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The health state of *Ginkgo biloba* L. in the presence of microfungi

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Abstract: The health state of *Ginkgo biloba* L. and damage caused by microscopic fungi were evaluated over the 2010–2011 time period, in selected localities of Slovakia and Czechia. The trees were assessed and put into two categories of health. Trees in very good (category 1) or good vitality and health with no or only light damage (category 2). A total of seven species of microscopic fungi were identified from samples taken from branches, fruits, and leaves. The following fungal genera were detected: *Epicoccum*, *Fusarium*, *Alternaria*, *Phomopsis*, *Cylindrosporium*, *Phyllosticta*, and *Cladosporium*. This present study is the first report about microscopic fungi determined on *G. biloba* for Slovakia.

Key words: damage, fungi, *Ginkgo biloba*, maidenhair tree

Introduction

Ginkgo biloba L., also called the ginkgo tree or maidenhair tree, is the sole surviving species of the genus *Ginkgo*, which belongs to the Ginkgoaceae family. Ginkgo is a long-lived tree (Commbes 1992).

According to Benčať (1982), *G. biloba* has been found on 80 localities in Slovakia. The ginkgo tree, *G. biloba*, is extra resistant not only to climatic changes, but also to natural elements. This tree is also extremely tolerant of air pollution, and is often planted in harsh city environments where most trees will not survive. The ginkgo has developed an amazing resistance to diseases and parasites, fungi, and other enemies of plants. In the young trees, the greatest harm comes from humans who irregularly water, and keep the young plants in high temperatures (direct sunlight) (Orwa *et al*. 2009).

"The Index of Plant Diseases in the United States" lists the following diseases for *G. biloba*: leaf spots, *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk (antracnose), *Phyllosticta ginkgo* Brunaud, sapwood or wound rot, *Fomes conchatus* (Pers.) Gillet, *Oxyporus populinus* (Schumach.) Donk, and *Polyporus* spp. (sometimes found on living trees following injuries). The basidiomycete *Bartheletia paradoxa* G. Arnaud (Scheuer *et al*. 2008) was redescribed based on freshly-fallen, collected leaf specimens of *G. biloba*.

The aim of this study was to evaluate the health state of *G. biloba* trees and to identify and characterise the microscopic fungi in different organs of this tree. This present study about damage and occurrence of microscopic fungi on *G. biloba* is the first report for Slovakia.

Materials and Methods

The health state of *G. biloba* and damage caused by microscopic fungi were evaluated from 2010 to 2011, in selected localities in Slovakia and Czechia (together in 17 localities). The health state evaluation was done visually according to Hrubík *et al*. (2011) during August and September. The categories for health state evaluation and their explanation are described in table 1. An inventory methodology for the field survey and for harmful factors was used for determination of the fungal diversity.

All the pathological changes on trees were noticed during the field surveys. Samples were taken from leaves, fruits, and branches. The samples were examined visually and microscopically in a laboratory.

To release spores from fruiting bodies in host tissues, damping with distilled water in a damp chamber was performed at room temperature. After damping with distilled water, and after 1 day of incubation, the fruiting bodies were removed for microscopic examination. The samples were observed under the binocular microscope.

The fungi were isolated from the collected samples. The samples with mycelium and/or stromata were surface disinfected in 0.15% NaOCl for 20 min and then washed in distilled water. A maltose agar medium (3%) was used for cultivation. The plates were incubated at 25°C in the dark.

The fungi were examined immediately for fungal fruiting structures, and fungal colonies growing in culture. The fungi were identified to the genus level and the species level where possible, based on cultural and morphological characteristics.

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Table 1. Scale for classification of qualitative signs on evaluated trees

Category	Vitality	Health state	Damage	Safety	Horticultural value
1	very good	very good	no	safe	very high
2	good	good	light	moderately dangerous	high
3	adequate	sufficient	medium light	medium dangerous	average
4	weak	bad	heavy	excessively dangerous	low
5	excessively weak	criticaldead	excessively heavy	critical	very low

According to Hrubík *et al*. (2011)

Table 2. List of evaluated localities, number of trees, and their diameter and health state

Locality	Coordinates	Number of trees	Health state*	Diameter of trees [cm]
Praha	N 50°0514′ E 14°2515′	20	1	124–280
Beladice	N 48°2042′ E 18°1737′	2	1	192–198
Bystrany	N 48°5658′ E 20°4528′	1	1	179
Galanta	N 48°1122′ E 17°4336′	2	1	267–285
Nenince	N 48°0850′ E 19°1549′	1	2	194
Hnúšťa	N 48°3444′ E 19°5713′	1	1	254
Jaklovce	N 48°5225′ E 20°5933′	1	1	203
Komárno	N 47°4544′ E 18°0750′	1	1	62
Komjatice	N 48°0925′ E 18°1012′	1	2	320
Košice	N 48°4314′ E 21°1527′	9	1	160–345
Malinovo	N 48°0927′ E 17°1756′	1	2	38
Modra	N 48°2003′ E 17°1825′	1	1	193
Nová Ves nad Žitavou	N 48°1721′ E 18°1922′	2	1	219–280
Palárikovo	N 48°0225′ E 18°0415′	3	1	55–238
Tomášov	N 48°0832′ E 17°2011′	1	1	280
Trenčín	N 48°5337′ E 18°0216′	3	1	202–250
Veľký Blh	N 48°2641′ E 20°0701′	1	2	183

*according to Hrubík *et al*. (2011) indicated in table 1

Spores proportions were measured using QuickPhotoMicro 2.2 software under 200 and 400 times enlargement. Enlarged scanned pictures of spores were used for measuring with an accuracy of 0.1 μm. The identification was performed using morphological keys.

Results and Discussion

All together, 54 *G. biloba* trees were evaluated from 17 different localities (Table 2). The trees were healthy and were assigned to category 1 or 2. These categories took into consideration whether the trees were in very good or good vitality, and what the health state of the trees was,

and whether there was no damage or light damage. A total of seven species of microscopic fungi were identified from samples taken from branches, fruits, and leaves.

The following fungi were identified:

1) *Epicoccum nigrum* Link
This fungus was detected immediately from the samples of the collected leaves. After isolation, the fungus was also identified in the fungal culture. It was very common, and it was observed in 12 localities out of the 17 evaluated localities (Table 3).

Conidiophores are slightly pigmented, and densely compacted. Conidia are formed singly, are globose pyriform, l5–25 µm in diameter, with a funnel-shaped base and broad attachment scar, often seceding with a protuberant basal cell. Conidia are multicellular (dictyoconidia), darkly pigmented, and have a verrucose external surface.

Colonies in culture proved to be fast-growing, suede-like to downy, with a strong yellow to orange--brown pigmentation. When sporulating, numerous black sporodochia were visible.

Bánhegyi *et al.* (1987) described this fungus on different dead or weakened plant tissues as conidia rounded, with transverse and longitudinal septa, and sized 7–65 × 6–54 µm.

The fungus was determined on the leaves of *G. biloba* in Germany (Lotz-Winter 2011) and also in the USA (Neely 1959).

2) *Alternaria alternata* (Fr.) Keissl.
Alternaria was the second most commonly identified fungus. It was determined in 10 localities (Table 3). *Alternaria* was identified immediately from samples and partly after isolation in culture. It was isolated from leaves and also from fruits. The fungus causes black spots in leaves and also on fruits.

Conidia are 30–300 µm long, club and pear shaped or ovate in shape, dry and thick-walled, and multicellular. They are often both transverse and longitudinal or have oblique septa, and often have a long tapered apical cell. The development of apical extensions was minimal.

Colonies in culture were olive green to olive grey with a white margin. The colony texture was woolly to cottony.

Two species of *Alternaria* were determined on leaves of *G. biloba*. In China, both species were determined: *A. alternata*, *A. tenuissima* (Kunze) Wiltshire (Zhang 2003a). Leaf spot on *Ginkgo* caused by *A. alternata* was also identified in India (Upadhyaya *et al.* 2008).

3) *Fusarium* sp.
This fungus was isolated from cracks on *Ginkgo* branches. It was identified only in one locality out of 17 (Table 3).

Colonies in culture were fast growing, pale or brightly coloured and had a cottony aerial mycelium. *Fusarium* produces colourless, elongate, multicelled conidia, which usually have curved and pointed ends and are sickle-shaped. Conidia with 1-septum are 18 × × 3 µm, with three and more septa 23–28 × 3–4 µm. The species are distinguished on the basis of characteristics in culture: size and shape of conidiophores; size, shape, and septation of conidia; formation of chlamydospores; and pigment formation (Sinclair and Lyon 2005). A determination into the species was not carried out during this research. All *Fusarium* species are anamorphs of ascomycetes in the Hypocreales (Sinclair and Lyon 2005).

Fusarium causes cankers and dieback of trees and shrubs. *Fusarium* species that cause plant diseases also include strains that are secondary invaders of weakened or dying plants. For this reason, a diagnosis of *Fusarium*-associated tree diseases often requires pathogenicity tests (Sinclair and Lyon 2005).

On *G. biloba*, the fungus *Fusarium* was identified in China (Chen 2002) and also in Japan (Kobayashi 2007), and from tissue explants from brown spots of *Ginkgo* tea flakes in the USA (Kuddus *et al*. 2008).

4) *Phomopsis occulta* var. *ginkgoina* Grove
This fungus was determined from fruits after isolation in one locality. The pycnidia are formed in dead tissues and are greyish black. For this genus, it is typical to find a formation of two kinds of conidia (α and β conidia) in each pycnidium. Both kinds of spores are

Table 3. List of determined microscopic fungi from different plant organs of *Ginkgo biloba* (determination culture – fungi identified from colonies growing culture, determination immediately – fungi identified from fungal fruiting structures in plant tissues)

Fungal genus	Plant tissue	Determination	Locality
Fusarium	branch	culture	P
Epicoccum	leaf	culture, immediately	Be, G, H, J, Km, Kj, Kc, Ma, Mo, Pa, To, Tr
Alternaria	fruit, leaf	culture, immediately	Be, G, H, J, Kj, Kc, Ma, Ne, No, Tr
Phomopsis	fruit	culture	Tr
Cylindrosporium	leaf	immediately	G, H, Km, Pa, To, VB
Phyllosticta	leaf	immediately	Kc
Cladosporium	leaf	immediately	By

P – Praha, Be – Beladice, By – Bystrany, G – Galanta, H – Hnúšťa, J – Jaklovce, Km – Komárno, Kj – Komjatice, Kc – Košice, Ma – Malinovo, Mo – Modra, Ne – Nenice, No – Nová Ves nad Žitavou, Pa – Palárikovo, To – Tomášov, Tr – Trenčín, VB – Veľký Blh

one celled and hyaline. The α conidia are ellipsoid to spindle-shaped cells, and usually contain two oil guttules – one near each end of the spores. The α conidia easily germinate, and their size is 5–8 × 2–3 μm. The β conidia are curved, linear cells that do not germinate, and their size is 20–29 × 1 μm.

Two species of *Phomopsis* were identified on *Ginkgo*: *P. ginkgonis* Z.D. Jiang, C.Q. Chang & M.M. Xiang (Chi *et al*. 2007), and *P. occulta* var. *ginkgoina* (Grove 1935; Mulenko *et al*. 2008). *Phomopsis occulta* var. *ginkgoina* occurred on twigs of *G. biloba* in Britain (Grove 1935). Grove (1935) described α spores as ellipsoid-oblong, often tapering below and obtuse above, biguttulate, hyaline 5–8 × 2 μm, β conidia straight or curved, rarely hooked, 18–25 × 1–1.5 μm. This species was recently determined in Poland (Mulenko *et al*. 2008).

5) *Cylindrosporium* sp.
The fungal species was detected immediately from leaves in samples collected from six localities (Table 3). Isolation was not successful.

The acervuli are discoid, separately formed, but sometimes can form a blended white layer and are under the epidermis. Spores are filiform, hyaline, often are flexuous or curved. They are aseptate, 18–33 × × 2–4(–5) μm. The fungus causes leaf spots. The teleomorph state where *Cylindrosporium* species belong is genus *Mycosphaerella*.

Cylindrosporium has not been described on *G. biloba* yet. Only one record of a teleomorph state has been published. Kobayashi (2007) described *Mycosphaerella* sp. on this host tree in Japan.

6) *Phyllosticta* sp.
The fungus was identified in one evaluated locality (Košice). It causes leaf spots. Lesions caused by *Phyllosticta* are round to angular or irregular spots, 1–6 mm across with tan to grey centres and with brown or reddish brown borders. Some spots become fragile and may drop out of mature lesions, leaving ragged holes in the leaf. Pycnidia arise in the tan portion of older lesions, and are black. Spores are small, ovoid, one celled, hyaline, and are 8–9 × 3–4 μm.

Compared to other fungi detected during this research, *Phyllosticta* leaf spot is quite commonly identified around the world – in China (Chen 2002), Japan (Kobayashi 2007), Korea (Cho and Shin 2004), the USA (Alfieri *et al*. 1984), Poland (Mulenko *et al*. 2008), Italy and Russia (Spaulding 1961). Three species of *Phyllosticta* were described on *Ginkgo*: *P. capitalensis* Henn., *P. ginkgo* Brunaud, and *P. salisburiae* Tassi. During the recent research concerning phylogenetic analyses of *Phyllosticta* species in Japan, *P. capitalensis* was detected on *Ginkgo* (Motohashi *et al*. 2009), which is the anamoprh state of *Guignardia endophyllicola* Okane, Nakagiri & Tad. Spots caused by P. *capitalensis* are oblong, pale, with a brown margin. Pycnidia are epiphyllous, gregarious, subglobose to lenticular, and black. Spores are subglobose to ellipsoidal, hyaline, and 6–8 × 5.5–6 μm (Saccardo 1931). *Phyllosticta* was also determined on other woody plant species (Motohashi *et al*. 2009).

In France, *P. ginkgo* was detected on the leaves of *G. biloba* (Berlese and Vogline 1886), and recently in China (Tai 1979; Bai 2000), the USA (Anonymous 1960), Korea (Cho and Shin 2004), Poland (Mulenko *et al*. 2008), and in the former USSR (Spaulding 1961). Berlese and Vogline (1886) described the spots caused by fungus as small, point form and black; spores small, ovoid, hyaline, 3–8.5 × 2 μm.

The third *Phyllosticta* species (*P. salisburiae*) was detected on the leaves of *G. biloba* in Italy (Sacardo 1902; Spaulding 1961), and recently in Poland (Mulenko *et al*. 2008). Saccardo (1902) described the spots caused by this fungus as wide-ranging, irregular, with creamy margins and dry centres; fruiting bodies are isolated, globose and black, and are 100 μm in diameter; spores are ellipsoid, hyaline, with two small droplets, and are 6–7 × 3 μm large. Brandenburger (1982) described two species of *Phyllosticta* (*P. ginkgo, P. salisburiae*) on the leaves of *Ginkgo* in no specified localities in Europe.

7) *Cladosporium* sp.
The fungus has been determined only in one locality, Bystrany (Table 3), and was detected immediately from leaves. The spores are pale brown, ovoid to ellipsoidal, 0–3-septate. The sizes of conidia without septum are 10–15 × 4–7 μm, with one septum 14–20 × 6–8 μm, with two septa 23 × 7–8 μm, and with three septa 21–25 × 7–9 μm. The most frequently found were conidia 0–1-septate.

Conidia are at first round. Later they are continuously typically 1-septate, ovoid, sometimes in chains at first, and also can be 2–3-septate (Saccardo 1886). Two different species of *Cladosporium* [*C. herbarum* (Pers.: Fr.) Link, *C. cladosporioides* (Fresen.) G.A. de Vries] have been detected on the maidenhair tree. The conidia of *C. herbarum* are near at the apex of growing hyphae, pale brown to olive, and variable in shape and size; oblong, ovoid, smooth, 1–3-septate, constricted at the septum (Saccardo 1886). Schubert *et al*. (2007) minutely described the morphological characteristics of *C. herbarum*. Intercalary conidia ellipsoid to cylindrical, 6–16 × 4–6 μm, 0–1-septate, secondary ramoconidia ellipsoid to cylindrical-oblong, 12–25(–35) × (3–)5–7(–9) μm, 0–1(–2)-septate, rarely with up to three septa, sometimes distinctly constricted at the septum, pale greyish brown or brown to medium brown or greyish brown (Schubert *et al*. 2007). According to Vries (1952), the size of 1-celled conidia of *C. herbarum* is (1)4.5–11(19) × (2)4–5(7) μm. The fungus was found on fading and decaying plant material as well as on living leaves, but also as a secondary invader, as an endophyte, and was isolated from numerous other materials. It is a cosmopolitan. Under favourable climatic conditions, *C. herbarum* also germinates and grows as an epiphyte on the surface of green, healthy leaves (Schubert *et al*. 2007). It was found on leaves of *G. biloba* in China (Zhang 2003b; Zhuang 2005). Also the other *Cladosporium* sp., *C. cladosporioides*, was detected on *G. biloba* in China (Zhang 2003b). *Cladosporium* sp. was detected

in two localities: Lijiang in Yunnan Province (in southwestern China) and Lanzhou in Gansu Province (in northwestern China) (Zhang 2003b). The most striking differences from *C. herbarum* are the smaller, usually 1-celled [2–7(11) × (1)2–4(6) µm], smooth conidia, the greater number of conidia per conidial head, the denser ramification of the conidial chains, and the absence of prolongations and inflations of the conidiophores. These morphological differences were considered sufficient enough to justify the establishment of *C. cladosporioides* as a distinct species (Vries 1952).

Kuddus *et al*. (2008) isolated the fungi *Phoma* sp. and *Curvularia* sp. from the affected leaves of *G. biloba* in the middle of the growth season. Also the fungi *Aspergillus* sp. and *Curvularia* sp. were isolated from brown spots of apparently healthy leaves. *Aspergillus* sp., *Chaetomium* sp., *Fusarium* sp., *Penicillium* sp., and *Aureobasidium* sp. were isolated from tissue explants from brown spots of *Ginkgo* tea flakes (Kuddus *et al*. 2008).

Many factors influence the incidence of leaf blight disease, such as *Ginkgo* varieties, tree years, gender, orchard location – including slope direction as well as soil condition, and cultivation measures – including water and fertiliser management, pruning and clearing, and climatic factors – including temperature and rainfall. Temperature and rainfall are the main factors affecting the leaf blight disease development. Cultivating improved varieties and fit stocks. Choosing a favourable orchard location and enhancing water and fertiliser management are effective ways to deter the disease (Zhiquan *et al*. 2001).

Although *G. biloba* is remarkably resistant to microbial colonisation (Major 1967; Huang *et al*. 2000), fungal colonisation of young *G. biloba* trees appears to be common because fungicides are routinely used in the agro-industrial propagation of *Ginkgo* (DeFeudis 1998). Previous reports indicated that several fungi (such as *Glomerella cingulata*, *P. ginkgo*, and *Epicoccum purpurascens* Ehrenb.) may colonise live *G. biloba* leaves (Hepting 1971; Hartman *et al*. 2000). It is possible, that fungi simply colonised tissues of environmentally stressed leaves or existing brown spots of the leaves of live trees without causing a primary infection. Whether any of the fungi we isolated are primary pathogens of *Ginkgo* trees remains to be investigated.

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