UNIVERSITY OF WEST HUNGARY

PHD THESIS

The new disease of ash caused by *Hymenoscyphus fraxineus*, the examination of spreading, growing and pathogenicity of ash die-back

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1. Introduction

Common ash (*Fraxinus excelsior* L.), spread almost all over Europe, was attacked by a previously unknown pathogen in the 1990s. In 2006 *Chalara fraxinea* was described as a new species, a conidia producing fungus that causes ash dieback of common ash trees. The pathogen was identified in several countries and nowadays it has become one of the most important issues of forest protection in Europe. The high degree of the infection and the considerable spread of the disease raises the question of the future cultivation of the economically important and valuable common ash. On the other hand it could arise problems in the function of some forest communities – eg. oak-ash-elm grove forest, rock forest, detritus forest, – and forest ecosystems because of its significant ecological role.

Since then it has been proved that the pathogen does not only attack and destroy common ash. New investigations show that narrow-leafed ash (*Fraxinus angustifolia* Vahl. subsp. *danubialis* Pouzar.), black ash (*Fraxinus nigra* Marshall.) from North -America and green ash (*Fraxinus pennsylvanica* Marshall.) are also susceptible. Moreover the natural contamination of flowering ash (*Fraxinus ornus* L.) is also proven.

Despite the fact that ash dieback spreads dramatically all over Europe, a small number of common ash specimen can resist the infection even in places where most of the trees are largely damaged. The natural resistance raises hopes to protect common ash and improve resistant specimen.

Thus the principal aim of my thesis is broadening ecological and plant protection knowledge of ash dieback.

2. Objective

At the beginning of my research I set following aims:

1. monitoring the presence and the spread of the pathogen in common ash stands with different site conditions and stand structures, as well as the alteration of the state of health of ash stands,
2. in laboratory tests the examination of the growing features of cultures collected from *Chalara fraxinea* isolates of different provenances, furthermore finding a connection between growth and colour as well as provenance of the cultures,
3. to test the effect of fungicides with different agents on cultures of the pathogen under laboratory conditions,

4. investigation of the pathogenicity of pathogen phyla in Hungary, after an artificial inoculation on seedlings of ash species present in Hungarian forests, – common ash, narrow-leafed ash, flowering ash, green ash – whereas there is the opportunity of natural infection, and defining the susceptibility of ash species.

My hypotheses were:

1. The pathogen *H. fraxineus* causes decay of the health state of common ash – both on specimen and on stand level.
2. The pathogen attacks common ash irrespectively of the site conditions.
3. Cultures collected from isolates of different provenances can be characterised by various qualitative (colour) and quantitative features (size, rate of growth).
4. By using some fungicides, the growth of the pathogen can be influenced, accordingly the spread can be moderated.
5. The pathogen *H. fraxineus* is able to attack other ash species apart from common ash, but they have different susceptibility to the pathogen.

3. Material and method

Monitoring ash dieback in common ash stands

The monitoring was carried out on the forest land area of the Szombathely Forestry Zrt., Sárvár Forestry Directorate, in 16 common ash forest subcompartments between 2010 and 2015. The purpose of the monitoring was observing the health state changes in common ash stands with different climatic and site conditions, stand structures, as well as observing the spread of the disease year by year.

In Sárvár 6 H subcompartment not only common ash but also green ash trees are present. Their health state was measured every July and August.

In natural common ash subcompartments, in randomly chosen 2 diameter regrowth groups, the health state of 3-4 trees next to each other were measured. This means 100 specimen in 25 regrowth groups each subcompartment, where the distance between the groups were 15-20 meter. In artificial forests and in older stands in every fifth row 20 specimen next to each other were measured, altogether yearly 100 trees in all subcompartments. During the survey characteristic symptoms were written down. The following <u>health state categories</u> were used: **0.:** On the surface <u>healthy, symptomless</u> specimen, crown dieback 0-10%.; **1.:** <u>Slightly</u>

infected specimen: first symptoms, or eventually symptoms from previous year, crown dieback 11-25%.; **2.:** Strongly infected specimen: symptoms originate from several years before, crown dieback 26-75%. Thin epicormic shoot appears.; **3.:** Dying specimen: crown dieback over 75%, strong epicormic shoot was observed.; **4.:** Dead specimen.

Rate of growth analysis of the pathogen

The aim of this analysis was establishing growth differences among *H. fraxineus* isolates from West-Transdanubia, finding correlation between the rate of growth and the place of origin, as well as between the colour of cultures and the rate of growth. 39 isolates of the pathogen were utilized, from each isolates 2 cultures were inoculate in Petri dishes. The cultures grew at 20 °C degrees, in a dark place, on potato-dextrose agar (PDA) substratum. Their surface was measured three times with the help of a section paper. The first measuring took place on the 25th day, the second 30 days later, the third on the 250th day. From the measured data daily growing rate was calculated (mm^2/day). The colour of the cultures was evaluated by the Kowalski and Bartnik (2010) scale.

Protection against the pathogen

The purpose of the protection experiments was blocking the growth of the pathogen cultures and destroying them under laboratory conditions. The strong growing number 196/19. culture from Sárvár was inoculated in malt extract agar. This experiment was repeated six times, from the cultures 6-6-6 malt extract substrata were prepared pro agent, as well as 6 control cultures. Three fungicide agents – ciprodinil, iprodion, fenhexamid – were used, which were sprayed on the cultures with the help of a pharmaceutical feeder when they were one week old. The cultures applied in this experiment were kept in dark, at permanent 24 °C degrees.

For spraying the pathogen inoculated in the substratum, the quantity of the fungicides was calculated according to the instruction sheet: thus 1 mg ciprodinil, 1,5 µl iprodion and 1 µl fenhexamid was spayed out. After the treating the rate of growth of the pathogen cultures were monitored every day, and they were compared with the cultures inoculated in the control substrata. The surface of the cultures was measured three times with the help of a section paper. The first measuring took place on the day of the treating, the second on the third day, the third three days later. From the measured surface data the effect of the individual agents on the growth of the pathogen was concluded.

Pathogenecity test

The aim of the test was investigating the susceptibility of ash species present in our climate against the pathogen, and establishing a susceptibility rank.

In 2010 an artificial inoculation experiment was started in the Bejcgyertyános nursery of the Szombathely Forestry Zrt., in order to gain knowledge of the pathogenicity of *H. fraxineus*. The experiment was carried out on one-year-old plants. In the artificial inoculations of 2010 and 2011 the strong growing *C. fraxinea* phyla with the identity numbers 196/18 and 196/21 from Sárvár were used, in 2014 the also strong growing *C. fraxinea* phyla with the identity numbers 196/8 and 196/21 from Kapuvár. For each species 10 uninfected control plants were monitored.

After the second artificial inoculation in 2011 no other infection was applied on common ash and narrow-leafed ash seedlings, as the natural infection could not be excluded any more. On all previously infected flowering ash and green ash seedlings the infection was repeated in 2014, whereas in case of common ash and narrow-leafed ash seedlings only the characteristic symptoms were recorded. In 2016 the symptoms were surveyed again both on the infected and on control specimen of all ash species.

During the artificial inoculation the method of stem wounding was applied. For the evaluation of the symptoms the degree of the wilting in addition the degree of the shoot dieback of the infected plants were recorded. While classifying the contamination of the individual plants, the following categories were stated: **1.:** At first sight healthy, <u>symptomless</u> specimen.; **2.:** <u>Slightly infected</u> specimen: first symptoms, or eventually symptoms from previous year.; **3.:** <u>Strongly infected</u> specimen: symptoms originate from several years before.; **4.:** <u>Dead</u> specimen.

4. Results

Results of the ash dieback survey

In the individual subcompartments significant differences can be shown in different degree among the health state data monitored yearly. It follows from the test results that several years later more and more significant differences appeared in the health state of the tree stands.

The tree stands can be grouped in three well distinguishable categories on the basis of the ash dieback symptoms in 2015. In tree stands belonging to the first group there are no dying or dead specimen. The proportion of the symptomless trees are between 72-90%. Common ash stands in the subcompartments of this category are least of all infected by ash dieback. In the second group there can be found dying or dead plants in a small per cent, the symptomless trees and the trees with a less than 25% crown dieback symptom are together about 75%. The proportion of the symptomless plants add up to 31-51%. Trees of subcompartments belonging here are moderately infected by the disease. Plants in the third group has a far deteriorated health state in 2015. The proportion of dying or dead plants is between 19-41%, the degree of specimen with a crown dieback under 25% is merely 18-38%, the rate of the symptomless trees falls between 10-16%. There are not any significant differences among the health state of tree stands in the same group in year 2015. (p=1,000).

Subcompartments rated in three groups by right of the ash dieback symptoms, show a great variety in stand structure features and site conditions. The age of the tree stands in the first category is 5-25 year, the mixture rate of common ash ranges from 20% to 30%. Both the genetic soil type and the depth of the soil differ. Tree stands in the second category are 11-84 year old, the mixture rate of common ash ranges between 60% and 100%. The diversity of the site conditions is perceptible here as well. Subcompartments in the third group are mostly affected by ash dieback. The age of tree stands is between 10 and 29 year, the mixture rate of common ash ranges from 5% to 60%. The genetic soil type and the depth of the soil are even here different. The hydrologic conditions of the three groups are similar.

On the basis of the results of the health state examinations in the tree stands ranked in three categories it can be determined that the appearance and degree of the symptoms of ash dieback, the contamination of the tree stands do not correlate with the age of the tree stands, the mixture rate of common ash, the hydrologic conditions, the genetic soil type and the depth of the soil.

In the subcompartment Sárvár 6 H green ash appears in 35% mixture rate, but it was not susceptible to the infection caused by the pathogen *H. fraxineus* despite the fact, that in the subcompartment sporadically infected common ash specimen arise among green ash trees.

Rate of growth of the pathogen

The growing examinations of the cultures supported the great variety of the pathogen in Hungary both in rate of growth and in colour.

In the repeated experiments the measured and averaged culture sizes were placed in growing order in all three growing periods. The rate of growth of the individual cultures compared to each other was varied. In respect of the absolute growing sizes the cultures took different places in each growing stage. After the first growth stage some cultures were growing more aggressive while others slower during the whole experiment. In the whole growing period three rate of growths were distinguished: the area of the weak growing cultures were less than 3500 mm^2, that of the medium growing rate were between 3500 mm^2 and 5000 mm^2, while the strong growing cultures took an area over 5000 mm^2.

The cultures grew in the first period in the largest measure, while they grew in the third period the least. Besides, in the third stage the growing differences between the individual cultures were the least different.

Some cultures grew slowly from the beginning of the survey, though others were definitely stronger in growing, and these features were true during the whole experiment. However, some cultures started with a slower growth but grew quicker later; others produced a contrary growth, that means after a strong growing they slowed down.

Significant differences ($R= -0,509$; $p=0,000$) were surveyed between the growth of cultures characterised by different colour scale. The white coloured cultures, marked with colour scale „A" grew the biggest during the same time, the orange coloured cultures, marked with colour scale „B" grew a bit smaller, the brow cultures marked with „C" developed the least.

Regarding the cultures from Kapuvár and Bakony, diverse colours developed from the same provenance. However, the cultures from Sárvár and Homorúd can be characterised by an only colour. The connection between provenance and rate of growth was supported by the correlation between growing and colour.

Significant differences ($p=0,003$) appeared also between the size of the cultures from other provenances. Cultures from Homorúd (merely brown coloured) showed the weakest rate of growth, then in order the cultures from Kapuvár, from Bakony, and the culture from Sárvár pointed at the strongest growing (only white coloured).

Results of the protection against the pathogen

The cultures treated with ciprodinil grew three day long after the spraying weakly, then the growing of the pathogen stopped soon. Up to the third measuring the cultures disappeared, the mycelia could be seen only on the infected agar pieces. Iprodion also blocked the pathogen, for the second measuring the growing of the pathogen stopped in case of all six repeats. A control after the third day showed that 4 of 6 repeats were completely blocked (R_1, R_3, R_4, R_5 cultures), mycelia were seen only on the infected agar pieces, while the mycelia of two other cultures (R_2 és R_6) were largely blocked. Using the agent fenhexamid in six-repeat-experiments the growth of 5 cultures were blocked on the third day. The only exception was culture „T_6”, but even it had merely a minimal rate of growth after the third day. On the contrary, the area of the control cultures grew strongly in all six repeats.

In laboratory tests all three used agents –ciprodinil, iprodion, fenhexamid–, blocked the growing of the mycelium of the pathogen, even if in different degree. The two agents with contact effect, iprodion és fenhexamid had similar effect to ciprodinil, with the difference that iprodion could completely block the growing only in 4 of 6 repeats; after using fenhexamid one culture grew on slightly. On the third day the growing average of the 6 cultures after treating with ciprodinil was 13,67 mm^2, using iprodion 9,5 mm^2, using fenhexamid 11,5 mm^2. It verifies the quicker effect of contact effect agents, as in this case the blocking of the area growth happened earlier than in case of the systemic ciprodinil. This difference regarding the three agents disappeared completely for the sixth day. It is supported by the results of Kruskal-Wallis statistic test, which says that the growing of areas treated by iprodion (p_i) and fenhexamid (p_f) significantly differs from the control cultures (p_i=0,008 and p_f=0,006) on the third day; but in this period there is no significant difference between the growth of cultures treated by ciprodinil (p_c) and the control cultures (p_c=0,133). To compare the final sizes on the sixth day after the spraying, the growing of cultures treated by fungicides differs significantly from the control cultures (p_c=0,016; p_i=0,011 and p_f=0,042).

Pathogenicity test

After the artificial inoculations in 2010 and 2011 the first symptoms appeared 2-3 weeks after the treatment in July on common ash and narrow-leafed ash specimen in form of shoot-and leaf wilting. In 2010 some common ash and narrow-leafed ash trees seemed to be susceptible to the pathogen: 24% as well as 21% of the inoculated plants produced wilting.

In 2011 75% of the inoculated common ash trees had symptoms, and on 10% of the seedlings symptoms from both years were visible. After the second inoculation 2% of the common ash seedlings died. However the two artificial infections and the opportunity of the natural infection, 25% of the common ash trees remained symptomless. The second artificial inoculation in 2011 did not notably increase the number of the susceptible narrow-leafed ash.

The inoculated flowering ash seedlings remained symptomless in 2010, but in 2011 symptoms appeared on 10% of them. Under laboratory conditions *C. frax-*

inea conidia producing fungus was confirmed from tissues collected from flowering ash with infectious symptoms. In the following years even these specimen with symptoms did not produce new symptoms. It proves that the symptoms of the disease do not spread in the infected flowering ash.

In 2010 and in 2011 the symptoms could not be observed on any of the control ash trees. Thus it can be stated that in 2010 and in 2011 symptoms appeared on the infected plants solely as a result of the artificial inoculation. From 2014 symptoms turned up on common ash and narrow-leafed ash control seedlings.

At the end of the survey the proportion of the infected common ash plants with symptoms were 90%, while the infected narrow-leafed ash were 88%. The susceptibility rank of the ash species present in this experiment was settled: common ash seems to be the most susceptible, it is followed by narrow-leafed ash and flowering ash. Green ash proved to be resistant; all infected and control specimen stayed symptomless during the whole experiment.

The degree of the symptomless plants reflects the susceptibility of each ash species that were exposed to natural infection after the artificial inoculation. The proportion of trees that stayed completely symptomless during the whole period: flowering ash 90%, narrow-leafed ash 12%, common ash 10%.

According to the opportunity of the artificial and natural infections 20% of common ash trees and 13% of the narrow-leafed seedlings died during the whole period. Till the end of the six-year-long survey the number of the infected plants raised up to 66% (common ash) and 67% (narrow-leafed ash).

5. Theses

According to the results I set the following theses:

1. **The health decay of the examined common ash stands caused by ash dieback was not influenced by the site conditions (hydrologic conditions, genetic soil type, thickness of soil), or tree stand structures (age, mixture rate).**

The examined common ash stands can be classified into three significantly different groups according to their contamination. The group with the largest infection rate was represent with the greatest number of subcompartments; where 4-19% of the trees died. The three distinguished groups are characterised by various site conditions and stand structures.

2.a **As a result of the health state examination of common ash stands, green ash is not susceptible to the infection caused by the pathogen *H. fraxineus.***

In the examined Sárvár 6 H forest subcompartment, the green ash trees – mixture rate 35% - did not show symptoms of ash dieback, although the neighbouring common ash trees - 20% of the stand – were infected.

2.b **The pathogenity examinations could not prove the susceptibility of green ash to the ash dieback pathogen *H. fraxineus.***

However the two artificial infections and the opportunity of the natural infection, both the infected and the control plants of green ash stayed symptomless in the examined period.

3.a **The growth experiments of the cultures demonstrated the great variability of the pathogen.**

The examinations pointed at the different rate of growth of the cultures from the same provenance. The Cluster-analysis supported that in many cases there is a closer similarity between cultures from different provenances than between cultures from the same origin.

3.b **There are significant differences between the rate of growths from different provenances.**

Cultures from Homorúd showed the weakest rate of growth, then in order the cultures from Kapuvár, from Bakony, and the culture from Sárvár pointed at the strongest growing.

3.c **White cultures had the strongest rate of growing, then the orange ones, whereas the brown cultures gained the weakest growing.**
The Spearman rank correlation examination resulted in significant differences between the cultures with different colour scale.

4. **The agents ciprodinil, iprodion, and fenhexamid block the growth of the pathogen.**
The two agents with contact effect, iprodion és fenhexamid could completely block the growing of the pathogen even on the third day of the spraying, while the systemic ciprodinil reached the same effect only on the sixth day. On the sixth day the growing of cultures treated by any of the three fungicides differs significantly from the control cultures.

5. **Common ash seems to be the most susceptible to the pathogen *H. fraxineus*, it is followed by narrow-leafed ash, then flowering ash.**
At the end of the survey in 2016 the proportion of the infected common ash plants with symptoms were 90%, the rate of the infected narrow-leafed ash were 88%, while that of the flowering ash was 0%.

6. **In pathogenic tests the susceptibility of flowering ash to the pathogen *H. fraxineus* was proved.**
After artificial inoculation in 2011 symptoms appeared on 10% of the flowering ash, but it proved that the symptoms of the disease did not spread in the infected flowering ash trees, as symptoms could not be monitored in 2014 and 2016. Symptoms only manifested in the form of wilting.

7. **Results of health state experiments and pathogenic tests verified that some common ash trees are resistant to the pathogen *H. fraxineus.***
At the end of the survey the range of the symptomless plants were between 10% and 16% even in the most infected common ash stands. Similarly, in the pathogenicity test 10% of the common ash trees stayed symptomless.

6. The list of publications related with the thesis

Scientific papers

1. Nagy L., Szabó I. (2013): A magas kőris hajtáspusztulását okozó gomba (*Chalara fraxinea*) járványdinamikai és patogenitási vizsgálata. Növényvédelem 49 (9): 389-396.
2. Szabó I., Németh L., Nagy L. (2009): A magas kőris hajtáspusztulása. Erdészeti Lapok, 144 (2): 46-48.

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1. Nagy L., Szabó I. (2012): A kőris hajtáspusztulását okozó *Chalara fraxinea* járványdinamikai és patogenitási vizsgálata. Kari Tudományos Konferencia Kiadvány. Nyugat-magyarországi Egyetem, Erdőmérnöki Kar. Konferencia helye, ideje: Sopron, Magyarország, 2011. 10. 05 Sopron: Nyugat-magyarországi Egyetem
2. Nagy L., Szabó I. (2011): A kőris hajtáspusztulását okozó *Chalara fraxinea* járványdinamikai és patogenitási vizsgálata. Tudományos Doktorandusz Konferencia, Nyugat-magyarországi Egyetem. Konferencia helye, ideje: Sopron, Magyarország, 2011. 04. 13.

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1. Nagy L., Szabó I. (2011): A kőris hajtáspusztulását okozó *Chalara fraxinea* járványdinamikai és patogenitási vizsgálata. 57. Növényvédelmi Tudományos Napok. Konferencia helye, ideje: Budapest, Magyarország, 2011. 02. 21 - 2011. 02. 22.
2. Nagy L., Szabó I. (2011): Epidemic and Pathogenicity of *Chalara fraxinea* Causing Ash Dieback in Hungary. IUFRO WP 7.02.02. conference Global change and forest diseases: new threats new strategies: Konferencia helye, ideje: Montesclaros Monastery, Spanyolország, 2011.05.23-2011.05.28.
3. Nagy L., Lakatos F., Szabó I. (2010): Ash dieback caused by *Chalara fraxinea* in Hungary. Biotic Risks and Climate Change in Forests. Konferencia helye, ideje: Freiburg, Németország, 2010. 09. 20 - 2010. 09. 23.

Conference publications/Book of abstract

1. Nagy L., Szabó I. (2012): A kőris hajtáspusztulását okozó *Chalara fraxinea* járványdinamikai és patogenitási vizsgálata. In: Lakatos Ferenc, Szabó Zilia (szerk.): Kari Tudományos Konferencia Kiadvány. Nyugat-magyarországi Egyetem, Erdőmérnöki Kar. 85-87.

2. Nagy L., Szabó I. (2011): A kőris hajtáspusztulását okozó *Chalara fraxinea* járványdinamikai és patogenitási vizsgálata. In: Kőmíves T, Haltrich A, Molnár J (szerk.): 57. Növényvédelmi Tudományos Napok. 72.
3. Nagy L., Szabó I. (2011): Epidemic and Pathogenicity of *Chalara fraxinea* Causing Ash Dieback in Hungary. In: IUFRO WP 7.02.02. conference Global change and forest diseases: new threats new strategies: Book of abstracts. 109.
4. Nagy L., Szabó I. (2011): A kőris hajtáspusztulását okozó *Chalara fraxinea* járványdinamikai és patogenitási vizsgálata. In: Lakatos F, Polgár A, Kerényi-Nagy V (szerk.): Nyugat-magyarországi Egyetem, Tudományos Doktorandusz Konferencia, Konferencia-kötet. 185-187.
5. Nagy L., Lakatos F., Szabó I. (2010): Ash dieback caused by *Chalara fraxinea* in Hungary. In: Horst Delb, Silvia Pontuali (ed.): Biotic Risks and Climate Change in Forests. 175-176.

Publications not connected to the thesis

Scientific publications:

1. Sárándi-Kovács J., Nagy L., Lakatos F., Sipos Gy. (2016): Sudden Phytophthora Dieback of Wild Cherry Trees in Northwest Hungary. Acta Silvatica et Lignaria Hungarica, 12 (2): 117-124.
2. Nagy L. (2005): Uradalmi erdőgazdálkodás Vas vármegyében. Erdészeti Lapok, 140 (6): 182-183.

Non-scientific publications:

1. Gángó Cs., Nagy L. (2013): Bajor és türingiai szálalóerdők mélyén. Erdészeti Lapok, 148 (9): 219-294.

2. Nagy L., Urbán P. (2006): A PRO SILVA EUROPA 2005. évi horvátországi rendezvénye. Erdészeti Lapok, 141 (1): 16-18.