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Screening resistance to chestnut blight in young chestnut trees derived from *Castanea sativa* × *C. crenata* hybrids

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Abstract

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In 2010, 2011 and 2012 four trials were carried out to prove a reliability of the new screening method for resistance to chestnut blight caused by *Cryphonectria parasitica.* In the selected trees of hybrid origin (*C. sativa* × *C. crenata)*) 10 cm long cut-branch sections were inoculated with mycelia of three virulent isolates and one hypovirulent isolate (in two last trials) and cultivated for 7 days in the dark at 25 °C with 95% humidity. Then the bark of branch segments was peeled off and the size of necrotic lesions formed on the wood tissue around the inoculation place was measured. The size of necrotic areas varied by sampling date, type of fungus strains and tested trees. In all three summer trials necrotic lesions were larger than lesions in a spring trial of 2011, in which still dormant stem sections were used. Unlike the summer trials in the spring trial higher differences in the size of necrotic lesions among tested trees and among used virulent strains were observed. In the spring trial interactions between tested trees and fungal isolates were not so frequent like in summer trials where more trees exhibited different response to the same virulent strain. Majority of trees showed different susceptibility in particular trials. Observed high variation of reactions of tested trees to both virulent and hypovirulent isolates has pointed at the need to prove other screening methods, and to find such one, that would be highly effective to reveal an inherited resistance and/or a lower degree of susceptibility to chestnut blight.

Key words

branch sections, *Cryphonectria parasitica*, hypovirulent isolate, necrotic lesions, seasonal variation, virulent isolate

Introduction

Since the first occurrence of chestnut blight in Slovakia in 1976 (Juhásová, 1983), this disease caused by *Cryphonectria parasitica* (Murrill) M. E. Barrhas has spread to all main chestnut growing areas and caused mass extinction of chestnut trees (Juhásová et al., 2011). As long as several decades in all Europe there has been an effort to reduce and minimize the dissemination and the devastating effect of chestnut blight disease. In affected areas, the attempts to control the disease by chemical, legislative and phytosanitary measures were reported as insufficient (Jaynes and Van Alfen, 1977; Elliston, 1981). Therefore the effort has been concentrated on biological control based on application of hypovirulent isolates of the fungus (Heiniger and Rigling, 1994).

In Slovakia no natural hypovirulent isolates were found out till now (Adamčíková et al., 2012), so French and Hungarian hypovirulent isolates were used to prepare suitable isolates for biological control in our area (Ondrušková et al., 2010). The success of biological control was very variable and it fluctuated from 5 to 72.7% (Juhásová et al., 1997 and 2005).

Successful managing bio-control of chestnut blight requires a rapid, convenient and reproducible virulence test. Standard tests involve inoculation of the fungus into living trees (Elliston, 1978; Griffin et al., 1978), excised stems (Elliston, 1985; Fulbright et al., 1983), or apple fruit (Fulbright, 1984) with incubation times

of 2–3 months, 5 weeks, and 3 weeks, respectively. Field research on living trees can be difficult because several healthy trees of similar size and developmental stage must be found for replicate inoculations with the fungus. Genetic variation among the trees can introduce another variable to this test. In addition, canker development from field inoculations can be poor during late fall and winter. The cut-stem method, in which comparable cut-stem sections from a tree (or trees) are inoculated with fungi in the laboratory, can be used to overcome the seasonal limitations of field tests and to minimize size, developmental-stage, and genetic variations. However, the cut-stem virulence-test incubation period is also long and requires constant, high humidity. Results from apple fruit inoculation test can be obtained more quickly, but because this tissue is substantially different from chestnut, the conclusions drawn from the fruit data may not always be directly applicable to chestnut. McCarroll and Thor (1985) found that inner-bark tissue from American chestnut has a detectable reaction to fungal products in vitro. As a result, this type of tissue has potential for use in fungal virulence tests. Following this finding a rapid *C. parasitica* virulence test was developed using excised bark- and wood-tissue sections with incubation time only of 4 days (Lee et al., 1992). Unlike the standard tests where canker area formed on the bark surface was evaluated, in this test necrotic areas formed on inner-bark- and wood tissue sections were measured.

In our study we have combined method of cut-stem sections with the method of bark- and wood-tissue sections and we evaluated size of necrotic areas formed on inner bark and wood tissue on the stem sections in which bark and wood tissues were not separated prior to mycelium inoculation. The main aim of this study was to assess reliability of the new, rapid method of screening chestnut for resistance and/or lower susceptibility to chestnut blight especially in relation to different date of sampling. At the same time we have attempted to assess: (i) interaction of fungal isolates with host trees in necrosis induction, (ii) interaction of necrotic lesions size with type of fungal isolates, (iii) seasonal effect on the necrotic lesions size.

Material and methods

Fungal isolates and cultural conditions

The following virulent strains of *C. parasitica,* each from a different vegetative-compatibility (vc) group, were used in the study: M1297 (MAT-1, vc group EU1, Pian Ne, Swiss), M1115 (MAT-2, vc group EU2, Gorduno, Swiss, Rigling, 1995), EU12 (SA 16 from European vc tester database, Tonara, Italy, Cortesi et al., 1998). These strains were found to be the most, medium and least virulent, respectively, following previous virulence tests on cut-stem sections. In two last trials (29th June 2011 and 13th July 2012) in addition to virulent strains also a hypovirulent one was used in the tests. This strain named as I22 × IHB2 (MAT-1, vc group EU12, Príbelce, Slovakia) was produced by pairing a virulent strain isolated from the tree I22 (a seedling *C. sativa* from the family TV21o.p./15 grown in Príbelce orchard) with hypovirulent strain IHB2 coming from Hungary (Radócz, 2001). Fungal strains were grown on Malt extract agar (MEA) in Petri dishes for 7 days at 25 °C.

Origin of host trees

Eight seedlings derived from open pollination of eight different *C. sativa* × *C. crenata* hybrids, one seedling derived from open pollination of a *C. sativa* × *C. sativa* tree and one graft *C. sativa* × *C. crenata* were used as host trees in the trials (Table 1).

Parental trees of the tested accessions were derived from both interspecific and intraspecific crosses of a *C. sativa* tree TV21 on locality Tlstý Vrch with *C. crenata* and *C. sativa* trees respectively grown in Spain, Pontevedra. In 1984 two-year-old seedlings of these crosses together with other seedlings derived from other crosses were planted in Arboretum Mlyňany on an experimental plot. In 1997 in village Príbelce a chestnut orchard was established by planting seedlings from interspecific and intraspecific hybrids grown on this experimental plot. In 1999 most of seedlings were grafted by scions coming from female parents of the seedlings. However a great part of grafting was unsuccessful so rootstocks remained as permanent accessions in the orchard. In 2010, first year of the experiment, seedlings were 15 years and the graft 13 years old.

Table 1. Host trees used in susceptibility test, their names, origin and parentage

Tree name	Tree origin	Parent origin / parent No
B4	Seedling	*C. sat.* 21 × *C. sat.* E/4
B10	Seedling	*C. sat.* 21 × *C. cren*. E/7
E4	Graft	*C. sat.* 21 × *C. cren*. E/6
F3	Seedling	*C. sat.* 21 × *C. cren*. E/13
F4	Seedling	*C. sat*. 21 × *C. cren*. E/4
H3	Seedling	*C. sat*. 21 × *C. cren*. E/7
H4	Seedling	*C. sat*. 21 × *C. cren*. E/3
I3	Seedling	*C. sat*. 21 × *C. cren*. E/5
I4	Seedling	*C. sat*. 21 × *C. cren*. E/11

All trees included to the experiment were of good health condition at the time of testing and showed good growth performance. As the experiment was not designed primarily for screening and selection of

the resistant chestnut accessions, the eight different genotypes were considered to be a sufficient number for the experiment.

Excised-branch inoculation

One or two branches with no signs of blight infection were excised from the selected trees and then cut to the sections of 10 cm in length. Number of branch sections per tree varied from 9 to 15 depending on the number of used *Cryphonectria* strains. Each strain was applied in 6 repetitions in single tree (three branch sections, each with two inoculations). In each tree, both thinner and thicker branch sections were included to the trial in order to assess the effect of branch thickness on the necrotic lesion size. Two bark plugs, each on the opposite site of the section, were removed from each branch section by using 7-mm diameter cork borer. Agar plugs of the same size from the edge of actively growing colonies of the isolates were inserted into holes with mycelium facing downwards. Sterile agar plugs as controls were inserted to the bored holes in the same number of sections as used for each fungal isolate. The inoculation sites and both cut ends of branch sections were sealed with Parafilm to prevent desiccation and the branch sections were placed to cultivation chamber in the dark at 25 °C with 85% relative humidity. In total, four trials were performed, each of them having started with collecting branch samples on the following dates: 6th July 2010, 19th April 2011, 29th June 2011 and 13th July 2012.

Seven days after inoculation, the branch sections were picked out from cultivation chamber and diameter of each section in its middle position was measured. Subsequently bark and wood tissues of branch sections were separated and on wood tissues the length (a) and width (b) of the necrotic lesions were measured. As area of necrotic lesions was in general of ellipsoid shape, formula for the ellipse area calculation was used ($a \times b \times 0.785$).

Data analysis

Data obtained in each of four trials were evaluated separately because the trials included both the same and also different trees. The size of necrotic lesions was evaluated by analysis of variance where as sources of variance were assumed the trees, isolates and interaction trees × isolates. Differences between the mean values were evaluated by Duncan's multiple range test. Statistical package Statgraphics 5.1 was used for data processing.

Results

Variation of necrotic lesions size among trees and dates

Correlations between diameter of branch segments and necrotic lesion size were very low and in individual trials varied from $r = -0.2658$ to 0.2365 and were not significant at $P = 0.05$. Following this finding, thickness of branch segments was not assumed as source of variation in analysis of variance of necrotic lesion size.

In particular dates a size of necrotic lesions varied among trees with different results. The trees with the smallest lesions in one date had the largest or medium large lesions in other dates. For instance trees H3 and H4, both seedlings derived from two *C. sativa* × *C. crenata* hybrids, had in 2010 the smallest lesions, next year in the spring of 2011 H3 the smallest and H4 the largest spot but in the summer of 2011 both trees had the largest lesions (Table 2). In the next year 2012 the tree H4 died out because of chestnut blight but H3 was healthy without any symptoms of disease and had medium large necrotic lesions in the test. The tree B10 with symptoms of chestnut blight in 2012 had in this year the smallest necrotic lesions on average among seven tested trees.

Table 2. Mean necrotic area on wood-tissue of cut-branch sections of the selected host trees after infection by all isolates at different dates

Infection date							
29-06-2010		12-04-2011		22-06-2011		13-07-2012	
Tree name	Necrosis [mm^2]	Tree name	Necrosis [mm^2]	Tree name	Necrosis [mm^2]	Tree name	Necrosis [mm^2]
H3	750.55 a	H3	347.30 a	E4	740.72 a	B10	566.87 a
H4	755.14 a	E4	430.38 b	I4	832.28 ab	I4	672.80 b
I3	806.14 a	B10	494.55 bc	I3	877.08 b	E4	703.80 b
B4	835.39 ab	B4	506.08 c	H4	993.80 c	H3	719.19 b
I4	865.39 ab	I4	582.80 d	F4	1,039.09 c	B4	738.97 b
E4	958.18 bc	H4	610.37 d	B10	1,039.57 c	F4	746.72 b
F3	1,008.29 c			H3	1,050.36 c	I3	862.56 c

Means followed by the same letter are not significantly different at $P = 0.05$. Numbers of observations (n) for the trees in individual dates were as follows: 16, 16, 36, 36.

The mean size of necrotic area on inoculated cut-branch sections differed in individual trials. The smallest necroses were observed in cut-branch sections inoculated on 12th April 2011 (on average 495.2 mm^2). Necrotic area increased during the remaining dates as follows: July 13, 2012 – 717.3 mm^2, June 29, 2010 – 875.6 mm^2 and June 22, 2011 – 939.0 mm^2. In the trial performed in April 2011 the most differences occurred among tested trees compared with other dates.

Variation in necrotic lesions size in relation to fungal isolates

Original grading of the virulent strains by intensity of their virulence was in accord with the observed results only during the last test in 2012 (Table 3). In June 2010 the strain EU12 classified as the least virulent was the most virulent and formed necrotic lesions significantly bigger ($P < 0.05$) than strain M1297 classified as most virulent. In June 2011 all three virulent strains induced necrotic lesions of the same size ($P > 0.05$). In April 2011 the strain EU12 gave rise significantly smallest ($P < 0.05$) necrotic lesions but M1297 classified originally as the most virulent initiated formation of significantly smaller ($P < 0.05$) lesions than M1115. A hypovirulent strain used in two last dates initiated significantly smaller ($P < 0.05$) necrotic lesions than virulent strains especially in the test conducted in June 2011.

Interaction of fungal isolates with host trees in necrosis induction

In each of four trials some host trees responded to the same fungal isolate in another way than remaining host trees. In the first trial where only two fungal isolates were used it was tree I4, which was more susceptible to the strain M1297 than to EU12. All remaining trees were more susceptible to EU12 than to M1297 forming necrotic lesions of bigger size. In the second trial where virulent isolates produced lesions of significantly different size ($P < 0.05$), trees I4, H3, H4 and B10 responded to fungal strains in a different way than was average for all trees (Fig. 1). In the third trial where all three virulent isolates produced necrotic lesions of the same size on average, their effect in each of single trees was different but most of differences were not significant. In all host trees necrotic lesions induced by hypovirulent strains were significantly smaller ($P < 0.05$) than those induced by virulent strains except fourth trial, tree I4. In this trial, the highest effect of M1297 calculated for all trees was proved only in three trees (B4, I4 and E4) and in other trees its effect was on the level of either second or third virulent isolate.

Discussion

Data on area of necrotic lesions varied in this study in relation to several factors. Dates of test performance showed the most significant effect on this variation. Lesions developed on stem sections collected in dormant period (beginning of April) were two times smaller than those developed on stem sections from late June. Similar results were also obtained by Guérin and Robin (2003) after inoculation of 4-year-old *C. sativa* sprouts *in situ* and *in vitro* on stem segments. In both trials, lesion length was lower in April than in June, more markedly in excised chestnut segments. Generally in all trials bark susceptibility was higher during the vegetative period of the chestnut than during the dormant season. The authors recommend using for inoculation tests chestnut segments excised during the vegetative period rather than dormant segments (higher percentage of infected segments when inoculated by spores and higher lesion development when inoculated by mycelium). However, the proposed method might not be reasonable in all cases. For instance, if we wish to distinguish host trees of moderate blight resistance

Table 3. Mean necrotic area on wood-tissue of cut-branch sections developed after infection of virulent (M1297, M1115, EU12), hypovirulent (I22 × IHB2) isolates and pure culture medium (control) at different dates of infection

Infection date							
29-06-2010		12-04-2011		22-06-2011		13-07-2012	
Isolate	Necrosis [mm^2]	Isolate	Necrosis [mm^2]	Isolate	Necrosis [mm^2]	Isolate	Necrosis [mm^2]
Control	184.9 a	Control	114.5 a	Control	271.8 a	Control	89.4 a
M1297	1,117.1 b	EU12	535.3 b	I22 × IHB2	536.5 b	I22 × IHB2	605.1 b
EU12	1,324.7 c	M1297	600.1 c	M1297	1,271.3 c	EU12	890.6 c
		M1115	731.1 d	EU12	1,275.6 c	M1115	956.8 c
				M1115	1,339.7 c	M1297	1,044.9 d

Means followed by the same letter are not significantly different at $P = 0.05$. Numbers of observations (n) for the isolates and control in individual dates were as follows: 48, 35, 42, 48.

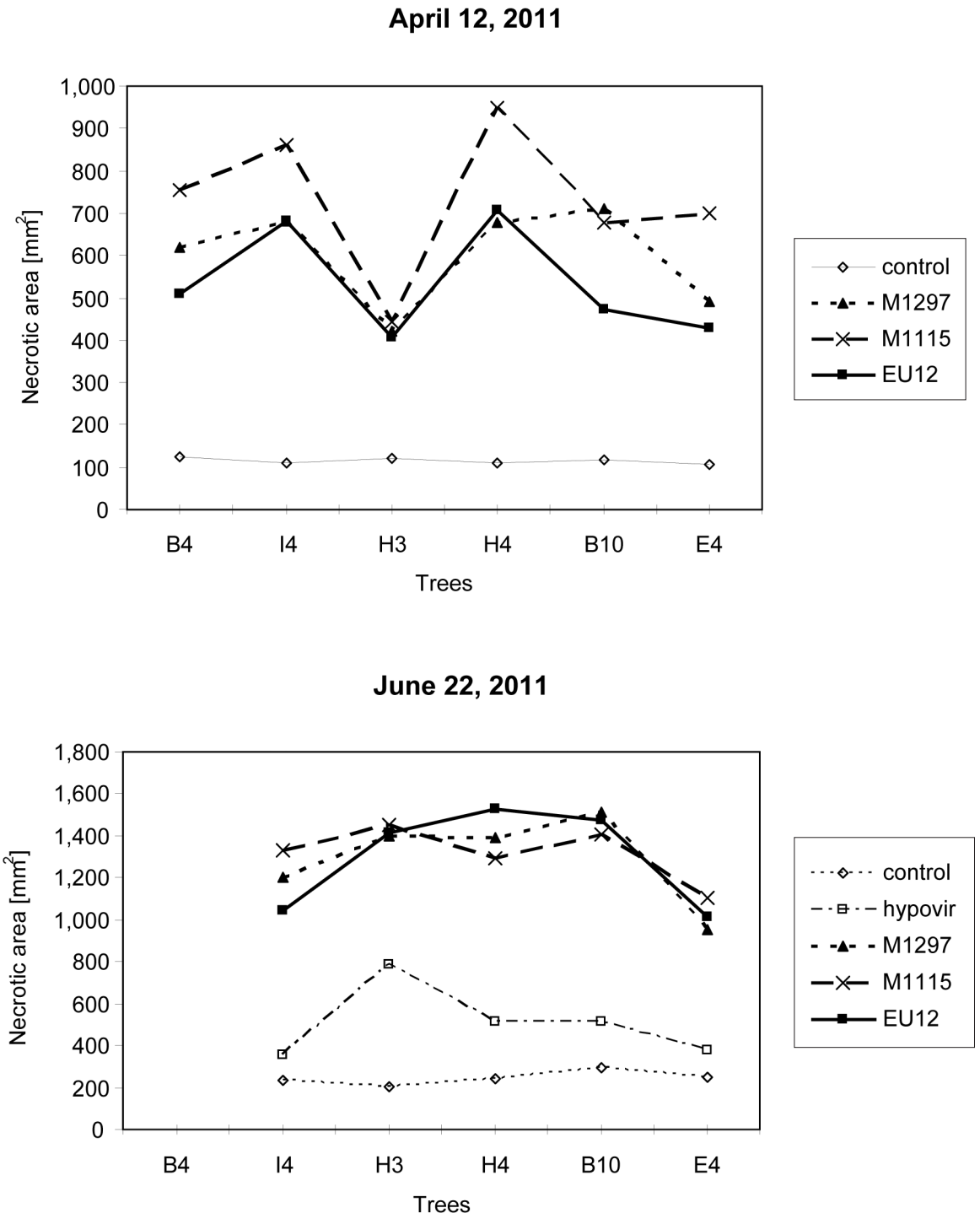

Fig. 1. Interaction of host trees and fungal isolates in forming necrotic area on in vitro cultivated branch segments in two different dates. M1297, M1115 and EU12 are virulent isolates and hypovir is the hypovirulent isolate I22 × IHB2.

from hosts of low or else high resistance, a more rapid growth of inoculated isolates may make even eventual genetic differences between tested trees in susceptibility to chestnut blight. Namely in our study just inoculations performed on branch sections from dormant period (early April 2011) resulted to the most distinct differences among host trees as well as among fungal isolates in the size of necrotic area. At the end of June 2011, when necrotic lesions were larger, differences among host trees were less distinct and

among tested virulent strains absent at all. Similarly, relatively uniform response of tested trees to inoculated isolates was also at end of June 2010 and in July 2012, when in five out of seven trees necrotic lesions were of the same size.

Seasonal variation in canker development appeared to result from seasonal variation of chestnut susceptibility particularly of intrinsic susceptibility of chestnut cortical tissues and from the direct effect of meteorological factors on *C. parasitica* development (Guerin and Robin, 2003). Just during summer, susceptibility of chestnut cortical tissues might be higher in all tested trees but in spite of this, individual trees may exhibit different response to different isolates. Although responses of particular host trees to different virulent isolates (interaction host tree × isolate) varied also in dormant period, the variation was higher in summer period. In this context, Elliston's (1978) recommendation for using dormant stems to carry out pathogenicity tests seems to be reasonable.

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