

THE DETERMINATION OF MICROSCOPIC FUNGI FROM CHESTNUT (*Castanea sativa* Mill.) FRUITS, LEAVES, CRUST AND POLLEN

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Abstract

KAČÁNIOVÁ, M., SUDZINOVÁ, J., KADÁSI-HORÁKOVÁ, M., VALŠÍKOVÁ, M., KRÁČMAR, S.: *The determination of microscopic fungi from Chestnut (Castanea sativa Mill.) fruits, leaves, crust and pollen.* Acta univ. agric. et silvic. Mendel. Brun., 2010, LVIII, No. 4, pp. 73–78

The plant-microbial interactive relations with respect to determination of the mycoflora of the *Castanea sativa* Mill. nuts, crust, leaves and pollen and their effect on the host organism in four Slovak regions were studied. In the experiments were isolated 7 genera and 10 species of microscopic fungi from the nut, crust and leaves. It was found, that isolates from the *Castanea sativa* Mill. pollen were represented by 8 genera and 11 species of microscopic fungi. *Alternaria*, *Cladosporium*, *Mucor* and *Rhizopus* appeared to be the most frequently occurring genera of nuts, leaves and crust. *Acremonium*, *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma* are the most frequently fungi of pollen. On the base of further taxonomic determination from the genera *Aspergillus* were isolated and identified representatives of species *A. flavus*, *A. fumigatus*, *A. ochraceus*, *A. terreus* and *A. versicolor*. From the genera *Fusarium* was isolated *F. oxysporum* and from *Penicillium* genera were isolated *P. crustosum* and *P. glabrum*. It is necessary to underline that the isolated genera *Aspergillus*, *Fusarium* and *Penicillium* are considered as the most important producers of mycotoxins.

Castanea sativa Mill., nuts, leaves, crust, pollen, microscopic fungi

The chestnut fruits produced in Slovakia are generally of small size and of lower quality because of less favorable climatic conditions and absence of breeding and selection work.

The European chestnut trees spread all over the world and produce wood and chestnuts that have considerable economical value. Specific literature describes some utilization for chestnuts in Europe being emphasized the marrons glaces production; some references also relate their use in baking, mainly under economically difficult condition (Demiate et al., 2001). Also it is generally known, that the main component of chestnuts is starch, there are a little studies related to chemical composition of the chestnuts. Many studies are oriented to the agricultural features of the trees and especially to the phytopathology (Rutter et al., 1991).

Moulds are distributed in nature ubiquitously and their spores can be found in the atmosphere even at high altitudes. They are drive and disseminate by

wind and air currents, as well as can be spread by insects, rodents, and other animals. The metabolic activities accompanying the growth and mould development decomposes organic substrate ensuring the recycling of elements that comprise organic matters. Food products, being organic substances and containing essential nutrients, are very suitable substrates for the mould growth (Leoni et al., 2001; Saleemullah et al., 2006; Carmo et al., 2008). Because of their hydrolytic enzymes powerful arsenal, moulds may cause a high degree of deterioration when they occur in/on foods and can be in charge of considerable economic losses (Overy et al., 2003; Kawashima and Valente Soares, 2006). Besides the possible food decaying caused by moulds and ultimate changes in it nutritional and organoleptic characters, the moldiness in foodstuffs is toxicologically significant since the mould species growing on such products is known as potentially mycotoxicogenic (Battilani et al., 2006; Carmo et al., 2008). Mycoto-

xins are secondarily toxic metabolites produced by filamentous fungi that have been detected in several food commodities. Levels of mycotoxins and mycotoxicogenic moulds, which may cause risk to population, are refused by consumers and many countries have set regulations in various agricultural foods. The consumption of mouldy products may cause human or animal mycotoxicoses, and some of more important mycotoxins are strong carcinogens (Rasooli and Abyaneh, 2004; Wangikar et al., 2005; Kawashima and Valente Soares, 2006; Carmo et al., 2008).

For the better understanding of ecological and practical interactions occurring on the nuts, crust, leaves and pollen of *Castanea sativa* Mill., some analyses including the study of the diversity and exact species identification are needs. Our study was intended on the survey of fungal community composition occurring on four different parts of *C. sativa* Mill. species from four different regions of Slovakia.

MATERIALS AND METHODS

The samples were taken from four different parts of *Castanea sativa* Mill. in localities of Krupina, Mlyňany, Príbelce and Radošiná. The samples as leaves, crust and pollen were collected in June and July of year 2007. The samples of nut were collected in September and October of year 2007.

1. Nut sample number: N1–N13.
2. Leaves sample number: L1–L13.
3. Crust sample number: C1–C13.
4. Pollen sample number: P1–P43.

The samples of nuts, leaves and crust numbered 1–4 were taken from locality Krupina, 5–9 were taken from Radošiná and 10–13 were taken from locality Príbelce. Sample of pollen numbered 1–14 were taken from Radošiná, 15–22 were taken from Krupina, 23–32 were taken from Príbelce and 33–43 were taken from Mlyňany.

All samples were taken with scalpel previously cleaned with 70% ethylalcohol, transferred to sterile test-tubes and directly transported to the laboratory for further microbiological processing.

Isolation and morphological characterization of fungi

One gram of samples was transferred to 10ml 0.01% Tween 80 solution and shaken for 30 min. Coarse-gained material was allowed to precipitate and 50 µl were homogeneously dispersed onto selective agar media in Petri dishes and the plates were incubated at room temperature. The isolates were purified in two or three steps by transfer to fresh medium; purity was checked and maintained by repetitive light microscopic observation. The fungi were grown in Petri dishes and slide cultures on 2% malt extract agar (MEA) and Czapek – Dox agar (CDA) (Imuna Šarišské Michalany, Slovakia) for the morphological studies. After 14–20 days of cultivation in darkness at room temperature, slide cultures

and preparations were made in solution of lactic acid. The pure cultures were identified according to method for diagnosis of fungi (Hoog et al., 2000; Klich, 2002; Samson et al., 2002).

RESULTS AND DISCUSSION

The study of mould spores have major importance because many fungi may cause considerable economic losses worldwide acting as plant pathogens or triggering respiratory diseases and allergenic processes in humans (Rodriguez-Rajo et al., 2005). The contamination of food products with micro-mycetes of the *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria*, *Paecilomyces*, *Trichotecium*, genera should receive particular attention. Some of the strains growing on a particular type of vegetable or fruit could synthesize and excrete different toxic secondarily metabolites (Lugauskas et al., 2005; Piecková and Jesenská, 1999).

The most frequently fungi found in nuts of *Castanea sativa* Mill. were *Alternaria alternata*, *Cladosporidium cladosporoides*, *Fusarium oxysporium*, *Mucor mucedo* and *Rhizopus stolonifer*. The less abundance was determined by *Aspergillus fumigatus*, *Aspergillus terreus*, *Mucor racemosus*, *Penicillium crustosum* and *Penicillium glabrum* (Tab. I). *Cladosporium cladosporoides* was the most common spoiler of nut samples contaminating 43.7%.

The nationwide survey was carried out to assess mould spoilage of *Castanea sativa* nuts sold in Canadian grocery stores. Three mycotoxigenic fungi were dominated (*Penicillium crustosum*, *Penicillium glabrum/spinulosum* and *Penicillium discolor*) and isolated at frequencies of 67.1%, 18.6% and 17.7%, respectively, from a total sample size of 350 nuts. *Aspergillus ochraceus* was also isolated, but at a much lower frequency (Overy et al., 2003). *Alternaria alternata* affect different cultivated and wild plants. Inhibitor from buckwheat also suppressed spore germination and mycelium growth of phytopathogenic fungi *Alternaria alternata* and *Fusarium oxysporium* (Valueva and Mosolov, 2004).

The most frequently fungi found in leaves of *Castanea sativa* Mill. were *Alternaria alternata*, *Cladosporidium cladosporoides*, *Mucor mucedo*, *Mucor racemosus* and *Rhizopus stolonifer*. The less abundance was determined by *Aspergillus fumigatus*, *Aspergillus terreus*, *Fusarium oxysporium*, *Penicillium crustosum* and *Penicillium glabrum* (Tab. I). *Alternaria alternata* was the most common spoiler of leave samples contaminating 49.7%. The leaf litter decomposition in streams is an important ecosystem level process, which depends on the activity of invertebrates and microorganisms. Both fungi and bacteria convert leaf carbon into microbial biomass, enhancing leaf palatability for shredding invertebrates (Pascoal and Cássio, 2004).

Alternaria alternata (100%), *Cladosporium cladosporoides* (100%), *Mycelia sterilia* (67%) and *Penicillium* spp. (67%) were the most frequently isolated species of fungi encountered on the surface of the *Sorbus do-*

I: Frequency of fungi species of *Castanea sativa* Mill.

| Species of fungi | Contaminated samples % | Number of isolates (range) |
|--|---------------------------|-------------------------------|
| Nuts | | |
| <i>Alternaria alternata</i> (Fr.) Keissl. | 36.4 | 0–45 |
| <i>Aspergillus fumigatus</i> Fresen. | 16.5 | 0–30 |
| <i>Aspergillus terreus</i> Thom. | 12.0 | 0–34 |
| <i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries | 43.7 | 0–65 |
| <i>Fusarium oxysporium</i> Schltdl. | 23.8 | 0–34 |
| <i>Mucor mucedo</i> Fresen. | 21.4 | 0–27 |
| <i>Mucor racemosus</i> Fresen. | 8.6 | 0–10 |
| <i>Penicillium crustosum</i> Thom. | 14.0 | 0–27 |
| <i>Penicillium glabrum</i> (Wehmer) Westling | 15.7 | 0–39 |
| <i>Rhizopus stolonifer</i> (Ehrenb.) Vuill. | 22.2 | 0–40 |
| Leaves | | |
| <i>Alternaria alternata</i> (Fr.) Keissl. | 49.7 | 0–39 |
| <i>Aspergillus fumigatus</i> Fresen. | 12.4 | 0–56 |
| <i>Aspergillus terreus</i> Thom. | 8.4 | 0–50 |
| <i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries | 34.7 | 0–87 |
| <i>Fusarium oxysporium</i> Schltdl. | 21.3 | 0–34 |
| <i>Mucor mucedo</i> Fresen. | 30.0 | 0–56 |
| <i>Mucor racemosus</i> Fresen. | 25.8 | 0–35 |
| <i>Penicillium crustosum</i> Thom. | 15.4 | 0–60 |
| <i>Penicillium glabrum</i> (Wehmer) | 17.5 | 0–45 |
| <i>Rhizopus stolonifer</i> (Ehrenb.) Vuill. | 30.5 | 0–25 |
| Crust | | |
| <i>Alternaria alternata</i> (Fr.) Keissl. | 35.1 | 0–60 |
| <i>Aspergillus fumigatus</i> Fresen. | 12.7 | 0–27 |
| <i>Aspergillus terreus</i> Thom. | 12.7 | 0–56 |
| <i>Cladosporium cladosporioides</i> (Fresen.) G. A. de Vries | 35.9 | 0–50 |
| <i>Fusarium oxysporium</i> Schltdl. | 23.6 | 0–45 |
| <i>Mucor mucedo</i> Fresen. | 15.4 | 0–70 |
| <i>Mucor racemosus</i> Fresen. | 12.7 | 0–47 |
| <i>Penicillium crustosum</i> Thom. | 10.7 | 0–7 |
| <i>Penicillium glabrum</i> (Wehmer) | 12.5 | 0–20 |
| <i>Rhizopus stolonifer</i> (Ehrenb.) Vuill. | 25.9 | 0–30 |
| Pollen | | |
| <i>Acremonium chrysogenum</i> (Thirum. & Sukap.) W. Gams | 38.9 | 0–87 |
| <i>Alternaria alternata</i> (Fr.) Keissl. | 54.8 | 0–48 |
| <i>Aspergillus flavus</i> Link | 23.5 | 0–90 |
| <i>Aspergillus fumigatus</i> Fresen. | 12.7 | 0–58 |
| <i>Aspergillus ochraceus</i> G. Wilh. | 17.9 | 0–67 |
| <i>Aspergillus versicolor</i> (Vuill.) Tirab. | 12.6 | 0–37 |
| <i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries | 50.9 | 0–57 |
| <i>Fusarium oxysporium</i> Schltdl. | 23.7 | 0–35 |
| <i>Mucor racemosus</i> Fresen. | 18.5 | 0–7 |
| <i>Penicillium crustosum</i> Thom. | 28.9 | 0–10 |
| <i>Trichoderma harzianum</i> Rifai. | 21.9 | 0–20 |

mestica L. leaf samples (Kačániová and Fikselová, 2007).

Alternaria was consistently isolated from disease leaves and fruits. Spots did not develop on leaves inoculated with isolate from *Castanea sativa* Mill. or necrosis spread very slowly on leaf blades treated with isolate from *Chamaecyparis lawsoniana* (Orlikowskij and Oszako, 2005). In Croatia research was carried out on the presence and variation in occurrence of fungal genera on plant samples collected from three different climatic regions in relation to temperature and relative humidity. The spores belonging to 15 fungal genera and sterile mycelium were identified. *Cladosporium* (65.3–74%), *Penicillium* (4.43–13.9%), *Alternaria* (2–4.7%), and sterile mycelium (7.4–15%) were the most prevalent fungi in the air of all three climatic regions, while *Fusarium* (20.3–36.2%) was the most prevalent fungal genera on plant samples especially in the modest continental climate (Pepeljnjak and Šegvič, 2003).

The most common fungi found in crusts of *Castanea sativa* Mill. were *Alternaria alternata*, *Cladosporidium cladosporioides*, *Fusarium oxysporium* and *Rhizopus stolonifer*. The less abundance was determined by *Aspergillus fumigatus*, *Aspergillus terreus*, *Mucor mucedo*, *Mucor racemosus*, *Penicillium crustosum* and *Penicillium glabrum* (Tab. I). *Cladosporidium cladosporioides* was by far the most common spoiler of crust samples contaminating 35.9%. *Alternaria alternata* (89%), *Cladosporium cladosporioides* (89%), *Mycelia sterilia* (89%), *Penicillium* sp. (89%) and *Trichoderma* sp. (67%) were the most frequently isolated fungal species encountered on the surface of the *Sorbus domestica* L. crust samples (Kačániová and Fikselová, 2007).

Some scientist identified in the chestnuts *Phomopsis castanea*, which occurred endophytically in plant parts of European chestnut including flowers, leaves, shoots and fruits. The apparently healthy trees of four cultivars were sampled seven times throughout 1 year. This fungus is associated with the important post-harvest disease *Phomopsis* nut rot (Washington et al., 1999). It was found, that the fungus *Phomopsis castanea* could be associate with both rotten and healthy nuts in all the North Island sam-

ples, although there was a large variation in incidence between varieties and locations. Other fungi isolated from rotten nuts were *Botrytis cinerea* and species belonging to *Penicillium*, *Fusarium* and *Pestalotia* (Wadia et al., 1999).

The most common fungi found in pollen of *Castanea sativa* Mill. were *Acremonium chrysogenum*, *Alternaria alternata*, *Aspergillus flavus*, *Cladosporidium cladosporioides*, *Fusarium oxysporium*, *Penicillium crustosum* and *Trichoderma harzianum*. Less common were *Aspergillus fumigatus*, *Aspergillus ochraceus*, *Aspergillus versicolor* and *Mucor racemosus* (Tab. I). *Cladosporidium cladosporioides* was by far the most common spoiler of pollen samples contaminating 35.9%. Six microscopic fungal species were isolated from *Sorbus domestica* L. pollen samples: *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Fusarium* sp. and *Penicillium* sp. The most frequently species found in the pollen samples were *Penicillium* sp. (100%), *Aspergillus niger* (67%) and *Cladosporium cladosporioides* (67%) (Kačániová and Fikselová, 2007).

Fungi belong to the genus *Aspergillus* were detected in 80% of pollen samples being clearly predominant *Aspergillus* section *Nigri*. Species of this section were found in 66.6% of the pollen samples. The group *A. flavus* plus *A. parasiticus* was found in 23.3% of the samples. Other species of *Aspergillus* were found but only in 10% of the samples. Among these species it is remarkable the presence of *A. ochraceus*, which was isolated in 6% of the pollen samples. The genus *Penicillium* contaminated 90% of the samples, although *P. verrucosum* was isolated in only 6.6% of the samples. Other *Penicillium* species were found contaminating 83.3% of the samples. Species of the genus *Fusarium* were less common. They were isolated in 53.3% of the pollen samples. *Alternaria* sp. was found in 86.6% of the pollen samples. Other fungi of low interest regarding mycotoxin production were isolated. *Cladosporium* and *Rhizopus* were found in 90% and 80% of the samples, respectively. *Mucor* was isolated in 46.6% (González et al., 2005).

SOUHRN

Stanovení mikroskopických hub z ořechů, listů, kůry a pylu kaštanovníka jedlého (*Castanea sativa* Mill.)

Ve čtyřech slovenských regionech byly studovány vztahy mezi rostlinou a mikroorganismy s ohledem na stanovení mykoflory u *Castanea sativa* Mill. (ořechy, kůra, listy a pyl) a jejich vliv na hostitelský organismus. V experimentech bylo izolováno 7 rodů a 10 druhů mikroskopických hub z ořechů, kůry a listů. Bylo zjištěno, že izoláty z pylu *Castanea sativa* Mill. byly zastoupeny osmi rody a jedenácti druhy mikroskopických hub. U izolátů ořechů se nejčastěji vyskytovaly druhy *Alternaria alternata* (36,4%), *Cladosporium cladosporioides* (43,7%), *Fusarium oxysporium* (23,8%) a *Rhizopus stolonifer* (22,2%). U izolátů listů se nejčastěji vyskytovaly druhy *Alternaria alternata* (49,7%) a *Cladosporium cladosporioides* (34,7%) a kůry se nejčastěji vyskytovaly druhy *Alternaria alternata* (35,1%) a *Cladosporium cladosporioides* (35,9%). Druhy *Alternaria alternata* (54,8%) a *Cladosporium cladosporioides* (50,9%) byly nejčastěji vyskytující se mikroskopické houby pylu. Na základě dalších taxonomických určení z rodů *Aspergillus* byly izolovány a identifikovány zástupci druhů *A. flavus*, *A. fumigatus*, *A. ochraceus*, *A. terreus* a *A. versi-*

color. Z rodu *Fusarium* byl izolován druh *F. oxysporum* a z rodu *Penicillium* byly izolovány druhy *P. crustosum* a *P. glabrum*. Zdůrazňujeme, že izolované rody *Aspergillus*, *Fusarium* a *Penicillium*, jsou považovány za nejvýznamnější producenty mykotoxinů. Typičtí producenti mykotoxinů z rodů *Penicillium*, *Alternaria*, *Fusarium* a *Aspergillus* byli detekováni na čerstvých ořechách, listech, kůře a pylu při laboratorní teplotě. U všech testovaných částí *Castanea sativa* Mill. bylo zaznamenáno vysoké procento plísňové kontaminace. Rody *Alternaria*, *Cladosporium*, *Penicillium* a *Fusarium* byly převládající ve všech izolátech rostliny. Některé z rodů (např. *Cladosporium*, *Alternaria* a *Penicillium*) jsou potenciálními alergeny, za předpokladu vysoké produkce konidií. Pokročilé kažení ořechů by mohlo mít nepříznivý účinek na zdraví personálu, který nakládá s těmito komoditami. Zvláštní opatření by měla být přijata během sklizně, čištění, třídění, balení, přepravy, skladování a uvádění zboží na trh. Chlazení je důležité pro udržení kvality a prodloužení trvanlivosti, i když některé druhy mohou růst při nízkých teplotách. Plody, které jsou citlivé na nízké teploty, by měly být uváděny na trh rychle, aby se zabránilo houbové kontaminaci a potenciální tvorbě mykotoxinů a tím souvisejícímu vzniku zdravotního rizika.

Castanea sativa Mill., ořechy, kůra, pyl, mikroskopické houby

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