

Diversity of endophytic fungi isolated from cherry (*Prunus avium*)

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Abstract Endophytic fungi are currently considered as symbionts which can colonize a wide range of hosts and do not induce any pathological signs. The main aim of this study was to identify the endophytic fungi colonizing different organs (root, twig and leaf) of cherry (*Prunus avium*). Samples were collected from eight-year-old trees of the Hungarian cultivar 'Péter' grafted on 11 different rootstocks. After surface sterilization samples were put on potato dextrose agar and outgrowing fungal colonies were visually classified after 1 and 2 weeks. Then monosporation and monohyphation technique was applied to generate single colonies of each individual fungus, and the isolated colonies were classified morphologically. Molecular identification at genus or species level was performed by applying the polymerase chain reaction (PCR) to amplify a nuclear ribosomal internal transcribed spacer region (ITS) and by sequence comparison of the PCR products. A total of 150 endophyte strains were isolated from 4500 tissue segments. About 25 species were identified as belonging to the genera *Acremonium*, *Alternaria*, *Botryotinia*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Embellisia*, *Epicoccum*, *Fusarium*, *Glomerella*, *Macrophomina*, *Neonectria*, *Phoma*, *Diaporthe/Phomopsis*, *Pyronema*, *Rhizoctonia*, *Rhizopycnis*, *Rosellinia* and *Xylaria*. Endomycota showed the highest diversity in roots, while in twigs and leaves we predominantly found *Alternaria*. Further analyses are being carried out to verify which endophytes can be used to improve the physiological functions and tolerance threshold of the grafted trees against environmental stresses.

Key words

Diversity, endophytic fungi, symbiosis, *Prunus avium*

Fungal endophytes colonize healthy plant tissues without causing visible symptoms in the host plant [5]. Thus, the plant - endophyte interaction can be considered as a sort of symbiosis. Endophytes may receive protection and nutrients within host tissues. The plant may benefit from endophyte infection through improved drought and flooding tolerance, increased biomass production and improved herbivore and pathogen resistance [7]. It is also believed that endophytes can live in plant tissues either as mutualists or antagonists, depending on the physiological state of the host. Moreover, both pathogenic and nonpathogenic fungi are routinely isolated from asymptomatic tissues, suggesting that pathogens may either have a nonpathogenic lifestyle or remain dormant after infection until plant senescence [11]. In spite of recent attempts, there is a lack of knowledge about the biological aspects in host plant - endophyte interaction and there are no specific estimates about the number of existing endophytes [10]. However, the

endophytes have cryptic lifestyle, ubiquity and richness within individual plants coupled with emerging evidence of their often-overlooked ecological importance, have inspired growing enthusiasm regarding these little known fungi over the past four decades. In particular, David Hawksworth's brilliant studies [8][9] on estimation of fungal diversities at a global scale, directed much research to understand endophytes' diversity. Since 1991, a growing number of surveys have been fulfilled to define the contribution of these cryptic microfungi to fungal systematics, plant and fungal ecology and evolutionary biology. Furthermore, it has been revealed that fungal endophytes may play a crucial role in pathophysiology of other coinfections and may improve resistance against environmental stresses in host plants. Sweet cherry (*Prunus avium*) originated in the region between Caspian and Black seas of Asia Minor, and is cultivated in all temperate areas of Northern Hemisphere, more largely in Europe and western Asia.

Sweet cherry is native in Hungary although it was not amidst the economically beneficial horticultural products before 1950. The sweet and sour cherry breeding program began in 1950 and right after many attempts were put to improve the products' commercial efficiency. Nowadays, Hungary is considered amidst the major exporters of sweet cherry in Europe. Accordingly, scientific studies, of any sorts which may produce results with implication to industry, are required to be undertaken effectively.

Concerning what has been mentioned above; the present study was designed to indicate the diversity of fungal endophytes in different organs of sweet cherry (*Prunus avium*) cultivar 'Péter' which was bred in Hungary [2]. The outcomes achieved by the study would be applied in further investigations for describing the contribution of predominant species of fungal endophytes in modulation of host physiological events occur during the environmental stresses.

Materials and Methods

Samples were taken three different times within the period of one year in the University's orchard in Soroksár, located in Budapest, Hungary. Leaf, twig and root samples were collected randomly from 110 of the 180 cherry trees of the cultivar 'Péter'. The scion was grafted on 11 different rootstocks, among them 8 cultivars of *Prunus mahaleb* ('Érdi V', 'Bogdány', 'SL 64', 'Egervár', 'Korponay', 'SM 11/4', 'CEMANY' and 'Magyar'), a *P. avium* ('Vadcserezsnye'), a *P. cerasus* x *P. canescens* hybrid ('Gisela6') and a *P. fruticosa* ('Prob'). After sterilisation in 70% ethanol and 1.5% hypochlorite, samples were put on PDA medium and incubated at 22°C. Depending on their growth rate, one or two weeks later colonies were counted and classified. To generate single colonies of each individual fungus the monosporation and monohyphation technique was applied. After morphological classification of the single colonies, their DNA was isolated and the ITS region was amplified by PCR (primers: ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3'; [13]). Molecular taxonomy and genotyping methods were used to identify fungal genera and species on the basis of the sequence of the amplified ITS.

Results

Fungal endophytes isolated from different tissues of cherry grafts were identified to the level of genera, occasionally to species. Different isolates derived from root, twig and leaf were classified separately, although some colonies could not be recognized by the morphology and gene sequencing. To estimate the diversity of fungal endophyte in different biologically active tissues, the number of unidentified colonies was

also considered. Table 1 shows the overall number of identified colonies which were isolated from three different tissues. As a total, 6235 colonies were isolated from roots, twigs and leaves of cherry grafts of 'Péter' cultivar and a number of 5773 fungi were identified by applying morphology and molecular taxonomy methods. The highest number of isolates were found in twigs (3053 isolates), while the lowest number (1078) was isolated from leaves. Besides, unidentified colonies were excluded from analysis (235 colonies in root, 86 colonies in twig and 141 colonies in leaf).

As for diversity of endophytic fungi, twelve different genera including 16 species of fungi were found in root. Seven genera with 16 species and nine genera with 13 species were isolated from leaf and twig tissue, respectively. The order Hypocreales was represented by the largest diversity of fungal endophytes, most frequently detected in root samples. *Fusarium* appeared as significantly dominant genus in all three tissues; however *F. solani* was the only member of the genus which was isolated from twigs.

Except *Fusarium*, the other three genera belonging to Hypocreales, i.e. *Neonectria radiculicola*, *Acremonium* sp. and *Hypocreales* sp. were found only in isolates obtained from root samples, indicating that the distribution of fungi varies in different tissues. Some species such as *Alternaria alternata*, *Fusarium* sp.3 and *Epicoccum nigrum* were, however, found in roots, in twigs as well as in leaves. Figures 1-3 indicate the frequency of isolated species in different tissues.

Conclusions

Understanding the diversity of endophytes has an essential implication for scientific approaches to fungal endophyte-host interactions. It can also provide reliable background for the studies on biological control programmes and represents new trends in proteomic and genomic features that underlie pathogenicity or evolutionary origin and stability of the species [6]. The plant-endophyte association has a dynamic nature in which many factors affect the structure and species composition of the microbial communities that colonize root, twig and leaf. It has been suggested that the diversity of endophytic fungi may also depend on the environmental effects and/or on the interaction with other endophytic or pathogenic microorganisms [1].

Endophytic fungi are ubiquitous and probably increase the fitness of the host plant by improving tolerance to heavy metals and drought, reducing herbivory or phytopathogen settling and promoting plant growth [3][7]. These characteristics have generated a growing interest about the biology of endophytes and their contribution to ecology, plant physiology and plant pathology. Recently many studies have focused on the diversity of endophytic fungi in different geographical regions, even though it is very difficult to determine a

certain pattern of endophytic symbionts which colonize the plants in a particular ecosystem.

Based on the results of previous studies, the genus *Fusarium* (Hypocreales) has been considered as a ubiquitous endophyte which has been isolated from many plants in most parts of the world [5] although the majority of the studies were carried out on tropical and subtropical plants. The present study showed the dominance of *Fusarium* in horticultural species on higher latitudes. According to the results, *Fusarium* was found the most diverse genus in the root and the twig but not in the leaf. Only one of the five identified species of *Fusarium* was isolated from the leaf samples which may show that *Fusarium* species preferentially colonize the roots and the twigs. In fact, *Fusarium* is the dominant genus in the plant which suggest, that this endophyte probably has a role in plant response to different biological and physical factors. This concept is supported by other studies which have demonstrated the role of nonpathogenic *Fusarium oxysporum* to induce resistance of the host plants against various pathogens in cucumber [4].

Molecular interactions between the host plants and their vertically inherited symbionts such as endophytes depend on a complex web of relations within the tissues. The main questions are how and when they manifest and what the advantages of these interactions are. Studying these, yet poorly understood aspects may well prove to be a successful approach to control plant diseases.

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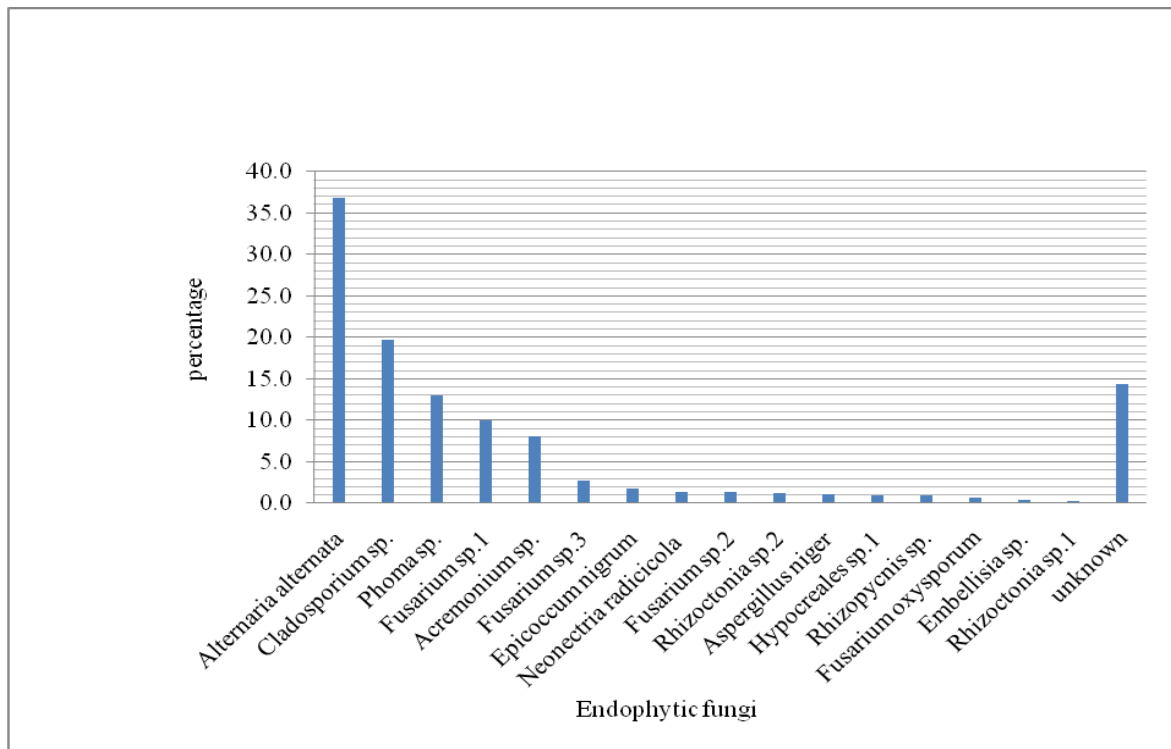


Fig.1 Frequency of Endophytic Fungi Outgrowth from Root Samples of Cherry Trees

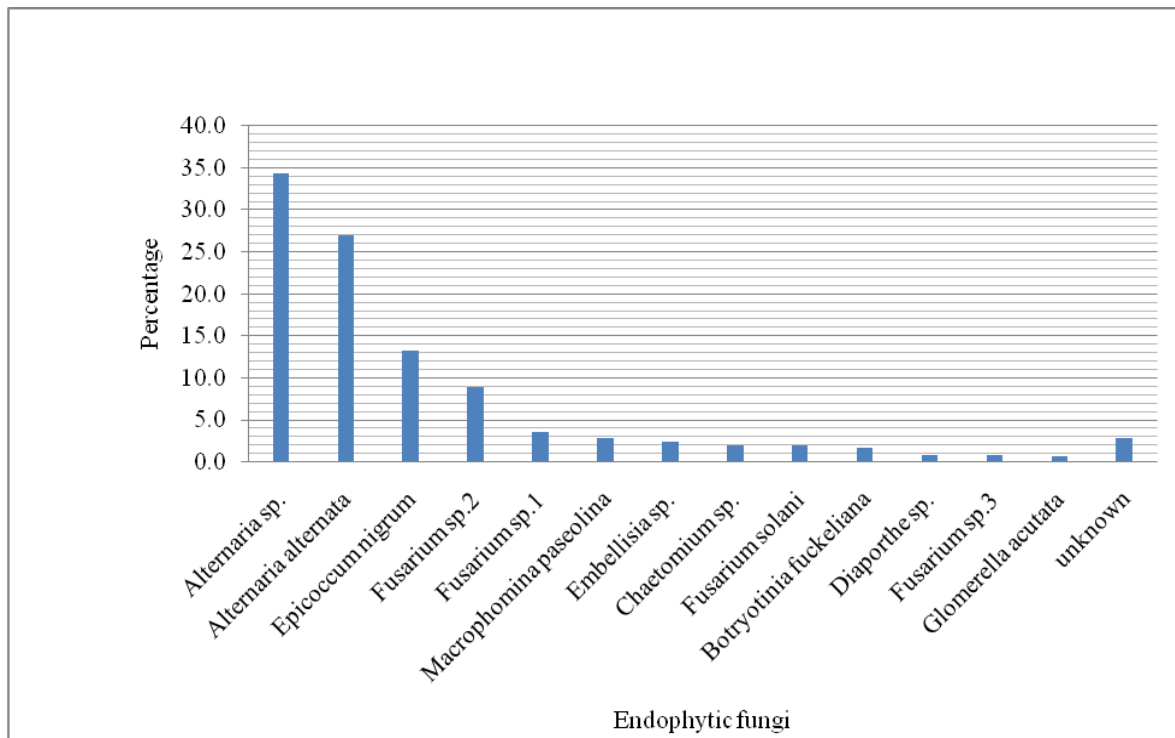


Fig. 2 Frequency of Endophytic Fungi Outgrowth from Twig Samples of Cherry Trees

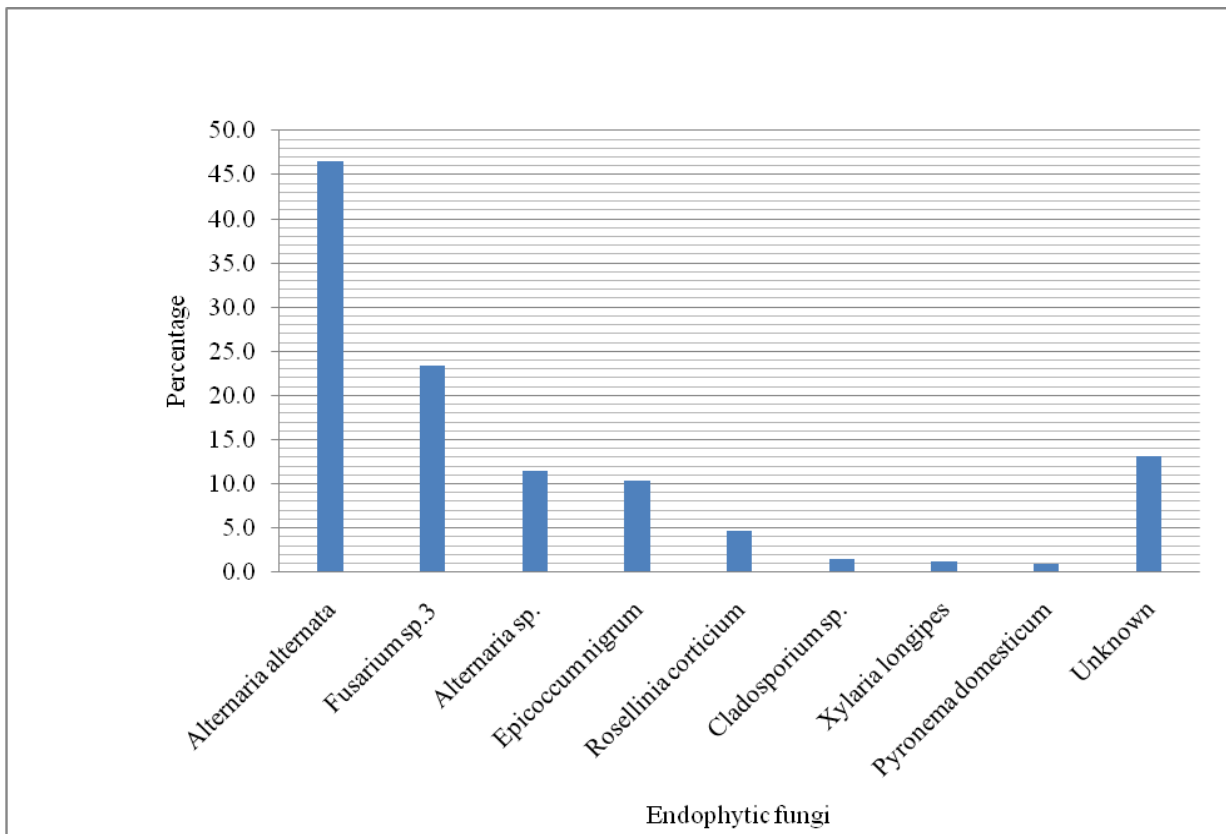


Fig.3 Frequency of Endophytic Fungi Outgrowth from Leaf Samples of Cherry Trees

Table 1

Diversity of endophytic fungi in different organs of cherry tree

Order	Genus/Species	Number of isolated colonies			Total
		Root	Twig	Leaf	
Pleosporales	<i>Alternaria alternata</i>	605	824	502	1931
	<i>Alternaria</i> sp.	-	1049	124	1173
	<i>Phoma</i> sp.	213	-	-	213
	<i>Embellisia</i> sp.	7	72	-	79
Hypocreales	<i>Fusarium solani</i>	-	59	-	59
	<i>Fusarium oxysporum</i>	11	-	-	11
	<i>Fusarium</i> sp.3	44	23	252	319
	<i>Fusarium</i> sp.1	163	110	-	273
	<i>Fusarium</i> sp.2	22	270	-	292
	<i>Acremonium</i> sp.	133	-	-	133
	<i>Neonectria radicularis</i>	23	-	-	23
<i>Hypocrea</i> sp.	15	-	-	15	
Incertae sedis	<i>Epicoccum nigrum</i>	28	404	111	543
	<i>Rhizopycnis</i> sp.	15	-	-	15
Botryosphaerales	<i>Macrophomina phaseolina</i>	-	88	-	88
Phyllachorales	<i>Glomerella acutata</i>	-	21	-	21
Capnodiales	<i>Cladosporium</i> sp.	323	-	16	339
Cantharellales	<i>Rhizoctonia</i> sp.2	4	-	-	4
	<i>Rhizoctonia</i> sp.1	19	-	-	19
Eurotiales	<i>Aspergillus niger</i>	17	-	-	17
Sordariales	<i>Chaetomium</i> sp.	-	59	-	59
Helotiales	<i>Botryotinia fuckeliana</i>	-	50	-	50
Diaporthales	<i>Diaporthe</i> sp.	-	24	-	24
Xylariales	<i>Rosellinia corticium</i>	-	-	50	50
	<i>Xylaria longipes</i>	-	-	13	13
Pezizales	<i>Pyronema domesticum</i>	-	-	10	10
Total		1642	3053	1078	5773