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A comparison of two methods for the study of microscopic fungi associated with oak roots

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Roots of four trees with symptoms of oak decline and roots of one healthy tree of *Quercus robur* were examined for the presence of fungi by using two methods (moist chamber method and strong surface sterilisation method). Forty-five species were isolated in this project. Significant differences in composition of mycobiota based on the used method were detected. *Fusarium solani*, *F. proliferatum*, *Sphaerostilbella aureonitens*, *Cylindrocarpon destructans*, *Penicillium simplicissimum*, *P. purpurogenum* var. *rubrisclerotium*, *Trichoderma viride*, *Ophiostoma piceae* s.l. and *Penicillium glandicola* were the most frequent fungi isolated by the moist chamber method. *Cryptosporiopsis radicicola*, dark sterile mycelium sp. 1, *Cylindrocarpon destructans*, *Chaetomium globosum*, *Cylindrocarpon didymum*, *Penicillium simplicissimum* and *Trichoderma koningii* were dominant species observed by the method of strong surface sterilisation.

Key words: *Quercus robur*, oak decline, Czech Republic, mycobiota, ophiostomatoid fungi.

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V této práci byla studována mykoflóra kořenů pěti stromů dubů letních (*Quercus robur*) v různém zdravotním stavu použitím metody vlhkých komůrek a metody založené na silné povrchové sterilizaci. Celkově bylo zaznamenáno 45 druhů hub a rozdíl v jejich spektru v závislosti na použité metodě. Použitím metody vlhkých komůrek byly nejčastěji izolovány *Fusarium solani*, *F. proliferatum*, *Sphaerostilbella aureonitens*, *Cylindrocarpon destructans*, *Penicillium simplicissimum*, *P. purpurogenum* var. *rubrisclerotium*, *Trichoderma viride*, *Ophiostoma piceae* s.l. a *Penicillium glandicola*. Metodou založenou na silné povrchové sterilizaci byly nejčastěji zjištěny *Cryptosporiopsis radicicola*, „dark sterile mycelium sp. 1", *Cylindrocarpon destructans*, *Chaetomium globosum*, *Cylindrocarpon didymum*, *Penicillium simplicissimum* and *Trichoderma koningii*.

INTRODUCTION

Dieback of oak (oak decline) is one of the most frequent "diseases" of oaks occurring in many countries of Europe (including the Czech Republic) in the last thirty-five years (Ragazzi et al. 2000, Siwecki and Liese 1991). A complex of abiotic and biotic factors (climate changes, insects, fungi, lack of nutrients and others) is

considered to be the cause of this phenomenon (Ragazzi et al. 1995). The role of fungi in oak decline was studied in many European countries and is not yet fully explained (Kehr and Wulf 1993; Kowalski 1991, 1996; Przybyl 1995, 1996).

So far, the composition of mycobiota of trees (including oaks) has been studied using two methods. Many mycologists (Collado et al. 1996; Kowalski 1991, 1996; Petrini and Fisher 1990; Przybyl 1995, 1996; and others) have used the strong surface sterilisation method. The observations by Eisenhauer (1991), Fassatiová et al. (1995), Kubátová and Prášil (1995), Novotný (2001) and Čížková and Švecová (1999) are based on the moist chamber method. Penicillia and ophiostomatoid fungi are reported very frequently when using the second method.

Schulz et al. (1993) compared the effectiveness of the various surface sterilisation methods in isolating endophytes in herbaceous material. Bills and Polishook (1992), who investigated endophytic mycobiota of leaves and twigs of *Chamaecyparis thyoides*, applied three different isolation media.

To date, no comparison of the moist chamber method and the strong surface sterilisation method during studies of the composition of mycobiota of trees has been made.

MATERIALS AND METHODS

The study was conducted in southwest Moravia (Czech Republic), near Moravské Budějovice, in a middle-aged oak (88 years old) stand called Dešov (forest number 119 A7). The stand was classified as loamy oak-beech forest.

In April 1996, one healthy tree and four trees (*Quercus robur*) which had recently died or with symptoms of decline in various stages were extracted (Table 1). Necrotic black or dark spots were not observed on branches, stems or roots of these trees. The trees were classified according to the health state of aboveground parts based on canopy cover (after Jančařík 1990). A tree marked 0 is healthy (without symptoms), a tree marked 4 is dead or missing >70 % of leaves of canopy cover.

The samples were taken from three skeleton roots (1–5 cm thick) of each selected tree. Two slices (about 1 cm thick) were cut from each root and brushed under running water. One slice from each root was used for each method performed in the present study as follows:

1) Strong surface sterilisation method. Slices of roots were sterilised by dipping them first in 96 % ethanol for 1 minute, then into a 5 % sodium hypochlorite solution (NaClO) for 3 minutes followed by 30 seconds in 96 % ethanol and were cut into segments (3–5 × 3–5 × 1 mm). Twenty segments from each slice were placed on 2 % malt extract agar and incubated at room temperature for up to four weeks.

2) Moist chamber method. Slices of roots were washed in 0.5 % sodium hypochlorite solution (NaClO) for five minutes and then in sterile water for 5 minutes. The slices were placed into a sterile glass moist chamber with sterile cotton wool and filter paper and incubated at room temperature for up to four weeks. Fungi isolated from slices were cultivated on 2 % malt extract agar (MA2), oatmeal agar (OA), potato carrot agar (PCA) and soil agar (SA) and identified.

For identification, the isolated fungi were cultivated on diagnostic agar media: (*Penicillium* – 2 % malt extract agar for *Penicillium* (MEA), Czapek yeast extract agar (CYA), glycerol nitrate agar (G25N), *Trichoderma* – potato dextrose agar (PDA), SA, *Fusarium* and *Cylindrocarpon* – PDA, synthetic nutrient agar (SNA), *Ophiostoma* – OA.

Table 1. Health state of roots of studied trees

Tree no.	Health category of aboveground part	Health state of roots
1	0	healthy
2	3–4	mostly healthy, some thin roots dried
3	2–3	healthy
4	4	healthy
5	4	rotting

RESULTS

Forty-five species of fungi (including sterile mycelia) were isolated from 15 roots of the five investigated trees. Twenty-six species and thirty-one species were found by the strong surface sterilisation method and by the moist chamber method, respectively (Table 2). Using both methods eleven species were isolated, but only *Cylindrocarpon destructans* was found in more than 30 % of roots of the studied trees by using both them.

The fungal community (twenty-six taxa) of root samples processed by the strong surface sterilisation method was dominated by three species (dark sterile mycelium sp. 1, *Cylindrocarpon destructans*, *Cryptosporiopsis radicicola*) which occurred in more than 30 % of roots of the studied oaks.

The mycobiota of root samples incubated in the moist chamber was composed of thirty-one species, but only about one third of them showed appreciable colonisation frequency. Eight dominant species (*Fusarium solani*, *Sphaerostilbella aureonitens*, *Cylindrocarpon destructans*, *F. proliferatum*, *Ophiostoma* sp. 1., *Penicillium simplicissimum*, *P. glandicola* and *P. purpurogenum* var. *rubrisclerotium*) were recorded in more than 30 % of roots of the studied oaks.

Two species of ophiostomatoid fungi (*Ophiostoma piceae* s. l. and *Ophiostoma* sp.) were observed during this study in roots of healthy and diseased trees.

They were isolated from samples processed by the moist chamber method only. Ophiostomatoid fungi were not recorded in roots of trees numbers four and five, the aboveground parts of which were very disturbed, but one species of these fungi occurred in a root of healthy tree "number one".

The roots of tree number five were rotting and the mycobiota of root samples of this tree by using the moist chamber method was dominated by *Chloridium* cf. *virescens*.

Table 2. Fungi recovered by two different methods from 15 roots of five studied oaks (S – Strong surface sterilisation method, M – Moist chamber method, R – number of tree roots colonised by fungi, R% – percentage of roots colonised by fungi, T – number of trees colonised by fungi).

Species of fungi	Strong surface sterilisation method			Moist chamber method		
	SR	SR%	ST	MR	MR%	MT
Acremonium curvulum W. Gams	2	13.33	2	4	26.67	3
Acremonium persicinum (Nicot) W. Gams	2	13.33	2	3	20	2
Alternaria alternata (Fr.: Fr.) Keissl.				1	6.67	1
Aspergillus niger van Tieghem				2	13.33	2
Aspergillus ustus (Bain.) Thom et Church				2	13.33	2
Chaetomium globosum Kunze: Fr.	3	20	3			
Chloridium cf. *virescens* (Pers.: Fr.) W. Gams et Hol.-Jech.				3	20	1
Chrysosporium sp.				1	6.67	1
Cladosporium cladosporioides (Fresen.) G. A. de Vries	2	13.33	2	2	13.33	2
Cladosporium sphaerospermum Penz.	1	6.67	1			
Clonostachys rosea (Link: Fr.) Schroers, Samuels, Seifert et W. Gams				2	13.33	2
Cryptosporiopsis radicicola T. Kowalski et C. Bartnik	7	46.7	5	2	13.33	2
Cylindrocarpon destructans (Zinssm.) Scholten	8	53.33	4	9	60	5
Cylindrocarpon didymum (Hartig) Wollenw.	3	20	3	3	20	2
Fusarium proliferatum (Matsush.) Nirenberg	2	13.33	2	9	60	5
Fusarium solani (Mart.) Sacc.				11	73.33	5
Fusarium sp. 1				1	6.67	1
Fusarium sp. 2	1	6.67	1			
Fusarium sp. 3				1	6.67	1
Hyalodendron lignicola Diddens	1	6.67	1			
Leptodontidium elatius (F. Mangenot) de Hoog	1	6.67	1			

Table 2. (continuation)

Species of fungi	Strong surface sterilisation method			Moist chamber method		
	SR	SR%	ST	MR	MR%	MT
Monodictys levis (Wilts.) S. Hughes				1	6.67	1
Monodictys putredinis (Wallr.) S. Hughes	3	20	2			
Mucor plumbeus Bon.	1	6.67	1			
Ophiostoma piceae (Münch) H. et P. Sydow s. l.				2	13.33	2
Ophiostoma sp. 1				5	33.33	3
Paecilomyces farinosus (Holm.: Fr.) A. H. S. Br. et G. Sm.	1	6.67	1	1	6.67	1
Paecilomyces lilacinus (Thom) Samson	1	6.67	1	2	13.33	2
Penicillium arenicola Chalab.				1	6.67	1
Penicillium glabrum (Wehmer) Westling				4	26.67	2
Penicillium glandicola (Oudem.) Seifert et Samson				5	33.33	4
Penicillium minioluteum Dierckx	1	6.67	1			
Penicillium purpurogenum Stoll var. *rubrisclerotium* Thom				5	33.33	4
Penicillium simplicissimum (Oudem.) Thom	3	20	3	7	46.67	4
Penicillium viridicatum Westling				1	6.67	1
Pilidium concavum (Desm.) Höhn.				1	6.67	1
Sordaria fimicola (Roberge) Ces. et De Not.				1	6.67	1
Sphaerostilbella aureonitens (Tul.) Seifert et al.	1	6.67	1	10	66.7	5
Stachybotrys chartarum (Ehrenb.) Hughes	1	6.67	1			
Trichocladium opacum (Corda) Hughes	2	13.33	2			
Trichoderma koningii Oudem.	4	26.67	3	3	20	2
Trichoderma stripticilis Bissett	1	6.67	1			
Trichoderma viride Pers.: Fr. agg.	4	26.67	2	4	26.67	4
Ulocladium botrytis Preuss	1	6.67	1			
dark sterile mycelium sp. 1	10	66.67	5			
Number of isolated species			26			31

Seven species from the genus *Penicillium* were isolated during the study. Six of them were recorded by using the moist chamber method and two of them were found by using the strong surface sterilisation method.

DISCUSSION

Strong surface sterilisation, presence of agar medium or absence of these cultivation conditions are responsible for the large differences in composition of the mycobiota recorded by the used methods. A study of the influence of each of these factors would be very useful to find out of their role but the present work wants to compare the methods used to study of the mycobiota associated with oak decline. The remarkable differences between composition of mycobiota incubated by using the two methods were detected. Each of the mentioned method showed a different assembly of dominant species.

Strong surface sterilisation method yields part of the endophytic mycobiota of plant only. Application of other incubation methods (e.g. cultivation on different agar media, incubation in CO_2 atmosphere) is necessary to obtain a complete survey of the composition of the endophytic mycobiota. The moist chamber method reveals the saprophytic mycobiota associated with surface of plants and facultative endophytic fungi.

Cryptosporiopsis radicicola, dark sterile mycelium sp. 1 and *Cylindrocarpon destructans* were the main colonisers of the samples studied using the strong surface sterilisation method. Some of these species were recorded in lower frequency by using the moist chamber method. Kowalski (1983) and Bartnik (1996) studied the mycobiota of roots of dead and living oak trees, respectively, using the strong surface sterilisation method. They recorded most frequently *Trichoderma viride*, *Mycelium radicis atrovirens*, *Cylindrocarpon destructans*, *Coniothyrium fuckelii*, *Phomopsis quercella* and *Cryptosporiopsis radicicola*, respectively.

Fusarium solani, *Sphaerostilbella aureonitens*, *Cylindrocarpon destructans*, *F. proliferatum*, *Ophiostoma* sp. 1., *Penicillium simplicissimum*, *P. glandicola* and *P. purpurogenum* var. *rubrisclerotium* were dominant fungi when using the moist chamber method. They occurred less or not at all when using the strong surface sterilisation method. *Cylindrocarpon destructans* was isolated from more than 50 % of roots when using both methods. A similar composition of dominant species was recorded by Fassatiová et al. (1995) (*Graphium* sp., *Ophiostoma piceae* s. l., *Penicillium glandicola*, *P. glabrum*, *P. minioluteum*, *Trichoderma* sp.) and Novotný (2001) (*Fusarium solani*, *Penicillium glandicola*, *P. glabrum*, *P. simplicissimum*, *Acremonium curvulum*) by using the moist chamber method.

A similar composition of dominant fungi in oak roots at the stands Dešov (present study), Bučina and Na Křivánkách (Novotný 2001) was recorded, but some differences were detected among them. *Fusarium proliferatum*, *Sphaerostilbella aureonitens* and *P. purpurogenum* var. *rubrisclerotium* occurred at Dešov only. *Penicillium daleae*, *P. spinulosum*, *Sesquicillium candelabrum*, *Gliocladium catenulatum* and *Trichoderma atroviride*, which were frequently isolated at Bučina and Na Křivánkách, were not found at Dešov.

Penicillium minioluteum frequently occurred at Bučina, but it was not found at Na Křivánkách (Novotný 2001). At Dešov this species was recorded in stems and roots of oak (Fassatiová et al. 1995). During the present study it was observed at Dešov by using the strong surface sterilisation method only.

Cryptosporiopsis radicicola is a root endophyte of different species of trees (Novotný 2003). It belongs to the dominant species of the endophytic mycobiota of oak roots (Novotný 2002, Bartnik 1996). In the present study, this taxon was recorded not only by using the strong surface sterilisation method but also observed in lower frequency using the by moist chamber method. Fassatiová et al. (1995), Kubátová and Prášil (1995) and Novotný (2001) used the moist chamber method and did not find this species in the mycobiota of oak roots.

Cylindrocarpon destructans is a common fungus of roots of different trees (Fassatiová et al. 1995 (Holdenrieder and Sieber 1992; Kowalski 1983; Fisher and Petrini 1990; Fisher et al. 1991a, 1991b; Kubátová and Prášil 1995; Novotný 2001). It is a dominant species of rhizosphere of *Fraxinus excelsior* (Kubíková 1963). In the present study, this species was recorded very frequently.

Fusarium solani is a common species associated with many species of plants (Booth 1971). It was detected in trees by using both methods (Fassatiová et al. 1995, Fisher et al. 1991b, Kowalski 1991, Novotný 2001, Przybyl 1996 and others), but it was found predominantly by using the moist chamber method (Fassatiová et al. 1995, Novotný 2001). When using the strong surface sterilisation method, *F. solani* was frequently isolated from discoloured or necrotic spots on branches and stems (Bohár 1996, Kowalski 1991, Przybyl 1996, Sieber et al. 1995), but it was rarely observed in trees without such symptoms (Petrini and Fisher 1990, Fisher et al. 1991b). This species is able to cause vessel discoloration of oaks (Bohár 1996). In the present study, this species belongs to the most frequently recorded fungi when using the moist chamber method but it was not found by strong surface sterilisation method. This species appears to be a rarely endophyte of trees and is probably predominantly a saprophyte or parasite of woody plants.

Fusarium proliferatum occurs on different part of woody (Motta et al. 1996, Nireberg 1976, Summerbell 1989) and herbaceous plants (Nireberg 1976). During the present study, this species was discovered in roots of all investigated oaks using the moist chamber method and rarely detected in roots of some oaks using the strong surface sterilisation method. It lives predominantly on the surface of plants. Motta et al. (1996) and Summerbell (1989) observed it on samples of seeds and roots without any surface sterilisation.

Penicillium spp. were recorded in the mycobiota of trees by using both methods (Collado et al. 1996; Fisher et al. 1991a, 1991b; Kowalski 1991; Kubátová 2000; Novotný 2001; Przybyl 1995, 1996; and others) but they were observed more frequently using the moist chamber method. These fungi were not identified to the species level in many of these studies. Novotný (2001) isolated 20 species of

Penicillium from roots of *Quercus robur* and most frequently observed *Penicillium glandicola, P. glabrum, P. simplicissimum, P. spinulosum, P. daleae* and *P. minioluteum*. Kubátová (2000) found 13 species of *Penicillium* in stems, branches and roots of *Quercus robur*. She found most frequently *Penicillium glandicola, P. glabrum* and *P. minioluteum*. Seven species from this genus were also recorded during the present study. *Penicillium simplicissimum, P. purpurogenum* var. *rubrisclerotium* and *P. glandicola* were isolated most frequently. Terrestrial roots of *Alnus glutinosa* harbour some Penicillia too. The species *P. simplicissimum* occurs frequently in the bark of this tree (Fisher et al. 1991b). In the present study *Penicillium* spp. were recorded less frequently using the strong surface sterilisation method than using the moist chamber method. They occur probably on the surface of the roots or may rarely have entered the root tissues.

Sphaerostilbella aureonitens is a fungicolous species (wood decaying fungi) (Seifert 1985), but it was recorded in stems and branches of *Quercus robur*, too (Fassatiová et al. 1995). In the present study, this taxon was frequently found using the method of moist chambers. It is probably associated with mycelium of wood decaying fungi, which colonise the surface of roots or stems and branches.

Dark sterile mycelium sp. 1 occurred very frequently in the studied roots. It is similar to the species *Phialophora* cf. *fastigiata*, which the author isolated during a study of the endophytic mycobiota of *Quercus petraea* in the Křivoklát region (Novotný 2002).

Ophiostomatoid fungi are the most prominent fungi associated with oak decline. They are frequently isolated using the strong surface sterilisation method from necrotic spots in bark (Kowalski 1991, 1996; Przybyl 1995), but they are seldom observed in wood or bark without any necrotic spots (Halmschlager et al. 1993, Holdenrieder and Sieber 1992, Przybyl 1995). These fungi are frequently recorded using the moist chamber method in bark or wood without any necrosis (Eisenhauer 1991, Kubátová and Prášil 1995, Novotný 2001, Prášil et al. 1998). Sieber et al. (1995) did not discover ophiostomatoid fungi in stems of studied living oaks, but they observed them after three weeks on the stumps. In the present study, ophiostomatoid fungi were isolated using the moist chamber method from six roots without necrosis, but were not recorded using the strong surface sterilisation method in the same roots. Prášil et al. (1998) obtained similar results in the study of the mycobiota of oak branches. They performed surface sterilisation of samples of branches by fire (dipping in ethanol and then sterilised by flame) and placed the samples in a moist chamber. No ophiostomatoid fungi were recorded on these branches. In the author's opinion, spores of these species occur in soil or on the surface of roots or branches and a sterilisation solution or fire kill them. These fungi probably predominantly colonise bark and wood of dead parts (branches, roots, stems) of trees. Some species of *Ceratocystis, Graphium* and *Leptographium* were isolated from soil (Harrington 1992, Kubátová and Váňová 2001, Tainter 1992).

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