University of West Hungary
Faculty of Forestry

Doctoral theses

Chestnut blight fungus [*Cryphonectria parasitica* (Murr.) Barr] in Sopron Hills

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1. Importance of the topic

In the last century several epidemic appeared in forest ecosystems, which were dangerous on surviving of tree species because of great pathogenicity and the area of the disease. In most cases the introduce of the pathogen or the movement of host caused pandemic.
Dutch elm disease, and white pine blister rust or chestnut blight were among these. Control of the dynamic spreading epidemic on forest covered areas needed extremely efforts, and usually was not possible by the traditional means of forest protection.

The causal agent of chestnut blight is *Cryphonectria parasitica*, a microscopic fungus introduced into Europe from Asia, mediated in America. A few decades before disease reached Sopron Hills and caused epidemic infections and destruction of chestnut trees in plantations and semi-natural forest associations also. The control of disease and the stop of the epidemic are possible by only one effective biological method. The application of the method: surveying the population of the pathogen, and with the help of this information the less virulent strains of the pathogen into the trees, thanks to the results of these treatment the process of healing is spreading onto chestnut stands and forest associations.

2. Scientific background

Because of the importance of pathogen and the pandemic started at the beginning of the last century, many of relevant sources are available on this theme.

2.1. International scientific background

At first in 1904, **Merkel** observed the pathogen, when the epidemic threatening with the destruction of the whole American chestnut population began in the USA. **Murrill**, **Anderson** and **Barr** gave the first morphological description of the species, and specificated the fungus. The pathogen endemic in the Far East was moved into Europe, where it also caused great destruction of European chestnut.

The control of the epidemic was not possible by the traditional plant protection methods. The goal in the control was the environment considerated biological protection, which based on spreading of less virulent strains of the pathogen. The cytoplasm of these less virulent (hypovirulent) strains contain double-stranded RNA, the European type of this dsRNA called *Cryphonectria hypovirus 1* (CHV-1)**.** CHV-1 is could be transmit into a virulent strains in laboratory and natural conditions also, after this transmission the recipient virulent strain will become hypovirulent. The tree can overcome the attack of hypovirulent strains, the cambium and phloem are living under the hypovirulent cankers. But the transmission of CHV-1 could be possible only between each other compatible strains. So the isolates of the pathogen was categorized as vegetative compatibility types: EU-tester strains were determined.

In Europe **Biraghi**, **Grente** and **Berthelay-Sauret** made the first steps of control based on hypovirulence, apart from them **Turchetti** also took part in improving of practical applications. **Anagnostakis** developed the methods of

vegetative compatibility tests. **Dodds**, **Fulbright** added to our knowledge in molecular biology: they detected double-stranded RNA in the cytoplasm of less virulent strains, and proved correlation between dsRNA and hypovirulence.

Heiniger, **Cortesi**, **Robin**, **Wilhelm** had great role in creating European cooperation.

In contact to this study in chestnut micropropagation **Wilhelm**, **Ballester**, **A. M. Vieitez** and **F. J. Vieitez** have reached great results.

2.2. Scientific background in Hungary

In Hungary at first in 1969 **Attila Körtvély** detected the fungus in Nemeshetés, Zala County. Surveying the contaminated area of *C. parasitica* is due to **István Eke** and **Tibor Gál**. They also searched the chemical control of blight. The application of biological control in Hungary is the work of **László Radócz**, **Mária Varga**, **Ilona Szabó**, **János Sótonyi**, **Ilona Garamvölgyi Aponyiné**. Investigations on vegetative compatibility types of the pathogen was carried out by **László Radócz** in the whole area of Carpathian basin.

In 1996, in Ágfalva situated in Sopron Hills, **László Radócz**, **Ilona Szabó** and **Mária Varga** started curative treatments, with converted hypovirulent strain originated from **László Radócz**.

3. Objectives

The aim of this study was to characterize the population of pathogen, and to adapt the only effective, biological control onto the Sopron Hills. In accordance, the main goals of the research were as follows:

- to investigate the area of the disease in Sopron Hills,

- to collect bark samples from the infected areas, to isolate from these samples the pathogen, and to maintain in strain collection until the following laboratory investigations,
- to determine from the isolates the different vegetative compatibility types of the pathogen, and to determine the area of these types in the hills,
- to investigate compatibility to EU-tester strains, to deduce the possible spreading of the epidemic from these results,
- to find endogenous hypovirulent strains, if possible,
- to investigate the possible hypovirulent strains in virulence tests,
- in lack of endogenous hypovirulent strains, to converse the virulent strains with Hungarian hypovirulent strains,
- to justify the hypovirulence in these strains by molecular biological methods, and to prove the same genotypes,
- to utilize hypovirulent converted strains in a curative biocontroll programme during years, to evaluate the treatments, to improve practical applications for more efficiency
- to examine micropropagation of sweet chestnut
- to investigate *in vitro* interaction between host and pathogen
- to develop a rapid and reliable *in vitro* virulence test that suitable to separate virulent and hypovirulent strains.

4. Materials and methods

4.1. Field investigations

In autumn 1996 investigations were carried out on 4070 hectares of Sopron Hills (Sopron, Ágfalva and Harka). The determination of infection was carried out by linear sampling in each forest division. Sample for further laboratory investigations were collected from each forest division in which disease was occurred.

Field trials with converted hypovirulent strains were done from autumn 1996 to 2000 in a chestnut stand in Ágfalva. In the treatments every canker were artificially inoculated roundly with mycelia of adequate hypovirulent strains. At the first step cork borer wounds were carried out in the living bark, in distances 4-5 cm from each other. These holes were filled with the hypovirulent inoculum (pieces of media including mycelial fans), and at last the holes were closed with ointment. At parallel the treatments diseased branches were cut. In every year the results of artificial inoculations were evaluated. Treatments were carried out on 37 diseased trees.

In a few cases when the healing was uncertain the treatment was different: the holes were filled by turns with two hypovirulent strains. Strongly diseased, severe cankered trees were treated differently: not only cankers were treated roundly, but the stem also was treated vertically from the stock to the great branches up to the crown. Infected bark of heavy sporulated cankers was removed, to decrease further infections.

4.2. Laboratory investigations

4.2.1. Classical investigations

At first step of the classical investigations the pathogen was isolated from the bark samples. All of isolates were maintained in a strain collection on later vegetative compatibility tests. 51 isolates were identified by vegetative compatibility test. The identified vegetative compatibility types were converted with Hungarian hypovirulent strains as it was possible.

A few isolates seemed to be hypovirulent because of their origin or morphological characteristics, these strains were tested for virulence.

4.2.2. Molecular biological investigations

Hypovirulence is could be verified by molecular biological methods in a strain of possible hypovirulent fungus (less virulent, or different colony morphology), or in a converted hypovirulent strain of the fungus. The investigated strains were: S11, S18, S18xSa3, S21, S21xR5, S21xIhb2, An – hypovirulent control strain, S23 – virulent control strain. Isolation and analisation of dsRNA was performed by phenolic extraction method. Samples of extractions were separated by electrophoresis using 0,8 % agarose gel in TBE buffer at 110 V, dsRNS molecules were stained with ethidium bromide.

RAPD technique was used to determine genotypes of the converted strains. RAPD amplification was carried out by using a version of Wronski et al. The DNA-containing supernatant was originated from the dsRNA extraction protocol. Polimerase chain reaction (PCR) was happened by six ten-base oligonucleotide primers. After PCR the DNA fragments were separated by electrophoresis using 1 % agarose gel in TBE buffer at 110 V, after staining with ethidium bromide, and examined under UV light. Two virulent isolates, and three, their converted hypovirulent isolates were investigated.

4.2.3. Pathophysiological investigations

The process expressed by the pathogen for the plant, or the plant for the pathogen is happening in the plant and characterized by biochemical changes. The metabolites of the pathogen expressed on the plant and the host-pathogen interaction were investigated *in vitro* trials described below.

The trials needed chestnut shoots maintained *in vitro* culture, these shoots were established by a multiplication

protocol. The basal source of the multiplication was own prepared P24 culture media supplemented with 0,2 mg/l benzyl-amino-purine. Later the different explants useful for the multiplication were maintained separately, to determine the multiplication coefficients of different explant sources. Explant sources were: 8 mm nodal shoot segments without apex, shoot segments with apex, whole axillary shoots with little callus tissue and callus. After four week incubation time in the cultures number of reliable segments for further multiplication and multiplication coefficients were determined.

In vitro virulence tests were carried out on four-week-old chestnut shoots multiplicated on P24 culture media supplemented with 0,2 mg/l benzyl-amino-purine. The stems of shoots were infected with mycelia of *C. parasitica* strains below: S21- virulent, R5- hypovirulent, S21xR5- converted hypovirulent strain, control – only wounding. Little, 2 mm long U-shaped wound was taken using blade on the stem, and 2 mm^3, actively growing mycelia was put into these artificial wounds. In every treatments 14 shoot cultures were infected. Cultures were maintained under a 16–h photoperiod at room temperature. Results were estimated after 30 days.

5. The summary of the results

The objective of this study was at first to characterize population structure of chestnut blight caused *Cryphonectria parasitica* in Sopron Hills, and investigate efficiency of hypovirulent control against blight during *in situ* trials.

1.) During the area surveying I recognized that in forest covered areas near the city, chestnut blight is common, infection rate is more than 30%, blight occurrence in the far areas is only focal.

2.) Different strains of the pathogen were isolated from bark samples collected at parallel the land investigations,

or originated from the chestnut stands and suburbs. From the isolates a collection was compiled for the further investigations.

3.) There are six vegetative compatibility types of the pathogen cause damage in the hills. Three vegetative compatibility types are dominant (SI, SII, SIII), the other types are represented with only a few isolates (SIV, SV, SVII). Near to the hills a seventh (SVI) type was detected.

4.) After the vegetative compatibility investigations resulted six types were compared with 30 EU-tester strains. According to the results and the European and Hungarian data I deducted to the occurrence and spreading the epidemic. The first wave of the blight was entered from the Kőszeg Hills to the Sopron Hills. Later the second wave of epidemic has reached the hills from far South-Western Danube areas because of possible antopogenic effects. Then a vegetative compatibility type dominant in Austria was occurred at the forest covered areas close to the border, this type has local area, but great infection capacity. The sexual propagation of the pathogen has begun.

5.) Control based on hypovirulent strains needs local hypovirulent strains. In lack of these – as in Sopron Hills – local virulent strains have to convert. Three successful conversions were carried out with hypovirulent strains originated from Zala and Somogy Counties, the conversions were verified by methods of molecular biology. In the converted strains the presence the cytoplasmic agent of hypovirulence, double-stranded RNA (molecular weight 12,7 kb) was detected. Hypovirulent straint are not yet occurred in the hills. Genetic identication of converted strains was verified by RAPD technique.

6.) Efficiency of hypovirulent control against blight *in situ* trials was investigated. In the experimental area Ágfalva, and the surrounding chestnut stands own converted strain and strain provided László Radócz for Institute of Forest and

Wood Protection were utilizes for curative treatments regularly from 1996 to 2000. In 8 years 90 % of treated trees recovered. If there area more vegetative compatibility types are present, apply of combined inoculation technique is efficient: the main point is that all of converted hypovirulent strains are used at inoculation one canker.

7.) Strongly diseased, severe cankered trees efficiency could be treated by vertically inoculation the stem from the stock to the great branches up to the crown, because a protection zone is created this way, which is not available for the virulent strains, so the water and nutrition transport is ensured and the tree survives the attack.

8.) During the treatments it was found, that year for year new vegetative compatibility types will occur, so biological control needs continuous investigations on population structure of the pathogen. After the treatments natural dissemination of hypovirulence starts, it means approx. 2 m a year. In the knowledge of population structure of the fungus the first experiments in Ágfalva have spread, and a practical biocontrol programme in the chestnut stands surrounding of Sopron has started.

9.) Biocontrol based on hypovirulence needs an exact method to determine the virulence the local collected strains, easy method to test possible hypovirulent strains. Investiagtions on interaction *in vitro* between chestnut and the pathogen give reliable and rapid results practically, and useful to identify hypovirulent strains.

10.) At first step investigation needs micropropagated chestnut shoots in sterile cultures. P24 basal media supplemented with 0,2 mg/l benzyi-amino-purine gave high multiplication coefficient (MC=4,68). Multiplication rate of whole shoots and callus are twice better than nodal or apical shoot segments.

11.) Chestnut shoots infected by protocol *in vitro* with *C. parasitica* produced a reliable way to separate the virulent and hypovirulent isolates of the pathogen

12.) The effect of *C. parasitica* culture-filtrate decreases the growing of chestnut shoots, and increases the number of apex necrosis.

13.) Since *C. parasitica* causes necrotic cankers and destructions on sessile oak, and in Sopron Hills the blight is common in oak forests mixed with chestnut, more investigations need on dissemination of the pathogen and the hypovirulence on oak species.

6. Relevant lectures, posters and list of publications

6.1. Publications

VIDÓCZI H., VARGA M., SZABÓ I., RADÓCZ L. (2000): A szelídgesztenye-kéregrák elleni biológiai védekezés lehetőségei a Soproni-hegyvidéken. Növényvédelem 36: 53-59. (in Hungarian) (Possibilities of biological control against chestnut blight in Sopron Hills)

VIDÓCZI H., VARGA M., SZABÓ I. (2005): A szelídgesztenye-kéregrák elleni biológiai védekezés tapasztalatai a Soproni-hegységben. Növényvédelem. Nyomtatás alatt (in Hungarian) (Experiences of biological control against chestnut blight in Sopron Hills) (being printed)

6.2. Posters

VIDÓCZI, H., VARGA, M., SZABÓ, I. (2001): Chestnut blight and its biological control in Western Hungary. COST G4 Multidisciplinary Chestnut Research, Ascona, Ticino, Switzerland. May 23-27, 2001: 91.

6.3. Lectures

VIDÓCZI H. (1999): A szelídgesztenye-kéregrák elleni biológiai védekezés lehetőségei Sopron környékén. 45. Növényvédelmi Tudományos Napok. MTA, Budapest. 1999. február 23-24: 133. (in Hungarian) (Possibilities of biological control against chestnut blight in surroundings of Sopron. 45. Scientific Days on Plant Protection)

SZABÓ, I., VARGA, M., RADÓCZ, L., VIDÓCZI, H. (1999): Chestnut stands and biological control in Ágfalva. COST G4 Multidisciplinary Chestnut Research, Sopron, Hungary, 5-9 May, 1999: 62.

VIDÓCZI, H., VARGA, M., SZABÓ, I. (1999): VC-type diversity of *Cryphonectria parasitica* in forests and plantations of Sopron, Hungary. COST G4 Multidisciplinary Chestnut Research, Sopron, Hungary, 5-9 May, 1999: 53.

VARGA, M., VIDÓCZI, H., RADÓCZ, L., SZABÓ, I. (1999): Results of the field inoculations of chestnut with converted hypovirulent strains of *Cryphonectria parasitica* in the surroundings of Sopron. COST G4 Multidisciplinary Chestnut Research, Sopron, Hungary, 5-9 May, 1999: 51.

VIDÓCZI, H. (1999). Effect of *Cryphonectria parasitica* culture-filtrate on the growing of micropropagated chestnut. COST G4 Multidisciplinary Chestnut Research, Sopron, Hungary, 5-9 May, 1999: 30.

SZABÓ I., VIDÓCZI H., JUHASOVA G. (2002): A kéregrák (*Cryphonectria parasitica*) gyógyítási lehetőségei. MTA-VEAB „Héjas gyümölcsök (dió, gesztenye, mandula, mogyoró) növényvédelmi problémái" tudományos ülés, Lengyeltóti, 2002. szeptember 26. (in Hungarian) (Plant protection problems of corticiferous fruits /walnut, sweet chestnut, almond, nuts/. Scientific session)

VIDÓCZI H. (1998): A szelídgesztenye-kéregrák Sopron környékén. Haracsi Lajos Emlékülés, Sopron, 1998. december 2. (in Hungarian) (Chestnut blight in surroundings of Sopron. Commemoration of Haracsi Lajos)

VIDÓCZI H., Szabó I. (2000): A szelídgesztenye pusztulása és az ellene alkalmazott biológiai védekezés, „Biológiai védekezés a gesztenyepusztulás megállítására” című PHARE CBC Projekt rendezvénye, Sopron, 2000. szeptember 29. (in Hungarian) (Destruction of chestnut, and biological control against the disease. “Biological control on stop the destruction of chestnut” Phare CBC Project Program)

VIDÓCZI H. (2003): A szelídgesztenye kéregbetegsége és az ellene való biológiai védekezés, „Gesztenye-napok” rendezvénye, Iharosberény, 2003. október 11. (in Hungarian) (Chestnut bark disease and biological control against it “Chestnut-days” Program)