Isolation and identification of L-asparaginase-producing endophytic fungi isolated from some medicinal plants of Iran

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Abstract

In this study, endophytic fungi from seven healthy medicinal plants including *Matricaria chamomilla, Matricaria parthenium, Anthemis triumfetii, Anthemis altissima, Achillea millefolium, Achillea filipendulina, Cichorium intybus* were investigated for the production of L-asparaginase using standard isolation methods. A total of 827 isolates belonging to 86 species were were isolated from the stems (56.6%), leaves (31.1%), roots (10.47%) and flowers (1.45%) of the plants. Initial screening of L-asparaginase-producing endophytes was performed by qualitative plate assay on modified Czapex dox's agar medium. L-asparaginase activity of active isolates was quantified by the nesslerization method. Of the 86 isolates, 38 species were able to produce L-asparaginase with activity ranging from 0.019-0.492 unit/mL. L-asparaginase producing endophytes were identified as *Plectosphaerella, Fusarium, Stemphylium, Septoria, Alternaria, Didymella, Phoma, Chaetosphaeronema, Sarocladium, Nemania, Epicoccum, Ulocladium* and *Cladosporium* genera morphologically and analysis of their sequenced data including ribosomal DNA regions of ITS (Internal transcribed spacer) and LSU (Large subunit), *tef1* (Translation elongation factor) and β-tubulin (*tub*) loci. The most potent L-asparaginase was produced by *Fusarium proliferatum*. This study showed that endophytic fungi have a high L-asparaginase producing potential and may be used as an alternative source for production of this anticancer enzyme.

Key words: Asparaginase activity, Colonization frequency, Endophytic fungi, *Fusarium proliferatum*

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Introduction

Endophytes are microorganisms that reside inside plant tissues without causing symptoms and obvious negative effects to host plants. Endophytic fungi from medicinal plants have been considered to be a rich source of novel natural products for medical and commercial exploitation (Gutierrez et al., 2012). The close symbiotic relationship between endophytic fungi and host plants gives endophytes a potent 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, enhanced competitive abilities and enhanced growth (Zhang et al, 2006). In recent years, endophytic fungi have been shown to be sources of secondary metabolites, including anticancer, anti-inflammatory, antibiotic and antioxidant agents (Guo et al, 2008; Bungihan et al., 2011; Debbab et al, 2009; Gutierrez et al., 2012; Strobel & Daisy, 2004).

Enzymes produced by microorganisms are used for medical and industrial purposes. L-asparaginase is one such enzyme that hydrolyzes asparagine to aspartic acid and ammonia (Palti et al, 2012). L-asparaginase enzymes in the food industry are used as an admixture to reduce the acrylamide produced by high temperature in starchy foods and reduce the risk of cancer (Xu et al, 2016). 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 (McCredie & Ho, 1973). 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 derived from bacteria include chills, fever, abdominal cramps and fatal hyperthermia (Hosamani and Kaliwal, 2011). L-asparaginase derived from eukaryotes may induce less toxicity and reduced immune response (Asthana and Azmi, 2003). 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 in large quantities that are easy to extract and purify, may be an ideal source for L-aspariginase (Serquis et al., 2004). L-asparaginase from endophytic fungi isolated from medicinal plants has been reported in recent years (Theantana et al., 2007).

The appropriate selection of host plants is important to increase the chances of isolation of novel endophytes which may produce new bioactive metabolites (Ratklao, 2013). In this study, seven Iranian medicinal anti-cancer plants including: *Matricaria chamomilla, Matricaria parthenium, Athemis triumfetii, Anthemis altissima, Achillea millefolium, Achillea filipendulina* and *Cichorium intybus* were selected for the isolation of fungal endophytes to screen for L-asparaginase activity.

Materials & Methods

Isolation and identification of fungal endophytes

From May until September 2016, plant specimens (stems, roots, leaves, and flowers) were obtained from seven healthy plants: *Matricaria chamomilla, Matricaria parthenium, Athemis triumfetii,, Anthemis altissima, Achillea millefolium, Achillea filipendulina* and *Cichorium intybus* growing in natural communities in Northeastern of Iran (Table 2). The samples were stored in polyethylene bags at 4 ºC (Waksman et al., 1916). Samples were washed thoroughly in distilled water. Surface sterilization was performed by sequential immersion of samples in sodium hypochlorite (1.5% - 2/5% NaOCl) for 3–5 min followed by 75% ethanol for 1 min and

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rinsed five times in sterile distilled water. The samples dried on sterile blotters under the laminar air flow. The surface-sterilized samples were cut into about 0.5×1 cm^2 using sterile scalpel. 200 segments from stem, leaf, root and flower (Four segments per plate) for each plant species were placed equidistantly on Potato Dextrose Agar medium (PDA, Merck, Darmstadt, Germany) supplemented with tetracycline (50 mg/L) to inhibit the bacterial growth. The plates were incubated at 28 ± 2ºC with 12 h light and dark cycles for up to 6 to 8 weeks. Controls were prepared by pouring aliquots of the water from final rinse solutions on water agar medium plates and incubated for 2 weeks. The absence of fungal growth indicated effective sterilization while observing mycelial growth from samples was identified as endophytes. Colonies that emerged from tissue segments were picked up and transferred to antibiotic-free potato dextrose agar medium (PDA) to enable identification. Colonization Frequency (CF) of endophytes was calculated as the number of isolates from each segment divided by the total number of segments plated × 100 (Khan et al., 2010).

Identification was done by morphological and molecular methods. Morphological identification of the isolates was performed based on the fungal colony morphology, characteristics of the spores and reproductive structures using standard identification manuals (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 plugs were 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 diameters of the pink zones were measured (Gulati, 1997).

Measurement of the L-asparaginase activity

The L-asparaginase 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 the absorbance of samples at 450 nm using UV-Visible spectrophotometer (Jenway model 6315). 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

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Fungal isolates were grown in 200 mL of PDB (potato dextrose broth) for 7 days at 28ºC. The mycelia washed with distilled water and ground with liquid nitrogen. Nucleic acid was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Dayle, 2001). Strains were sequenced for four loci: ITS (Internal transcribed spacer), 28S rDNA (Large subunit), *tef1* (Translation elongation factor) and β-tubulin (*tub*); 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 (Table 4).

Results

Identification of Fungal Endophytes

Endophytes were obtained from all seven plant species with a total of 827 isolates from 200 each of leaf, stem and root segments. Endophytes were mostly recovered from *A .altissima* (229 isolates), followed by *A. millefolium* (163 isolates), *A. triumfetii* (121 isolates), C. intybus (121 isolates), *A. filipendulina* (90 isolates) *M. chamomilla* (60 isolates) and *M. parthenium* (30 isolates) (Table 3). Eighty five endophytic fungal species belonging to Ascomycota and Basidiomycota were identified using morphological and molecular methods. Few endophytic fungi had wide distributions in the host plants and were isolated from most plants such as *Acremonium sclerotigenum, Alternaria burnsii*, *Bjerkandera adusta, Colletotrichum tanaceti, Epicoccum nigrum*, *Fusarium acuminatum*, *Paraphoma chrysanthemicola*, *Plectosphaerella cucumerina* and *Stemphylium amaranthi.* Also a higher number of endophytes were recovered from stem tissues of all seven plant species (Fig. 3). The percent colonization frequency of endophytes varied in the plant parts with stem fragments harboring 56.6% of endophytic isolates followed by leaves with 31.1% and least for the isolates of flower samples (Table 3). Many isolates belonged to the genera *Alternaria, Fusarium*, *Phoma, Chaetosphaeronema* and *Plectosphaerella* which colonized more than one plant part. The isolates of *Fusarium* were recovered from stem, leaf, flower and root segments while *Phoma* spp. were obtained from stem and leaf samples. Tissue specificity was also observed for some endophytes. This was most evident in the *Septoria* species that were found only in stem tissues. basidiomycetous endophytes such as *Trametes versicolor*, *Bjerkandera adusta, Trichaptum biforme* and *Schizophyllum commune* were isolated from stem tissues. *Fusarium* spp. were found as the dominant endophyte with 140 isolates followed by *Alternaria* spp. (105 isolates). The results indicated that the species composition and frequency of endophyte species were found to be dependent on the tissue and host plant species.

Screening of L-asparaginase-producing endophytes by qualitative plate assay

All fungal isolates were screened for their ability to produce L-asparaginase by qualitative rapid plate assay. Of the eighty-five fungal endophyte species tested for L-asparaginse activity (Table 3), thirty-eight isolates were positive for extracellular L-asparaginase, and formation of pink

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zones were evident on Modified Czapex Dox (MCD) medium. The pink zone diameters varied from 17.9 to 58.2 (Table. 3). *Fusarium proliferatum* showed maximum enzyme activity followed by *Plenodomus tracheiphilus*. All six *Fusarium* species in this study had the ability to produce L-asparaginase, where the pink zone was measuread above 1.87mm. Forty-eight isolates did not have the ability for L-asparaginase production under conditions of our experiments. None of the endophytes isolated from *M. parthenium* were able to produce L-asparaginase. Also, the basidiomycetous endophytes did not show L-asparaginase activity. Thirty eight fungal strains exhibiting positive enzyme activities were selected for quantitative assay of L-asparaginase.

Estimation of L-asparaginase production by Nesslerization

L-asparaginase activities of thirty-eight fungal endophytes were measured to range of 0.019–0.492 unit/mL^{-1} (Table 4). The isolates of *F. proliferatum*, obtained from *A. altissima*, exhibited a maximum enzyme activity with 0.492 unit/mL^{-1} followed by *P. tracheiphilus*, isolated from *A. altissima* (0.481 unit/mL^{-1}). *F. oxysporum* and *Cladosporium limoniforme* exhibited moderate enzyme activity while *Septoria tormentillae* showed least activity with 0.019 unit/mL^{-1} of enzyme. Results showed that there were statistically significant differences among the isolates at 1% level of significance (Table 3). The percentage of L-asparaginase-producing fungal endophytes was 44.7% of the total isolated endophytes (85 isolates) with 2.5%, 3.5%, 15%, 14.2%, 3.5% and 6% respectively for *M. chamomilla, A. triumfetii, A. altissima, A. millefolium, A. filipendulina* and *C. intybus*. The fungal endophytes that were obtained from *M. parthenium* did not show the capacity to produce L-asparaginase.

Discussion

Eighty-five fungal endophytes belonging to Ascomycota (95%) and Basidiomycota (5%) were obtained from seven medicinal plants in Iran. All species obtained in the present study are reported for the first time here as endophytes of *M .chamomilla, M. parthenium, A .triumfetii, A .altissima, A millefolium, A filipendulina* and *C .intybus*. In recent years, most records of fungal endophytes have been ascomycetes (Carroll, 1988; Rodrigues, 1994; Gonthier et al., 2006; Arnold, 2007), with a few species of basidiomycetes (Petrini, 1986; Chapela and Boddy, 1988; Oses et al., 2006; Sánchez Márquez et al., 2007). Endophytic basidiomycetes including *T. versicolor*, *B. adusta, T. biforme* and *S. commune* are white- rot fungi. Researchers have previously shown that most basidiomycetous endophytes are white-rot species (Oses et al., 2006; Thomas et al., 2008).

The frequency of colonization by endophytic fungi was higher in stem tissues than in other plant parts. Similar results were found by Bezerra et al (2015) where 51% of the fungi were isolated from stems. These authors suggested that the highest frequency of colonization in stems may be due to spore abundance of a few dominant endophytes in stem tissues. Similarly, Verma et al (2013) demonstrated that the diversity of endophytic fungi was highest in stems. The diversity and frequency of colonization of fungal endophytes are influenced by the host tissue (Rodrigues 1994) and environmental factors (Clay 1986). However, most studies reported that leaf tissues yield a higher diversity of endophytes (Verma et al., 2007; Gond et al., 2012).
In our study, most of the species isolated were selective to single host species, for example *T. versicolor*. In contrast, a few fungal species, such as *A. sclerotigenum*, were common in several host plants. Endophytic assemblages have tended to be distributed in specific hosts and specific tissues (Siqueira et al., 2011; Xing et al., 2010). Some dominant endophytes have been isolated

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from all parts of plants (e.g., *F. avenaceum*), perhaps due to the ability of endophytes to move from one plant part to another (Manasa et al., 2014).

L-asparaginase is an enzyme that is used as an effective antineoplastic agent for treatment of acute leukemia (Nakamura et al., 1999). It is produced by plants and a variety of microbial sources including fungi (Serquis et al., 2004). Endophytes from medicinal plants are producers of novel compounds (Hwang et al., 2011). In this study, L-asparaginase activities of fungal endophytes from several medicinal plant species were evaluated. Eighty-six fungal endophytes were examined for L-asparaginase activity. Thirty-eight of the endophytes demonstrated ability to metabolize L-asparagine. The fungi that were good producers of L-asparaginase enzyme belonged in the genus *Fusarium*, followed by species of *Alternaria* and *Cladosporium*. Furthermore, these species have been reported previously to produce L-asparaginase (Serquiset al., 2004). Species of the genus *Fusarium* have been reported as a good L-asparaginase producers by Theantana et al. (2009). L-asparaginase activity was not observed in endophytes of *M. parthenium*. This may be due to low diversity of endophytes that were obtained from this medicinal plant. Although, *S. 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 differences in the ability of the fungi to produce the enzyme in solid and liquid growth states (Hölker et al., 2004).

According to available literature, this is the first record of L-asparaginase production by endophytic fungi of the host plants we examined.

Conclusions

We isolated a good number of fungal endophyte from seven healthy medicinal plants of Iran 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. The production of an enzyme L-asparaginase by endophytic fungi may provide an alternative source for production of L-asparaginase enzyme for the pharmaceutical market.

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