Zbornik gozdarstva in lesarstva 68, s. 61 - 76

GDK 176.1 *Castanea sativa* Mill.+411.16 *Cryphonectria parasitica*:(439)
Prispelo / *Received*: 19.03.2002 Izvirni strokovni članek
Sprejeto / *Accepted*: 06.06.2002 *Original professional paper*

DISTRIBUTION OF CHESTNUT BLIGHT IN HUNGARY AND CHARACTERIZATION OF THE HUNGARIAN CHESTNUT BLIGHT (*CRYPHONECTRIA PARASITICA* (MURILL) BARR) STRAINS

Ilona APONYI*, Gizella IZSÁNYI**, János SÓTONYI**, Krisztina VARGA*, Zsolt VENDREI*

Abstract

During surveys made in the main sweet chestnut growing regions in Hungary we observed that one of the most serious "adversaries" of sweet chestnut trees is chestnut blight caused by *Cryphonectria parasitica*. Our main goals were to survey the plant health status of the Hungarian sweet chestnut stands, with special attention to chestnut blight, and to implement a biological control method against chestnut blight based on hypovirulent strains of *C. parasitica*. The virulence of the strains was characterised by different laboratory tests: morphological examination, virulence test, phenol oxidase test, dsRNA extraction, VCG test and artificial inoculation were performed on experimental plots.

Key words: biological control, *Cryphonectria parasitica*, Hungary, hypovirulence, *Castanea sativa*, forest health status, Hungary

RAZŠIRJENOST IN KARAKTERIZACIJA SEVOV KOSTANJEVEGA RAKA (CRYPHONECTRIA PARASITICA (MURILL) BARR) NA MADŽARSKEM

Izvleček

Med popisi, ki smo jih opravili v območjih razširjenosti pravega kostanja na Madžarskem, smo ugotovili, da je kostanjev rak (povzroča ga Cryphonectria parasitica) glavni "sovražnik" kostanjevih dreves. Cilja popisa sta bila: (a) ugotoviti zdravstveno stanje dreves pravega kostanja na Madžarskem (s poudarkom na kostanjevem raku); (b) vpeljati biološko kontrolo kostanjevega raka z uporabo hipovirulentnih sevov C. parasitica. Virulenco sevov smo določili z različnimi laboratorijskimi testi: analizo morfoloških lastnosti, testom virulence, fenol oksidaznim testom, ekstrakcijo dsRNA, testom vegetativne kompatibilnosti in umetno inokulacijo na poskusnih ploskvah.

Ključne besede: biološka kontrola, Cryphonectria parasitica,, hipovirulenca, Castanea sativa, zdravstveno stanje gozda, Madžarska

* Central Service for Plant Protection and Soil Conservation, 1118 Budapest, Budaörsi út 141-145, Hungary

** Plant Protection and Soil Conservation Service of Zala County, 8900 Zalaegerszeg, Kinizsi út 81, Hungary

CONTENTS
VSEBINA

1 INTRODUCTION
UVOD

1.1 ECONOMIC IMPORTANCE OF SWEET CHESTNUT
GOSPODARSKI POMEN PRAVEGA KOSTANJA

European sweet chestnut is a tree native to the Carpathian basin. It is a natural and valuable tree species in Hungary, known through the centuries as a forest tree. There are no large continuous sweet chestnut stands; the largest one is at Zengővárkony, Baranya County (38 hectares). Unfortunately, a lot of stands are abandoned and visited only at harvesting time. However, there are also well-managed stands.

The most important stands are situated in the western part of the country, in the counties of Somogy, Vas, Veszprém and Zala, as well as in the Danube bend (Figure 1).

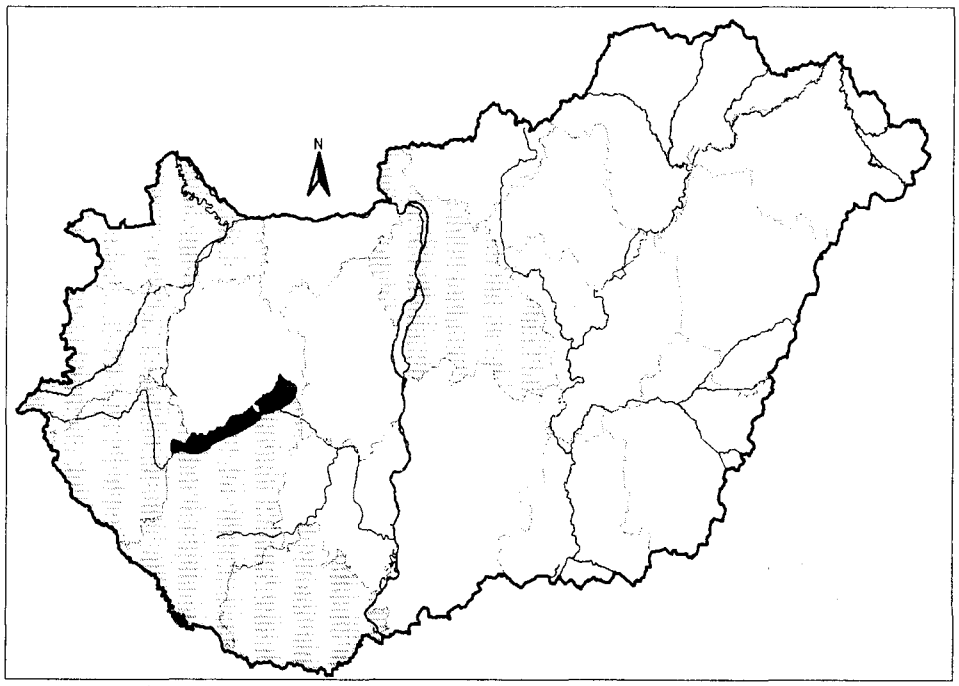

Figure 1: Hungarian sweet chestnut growing regions
Slika 1: Območja razširjenosti pravega kostanja na Madžarskem

The sweet chestnut area is not significant: approximately 200.000 ha. The decrease has mainly been caused by *Cryphonectria parasitica*, which is responsible for chestnut blight. As the quality of trees is not suitable for timber production, the trees are mostly used for fruit and tannin extraction.

The utilization of sweet chestnut is as follows:

- orchards: a highly nutritive food rich in vitamins,
- forestry trees: in the ship, furniture and upholstery industries,
- one of the most beautiful ornamental trees,
- honey-bearing and pharmaceutical raw materials,
- tannin extraction.

1.2 ECONOMIC IMPACT OF THE CHESTNUT BLIGHT
GOSPODARSKI POMEN KOSTANJEVEGA RAKA

C. parasitica caused almost complete destruction of *Castanea dentata* in America between 1904 and 1950. In Europe, it has been extensively spreading from Italy on *C. sativa* since 1938. The pathogen was first found in Hungary in 1969 at Nemeshetés, Zala county (KÖRTVÉLY 1970).

During surveys, *C. parasitica* was detected in all significant chestnut groves, orchards and in several plantations producing propagation material. As a result, in 1972 *C. parasitica* was declared a quarantined pest. In the 1970s, 1.100 ha of sweet chestnut orchards were planted in the western part of the country. These stands have almost completely disappeared due to planting deficiencies, frost damage and chestnut blight. Natural and scattered stands are also infected and degraded.

According to surveys conducted during the past few years, the presence of hypovirulent strains was observed in every sweet chestnut growing region in the country except the Danube bend.

1.3 BIOLOGICAL CONTROL OF CHESTNUT BLIGHT
BIOLOŠKI NADZOR KOSTANJEVEGA RAKA

Lacking an effective pest management programme, biological control seems to be one possible solution against chestnut blight. This method is based on using the hypovirulent strains of *C. parasitica* that are the cause of superficial canker. The virulence of these strains is low due to the dsRNA mycovirus in the cytoplasm (SHAPIRA *et al.* 1991). Trees infected by hypovirulent strains can survive the infection by callus formation.

Hypovirulence can be transmitted by hyphal anastomosis from hypovirulent strains to virulent ones of the same vegetative compatibility group. The virulent strains can be altered by the hypovirulent ones from the same vegetative compatibility group through hyphal anastomosis. Thus, the virulent strains lose their highly pathogenic character and become in their turn "hypovirulent".

In stands where the hypovirulent pressure is higher than the virulent one, the problem is solved naturally in a suitable silvicultural management system. Prunings and cleanings in chestnut fruit orchards are necessary.

Our purpose is to artificially spread hypovirulence where it is not naturally present or its ratio is low.

2 MATERIAL AND METHODS
MATERIAL IN METODE

We conducted surveys in sixteen plots located in five counties: Zala, Vas, Veszprém, Baranya and Pest.

2.1 FIELD EXPERIMENTS
TERENSKI POSKUSI

2.1.1 Selection of the experimental plots
Izbor poskusnih ploskev

Sixteen experimental plots were established in the main sweet chestnut growing regions in the western part of the country (Baranya, Vas, Veszprém and Zala counties) and in the Danube bend region (Pest county).

The types of experimental plots are different: natural sweet chestnut stand, mixed forest, scattered plot and orchard (cultivated and abandoned) (Table 1).

Table 1: Hungarian sweet chestnut experimental plots
Preglednica 1: Poskusne ploskve pravega kostanja na Madžarskem

Place *Kraj*	Area *Površina* (ha)	Type of the stand *Sestojni tip*	Natural hipovirulence *Naravna hipovirulentnost*
Zala county			
Nagykutas	5	Planted orchard / *Nasad*	Present / *Prisotna*
Nagylengyel	1	Planted orchard / *Nasad*	Present / *Prisotna*
Nemeshetés	4	Mixed forest / *Mešan gozd*	Present / *Prisotna*
Palin	16	Planted orchard / *Nasad*	Present / *Prisotna*
Pátró	2	Planted orchard / *Nasad*	Present / *Prisotna*
Miháld	2	Scattered / *Posamezno drevje*	Present / *Prisotna*
Zalamerenye	10	Mixed forest / *Mešan gozd*	Present / *Prisotna*
Zalaújlak	20	Mixed forest / *Mešan gozd*	Present / *Prisotna*
Rezi	30	Natural sweet chestnut *Naravni pravi kostanj*	Present / *Prisotna*
Vas county			
Csepreg	36	Mixed forest / *Mešan gozd*	Present / *Prisotna*
Velem	27	Scattered / *Posamezno drevje*	Present / *Prisotna*
Csipkerek	23	Planted orchard / *Nasad*	Present / *Prisotna*
Veszprém county			
Veszprém	0,1	Planted orchard / *Nasad*	Present / *Prisotna*
Tés	0,1	Planted orchard / *Nasad*	Present / *Prisotna*
Baranya county			
Zengővárkony	38	Natural sweet chestnut *Naravni pravi kostanj*	Present / *Prisotna*
Pest county			
Nagymaros	14	Natural sweet chestnut *Naravni pravi kostanj*	Not present / *Ni prisotna*

2.1.2 Evaluation of plant health status
Ocena zdravstvenega stanja dreves

We evaluated plant health status with special attention to ink disease and chestnut blight. We assessed the different types of blight infection: old damage or new infection and the percentage of the new, possibly hypovirulent infections was determined. Fifteen to fifty trees were evaluated by morphological observation per each plot. We focused mainly for the new hypovirulent infection.

2.1.3 Characterization of the different types of chestnut blight infection
Karakterizacija različnih tipov infekcij s kostanjevim rakom

All kinds of cankers were determined according to groups such as: abnormal (hypovirulent), intermediate and normal (virulent) canker. Detecting the percentage of abnormal canker is very difficult and not reliable in the field, therefore laboratory tests were performed.

C. parasitica is a wound parasite and can cause infection on the shoots, branches and bark of *Castanea sativa*. There are three different strains of the fungus: virulent, intermediate and hypovirulent. Virulent strains can cause serious sunken cankers. Cankers may enlarge so rapidly that the stem becomes girdled without callus formation. Typical symptoms of early infection on young shoots are: reddish, sunken bark, cracks, formation of epicormic shoots, fungal stroma production.

Hypovirulent strains are responsible for superficial infection. Callusing may occur only as a healing phenomenon.

A typical characteristic of the infection is a mycelial fan of *C. parasitica* in the inner bark of the chestnut tree. In humid weather, masses of yellow-orange to reddish-brown pustules develop on the infected bark and exude long orange tendrils of spores.

2.1.4 Sampling of *C. parasitica* strains from different types of cankered wounds
Vzorčenje sevov *C. parasitica* iz različnih tipov rakastih ran

We collected more than 300 samples during the surveys from each type of cankered wound; about 15 samples were collected per each plot. Samples approximately 2 x 2 cm were taken from the bark, from the margin of the infected and healthy part of the plant tissue by a disinfected knife or scalpel. We focused mainly on the new hypovirulent infections.

Artificial inoculation: After the laboratory experiment, artificial inoculations were performed with selected hypovirulent strains in two experimental plots: in the western part of the country, Zala county, where natural hypovirulence is present, and in the Danube bend, which is the only region where the natural hypovirulent strains are not present. The main reason for this work was to artificially spread hypovirulence at the stand level.

Holes (with a diameter of 8 mm) were made in the bark tissue by electric drills in four sets, with four different hypovirulent strains, 6 cm far from each other on the same tree. Five day-old mycelia of the hypovirulent strain from PDA were put in the wound and the surface was closed with transparent adhesive tape to prevent rapid drying.

At first, we used two differently compatible hypovirulent strains in the plots (instead of four), according to Turchetti's method (TURCHETTI / MARESSI 1991). The inoculation was done while the chestnut trees were flowering. Mycelium of hypovirulent strains was introduced into the holes at the margin of infections.

2.2 LABORATORY EXPERIMENTS
LABORATORIJSKI POSKUSI

2.2.1 Isolation of *C. parasitica* from infected samples
Izolacija *C. parasitica* iz okuženih vzorcev

5x5 mm sections were cut out from the collected samples on the day after sampling and were placed on difco potato dextrose agar containing 100 mg/l methionine, 1 mg/l biotin (PDAmb) after disinfection by 96% ethanol and flaming. Culturing was done in the dark at 28°C.

2.2.2 Maintenance of the isolates
Vzdrževanje izolatov

Isolates were maintained on slant potato dextrose agar (PDA difco) without methionin and biotin under sterile paraffin oil at 4°C and in four replicates.

2.2.3 Characterization of the strains based on morphology
Karakterizacija sevov na osnovi morfologije

Determination based on the colour of the colony and the quantity of the fruiting bodies is not reliable. The virulent isolates are mainly reddish, yellow and the hypovirulent isolates have little or no pigment. These grow more slowly than normal virulent strains at 28°C on PDA media in the laboratory. However, there are many exceptions, therefore other tests are required.

2.2.4 Virulence test
Test virulence

Two replicates of 100 isolates were used in this experiment. After artificial inoculation, shoots (length: 30 cm, diameter: 1,5-2 cm) were put in the incubation tubes for two

weeks at 28°C under conditions of high relative humidity until evaluation. During the evaluation, the diameter of the infected part was measured and pycnidia production was observed.

2.2.5 Bavendamm (phenol oxidase) test
Bavendammov (fenol oksidazni) test

To test for phenol oxidase activity (RIGLING / HEINIGER / HOHL 1989), mycelia were cut out from the five to six days old colonies on potato dextrose agar and were placed on malt extract agar. It contains 0,5% tannic acid, 1,5% Difco malt extract, and 2% Difco bacto-agar, adjusted with NaOH to pH 4,5. After a few days of incubation at room temperature in the dark, all virulent strains produced a strong colour reaction, indicating phenol oxidase activity, but the hypovirulent strains showed weak or no reaction in the lack of or blocked by this enzyme. Over three years, 100 isolates were tested. Evaluation was based on positive control isolates (Table 2).

Table 2: Control isolates used for Bavendamm test
Preglednica 2: Kontrolni izolati, uporabljeni za Bavendammov test

Laboratory code *Laboratorijska oznaka*	Origin *Izvor*	dsRNA extraction *dsRNA ekstrakcija*	Bavendamm test Bavendammov test	Virulence *Virulenca*
Nl HV/1	Nagylengyel	+	-	weak hypovirulent *šibko hipovirulenten*
HLHV/4	Nagylengyel	+	--	hypovirulent *hipovirulenten*
NlHV/2	Nagylengyel	+	---	strong hypovirulent *močno hipovirulenten*
NpV/3	Nemespátró	-	+	weak virulent šibko virulenten
NgV/4	Nagygerind	-	++	virulent / *virulenten*
NmV/1	Nagymaros	-	+++	strong virulent *močno virulenten*

2.2.6 ds RNA extraction
dsRNA ekstrakcija

ds RNA extraction was performed according to MORRIS / DODDS (1979) method, but was later modified to make the extraction faster by excluding DNA digestion, because all of the DNA was eliminated during the test.

The strains were grown on PDAmb. Mycelia were harvested from 7-10 day-old colonies. 1g of fresh mycelia was homogenised in 2 x STE buffer (10 x STE: 100mM NaCl, 50mM Tris, 1mM EDTA) with 10% SDS, 0,5% bentonite. Incubation took place for 5 min. at room temperature. Before shaking for 40 min., at 300 rpm, room temperature, 6 ml water-saturated phenol and 6 ml of chloroform were added. After centrifugation, the ethanol content of the supernatant was adjusted to 17 % and loaded to the freshly prepared CF11(Whatman) column. The column was washed through with 50 ml 1xSTE-17% ethanol and at the end, the RNA was harvested in 1 x STE buffer. After precipitation, the RNA was separated by agarose gel electrophoresis in 1% agarose gel containing ethidium bromide, at 90V for 40 min. and evaluated under UV light.

2.2.7 Determination of vegetative compatibility (V-C) groups
Določitev vegetativno kompatibilnih (V-C) skupin

The importance of this test is to determine the vegetative compatibility (V-C) properties between the isolates based on the presence of hyphal anastomosis. The isolates were placed on potato dextrose agar at a 1-1,5 cm distance from each other. The test was evaluated after one week of incubation in light at 28°C. The dsRNA is transmitted through hyphal anastomoses and rapidly converts the virulent strain if the virulent strain is compatible to the hypovirulent one. In cases of incompatibility, barrage lines with many pycnidia develop between the strains.

3 RESULTS
REZULTATI

We established and conducted surveys in the main sweet chestnut growing areas of the country; in sixteen plots located in five counties in the western part of the country and in the Danube bend. Among the stands there were plantations producing propagation material, planted groves, coppices, 'natural' (more than 100 years old trees) sweet chestnut trees and mixed forests.

Unfortunately, many stands were abandoned and visited only at harvesting time but there were well-managed stands, too.

During the surveys, we evaluated the plant health status of these stands. We confirmed that in Hungary chestnut blight is one of sweet chestnut's main problems. Natural presence of the hypovirulent strains was observed in all sweet chestnut growing areas except one region (Table 3).

Table 3: Shares of the hypovirulent, virulent and intermediate infections in the different counties

Preglednica 3: Deleži hipovirulentnih, virulentnih in vmesnih infekcij v posameznih okrajih

Site *Ploskev*	Hypovirulent (%) *Hipovirulentni (%)*	Virulent (%) *Virulentni (%)*	Intermediate (%) *Vmesni (%)*
Baranya county	20	60	20
Pest county	-	85	15
Vas county	18	53	29
Veszprém county	25	40	35
Zala county	28	15	57

Ink disease was not found, probably due to the extremely dry weather of the last few years. Other foliage diseases, such as *Mycosphaerella maculiformis*, were not significant at the time of the surveys (flowering and fruit development period).

For laboratory examinations, we collected about 300 samples infected with *C. parasitica* during the three-year work. Samples from each type of cankered wounds were collected but we focused mainly on the new hypovirulent infections. Isolation of the fungus is not

difficult, only 10 % of the samples were lost during the isolation. The first stage of isolate observation was based on morphological properties, but as the morphological character is not reliable in itself, other tests were required.

Virulence testing was carried out on 50 isolates to observe the virulence of the isolates but this test was not suitable for hypovirulence selection.

Bavendamm (phenol oxidase) testing was performed using 100 isolates. Clear differences in phenol oxidase activity were found between virulent and hypovirulent strains of *C. parasitica.* This enzyme, responsible for the colour reaction, was identified as phenol oxidase of the laccase type. Laccase activity has been suggested of being involved in degradation of lignin, in pathogenesis, in formation of fruiting bodies and in pigmentation (RIGLING / HEINIGER / HOHL 1989).

Mainly morphologically positive, seemingly hypovirulent isolates were tested by the extraction of the 12.000 base pairs long double-stranded RNA (dsRNA) mycovirus (Figure 2). Strong correlation was observed between the results of phenol oxidase test and dsRNA extraction.

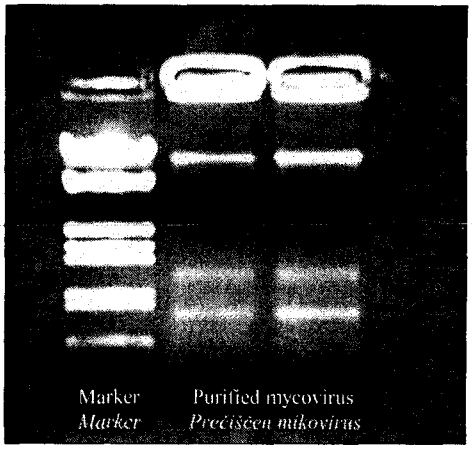

Figure 2: Purified mycovirus (12.000 bp in size and sub-units: 1.700 bp and 600 bp) on 1% agarose gel (Marker: NBL GENE SCIENCE EcoRI/Hind III)

Slika 2: Prečiščeni mikovirusi (velikosti 12.000 bp; podenote velikosti 1.700 bp in 600 bp) na 1 % agarju (Marker: NBL GENE SCIENCE EcoRI/Hind III)

Vegetative Compatibility Groups (V-CG) were determined within the plots and the proper hypovirulent strains were also selected for artificial inoculations.

Finally artificial inoculation was performed with the proper hypovirulent strains in two experimental plots: in the western part of the country, in Zala county where natural hypovirulence is present and in the Danube bend which is the only region where the natural hypovirulent strains are not present.

For reference, we have an experimental plot at Nagylengyel, county Zala. Twenty-seven trees out of 90 were inoculated with two different hypovirulent strains. The healing process was observed in this plot, but it is slow due to the high virulent pressure.

In the Danube bend, nearly 300 trees were inoculated with four different hypovirulent strains in the last year during sweet chestnut flowering.

4 DISCUSSION
RAZPRAVA

Studies of sweet chestnut trees have been performed by the Hungarian Plant Protection Service since 1993. In the framework of INCO-Copernicus project "CHESUD", we could continue this work with the implementation of the biological control method against chestnut blight.

We collected more than 300 samples during surveys from each type of cankered wounds for laboratory examination.

The virulence of the strains was characterised by different laboratory tests: morphological examination, virulence test, phenol oxidase test, dsRNA extraction and VCG test.

Determination based on the colour of the colony and the quantity of the fruiting bodies is not reliable and several studies suggest that no clear relationship exists between the white cultural phenotype and hypovirulence.

After the laboratory experiment, artificial inoculations were performed with the selected hypovirulent strains in two experimental plots: in the western part of the country, Zala county where natural hypovirulence is present and in the Danube bend which is the only region where the natural hypovirulent strains are not present. The main reason for this work was the artificial spread of the hypovirulence at the stand level.

Inoculations of active cankers with hypovirulent strains healed the cankers when strains with the appropriate V-C groups were used.

Biological control of chestnut blight in Hungary is very promising. Natural hypovirulence is present in almost every sweet chestnut stand. In the seventies 1.100 hectares of sweet chestnut orchards were planted in the western part of the country. These stands have been almost totally destroyed due to planting deficiencies, frost damage and chestnut blight. Sweet chestnut grove owners plan to establish new plantations and are hoping for a successful biological control method.

5 POVZETEK

Med popisi, ki smo jih opravili v območjih razširjenosti pravega kostanja v zahodnem delu Madžarske (okraji Baranya, Vas, Veszprém in Zala) ter v Podonavju, smo ugotovili, da kostanjev rak (povzroča ga Cryphonectria parasitica) resno ogroža kostanjeva drevesa. V popis smo vključili različne tipe ploskev: naravni sestoji pravega kostanja, mešan gozd, nasadi (kultivirani in opuščeni).

Cilja popisa sta bila: (a) ugotoviti zdravstveno stanje dreves pravega kostanja na Madžarskem (s poudarkom na kostanjevem raku); (b) vpeljati biološko kontrolo kostanjevega raka z uporabo hipovirulentnih sevov C. parasitica.

Prisotnost naravnih hipovirulentnih sevov smo ugotovili na vseh popisanih lokacijah, razen v Podonavju.

Za karakterizacijo reprezentativnega števila izolatov s poskusnih ploskev smo izvedli pet različnih laboratorijskih testov. Med popisi smo nabrali več kot 300 vzorcev vseh tipov

rakastih ran (po 15 vzorcev na ploskev), z namenom, da bi umetno vnesli hipovirulenco na ploskve, kjer le-ta po naravi ni prisotna oziroma je njen delež majhen.

S testom vegetativno kompatibilnih skupin (VGC test) smo na ploskvah določili vegetativno kompatibilne skupine in izbrali hipovirulentne seve, primerne za umetno inokulacijo.

Po laboratorijskih poskusih smo izvedli umetno inokulacijo izbranih hipovirulentnih sevov na dveh poskusnih ploskvah: (a) na zahodnem delu okraja Zala, kjer je prisotna naravna hipovirulenca; (b) v Podonavju, ki je izmed popisanih območij edino, kjer nismo našli naravnih hipovirulentnih sevov. Namen inokulacije je bila umetna razširitev hipovirulentnih sevov v sestoju. Inokulacija s hipovirulentnimi sevi je uspešno zavrla razvoj raka v primerih, ko smo uporabili ustrezne vegetativno kompatibilne (V-C) skupine.

6 REFERENCES
VIRI

KÖRTVÉLY, A., 1970. A gesztenye endotiás kéregelhalása. (Bark Destruction Caused by *Endothia parasitica* (Murr) Anderson, on Chestnut Trees).- Növényvédelem 6: 358-361.

MORRIS, T. J. / DODDS, J. A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue.- Phytopathology 69: 854-858.

RIGLING, D. / HEINIGER, U. /HOHL, H. R., 1989. Reduction of laccase activity in dsRNA-Containing Hypovirulent strains of *Cryphonectria (Endothia) parasitica*.- Phytopathology 79: 219-223.

SHAPIRA, R. / CHOI, G. H. / HILLMAN, B. I. / NUSS, D. L., 1991. The Contribution of Defective RNAs to the Complexity of Viral-Encoded Double-Stranded RNA Populations Present in Hypovirulent Strains of the Chestnut Blight Fungus *Cryphonectria parasitica*.- EMBO Journal 10, 4: 741-746.

TURCHETTI, T. / MARESSI, G., 1991. Inoculation Trials with Hypovirulent Strains of *Cryphonectria papasitica* (Murr.) Barr.- Eur. J. Path. 21: 65-70.