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Contribution to the endophytic mycobiota of aerial parts of oaks

DAVID NOVOTNÝ

Crop Research Institute, Drnovská 507, CZ-161 06 Praha 6 - Ruzyně, Czech Republic;
Czech University of Life Sciences, Faculty of Agrobiology, Food and Natural Resources,
Kamýcká 129, CZ-165 21 Praha 6 - Suchdol, Czech Republic;
novotny@vurv.cz

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The endophytic mycobiota inhabiting branches of *Quercus petraea* from two localities in the Křivoklát area, and branches, twigs and acorns of *Quercus robur* from one locality near the town of Semily were studied (all sites in the Czech Republic). Seventeen fungal taxa were isolated from branches of *Q. petraea*. Dominant fungi were found to be *Colpoma quercinum* and *Phoma* sp. as well as sterile dark mycelium and a black yeast-like fungus close to *Sarcinomyces crustaceus*. Eighteen fungal taxa were found in samples of aerial parts in *Quercus robur*. The most frequently isolated taxa were *Pezicula cinnamomea*, *Colpoma quercinum*, *Alternaria alternata* agg. and *Acremonium* sp.

Key words: endophytes, Czech Republic, *Quercus robur*, *Quercus petraea*, acorns, seeds, twigs.

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V rámci této práce byla studována endofytická mykobiota větví dubu zimního (*Quercus petraea*) ze dvou lokalit na Křivoklátsku a větví, větévek a žaludů dubu letního (*Q. robur*) z lokality blízko Semil. Všechny lokality se nacházejí v České republice. Z živých větví *Q. petraea* bylo izolováno 17 taxonů hub. Dominantními houbami byly *Colpoma quercinum*, *Phoma* sp., sterilní tmavé mycelium a černá, kvasince podobná houba, blízká *Sarcinomyces crustaceus*. Při studiu nadzemních částí *Q. robur* bylo nalezeno 18 taxonů endofyticky žijících hub. Nejhojněji se vyskytovaly *Pezicula cinnamomea*, *Colpoma quercinum*, *Alternaria alternata* agg. a *Acremonium* sp.

INTRODUCTION

Oaks are common trees in many parts of Europe, Asia and other parts of the world, including the Czech Republic. Approximately 20–30 species of this genus grow in Europe. *Quercus robur* and *Q. petraea* are the most abundant and economically most important oak species in Central Europe (Koblížek 1990, Boratyński 1991).

Plants harbour many species of asymptomatically living endophytic fungi. Their occurrences depend on many abiotic and biotic conditions. Endophytic communities are often specific at the host species, organ and tissue level. Different species of plants harbour distinct spectra of fungi (Petrini 1996, Rodriguez et al. 2009).

The composition of mycobiota in oak branches and stems has been studied by several authors. So far, three methods of sample processing (strong surface sterilisation, moist chamber technique, next generation sequencing) have been used (e.g. Petrini et Fisher 1990, Kowalski 1991, 1996, Kubátová et Prášil 1995, Agostinelli et al. 2018, Fort et al. 2021, Menkis et al. in press).

The diversity of fungi living in aerial parts of oaks (especially *Quercus robur* L. and *Q. petraea* L.) with symptoms of oak decline has been studied in different countries of Europe and in North Africa (Kowalski 1991, 1996, Kehr et Wulf 1993, Przybył 1995, 1996, Gennaro et al. 2003, Linaldeddu et al. 2011, Moricca et al. 2012, Hasnaoui et al. 2017). In the Czech Republic, Fassatiová et al. (1995) and Kubátová et Prášil (1995) investigated the occurrence of microscopic fungi in stems and branches of oaks (*Q. petraea*, *Q. pubescens*, *Q. robur*) with symptoms of oak decline. However, they used the moist chamber technique, which does not provide reliable information concerning composition of endophytic mycobiota. Kowalski (1983) investigated changes in the composition of mycobiota of oaks damaged by air pollution.

In Central, Western, Southern and Northern Europe, endophytes of leaves, twigs and branches of *Q. petraea* (Halmschlager et al. 1993, Fort et al. 2021) and *Q. robur* (Griffith et Boddy 1990, Petrini et Fisher 1990, Kowalski et Kehr 1996, Gennaro et al. 2003, Ragazzi et al. 2003, Gonthier et al. 2006, Agostinelli et al. 2018, Matule 2018) have been studied. The endophytic mycobiota of other *Quercus* species was investigated in Southern and Western Europe (*Quercus ilex* – Fisher et al. 1994, Collado et al. 1996, 1999, 2000; *Q. faginea* – Collado et al. 1996, 2000; *Q. cerris* – Ragazzi et al. 2001, 2003, Gennaro et al. 2003, Moricca et al. 2012; *Q. pubescens* – Ragazzi et al. 2003, Moricca et al. 2012; *Q. suber* – Linaldeddu et al. 2011, Costa et al. 2018, 2020) and in Asia (*Q. liaotungensis* – Sun et al. 2012; *Q. macranthera* and *Q. brantii* – Ghasemi-Esfahlan et al. 2019; *Q. acuta* – Hashizume et al. 2008).

Endophytic fungi are thought to take part in natural pruning of branches of trees including oaks. The mechanisms of parasite-host and endophyte-host interactions are apparently similar (Kehr 1998). It seems that some endophyte species change their life strategy, being able to become weak parasites. The presence of endophytic fungi may prevent more aggressive parasites from colonising trees (Butin et Kowalski 1983, Kowalski et Kehr 1996).

Many fungal species are known to be pathogens of stored acorns (Procházková 1991, Kehr et Schröder 1997, Washington 2003, Oskay et al. 2019). Fort et al.

(2021) investigated endophytic mycobiota of *Q. petraea* acorns by using next generation sequencing (NGS) and confocal microscopy analysis.

The endophytic mycobiota of healthy oak branches, twigs and acorns has not yet been investigated in the Czech Republic. The present study is the first contribution providing information about endophytic mycobiota of oaks in the Czech Republic, thus obtaining a better overview of the mycobiota of oaks.

The present work gives results of a study of the endophytic mycobiota of branches of sessile oak (*Quercus petraea*) and branches, twigs, acorn stalks, cupules and acorns of pedunculate oak (*Quercus robur*).

MATERIAL AND METHODS

The first part of the study was conducted in the Křivoklát area (Central Bohemia, Czech Republic), in two middle-aged oak stands. The study site Dřevíč (50°01'23" N, 13°58'25" E) is located approximately 600 m north-west of Dřevíč castle, at approximately 430 m a.s.l. The study site Křivoklát (forest number 127B2; 50°02'00" N, 13°52'40" E) is located approximately 800 m south-east of Křivoklát castle, at approximately 360 m a.s.l. In April 1997 and 1998, a total of 28 living branches (1.5–2 cm thick) of three *Quercus petraea* trees were cut off. One sample was taken from each selected branch. The branches were healthy, lacking any necrotic spots.

In August 1998, samples were collected in a mixed wood at the village of Bítouchov near the town of Semily (North Bohemia, Czech Republic). This site (50°37'09" N, 15°19'10" E) is located approximately 900 m north of the town of Semily near a gorge with a stream, at approximately 380 m a.s.l. Two *Quercus robur* trees were sampled. The samples were taken from one branch (1.5–2 cm thick), three twigs (0.5 cm thick), five stalks bearing acorns (2 mm thick), 12 cupules of acorns and 12 acorns of each selected tree.

Branches, twigs and cupules were brushed under running water, surface-sterilised (96% ethanol 1 min., sodium hypochlorite 3 min., 96% ethanol 0.5 min.) and cut into small pieces (3–5 × 3–5 × 1 mm), further referred to as 'segments'. The branch samples were separated into wood and bark. Five segments were taken from each sample.

Acorns and acorn stalks were brushed under running water. Acorn stalks were separated into wood and bark, surface-sterilised (as mentioned above) and cut into segments (3–5 × 3–5 × 1 mm). Five segments were taken from each sample.

Segments of all samples were placed on 2% malt extract agar in Petri dishes and incubated at a temperature of 20–22 °C for up to four weeks.

Identification of the recorded fungi was based on their micro- and macromorphology. The recorded fungi were divided into morphotypes. Only representative strains were identified to species level. To make identification possible, some isolated fungi were cultivated on diagnostic agar media: for *Talaromyces* 2% malt extract agar (MEA), Czapek yeast extract agar (CYA), glycerol nitrate agar (G25N) (Pitt 1979), for *Trichoderma* potato dextrose agar (PDA) and soil agar (SA) (Anonymus 1996).

Sequencing of selected regions of DNA would have been useful for identification on the species level, but the author did not have possibility to use this method at the time the obtained strains were to be identified. Therefore, not all taxa are assigned to the species level and such taxa are indicated using the abbreviations agg. or cf.

Results

Endophytic mycobiota of *Quercus petraea* branches from the Křivoklát area

Endophytic fungi were detected in 50% of branch segments. The bark was colonised more frequently (84.3% of segments) than the xylem (15.7%). Bacteria occurred in 9.3% of branch segments. They were observed in higher frequency in wood (11.4% of segments) than in bark (7.1%).

Seventeen fungal taxa (including sterile mycelia) were isolated from the branches. Bark and wood harboured 14 and 11 fungal species, respectively. Twelve taxa were found in branches from Dřevíč and thirteen taxa were isolated from branches from Křivoklát. *Colpoma quercinum*, sterile dark mycelia, *Phoma* sp. and a black yeast-like fungus close to *Sarcinomyces crustaceus* were the main colonisers of the oak branches examined. The mycobiota composition of the branches was similar at both localities. The dominant fungal species occurred at both study sites (Tab. 1).

Mycobiota of branches, twigs, acorn stalks, cupules and acorns of *Quercus robur* from Bítouchov

Eighteen species were recorded in this study. Twigs were most frequently colonised (90% of segments). They harboured eleven fungal species, but only two taxa (*Pezicula cinnamomea*, *Colpoma quercinum*) were recorded in more than one segment. Three species were found in bark and one species was isolated from xylem. The bark of acorn stalks harboured three fungal species, but no fungus was detected in the xylem of the stalks. Six taxa were isolated from cupules. *Alternaria alternata* agg., *Nemania serpens* and sterile black-white mycelium were found most frequently in this plant organ. One species was detected in acorns.

Pezicula cinnamomea was the most frequent taxon detected. It was recorded in twigs, bark of branches and bark of acorn stalks. *Colpoma quercinum*, *Alternaria alternata* agg. and *Acremonium* sp. were detected in two or three types of organs. The other species were isolated in one type of organ only (Tab. 2).

Tab. 1. Number and frequency of occurrence (number of isolations from segments) of endophytic fungi in *Quercus petraea* branches from two localities in the Křivoklát area.
The letter combinations in the column headings indicate the provenance of the samples: b – bark, w – wood; D – Dřevíč, K – Křivoklát, all – both localities; % – percentage of segments colonised by fungi.

Taxon / Samples	Db	Db%	Dw	Dw%	D	D%	Kb	Kb%	Kw	Kw%	K	K%	b	b%	w	w%	all	all%
Number of studied segments	110		110		220		30		30		60		140		140		280	
Black yeast-like fungus close to *Sarcinomyces crustaceus*	9	8.18	1	0.91	10	4.55	1	3.33			1	1.67	10	7.14	1	0.71	11	3.93
Cladosporium cladosporioides agg.									1	3.33	1	1.67			1	0.71	1	0.36
Colpoma quercinum (Pers.: Fr.) Waller	45	40.91	4	3.64	49	22.27	11	36.67			11	18.33	56	40.00	4	2.86	60	21.43
Epicoccum nigrum Link							2	6.67			2	3.33	2	1.43			2	0.71
Nemania serpens (Pers.) Gray	3	2.73	1	0.91	4	1.82	1	3.33			1	1.67	4	2.86	1	0.71	5	1.79
Nodulisporium sp.									1	3.33	1	1.67			1	0.71	1	0.36
Oidiodendron griseum Robak	2	1.82			2	0.91							2	1.43			2	0.71
Pezicula cinnamomea (Pers.: Fr.) Sacc.	1	0.91			1	0.45							1	0.71			1	0.36
Phialocephala sp.	1	0.91			1	0.45							1	0.71			1	0.36
Phoma sp. 1	10	9.09	1	0.91	11	5.00	2	6.67	1	3.33	3	5.00	12	8.57	2	1.43	14	5.00
Sterile basidiomycetes sp. 1			2	1.82	2	0.91									2	1.43	2	0.71
Sterile black-white mycelium							3	10.00			3	5.00	3	2.14			3	1.07
Sterile dark mycelium	17	15.45	2	1.82	19	8.64	8	26.67	3	10.00	11	18.33	25	17.86	5	3.57	30	10.71
Sterile hyaline mycelium	2	1.82	2	1.82	4	1.82	3	10.00	3	10.00	6	10.00	5	3.57	5	3.57	10	3.57
Talaromyces purpureogenus Samson, N. Yilmaz, Houbraken, Spierenb., Seifert, Peterson, Varga et Frisvad	1	0.91			1	0.45			1	3.33	1	1.67	1	0.71	1	0.71	2	0.71
Trichoderma viride agg.	5	4.55			5	2.27	1	3.33			1	1.67	5	3.57	1	0.71	6	2.14
Xylaria sp.							1	3.33			1	1.67	1	0.71			1	0.36
Number of segments colonised by fungi	89	80.91	13	11.82	102	46.36	29	96.67	9	30.00	38	63.33	118	84.29	22	15.71	140	50.00
Number of segments colonised by bacteria	9	8.18	15	13.64	24	10.91	1	3.33	1	3.33	2	3.33	10	7.14	16	11.43	26	9.29
Number of fungal taxa	11		7		12		9		6		13		14		11		17	

Tab. 2. Number and frequency of occurrence (number of isolations from segments) of endophytic fungi in *Quercus robur* branches, twigs, acorn stalks, cupules and acorns from Bítouchov, Semily District. The letter combinations in the column headings indicate the provenance of the samples: b – bark, w – wood; B – branches, T – twigs, S – acorn stalks, C – cupules, A – acorns; % – percentage of segments colonised by fungi.

Taxon / Samples	**bB**	**bB%**	**wB**	**wB%**	**T**	**T%**	**bS**	**bS%**	**wS**	**wS%**	**C**	**C%**	**A**	**A%**
Number of segments	10		10		30		50		50		120		120	
Black yeast-like fungus close to *Sarcinomyces crustaceus*			1	10.00										
Acremonium sp.					1	3.33					1	0.83		
Alternaria alternata agg.					1	3.33	1	2.00			8	6.67		
Cladosporium cladosporioides agg.					1	3.33								
Camarosporium cf. *quaternatum* (Hazsl.) Schulz					1	3.33								
Colpoma quercinum (Pers.: Fr.) Wallr.	1	10.00			5	16.67								
Epicoccum nigrum Link					1	3.33								
Microsphaeropsis sp.					1	3.33								
Nemania serpens (Pers.) Gray											4	3.33		
Oedocephalum sp.													1	0.83
Oidiodendron griseum Robak											1	0.83		
Pezicula cinnamomea (Pers.: Fr.) Sacc.	5	50.00			15	50.00	6	12.00						
Phialocephala sp.	1	10.00			1	3.33								
Phoma sp.					1	3.33								
Phomopsis sp.											3	2.50		
Pseudopithomyces chartarum (Berk. & M.A. Curtis) Jun F. Li, Ariyaw. & K.D. Hyde							3	6.00						
Sterile black-white mycelium											5	4.17		
Sydowia cf. *polyspora* (Bref. & Tavel) E. Müll.					1	3.33								
Number of segments colonised by fungi	7	70.00	1	10.00	27	90.00	10	20.00	0	0.00	22	18.33	1	0,83
Number of segments colonised by bacteria			2	20.00			5	10.00			1	0.83	1	0.83
Number of fungal taxa	3		1		11		3		0		6		1	

DISCUSSION

The composition of endophytic mycobiota of branches of the investigated oaks (*Q. robur* and *Q. petraea*) is similar. The most frequently recorded fungi in the present study were also recorded by Halmschlager et al. (1993), Przybył (1996), Kowalski & Kehr (1996), Ragazzi et al. (2001, 2003), Agostinelli et al. (2018) in their investigations of endophytic mycobiota of *Q. robur*, *Q. petraea*, *Q. cerris* and also *Q. pubescens*. Since 2010, only three studies (Agostinelli et al. 2018, Matule 2018, Fort et al. 2021) dealing with the endophytic mycobiota of

Q. robur or *Q. petraea* have been published. This is less in comparison with the years 1990–2009. Approximately 10–15 years ago (around 2010), molecular genetic methods (in particular sequencing) began to be massively used, making it possible to identify fungi exclusively creating sterile colonies in vitro to genus or species levels. Therefore, it is difficult to compare older results with new studies in which sequencing is standardly used to identify observed fungi.

Using the strong surface sterilisation method, no or one fungus was recorded per segment on average. Less frequently, two fungal taxa per segment were detected. Differences in the number of fungi per segment were found between the various tissues. However, the number of fungi recorded is influenced by the competitive ability of each fungal species to grow on agar medium under in-vitro conditions. If next-generation sequencing had been used, a higher number of fungal species per segment would probably have been detected.

During the present study, sterile mycelia were frequently isolated from branches of particularly *Q. petraea*. Sterile mycelia were and are often isolated during the study of endophytic mycobiota of oaks (e.g. Przybył 1995, Collado et al. 1996, 2000, Ragazzi et al. 2003). In all studies published since 2010, some recorded fungi are identified at species or genus level (indicated as e.g. Ascomycetes sp. 1, Unknown, Sordariomycetes sp. 2), although sequencing was used, and therefore the data for these taxa cannot further be used.

Colpoma quercinum, which was frequently found in branches of *Q. petraea* and *Q. robur* in the present study, is a common endophyte of branches and stems of *Q. robur*, *Q. petraea*, *Q. cerris* and *Q. pubescens* (Halmschlager et al. 1993, Przybył 1996, Kowalski et Kehr 1996, Ragazzi et al. 2001, 2003, Moricca et al. 2012, Agostinelli et al. 2018), and it was also recorded in *Q. liaotungensis* in northern China (Sun et al. 2012). This fungal species often occurs in attached or fallen dead branches of *Q. robur* (e.g. Butin et Kowalski 1983), in branches and stems of oaks with symptoms of oak decline (e.g. Przybył 1995), and in branches of *Q. rubra* affected by air pollutants (Kowalski 1983). *Colpoma quercinum* was not recorded in branches of *Q. macranthera*, *Q. brantii* (Ghasemi-Esfahlan et al. 2019), *Q. suber* (Linaldeddu et al. 2011, Costa et al. 2020), *Q. faginea* (Collado et al. 2000) and *Q. ilex* (Fisher et al. 1994, Collado et al. 1999, 2000) which were sampled in relatively warm parts of the world (except for the branches investigated by Fisher et al. 1994).

Six fungal species were recorded in cupules and one taxon was isolated from acorns of *Q. robur*. Sterile black-white mycelium was frequently recorded in acorns. This fungus was also isolated from the bark of *Q. petraea* branches from Křivoklát. Many of the isolated fungi are known as plant endophytes (*Nemania serpens*, *Oidiodendron griseum*, *Alternaria alternata* agg., *Phomopsis* sp.) or epiphytes (*Alternaria alternata* agg.). So far, however, mainly the mycobiota of stored acorns has been studied and only Fort et al. (2021) investigated

endophytes of acorns picked from *Quercus petraea* trees in the Pyrenees (south-western France). These authors found fungi in all tissues of acorns and ascomycetes dominated the fungal community. *Gnomoniopsis paraclavulata*, *Stromatoseptoria castaneicola*, *Taphrina carpini*, *Polyscytalum algarvense*, *Mycosphaerella tassiana*, *Cylindrium elongatum*, *Fusarium pseudensiforme*, *Cladosporium delicatulum* and *Curvibasidium cygneicollum* were the most frequently recorded fungi. The majority of these fungal species have not yet been recorded in the Czech Republic, but most of them have already been found in nearby countries (Germany, Austria, Hungary) (Anonymus on-line 1, on-line 2). It can be expected that these fungi will also be found in the Czech Republic in the coming years.

Kehr et Schröder (1997) isolated 24 species of fungi from acorns of *Q. robur*. *Alternaria alternata*, *Epicoccum nigrum* and *Cladosporium cladosporioides* were the dominant taxa. Procházková (1991) identified 27 species of microscopic fungi on stored acorns. The most frequently recorded taxa were *Penicillium* spp., *Ciboria batschiana*, *Botrytis cinerea*, *Fusarium* spp. and ophiostomatoid fungi. Some species of fungi of acorn stalks and cupules, detected in this study were also recorded in the fungal community of stored acorns (Kehr et Schröder 1997) and a similar composition of fungal communities of branches and acorns was found by Fort et al. (2021). The fungal community of acorns changes with storage duration. Some species recorded in storage already colonise acorns during their development into trees (endophytic fungi). Saprophytic and pathogenic fungi (e.g. *Botrytis*, *Ciboria*, *Penicillium*) are more frequently observed with storage time.

The fungi isolated in the present study are considered to be endophytes because they were isolated from apparently healthy, living organs. The low frequency of occurrence of fungi in the studied acorns is surprising and could have been caused by application of a modified form of surface sterilisation during which the fungi may have been killed by the sterilisation solution. However, the acorn stalks were processed in the same way, but endophytes were nonetheless detected in them, as well as in the bark of branches. The frequency of occurrence and species diversity would probably have been higher if acorn samples had been taken later, because older plant tissues are colonised more than younger ones. Climatic conditions (e.g. higher rainfall) affect the endophytic mycobiota of trees and may have influenced the mycobiota of the samples studied.

To date, we lack studies of the endophytic mycobiota of *Q. robur* and *Q. petraea* branches and acorns and their changes over time based on strong surface sterilisation followed by identification of the recovered strains by sequencing and parallel next-generation sequencing. Such studies would provide the best insight into the endophytic fungal community of these oaks.

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