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Full Length Research Paper

Diversity of *Cryphonectria parasitica*, hypovirulence, and possibilities for biocontrol of chestnut canker in Albania

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Since 1967 when chestnut blight (*Cryphonectria parasitica*) was observed for the first time in Albania several field surveys have been carried out in chestnut (*Castanea sativa*) stands and have emphasized the widespread presence of the disease. About 30% of surveyed trees showed different stages of decline. Different proportions of *C. parasitica* morphotypes (normal/orange, intermediate, white) were detected when isolates were grown on agar media in the laboratory. About 57% of isolates showed normal pigmentation suggesting virulence, while 42% showed intermediate morphology. Less than 1% (or five isolates) was identified as hypovirulent based on detection of dsRNA of virus. Five European vegetative compatibility (vc) groups, EU-1, EU-2, EU-3, EU-10 and EU-12 were identified in four districts (Pogradec, Tropoje, Tirana and Elbasan) with dominant chestnut forests. EU-12 was the dominant vc type making up 39% of isolates studied. Transmission of dsRNA between virulent and hypovirulent isolates was successful in laboratory conditions. Biological control using hypovirulent strains to inoculate virulent cankers on chestnut trees in the field yielded between 46 and 84% of heavily calloused cankers, while around 30% of healthy trees that had not been inoculated produced heavily calloused cankers, suggesting natural spread of hypovirulence.

Key words: Chestnut blight (*Cryphonectria parasitica* (Murrill) Barr.), diversity, hypovirulence, possibility, biocontrol, Albania.

INTRODUCTION

Chestnut blight is one of the major diseases of chestnut (*Castanea* spp.) and has caused serious losses in forest stands and orchards since *C. parasitica* (Murrill) Barr. was first introduced into the North America (in 1904, in New York City), and later into Europe (in 1938 near Genoa, in Italy) (Robin and Heiniger, 2001). The epidemic of chestnut blight is a great example of a tree disease that man was not able to stop after its introduction into a new area (Jurc, 2002). By the 1970's the diseases had spread throughout the European continent and had started to cause dieback of *C. sativa* Mill in Asia Minor (Robin and Heiniger, 2001).

In Albania, chestnut trees cover about 14,620 ha and are found in two main ecological zones: the hilly transition

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Table 1. Locations of studied sites with elevation and geographical position.

District	Site	Year of sampling	Year of inoculation	Elevation in m	Geographical coordinates	
Tropoja	Gri	1994	1996	550-1100	42° 19' 23"	20° 3' 33''
	Markaj			450-1250	42° 21' 3"	20° 3' 24''
	Margegaj			350 - 1100	42° 22' 3"	20° 4' 35''
	Kerrnaje			700-1250	42° 24' 37"	20° 7' 15''
Tirana	Qafe Molle 1	1996	1998	550-700	41° 21'32"	19° 58' 37"
	Qafe Molle 2			550-700	41° 20'11"	20° 0' 13"
Elbasan	Gurakuq 1	1996	1998	500-550	41° 17'27"	20° 11' 12''
	Gurakuq 2			500-550	41° 17'28"	20° 11' 13''
Pogradec	Zervaske	1996	1998	750 - 950	40° 52'23"	20° 40' 14"
	Stropske			750 - 950	40° 51'28"	20° 35' 16"

(sub-mountainous) zone and the mountainous zone. Here the climatic and soil conditions are favourable for chestnut growth. Chestnut blight was first reported in Albania in the village of Markaj (Buçpapaj), Tropoja in 1967 (Elezaj, 1970), and it can now be found in all the chestnut growing areas in Albania. In 1983 heavily calloused cankers were observed and the next year the first white isolates were obtained from heavily calloused cankers. Hypovirulence, which is caused by the double-stranded (ds) RNA *Cryphonectria* hypovirus, has reduced the impact of this disease and has been used as a biological control method in Europe (Nuss, 1992; Heiniger and Rigling, 1994). Natural spread of hypovirulence in European countries has resulted in the recovery of many chestnut trees (Grente and Sauret, 1969; Bonifacio and Turchetti, 1973; Milgroom and Cortesi, 2004). Still there are many determining factors, that are not fully understood, which can influence the success of biocontrol of the disease (Milgroom and Cortesi, 2004). Some control of *C. parasitica* (Murrill) Barr. in chestnut have been achieved using agro-silvicultural practices and by minimizing conditions suitable for the development of chestnut blight (Lushaj et al., 1999). Different techniques, developed by French, Italian, and Greek groups giving promising results were employed in order to introduce hypovirulent isolates into chestnut stands (Heiniger and Rigling, 1994). The natural occurrence of hypovirulent strains was detected in Albania in 1984 (Heiniger and Rigling, 1994). Subsequently we have carried out experiments on biological control of chestnut blight, using Albanian strains of hypovirulent *C. parasitica* (Lushaj, 1999).

The aims of this study were: (i) to provide a survey of the chestnut blight incidence and severity in chestnut populations of selected regions of Albania; (ii) to identify the vegetative compatibility (vc) types present in Albania; and (iii) to test the effect of biological treatments with hypovirulent strains.

MATERIALS AND METHODS

Disease survey

Research was carried out at 10 study sites with 40 permanent plots in four districts throughout Albania (Tropoja, Tirana, Elbasan, and Pogradec), at elevations ranging from 300 to 1250 m (Table 1) (Figure 1). Dominant chestnut stands, which were part of Forest Condition Monitoring network in Albania, were chosen for this study. Distance between the two farthest districts is around 200 km, while stands inside one region were around 10 to 20 km-apart. Square plots of ca. 2.1 ha (150 × 140 m) were selected and all trees were examined.

Several experiments have been conducted since 1990 with artificial inoculations of healthy and diseased trees. Four districts with dominant chestnut forests were chosen to test the chestnut blight control. In total 10 study sites with 40 permanent plots were chosen based on silvicultural features. The following four management were distinguished: a. high forest - naturally regenerated stands of different ages (HF), b. managed coppice forests – stand consisting of uneven-aged over story of planted trees or seed trees and coppice understory managed for short rotation (MCF), c. simple coppice forest – stands regenerated from shoots formed at the stumps of the previous crop trees (SCF) and d. untreated forests (UF), forests where no artificial inoculations were done, which acted as a control. Four permanent plots (150 m × 140 m) were set up at each study site one for each of the different forest types. The trees in each plot were marked for disease assessment and treatment: 1. Incidence, 2. Disease severity 3. Presence of hypovirulent cankers of *C. parasitica* was scored.

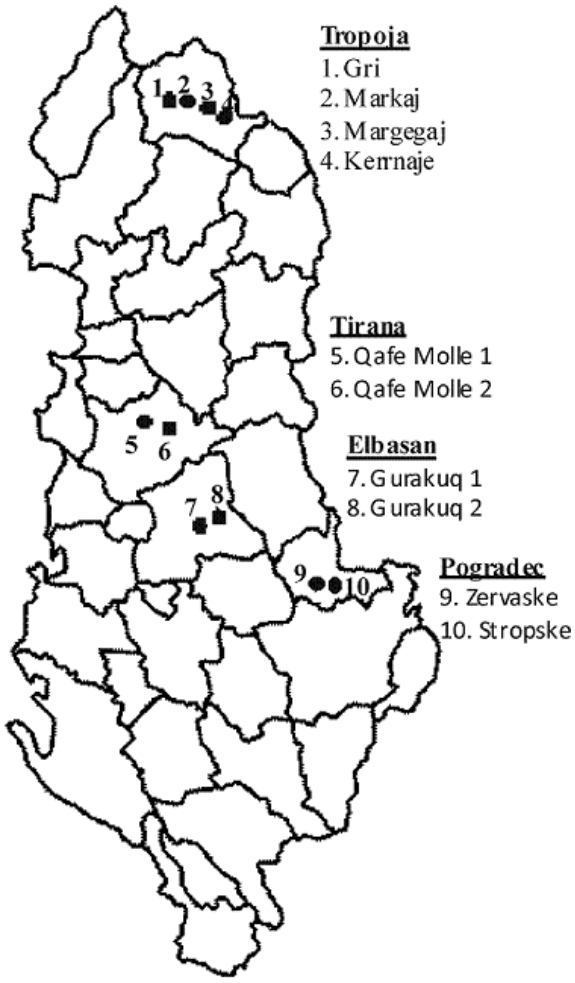

Figure 1. Map of Albania showing distribution of chestnut trees and locations of study sites.

4. Artificial inoculation experiments were performed. Four sites were established in Tropoja from 1990 to 1994 with four replicates of each of the forest types. From 1994 to 1996, three sites were established in Pogradec, Elbasan and Tirana with two replicates of each of the forest types.

The trees in each of the four forest types were examined for the presence of lethal *C. parasitica* cankers and for cankers with intermediate or/and heavily calloused appearance (Grente and Sauret 1969). The occurrence of blight was noted and different types of cankers recorded in the chestnut stands surveyed. Samples were collected at random in each stand from normal (able to kill branches and sprouts) and abnormal (heavily calloused) cankers, and from intermediate ones (Turchetti and Maresi, 1990; Turchetti and Maresi, 1991; Conedera, 1993; Turchetti, 1994; Lushaj et al., 1996). Infected chestnut trees were classified in one of five different classes according to the severity scale by foliar symptoms (observed damage 0= 0 to 10%, 1= ›10 to 20%, 2= › 20 to 50%, 3= › 50 to 90%, 4= › 90 to 100%) and the number of cankers (Lushaj et al., 1996). Disease incidence and severity was assessed on each tree. The disease severity was classified with a 5-point scale designed and based on estimation of the proportion of attacked (necrotized) leaves and dead branches and the number and size of cankers:

0. From healthy trees to concave cavities on branches or stump sprouts, smaller and yellow leaves are from 0 to10%
1. Dry brown-colored leaves on branches or stump sprouts. Considerable colour changes (reddish discoloration) in affected spots on smooth stem bark and smooth branches. From 10 to 20% of tree crown with dry branches
2. Progressive foliar necrosis on branches and/or stump sprouts. Longitudinal cracks in infected stem and branch bark. Fan-shaped mycelium of the fungus is easy-to-detect under the bark. From 20 to 50% of tree crown with dry branches
3. From 50 to 90% of tree crown with dry branches. Longitudinal strips of affected bark peel off from tree stem and branches. The fungus produces pycnidia and also perithecia.
4. The whole tree crown is dry and manifests many large cankers on stem and branches and drying of tree crown is from 90 % to 100% (Lushaj et al., 1996).

Isolates

Sampling was done on 40 permanent plots, one only canker was sampled per tree in each plot, in total 1200 samples. Numbers of sampled cankers were 483 for Tropoja, 238 for Pogradec, 232 for Tirana, 247 for Elbasan and in total 1200. Selection of infected trees was non-random; the aim was to select representative stands from four districts and from the four mentioned forest management types (HF, MCF, SCF and UF).

One to two samples of infected bark (approximately 4 × 4 cm) from canker margin with evident stromata were excised from the margin of the cankers using a surgical scalpel and forceps. Before, isolation each sample was observed under stereo microscope (magnification × 50) for the presence of picnidia and perithecia.

Samples collected from 1200 cankers, mentioned earlier that were virulent, intermediate or heavily calloused in appearance were taken to the laboratory to isolate the pathogen. Bark samples were taken one per canker, and concretely: 500 cankers appeared to be virulent, 600 had an intermediate and 100 were heavily calloused. *C. parasitica* was isolated from bark tissue, which was surface sterilized and aseptically placed in Petri dishes with water or with water-agar and incubated at 22° to 25°C for 5 to 6 days. Mycelium was then

aseptically transferred to potato-dextrose-agar (PDA, Difco) supplemented with methionine (100 mg/L) and biotin (10 mg/L) (PDA+mb) (Anagnostakis, 1977). Cultures were incubated in the dark at 27°C. After 3 to 5 days in culture, the hyphal tips of developing colonies of *C. parasitica* isolates were transferred onto fresh PDA + mb medium in 9-cm-diameter Petri dishes and incubated at 27°C for 10 days in the dark. The isolates were then classified as virulent, intermediate and hypovirulent types, on the basis of their morphological characteristics and pycnidia production, as described by Grente and Sauret (1969), Bonifacio and Turchetti (1973). White isolates obtained from heavily calloused cankers were later assayed for the presence of dsRNA hypoviruses. Hypovirulent isolates often lack pigmentation and therefore appear white in culture. Isolates that originated from Italy were used as standards for comparative morphological studies with the Albanian isolates.

Vegetative compatibility type

Vegetative compatibility (vc) types of 147 isolates of *C. parasitica* obtained from 4 districts (zones), 10 sites in 40 permanent plots in the Albania, were assessed according to the mycelial-barrage response (Anagnostakis 1988; Bissegger et al. 1997; Cortesi et al. 1998).

Vegetative compatibility (vc) types of 147 isolates of *C. parasitica* were detected from 1200 samples of *C. parasitica.* To identify the vc types present at each site we first randomly selected some strains of different vc types from each site to use as Albanian tester strains. Test for vegetative compatibility type was performed on PDA-Powell media (Powell 1995). The vc types for each site were identified according to the Bissegger et al. (1997). If the two isolates belonged to different vc types, a barrier zone formed between the two colonies with one or two lines of pycnidia. The vegetative compatibility types of the Albanian isolates were identified by comparing them with European tester strains (EU-1 to EU-31), which represented the European nomenclature system for *C. parasitica* (Cortesi et al., 1998) and are stored in the Istituto per la Protezione delle Piante by Dr. Turchetti and Dr. Maresi (Florence, Italy) and in the ex-Forest and Pasture Research Institute by Prof. Lushaj (Tirana, Albania) collection.

The vc types of the intermediate and virulent isolates were determined (Kuhlman and Bhattacharyya, 1984) by paring with all tester strains. The white isolates were included to test their conversion ability (Maresi et al., 1995). There were three replicates of each pairing on PDA + mb. The plates were incubated in the dark at 27°C for 20 days. The vc tester strains were used only in the laboratory and test plates were autoclaved before disposal.

Vegetative incompatibility in the chestnut blight fungus, *C. parasitica*, in Europe is controlled by six unlinked *vic* loci, each with two alleles. Four previously identified *vic* loci (*vic1*, *vic2*, *vic3*, and *vic4*) were polymorphic in European vegetative compatibility (vc) types (Cortesi and Milgroom, 1998). Vegetative incompatibility and the mycelial death of *C. parasitica* were indicated with a pH-indicator (bromocresol-green) in this type of media. Agar plugs containing mycelia were removed from the margins of seven-day-old PDA cultures and were paired 1 mm apart on the PDA-Powell media (1 cm from the edge of a 9-cm Petri dish).

The hypovirulent strains from Albania (H_{54} TROP, H_{09} TIR, H_{10} POGR, H_{l4} ELB. and H_{55} TROP) were paired with each single conidial virulent strain. Virulent ones derived from each hypovirulent culture by selection of single spore on PDA + mb.

Detection of dsRNA

Thirteen isolates selected from four districts were tested for the presence of hypovirus infection by assessing culture morphology. Isolates were grown on PDA + mb overlaid with cellophane. The mycelium was immersed in liquid nitrogen, ground to a fine powder using a mortar and pestle and the nucleic acid extracted using a phenol extraction method (Elliston, 1985). The dsRNA was isolated from 7 to 10 day-old cultures using CF-11 chromatography as described by Morris and Dodds (1979).

The presence of dsRNA in each isolate was assessed by agarose gel electro-phoresis. The dsRNA was electrophoresed using 1.2% agarose gels in 1 × TBE at 80 volts, stained with ethidium-bromide (0.25 mg/ml) and visualised by 300 nm UV-light, according Hilman et al. (1990). The American and Italian isolates EP713 and EP155 were used as positive and negative controls in each test (Istituto per la Patologia degli Alberi Forestali del Consiglio Nationale delle Ricerche, Firenze Italia). Under these conditions, CHV-1-free strains produce orange-pigmented cultures with abundant asexual sporulation. In contrast, CHV-1-infected strains show a white cultural appearance with no or very weak sporulation.

Phenol oxidaze activity

The 508 strains showing intermediate morphology were tested for phenol oxidaze activity using the Bavendamm test. The presence of dsRNA affects phenol oxidaze activity (Rigling et al., 1989). Isolates showing an intermediate morphology were tested for phenol oxidize on a medium containing 0.5% (w/v) tannic acid (Merk AG, Germany), 1.5% (w/v) Difco malt extract and 2% (w/v) Difco bacto, adjusted with NaOH to pH. 4.5 (Bavendamm, 1928). Plates were incubated in the dark at 24°C for four days. Phenol oxidize activity was recorded after 7 and 14 days.

Table 2. Phytosanitary status of chestnut trees in four districts of Albania: total number (%) of trees showing symptoms of disease.

Health status of trees	Total numbers (%) of trees showing disease symptoms				Total number of trees
	Tropoja	Pogradec	Tirana	Elbasan	
Healthy	556 (55)	409 (64)	643 (74)	904 (80)	2512
With virulent cankers	305 (30)	168 (26)	123 (14)	172 (15)	768
With intermediate cankers	11 (1)	14 (2)	18 (2)	15 (1)	58
With hipovirulant cankers	98 (10)	35 (6)	60 (7)	20 (2)	213
With other symptoms	38 (4)	14 (2)	26 (3)	19 (2)	97
Total	1008 (100)	640 (100)	870 (100)	1130 (100)	3648

Table 3. Blight severity (*C. parasitica*) on chestnut trees and canker types at four sites.

District	Total number of trees	Distribution of trees according to damage classes, defined according to foliar symptoms and the number of cankers					Number of sampled cankers	Canker type		
		1	2	3	4	5				
		0–10%	› 10–20%	› 20–50%	› 50–90%	› 90–100%		V	I	H
Tropoja	1008	556	208	145	91	8	483	253	190	40
Pogradec	640	409	141	64	24	2	238	119	100	19
Tirana	870	668	121	70	31	5	232	112	100	20
Elbasan	1130	904	145	62	14	5	247	116	110	21
Total	3648	2512	615	341	160	20	1200	600	500	100
	100%	69%	17%	9%	4.5%	0.5%				

Damage class: 1. no damage; 2. slight damage; 3. moderate (medium) damage; 4. severe damage; 5. dead. Canker types: V- virulent appearance; I- intermediate appearance; H- heavily calloused appearance.

Biological control experiment

The vc type of the virulent cankers was determined before inoculations and hypovirulent strains of the same vc type were used. After identification of vc types for virulent and hypovirulent isolates the following inoculation experiments were accomplished: 1,100 field inoculations using hypovirulent strains around virulent cankers and inoculated 1,050 canker-free (healthy) trees were inoculated with hypovirulent strains. The field inoculations were performed in July 1996 in Tropoja and in July 1998 in the Pogradec, Tirana and Elbasan districts. Inside permanent plots, the trees of varying diameters (dbh 15 to 50 cm), ages (10 to 70 year), stand positions and wound sizes (5 to 15 cm) were chosen randomly for inoculation (Lushaj, 1999).

After evaluating the affected trees, the margin of the cankers were marked. Hypovirulent strains were then inoculated 2 to 3 cm from the canker margin, at 5 places evenly distributed around the canker. Fresh wounds (1 × 2 × 2 cm), on the stems and branches of chestnut trees, were inoculated with mixture of equal parts of all five hypovirulent Albanian strains grown on PDA, belonging to the five EU vc-groups present in Albania.

Wounds were immediately covered with excised bark and sealed with Parafilm tape (Pechiney, Chicago, Ill., USA). All equipment was disinfected carefully between inoculations. The healthy trees were inoculated only in one place (Lushaj, 1999).

In August 2002, the field inoculation experiments were assessed, for changes in status from virulent to hypovirulent cankers, or from healthy to hypovirulent cankers, at the sites in the Tropoja district (6 years after inoculation) and at the sites in the Pogradec, Tirana and Elbasan districts (4 years after inoculation) and all sites were reassessed in July 2010 (Lushaj and Lushaj, 2010; Myteberi et al., 2011).

RESULTS

Phytosanitary status of chestnut trees at field sites

The survey of chestnut trees at the field sites in the four districts of Albania recorded both healthy (canker free) trees and cankered trees with *C. parasitica* cankers that appeared to be virulent, intermediate and healing. In addition, some other pathogens as *Phytophthora* spp. causing ink disease, semi-parasitic plants (*Loranthus* sp.) and abiotic stress symptoms were recorded (Table 2).

An assessment of the severity of disease caused by *C. parasitica* showed that ca. 69% of trees showed no defoliation, 17% showed slight damage; 9% showed moderate damage; 4% showed severe damage; and 0.5% of trees were dead when the field sites were established (Table 3). The incidence of cankered trees

Table 4. Number of *C. parasitica* isolates showing different cultural morphology.

District	Number of isolates tested	Number of isolate types showing different cultural morphology characteristics of *C. parasitica*		
		O	I	W
Tropoja	196	280	194	2
Pogradec	102	139	101	1
Tirana	107	132	106	1
Elbasan	108	136	107	1
Total	513	687	508	5

Abbreviations: O, orange; I, intermediate; W, white isolates contain dsRNA.

Table 5. Vegetative compatibility groups detected from the Albanian isolates of *C. parasitica.*

District	Sites	European vc strains					
		EU-1	EU-12	EU-3	EU-10	EU-2	Total
Tropoja	Gri	4	3	6	3	2	18
	Margegaj	6	10	8	3	3	30
	Markaj	7	15	6	4	3	35
	Kerrnaje	3	10	3	1	1	19
	Subtotal	20	38	23	11	9	101
Pogradec	Zervaske	2	6	1	–	1	10
	Stropske	2	5	1	–	2	10
	Subtotal	4	11	2	0	3	20
Tirana	Qafe Molle-1	2	3	2	2	4	13
	Qafe Molle-2	2	3	–	–	1	6
	Subtotal	4	6	2	2	5	19
Elbasan	Gurakuq -1	–	1	1	2	1	5
	Gurakuq-2	–	1	–	–	1	2
	Subtotal	0	2	1	2	2	7
	Total	28 (19%)	57 (39%)	28 (19%)	15 (10%)	19 (13%)	147

recorded as damaged or dead was 31% (Table 3).

Isolates

Of the 1200 collected samples of *C. parasitica* from three canker types (Table 3), 687 isolates showed typical morphological characteristics of orange culture, which produce virulent cankers, while 508 isolates were intermediate and only 5 isolates formed white colonies typical characteristics of hypovirulent isolates similar to those observed for the Italian hypovirulent strains (Table 4). These isolates as well as the 508 intermediate isolates were obtained from heavily calloused and intermediate cankers. Despite close examination of all 1200 bark samples, we could not find any perithecia, suggesting that only one mating type is present in these areas.

Vegetative compatible (vc) types

Vegetative compatibility (vc) types of 147 isolates of *C. parasitica* were detected from 1200 samples of *C. parasitica.* To identify the vc types present at each site we first randomly selected some strains of different vc types from each site to use as Albanian tester strains.

At all sites diversity of the vegetative compatibility (vc) of *C. parasitica* is present and the results for vc groups detected from the Albanian isolates of *C. parasitica*. The test showed at least 5 vc types occurring in Albania. Among the 147 isolates from four populations that were analysed, only five vc types were identified. The dominant vc type was EU-12 (39% of all isolates). The other vc types were present at lower levels (EU-1 = 19%, EU-3 = 19%, EU-10 = 10% and EU-2 = 13%, (Table 5). The five Albanian hypovirulent isolates to the five dominant vc types, that they belong to five different EU

Table 6. Field inoculation of virulent cankers and healthy chestnut trees with hypovirulent strains in 1996 for Tropoja and in 1998 for Elbasan, Pogradec and Tirana: assessment of calloused and heavily calloused cankers in 2002.

Forest types	Calloused or heavily calloused cankers (%)									
	Tropoja*		Pogradec**		Tirana**		Elbasan**		Total	
	VC	HC	VC	HC	VC	HC	VC	HC	VC	HC
HF	56	76	52	42	42	42	40	42		
MCF	63	80	56	48	54	46	46	46		
SCF	66	83	60	53	55	50	48	53		
UF ***	23	25	18	26	15	28	11	26		
No. of inoculations and assessed chestnut trees	440	420	220	210	220	210	220	210	1100	1050
p^a	0.001	0.001	0.001	0.022	0.001	0.089	0.001	0.026		
p^b	0.323	0.164	0.738	0.565	0.384	0.757	0.685	0.536		

Field sites inoculated in 1996. **Field sites inoculated in 1998. ***Untreated forests were not inoculated (control sites). VC, virulent cankers inoculated with hypovirulent strains; HC, healthy chestnut tree inoculated with hypovirulent strains. FVC, virulent cankers free inoculated with hypovirulent strains; FHC, healthy chestnut tree free inoculated with hypovirulent strains. Forest types: HF, high forest; MCF, managed coppice forest; SCF, simple coppice forest; UF, untreated forest. [a] Chi^2 tests were performed on the four forest types; [b] Chi^2 tests performed on HF, MCF and SCF.

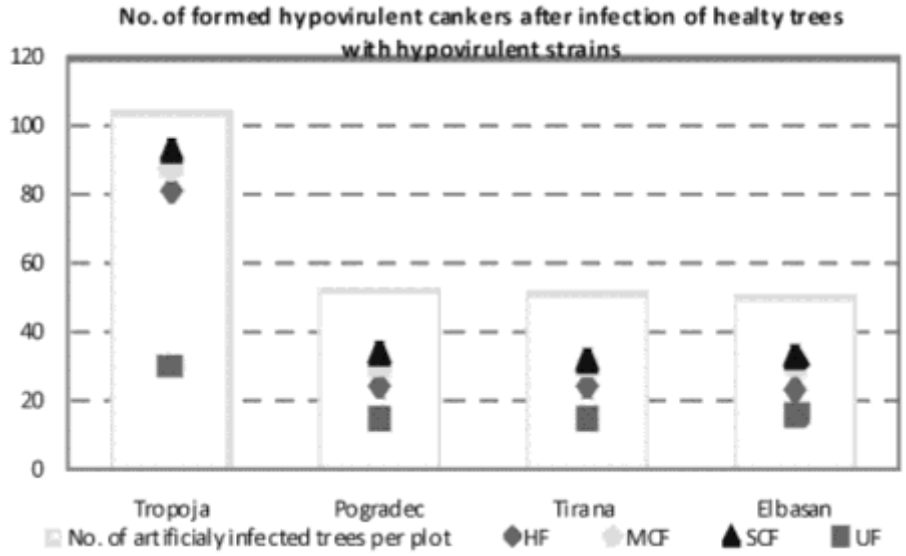

Figure 2. Number of healed virulent cankers after inoculation with hypovirulent strains, assessed in 2010.Note: Number of artificially trees per plot times four treatment (HF, MCF, SCF and UF) give the same number as No. of inoculations and assessed chestnut trees in Table 6.

groups.

dsRNA

dsRNA was found in all five white (hypovirulent) Albanian strains (H_{54} TROP, H_{09} TIR, H_{10} POGR, H_{14} ELB. and H_{55} TROP), which was isolated from five plots in four districts.

Phenol oxidize activity

One hundred and forty seven strains (or 29%) showed a positive test, suggesting that these strains were virulent and 356 were negative, suggesting the presence of the hypovirus.

Biological control of chestnut blight

During assessments of changes in status from virulent to heavily calloused cankers in 2002 and 2010 it was found that between 40 to 66% and 43 to 68%, respectively cankers were heavily calloused suggesting that they contained hypovirulent strains (Table 6 and Figures 2 and 3).

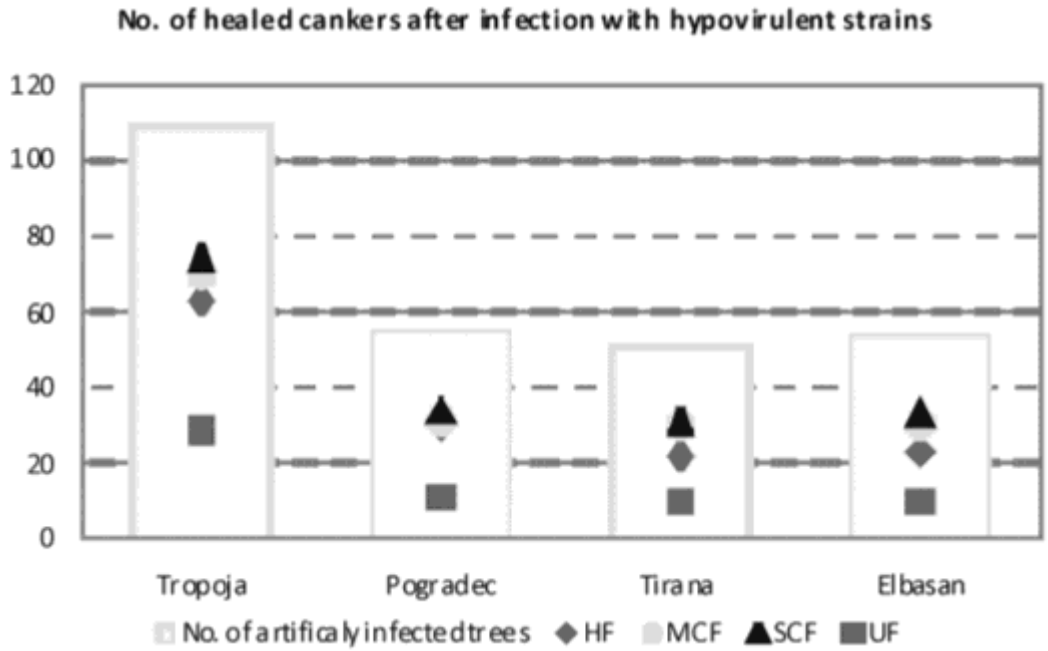

Figure 3. Number of formed hypovirulent cankers after inoculation of healthy trees with hypovirulent strains, assessed in 2010.

After four years, 46% (Elbasan) of the treated virulent cankers were calloused and over 11% of the untreated cankers were heavily calloused (Table 6). After 12 years, 57% of the treated virulent cankers in Elbasan had completely calloused, and over 19% of the untreated virulent cankers were heavily calloused (Figure 3). After four years over 50% (average for the three forest types (Table 6) of the treated virulent cankers in Tirana were calloused and 15% of the untreated virulent cankers were heavily calloused. After 12 years, 56% of the treated virulent cankers in Tirana were completely calloused and 18% of the untreated virulent cankers were heavily calloused. After four years, over 56% of the treated lethal cankers in Pogradec were calloused and over 18% of the untreated virulent cankers were heavily calloused. After 12 years, 58% of the treated virulent cankers in Pogradec were calloused and 21% of the untreated virulent cankers were heavily calloused. After six years, about 62% of the treated virulent cankers in Tropoja had completely calloused and over 23% of the untreated virulent cankers were heavily calloused. After 14 years, 63% of the treated virulent cankers in Tropoja had completely calloused (Table 6), and 27% of the untreated virulent cankers were heavily calloused (Figure 2). In addition, other virulent cankers inside and outside the plots showed signs of the spread of hypovirulence.

After 12 years, between 46 and 66% of healthy trees in Pogradec, Elbasan and Tirana that were treated with hypovirulent strains showed only hypovirulent cankers, and 29 to 31% of the non-inoculated trees had hypovirulent cankers.

After six years, about 80% of the originally healthy chestnut trees in Tropoja had developed calloused cankers, and 25% of the trees that had not been inoculated had heavily calloused cankers. After 14 years, about 84% of the originally healthy chestnut trees in Tropoja had developed calloused cankers, and 29% of the trees that had not been inoculated had heavily calloused cankers (Figure 3). Between 46 and 50% of healthy trees in Pogradec, Elbasan and Tirana that were treated with hypovirulent strains showed only heavily calloused cankers after 4 years, and approximately 27% of the non-inoculated trees had heavily calloused cankers.

Biological control by field inoculation using the hypovirulent strains into virulent cankers and
into canker free (healthy trees) on chestnut tree yielded excellent. The use of natural and biological control makes it possible to limit the damage caused to chestnut trees by this disease.

DISCUSSION

In Albania, chestnut grows in its optimal range and favourable climatic conditions enable development of very ecologically valuable and economically important forest ecosystems. Four distinct populations of chestnut in the regions of Tropoja, Pogradec, Tirana, and Elbasan have been thoroughly studied since 1967 when *C. parasitica* have been reported for the first time in Albania. At all sites diversity of the vegetative compatibility (vc)

of *C. parasitica* is present and the results for vc groups detected from the Albanian isolates of *C. parasitica*. Five European vc types, EU-1, EU-2, EU-3, EU-10 and EU-12, were identified among Albanian isolates. Around 40% of isolates belong to the EU-12, while the other four appear ranging between 10 to 19%. The results presented are in agreement with existing data for the geographic distribution of vc types in Europe.

The overall picture for SE Europe, as seen in Romania, Hungary Ukraine (Carpathian basin), Bosnia and Herzegovina, Greece, the Former Yugoslav Republic of Macedonia, Turkey, Slovakia, Czech Republic and Southern Italy is that EU-12 is the dominant vc type (Robin and Heiniger, 2001;
Radócz, 2001; Sotirovski et al., 2004; Juhásová et al., 2005; Perlerou and Diamandis, 2006; Adamčíková et al., 2006; Akıllı et al., 2009; Adamčíková et al., 2009; Jankovský et al., 2010; Erincik et al., 2010, Milanović, 2010), while the dominant vc type was EU-13 in Slovenia (Krstin et al., 2011), Bosnia and Herzegovina (Treštić et al., 2001), Hungary (Radócz, 2001), Slovakia (Juhásová, et al., 2005; Adamčíková et al., 2009) and also in the Czech Republic (Jankovský et al., 2010). In Western Europe, EU-12 is almost entirely absent and the dominant vc type is EU-2 (Robin and Heiniger, 2001), while the dominant vc type in Portugal was EU-11 (Braganca et al., 2005; Braganca et al., 2007). Outside Portugal, EU-11 is a very rare vc type that has only been detected in a few locations in Italy (Cortesi et al., 1996), France (Robin et al., 2000), and Hungary (Radocz, 2001). The dominance of EU-11 in Portugal may be pure chance, as a result of a founder effect. Vc type EU-10, which is very rare in Europe and was found only occasionally in Macedonia (Sotirovski et al., 2004) and Slovenia (Krstin et al., 2011) has a wide distribution in Albania, suggesting that introduction of this vc type happened through Albania.

High vc-type diversity among isolates of *C. parasitica* (Murrill) Barr. has been found in some European countries, such as France, Italy, Spain, Switzerland, Bosnia and Herzegovina, Hungary (Robin and Heiniger, 2001; Treštić et al., 2001; Radócz, 2001), Croatia (Krstin et al., 2008) and Slovenia (Krstin et al., 2011), where *C. parasitica* (Murrill) Barr. is known to reproduce sexually. The frequent occurrence of perithecia and the mating type ratios observed indicate that sexual reproduction of *C. parasitica* is common in Portugal (Braganca et al., 2005; Braganca et al., 2007). In contrast, in Macedonia, Greece and Albania, where a single vc type (EU-12) is dominant, *C. parasitica* does not reproduce sexually because of a lack of polymorphism for mating type (Sotirovski et al., 2004; Perlerou and Diamandis, 2006; Lushaj and Lushaj, 2010). Similarly, sexual reproduction was also very rare in newly established populations in Switzerland north of the Alps (Hoegger et al., 2000). In Balkan countries, such as FYR Macedonia (Sotirovski et al., 2004) and Greece (Perlerou and Diamandis, 2006), and also in Albania (Lushaj and Lushaj, 2010), where the sexual stage was not found, lower vc-type diversity is reported.

Biological control of chestnut blight is based on the transmission of the *Cryphonectria* hypovirus into the virulent strain of the fungus and its conversion to a hypovirulent one (Milgroom and Cortesi, 2004; Hillman and Suzuki, 2004; Nuss et al., 2005). In the field, the conversion is achieved either naturally or by artificial inoculation of the cankers using hypovirulent inocula of a compatible vc type. Transmission is also possible between isolates with closely related vc types (Anagnostakis et al., 1986; Cortesi et al., 2001). Therefore, the variability of the *C. parasitica* vc types in the field crucially affects the transmission of the *Cryphonectria* hypovirus (Anagnostakis et al., 1986; MacDonald and Fulbright, 1991; Heiniger and Rigling, 1994). Biological control of chestnut blight in France and Italy has demonstrated the effectiveness of the technique (Bisiach, 1978; Intropido et al., 1987; Heiniger and Rigling, 1994). However, in the USA, where the number of vc types is much higher than in Europe (Anagnostakis et al., 1986), the technique does not seem to halt the disease (Anagnostakis, 1987; MacDonald and Fulbright, 1991; Milgroom and Cortesi, 2004).

The probability of hypoviruses transmission depends on the regional vc-type diversity (Cortesi et al., 2001). In regions with low vc-type diversity, as in FYR Macedonia, a transmission probability between 60 and 80% can be expected for populations with few vc-types (Sotirovski et al., 2004) and up to 100% for populations with only one vc-type (Papazova-Anakieva et al., 2008). In regions with low vc-type diversity, as in Albania too, a transmission probability between 43 and 68% can be expected for populations with few vc-types. Our study showed that inoculations of hypovirulent strains are also promising in Albania, but less successful because of higher diversity (2-5) of vc types (Lushaj and Lushaj, 2010).

Artificially induced hypovirulence during years 1996 to 1998, on approximately 20 to 25 trees/ha in more or less uniform network, resulted in formation of dense network of trees with hypvirulent cankers and enhanced spread of hypovirulence in comparison with its spontaneous appearance and natural spread.

The low diversity in vc types for most of the *C. parasitica* populations provides good opportunities for natural regulation in high forests, managed coppice forests and in particular coppice forests and for biocontrol with CHV-1 (Robin et al., 2009).

Greek people have concluded that the systematic inoculation in a dense network of cankered trees may contribute to the establishment of hypovirulence and significantly enhance its spread in comparison with spontaneous appearance and natural spread of hypovirulence (Perlerou and Diamandis, 2009).

Moreover, knowledge of the vc type diversity in each region will allow us to determine the best strategies for

biological control, which should always be performed with local hypovirulent isolates. According to the presented results we can say that it is the responsibility of the forest manager/orchard owners to decide whether to wait for natural hypovirulence to occur and spontaneously spread or to introduce hypovirulence for quicker results (Perlerou and Diamandis, 2009; Lushaj & Lushaj, 2010). However, chestnut blight: an epidemic checked by biological control (Locci, 2010).

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