine activity ranged from 0.019 to 0.492unit/mL^{-1}. The isolates that were able to produce L-asparaginase belonged to the genera *Plectosphaerella, Fusarium, Stemphylium, Septoria, Alternaria, Didymella, Phoma, Chaetosphaeronema, Sarocladium, Nemania, Epicoccum, Ulocladium and Cladosporium*. Among these, *Fusarium proliferatum* was found to have the highest enzyme activity with 0.492unit/mL^{-1}. This is the first report of the production of L-asparaginase by these endophytic fungi isolated from medicinal plants.

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Abstract

In this study, endophytic fungi isolated from medicinal plants were studied for the production of L-asparaginase enzymes. Endophytic fungi from seven medicinal plants including *Matricaria chamomilla, Anthemis triumfetii, Anthemis parthenium, Anthemis altissima var. altissima, Achillea millefolium, Achillea filipendulina, Cichorium intybus* were investigated for production of L-asparaginase. Asparaginase activity was assayed by the nesslerization method. Isolated endophytic fungi were identified by morphological and molecular. Of the 104 species of endophytic fungi isolated from seven species of medicinal plants, 37 isolates were able to produce L-asparaginase. Asparagine activity ranged from 0.019 to 0.492unit/mL^{-1}. The isolates that were able to produce L-asparaginase belonged to the genera *Plectosphaerella, Fusarium, Stemphylium, Septoria, Alternaria, Didymella, Phoma, Chaetosphaeronema, Sarocladium, Nemania, Epicoccum, Ulocladium and Cladosporium*. Among these, *Fusarium proliferatum* was found to have the highest enzyme activity with 0.492unit/mL^{-1}. This is the first report of the production of L-asparaginase by these endophytic fungi isolated from medicinal plants.

Key words: Asparaginase activity, Asteraceae, Endophytic fungi, *Fusarium proliferatum*

Introduction

Endophytic fungi colonize plant tissue without causing symptoms of disease in the host plant. Endophytic fungi from medicinal plants have been considered to be a rich source of functional metabolites (Arnold et al., 2011). The close symbiotic relationship between endophytic fungi and host plants gives endophytes a powerful ability to produce novel bioactive compounds whose production is fueled by host plant carbohydrates (Aly et al., 2011). These bioactive compounds increase plant resistance to pathogens and herbivores. In the recent years, endophytic fungi have been viewed as a source of secondary metabolites, including anticancer, anti-inflammatory, antibiotic and antioxidant agents (Bungihan et al., 2011). One of the well-known compounds produced by endophytes is paclitaxel. Initially, this anticancer compound was derived from the Pacific yew tree (Zhou et al., 2010). With the discovery that the yew tree endophyte *Taxomyces andrenae* also produces paclitaxel, researchers have become interested in discovering new secondary metabolites from other endophytic fungi.

L-asparaginase is an extracellular enzyme that hydrolyzes asparagine to aspartic acid and ammonia. L-asparaginase enzymes are used for medical and industrial purposes. Asparaginase enzymes in the food industry are used as an admixture to reduce the acrylamide produced by the high temperature in starchy foods and reduce the risk of cancer. This enzyme is one of the most important biochemical therapeutic enzymes used in the treatment of various types of leukemia, such as acute lymphoblastic leukemia in children. In cancer treatment, L-asparaginase removes L-asparagine in the serum, depriving tumor cells of the large amounts of asparagine required for growth (Asthana and Azmi, 2003). Currently, L-asparaginase derived from *Escherichia coli* is the main source of asparaginase. However, side effects of this enzyme from *E. coli* include chills, fever, abdominal cramps and fatal hyperthermia (Hosamani and Kaliwal, 2011). Considering the importance of L-asparaginase in the treatment of leukemia, finding new sources of this enzyme that can produce high levels of enzyme with minimum side effects is a priority (Theantana et al., 2009). Microorganisms such as fungi, due to the ability to produce extracellular enzymes, produce a high amount of product, have easy extraction and purification of the product, and easy genetic manipulation to achieve the desired product, provide appropriate resources of production of L-asparaginase enzyme. (Serqis, 2004). L-asparaginase from endophytic fungi isolated from medicinal plants has been reported in the recent years (Theantana et al., 2007). There are few studies on the production of asparaginase enzymes from endophytic fungi (Strobel et al., 2004; Sarquis et al., 2004; Mahajan et al., 2014). In this study, endophytic fungi were isolated from seven Iranian medicinal plants: *Matricaria chamomilla, Athemis triumfetii, Tanacetum parthenium, Anthemis altissima var. altissima, Achillea millefolium, Achillea filipendulina* and *Cichorium intybus*. These species of plant family Asteraceae have antibacterial, anti-cancer and anti-viral properties. The present work explores the potential of endophytes to produce L-asparaginase

Materials & Methods

Isolation endophytic fungi from plants

Endophytic fungi were isolated from seven medicinal plant species: *Matricaria chamomilla, Anthemis triumfetii, Tanacetum parthenium, Anthemis altissima var. altissima, Achillea millefolium, Achillea filipendulina* and *Cichorium intybus*. Sampling was conducted from Roots, leaves and stems of the healthy and mature plants from Northeastern of Iran (Table2). The plants were rinsed gently in running water. After washing, samples were cut into 0/5-1cm pieces. The surface sterilization was done by sodium hypochlorite (1.5% - 2/5% NaoCl) and then followed 75% ethanol. The surface sterilized samples were placed on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) plates with 50 mg/L tetracycline to suppress the bacterial growth and incubated at 28 ± 2ºC up to 14 days. Colonization Frequency (CF) of an endophyte species is equal to the number of segments colonized by a single endophyte, divided by the total observed number of segments × 100 (Khan et al., 2010).

Morphological Identification of Isolates

Prior to taxonomic studies, the isolates were placed in morphotypic groups based on the characteristics such as color, colony shape and growth rate. For the microscopic observations, representative of each morphotype was selected and its morphological characteristics were evaluated under microscope. Morphological identification was performed with fungi identification keys (Barnett and Hunter, 1999; Bensch et al., 2012; Boerema et al., 2004; Simmons, 2007; Booth, 1971).

Screening of L-asparaginase-producing endophytes

The isolated endophytic fungi were screened for their ability to produce asparaginase. Mycelial plug was inoculated onto Modified Czapex Dox (McDox) agar [agar powder (20.0 g/ L^{-1}), glucose (2.0 g/L^{-1}), L-asparagine (10.0 g/L^{-1}), KH_2PO_4 (1.52 g/L^{-1}), KCl (0.52 g/L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.52 g/L^{-1}), $CuNO_3{\cdot}3H_2O$ (0.001g/L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.001 g/L^{-1}), FeSO4·7H2O (0.001 g/L^{-1})], l-asparagine (10.0 g/L^{-1}) and 0.3 mL of 2.5% phenol red dye (indicator). Controls were prepared by inoculating mycelial plugs on Czapex Dox agar without asparagine. Triplicates for each isolate were prepared. All plates were incubated at 26 ± 2°C. After 5 days of incubation, the diameter of the pink zone was measured (Gulati, 1997).

Measurement of the L-asparaginase activity

The positive fungal isolates were inoculated in 200 mL of McDox broth and incubated for 5 days at 36 ± 2 °C and 120 rpm. L-asparaginase was estimated by Nesslerization as described by (Imada et al., 1973). After incubation, 100 µl of broth (crude enzyme) was pipetted into 2 ml tubes. After that, 100 µl of Tris HCl (pH 7), 200 µl of 0.04 M asparagine and 100 µl of sterile distilled water (SDW) were added. The mixture was incubated at 37±2 °C for 1 h. After incubation, 100 µl of 1.5 M Trichloroacetic Acid (TCA) was then added to stop the enzymatic reaction. Finally, 100 µl of the mixture was pipetted into fresh tubes containing 750 µl SDW and 300 µl of Nessler's reagent and incubated at 28± 2°C for 20 min and the amount of enzyme activity was measured by determining absorbance of samples at 450 nm using UV-Visible spectrophotometer. One unit of asparaginase is expressed as the amount of enzyme that catalyzes the formation of 1 µmol of ammonia per minute at 37 ± 2°C (Theantana et al., 2007).
The experiment was conducted in a Complete Randomized Design (CRD) with triplicates and data were statistically analyzed using the software Statistical Package for the Social Sciences (SPSS) version16.0.

Molecular identification of the endophytic fungi

Selected fungal isolates were grown in 200ml PDB for 7 days at 28ºC. The mycelia washed with distilled water and ground with liquid nitrogen. The nucleic acid was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Dayle, 2001). Strains were sequenced for four loci: β-tubulin (B tub), internal transcribed spacer (ITS), Translation elongation factor 1-alpha (EF) and 28S rDNA (LSU); the primer sets listed in (Table 1). The PCR amplifications were performed in a total volume of 12.5 µL solution containing 10–20 ng of template DNA, 1 × PCR buffer, 0.7 µL DMSO (99.9 %), 2 mM MgCl2, 0.5 µM of each primer, 25 µM of each dNTP and 1.0 U Taq DNA polymerase. Amplification process was initiated by pre-heating at 95 °C for1 min, followed by 40 cycles of denaturation at 95 °C for 30s, primer annealing at the temperature stipulated in Table 1, extension at 72 °C for 10s and a final extension at 72 °C for 5 min. The products of the PCR reaction were then examined by electrophoresis using 1% (w/v) agarose gel, stained with gel red (Biotium®) and visualized with a UV transilluminator. BLAST analysis was carried out in the NCBI database. All sequences were deposited in NCBI's GenBank Database.

Results

Identification of Fungal Endophytes

A total 827 isolates of endophytic fungi were obtained from seven plant species. The percent colonization frequency of endophytes differed in the plant parts(Table 3). Diversity of species were higher in the stem tissues (58%) and after flowering stage (17% more than the before flowering stage). The results indicated that the species composition and frequency of endophyte species was found to be dependent on the tissue and the stage of plant growth. Due to the abundance of endophytes, the isolates were divided into 104 morphotypes. Identification was performed based on the morphological characteristics of fungal species and BLAST of sequencing results of the 37 selected endophytes to the NCBI database (Bethesda, MD, USA) (Table 4). The abundance of *Fusarium* and *Alternaria* were higher than other isolates which colonized more than one plant part. In contrast, some endophytes were observed to be only in one tissue, such as several *Septoria* spp. were obtained from stems only. The isolates of *Fusarium* and *Alternaria* were found in stem, leaf and root.

Screening of L-asparaginase-producing endophytes

Based on the fact that the L-asparaginase produces ammonia during its reaction, ammonia increases pH, the pH index can be used to identify the microorganisms that produce L-asparaginase. The preliminary results showed that 37 isolates from seven plant species are able to produce L-asparaginase enzymes and formation of pink zone was evident on MCD medium as a result of this enzyme produced by the endophytes, which hydrolyzes asparagine into aspartic acid and ammonia, the phenol red dye indicator change color from yellow (acidic condition) to pink (alkaline condition). ~~All isolates produce a pink zone around the colonies, which indicates the production of extracellular L-asparaginase enzymes.~~ Results showed that there was a significant difference between the isolates at 1% level, and the isolate of *F. proliferatum* had the most enzymatic activity (Fig1). All of the *Fusarium* species in this study have the ability to produce the L-asparaginase.

Estimation of L-asparaginase production by Nesslerization

The L-asparaginase enzyme activities of endophytic fungi were found to occur in the range of 0.019–0.492unit/mL^{-1} (Table 4). The isolates of *F. proliferatum* from stem of *Anthemis altissima* exhibited a highest activity among all the endophytic fungi with 0.492unit/mL^{-1}, after that *Plenodomus tracheiphilus* from the root of *A. altissima* with 0.481 of enzyme had high asparaginase activity. *Septoria tormentillae* isolated from leaf of *Achillea millefolium* showed least enzyme activity with 0.019 unit/mL^{-1}. The amount of enzyme activity of the fungal endophyte was different and there were significant differences between them (Table2, 4). Most of the asparaginase activity was in endophytic fungi isolated from *Anthemis altissima*.
The results showed that the percentage of endophytes with L-asparaginase production was 35% of the total number isolated, with 3%, 2%, 12%, 12%, 3% and 5% for *Matricaria chamomilla, Athemis triumfetii, Anthemis altissima var. altissima, Achillea millefolium, Achillea filipendulina* and *Cichorium intybus.*

Discussion

This is the first report of L-asparaginase-producing endophytic fungi from *Matricaria chamomilla, Athemis triumfetii, Anthemis altissima* var. *altissima, Achillea millefolium, Achillea filipendulina* and *Cichorium intybus*. Our results suggest that these species of Asteraceae family are potential hosts of endophytes that produce L-asparaginase. Enzymes and secondary metabolites may be produced in higher levels in strains of endophytic fungi because plants provide abundant nutrients internally to support their production ~~(Gutierrez et al., 2012). The production of enzymes and secondary metabolites may also vary due to environmental, genetic and evolutionary factors (Pradeep et al., 2007).~~ Endophyte-derived metabolites have attracted researchers because of their potential to provide new medicines. We found that the highest amount of the asparaginase activity in the endophytic fungi isolated from *A. altissima* var. *altissima*.. L-asparaginase activity was highest in isolates belonging in the genus *Fusarium*, followed by species of *Alternaria, Cladosporium* and *Septoria*. Other investigators have also identified isolates of the genus *Fusarium* as producers of the L-asparaginase (Serquis and Oliveira., 2004). Although *Septoria tormentillae* showed pink zones in the agar assay, enzymatic activity was low based on further quantitative analysis. The reason for the absence of enzyme activity in the quantitative estimation may be attributed to the differences in the ability of the fungi to produce the enzyme in solid and liquid states (Hölker et al., 2004)

~~The study also revealed that all *Fusarium* species were able to produce L-asparaginase enzymes. These results are consistent with the results of Theantana et al.(2009). In the study done by these researchers, the genus *Fusarium* was considered to be one of the dominant producers of L-asparaginase.~~

We found that the frequency of endophytes in stem tissues was higher than other tissues, this is consistent with the results of some studies done by other investigators; however, most other studies reported that leaf tissues yield a higher diversity of endophytes (Verma et al., 2007). We also observed that the diversity and colonization frequency were higher after flowering, with 17% more endophytes than before flowering. The age of the host plant is one of the factors that may influence endophyte distribution and colonization frequency.

In future studies, bioactivity of the L-asparaginases from endophytes should be further tested against cancer cell lines to determine their potential application as anticancer agents.

Conclusions

The isolates that were able to produce L-asparaginase belonged to the genera *Plectosphaerella, Fusarium, Stemphylium, Septoria, Alternaria, Didymella, Phoma, Chaetosphaeronema, Sarocladium, Nemania, Epicoccum, Ulocladium and Cladosporium*. *Fusarium proliferatum* was found to have the highest enzyme activity with 0.492unit/mL^{-1}. Endophytic fungi from Asteraceae may provide a rich sources of L-asparaginase-producing fungi that may be superior to the currently used the bacterial-produced L-asparaginase. In the present study, the production of an enzyme l-asparaginase from endophytic fungi, due to its lower side effects and high levels of production, reduces the incidence of drug immunity, can be used as an alternative source for the L-para-gazanase enzyme present in the pharmaceutical market.

In the following, it is suggested that purification of the enzyme L-aspagazinase from these endophytic fungi should be performed and clinical trials should be carried out on this enzyme.

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Figure 1

A maximum parsimony phylogeny for *Fusarium proliferatum* from combined ITS. Bootstrap tests were performed with 1,000 replications. *Fusarium staphyleae* obtained from GenBank was treated as the outgroup.

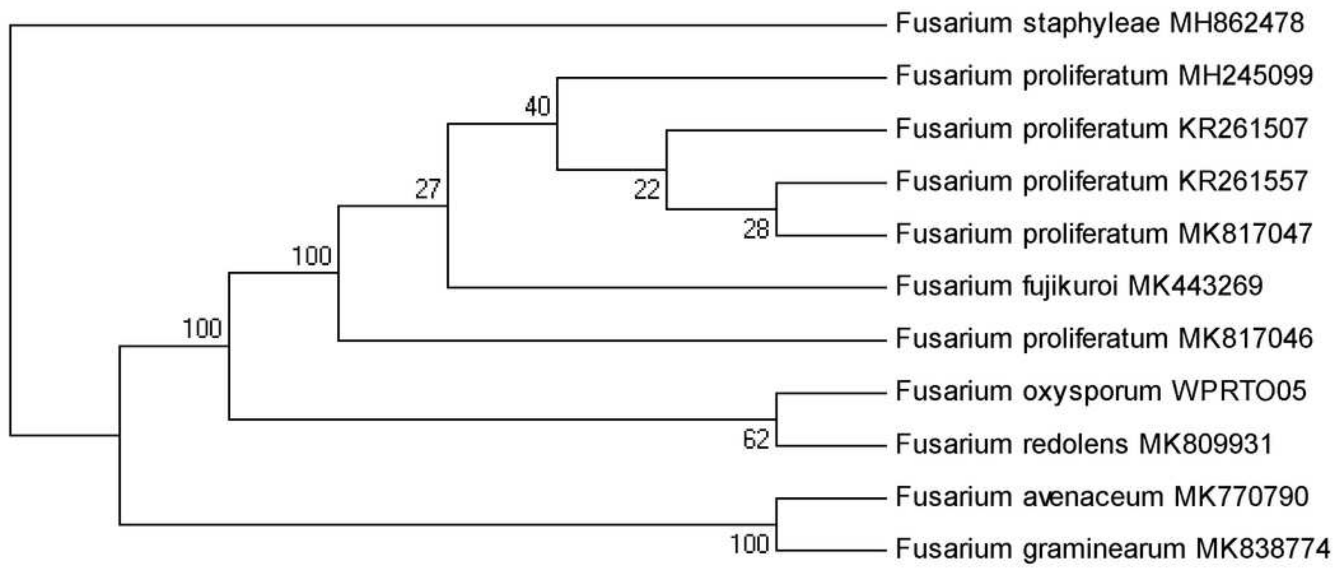

Figure 2

L-asparaginase activity detected by plate assay. Colour change in the medium (yellow to pink) around colony indicates production of enzyme, (A) Isolates have the highest production of L-asparaginase (B) non producer isolates

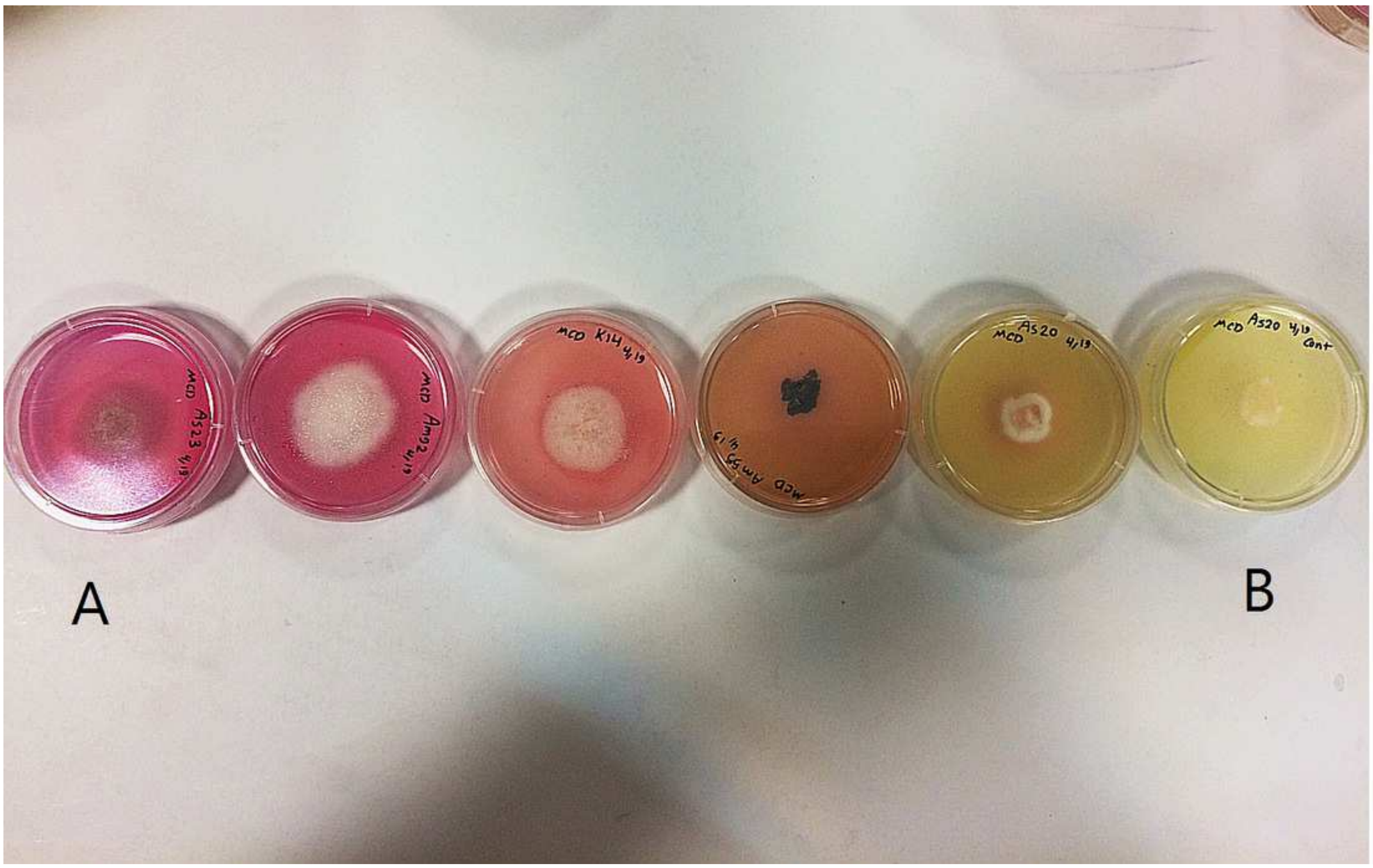

Table 1(on next page)

Primer combinations used for molecular identification.

Locus	Primer	Primer sequence5' to 3':	Orientation	Reference
TEF-1α	EF1-983F	GCCYGGHCAYCGTGAYTTYAT	Forward	Rehner & Buckley (2005)
	Efgr	GCAATGTGGGCRGTRTGRCARTC	Reverse	Rehner & Buckley (2005)
β-tubulin	T1	AACATGCGTGAGATTGTAAGT	Forward	O'Donnell & Cigelnik (1997)
	β-Sandy-R	GCRCGNGGVACRTACTTGTT	Reverse	Stukenbrock *et al*. (2012)
LSU	LROR	CC CGC TGA ACT TAA GC	Forward	Vilgalys & Hester (1990)
	LR5	TCCTGAGGGAA ACTTCG	Reverse	Vilgalys & Hester (1990)
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	Forward	White *et al*. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	Reverse	White *et al.* (1990)

Table 2(on next page)

Variance analysis of L-asparaginase producing endophytic fungi

.

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.331	30	.044	15.147**	.000
Error	.261	89	.003		
Total	5.622	120			
Corrected Total	1.592	119			

** Significant at less than 1% probability level.

Table 3 (on next page)

Colonization frequency of endophytic fungi from various plant parts

Endophytic Fungi	Host Plant	Stem	Leaf	Root	Flower	Total
Fusarium redolens	*Achillea millefolium*	5.5	-	-	-	11
Septoria saposhnikoviae	*Achillea millefolium*	1	-	-	-	2
Paraophiobolus arundinis	*Achillea millefolium*	4.5	-	-	-	9
Stemphylium amaranthi	*Achillea millefolium*	2.5	-	-	-	5
Cladosporium ramotenellum	*Achillea millefolium*	-	5	-	-	10
Septoria tormentillae	*Achillea millefolium*	1.5	-	-	-	3
Septoria lycopersici var. lycopersici	*Achillea millefolium*	1	-	-	-	2
Septoria sp.	*Achillea millefolium*	2.5	-	-	-	5
Fusarium oxysporum	*Achillea millefolium*	5	-	-	-	10
Septoria lycopersici var. malagutii	*Achillea millefolium*	1.5	-	-	-	3
Fusarium sp.	*Achillea millefolium*	-	5.5	-	-	11
Septoria tormentillae	*Achillea millefolium*	3.5	-	-	-	7
Alternaria infectoria	*Achillea millefolium*	5.5	-	-	-	11
Leptosphaerulina saccharicola	*Achillea millefolium*	2.5	-	-	-	5
Alternaria burnsii	*Achillea millefolium*	-	4.5	-	-	9
Alternaria sp.	*Achillea millefolium*	-	5.5	-	-	11
Nemania serpens	*Achillea millefolium*	3.5	-	-	-	7
Stemphylium vesicarium	*Achillea millefolium*	4	-	-	-	8
Fusarium avenaceum	*Achillea millefolium*	-	-	6	-	12
Fusarium sp.	*Achillea millefolium*	-	6	-	-	12
Paraphoma chrysanthemicola	*Achillea millefolium*	-	5	-	-	10
Fusarium oxysporum	*Achillea filipendulina*	8	-	-	-	16
Fusarium sp.	*Achillea filipendulina*	5.5	-	-	-	11
Preussia africana	*Achillea filipendulina*	1.5	-	-	-	3
Plectosphaerella cucumerina	*Achillea filipendulina*	6.5	-	-	-	13
Antennariella placitae	*Achillea filipendulina*	5	-	-	-	10
Fusarium acuminatum	*Achillea filipendulina*	-	6.5	-	-	13
Acremonium sclerotigenum	*Achillea filipendulina*	-	6	-	-	12
Colletotrichum tanaceti	*Achillea filipendulina*	4.5	-	-	-	9
Trametes versicolor	*Achillea filipendulina*	1.5	-	-	-	3
Alternaria burnsii	*Anthemis altissima*	4	-	-	-	8
Lewia infectoria	*Anthemis altissima*	-	-	8	-	16
Paraphoma chrysanthemicola	*Anthemis altissima*	6	-	-	-	12
Aspergillus calidoustus	*Anthemis altissima*	-	5.5	-	-	11
Bjerkandera adusta	*Anthemis altissima*	2.5	-	-	-	5
Schizophyllum commune	*Anthemis altissima*	3.5	-	-	-	7
Alternaria infectoria	*Anthemis altissima*	3	-	1.5		9
Paraphoma sp.	*Anthemis altissima*	4.5	1	-	-	11

Fusarium acuminatum	*Anthemis altissima*	8	-	-		16
Stemphylium botryosum	*Anthemis altissima*	-	6.5	-	-	13
Nemania serpens	*Anthemis altissima*	4	-	-	-	8
Fusarium proliferatum	*Anthemis altissima*	8.5	-	-	-	17
Plenodomus tracheiphilus	*Anthemis altissima*	4.5	-	-	-	9
Phoma tracheiphila	*Anthemis altissima*	6	1.5	-	-	15
Ulocladium consortiale	*Anthemis altissima*	-	6.5	-	-	13
Plectosphaerella cucumerina	*Anthemis altissima*	-	-	6	-	12
Cladosporium limoniforme	*Anthemis altissima*	6	-	6	-	12
Sarocladium strictum	*Anthemis altissima*	-	-	5.5	-	11
Verticillium dahliae	*Anthemis altissima*	4.5	-	-	-	9
Fusarium avenaceum	*Anthemis altissima*	-	-	-	5.5	11
Didymella tanaceti	*Anthemis altissima*	2	-	-	-	4
Chaetosphaeronema sp.	*Athemis triumfetii*	-	6	-	-	12
Chaetosphaeronema hispidulum	*Athemis triumfetii*	-	7	-	-	14
Paraphoma chrysanthemicola	*Athemis triumfetii*	6.5	-	-	-	13
Chaetosphaeronema achilleae	*Athemis triumfetii*	5	1	-	-	12
Chaetosphaeronema achilleae	*Athemis triumfetii*	-	4	-	-	8
Stemphylium amaranthi	*Athemis triumfetii*	-	7	-	-	14
Paraphoma sp.	*Athemis triumfetii*	7	-	-	-	14
Alternaria sp.	*Athemis triumfetii*	6	2	-	-	16
Alternaria sp.	*Athemis triumfetii*	7	2	-	-	18
Stemphylium vesicarium	*Matricaria parthenium*	-	4.5	-	-	9
Arthrinium phaeospermum	*Matricaria parthenium*	-	-	-	1	2
Epicoccum nigrum	*Matricaria parthenium*	4	-	-	-	8
Aspergillus chevalieri	*Matricaria parthenium*	-	4.5	-	-	9
Trichaptum biforme	*Matricaria parthenium*	1.5	-	-	-	3
Phoma haematocycla	*Matricaria chamomilla*	5	1	-	-	12
Paramyrothecium roridum	*Matricaria chamomilla*	-	-	6.5	-	13
Stemphylium amaranthi	*Matricaria chamomilla*	-	7	-	-	14
Xylariaceae sp.	*Matricaria chamomilla*	6	-	-	-	12
Epicoccum nigrum	*Matricaria chamomilla*	4	-	-	-	8
Cladosporium tenuissimum	*Cichorium intybus*	-	5.5	-	-	11
Epicoccum nigrum	*Cichorium intybus*	3.5	-	-	-	7
Septoria cerastii	*Cichorium intybus*	3.5	-	-	-	7
Plectosphaerella cucumerina	*Cichorium intybus*	-	-	5	-	10
Colletotrichum tanaceti	*Cichorium intybus*	7.5	-	-	-	15
Stephanonectria keithii	*Cichorium intybus*	-	2	-	-	4
Alternaria solani	*Cichorium intybus*	6	-	-	-	12
Bjerkandera adusta	*Cichorium intybus*	3.5	-	-	-	7
Torula herbarum	*Cichorium intybus*	-	3.5	-	-	7

Alternaria embellisia	*Cichorium intybus*	-	5.5	-	-	11
Stemphylium globuliferum	*Cichorium intybus*	4	-	-	-	8
Acremonium sclerotigenum	*Cichorium intybus*	-	-	-	5	10
Penicillium canescens	*Cichorium intybus*	-	2	-	-	4
Diaporthe novem	*Cichorium intybus*	9.5	-	-	-	19
Number of isolates		468	258	49	12	827

200 segments of each sample were plated for frequency analysis. (n=10) Not detected: —

Table 4(on next page)

Fungal endophytic strains from various medicinal plants and their L-asparaginase activity.

1

Isolate code	Fungus	Host plant	GenBank accession number	Enzyme in unit/mL	LSD test
Br08	*Fusarium proliferatum*	*Anthemis altissima*	MH245099	0.492	a
Br12	*Plenodomus tracheiphilus*	*Anthemis altissima*	MH245100	0.481	b
k100	*Torula herbarum*	*Cichorium intybus*	MH258980	0.442	c
Br18	*Fusarium avenaceum*	*Anthemis altissima*	MH245076	0.424	d
Am72	*Fusarium oxysporum*	*Achillea millefolium*	MH259174	0.332	e
Br15	*Cladosporium limoniforme*	*Anthemis altissima*	MH245072	0.309	f
Am13	*Fusarium redolens*	*Achillea millefolium*	MH259166	0.252	g
Am91	*Alternaria infectoria*	*Achillea millefolium*	MH259179	0.244	h
AS26	*Fusarium sp.*	*Achillea filipendulina*	MH250005	0.242	h
AM55	*Cladosporium ramotenellum*	*Achillea millefolium*	MH259170	0.232	i
BB05	*Chaetosphaeronema hispidulum*	*Anthemis triumfetii*	MH245081	0.224	ij
Am03	*Septoria sp.*	*Achillea millefolium*	MH259176	0.208	k
k11	*Alternaria embellisia*	*Cichorium intybus*	MH258981	0.203	l
BB28	*Alternaria sp.*	*Anthemis altissima*	MH245085	0.202	l
K24	*Plectosphaerella cucumerina*	*Cichorium intybus*	MH258974	0.192	m
Am87	*Fusarium sp.*	*Achillea millefolium*	MH259177	0.187	mn
BA18	*Epicoccum nigrum*	*Matricaria chamomilla*	MH245107	0.166	0
Br42	*Didymella tanaceti*	*Anthemis altissima*	MH245108	0.157	p
Br09	*Verticillium dahliae*	*Anthemis altissima*	MH245075	0.155	p
Am39	*Paraophiobolus arundinis*	*Achillea millefolium*	MH259168	0.146	q
Br41	*Ulocladium consortiale*	*Anthemis altissima*	MH245090	0.145	q
Am04	*Septoria lycopersici var. lycopersici*	*Achillea millefolium*	MH259172	0.144	q
Ba24	*Didymella tanaceti*	*Matricaria chamomilla*	MH245097	0.143	q
BB26	*Stemphylium amaranthi*	*Anthemis triumfetii*	MH245085	0.132	r
Br38	*Aspergillus calidoustus*	*Anthemis altissima*	MH245078	0.131	r
Am64	*Nemania serpens*	*Achillea millefolium*	MH259183	0.125	s
k29	*Alternaria solani*	*Cichorium intybus*	MH258977	0.123	s
BA06	*Phoma haematocycla*	*Matricaria chamomilla*	MH245096	0.112	t
As01	*Antennariella placitae*	*Achillea filipendulina*	MH250008	0.106	w
Am88	*Alternaria burnsii*	*Achillea millefolium*	MH259181	0.107	w
Am28	*Stemphylium amaranthi*	*Achillea millefolium*	MH259169	0.108	w
As16	*Acremonium sclerotigenum*	*Achillea filipendulina*	MH250010	0.106	w
Br92	*Lewia infectoria*	*Anthemis altissima*	MH245070	0.105	w
BR25	*Paraphoma sp.*	*Anthemis altissima*	MH245091	0.083	x
Br31	*Sarocladium strictum*	*Anthemis altissima*	MH245074	0.079	x
K15	*Cladosporium tenuissimum*	*Cichorium intybus*	MH258971	0.029	y
Br34	*Stemphylium botryosum*	*Anthemis altissima*	MH245094	0.027	y
Am51	*Septoria tormentillae*	*Achillea millefolium*	MH259171	0.019	z

LSD test; The results with different superscripts were different significantly ($p<0.01$) according to LSD test.