

Isolation of medically important fungi from Ginkgo biloba leaves and crude ginkgo supplements

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Citation

R Kuddus, J Oakes, C Sharp, J Scott, K Slater, J Kirs, O Kopp, W Burt. *Isolation of medically important fungi from Ginkgo biloba leaves and crude ginkgo supplements*. The Internet Journal of Microbiology. 2007 Volume 5 Number 2.

Abstract

We observed fungal growth in Ginkgo biloba leaves in the middle of the growth season and isolated *Phoma* sp. and *Curvularia* sp. from the affected leaves. We also isolated *Aspergillus* sp. and *Curvularia* sp. from brown spots of apparently healthy leaves; *Aspergillus* sp., *Chaetomium* sp., *Fusarium* sp., *Penicillium* sp. and *Aureobasidium* sp. from tissue explants from brown spots of Ginkgo tea flakes (from three different suppliers); and *Chaetomium*

sp., *Penicillium*

sp., *Aspergillus* sp. and *Aureobasidium pululans* from cold-brewed Ginkgo flake tea (from one supplier). Fungus was not recovered from any of the eight samples of hot-brewed (at 75°C or 90°C for 5 minutes) Ginkgo tea tested, cold- or hot-brewed powdered Ginkgo tea (from two suppliers) and three different brands of Ginkgo tea bags. This data indicated that *G. biloba* leaves and crude supplements derived from crushed Ginkgo leaves could be contaminated by fungi known to be opportunistic pathogens.

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The source of any support received: Partially supported by a grant from UVSC Foundation, Orem, UT to RHK.

INTRODUCTION

Ginkgo biloba is among the most ancient surviving trees. The tree is remarkably resistant to microbial infections (Major, 1967; Huang et al., 2000; Mazzanti et al., 2000). While examining genetic diversity of Ginkgo trees introduced to the United States (Kuddus et al., 2002) we observed fungal growth in prematurely dying leaves of some adult Ginkgo trees. We also observed brown spots and mildew-like growth in the leaves of Ginkgo trees (all in the middle of Ginkgo growth season) and isolated fungi from the affected tissues.

Various *G. biloba* preparations are used in complementary and alternative medicines (CAM) as well as (experimental) conventional medicine (reviewed in DeFeudis, 1998; Sierpina et al., 2003; Kuddus, 2005; Carlson et al., 2007). Ginkgo products come as crude preparations such as Ginkgo tea and capsules or in the form of pharmacologically standardized extracts. Crude supplements such as Ginkgo tea

are made simply by crushing dried Ginkgo leaves. Thus microbes (and their toxins) present in leaf tissues may also be present in the supplements. Since *G. biloba* is known to be highly resistant to microbial infections, infection or intoxication risks from consumption of *G. biloba* supplements could be underestimated. Use of CAM has increased significantly and *G. biloba* preparations are among the highest selling CAM (Eisenberg et al., 1998; Jones, 2007). Here we describe isolation of fungi, including some known opportunistic pathogens, from both live and prematurely dying *G. biloba* leaves and crude *G. biloba* supplements.

MATERIALS AND METHODS

FIELD WORK

Two urban *G. biloba* populations, one located at Pittsburgh, Pennsylvania (Lat 40° 21' N, Long 79° 55' W, Alt 382 meter) and another at Orem, Utah (Lat 40° 17' N, Long 111° 41' W, Alt 1448 m) were observed year-round for about two years (June 2003 to May 2006). *G. biloba* leaves emerge in February-March and become senescent by late October to early November in both regions. Trees with excessive numbers of prematurely dry leaves before August and green leaves with visible fungal growth (before September) were collected and inspected by stereomicroscope. Loose Ginkgo

tea flakes (from three suppliers), loose powdered tea (two suppliers) and individually bagged Ginkgo tea (three brands) were purchased from local health food stores (between June 2005 and June 2007).

TISSUE STAINING FOR DETECTION AND ISOLATION OF FUNGI

Affected leaves, tea flakes and healthy leaves (as control) were cleared of superficial microbes by boiling in 2.5% KOH. The treated leaves were stained with trypan blue and counter-stained with Sudan IV to detect intercellular fungi as described previously (Barrow and Aaltonen, 2004). The treated samples were examined using a Labomed CXR3 microscope at 400-1,000x magnification and photographed. At least 20 fields were examined for each leaf sample.

To isolate fungi, leaves or tea flakes were initially cleaned using sodium hypochlorite, distilled water and ethyl alcohol as described by Tuite (1969). Tissue explants were harvested aseptically under a dissection microscope and then placed on potato dextrose agar (PDA) plates. Fungi in brewed loose Ginkgo tea were examined as described previously (Halt, 1998; Wilson et al., 2004). Pre-packed tea bags were brewed in hot (rolling-boiled) distilled water in sterilized cups following standard brewing methods (Rombauer et al., 2006). Tea was steeped for 2-3 minutes and the brew was serially diluted with sterile water and 0.1 ml was plated in two PDA plates. Similarly treated sterile distilled or tap water was plated as the controls. The plates were incubated at room temperature for 4-10 days. All experiments were done at least twice. Fungal (and bacterial) colonies were counted as colony forming units (cfu)/gm of tea using the formula- (number of colonies multiplied by dilution factor multiplied by total volume of water used in brewing)/grams of tea used.

IDENTIFICATION OF FUNGI

Fungal hyphae were transferred to new plates to establish pure culture and then identified to the level of genera using conventional methods (data not shown). *Phoma* sp. and *Curvularia* sp. were identified to species level by cloning and sequencing 18s rDNA. DNA was extracted from hyphae using cetyl trimethylammonium bromide extraction method as described (Carlson et al., 1991). Crude DNA was further cleaned using GenElute Plant Genomic DNA Miniprep DNA kit (Sigma, Saint Louis, MO). A ~900bp fragment of 18S rDNA was PCR-amplified using primers (5'GCAAGTCTGGTGCCAGCAGCC3' and 5'GCATCACACCTGTTATTGCCCT3') as described

previously (White et al., 1990). The amplified PCR products were resolved in 1% agarose gel (data not shown), gel-purified using QIAquick Gel Extraction kit (Qiagen, Santa Clarita, CA) and cloned in pCR4-TOPO cloning vector (Invitrogen, Carlsbad, CA). DNA extracted from at least three independent transformed bacterial colonies harboring the recombinant pCR4-TOPO plasmids (carrying fungal rDNA inserts) was sequenced from both ends of the inserts using an external vendor (Gene Gateway, Hayward, CA). The electropherograms of the sequencing reactions were studied carefully using Chromas software (<http://www.techneleysium.com.au/>). The DNA sequences were analyzed using BLAST (<http://www.ncbi.nlm.nih.gov/>) and MultAlin software (<http://bioinfo.genopole-toulouse.prd.fr/multalin/>). Bacteria (mostly *Bacillus* sp.), non-sporulating coenocytic fungi and yeasts that grew from some samples were not identified.

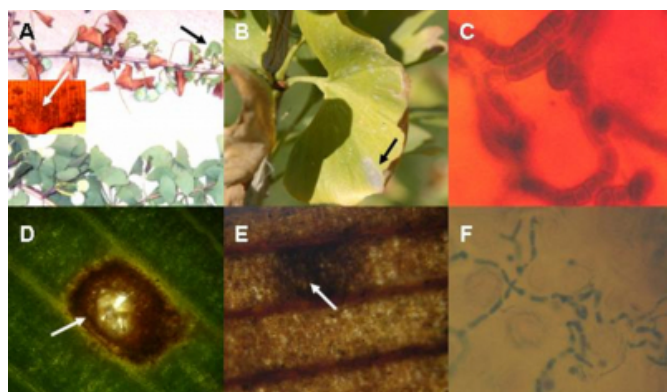
RESULTS

Several adult trees (about 20 meters in height) in Pittsburgh were found with drying leaves in June and July (midway of the growth season). The affected female trees had green seeds yet the plants were attempting to sprout new buds (Fig. 1A). Normally *G. biloba* sprouts buds only once and very early (March-April) in the growth season. Leaf samples were isolated from three different affected trees for further examination. In August of the same year, a young *G. biloba* tree (about two meters in height) was found at Orem, Utah having green leaves with drying margins and mildew-like growth in the green tissues (Fig. 1B). Stereomicroscopic examination of the leaves confirmed fungal growth in the leaves of the affected trees from both locations (Fig. 1A, inset). Endophyte staining also revealed the presence of fungi in the tissue of the affected leaves (Fig. 1C). When aseptically removed tissue explants from the affected leaves were placed on PDA plates, fungi grew profusely (data not shown). Fungus grown from leaves of the affected trees from Pennsylvania was identified as *Phoma* sp. (data not shown). The sequence of an 888 bp fragment of 18s rDNA cloned from this sample (GenBank Accession Number EF152573) was identical to the 18s rDNA sequence of *P. herbarum*. Fungus grown from the affected leaves of tree from Utah was identified as *Curvularia* sp. (data not shown) and the sequence of an 887bp fragment of 18s rDNA cloned from this sample (GenBank Accession Number EF152574) was >95% similar to the 18s rDNA sequence of *C. brachyspora*. In the following growing season, the affected trees at Pittsburg recovered and produced leaves and cones but the affected young tree at Orem attempted to recover but died by

the month of June, the middle of the growth season (data not shown).

Figure 1

Figure 1: Presence of fungi in leaves and tea flakes. Photograph showing twigs of an affected tree (top) and an unaffected tree (bottom) from Pittsburgh, PA. Note the presence of dry leaves and wilted seeds along with (newly sprouted) young leaves in the affected twig (indicated by an arrow). Stereomicroscopic image (x4) of an affected leaf is shown in the inset. Photograph showing twig of an affected tree from Orem, UT (mildew-like growth on leaves is indicated by arrows). Fungal hyphae in the tissue of a affected leaf of a tree from 8 Pittsburgh, PA (x1,000). Brown spots in healthy leaf (x40). Brown spots in Ginkgo tea flakes (x40). Tea flake stained for endophytes and examined microscopically. Fungal hyphae are shown growing in and around stomata (x400).



We observed the presence of brown spots in many healthy adult green leaves of every *G. biloba* tree we examined in July and August (Fig. 1D and data not shown) in both locations. Similar spots were also present in some Ginkgo tea flakes (Fig. 1E). Endophyte staining revealed the presence of fungal hyphae in the tea flakes (Fig. 1F and data not shown). Fungi grew out when aseptically removed tissue explants from the brown spots of live leaves or the flakes were placed on PDA plates (data not shown). Fungi recovered from brown spots of live *G. biloba* trees were identified as *Aspergillus* sp. and *Curvularia* sp. All three flake tea samples examined were positive for fungal growth (from tissue explants). The fungi were identified as *Aspergillus* sp., *Chaetomeum* sp., *Fusarium* sp. and *Penicillium* sp.

To test if microbes are present in the brewed Ginkgo tea, cold- and hot-brewed tea samples were plated in PDA plates. Fungi grew out from cold-brewed tea derived from one of the three flake tea samples and the fungal load was estimated to 8×10^3 fungal cfu/gm of tea. All water controls for cold brewing experiments were negative for fungal growth. The

sporulating fungi were identified as *Chaetomium* sp., *Penicillium* sp. *Aspergillus* sp. and *Aureobasidium pululans*. No fungus was recovered from hot-brewed loose flake tea, cold or hot-brewed loose powdered tea or bagged tea samples.

DISCUSSION

Although *G. biloba* is remarkably resistant to microbial colonization (Major, 1967; Huang et al., 2000), fungal colonization 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 *Clomerella cingulatea*, *Phyllosticta ginkgo*, and *Epicoccum purpurascens*) may colonize live *G. biloba* leaves (Hepting, 1971; Hartman et al., 2000). It is possible that the fungi simply occupied (colonized) 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 remained to be investigated. However, if the fungi could survive in the leaf tissues, the microbes and their metabolites (mycotoxins) could pass on to the crude CAM derived from such leaves (Rader et al., 2007).

We observed that brown spots are common in adult healthy Ginkgo leaves particularly during the later half (August-September) of the growing season. Brown spots were also common in Ginkgo tea flakes sold in health food stores. We isolated several species of fungi from aseptically explanted tissues of such brown spots and cold-brewed Ginkgo tea derived from one of the three flake tea brands tested. Cold-brewed teas from two other samples were negative for fungal growth although staining showed presence of fungus in both of tea flake samples. No fungi grew from hot-brewed tea. This data indicated that the colonizing fungi are not readily released by the standard brewing conditions.

We also noticed that bacteria (and occasionally yeast) grew from both cold and hot-brewed tea derived from all three flake Ginkgo tea samples and two of the three bagged ginkgo tea samples with bacterial count ranging 0.1×10^3 - 1.1×10^6 bacterial cfu/gm of tea (data not shown). No bacterial growth was observed in cold or hot brewed tea derived from the powdered loose Ginkgo tea and one of the three brands of bagged ginkgo tea (data not shown). Wilson et al. (2004) reported the presence of bacteria at 1×10^1 - 1.6×10^6 cfu/100 ml of herbal tea brewed for 5 minutes at 90°C. Bacteria we isolated were mostly spore

formers (*Bacillus* sp.). An investigation of bacterial contamination and presence of mycotoxins in ginkgo products is currently being planned. Bacterial contamination of CAM is quite common and considered a significant health hazard (Bouakline et al., 2000; Kneifel et al., 2000; Raman et al., 2004; Wilson et al., 2004; Tournas et al., 2006).

All of the fungal species we isolated from live *G. biloba* leaves and supplements have been previously reported to cause (fatal) mycoses (Everett et al., 2003; Tessari et al., 2003; Teixeira et al., 2003; Varghese et al., 2004; Mohammedi et al., 2005; Petit et al., 2005). Microbial contamination of CAM could be an infection risk to immunocompromised populations (Halt, 1998; Bouakline et al., 2000; Kneifel et al., 2000; Wilson et al., 2004) given that people with poor health are among the major consumers of CAM (Austin, 1998; Upchurch and Chyu, 2004). Manufacturing of microbe-free Ginkgo supplements is possible as three of the Ginkgo tea brands we examined were found free of viable microbes (although not shown on the labels, it is possible that some of the tea samples were treated with antimicrobial agents). Dry herbs can be radio-sterilized without significant loss of active ingredients (Crawford and Ruff, 1996; Murcia et al., 2004). With the rapid increase in the use of CAM, appropriate quality control, some oversight and public education could be helpful in protecting the consumers and safeguard the industries.

ACKNOWLEDGEMENTS

This work supported in part by UVU Presidential and Foundation grants to RHK. The authors are grateful to Drs. S. Rushforth, R. Van Buren, M. Bracken, and R. Robbins for helpful discussions and Dr. B. Barger for help with photography.

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