

Thesis of PhD Dissertation

**APPEARANCE OF *MONILINIA FRUCTICOLA*  
AND *MONILIA POLYSTROMA* IN HUNGARY  
AND NEWER POSSIBILITY  
OF THE PROTECTION**

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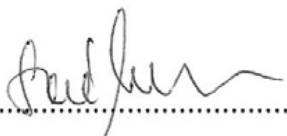
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# 1. INTRODUCTION

The distance between the Continents is getting much closer in the past century. Travelling from large distances now it is possible for plant pathogens all over the world. In the past decades lot of new pathogens have appeared in Hungary, some cases locally, but in some cases seriously endangering the fruit-, vegetable- and ornamental plant production. The aggressiveness of the pathogens could be change, new biotypes could develop, host range could get wider or its can cause new, unusual symptoms, endemics or pandemics (**Vajna 2007**).

Fungi belonging to *Monilinia* genus are well-known pathogens of fruit trees affecting fruit production all over the world. They are particularly important with regard to fruit trees and ornamentals because they cause serious blossom and twig blight and brown rot on fruits (**Byrde és Willetts, 1977**).

Blossom and twig blight and brown rot caused by *Monilinia laxa* and *Monilinia fructigena* is well known in our country, but unfortunately they cause increasing economical losses in our orchards. In my study I tried to find the answer for this increasing damage. Are there any differences between isolates from different host considering morphological, cultural and genetic properties? Have these pathogens been changed during the past decades or have new species been appeared in Hungary?

Application of conventional pesticides in plant protection poses a significant risk to the environment and human health. For this reason, the permission of several pesticides has been cancelled. Nowadays, the demand of the use of plant protection products containing natural agents, e. g. plant extracts, is increasing. The possibility of applying essential oils against pests became the focus of interest in the past years.

In the international literature numerous publications are available in connection with fungistatic and fungicide effect of medicinal and aromatic plants (**Paranagama et al., 2003**), but only a few data available about the effect of essential oils against *Monilinia* species and *in vivo* experiments were rarely carried out (**Neri et al., 2007**).

**Our aims during the experiments were the followings:**

- Collection of *Monilinia* isolates from different hosts and localities;
- Identification and characterization of the species by classical mycological methods (after morphological and culture characteristics);
- Identification and characterization of *Monilinia* isolates by molecular methods and phylogenetic analysis;
- Comparison of aggressiveness of *Monilinia laxa* isolates causing blossom blight *in vitro* and *in vivo*;
- Analyzing antifungal activity of essential oils against *Monilinia fructigena* and *Monilinia laxa in vitro* and *in vivo*.

## 2. MATERIALS AND METHODS

### Isolates

Ninety-three *Monilinia* isolates have been collected from fruit trees and ornamental plants between 2004 and 2008 from public places, from gardens, from a market of Budapest and from two supermarkets.

### Classical mycological experiments

After describing the symptoms, the size, colour and berth of stromata were observed, than length and diameter of 100 conidia from infected were measured. Analysis of variance of conidial dimensions was performed using ROPstat program package. For one-way comparison of independent samples Games-Howell pairwise comparison was used ( $p < 0.01$ ).

For isolation Potato dextrose agar (PDA) and Leonian malt agar (LMA) media were applied. All pure cultures were grown in plastic Petri dishes sealed with parafilm, and incubated at 24°C, continuous darkness. Culture characteristics (shape, colour, margins, air-mycelia, sporulation) and colony growth rates (mm/24 h) were compared.

Pathogenicity was tested of each isolate by inoculation of 5–5 db surface disinfested fruits of the host plant. Plugs of mycelia from fungal colony margins were inserted into a wound, made by puncturing a sterile needle into the fruit skin. Fruits were examined at 7-12 days after inoculation. Pathogenicity of UFT isolate were confirmed on apple shoots (cv. Mutsu and Granny Smith) based on Koch's postulates as well.

Aggressiveness of *Monilinia laxa* isolates (M38/JÁ – M61/JÁ) were compared *in vitro* by growth rate of colonies and *in vivo* by symptoms of inoculated apricot fruits (KPI - Jedlik Ányos program).

### Molecular mycological experiments

During molecular identification and characterization two genomic regions were examined: the ITS (Internal Transcribed Spacer)-region and a more variable non-coding region with unknown function.

Primers (synthesized by Biomi Kft. Gödöllő, Hungary) were used to amplify the ITS regions of ribosomal DNA (ITS1-5.8S-ITS2): ITS4Mfgn reverse primer (5'-GGTGTTTTGCCAGAAGCACACT-3' by **Ioos and Frey [2000]**) and ITSMonilia forward primer (5'-GGTAGACCTCCCACCCTTGTGTA-3') designed by us based on sequence data from NCBI database, is specific to *M. fructigena*, *M. laxa*, *M. fructicola* and *Monilia polystroma*.

To recover a “genomic sequence with unknown function”, we designed primers using sequence data from **Côté et al. (2004)**. This genomic region was chosen because it was the only available sequence data for *Monilia polystroma* except for the ITS regions; as well, this region is more diverse among the different *Monilinia* species. UniMon\_Rev (5' – AAGGATCCGAGCAAGGTGTCAAACCTTCAT–3') and UniMon\_Forw: (5'– TTGAATTCATCGGCTTGGGAGCGG – 3') are universal primers for multiplex PCR for four species, *Monilinia fructigena*, *Monilia polystroma*, *Monilinia laxa*, and *Monilinia fructicola*. The species can be distinguished by the size of the PCR product.

DNA extraction was carried out by CTAB method. After polymerase chain reaction, amplified DNA products were purified using High Pure PCR Product Purification Kit and were ligated into pGEM-T Easy following the manufacturer's instructions. The vector was transformed into *Escherichia coli* DH5 $\alpha$ , white colonies were selected, and recombinant plasmids were purified. Sequences of the isolates were compared with other isolates from international database (NCBI).

### **Antifungal activity of essential oils against *Monilinia fructigena* and *Monilinia laxa***

In our experiments, 28 essential oils extracted from Mediterranean, tropical and continental plant species were tested *in vitro* and *in vivo* for their effectiveness against *Monilinia fructigena* and *Monilinia laxa*. Essential oils were ensured by Aromax ZRt (GVOP 3.3.3 -5/1.-2005-05-0016. program). Oils were encoded by numbers during the tests. The antifungal activity *in vitro* was compared on the basis of the inhibition of mycelial growth (agar diffusion hole test and agar dilution technique) and germination of conidia in different concentrations (1%–0.01%). The effective oils were tested *in vivo* against brown rot of sour cherry fruits, and blossom blight in sour cherry orchards (at Soroksár, Alsóörs and Ökörítófülpös).

### 3. RESULTS AND DISCUSSION

#### Identification and characterization of *Monilinia fructicola*

In early October 2005, brown rot was observed on imported peaches from Italy and Spain at a vegetable market and some supermarkets in Budapest. Isolates M11–M13 were identified as *Monilinia fructicola* on the basis of morphological and molecular characteristics.

Symptoms began with a small, circular brown spot, and the rot spread rapidly. At the same time, numerous small, greyish stromata developed. Finally, the whole surface of the fruit was covered with conidial tufts. The conidia were one-celled, lemon-shaped, hyaline, and produced in branched monilioid chains. Conidia from infected fruit were transferred to potato dextrose agar. The colour of the colony was greyish, and the sporulation showing concentric rings was abundant. The colony was not rosetted and the margin was not lobed. Pathogenicity was tested by inoculating surface-sterilized, mature peach fruits with conidia. After 5 days of incubation, typical brown rot symptoms developed on inoculated fruits while control fruits remained healthy. *M. fructicola* was reisolated from the inoculated fruits.

By the molecular results of ITS- region and the genomic region with unknown function, the pathogen was identified as *Monilinia fructicola*. After analyzing the sequences it can be stated that M12 isolate differed in one base from M13 and *Monilinia fructicola* reference isolate. In 5.8S ribosomal gene at the position of 281 was thymine instead of cytosine, causes change of serine (TCG) to leucine (TTG). A 592 bp long fragment could be amplified in case of *Monilinia fructicola* using universal primer pairs targeted the genomic region with unknown function. For comparison in this region these primer pairs can amplify 395 bp and 415 bp long fragments from *Monilinia laxa* and *Monilinia fructigena* respectively. Comparing our isolates with the reference sequences 17 bases substitution and deletions in two positions have been found.

It was demonstrated that *Monilinia fructicola* got into trade by export shipments and it might got to other countries as well. The quickly rotten fruits might were thrown to communal waste by the consumers. The pathogen might overwinter in the mummies and in spring - among favourable weather conditions - could produce conidia on the surface of the mummies, which cause infections in the orchards. It is conceivable, that these sources of inoculums, or the infected exported propagating material caused the rapid spread of the

pathogen at different areas of the country, which was confirmed by the tests of the plant protection authority.

After identifying the quarantine pathogen by EPPO standard methods it was immediately reported to the Hungarian Plant Protection Service in order to perform the necessary measure.

After reporting the occurrence of the quarantine pathogen (**Petróczy és Palkovics, 2006**) the Spanish Plant Protection Authority (NPPO) confessed the presence of *Monilinia fructicola* in Ivars de Noguera (Lleida, Catalonia) and in Castillonroy (Huesca, Aragon). Orchards within a radius of 5 km were quarantined, and another zone of 10 km radius was also delimited and phytosanitary measures will be applied in orchards, packing stations and nurseries to prevent any further spread of the disease. (**EPPO, 2006; Gell és mtsai., 2007**).

#### **Identification and characterization of *Monilia polystroma***

In April 2006, unusual symptoms were observed on ‘Ashton Bitter’ apple trees in Újfehértó. Brownish die back was present on the leaf petioles and laminae and on the small fruits and fruit pedicels. Infected areas were covered with yellowish exogenous stromata. Colonies of the UFT isolate grown on PDA were yellowish in colour and irregular black stromatal crusts occurred on the edges of the colonies after 10-12 days of incubation. The margins of the colonies were slightly undulate. The growth rate of the colony was 7.4 mm/24h. Pathogenicity testing was successful on apple shoots and fruits, and the fungus was reisolated from inoculated tissues.

The ITS region was cloned, sequenced, and deposited in the GenBank database. The sequence of the UFT isolate was almost identical (only one base difference) to that of *Monilia polystroma*, containing all five nucleotides that distinguish it from *M. fructigena* (**Fulton et al., 1999; van Leeuwen et al. 2002**). The only difference detected between the UFT isolate and the published sequence of *Monilia polystroma* occurred at nucleotide 372, where the UFT sequence was identical to the three other species, but the published *Monilia polystroma* sequence contained an extra ‘T’. A “genomic sequence with unknown function” was also deposited in the GenBank database. Comparing *M. fructigena* and *Monilia polystroma* sequences, insertions and substitutions were detected in the *Monilia polystroma* sequence. The Hungarian UFT and the published *Monilia polystroma* sequences were almost identical. Three repetitive sequence motifs (CAT, CCT, TAGTCCA or TAGTCCC) were identified.



The CAT and CCT motifs occurred twice in all *M. fructigena* isolates and three times in *Monilia polystroma* and UFT isolates, while the TAGTCCA or TAGTCCC motif occurred three times in all *M. fructigena* isolates, five times in *Monilia polystroma*, and four times in the UFT isolate

*Monilia polystroma* is currently not included in EPPO lists of quarantine pathogens. As a result of the report and existence of this new pathogen in this region, EPPO might plan to perform a pest risk analysis to determine whether to place *Monilia polystroma* in one of the lists.

### **Identification and characterization of *Monilinia fructigena* and *Monilinia laxa* isolates**

*Monilinia fructigena* was identified in case of 37 isolate and *Monilinia laxa* in 52 cases. Considering hosts of *Monilinia fructigena* no variance has been found according to the literature. The brown rot of grapes was caused by *Monilinia laxa*. This was the first data about this host-pathogen interaction in Hungary and in Europe, because it was only reported from New-Zealand (**Pennycook, 1989**).

In connection of the symptoms, the size, colour and berth of stromata, the colour, shape, formation of conidia no differences were observed according to the literature.

Considering end values and averages of conidial size small deviations were observed according to data in the literature. Culture characteristics of the pathogens were similar those of noticed in the literature, but in some cases difficulties occurred during the identification based on culture morphology as it was mentioned by **Muñoz et al (2008)**. By **OEPP/EPPO (2003)** the colonies of *Monilinia laxa* are hazel, while those of *Monilinia fructigena* are yellowish or creamy. In case of M26, M88 and M91 *Monilinia fructigena* isolates, colour of the colonies were hazel, which is typical to *Monilinia laxa* or *Monilinia fructicola* pathogens, but not to *Monilinia fructigena* by the literature. The colony of M83 *Monilinia laxa* isolate was creamy on LMA media, which is characteristic to *Monilinia fructigena* according to the literature. Colonies of *Monilinia laxa* are rosetted and zones develop. On the other hand the same was observed at M88 *Monilinia fructigena* isolate's colony on PDA. By the literature and according to our notification neither *Monilinia laxa*, nor *Monilinia fructigena* produce conidia on culture media in complete darkness. Sporulation has been observed only in case of M33 (*Monilinia laxa*), which was isolated from 'Bluefre' plum fruit. Both media (PDA and

LMA) are suitable for cultivation of the pathogens, but on account of more intensive growth of mycelia, we prefer and suggest PDA media for cultivation and identification.

Species specific primers of **Ioos és Frey (2000)** designed to the ITS1 region, 5.8S rRNA gene and to ITS2 region of *Monilinia fructigena*, *Monilinia laxa* and *Monilinia fructicola*, did not work specifically causing cross reactions between species at the annealing temperature given by the authors (55°C). Raising the temperature to 70°C yielded specific PCR products, so these oligonucleotides are suitable for identification, but they are reliable only at this higher temperature. This temperature substantially exceeded the melting temperature of the primers, accordingly the quantity of PCR products decreased significantly. During the sequence analysis, we determined that this genome section is slightly variable at nucleic acid level, so it is an appropriate method for identification of *Monilinia* species.

Multiplex PCR was found a suitable method for separation of *Monilinia* species by the different length of the fragments, as it was mentioned by **Côté et al. (2004)** as well. Molecular verification is required in every case to complete classical identification, because the latter is not reliable in all samples, as it was observed by **Sonoda et al. (1982)** as well. By the results of sequence analysis, the isolates showed complete identity at the genomic region with unknown function on nucleic acid level. This region is suitable for identification of *Monilinia* species, but not for quest evolutionary differences among isolates.

### **Antifungal activity of essential oils against *Monilinia fructigena* and *Monilinia laxa***

Hole test was not suitable for selection of effective essential oils, because all oils caused complete inhibition of mycelial growth of *Monilinia fructigena* and *Monilinia laxa*. In case of the agar dilution method, great differences could be observed among the oils in effectiveness. At 1% concentration all of the oils inhibited the growth of mycelia. At the used lowest concentration (0.01%) a few essential oils yielded complete inhibition.

In case of *Monilinia fructigena* the 2, 3, 16, 21 and 23; in case of *Monilinia laxa* 2, 3, 7, 16, 21, 22, 23 and 27 essential oils gave the best results considering inhibition of mycelial growth. During *in vivo* tests in flowering sour cherry orchards the some oils gave significantly better results than applied fungicide against blossom and twig blight caused by *Monilinia laxa* (2, 3, 16, 18, 21, 23 and 27). In reference to *in vivo* studies there is no data available in the literature.

## 4. SUMMARY OF NEW SCIENTIFIC RESULTS

- *Monilinia fructicola* - declared as quarantine organism in Europe - was firstly identified from imported peach fruits from Italy and Spain.
- *Monilia polystroma* was firstly reported from Hungary and Europe on apple shoots and fruits. According to the literature the pathogen has been existed only in Japan.
- *Monilia polystroma* caused twig blight on apple shoots of 'Ashton bitter' cultivar. By the literature only fruit rot was reported.
- Comparison of the sequences of the „genomic region with unknown function” revealed that repetitive motifs TAGTCCA and TAGTCCC occurred in different numbers in UFT isolate and *Monilia polystroma* reference isolate. These exist five times in the reference isolate, and four times in UFT isolate.
- Brown rot of grapes was caused by *Monilinia laxa*. This was the first data about this host-pathogen interaction in Hungary and in Europe, because it was only reported from New-Zealand.
- During *in vivo* tests in sour cherry orchards some oils gave significantly better results than applied fungicide against blossom and twig blight.

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