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Diversity of fungal endophytes in leaves of *Mentha piperita* and *Mentha canadensis*

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Abstract
Endophytic fungi are microorganisms that colonize internal plant tissues without causing immediate negative effects, being present in virtually the entire plant kingdom. Despite this, much of its biotechnological potential is unknown, and its identification is still recent. Peppermint, popular name of the genus *Mentha*, is a perennial plant native of Europe with various applications in cosmetics, pharmaceuticals, food and hygiene products. The objective of this work was the isolation and identification of endophytic fungi from leaves of two peppermint species, *Mentha piperita* and *Mentha canadensis*. Identification was performed by macro and micromorphology and by the sequencing of the ITS1-5.8S-ITS2 region of ribosomal DNA and partial ß-tubulin gene. Thirty-five endophytes were identified in total, belonging to the genus *Alternaria, Aspergillus, Curvularia, Epicoccum, Fusarium, Penicillium, Periconia* and *Phoma* and the order *Xylariales*. This is the first report of endophytic fungi diversity in *M. canadensis,* and provides knowledge on their future use on biotechnological processes.

Keywords: peppermint, isolation, endophytic

1. Introduction

Endophytic microorganisms are organisms that colonize the interior of plants without causing any visible negative effects at first, being present in practically all plant species [1]. Although it is a propagated concept, there are still doubts about the definition of endophytic, wherein some of which may be latent pathogens or cause damage to the plant [2,3]. However, the great majority presents themselves as microorganisms that develop in the apoplastic fluid of the plants in an asymptomatic way, acting commonly in a mutualistic way and often increasing the resistance of the plant against drought and diseases, facilitating its growth. Thus, the definition of endophyte has been extended to include all microorganisms that colonize internal tissues of plants, either for benefit, detriment or in a neutral way [2, 4, 5].
Potentially promising, endophytic microorganisms can be used as sources of primary metabolites [6] and secondary metabolites. Some advantages are attributed to the presence of endophytes, such as the ability to produce antibiotics [7] and other secondary metabolites of pharmacological interest; employment as agents of biological control of diseases and pests (bio-pesticides) [8]; bioremediation of soil contaminated with pollutants; and use as vectors for introducing genes into host plants [3, 5, 9].
The peppermint, as it is popularly known the genus *Mentha*, is aneudicotyledone plant which belongs to the *Lamiaceae* family, cultivated by the ancient Egyptians and documented since before the thirteenth century [10]. It is a perennial medicinal plant, native of Europe and naturalized in several parts of the world, and it is often used on a large scale as a component of products intended for cosmetic and pharmaceutical use [11]. In addition to its high morphological variability, most peppermint species are characterized by their high chemical diversity in terms of the constituents present in their leaves [12]. The peppermint essential oil is composed mainly of mentone, menthol, eucalyptol, linalool and alpha-terpinene, and it has antibacterial activity against *Candida albicans, Escherichia coli, Salmonella choleraesuis* and *Staphylococcus aureus* [13].
Its properties are well known in traditional medicine, and its antimicrobial, anti-viral, antifungal, anti-oxidant and anti-allergic characteristics have been tested and proven, either as essential oil [10, 14], aqueous extract [12, 15] or tea [11]. There are reports in the literature about tests performed with essential oil of peppermint to verify its effectiveness against the growth of *Aspergillus niger* and *A. carbonarius*, ochratoxin A producers [16], and *A. flavus*, aflatoxin producer [17]. All these properties may associate peppermint to a possible source of endophytic fungi with biotechnology capability.
Therefore, the objective of this work was to isolate endophytic fungi from leaves of two peppermint species, *Mentha piperita* and *Mentha canadensis*, and to identify them by molecular techniques.

2. Materials and methods

2.1 Isolation of endophytic fungi

The endophytic fungi were isolated from healthy peppermint leaves (*M. piperita* and *M. canadensis*) collected at the Canguiri Experimental Station Center of the Federal University of Paraná (UFPR) (25°23'30"S and 49°07'30" W), located in the municipality of Pinhais, PR, Brazil. The leaves were initially washed with water to remove soil and dust and then dipped in 70% alcohol for 30 seconds and 1% sodium hypochlorite for 3 minutes [18]. Subsequently, the material was washed twice in sterile distilled water and cut into 4 mm² fragments, and 5 fragments were placed in each Petri dish (90 x 15 mm) containing potato dextrose agar (PDA) with tetracycline (100 µL mL^{-1}). The abaxial part of the leaves was in contact with the medium. The plates were placed in a BOD incubator at 28 ± 1 °C for 7 days. The grown fungi were isolated in Petri dishes containing PDA medium and the isolation frequency was calculated [19].

2.2 Morphological characterization

Single-spore colonies were cultivated in PDA and incubated at 28 ± 1 °C for 14 days. The isolates were separated into morphogroups according to their macro and micromorphological characteristics. The isolates are deposited in the Microbiological Collection of Paraná Network - Taxonline (CMRP), Basic Pathology Department, Federal University of Paraná, Curitiba, Brazil.

2.3 Molecular identification of endophytic fungi

For molecular identification, the ITS1-5,8S-ITS2 region of ribosomal DNA and partial ß-tubulin gene were sequenced. The DNA was extracted according to Badali [20], and PCR was made using the pairs of primers ITS1 and ITS4 [21] and Bt2a and Bt2b [22]. Sequencing was performed by the chain termination method [23], in ABI 3500 automatic DNA sequencer (Applied Biosystems). The sequences were aligned and edited with the Staden program version 1.6 [24], aligned with the MEGA 7 program [25] and compared with other sequences in the NCBI database by the BLAST program [26].

3. Results

From *M. piperita*, 220 fungi belonging to 50 different morphogroups were isolated, and the isolation frequency was 40.8%. For *M. canadensis*, 73 fungi belonging to 19 morphogroups were isolated, with a frequency of 14.6%. In total, 33 fungi were identified from the ITS region and 2 from partial ß-tubulin gene sequencing (TABLE 1), being 10 of *M. canadensis* and 25 of *M. piperita.*

Table 1: Endophytic fungi isolated from leaves of Mentha canadensis and Mentha piperita, identified by the ITS region and partial ß-tubulin gene sequencing.

Host	Identification	Frequency (%)	Isolate	Genbank acession number	Homology (%)
Mentha canadensis	*Alternaria alternata*	67,7	100	MK370625	100
			92-E1	MK370626	100
			83-D1	MK370627	100
			11-B1	MK370628	100
			41-B1	MK370629	99
			28-C1	MK370630	100
	Aspergillus fumigatus	9,7	70-E1	MK370631	100
	Curvularia trifolii	12,9	89-E1	MK370632	100
	Fusarium equiseti	3,2	74-D1	MK370633 / MK373039	100
	Phoma sp.	6,5	65-E1	MK370634	100
Mentha piperita	*Alternaria alternata*	52,8	75-D1	MK370635	100
			20-A1	MK370636	100
			63-A1	MK370637	100
			86-A1	MK370638	100
			93-D1	MK370639	100
			94-C1	MK370640	100
			96-C1	MK370641	100
			47-A2	MK370642	99
	Alternaria solani	8,7	61-C1	MK370643	100
			52-B1	MK370644	99
			91-B1	MK370645	99
	Alternaria sp.	23,6	36-C1	MK370646	100
			14-B1	MK370647	100
			57-D1	MK370648	99
	Aspergillus nomius	1,2	89-B1	MK373042	99
			50-C1	MK373043	99
	Curvularia trifolii	0,6	64-C1	MK370649	100
	Epicoccum nigrum	0,6	09-B1	MK370650	100
	Fusarium equiseti	0,6	31-E1	MK370651 / MK373040	100
	Penicillium crustosum	1,2	95-A1	MK370652 / MK373041	100
	Periconia byssoides	3,1	86-B1	MK370653	100
			39-A1	MK370654	99
	Xylariales sp.	7,5	31-C1	MK370655	99
			94-E1	MK370656	99
			03-D1	MK370657	98

4. Discussion

This is the first study of endophytic fungi isolated from leaves of *M. canadensis* and the first record of the genus *Curvularia* and *Periconia* and the order *Xylariales* in *M. piperita*. The identified endophytic fungi were *Alternaria*, *Aspergillus*, *Curvularia*, *Epicoccum*, *Fusarium*, *Penicillium*, *Periconia*, *Phoma* and *Xylariales*.

The genus *Colletotrichum*, *Drechslera*, *Fusarium*, *Guignardia*, *Nigrospora* and *Xylaria* were isolated from *Mentha spicata* [27], among them only the genus *Fusarium* and the order *Xylariales* were identified in this paper in the leaves of *M. piperita* and *M. canadensis*. Another study reported the presence of an endophytic ascomycete isolate in stems and leaves of *M. piperita* without causing damage to the host plant [28], which may indicate interactions similar to those of the fungi identified in this study.

Zimowska [29] isolated endophytes from several parts of the *M. piperita* plant, and identified the genus *Alternaria*, *Epicoccum*, *Fusarium*, *Penicillium* and *Phoma* in common with the present paper, as well as other genus such as *Cladosporium* and *Trichoderma*. Also in this study, the genus *Phoma* was identified as a possible cause of necrosis. In previous studies, Zimowska [30] reports that *Phoma* and *Fusarium* species were less frequent in peppermint leaves, wherein the *Alternaria alternata* species was predominant, which is in agreement with our results.

In a study with *M. piperita* samples from several regions of India, it was identified 63 endophytes. The genus in common with the present study are *Alternaria, Aspergillus, Fusarium* and *Penicillium*, emphasizing again the species *Alternaria alternata* [31].

The genus of fungi presented in this paper have been isolated from several species of plants, however studies with peppermint are still scarce. The species *A. alternata* was isolated from coffee and presented antimicrobial and antioxidant activity [32]. Lin [33] isolated 174 endophytic fungi from *Camptotheca acuminata*, a medicinal plant, with 12.6% of *Alternaria* sp. This genus was also isolated from tangerine, lemon [34], soybean [35], eucalyptus [36], *Crambe* sp. [37], peach palm [38], tomato, arugula, cauliflower, wheat, grape, apple, among others [39]. It is important to emphasize that the presence of *Alternaria* spp. in peppermint leaves may be an indicator of the presence of mycotoxins such as alternariol, alternariol monomethyl ether, tenuazonic acid and altertoxins [40].

In addition to *Alternaria*, other endophytic fungi from peppermint were the genus *Epicoccum*, *Curvularia* and *Periconia*, as well as the order *Xylariales*. The fungus *Epicoccum nigrum* has been isolated from peach palm (*Bactris gasipaes*) [38], rubber tree [41], maize [42], cabbage [43] and olive [44]. Madrigal and Melgarejo [45] showed that this species has antifungal activity, producing compounds such as flavipin.

The genus *Curvularia* is associated with plant diseases, such as leaf spot [46], and humans diseases [47]. The same genus has already been isolated from dwarf papyrus sedge, *Cenchrus ciliaris* [48], ginger [49], cacao [50], carrot [51], *Lippia sidoides* [52] and rice [53]. *Hypoxylon*, one of the genus that belong to *Xylariales*, can be found as an endophytic of winter cherry [54] and *Persea indica*, producing volatile compounds with potential as biofuel [55,56].

Two antitumor substances (pericosines A and B), effective against leukemia cells, have been isolated from *Periconia byssoides* extracts [57]. The endophytic genus was also found in medicinal plants such as *Lippia sidoides* [52], *Vitex negundo* [58], bear's breeches (*Heracleum sosnowskyi*) [59], ginger [49], *Ougeiniao ojeinensis* [60] and grasses [61].

The genus *Fusarium*, found in this study, is also considered a phytopathogen and producer of secondary metabolites toxic for humans and animals [62, 63]. Species of this genus were isolated from barley, wheat, onion, beet, straw [62], plane trees, *Artemisia annua*, winter cherry [54], rubber tree [41], maize [64], *Apodytes dimidiata* [65] and peach palm [38].

Another producer of mycotoxins is the genus *Phoma*, associated with the leaf spot of *Phaeosphaeria*, a disease that attacks tropical and subtropical maize producing areas [66]. Some species have already been isolated from mangrove trees [67], *Cyperus laevigatus*, *Chloris inflata* [48], *Fallopia japonica* [68], maize [42], carrot [51], rubber tree [41], and baobab [69].

It is important to emphasize that the species *Penicillium crustosum*, producer of penitrem toxin A [70], was first identified in peppermint. Different *Penicillium* species have already been found in mangrove trees [67, 71], rubber tree [41], *Pappophorum krapovickasii* [48], African oil palm [72], *Vitex negundo* [58] and cabbage [43], evidencing the diversity of the genus in plants.

There are no records in the literature of the presence of *Aspergillus fumigatus* in peppermint plants. This genus, isolated from mangrove, has been used in the production of drugs, such as lovastatin [73] and as phosphate solubilizers [71]. Besides, it has been isolated from coffee beans [74, 75], dwarf papyrus sedge [48], African oil palm [72], *Vitex negundo* [58] and *Anemopsis californica* [76].

5. Conclusions

This is the first report of endophytic fungi diversity in *M. canadensis*, and the presence of the genus *Curvularia* and *Periconia* and the order *Xylariales* in *M. piperita*, which provides knowledge on their future use on biotechnological processes.

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