**PhD thesis**

# Investigations on some aspects of genetic variability, epidemiology, protection possibilities and fungicide resistance of *Monilinia* species

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# 1. INTRODUCTION AND THE AIM OF RESEARCH

## 1.1. Importance of the topic

Fruit fungal pathogens cause significant damage every year in domesticated fruit tree species all over the world where fruit are grown. *Monilinia* species are the most damaging fungal species worldwide causing serious diseases of apple and stone fruit. Certain species of the fungus cause regular epidemic on fruit trees in Hungary. Four species are known to occure in Hungary: *Monilinia fructigena*, *M. laxa*, *M. fructicola* and *Monilia polystroma*. In Europe the first two species cause significant brown rot symptoms on fruit, twigs and blossom. In wet periods fungus cause serious yield loss in Hungarian orchards. Control of the diseases is complex, it can be successful only with integrated methods especially in wet years. The complexity of protection can be originated in rapid formation of epidemic, genetic features of species and their resistance to fungicide. In these aspects several former achievements are known in international literature, however, new genetic diversity, epidemic and protection examination are necessary in order to delay *Monilinia* epidemic and evolve successful protection against the disease. By these general principles we focused some more important elements, which became aims of our study.

**Aims:**

- Identification of *Monilinia* isolates from contaminated plant parts collected all over the country with classical and molecular biological methods. Demonstration of genetic diversity among the isolates and distinguishing and identification of genotypes.
- Temporal development of disease incidence caused by *M. fructigena* and analysis of epidemic in integrated and organic apple orchards.
- Working out and examination on fields of disease warning and control strategies against *M. fructigena* in organic apple orchards.
- Examination of effects of reduced spray programmes against three fungal diseases – among them brown rot caused by *M. fructigena* – in integrated and organic apple orchards.
- Examination of sensitivity of some *M. laxa* isolates to different fungicides.

## 2. MATERIALS AND METHODS

### 2.1. Origin, isolation and identification of *Monilinia* isolates

#### 2.1.1. Collection of samples, isolation of causative agents and reserve of isolates

Contaminated fruit with characteristic symptoms of *Monilinia* species were collected from settlement all over the country in 2009, 2010 and 2012. Reference *M. laxa, M. fructigena* and *M. fructicola* strains were used for molecular identification. Contaminated fruit was collected into paper bags with label and stored on 4 °C in fridge until examinations. Conidia and/or mycelia from collected fruit were incubation on Petri dishes with steril PDA medium on 22 °C in dark. Pure yard was made, which was put on mitre agar and paraffin oil on 4 °C. Medium with mycelia was also stored on -20°C in 10% glycerol.

#### 2.1.2. Identification of morphological and growing characteristics

On contaminated fruit species were identified by colours and morphology of exogen stroma. Then they were identified by morphology of isolates on PDA media (colour, tickness, shape and edge, size, propagation material) according to describing methods of Van Leeuwen et al. (2002). *Monilinia* species were also identified with Koch postulate as apples were reinfected, and hyphae were placed into wounded apples, then symptoms were identified after one week incubation. Identification was also confirmed by PCR reaction.

#### 2.1.3. Isolation of genomic DNA

DNA isolation was done with GenElute$^{TM}$ Plant Genomic DNA Miniprep Kit (SIGMA-ALDRICH G2N70). *Monilinia* species were incubated in 50 ml PDB (27g Potato Dextrose Broth, final volume 1000 ml with distilled water) for 2 days on 22 $^{o}$C-on (150 rpm). DNA was isolated by instruction of KIT protokol. Pure DNA was stored on -20 $^{o}$C.

#### 2.1.4. Identification of species with PCR reaction

Identification of *Monilinia* isolates was carried out by the following method: reaction compound (50 µl): 1 µl genomic DNA; 2 µl primer, forward 25 µM (Primer: UniMon_Forw); 2 µl primer, reverse 25 µM (Primer: UniMon_ Rev:); 4 µl dNTP Set 5mM (Thermo Scientific R0181); 5 µl 5x Green GoTaq® buffer (Promega M3171) with 7,5 mM MgCl2; 0,5 µl GoTaq® DNA-polymerase (Promega M3171); 31,5 µl Milli-Q water. Applied primers:

UniMon_Forw: sequence: 5'-TTGAATTCATCGGCTTGGGAGCGG-3'; UniMon_Rev: sequence: 5'-AAGGATCCGAGCAAGGTGTCAAAACTTCCAT-3' (Cote et al., 2004).

Steps for PCR reaction:

1. 94 °C → 5 min
2. 94 °C → 30 sec
3. 65 °C → 30 sec
4. 72 °C → 30 sec
5. 72 °C → 5 min
6. 4 °C → ∞

} 35 cycles

10 µl of PCR product was run (with 10 µl Milli-Q water and 4 µl 6x loading buffer) in 1 % agarose gel, next to 10 µl 1 kb DNS-marker. For identification PD 2.96 *M. laxa*, PD 4.96 *M. fructigena* and SF-BSZ *M. fructicola* were used as references.

## 2.2. Genetic variability

### 2.2.1. ISSR (Inter Simple Sequence Repeat) PCR reaction

Primers as ISSR-markers (altogether 27) were chosen, which had been used for *M. fructicola* before. The following reaction compound was composed (50 µl): 1 µl genomic DNA; 4 µl dNTP Set 5mM (Thermo Scientific R0181); 4 µl primer 25 µM; 5 µl 5x Green GoTaq® buffer (Promega M3171) with 7,5 mM $MgCl_2$; 0,5 µl Green GoTaq® DNA-polymerase (Promega M3171); 35,5 µl Milli-Q water.

Steps for PCR reaction:

1. 94 °C→ 3 min
2. 94 °C→ 1 min
3. 45 °C →1 min
4. 72 °C →1:15 min
5. 72 °C→ 5 min
6. 4 °C →∞

} 30 cycles

50 µl of PCR product was run (with 10 µl 6x loading buffer) in 1,4 % agarose gel, next to 15 µl 1 kb DNS-marker (1h, 110 mV).

### 2.2.2. RAPD (Random Amplified Polymorfic DNA) PCR analysis

The following compound was used (50 µl): 1 µl genomic DNA; 4 µl dNTP Set 5mM (Thermo Scientific R0181); 4 µl primer 25 µM; 5 µl 5x Green GoTaq® buffer (Promega M3171) with

7,5 mM $MgCl_2$; 0,5 µl Green GoTaq® DNA-polymerase (Promega M3171); 35,5 µl Milli-Q water. Altogether 20 primers were applied.

Steps for PCR reaction:

1. 94 °C →5 min
2. 94 °C → 1 min
3. 35,5 °C→1:10 min
4. 72 °C →1:10 min
5. 72 °C → 10 min
6. 4 °C → ∞

} 40 cycles

50 µl of PCR product was run (with 10 µl 6x loading buffer) in 1,4 % agarose gel, next to 15 µl 1 kb DNS-marker (1h, 110 mV).

### 2.2.3. ITS (Internal Transcriped Spacer) sequence

The following compound was used (50 µl): 1 µl genomic DNA; 2 µl ITS1Mlx primer 25 µM; 2 µl ITS4Mlx primer 25 µM; 4 µl dNTP Set 5mM (Thermo Scientific R0181); 5 µl 5x Green GoTaq® buffer (Promega M3171) with 7,5 mM $MgCl_2$; 0,5 µl Green GoTaq® DNA-polymerase (Promega M3171); 31,5 µl Milli-Q water. Applied primers: ITS1Mlx: 5'-TATGCTCGCCAGAGAATAATC-3' and ITS4Mlx: 5'-TGGGTTTTGGCAGAAGCACACC -3' (Ioos and Frey, 2000).

Steps for PCR reaction:

1. 94 $^{o}$C → 5 min
2. 94 $^{o}$C →30 sec
3. 65 $^{o}$C → 30 sec
4. 72 $^{o}$C → 30 sec
5. 72 $^{o}$C → 5 min
6. 4 $^{o}$C → ∞

} 35 cycles

10 µl of PCR product was run (with 4 µl 6x loading buffer) in 1 % agarose gel, next to 15 µl 1 kb DNS-marker (1 h, 120 mV).

### 2.2.4. Data analysis of PCR bands

After ISSR és RAPD PCR reactions the results of gel electrophoresise were analysed with GelAnalyzer 2010 programme and polimorf bands were paired with binary variables (present 1, absent 0). Binary matrices were then constructed from data and dendrograms were generated after UPGMA cluster analysis.

### 2.2.5. Nei indexes

ISSR data was also used to calculate gene diversity for *M. laxa* isolates ($H_T$) according to Nei (1975). $H_T$ was divided into gene diversities within ($H_S$) and between ($D_{ST}$) subpopulations, $H_T = H_S + D_{ST}$ and each component was calculated (Nei, 1987). Genetic differentation relative to the total population was calculated by the coefficient of gene differentation (Nei, 1987): $G_{ST} = D_{ST} / H_T$. $G_{ST}$ can take values between 0.0 (no differentiation between subpopulations) and 1.0 (complete differentation between subpopulations). The amount of gene flow between populations, *Nm*, where *N* is the effective population size and *m* is the fraction of individuals in a population that are immigrants, was estimated by using the following formula (Wright, 1951): $Nm = 0{,}5 \times (1/ G_{ST} - 1)$. If Nm<1, then the local populations tend to differentiate, if Nm>1, little differentiation among populations is indicated (Wright, 1951).

## 2.3. Epidemic dynamic and forecasting models

### 2.3.1. Orchard sites and disease assessment

Experiments were carried out in two orchards in East- Hungary (Nagykálló és Eperjeske) between 2002 and 2006. Planting was in 4 x 1,5 m spacing on rootstocks M9 at Nagykálló and M26 at Eperjeske in 1996. Hungarian organic production guidelines were applied (Anon, 1997). All sprays were applied with a Kertitox 2000 axial fan sprayer with a volume of 1000 l/ha. Rainfall and temperature were recorded with METOS agrometeorological station from 1 May to 10 October. At both locations, the same three apple cultivars, an early-season cultivar (Prima) and two late-season cultivars (Idared and Mutsu) were selected for disease assessment. Fruit size in month-periods was measured. In both orchards assessment was carried out four times. Twenty trees were selected randomly. On each selected trees, 100 fruit were assessment on a weekly basis from 20 May until 30 August for cv. Prima and until 10

October for cvs. Idared and Mutsu. Brown rot incidence was calculated as the percentage of diseased fruits.

### 2.3.3. Curve fitting and disease variables

The weekly data were graphed in a x and y coordinate curve, then models were fitted based on three-parameter logistic function: $Yt= Yf/(1+e^{-\beta(t-M)})$. Parameters of the model: *Yt* disease incidence in *t* moment, *Yf* the final disease incidence (%), *ß* the relative rate of disease and *M* the inflection point, at which disease progress is the fastest. Model was fitted by R 2.8.1 „nlme" (nonlinear mixed-effect) statistic programme (Anon, 2008). For selecting the best fitted models, both the Akaike's Information Criteriont (AIC) and Bayesian Information Criteriont (BIC) were used.

### 2.3.4. Brown rot forecasting and management strategy (BRFMS)

The BRFMS was developed in three steps: firstly a fundamental model was created, secondly occurence of first fruit rot symptoms was predicted, and thirdly insect injury prediction related brown rot development was prepared and attached to the fundamnetal model. The fundamental model contained four parts: data insertion and analysis by computer simulation of pathogen submodels, calculation of yield loss threshold levels based on disease incidence, determination of epidemic intensity levels, and a decision module with suggestions of disease management practises for each epidemic intensity level.

### 2.3.5. The fundamental model

In the fundamental model, data insertion and analysis were carried out by computer simulation of pathogen submodels. Seasonal spore dispersal was predicted with using autoregressive models with six autoregressive parameters, ($\Phi_i$) developed for *M. fructigena*: $Y_t=\sum\Phi_i Y_{t-1}+a_t$, by Holb (2008). Based on this, Holb (2008) used the Gompertz function for describing the relationship between seasonal spore dispersal and corresponding disease incidence, and with this brown rot incidence was able to forecast based on spore dispersal data. For calculation of yield threshold levels and determination of epidemic intensity levels in the fundamental model, threshold values of brown rot development were determined by maximum values of parameters *AUDPCs*, $Y_f$ and *ß* derived from the three-parameter Gompertz function.

### 2.3.6. Prediction of first symptom occurence and prediction of insect

Prediction of first symptom occurence (To) in the first level of epidemic intensity was based on using the method developed by Holb and Scherm (2007). A majority of insect injury was assumed to be caused by codling moth larvae according to the study of Holb and Scherm (2008) In this study, regression equations between codling moth trapping and insect injury, as well as between insect injury and brown rot incidence were developed, were applied in our forecasting model to predict the insect injury in the further levels of threshold.

### 2.3.7. Practical evaluation of BRFMS in season-long spray programmes

At Eperjeske and Nagykálló in three equally sized treatment blocks effects of BRFMS were studied with cv. Idared. In block 1, brown rot management was applied based on the newly developed BRFMS, in block 2, general brown rot management was applied according to IFOAM standards, and in block 3, no brown rot and insect management was applied. Assessments were carried out between 2006 and 2008 in four replications. Treatment blocks were prepared on 15 February 2006, 19 February 2007 and 11 February 2008 and one spray was applied with Funguran-OH 50 WP (77% copper hydroxide) at 1kg/ha at the same dates in blocks 1 and 2. Other sprays in both blocks were applied from 5 weeks after fruit set until harvest with Kumulus S (80% wettable sulfur) at 4 kg/ha and Dipel ES (3,2% *Bacillus thuringiensis*) against codling moth. Brown rot incidence and percentage of injury incidence were assessed at harvest according to Holb és Scherm (2008). Differences among three blocks were evaluated by variance analysis at 5% significance level.

## 2.4. Effects of reduced spray programmes on important causative agents of apple in organic orchards

Assessments were carried out at two apple cultivars at Eperjeske in 2008 and 2009. Cultivar 1 was treated according to organic rules, cultivar 2 was tretaed according to integrated rules from 1996. Assessments were carried out on cv. Idared. Planting was in 5 x 2 m spacing on rootstocks M26. Four spray programmes were compared. In integrated orchard there were standard spray programme and reduced spray programme. In reduced spray programme the same active substances were applied, but number of sprays were reduced by 25%. In organic orchards there also were standard spray programme and reduced spray programme by 40%. In August in 2008 and 2009 incidence of scab of leaves and fruit were assessed from 5 trees.

With 50 randomly selected leaves and fruit from each tree incidence of disease was defined. On the same days powdery mildew infection of 20 randomly selected shoots and 50 randomly selected fruit per tree was defined on 5 trees. *Monilinia* infection of 50 randomly selected fruit per tree were assessed on 5 trees. Statistic analysis were carried out by variance analysis at 5% significance level. Differences among spray programmes were separately analysed in integrated and organic orchards.

### 2.5. Studying of fungicide resistance

Ten fungicides, registered for brown rot control were used (Table 1). Sensitiviy to fungicides was determind in Petri dishes on fungicide-amended PDA 1x (according to minimum label rate), 0.5x and 2x dosages of each fungicide were used for each isolates replicated four times. Inoculum plugs (6 mm in diameter) with actively growing mycelium of each isolates were taken from the periphery of 3-day-old cultures and placed upside down PDA amended with fungicide. 12 *M. laxa* isolates were tested (MLX-SP-P18, MLX-FO-P7, MLX-DBP-O1, MLX-SF-SC3, MLX-VSZ-P21, MLX-BF-P1, MLX-KL-P10, MLX-EP-P6, MLX-KNY-P9, MLX-KB-SC1, MLX-BE-P3, MLX-MV-P12). Colony diameters were measured after 5 and 10 days of incubation in the dark at 22 °C and inhibition of mycelium growth was calculated at percentage.

**Table 1:** Fungicides used for studying fungicide resistance.

| Fungicide | Active ingredient | Manufacturer | Treated plant | Dosage |
|---|---|---|---|---|
| Captan 50 WP | 50 % captan | Arysta (US) | apples | 2-3.2 kg/ha |
| Chorus 50 WG | 500 g/kg ciprodinil | Syngenta (CH) | apple, pear | 0.4-0.45 kg/ha |
| Indofil M-45 | 80% mankoceb | Indofil Ch. (I) | apples | 2-3.2 kg/ha |
| Kén 800 FW | 800 g/l sulfur | Agrokémia Sellye Rt. | apricot | 6-7 l/ha |
| Mirage 45 EC | 450 g/l prochloras | Makhteshim (IL) | stone fruit | 0.3-0.5 l/ha |
| Signum WG | 27% boscalid+7% piraclostrobin | BASF (DE) | stone fruit | 0.75-1 kg/ha |
| Systhane Duplo | 240 g/l miklobutanil | Dow AgroSciences | stone fruit | 0.13 l/ha |
| Teldor 500 SC | 500 g/l fenhexamid | Bayer (DE) | stone fruit | 1 l/ha |
| Topas 100 Ec | 10% penconazol | Syngenta (CH) | stone fruit | 0.5 l/ha |
| Topsin M 50 WP | 70% tiofenat-metil | Nippon Soda (JP) | apples | 0.8-1.6 kg/ha |

## 3. RESULTS

### 3.1. Identification by classical and molecular biological methods

138 *M. fructigena* és 64 *M. laxa* from 202 from all over the country were identified. On apples infected by Koch postulate 1-2 mm, ochre and grey exogen stroma was seen, which was *M. fructigena* (Figure 1a). On PDA characteristics of *M. fructigena* and *M. laxa* were observed (Figure 1b,c).

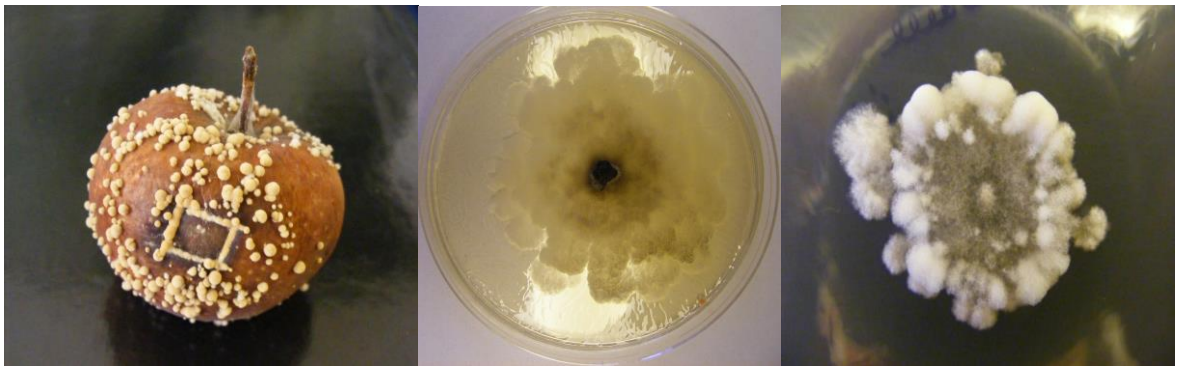

**Figure 1: a)** epple infected by *M. fructigena* **b)** *M. laxa* on PDA media **c)** *M. fructigena* on PDA media (Photos: Fazekas M).

Molecular biological method supported that isolates belong to *M. laxa* and *M. fructigena* species (Figure 2).

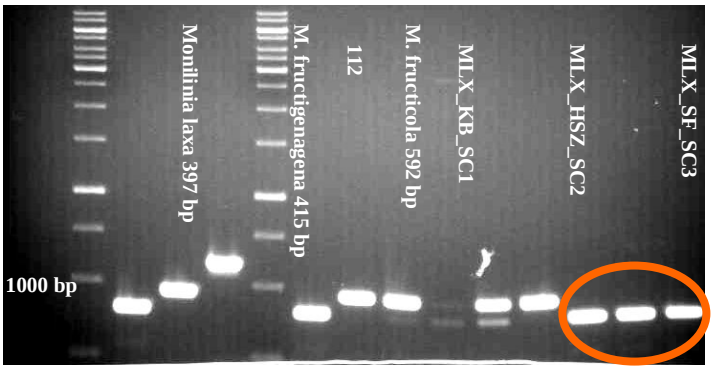


**Figure2:** Identification by molecular biological method.

### 3.2. Genetic variability

#### 3.2.1. Evaluation of ISSR-PCR

Among the microsatellite primers tested, four primers produced reproducible polymorphic bands. A total 53 bands were amplified and 20 of them were monomorphic and 33 of them

were polymorphic. This means that (AC)8T gave 8 bands, 4 of them were monomorphic and 4 of them were polymorphic. (AG)8TC gave 13 bands (5 monomorphic, 8 polymorphic). (AC)8TC primer gave 12 bands (2 monomorphic, 10 polymorphic) and (AC)8CT gave 20 bands (9 monomorphic, 11 polymorphic). The bands samples were analysis (GelAnalyzer 2010) (http://www.gelanalyzer.com) and binary matrices were then constructed from data. We paired polimorf bands with binary variables (present 1, absent 0) and dendrograms were generated after UPGMA cluster analysis (UPGMA) (http://genomes.urv.cat/UPGMA/index.php?entrada=Example3) (Figure 3). On the dendrogram there were two big clusters, which diverge smaller branches. There was no relationship between clustering among isolates from different locations and hosts.

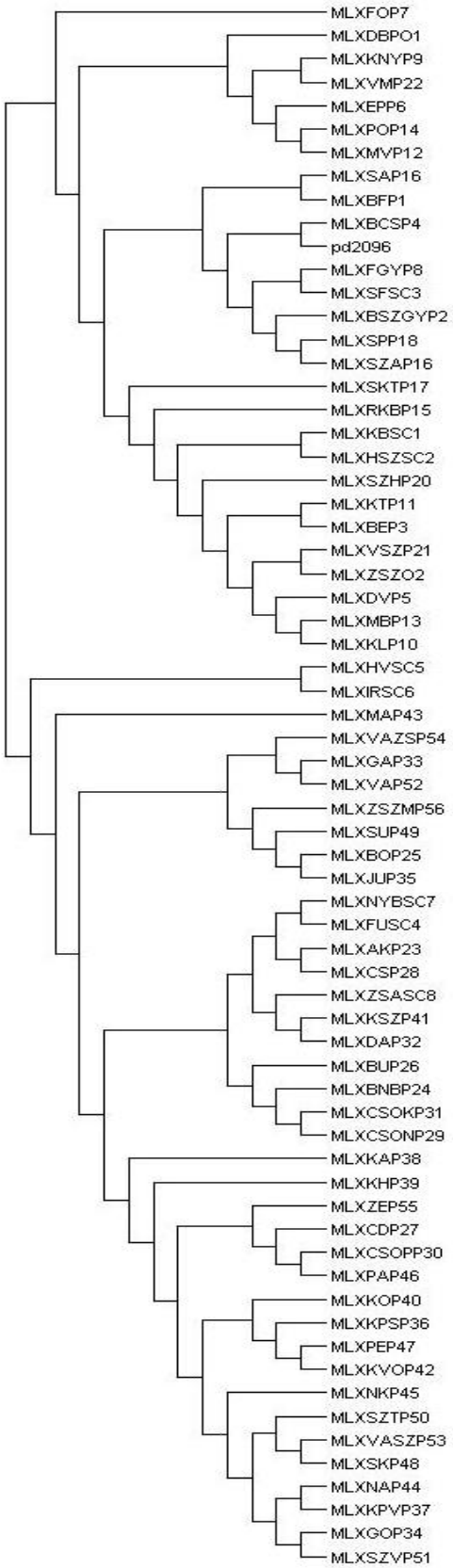


**Figure 3:** A dendrogram showing the relationships among isolates of Monilinia laxa sampled in different locations from Hungary, using data from the ISSR genotyping analysis.

### 3.2.2. Evaluation of RAPD-PCR

Twenty primers out of 39 tested were selected for further analysis because they generated clear and reproducible bands that could be used as RAPD markers. Each of these primers generated one to eight bands. These were ranged from 200 to 1500 bp in size. 82 markers (28 polymorphic and 54 monomorphic) were obtained. The RAPD grouping of isolates in the dendrogram showed no relationship between clustering among isolates from different locations and hosts (Figure 4).

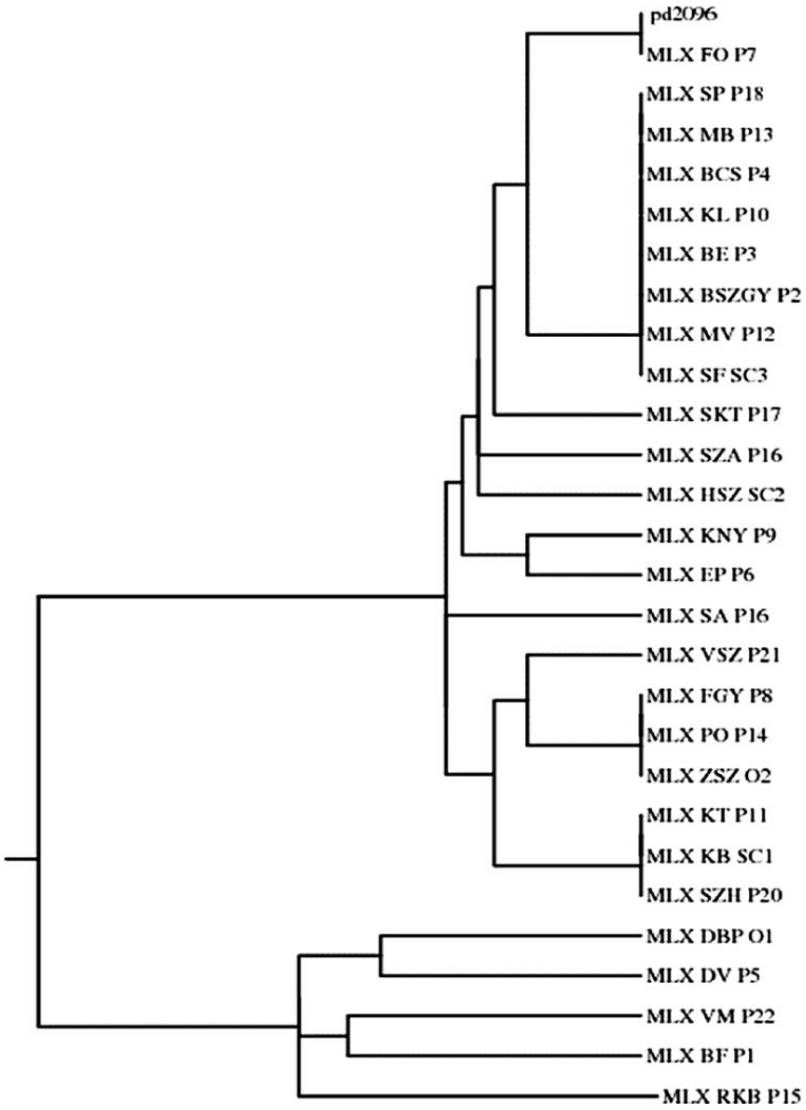


**Figure 4:** A dendrogram showing the relationships among isolates of Monilinia laxa sampled in different locations from Hungary, using data from the RAPD genotyping analysis.

### 3.2.3. Evaluation of ITS sequence

We had sequence of 356 bp fragments (ITS1-5,8S rRNS gén-ITS2) determined. Nucleotid sequence of each isolate was the same.

### 3.2.4. Analysis of genetic structure

An analysis of the population structure for ISSR-PCR and RAPD-PCR data set revealed that genetic diversity within subpopulations of geographical locations, host plants and fruit maturity stages ($H_S$=0.824-0.382 and 0.956-0.961) accounted for 99% of the total genetic diversity ($H_T$=0.832-0.838 és 0.962-0.968) (Table 2).

**Table 2:** Genetic diversity data according to Nei (total genetic diversity, genetic diversity within and between subpopulations, gen differentiation koefficiens) gene flow among *M. laxa* populations in case of 3 geographical locations (West-Hungary, Northeast-Hungary, South-Hungary), 3 host plants (plum, peach, sour cherry) and 3 fruit maturity stages (young, mature, mummified).

| Populations | $H_T$ | $H_S$ | $D_{ST}$ | $G_{ST}$ | Nm |
|---|---|---|---|---|---|
| **ISSR-PCR** | | | | | |
| 3 geographical locations | 0.838 | 0.832 | 0.006 | 0.007 | 70.29 |
| 3 host plants | 0.832 | 0.824 | 0.007 | 0.009 | 53.86 |
| 3 fruit maturity stages | 0.836 | 0.831 | 0.004 | 0.005 | 99.15 |
| **RAPD-PCR** | | | | | |
| 3 geographical locations | 0.962 | 0.957 | 0.005 | 0.005 | 93.98 |
| 3 host plants | 0.962 | 0.956 | 0.006 | 0.006 | 81.31 |
| 3 fruit maturity stages | 0.963 | 0.957 | 0.006 | 0.006 | 83.06 |

Genetic diversity between subpopulations ($D_{ST}$=0.004-0.007 and 0.005-0.007) represented only 1% for the same conditions. The relative magnitude of gene differentation between subpopulations ($G_{ST}$) was between 0.005 and 0.009 and between 0.005 and 0.007. The estimated numbers of migrants per generation (Nm) were between 53.9 and 99.2 and between 73.8 and 93.0 indicating little differentation among populations. This result suggested that the three populations were very similar to each other.

## 3.3. Development of brown rot forecasting and management strategy

### 3.3.1. Weather conditions and analysis of temporal brown rot progress

The year 2003 was favourable for codling moth damage and especially years of 2005, 2006, 2007 and 2008 were conductive for fruit rot. In cv. Prima, disease progress started at the end

of June or at the beginning of July in all years and both locations. For cvs. Idared and Mutsu, disease progress started only in late July or early August. Disease increased continuously from 6 to 8 weeks before harvest up to harvest depending on the year, cultivar and location (Figure 5). Brown rot incidence was influenced by the year, the cultivar and the location. In all cases the best fitting model was the three-parameter logistic model, so our model was based on this.

### 3.3.2. Brown rot forecasting and management strategy

A computer simulated submodel was used for our brown rot forecasting and management strategy, which requires insertion of field data and methods of data analysis for prediction purposes (mathematic curves, and their parameters). These were the following: AR6 model for spore dispersal, correlation coefficients for weather parameters, three-parameter Gompertz function for relationship between spore dispersal and responding disease incidence, and values of $AUDPC_s$, $Y_f$ és $\beta$ derived from three-parameter logostic curve at given time. These values were compared with the maximum values inserted into the simulation program at the beginning.

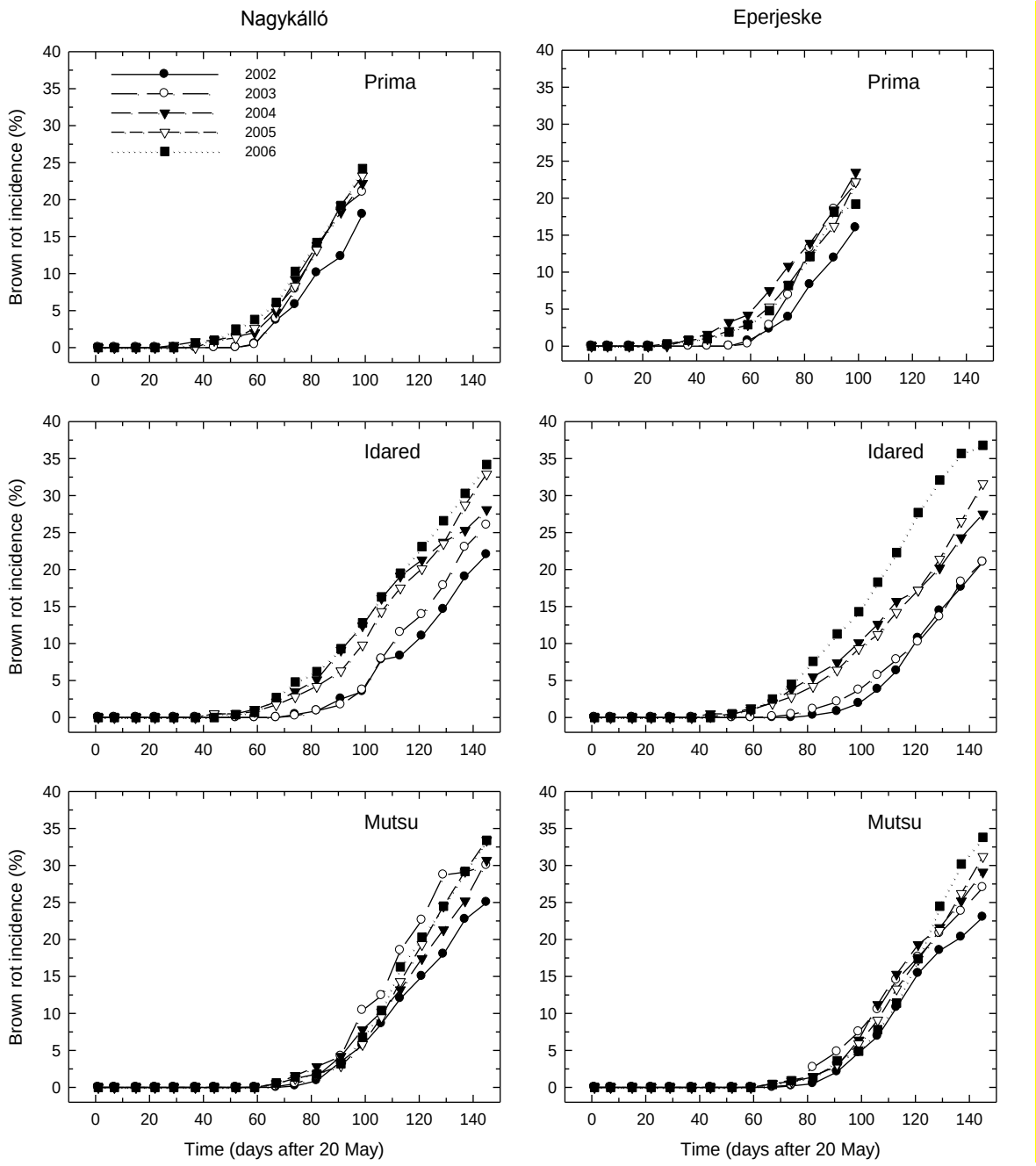


**Figure 5:** Disease progress curves of brown rot incidence caused by *Monilinia fructigena* assessed on three apple cultivars (Prima, Idared and Mutsu) in two organic apple orchards (Nagykálló és Eperjeske) from 2000 to 2006.

If the calculated values reached the given values, then three threshold values of the brown rot development could be calculated. The first threshold value was the time when disease incidence reached 1.5% ($T_{1.5}$), the second threshold value was the inflection point ($M$), the third threshold value was the final disease incidence ($Y_f$). Each threshold value corresponded to an epidemic intensity level (Figure 6).

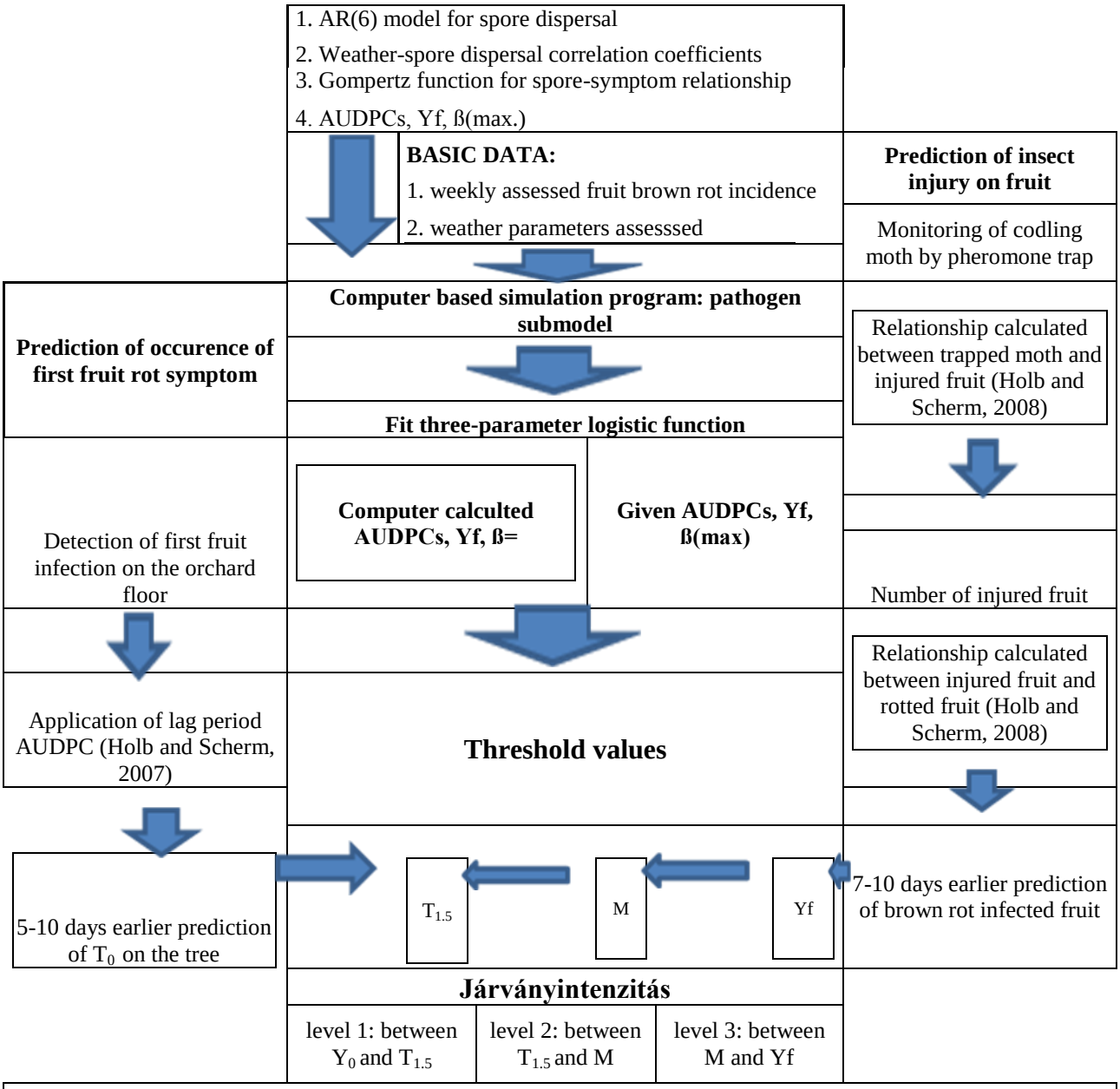


**Figure 6:** A brown rot forecasting and management strategy (BRFMS) against *Monilinia fructigena* for organic apple orchards. Explanations: AR6 model: autoregressive model with six autoregressive parameters, *AUDPC*: area under the disease progress curve (% days); $AUDPC_S$: standardized area under the disease progress curve (% days); $Y_f$: is the final disease incidence (%); $\beta$: is the relative rate variable ($day^{-1}$); $T_0$: is the time when the first fruit symptoms occur on the tree (day); $T_{1.5}$: is the time when disease incidence reaches 1.5 %-ot (day), and *M*: is the inflection point, measured in days.

The first threshold of BRFMS is suit for the forecasting of first appearance of infected fruit According to study of Holb and Scherm (2007), which was based on time lag period of *AUDPC* determined by the initial point of the fruit incidence on the orchard floor and by the point of the appearance of fruit incidence on the tree ($T_0$). With this approach, 5-10 days prediction of appearance of first symptoms was achieved.

The success of BRFMS was largely dependant upon the prediction of insect injury. In our model, counts of codling moth adults from pheromone traps were used as the basic data sets. The incidence value of fruits injured by insects were predicted based on the regression models (see Holb and Scherm 2008) in which relationship between trapped moth, injured fruit and actual brown rot incidence was explained. With this approach, 7-10 days earlier prediction of a newly infected, injured fruit could be achieved.

Then, in the decision module, brown rot management practises were suggested throughout the three epidemic intensity levels. In the period of first epidemic intensity level, timing of the first fungicide programs based on injury prediction as well as the removal of dropped fruit according to the study of Holb and Scherm (2007) were applied. In the period of the second epidemic intensity level, fungicide sprays based on model prediction, insect spray programs based on injury prediction, and weekly removal of infected fruits from the tree and from the orchard floor were needed. In the period of third epidemic intensity level, continuous insect spray programs based on injury prediction, intensive brown rot spray programs to reduce fruit rot and the removal of infected fruit were needed.

### 3.3.3. Practical evaluation of BRFMS in the general spray programme

The number of sprays against brown rot was between 8 and 12 in the general spray schedules while the number of sprays was significantly reduced 6-8 in the new disease management strategy in both locations and in the 3 years. (Table 3). In general, the number of sprays against brown rot was reduced by 22.2-33.3% in the new management strategy compared with the IFOAM general spray schedules. Despite the spray reduction in the BRFMS treatments, both incidence types were not significantly different from each other in the two spray programmes, brown rot incidence and percent insect injury incidence on fruit were significantly ($P<0,005$) lower compared to untreated control plots.

Field application of the BRFMS reduced number of sprays up to one third, which meant 2-4 fewer applications during summer. Summer spray reduction against brown rot coincides with possibilities for omission of sprays against apple scab as ontogenetic resistance of fruit of scab occurs at the second half of the season.

**Table 3:** Number of sprays, final brown rot incidence on fruit and percent injury incidence at harvest (%) in three season-long spray programme sin two organic apple orchards (Nagykálló and Eperjeske, 2006-2008).

| | Number of sprays | | | Brown rot and injury incidence (%)[a] | | |
|---|---|---|---|---|---|---|
| Treatments[b] | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| NAGYKÁLLÓ | | | | | | |
| GEVS | 8.3[c] b[d] | 7.5 a | 7.5 b | 26.1 (30.3) a | 28.6 (33.1) a | 23.3 (20.3) a |
| General BRDM | 12.0 b | 9.3 b | 10.5 b | 23.3 (28.6) a | 27.1 (30.2) a | 26.4 (22.2) a |
| Control | -[e] | - | - | 52.5 (58.9) b | 58.4 (55.5) b | 45.1 (41.8) b |
| $LSD_{0.05}$[f] | 2.6 | 1.4 | 2.0 | 10.7 (11.2) | 12.1 (10.1) | 8.9 (9.6) |
| EPERJESKE | | | | | | |
| BRFMS | 7.3 a | 6.5 a | 8.3 a | 24.2 (28.3) a | 22.6 (27.2) a | 25.2 (20.4) a |
| General BRDM | 9.5 b | 8.8 b | 11.8 b | 26.5 (32.1) a | 26.4 (30.1) a | 24.4 (21.2) a |
| Control | - | - | - | 52.6 (61.4) b | 50.8 (58.7) b | 57.1 (48.2) b |
| $LSD_{0.05}$ | 1.6 | 1.4 | 2.8 | 14.2 (15.1) | 13.1 (9.9) | 16.1 (11.9) |

[a] Percent insect injury incidence on fruits at harvest are in brackets.
[b] BRFMS sprays applied according to brown rot forecasting and management strategy, General BRDM general brown rot disease management, control: no brown rot and insect management
[c] Data means of four replications.
[d] Values within columns and location followed different letters are significantly different.
[e] No spray was applied. Control is not included in the statistical analysis.
[f] Means in each spray treatment were compared using Least Significanc Difference test at P = 0.05.

### 3.4. Effects of reduced spray programmes on important causative agents of apple in organic orchards

Incidence of apple scab reached 10% in integrated orchards, while it was above 30% in organic orchards in 2008. In reduced spray programmes of organic orchards disease incidence increased above 50%. Incidence of powdery mildew was low both in standard spray programmes and in reduced spray programmes (below 2%). It was above 10% in organic orchards. Therefore incidence of powdery mildew increased significantly in reduced spray programmes. Incidence of brown rot was minimal in integrated spray programmes. At the same time incidence of brown rot was high in both organic orchards.

Incidence of apple scab was not above 10% in integrated orchards, while in organic orchards it was above 20% in 2009. In reduced spray programmes of integrated orchards disease incidence did not increase, while in organic orchards it was above 30%. Incidence of powdery mildew was low both in standard spray programmes and in reduced spray programmes in integrated orchards (below 5%). It was above 20% in organic orchards. In organic orchards incidence of powdery mildew increased significantly. Incidence of brown rot was minimal in integrated orchards. At the same time it was high in organic orchards and in reduced spray programmes it doubled.

Our results showed, that reduced spray programmes at the second half of the season could be applied only in integrated orchards. In organic orchards reduced spray programmes would be at serious risks which is due to significant increased incidence of disease, therefore practical application was not suggested.

### 3.5. Studying of fungicide resistance

Inhibiting effects of fungicide on *M. laxa* isolates on PDA media are seen in Table 5. Four replicates were carried out, so average figures are in the table 5. Percent inhibition of 5 fungicide active ingredients (tiophanat-methyl, captan, penconazole, myclobutanil, mancozeb) was 100% for all the selected 12 isolates at all dosages after 5 days incubation. However, isolates showed different sensitivities to the other five fungicide active ingredients (boscalid+piraclostrobin, elementary sulphur, cyprodinil, fenhexamid and prochloraz, table 5). Sensitivity differences of the isolates were larger after 10 days incubation compared with after 5 days incubation. Almost all isolates were significantly insensitive to elementary sulphur at $P<0,05$. Isolates of MLX-FO-P7, MLX-SF-SC3, MLX-KL-P10 and MLX-BE-P3 were also partly insensitive to boscalid+piraclostrobin, cyprodinil and fenhexamid at $P<0.05$. In addition, one isolate (MLX-BE-P3) was also insensitive to prochloraz at $P<0.05$.

| Active ingredient | boscalid+piroclastrobin | | | elementary sulphur | | | cyprodinil | | | fenhexamid | | | prochloraz | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dosage** | **0.5x** | **1x** | **2x** | **0.5x** | **1x** | **2x** | **0.5x** | **1x** | **2x** | **0.5x** | **1x** | **2x** | **0.5x** | **1x** | **2x** |
| **Day 5** | | | | | | | | | | | | | | | |
| **isolates** | | | | | | | | | | | | | | | |
| **MLX-SP-P18** | 95 | 100 | 100 | 70 | 85 | 90 | 95 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-FO-P7** | 81 | 88 | 94 | 68 | 84 | 92 | 94 | 100 | 100 | 88 | 100 | 100 | 100 | 100 | 100 |
| **MLX-DBP-O1** | 100 | 100 | 100 | 63 | 73 | 90 | 67 | 83 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-SF-SC3** | 100 | 100 | 100 | 47 | 60 | 85 | 67 | 83 | 100 | 33 | 50 | 67 | 100 | 100 | 100 |
| **MLX-VSZ-P21** | 100 | 100 | 100 | 70 | 80 | 91 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-BF-P1** | 100 | 100 | 100 | 72 | 80 | 93 | 83 | 92 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-KL-P10** | 92 | 100 | 100 | 72 | 84 | 92 | 83 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-EP-P6** | 100 | 100 | 100 | 70 | 82 | 94 | 90 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-KNY-P9** | 100 | 100 | 100 | 93 | 97 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-KB-SC1** | 87 | 93 | 100 | 93 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-BE-P3** | 70 | 90 | 100 | 60 | 80 | 90 | 90 | 100 | 100 | 70 | 80 | 90 | 50 | 80 | 90 |
| **MLX-MV-P12** | 100 | 100 | 100 | 73 | 87 | 93 | 93 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **Day 10** | | | | | | | | | | | | | | | |
| **MLX-SP-P18** | 93 | 100 | 100 | 73 | 83 | 97 | 87 | 90 | 93 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-FO-P7** | 88 | 92 | 96 | 74 | 78 | 92 | 88 | 92 | 92 | 80 | 93 | 100 | 100 | 100 | 100 |
| **MLX-DBP-O1** | 100 | 100 | 100 | 78 | 86 | 96 | 76 | 88 | 92 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-SF-SC3** | 88 | 93 | 100 | 57 | 65 | 92 | 50 | 67 | 83 | 27 | 43 | 52 | 100 | 100 | 100 |
| **MLX-VSZ-P21** | 100 | 100 | 100 | 60 | 80 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-BF-P1** | 90 | 95 | 100 | 75 | 85 | 95 | 75 | 85 | 90 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-KL-P10** | 80 | 87 | 93 | 77 | 83 | 93 | 80 | 87 | 93 | 87 | 94 | 100 | 100 | 100 | 100 |
| **MLX-EP-P6** | 98 | 93 | 97 | 77 | 96 | 100 | 97 | 97 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-KNY-P9** | 100 | 100 | 100 | 81 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-KB-SC1** | 84 | 93 | 97 | 80 | 91 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **MLX-BE-P3** | 30 | 50 | 90 | 50 | 73 | 82 | 80 | 90 | 100 | 40 | 50 | 70 | 0 | 40 | 60 |
| **MLX-MV-P12** | 100 | 100 | 100 | 83 | 87 | 93 | 93 | 97 | 97 | 97 | 100 | 100 | 100 | 100 | 100 |

**Table 5:** Values of inhibiting effects of fungicide on *M. laxa* isolates at % by dosages of 0.5x 1x and 2x on PDA media *in vitro* PDA after 5 and 10 days incubation on 22 C°.

## 4. NEW SCIENTIFIC RESULTS

The following results of our study were new scientific results:

- Results of genetic studying of the selected *M. laxa* isolates showed that genetic variability of *M. laxa* populations was independent on geographic location, inoculum source, host and sensitivity of the fungus to fungicides. However, genetic diversity within subpopulations was high, about 99%.

- Temporal dynamics of disease was described with three-parameter logistic curve with which development of brown rot and dynamics of temporal epidemic were characterized and used for forecasting of disease.
- Brown rot forecasting and management strategy (BRFMS) was set out, which reduced annual number of sprays against brown rot by 15-25% in organic orchards compared with standard spray programmes.
- Effects of reduced spray programmes on three important apple causative agents were studied and it was concluded that reduced spray programmes at the second half of the season could be applied only in integrated orchards. In organic orchards, reduced spray programmes cause serious risks which is due to significant increase of disease incidence, therefore practical application was not suggested.

- Studying fungicide resistance of *M. laxa* isolates, percent inhibition of 5 fungicide active ingredients (tiophanat-methyl, captan, penconazole, myclobutanil, mancozeb) was 100% for all the selected isolates. While isolates showed different sensitivities to the other five fungicide active ingredients (boscalid+piraclostrobin, elementary sulphur, cyprodinil, fenhexamid és prochloraz a boscalid+piraclostrobin).

## 5. APPLICABLE RESULTS IN PRACTICE

- Our newly developed brown rot forecasting and management system (BRFMS) reduced the number of annual sprays with 15-25 % against brown rot caused by *M. fructigena* with which more cost-effective production can be carried out.

- Results on our tested reduced spray programmes shows that the number of annual sprays can be succesfully reduced in integrated orchards, while spray reduction are not recomemended for organic production system as it caused significant increase in disease incidences in organic orchards.

- Fungicide sensitivity experiments showed that five out of ten active ingredients (thiophanate-methyl, captan, penconazole, myclobutanil and mancozeb) could fully reduce mycelium growth of *M. laxa* isolates so these are can be succesfully used in the practice in fruit orchards. However, isolates showed various sensitivity to the other five fungicide ingredients (boscalid+pyraclostrobin, elementary sulphur, cyprodinil, fenhexamid and prochloraz) these fungicides are not recommended for commercial use as they have a high risk of field resistance potential.

## 6. REFERENCES


*Anon*: 1997. Biotermékek előállításának és minősítésének feltételrendszere. [Processing and qualifying organic products.] Budapest: Biokultúra Egyesület. In Hungarian.

*Anon*: 2000. Basic standards for organis production and processing. New York: Tholey-Theley.

*Cekic, C., Battey, M. H. and Wilkinson, M. J.*: 2001. The potential of ISSR-PCR primer-pair combinations for genetic linkage analysis using the seasonal flowering locus in *Fragaria* as a model. *Theoretical and Applied Genetics* 103: 540–546.

*Cote, M. J., Tardif, M. C. and Meldrum, A. J.*: 2004. Identification of *Moniliniafructigena*, *Monilinia fructicola*, *Monilinia laxa* and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant Disease* 99 (11): 1219-1225.

*Fan, J. Y., Guo, L. Y., Xu, J. P., Luo, Y. and Michailides, T. J.*: 2009. Genetic diversity of populations of *Monilinia fructicola* (*Fungi, Ascomycota, Helotiales*) from China. *Journal of Eukaryotic Microbiology* 1-7.

*Gell, I., Larena, I. and Melgarejo, P.*: 2007. Genetic diversity in *Monilinia laxa* populations in peach orchards in Spain. *Journal of Phytopathology* 155: 549-556.

*Groppe, K., Sanders, I., Wiemken, A. and Boller, T.*: 1995. A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichlöe* spp.) in grasses. *Applied and Environmental Microbiology* 61: 3943–3949.

*Holb, I. J.:* 2008. Monitoring conidial density of *Monilinia fructigena* in the air in relation to brown rot development in integrated and organic apple orchards. *European Journal of Plant Pathology* 120: 397–408.

*Holb, I. J. and Scherm, H.:* 2007. Temporal dynamics of brown rot in different apple management systems and importance of dropped fruit for disease development. *Phytopathology* 97: 10004-1111.

*Holb, I. J. and Scherm, H.*: 2008. Quantitative relationships beteween different injury factors and development of brown rot caused by *Monilinia fructigena* in integrated and organic apple orchards. *Phytopathology* 98: 79-86.

*Ioos, R. and Frey, P.:* 2000. Genomic variation within *Monilinia laxa*, *M. fructigena* and *M. fructicola*, and application to species identification by PCR. EPPO Conferences on diagnostic techniques for plant pests, 1-4 February 2000, Wageningen, the Netherlands, 52. (Abstract).

*Lim, S., Notley-McRobb, L., Lim, M. and Carter, D. A.:* 2004. A comparison of nature and abundance of microsatellite in 14 fungal genomes. *Fungal Genetics and Biology* 41:1025–1036.

*Nei, M.:* 1975. Molecular Population Genetics and Evolution. *New York, USA, American Elsevier:* 175 pp.

*Nei, M.* 1987. Molecular Evolutionary Genetics New York, NY, USA, Columbia University Press, 187 pp.

*Petróczy M.:* 2009. A *Monilinia fructicola* és a *Monilia polystroma* megjelenése Magyarországon és a védekezés újabb lehetősége (doktori értekezés). Budapesti Corvinus Egyetem, Kertészettudományi Kar 180 pp.

*Van Leeuwen, G. C. M., Stein, A., Holb, I. J. and Jeger, M. J.*: 2000. Yield loss caused by *Monilinia fructigena* (Aderh. & Ruhl.) Honey, and spatio-temporal dynamics of disease development. *European Journal of Plant Pathology* 106: 519-528.

*Van Leeuwen, G. C. M., Holb, I. J. and Jeger, M. J.*: 2002. Factors affecting mummification and sporulation of pome fruit infected by *Monilinia fructigena* in Dutch orchards. *Plant Pathology* 51: 787-793.

*Villarino, M, Larena, I, Martinez, F, Melgarejo, P. and De Cal, A.*: 2011. Analysis of genetic diversity in *Monilinia fructicola* from the Ebro Valley in Spain using ISSR and RAPD markers. *European Journal of Plant Pathology*. volume 132, number 4. 511-522(14).

*Wright, S.:* 1951. The genetical structure of populations. *Annals Eugenics* 15: 323–354.

*Xu, X. M., Robinson, J. D., Berrie, A. M. and Harris, D. C.:* 2001. Spatio-temporal dynamics of brown rot (*Monilinia fructigena*) on apple and pear. *Plant Pathology* 50: 569-578.

*Garcia-Vallvé, S. and Puigbo P.* 2002. DendroUPGMA: A dendrogram construction utility. Universitat Rovira i Virgili (URV). Tarragona. Spain http://genomes.urv.cat/UPGMA/index.php?entrada=Example3

*Anon:* http://www.gelanalyzer.com

## 7. PUBLICATIONS



Register number: DEENKÉTK/194/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Mónika Fazekas
Neptun ID: NXKPNG
Doctoral School: Kerpely Kálmán Doctoral School of Corp Production, Horticulture and Regional Sciences

### List of publications related to the dissertation

Hungarian scientific article(s) in Hungarian journal(s) (3)

1. **Fazekas M.**, Abonyi F., Balla B., Lakatos P., Holb I.: Előzetes vizsgálatok a Venturia inaequalis populációk in vitro érzékenységére egyes fungicid-hatóanyagokkal szemben. *Agrártud. közl. - 50*, 165-168, 2012. ISSN: 1587-1282.

2. **Fazekas M.**, Szalóki S., Madar A., Miklós I., Sipiczki M., Holb I.: Néhány hazai Monilinia faj azonosítása és biológiai vizsgálata. *Agrártud. közl. 46*, 27-29, 2012. ISSN: 1587-1282.

3. Balla B., **Fazekas M.**, Lakatos P., Abonyi F., Holb I.: Csökkentett permetezési programok hatása az alma jelentősebb gombakórokozóira környezetkímélő termesztési rendszerekben. *Agrártud. Közl. 42*, 13-16, 2010. ISSN: 1587-1282.

Foreign language scientific article(s) in international journal(s) (6)

4. **Fazekas, M.**, Madar, A., Sipiczki, M., Miklós, I., Holb, I.J.: Genetic diversity in Monilinia laxa populations in stone fruit species in Hungary. *World J. Microbiol. Biotechnol. 30* (6), 1879-1892, 2014. ISSN: 0959-3993. DOI: http://dx.doi.org/10.1007/s11274-014-1613-4 IF:1.353 (2013)

5. Holb, I.J., **Fazekas, M.**, Abonyi, F.: In vitro sensitivity of Monilinia laxa to fungicides approved in integrated and organic production systems. *Int. J. Hortic. Sci. 17* (4-5), 59-62, 2011. ISSN: 1585-0404.



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6. Holb, I.J., Balla, B., Abonyi, F., **Fazekas, M.**, Lakatos, P., Gáll, J.M.: Development and evaluation of a model for management of brown rot in organic apple orchards.
*Eur. j. plant pathol. 129* (3), 469-483, 2011. ISSN: 0929-1873.
DOI: http://dx.doi.org/10.1007/s10658-010-9710-1
IF:1.413

7. Holb, I.J., **Fazekas, M.**, Lakatos, P., Balla, B., Gáll, J.: Possibilities of blossom and twig blight management in organic stone fruit production.
*Int. J. Hortic. Sci. 16* (3), 103-105, 2010. ISSN: 1585-0404.

8. **Fazekas, M.**, Abonyi, F., Madar, A., Lakatos, P., Gáll, J., Holb, I.: Some aspects of reduced disease management against Monilinia spp. In sour cherry production.
*Int. J. Hortic. Sci. 16* (1), 51-53, 2010. ISSN: 1585-0404.

9. Holb, I.J., **Fazekas, M.**, Abonyi, F., Lakatos, P., Thurzó, S., Nyéki, J., Szabó, Z., Kruppa, J., Balla, B.: Effect of reduced spray programmes on incidences of apple scab, powdery mildew and codling moth damage in environmentally friendly apple production systems.
*Int. J. Hortic. Sci. 15* (4), 75-78, 2009. ISSN: 1585-0404.

Hungarian conference proceeding(s) (1)

10. Madar A., **Fazekas M.**, Miklós I., Sipiczki M., Abonyi F., Lakatos P., Balla B., Holb I.: Monilinia laxa izolátumok ISSR-markeren alapuló polimorfizmus vizsgálata.
In: IX. Magyar Genetikai Kongresszus és, XVI. Sejt- és Fejlődésbiológiai Napok : Siófok ..., 2011. március 25-27. : program, összefoglalók. Szerk.: ... Hohol Róbert ; rend. a Magyar Genetikusok Egyesülete és a Magyar Biológiai Társaság Sejt- és Fejlődésbiológiai Szakosztálya, Diamond Congress Kft., Budapest, 182, [2011]. ISBN: 9789638801944

Foreign language conference proceeding(s) (3)

11. Madar, A., **Fazekas, M.**, Sipiczki, M., Miklós, I., Holb, I.J.: Genetic diversity of Monilinia laxa populations from three geographic areas of Hungary.
*Acta Microbiol. Immunol. Hung. 60* (Suppl.), 48, 2013. ISSN: 1217-8950.

12. Madar, A., **Fazekas, M.**, Miklós, I., Sipiczki, M., Abonyi, F., Lakatos, P., Balla, B., Holb, I.J.: Are ITs sequences suitable for detecting intespecific polymorphism of Monilinia laxa?
In: A Magyar Mikrobiológiai Társaság 2010. évi Nagygyűlése : Absztraktfüzet. Közread.: Magyar Mikrobiológiai Társaság és a MMT Alapítványa, Magyar Mikrobiológiai Társaság és a MMT Alapítványa, Keszthely, 57-58, 2011.

13. **Fazekas, M.**, Madar, A., Sipiczki, M., Miklós, I., Abonyi, F., Lakatos, P., Balla, B., Holb, I.: Genetic diversity of Monilinia fructigena population from Hungary.
*Acta Microbiol. Immunol. Hung. 58* (Suppl. 1), 26, 2011. ISSN: 1217-8950.
DOI: http://dx.doi.org/10.1556/AMicr.58.2011.Suppl.1

## List of other publications

Foreign language scientific article(s) in international journal(s) (1)

14. Holb, I., Fodor, B., Lakatos, P., Balla, B., **Fazekas, M.**, Gáll, J.: Effect of production system and pruning on Aphis sambuci dynamics over time and on elderberry yield.
*J. Appl. Entomol. 134*, 615-625, 2010. ISSN: 0931-2048.
DOI: http://dx.doi.org/10.1111/j.1439-0418.2010.01511.x
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**Total IF of journals (all publications): 4,042**
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