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Project

State and prospects of the Castanea sativa population in Belasitsa mountain: climate change adaptation; maintenance of biodiversity and sustainable ecosystem management.

Funded by: EEA and Norway Grants.

Beneficiary: Forest Research Institute at the Bulgarian Academy of Sciences

Report

Health condition of European chestnut dominated forests in the Bulgarian part of Belasitsa Mountain

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the Bulgarian part of Belasitsa Mountain**

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Recommended citation:

Georgieva M, P Petkov, G Georgiev, P Mirchev, B Rossnev, T Zlatanov. 2011. Health condition of European chestnut dominated forests in the Bulgarian part of Belasitsa Mountain. In: Zlatanov T, I Velichkov, B Nikolov (eds.) State and prospects of the *Castanea sativa* population in Belasitsa mountain: climate change adaptation; maintenance of biodiversity and sustainable ecosystem management. Project BG 0031 EEA report.

INTRODUCTION

INTRODUCTION

Due to its multipurpose character, sweet chestnut (*Castanea sativa* Mill.) used to be one of the most important tree species in certain regions of South West Bulgaria. The species is still of interest even though both socio-economic changes and subsequent outbreaks of the exotic pathogen *Cryphonectria parasitica* (Murrill) Barr (= *Endothia parasitica* (Murrill) Anderson) (Rossnev and Zachov 1982; Petkov and Rossnev 2000; Petkov and Rossnev 2001) reduced the importance it had until the first decades of the last century.

Chestnut blight fungus is a classic example of an invasive species and causes one of the most destructive diseases both of American (*Castanea dentata* (Marsh.) Borkh.) and European chestnut (Anagnostakis 1987; Anagnostakis and Kranz 1987; Milgroom et al. 1996; Elliott and Swank 2008). The chestnut blight agent was most likely introduced from Asia to North America

around 1900, either through imported chestnut lumber or trees (Milgroom et al. 1996). In Europe, chestnut blight was first recorded on *C. sativa* trees in 1938 near Genoa, Italy (Biraghi 1946). In France, the disease was reported in 1956 (Darpoux et al. 1957) and the parasite was found on Japanese chestnut in Spain as early as 1950, but only later (1972–1974) on European chestnut, as reported by Munoz and Cobos (1991). In short period, the fungus spread through southern Europe, causing significant epidemics on chestnut stands and playing an important role in their evolution and affecting management (Turchetti and Maresi 2000; Turchetti et al. 2000; Robin and Heiniger 2001). *C. parasitica* was found for the first time in Greece in 1963 in Mount Pelion (Central Greece) (Perlerou and Diamandis 2009). The pathogen was observed in Greece again in 1964 (Xenopoulos 1982) and in the Republic of Macedonia in 1974 (Sotirovski et al. 2004).

In the middle of the 20th century the Bulgarian National Governments multiplied the number of quarantine laws and inspection regulations governing imported chestnut wood and non-wood products (Zashev 1969). Although quarantine measures had been enforced, the virulent disease caused by *C. parasitica* was introduced in Bulgaria. The fungus was most likely imported by the introduction of Asian chestnut species (*C. mollissima* and *C. crenata*). The latter are considered as less susceptible to cryphoncrosis and rarely die from this disease (Anagnostakis and Hillman 1992, Popov et al. 2009). *C. parasitica* was first observed in Bulgaria in 1993 on chestnut trees in pure and mixed stands on the northern slopes of Belasitsa mountain and Eastern Rhodopes mountain (Petkov and Rossnev 2000). According to the results obtained by the field observations, the incidence and distribution of chestnut blight presumably began several years earlier. In the subsequent years, the disease spread by natural pathways or by the influence of man in stands of both seed and coppice origin (Petkov and Rossnev 2001; Bratanova-Doncheva and Mihaylov 1995).

Mass die-back of chestnut trees was observed in 1930s in the second largest locality of chestnut in Bulgaria which is situated in the Western Balkan Range (Zashev 1969). As no disease symptoms were found the decline was considered to be a result of lack of enough air and soil moisture (Zashev 1969). Since then, a great number of investigations had been initiated in order to clarify the main factors that provoked chestnut decline in that particular locality. In

1990s it was ascertained that both blight disease caused by *C. parasitica* and ink-disease caused by *Phytophthora* spp. led to the decline of the chestnut stands (Iliev and Mirchev 1992; Petkov and Rossnev 2001).

In North America and Europe chestnut blight disease has been controlled by means of a biological method based on the use of hypovirulent strains of the pathogen as stated in 1965 in France (Grente 1965). Abnormal cankers, from which the hypovirulent strains of the fungus were isolated, consist of exposed sapwood bordered by vigorous callus, with superficial infections radiating from the margins of the openings. The final effect of the hypoviruses is to reduce mortality of infected trees (Anagnostakis and Day 1979; Anagnostakis 1984; Heiniger and Rigling 1994; MacDonald and Fullbright 1991). France and Italy have applied such control in the 1980s and 1990s with satisfactory results. Greece has started applying the technique since 1998 to culminate with a nationwide project in 2007–2009 (Perlerou and Diamandis 2006). According to Turchetti and others (1999), the level of chestnut blight damage in Europe is decreasing as a consequence of the development of hypovirulent strains, resulting from a viral hypoparasite that attacks the fungus.

Chestnut fruits are rich in nutrition which is suitable medium for growth of numerous fungi organisms and insects. Insect larval development and fungi infections are considered as major factors in depreciation of *Castanea sativa* seeds (Wells and Payne 1980; Breisch 1993; Bassi et al. 2001). Insect damage is usually due to infestations of the chestnut moth (*Cydia splendana* (Hübner)) and the chestnut weevil (*Curculio elephas* (Gyllenhal)), which attack the fruits while still on the tree. Fungal infections often start in the larval galleries of insects (Wells and Payne 1980), many nuts becoming infected on the ground before picking. Some moulds are considered endophytes that colonize the fruits at various stages during their development but do not cause any symptoms of disease until after fruit fall (Washington et al. 1997; 1998). Expansion of fungal mycelia in the fruits and degradation of the cotyledons occur mainly during storage (Rutter et al. 1990; Giacalone and Bounous 1993). Visual differentiation of slightly mouldy or parasitized nuts from the good ones is often not obvious at early infection stages and requires post-processing (Rutter et al. 1990).

The objective of the current study was to evaluate the health condition of European chestnut (*Castanea sativa* Mill.) population in the Bulgarian part of Belasitsa Mountain and to define the most important damaging agents across the environmental gradient.

MATERIALS AND METHODS

FIELD DATA COLLECTION

The health condition of European chestnut in Belasitsa Mountain was evaluated in 67 permanent sample plots (PSPs), with a special attention to chestnut blight disease. On the field, the health condition of the trees was surveyed twice: (i) in the dormant season, to evaluate the occurrence of symptoms or signs of the blight, and (ii) in the vegetative season, to assess the degree of crown defoliation (by 10% steps). Five defoliation classes were also defined, using UNECE and EU standardized criteria (Eichhorn et al. 2010) (Table 1):

Table 1. Defoliation classes according to UNECE and EU classification

Defoliation class	Degree of defoliation	Percentage of leaf loss
0	not defoliated	0–10%
1	slightly defoliated	>10–25%
2	moderately defoliated	>25–60%
3	severely defoliated	>60–90%
4	dead tree	100%

A defoliation of 10 to 25% was considered a warning stage, and a defoliation of more than 25% was taken as a threshold for damage. Dead trees were indicated by defoliation values of 100% (Eichhorn et al. 2010).

The occurrence of chestnut blight was revealed by the presence of numerous completely dry branches in the crowns of infected trees. Incidences caused by recent virulent infections were detected by the wittings in young branches and dry leaves remaining on the killed twigs. The

presence of infection symptoms such as bark necrosis, reproductive fungal structures and a mycelia fan of *C. parasitica* in the inner bark of the chestnut trees were also considered. In humid weather, masses of yellow-orange to reddish-brown pustules developed on the infected bark and exuded long orange tendrils of spores. Biological material for identification of pests was collected during the vegetative period in 2010 by hand collections of adults and larvae on chestnut trees (in wood, on leaves, twigs and branches).

In order to initiate a systematic study on the *C. parasitica* hypovirulent stain, a total of 80 cankers yielded the fungus were isolated. The pathogen fruit bodies were collected from the canker margins on live infected trees in 38 permanent sample plots at altitude between 400 and 900 m a.s.l.

During the period October 2009 – March 2010, 30 samples of chestnuts were gathered from stands at altitude between 400 and 900 m a.s.l. For each sample, one hundred nuts were randomly collected from the ground inside the crown projection area of 10 randomly chosen trees. A total of 3000 nuts were obtained for further analysis.

LABORATORY TESTS

In order to identify fungal pathogens, microscopic fungus were isolated from samples of bark, sprouts and leaves of chestnut trees. The samples were surface sterilized with 70% ethanol for 10s. Then they were washed by 0,5% sodium hypochlorid and sterile water. The samples were placed on surface of Saburo's nutrient medium. One isolate per sample was randomly chosen and transferred to Saburo's nutrient medium. All developed colonies of *C. parasitica* were transplanted in pure medium. The species identification was carried out on the basis of their morphological and cultural properties. In the laboratory tests for isolation of virulent and hypovirulent stain of *C. parasitica* bark samples were put in water chambers at temperature 22°C and humidity 70% after surface disinfection for 10 s in 70% ethanol then 0,5% sodium hypochlorid. One isolate per canker was randomly chosen and transferred to Saburo's nutrient medium. All developed colonies of *C. parasitica* were transplanted in pure medium. Mycellium color of each isolate was scored after 7-10 days of incubation. Isolates with white phenotype were presumed to be infected with hypovirulent stain collected from abnormal (healing)

cankers, whereas isolates with yellow phenotype were presumed to be infected with virulent stain (able to kill branches and sprouts).

Chestnuts were bisected and visually examined for the presence of insects and pathogens. Insect species were identified on the basis of both the morphology of the larvae present and the type of damage. The number of dead and live immature (larvae) and exit holes was recorded. Nuts with rot or mould was placed in a humidity chamber in thermostat (temperature 22°C and humidity 70%). The colonized fungi were further identified to genus or species by combination of colony macroscopic (colour, morphology and growth rate) and microscopic characteristics (spore shape, size, colour and hypha morphology).

DATA ANALYSES

Multiple regression analyses were used to explain the variation in the crown defoliation of chestnut trees at plot scale, Eq. 1

$$CD_{30-100}(CD_{70-100}) = f(Alt, T_i, E_z, CP, Age_m, M) \quad (1)$$

where CD_{30-100} and CD_{70-100} represent the relative proportion of trees, at plot scale, with 30–100% and 70–100% of crown defoliation, Alt is the altitude, T_i and E_z are the terrain inclination and standardized exposure, Age_m is the mean age of chestnut, CP is the relative proportion of trees infected by chestnut blight disease caused by *C. parasitica* and M represents management at plot scale. M is binary coded variable ($M = 0$ or $1 \rightarrow$ unmanaged vs managed stands during last two decades). All other predictors are continuous variables. The standardized exposure of the terrain (E_z) was counted by Eq. 2:

$$E_z = \frac{|180 - |202.5 - \alpha||}{180} \quad (2)$$

where α is the exposure magnetic north azimuth. E_z obtains its minimum value, zero, at 22.5° magnetic north azimuth. The Age of the trees was estimated according to Zlatanov et al. (2011), Eq. 3

$$Age = 137.39[1 - \exp(-0.0114DBH)]^{1/1+15.1830} \quad (3)$$

where DBH is the diameter of breast height of the tree.

Logistic regression was conducted to predict the probability of whether or not a tree was infected by chestnut blight disease, $P(CPI)$. The general form of the model was, Eq. 4

$$P(CPI) = f(Alt, T_i, E_z, TR_{age}, TR_{cp}, M) \quad (4)$$

where TR_{age} is the tree age and TR_{cp} is the position of the tree crown in the main canopy. TR_{cp} is binary coded variable ($TR_{cp} = 0$ or $1 \rightarrow$ dominant and co-dominant trees vs intermediate and suppressed trees, Nyland 1996). The goodness of fit of all regression models was assessed through the coefficient of determination (R^2), F -test for significance of the regression and t -tests for significance of the coefficients of the models. Plots of the predictor variables against the residuals and the predicted values against the residuals were examined to check for model deficiencies (Draper & Smith, 1981). Cook's distance, Leverage and DFFit residual statistics were employed to identify potential influence cases. The linearity assumption of the logistic regression model was assessed by checking whether the interaction term between the predictor and its log transformation is significant (Hosmer and Lemeshow 1989).

RESULTS

FUNGAL PATHOGENS ON CHESTNUT TREES

All recorded parasitic and saprotrophic fungal species are listed in Table 2 in alphabetical order. Most of detected pathogens were parasites, causing damages of roots, branches, stems and leaves. Among parasitic fungi, the introduced pathogen *C. parasitica* caused the most serious damages.

DISEASES OF THE ROOT AND COLLAR

White rot of roots and collar was caused by *Armillaria mellea* (Fr.) Quel sensu lato. The fruit bodies of the fungus were noticed in the cortex on the old trees. The affected plants showed a progressive yellowing and wilting of foliage. The pathogen was characterized by the fruit bodies, white mycelia and rhizomorphs of the pathogen.

Ink disease caused by *Phytophthora* spp. is one of the most damaging diseases of the chestnut tree in Europe (Vettraino et al. 2005). The disease is not a serious problem in the forests of the Belasitsa mountain, and is not a limitation to planting on bare land. Symptoms of ink disease

were occasionally observed in the PSPs and were manifested on individual trees with thinning of the crown that appeared with smaller leaves and dieback, necrosis of the collar and trunk. Dark to black exudates on the collar bark was recorded on the infected trees.

DISEASES OF THE TRUNK AND BRANCHES

Canker and necrosis. A number of less dangerous fungal pathogens causing canker or necrosis on the chestnut stem and branches were found such as *Cytospora intermedia* Sacc., *Melanconis modonia* (Tul.) Hohn, *Phomopsis castaneae* Woronin, *Diplodina castanea* Prill. et Del. (Table 2).

Chestnut blight disease. Symptoms of chestnut blight disease were detected in all locations surveyed and showed the ability of *C. parasitica* to spread through chestnut stands. In our phytopathological study on mixed stands of chestnut and other broadleaved trees, damages of *C. parasitica* were recorded only on chestnut trees. Chestnut blight was one of the most important mortality factors of the European chestnut in studied stands. It affected the shoots, branches and stems of European chestnut, forming cankers inside the inner bark and cambium (Fig. 1). The symptoms of the disease were well observed: yellow or brown leaves of dead branches, standing out the trees and contrasting to the green foliage (Fig. 2A). The dead leaves and branches, which usually remain on the tree during the winter, afforded us to detect the blight infections during the dormant season of the year. Dead limbs without leaves often indicated the presence of the blight. Occasionally the close examination of dead stems or branches revealed the presence of a canker located on the branch or the trunk. Cankers on smooth bark were easily recognized by their yellowish and brown surface color, which contrasts sharply with the grayish green color of normal bark (Fig. 2B). Cankers on chestnut trees killed branches and entered trees.

Table 2. Pathogens found on chestnut trees in the sample plots

N	Pathogens by scientific name	Damages and affected parts of the tree	Dissemination
1	<i>Armillaria mellea</i> (Fr.) Quel sensu lato	white rot of roots and collar	+
2	<i>Cryphonectria parasitica</i> (Murr.) Barr Synonyms: <i>Endothia parasitica</i> (Murr.) Anderson	dieback and canker of stem and branches	+++
3	<i>Cryptodiaporthe castanea</i> Prill. et Del.	dieback and canker of stem and branches	+
4	<i>Cytospora indermedia</i> Tul.	dying of thin branches	+++
5	<i>Diplodina castaneae</i> Prill. et Del.	dieback with brown necroses on the bark of thinner branches	+++
6	<i>Exidia glandulosa</i> (Bull.) Fr.	saprotrophic organism on dead wood	+
6	<i>Laetiporus sulphureus</i> (Bull.) Murrill <i>Melanconis modonia</i> Tul.	brown cubical rot of roots, base and stem	+
7	(anamorph <i>Coryneum modonium</i> (Sacc.) Griffon)	dying of branches	+
8	<i>Mycosphaerella maculiformis</i> (Pers.) Schöet	leaves spots	+++
9	<i>Phomopsis castaeae</i> Voronin	necroses on the bark of thinner branches	++
10	<i>Phytophthora</i> spp.	rot of roots and collar	+
11	<i>Polyporus sulphureus</i> Bull. ex Fr.	rot of stem	+
12	<i>Polyporus squamosus</i> (Huds.) Fr.	white rot in the heartwood of living and dead hardwood trees	+
13	<i>Sarcoscypha coccinea</i> (Scop.) Lambotte	saprotrophic organism growing on dead wood	+
14	<i>Schizophyllum commune</i> Fr.	saprotrophic organism growing on dead wood	++
15	<i>Vuilleminia comedens</i> (Pers. ex Fr.) Maire	saprotrophic organism growing on dead wood	+



Fig. 1



Fig. 2A



Fig. 2B

The stems often showed epicormic shoots, which frequently developed just below an infection that has girdled the stem. The sprouts or suckers that frequently develop below cankers aid in locating the latter (Fig. 3). The mature and thick bark may lack the symptoms of bark discoloration. Mycelial fans may be found throughout the inner and/or outer layers of loose/cracked bark and/or cambium layer. Some annual rings of sapwood can be also infected, although mycelial fans do not form there.

Stems of young chestnut trees infected with hypovirulent strains may exhibit early symptoms similar to virulent forms. Older infections are limited to superficial bark layers and show callus production. Small plants, or parts of them, may be killed. Seedlings, young and grafted trees of chestnut are seldom infected and hypovirulent strains rarely causes dieback of branches.



Fig. 3

Sings of the disease were the fruiting stage of the pathogens. Stromata with conidiomata and ascomata are formed in bark cracks. Pathogen infected the wound or cracked areas by means of its ascospores from peritecia and conidia from pycnidium. The disease in its typical virulent form was recognized by the necrosis of the bark. Cracks can also form on the infected surface and stromata were formed in bark cracks (Fig. 3). The fungus could entrance the tree at any

point where the bark is broken and the disease spread primary in the inner bark and produced characteristic lesions which girdled the trees.

ROT DISEASES

Many of the recorded fungi affecting the chestnut trees caused white rot (*P. squamosus*, *V. comedens*, *P. sulphureus*, *S. commune*) or brown rot (*Laetiporus sulphureus*) on living or dead trees. Saprotrophic organisms growing on dead wood were also detected (Table 2).

DISEASES ON THE LEAVES

The most frequently occurring fungal pathogens that cause damages of chestnut leaves in the PSPs was *Mycosphaerella maculiformis* or its conidium stage *Cylindrosporium costanae* (Fig. 4).



Fig. 4

The symptoms of the infected trees appeared in June as tiny white spots on the leaves, which increased in size and turn brown over time (Fig. 4). The infected leaves started to look curly reddish but intensive premature defoliation was not observed. *M. maculiformis* attacked leaves of seedlings, adult trees and stump sprouts. Seedlings, young trees, stump sprouts and low part of tree crown are being attacked most intensively.

LORANTHUS EUROPAEUS JACQ.

The yellow mistletoe (*L. europaeus*) is a hemiparasite growing specifically on oaks (*Quercus* spp.) and on chestnut (*C. sativa*) (Ebermann and Lickl 1985). Out of 2022 European chestnut trees examined in the current study, 18.1% were infected with yellow mistletoe. On the average there were 8 mistletoe plants on an infected trees, with a maximum of 60 plants on one tree. The relative proportion of trees infected by mistletoe (26.4%) was highest between 700-800 m a.s.l. (Fig. 5)

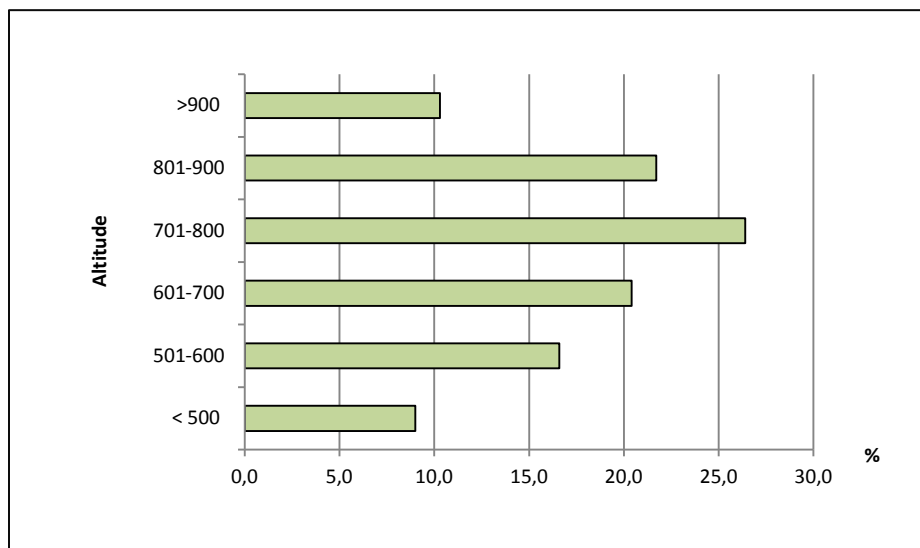


Fig. 5. Relative proportion of chestnut trees infected by *Loranthus europaeus*

PESTS

Insects known until now to have a trophic link with European chestnut in Bulgaria are presented in the following checklist. The species of which such links are reported for the first time in Bulgaria are marked with an asterisk.

COLEOPTERA: CERAMBYCIDAE

****Poecilium alni* (Linnaeus, 1767)**

New data: Belasitsa Mnt., PSP 11, 548 m a.s.l., on twigs of Chestnut sapling, 18.06.2010, Georgiev G.

****Axinopalpis gracilis* (Krynicky, 1832)**

New data: Belasitsa Mnt., PSP 8, 853 m a.s.l., on twigs of Chestnut sapling, 17.06.2010, Georgiev G. ***Leiopus nebulosus* (Linnaeus, 1758)**

Georgiev et al., 2005

New data: Belasitsa Mnt., PSP 4, 706 m a.s.l., on twigs of Chestnut sapling, 05.05.-05.08.2010 and PSP 11, 548 m a.s.l., Georgiev G.

***Pogonocherus hispidulus* (Piller et Mitterpacher, 1783)**

Georgiev et al., 2005; Ovcharov et. al. 2004

Belasitsa Mnt., PSP 4, 706 m a.s.l., 02.07.-03.08.2010 r.; 2 ex., PSP 11, 548 m a.s.l. 01-19.08.2010 r.

COLEOPTERA: BUPRESTIDAE

****Agrilus hastulifer* Ratzeburg, 1837**

New data: Belasitsa Mnt., PSP 4, 706 m a.s.l., on twigs of Chestnut sapling, 28.06.2010 and PSP 11, 548 m a.s.l., 17.06.2010, Georgiev G.

DEFOLIATION OF *CASTANEA SATIVA* TREES

During the period 2009-2010, the crown defoliation of a total of 4053 trees were assessed. Besides chestnut, the following tree species were evaluated: *Fagus sylvatica* L., *Quercus petraea* Liebl., *Acer campestre* L., *Acer heldreichii* Orph., *Acer platanoides* L., *Acer pseudoplatanus* L., *Betula pendula* Roth, *Carpinus betulus* L., *Carpinus orientalis* Mill., *Cerasus avium* (L.) Moench, *Fraxinus ornus* L., *Juglans regia* L., *Ostrya carpinifolia* Scop., *Platanus orientalis* L., *Prunus cerasifera* Ehrh., *Sorbus torminalis* (L.) Crantz, *Tilia tomentosa* Moench, *Ulmus glabra* Huds (Zlatanov et al. 2011). Chestnut, beech (*F. sylvatica*) and oak (*Q. petraea*)

were evaluated by species basis while the remaining species were grouped as “other broadleaved”.

The relative proportion of trees by crown defoliation classes are summarized in Table 3. Distinct decline in crown condition was observed in sampled chestnut trees. The decline was widespread and its effect varied from partial crown dieback to completely death trees (16.4%). Only 26.5% of chestnut trees were classified as healthy (defoliation of up to 25%). The beech (*F. silvatica*) was the second frequent tree species among all broadleaved. The highest proportion of healthy trees (89%) was observed for this species. The share of damaged trees of oak (*Q. petraea*) amounted to 20.9%.

Table 3. Relative proportion of trees by crown defoliation classes for observed tree species

Species type	class 0	class 1	class 2	class 3	class 4	Number of trees
	(0-10%)	(>11-25%)	(>25-60%)	(>60-90%)	(dead tree)	
<i>Castanea sativa</i>	5.4	21.1	35.9	21.3	16.4	2022
<i>Fagus silvatica</i>	64.3	24.3	8.2	2.3	0.9	788
<i>Quercus petraea</i>	38.8	40.3	17.0	2.5	1.4	722
Other broadleaves	48.4	33.4	13.6	3.8	0.8	521

Chestnut blight incidence ranged from 18% to 100% of the trees in the PSPs, and mortality caused by the fungus was between 2% and 80%. Healed cankers represented 1% of the total infections recorded, whereas normal cankers represented 99%. The results obtained indicated that chestnut blight had spread into most chestnut stands of Belasitsa mountain the most affected stands growing between 400-800 m. a.s.l. (Fig. 6).

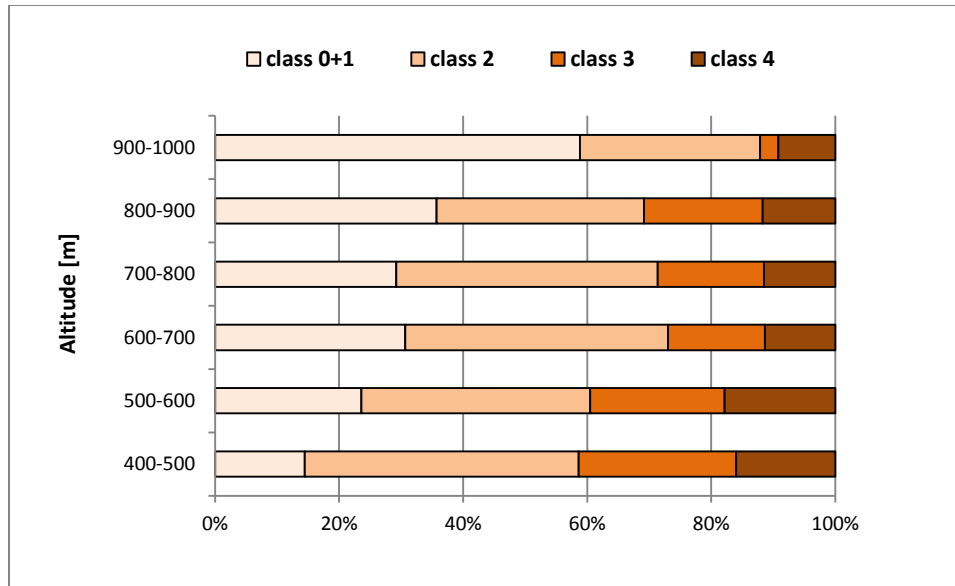


Fig. 6. Relative proportion of defoliation classes for chestnut

The variation in the relative proportion of trees with 30–100% (CD_{30-100}) and 70–100% (CD_{70-100}) of crown defoliation at plot scale was significantly predicted by the hierarchical regression model at both step 1 when site defining variables (Alt , T_i and E_z) were entered into the model, and Step 2 when all other predictors (CP , Age and M) were included. Only altitude (Alt) and relative proportion of trees infected by chestnut blight disease (CP) significantly ($\alpha < 0.05$) contributed for the prediction of CD_{30-100} and CD_{70-100} . Altitude alone accounted for 22% of the variance in CD_{30-100} and for 12% in CD_{70-100} . Addition of CP improved the prediction by 40% for CD_{30-100} and by 12% for CD_{70-100} . In accordance, the equations selected were, Eq. 6 and Eq. 7

$$CD_{30-100} = 28,80 - 0,03Alt + 0,77CP \quad (6)$$

$$CD_{70-100} = 17,40 - 0,03Alt + 0,44CP \quad (7)$$

The models significantly ($\alpha < 0.01$) accounted for 62% (large effect, Cohen 1983) of the variation in CD_{30-100} ($F_{2;66} = 53,63$; $Adj. R^2 = 0,62$) and for 24% (small to medium effect) in CD_{70-100} ($F_{2;66} = 11,65$; $Adj. R^2 = 0,24$). Means and 95% confidence intervals for the relative proportion of trees with 30–100% and 70–100% of crown defoliation are depicted in Fig. 7. For both subsets, only mean values for altitude of more than 800 m a.s.l. significantly

differed from mean values for other altitudes at $\alpha < 0.05$ (Turkey post hoc test was employed to identify significantly different substrata) indicating generally better health condition of chestnut at that altitude.

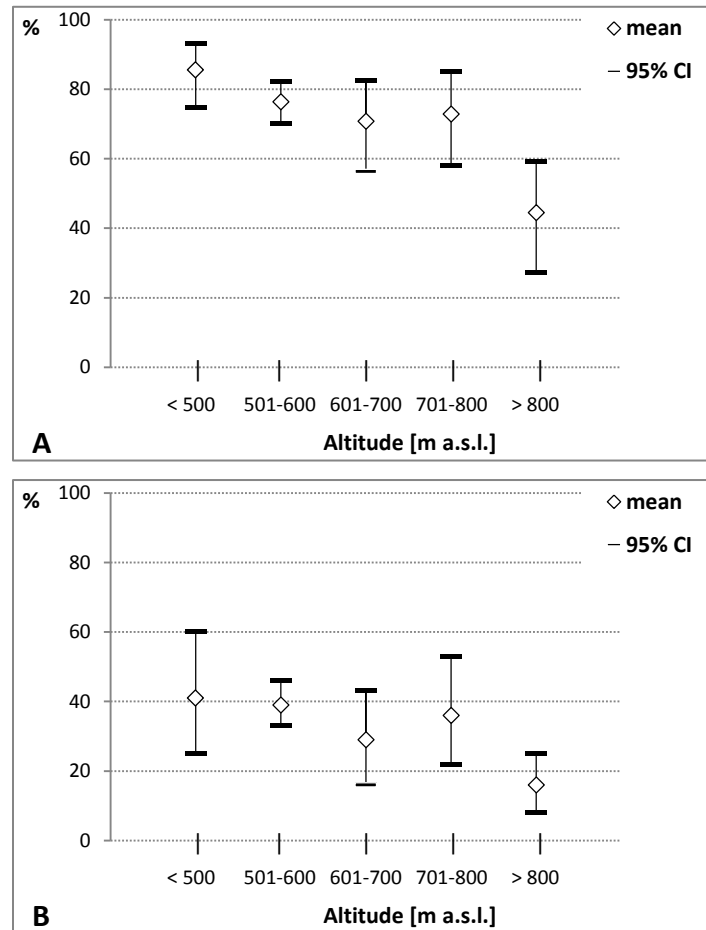


Fig. 7. Means and 95% confidence intervals for the relative proportion of trees at plot scale with (A) 30–100% and (B) 70–100% of crown defoliation. Bootstrapping option was used in order to derive the 95% CI.

The probability of whether or not a tree was infected by chestnut blight disease $P(CPI)$ was significantly predicted by the hierarchical logistic regression model at both step 1 when site defining variables (Alt , T_i and E_z) were entered into the model, and Step 2 when all other predictors (TR_{age} , TR_{cp} and M) were included. However, only altitude (Alt), tree age (TR_{age}) and management (M), significantly contributed for the prediction ($\chi^2 = 212, df = 3, N =$

2022, $p < 0.001$), accounting for 14% (Cox&Snell $R^2 = 0,14 \rightarrow$ small effect) out of the total 15% accounted for by the model when all variables were included into the equation. In accordance, the final equation selected was, Eq. 8

$$P(CPI) = \frac{1}{1 + e^{-(2,18 - 0,01Alt + 0,04TR_{age} + 0,91M)}} \quad (8)$$

The odds ratios equals to 0,97 for Alt , 1,04 for TR_{age} and 2,49 for M , which suggests that the odds of estimating correctly whether a tree was infected by chestnut blight disease improved by 4% if TR_{age} was known and by 149% if M was known. Results of the model suggest that the occurring of chestnut blight disease is quite a chaotic process, hence difficult to predict. Means and 95% confidence intervals for the relative proportion of trees at plot scale infected by chestnut blight disease is shown on Fig. 8.

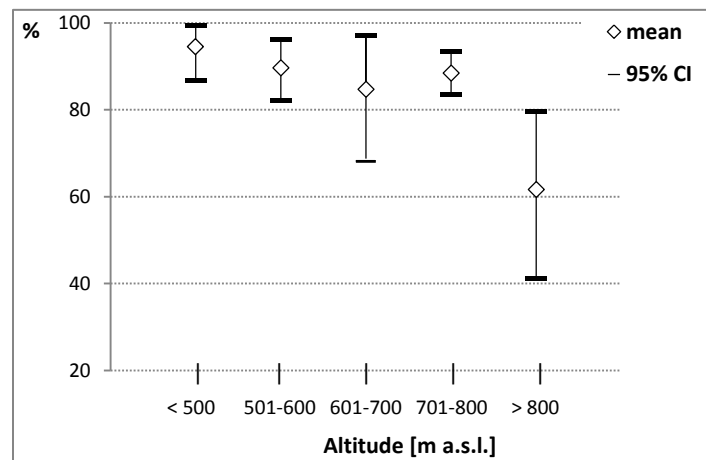


Fig. 8. Means and 95% confidence intervals for the relative proportion of trees at plot scale infected by chestnut blight disease. Bootstrapping option was used in order to derive the 95% CI.

Only mean values for altitude of more than 800 m a.s.l. significantly differed (Turkey post hoc test) from mean values for altitudes of less than 500, 501–600 and 701–800 m a.s.l. at $\alpha < 0.05$.

Field observations have shown the presence of abnormal disease symptoms at some localities. Isolates of *C. parasitica* from these sites varied widely in cultural characteristics and pathogenic properties, some showing the characteristics of hypovirulent strains. Generally the virulent

isolates are mainly reddish, yellow and the hypovirulent isolates have little or no pigment. The latter grow more slowly than normal virulent strains at 28°C on PDA media in the laboratory. As depicted in Fig. 9 white phenotypes (possible hypovirulence) predominated in the isolates extracted from pathogen fruit bodies at altitudes of more than 800 m. However, determination based upon morphological observations (e.g. the colour of the colony) only is not reliable as there are many exceptions, hence other tests are required as well.

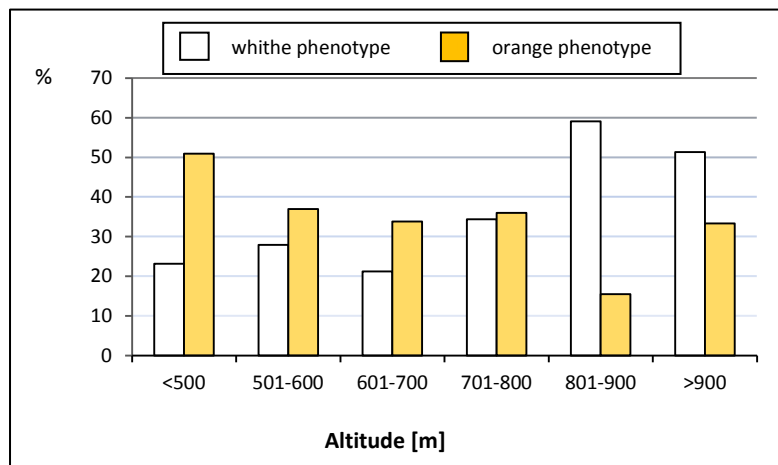


Fig. 9. Relative proportion of white and orange phenotypes for the 80 isolates.

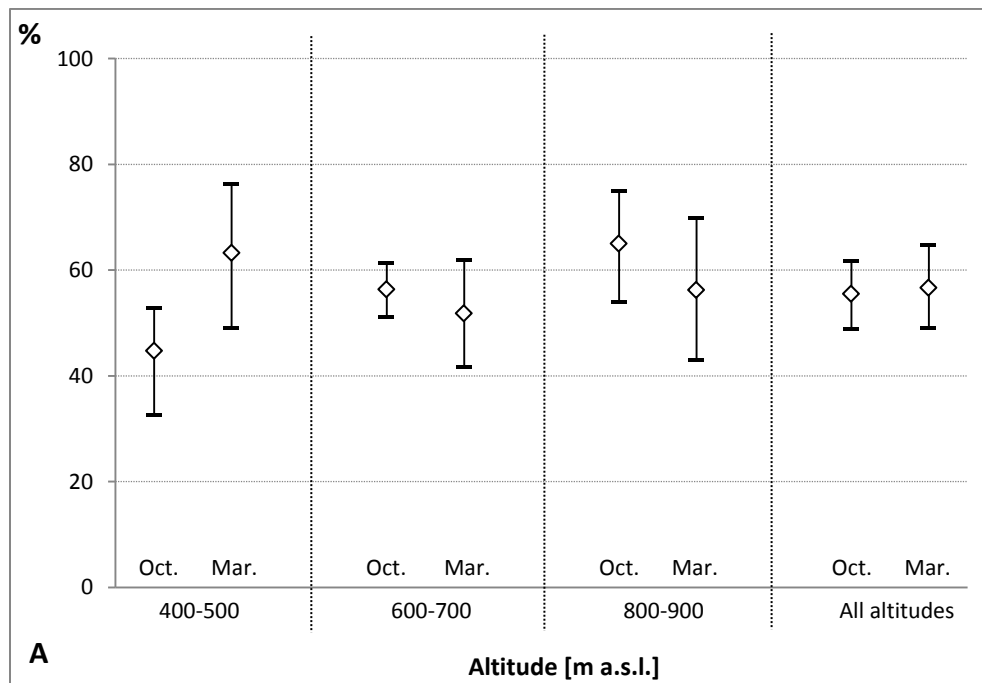
Application of biological control by the *C. parasitica* hypovirulent strain is beyond the targeted deliverables of the current project (BG0031-PIP-00110-E-V1). Still, it is strongly recommended that further isolation of the strain is performed by the method of vegetative compatibility, followed by the strain propagation and transmission.

Health condition of chestnut seeds

Dissected chestnuts displayed a range of disease symptoms representing several genera and species including *Alternaria alternata* (Fr.) Keissl, *Ciboria batschiana* (Zopf) Buchwald, *Mucor mucedo* de Bary & Woron., *Mucor racemosus* Bull, *Penicillium glaucum* Link, *Penicillium italicum* Wehmer, *Phomopsis endogena* (Speg.) Cif., *Phomopsis castanea* (Sacc.), *Rhizopus nigricans* Ehrenberg and *Trichotecium roseum* (Pers.) Link. Contamination and infection of chestnuts by fungi may have occurred while the nuts were still on the tree. At this stage, particularly when

the nuts have ripened and the hulls have opened, they are often attacked by airborne and insect-borne spores of fungal species. Contamination may have also occurred after the nuts were dropped on the ground. During this period they are wet and highly susceptible to mould growth and mycotoxin contamination (Khayria et al. 1993). Infestation by the following insects was also found: *Curculio elephas* Gyll., *Cydia splendana* Hübner and *Cydia amplana* Hübner. Individuals of *Pammene fasciana* was occasionally observed.

The relative proportion of healthy seeds was substantial, averaging 56% and 57% in October and March respectively (Fig. 10A). The relative proportion of healthy germs was even higher (Fig. 10B). There were no statistically significant differences (at $\alpha < 0.05$) between the relative proportion of healthy seeds/ germs in October and March.



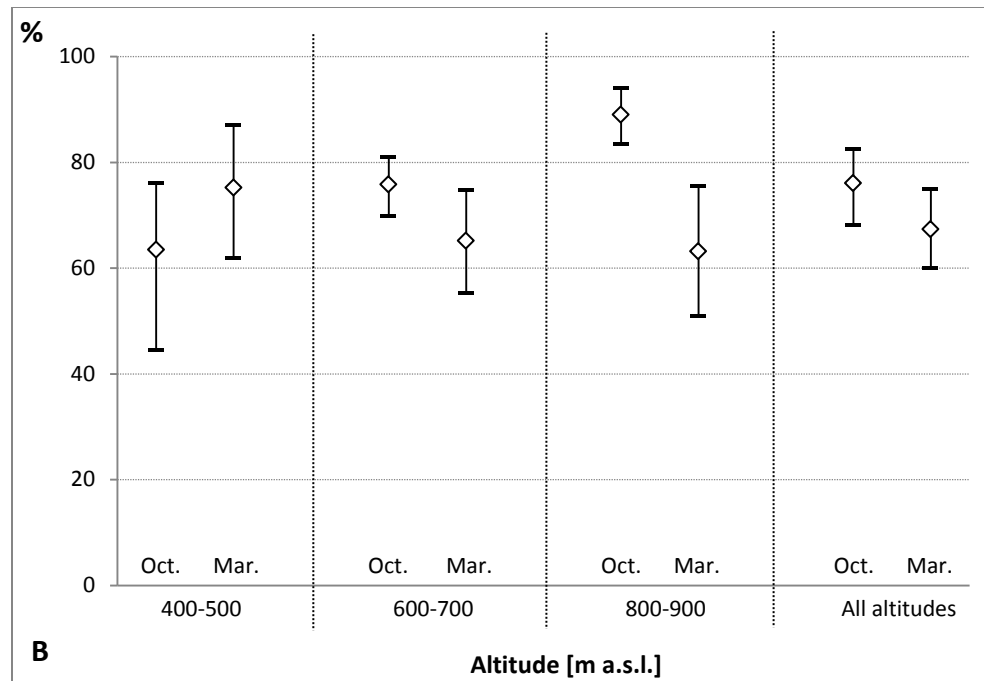


Fig. 10. Relative proportion of healthy seeds (A) and germs (B) along the altitudinal gradient in October and March

The Infestation by insects led to significant increase (at $\alpha < 0.05$) of the average relative proportion of nuts contaminated by pathogens in both October (Fig. 11 A) and March (Fig. 11 B) samples. The difference was greater in March and pronounced at all altitudes.

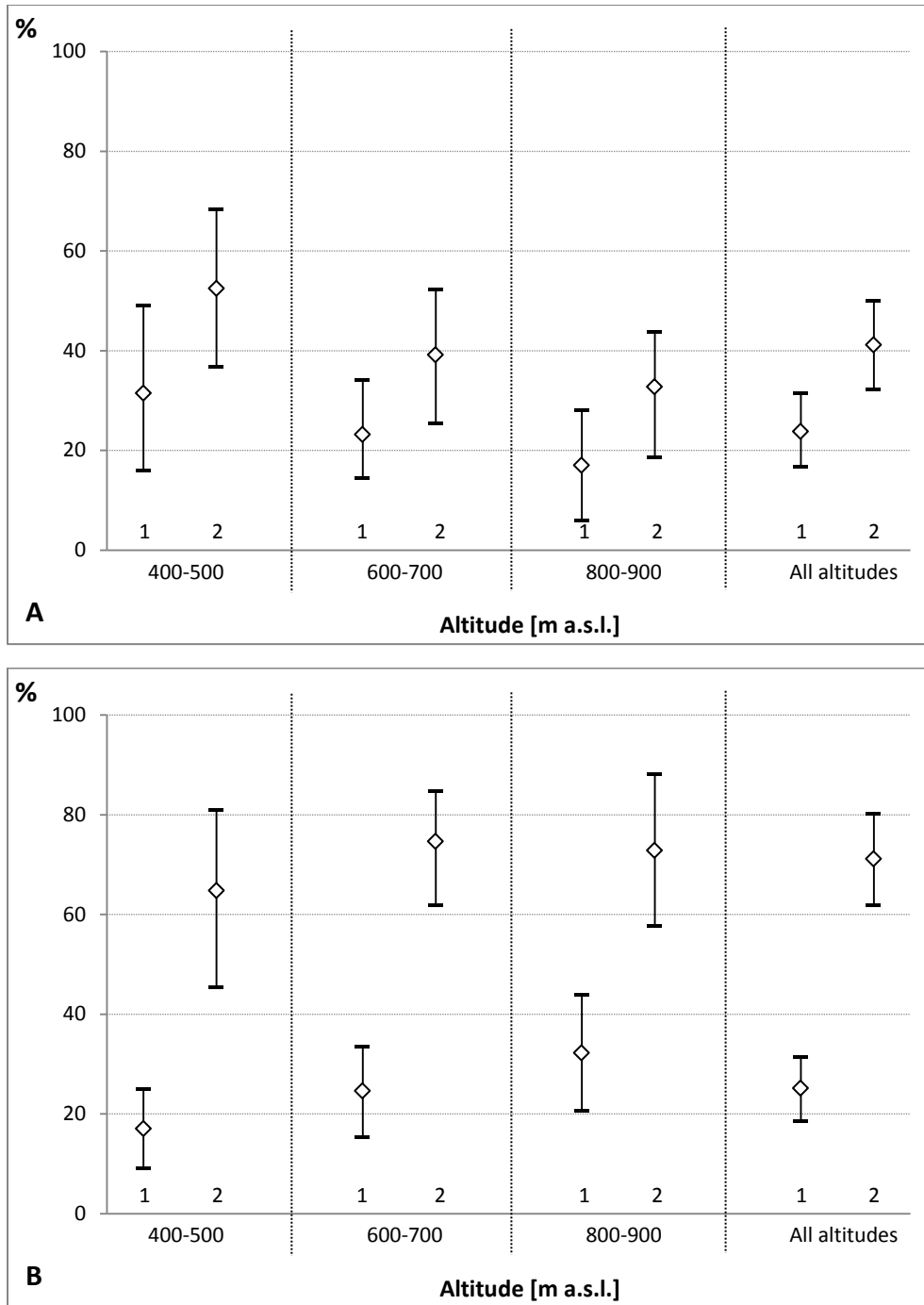


Fig. 11. Relative proportion of seeds contaminated by pathogens in October (A) and March (B):
1. Presence of infestation by insects; 2. No Infestation by insects

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