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Isolation, identification and biodiversity of endophytic fungi of *Thymus*

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ABSTRACT

Endophytes are considered as an important source of bioactive natural products because these microorganisms are able to occupy unique biological niches and grow in non-ordinary environments. Therefore, endophytic fungi of medicinal plants can be developed for medicinal applications. Besides, medicinal properties of plant can be ascribed to its endophytic fungi. *Thymus* sp. is widely used as a medicinal plant and in this research, the endophytic microflora of this plant was studied. In order to isolate endophytic fungi, 800 segments of 6 *Thymus* species were collected from its natural habitats in Hamedan province (Iran) and medicinal plants garden during spring, summer and fall 2011. Finally, 95 isolates consisting of 11 different fungal genera, 6 yeast isolates and 19 sterile mycelia were obtained. Among the identified isolates, the most abundant genera were *Alternaria*, *Phoma*, and *Fusarium*. The above mentioned genera and the genera of *Cladosporium* and *Colletotrichum* were first reported from *Thymus* in Iran. The genera *Curvularia, Cylindrocarpon*, *Drecheslra, Aspergillus, Stemphylium* and *Ulocladium* were also, first reported from *Thymus* in the world.

Keywords: endophyte, medicinal plant, microflora, *Thymus*.

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Introduction

Endophytes consist a set of microorganisms which live in the tissues of higher plants through intra or intercellular growth without any symptom and they are rich sources of bioactive compounds (1, 2, 3). It seems that plants provide nutritional materials for endophytes while endophytes may help the host to resist pests and diseases or do other useful tasks. Among other benefits of endophytes to its hosts, tolerance to heavy metals, enhancement of resistance against herbivores, protection against nematodes, systematic resistance against disease factors, and growth increase can be mentioned (4, 2).

Medicinal plants have been known act as a harbour for endophytic fungi because they are able to produce bioactive compounds similar to those of their host (5, 6). The ubiquity of these symbiotic microorganisms is clear, but variety, host range and geographical distribution have been unknown (7). So far, only a few numbers of plants have been studied for their endophyte biodiversity and the potential to produce secondary bioactive metabolites (8).

Thymus sp. belongs to Lamiaceae family with a long history of medicinal application widely used in conventional and modern medicine. It is also used in pharmaceutical, food and cosmetic industries (9, 10, 11). Eighteen species of *Thymus* have been reported from all over Iran (12) and the greatest distribution is believed to be in the north and west of the country (11). The pharmaceutical properties of this plant can be attributed to its endophytes and there appears to be different endophytes with bioeffects. This research was to isolate and identify the endophytic fungi of *Thymus* sp. in Hamedan, Iran.

Materials and Methods

Sampling

During spring, summer and fall, 2011, the plant samples of *Thymus* sp. were collected from their natural habitats in the west of the country especially in Hamedan and its medicinal plants garden. Sampling was randomly taken from different healthy *Thymus* sp. In all cases, the samples collected were separately placed in paper bags. The samples were then transferred to the laboratory and *Thymus* species were identified.

Isolation of endophytes

To isolate fungal endophytes, plant samples were rinsed gently with tap water (to remove dust). The samples were cut into 0.5 to 1cm segments and surface disinfection was carried out based on the procedure of Fisher *et al*. (13). The samples were disinfected with ethanol (75%) for one minute, hypochlorite sodium (1-1.5%) (depending on tissue) for 2 min and they were re-immerse in ethanol (75%) for 30 s. The samples were washed with distilled water and the plant segments were dried with sterile filter paper. Plant segments were then placed (4-5 segments in each Petri dish) on plate containing potato dextrose agar (PDA) and kept in incubator at 27±2ºC for 4-6 weeks in the dark. Purification of fungi was done using hyphal tip method.

Identification of fungi

The fungi were identified based on their morphological and cultural characteristics. They were grown on PDA and examined for colony morphology with the naked eye and characteristics of asexual spores under compound microscope (Leica DMLB and LETIZ).

Fungal identification was done through available keys (14, 15, 16, 17, 18, 19, 20, 21) and based on apparent characteristics such as colony color, existence or lack of aerial mycelia,

color and density, presence or lack of acervulus, pycnidium and other asexual reproductive organs in cultural media, and microscopic properties consisting of shape and size of conidia, conidiophores, pycnidia, and formation of hair in pycnidium. The fungi which were unable to produce sexual and asexual organs were classified as sterile mycelium. The colonization frequency of each plant tissue by endophytic fungi was calculated by suryanarayanan's formula (22) as follows:

Colonization frequency (CF%) = number of segments colonized by a single fungi/ total number of segments observed ×100

Results

This was the first research regarding the isolation and identification of endophytes of *Thymus* species in Hamedan (Iran) to provide fungal endophytes for further researches.

To investigate the endophytic microflora of *Thymus*, plant materials were collected from the west of Iran especially Hamedan province. Six species of this genus including *T. eriocallyx*, *T. lancifolius*, *T. fallax*, *T. kotschyanus*, *T. vulgaris* and *T. daenensis* were identified. The isolation of endophytic fungi was performed from different parts of the plant. A total of 800 segments, including 300 segments of leaves, 300 segments of stems, 150 segments of roots and 50 segments of flowers, from 6 different species of *Thymus* 95 isolates of 11 different fungal genera (in 25 species), 6 yeast isolates and 19 isolates in form of sterile mycelia were obtained (Table 1). Thirty four fungi were isolated from leaves, 41 from stems, 14 from roots and 6 from flowers. All isolated endophytic fungi belonged to imperfect fungi.

Table 1. Colonization frequency of endophytic fungi in different parts of *Thymus* spp

Fungi	exactly on the top of numbers	% Frequency of Colonization			
		Leaf	Stem	Root	Flower
Alternaria spp.	26	57.6	34.6	-	7.6
Phoma spp.	16	31.25	37.5	12.5	18.75
Fusarium spp.	13	15.38	46.1	38.4	-
Cladosporium spp.	4	75	25	-	-
Stemphylium spp.	1	100	-	-	-
Ulocladium spp.	1	-	100	-	-
Colletotrichum spp.	2	50	-	50	-
Cylindrocarpon spp.	1	-	-	100	-
Drecheslra spp.	2	-	100	-	-
Curvularia spp.	3	-	100	-	-
Aspergillus spp.	1	100	-	-	-
Yeast	6	16.66	83.33	-	-
infertile mycelium	19	21.05	47.36	26.31	5.26

Among the identified isolates, the most frequent strains belonged to three genera of *Alternaria*, *Phoma* and *Fusarium* (Table 1). The most frequent species was *A. alternata* which was isolated from 4 species (*T. lancifolius, T. eriocallyx, T. fallax, T. kotschyanus*). Although *A. alternata* was isolated from *T. richardii*, *T. capitatus,* and *T. herba-barona* by Fisher *et al*. (13), this is the first time that *A. alternata* is isolated from *T. lancifolius, T. eriocallyx, T. fallax* and *T. kotschyanus*. Other species of *Alternaria* are first reported from *Thymus* in the world (Table 2). Species of *Phoma* and *Fasarium* are first reported from *Thymus* species which is mentioned in Table 2. In this research, the genera of *Curvularia, Cylindrocarpon, Drechslera, Aspergillus, Stemphylium* and *Ulocladium* are first reported from *Thymus* and are new for fungal flora of this plant in the world.

Table 2. Biodiversity of endophytic fungi in *Thymus* species

Taxon	L l	L s	E s	E l	E f	E r	K f	K l	K s	K r	F l	F r	F s	D l	D r	V s
Alternaria alternata	4	-	2	6	-	-	1	-	2	-	1	-	-	-	-	-
Alternaria tangelonis	1	-	1	1	-	-	-	1	1	-	-	-	-	-	-	-
Alternaria franseriae	1	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-
Alternaria sp.1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alternaria sp.2	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Phoma glomerata	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Phoma valerianae	-	-	-	-	-	1	-	1	1	-	-	-	-	-	-	-
Phoma pimpinellae	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-
Phoma pereupyrena	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Phoma selaginellicola	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Phoma haematocycla	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-
Phoma tropica	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
Phoma capitulum	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Phoma chrysanthemicola	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Phoma sp.	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium equiseti	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium acuminatum	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium oxysporum	-	-	-	-	-	-	-	-	-	2	-	-	-	1	-	-
Fusarium reticulatum	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-

Table 2. Biodiversity of endophytic fungi in *Thymus* species

Taxon	L l	L s	E s	E l	E f	E r	K f	K l	K s	K r	F l	F r	F s	D l	D r	V s
Fusarium javanicum	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Fusarium lateritium	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium subglutinans	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium sp.1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium sp.2	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Cylindrocarpon sp.	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Cladosporium cladosporioides	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Cladosporium variabile	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-
Curvularia akaii	-	-	2	-	-	-	-	-	1	-	-	-	-	-	-	-
Drechslera tetrarrhenae	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Drechslera dematioides	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Ulocladium atrum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Stemphylium sp.	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colletotrichum sp.	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-
Aspergillus sp.	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Yeast	-	1	3	1	-	-	-	-	-	-	-	-	1	-	-	-
infertile mycelium	1	-	6	3	1	1	-	-	2	3	-	-	-	-	1	1

E: *T. eriocallyx*, L: *T. lancifolius*, F: *T. fallax*, K: *T. kotschyanus*, V: T. vulgaris, D: *T. daenensis*.; l: leaf, s: stem, r: root, f: flower.

In this study, evidence of specificity of tissue in some isolated fungal genera has been found upon which the genus *Stemphylium* and *Aspergillus* are just isolated from leaf and genera *Ulocladium, Drechslera* and *Curvularia* from stem and *Cylindrocarpon* from root, which is confirmed by Petrini *et al*. (23) who regarded the specificity of endophytic fungi from compatibility with physiological and microecological conditions in special organs.

Carroll and Carroll (24) observed some specificity in endophytic species within needles (petiole or blade) of conifers in northwest of the Pacific and they stated that endophytes mostly colonize the petiole. It has also been observed that endophytic fungi isolated from *Alnus glutinosa* show organ specificity so that aquatic and terrestrial roots of this plant are colonized by two different populations (25). The degree of tissue specification in endophytes has been confirmed by a number of studies (26, 27).

The greatest number of endophytic fungi

(52 isolates) was obtained from *T. eriocallyx* species, therefore biodiversity within this species seems to be higher than other species. In this study, 11 isolates were obtained from *T. lancifolius*, 21 isolates from *T. kotschyanus*, 4 isolates from *T. fallax* and 5 isolates from *T. daenensis* and 2 isolates from *T. vulgaris* (Table 2).

Most endophytic fungi (41 isolates) were obtained from the stems of *Thymus*, majority of them (27 isolates) were isolated from the stems of *T. eriocallyx* (Table 2).

Discussion

Endophytes are considered to be an important source of bioactive natural products because they can occupy unique biological niches and grow in non-ordinary environments (28, 29). Therefore, endophytic fungi of medicinal plants can be developed for medicinal applications. Besides, medicinal properties of plant can be ascribed to its endophytic fungi (5). Debbab *et al*. (30, 31) examined the endophytes of *Mentha pulegium* and *Salvia officinalis* from Lamiaceae family and isolated endophytic fungi *Stemphylium globuliferum* and *Chaetomium* sp. for the first time, respectively. The genus *Stemphylium* was also isolated in the present research.

Banerjee *et al*. (32) examined the endophytes of three medicinal plants (*Ocimum sanctum, Ocimum bacilicum* and *Leucas aspera*) from Lamiaceae family and reported yeast isolates as the endophytes of these plants.

Among the Lamiaceae family, *Thymus* sp. is a well-known medicinal plant and its different parts have antispasm and antimicrobial properties. Fisher *et al*. (13) reported 24 fungal genera from species of *T. richardii*, *T. capitatus*, *T. polytrichum, T. herba-barona* and *T. serpyllum.*

The living environment is believed to play an important role in biodiversity of endophytes and variety of species. For example, the endophytic fungi of ligneous plants are commonly found in tropical regions (33). The endophytic flora is different in terms of number and type in each individual host and depends on geographical situation of the host (34, 4). Therefore, *Thymus* sp. with species of *T. eriocallyx, T. lancifolius, T. fallax, T. kotschyanus, T. vulgaris* and *T. daenensis* was subjected for the first time to identify their endophytic microflora.

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