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Assessment and characterisation of Turkish hypovirulent isolates of *Cryphonectria parasitica* (Murr.) Barr.

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Summary. Hypovirulent isolates of *Cryphonectria parasitica* on chestnut trees were collected from abnormal cankers in eastern and western Turkey. Laboratory tests showed differences between these isolates in morphological and physiological characters and in their virulence in culture. The majority of selected isolates of *C. parasitica* contained dsRNA, but some of the hypovirulent ones were dsRNA-free. The decrease in damage severity in Turkey is possibly a consequence of the occurrence of hypovirulent isolates and the biological control resulting from their natural spread.

Key words: chestnut blight, hypovirulence, dsRNA.

Introduction

Chestnut blight caused by *Cryphonectria parasitica* (Murr.) Barr. has been spreading in Turkey since 1967, producing damage to chestnut stands and orchards (Akdogan and Erkam, 1968; Karaca, 1968; Delen, 1975, 1979; Coskun *et al*., 1999).

The natural spread of hypovirulence in *C. parasitica* has reduced in Europe the severity of the disease, and regrowth of many blighted trees is frequently observed in stands of *Castanea sativa* (Mill.) (Grente and Sauret, 1969; Bonifacio and Turchetti, 1973; Heiniger and Rigling, 1994; Turchetti, 1994). Recent investigations into chestnut blight in Turkey have revealed the occurrence of hypovirulent (H) strains of *C. parasitica* in this country as well (Coskun *et al*., 1999).

The objectives of this study were to increase knowledge of hypovirulence in Turkey and to examine Turkish hypovirulent isolates for culture morphology, virulence and dsRNA presence, focused on possible programs of biological control.

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Materials and methods

Field observations and sampling

Turkish chestnut stands were surveyed in districts located in Marmara Region (Bursa, Canarkçi, Golcuk, Kefken and Tepekoy) and Black Sea Region (Akçakoca, Kesap).

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 (healing) cankers, and from intermediate ones (as described in Turchetti and Maresi, 1990, Conedera, 1993, Turchetti, 1994).

Isolation and culture of the pathogen

C. parasitica was isolated from all collected samples by placing small fragments of infected bark on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) amended with methionine (100 mg l^{-1}) and biotin (10 mg l^{-1}) (PDAmb) as indicated by Anagnostakis (1977). All the isolates were subcultured and tested on PDAmb.

Isolates from abnormal cankers used for the study, were grown on PDAmb at 25°C for 10 days in darkness and their morphology, colony diameter and pycnidia production were recorded. The isolates were considered hypovirulent when they exhibited white mycelium and a few and large pycnidia as indicated by Grente and Sauret (1969) and Bonifacio and Turchetti (1973). One Turkish virulent (V) strain (TVM1), two Italian V strains (IV5, IV7) and three Italian H strains (IH1, IH2, IH3), previously selected and studied (Coskun *et al*., 1999) were employed as standards for comparison with the new Turkish isolates. Three replications were carried out for each laboratory test. The stability of the hypovirulent phenotypes was assayed over seven transfers on PDAmb.

H isolates that did not produce pycnidia on PDAmb were then tested on small autoclaved stem segments of *Castanea sativa*. These fragments were placed on PDAmb in Petri dishes, inoculated with a mycelial plug of each isolate and maintained for fifteen days at room temperature near a window of the laboratory. Controls were: one Italian V isolate and one H isolate, both able to produce pycnidia.

Statistical computations were carried out and normal colony diameter data were statistically determined using the univariate procedure. Homogeneity of variance was performed using the general linear model. If the F value from ANOVA was significant, cluster analysis (P=0.05) was used to separate the means of unrelated treatments (Gates and Bilbro, 1978; Dunn and Boland, 1993).

Laboratory assays

Phenol oxidase test (Bavendam test). The selected H isolates were grown on a medium containing 0.5% tannic acid (Merck AG, Darmstadt, Germany), 1.5% Difco (Difco Laboratories) malt extract and 2% Difco bacto agar, adjusted with NaOH to pH 4.5, as described by Rigling *et al*. (1989). The tannic acid solution and malt extract agar suspension were autoclaved separately and mixed before being poured into the Petri dishes. Plates containing this medium were inoculated with mycelial plugs taken from the growing edge of 7-day-old PDAmb cultures. Plates were incubated for 4 days in the dark at 25°C and colouring of the agar medium indicated phenol oxidase activity. Controls were: three European H isolates, two Italian V isolates and one Turkish V isolate.

dsRNA assays. All Turkish isolates showing hypovirulent morphological characters were tested for dsRNA. Agar cultures were harvested on PDAmb overlaid with cellophane in 9-cm-diam. Petri dishes. Mycelium was prepared from 10-day-old colonies. The mycelium was immersed in liquid nitrogen, ground to a fine powder using a mortar and pestle, and nucleic acids were extracted with the phenol extraction method. dsRNA was extracted using the cellulose column method as described by Morris and Dodds (1979). The dsRNAs were separated on agarose gels (1%) according to Hillman *et al*. (1990).

Conversion tests and transmission of hypovirulence to conidia. The Turkish H isolates were used to transmit hypovirulence (hypovirulence conversion) to three V isolates, one Turkish (TVM1) and two Italian (IV5, 7). Small mycelium disks of the H and V isolates were transferred to plates of PDAmb and paired. Pairings were incubated for 15 days at 27°C in darkness.

Plugs of V-paired colonies were transferred to sterilised PDAmb and checked for morphological characteristics. The converted V isolates were subcultured seven times to ascertain the stability of their morphological characters.

Conidia were harvested from colonies of the H isolates A17, G6, G7 and Kf3. Dilute spore suspensions of individual isolates were spread on PDA plates and one hundred single-conidial isolates were selected and subcultured as indicated by Melzer *et al*. (1997).

Virulence tests

In the laboratory. The Turkish H isolates were inoculated on 30-cm-long dormant chestnut stems (3 cm diameter) collected from a single stump and excised as described by Fulbright (1984), Tattar *et al*. (1992) and Dunn and Boland (1993). Agar

plugs with mycelium were inserted into three bark wounds (8 cm distant from each other) made in each stem with a 5-mm cork-borer. Sterile agar plugs were used as controls; all inoculation sites were sealed with Parafilm to prevent desiccation. The high stem section was protected with paraffin. The inoculated samples were placed in the dark at 25°C. After 4 weeks, the length and width of the cankers were measured, pycnidia production was recorded and the infected canker areas (cm^2) were calculated using the formula for an ellipse (Elliston, 1978). TVM1 was used as a control.

In the field. Inoculations were performed in the spring of 1999 in a Turkish chestnut stand at Bursa-Karacabey. Coppice shoots (70 to 90 mm diam.) growing from the same stump were inoculated as described by Elliston (1978) and Turchetti and Maresi (1988, 1991). Small plugs of mycelia from each isolate were placed in 8-mm cork-borer wounds at breast height and covered with masking tape. Each sprout was inoculated with one of the isolate in three replications. Controls were: wounds inoculated with a V isolate (TVM1) in one stump sprout, uninoculated wounds in another sprout.

The cankers were measured after 2, 6 and 12 months. Their surface area was calculated as above, and healing processes and pycnidia production were noted.

Results

Field observations

Blight was found in all the locations surveyed and showed the ability of *C.parasitica* to spread through Turkey's chestnut stands (Fig. 1). Different types of cankers were observed, including abnormal 'healing' cankers, intermediate cankers and typical normal cankers, able to kill the infected sprouts or branches. These observations confirmed earlier surveys in different districts of Turkey by Coskun *et al*. (1999). Abnormal 'healing' cankers occurred on apparently healthy trees and were similar to those described in Italy and other European countries (Bonifacio and Turchetti, 1973; Bazzigher *et al*. 1981; Turchetti and Maresi, 1990; Heiniger and Rigling, 1994) (Fig. 2). With abnormal cankers, although in its initial stages the infection seemed normal, there was more or less pronounced swelling of the bark. Abnormal cankers fully surrounded the sprouts growing from the stumps or branches, but did not kill them. Such cankers were often found on living sprouts, but bark lesions were few, pycnidia production was low and perithecia were not observed on the diseased bark. The buds below the canker did not vegetate and epicormic shoots were not produced. Under the bark, the tissues were alive because growth of the fungus was superficial.

Intermediate cankers exhibited epicormic shoots with abundant pycnidia production in the central zone. This type of canker was often unable

Fig. 1. Map of Turkey showing the location of the seven chestnut stands surveyed: 1, Bursa; 2, Canarkçi; 3, Golcuk; 4, Kefken; 5, Tepekoy; 6, Akçakoca; 7, Kesap.

Fig. 2. Healing canker on a chestnut stem.

to kill affected branches or sprouts and became a healing canker on vigorous stems.

Healing cankers were observed in all the chestnut stands surveyed. They comprised 10 to 20% of the total number of cankers.

Characterisation of Turkish *C. parasitica* isolates

Twenty-three isolates of *C. parasitica* were obtained from abnormal cankers. All these isolates had typical H colony morphologies as compared to the standard H and V strains (Table 1).

Fourteen of these isolates produced colony diameters similar to those of standard H strains (IH1, IH2 and IH3) and of two V strains (Table 2). With nine H isolates colony diameters were smaller than those of the standard H strains.

Eleven isolates (B3, B6, B43; C6; G2, G7, G9, G16, G24; Kf40; T9) were typical H isolates, characterised by white flocking mycelium without pycnidia. Pycnidia also did not occur in four cream-coloured isolates (G10, G20; Kf19; Ks2), while seven whitish and cream-coloured isolates (A17; C12; G4, G6; Kf3, Kf40; T3) produced a few large pycnidia. Isolate G15 grew in a hypovirulent manner but produced abundant pycnidia (Table 2). Pycnidia were observed after 15 days on the fifteen Turkish white or cream-coloured isolates when transferred to fragments of chestnut stems placed on Petri dishes containing PDAmb and stored in daylight (Fig. 3). The other isolates produced a few large pycnidia in the same conditions.

Table 1. Hypovirulent (H) isolates from abnormal cankers collected in different districts in Turkey.

Region	District	No. of H isolates recovered
Marmara	Bursa (B)	3
	Canarkçi (C)	2
	Golcuk (G)	10
	Kefken (Kf)	4
	Tepekoy (T)	2
Black sea	Akçakoca (A)	1
	Kesap (Ks)	1

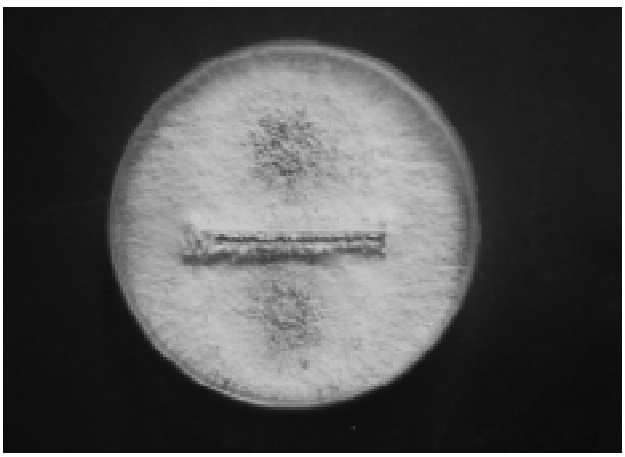

Fig. 3. Pycnidia production by a hypovirulent isolate inoculated on a stem segment of *Castanea sativa*.

Table 2. Some characters of *Cryphonectria parasitica* isolates grown on PDAmb after 10 days.

Hypovirulent isolates	Colony colour	Diameter[a]	Pycnidia production		H conidia %	Bavendam test[b]	dsRNA occurrence
			on PDAmb	on fragments of chestnut stems			
From Black sea Region							
A17	cream (s)	5.0 a	+	n.d.	63	-	+
Ks2	cream (s)	5.2 a	-	+	n.d.	+	+
From Marmara Region							
B3	white (s)	3.7 a	-	+	n.d.	-	+
B6	white (s)	6.0 b	-	+	n.d.	-	+
B43	white (s)	5.0 a	-	+	n.d.	-	+
C6	white (s)	5.0 a	-	+	n.d.	-	+
C12	cream (s)	6.6 c	+	n.d.	n.d.	-	+
G2	white (s)	6.4 c	-	+	n.d.	-	+
G4	cream (s)	5.7 b	+	n.d.	n.d.	+	+
G6	cream (s)	5.7 b	+	n.d.	75	-	+
G7	white (s)	5.8 b	-	+	52	+	+
G9	white (s)	5.0 a	-	+	n.d.	-	+
G10	cream (s)	5.5. b	-	+	n.d.	-	+
G15	cream (s)	5.2 a	++	n.d.	n.d.	-	+
G16	cream (s)	6.0 b	-	+	n.d.	-	+
G20	cream (s)	5.0 a	-	+	n.d.	+	+
G24	white (s)	6.6. c	-	+	n.d.	-	+
Kf3	cream (s)	7.1 c	+	n.d.	67	-	+
Kf19	cream (s)	4.4 a	-	+	n.d.	-	-
Kf20	white (s)	6.2 b	+	n.d.	n.d.	+	+
Kf40	white (s)	6.8 c	-	+	n.d.	++	-
T3	white (s)	5.5 b	+	n.d.	n.d.	-	+
T9	white (s)	6.6 c	-	+	n.d.	-	+
Standard isolates used as controls							
IV5	orange/cream	6.7 c	++	++	n.d.	+++	-
IV7	orange/cream	7.2 c	++	++	n.d.	+++	-
TVM1	orange/cream	8.0 d	++	++	n.d.	+++	-
IH1	white	6.7 c	+	+	80	-	+
IH2	cream	5.7 b	-	-	n.d.	-	+
IH3	white	7.1 c	+	+	72	-	+

[a] Average of three replications. Means followed by the same letter are not significantly different according to cluster analysis (P=0.05).
[b] +, positive; ++, medium positive; +++, strongly positive; -, no reaction.
(s), stable after 7 transfers on PDAmb.
n.d., not determined.

Laboratory assays

When tested for phenol oxidase activity, the 23 Turkish isolates produced different reactions (Fig. 4). The V strains used as controls produced a dark area, but of the H Turkish isolates tested, 5 isolates showed a weak reaction and produced a colour reaction as defined by Rigling *et al.* (1989) and only one (Kf40) had a medium - high colour reaction. No reaction was observed for the remaining group of seventeen H isolates.

The assays revealed dsRNA in twenty-one Turkish isolates and in the H control strains. A large

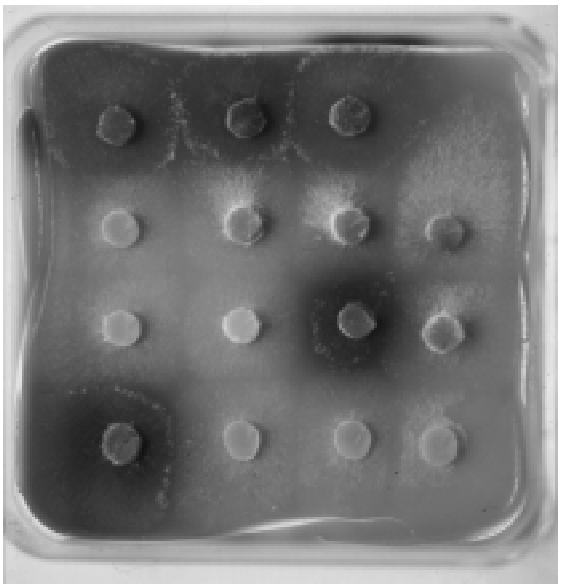

Fig. 4. Bavendam reaction of nine Turkish hypovirulent (H) isolates. Three virulent tester strains are at the top, three H tester strains on the left and nine H isolates in the remaining surface of the plate.

band (L-dsRNA) of about 12 kb similar to that in the controls, was observed in 21 tested isolates (Fig. 5). Additional bands were occasionally found. No dsRNA was found in the cytoplasm of isolates Kf19 or Kf40.

The morphological characters of all Turkish H isolates remained stable over 7 transfers on PDAmb independently from their dsRNA content. The two dsRNA-free isolates were also unchanged.

The percentage of H conidia from colonies of H isolates selected from the 21 dsRNA-containing Turkish isolates ranged from 52 to 75%. The Italian H strains used as controls produced 72 to 82% of H conidia while the V Turkish (TVM1) and two Italian V strains (IV 5 and 7) produced 100% V conidia.

In the hypovirulence conversion tests, five Turkish H isolates (B3, B6, G2, G20, Ks2) failed to convert the VC testers (IV5, IV7, TVM1). dsRNA was transmitted to all the V isolates assayed by six Turkish H isolates (A17, C12, G6, G7, G24, Kf3) (Table 3). Seventeen Turkish H isolates converted the V Turkish strain TVM1, ten the V European tester (IV5), and only seven the V Italian tester IV7.

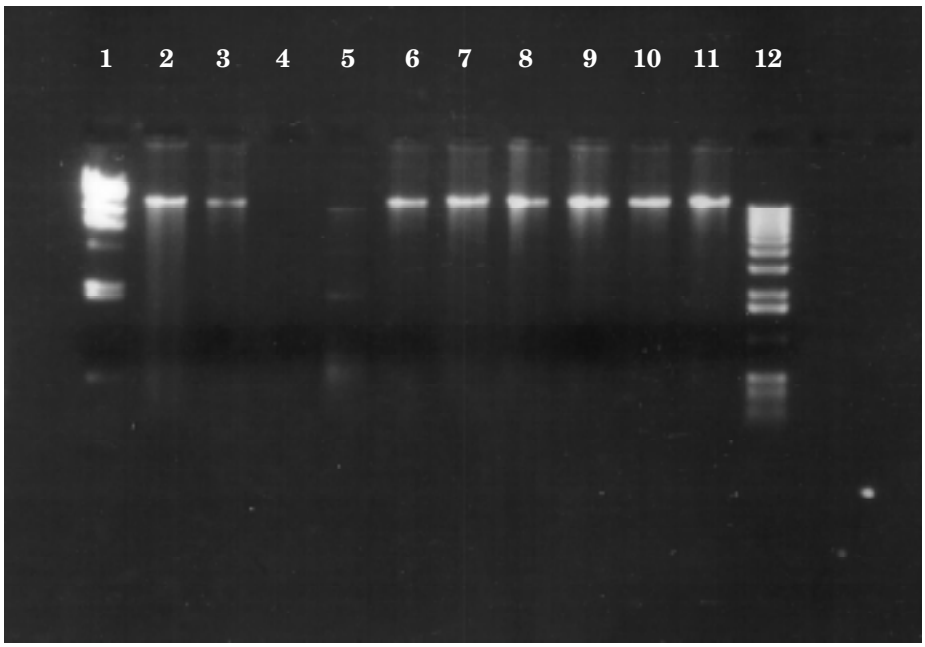

Fig. 5. Occurrence of dsRNA in Turkish hypovirulent isolates. From left to right: Marker II (Boehringer Mannheim, Germany) (lane 1), A17 (lane 2), Ks2 (lane 3), Kf40 (lane 4), B3 (lane 5), C6 (lane 6), G4 (lane 7), G20 (lane 8), Kf20 (lane 9), T3 (lane 10), IH1 (lane 11), 1 kb Ladder Plus (Life Technologies, Rockville Maryland, USA) (lane 12).

Table 3. Compatibility and conversion results among *Cryphonectria parasitica* vegetative compatibility testers (V-C Testers) and hypovirulent Turkish isolates.

Hypovirulent isolates	V-C Testers					
	IV5 [a]		IV7 [b]		TVM1 [c]	
	X	Y	X	Y	X	Y
A17	+	+	+	+	+	+
B3	-	-	-	-	-	-
B6	-	-	-	-	-	-
B43	+	+	-	-	+	+
C6	-	-	-	-	+	+
C12	+	+	+	+	+	+
G2	-	-	-	-	-	-
G4	+	+	-	-	+	+
Kf3	+	+	+	+	+	+
Kf19	-	-	-	-	+	+
Kf20	-	-	-	-	+	+
Kf40	-	-	-	-	+	+
Ks2	-	-	-	-	-	-
T3	-	-	-	-	+	+
T9	-	-	+	+	+	+

[a] European tester found in Turkey, Spain, Switzerland, Hungary and Italy.
[b] Italian tester.
[c] Turkish tester.
X, Compatibility test: +, compatible isolate; -, incompatible isolate.
Y, Conversion test (morphological control): +, converted isolate; -, unconverted isolate.

Conversion also occurred when the dsRNA-free isolates Kf19 and Kf40 were paired with the Turkish virulent strain (TVM1).

Virulence tests

All the isolates produced cankers of different sizes when inoculated into 30-cm-long dormant chestnut stems.

Two groups of isolates were identified, the first (A17, B3, B6, B43, C6, G2, G9, G15, G16, Kf19, Kf20, Ks2, T3, T9) producing small cankers ranging from 2.0 to 11.0 cm^2 (Table 4), the second (C12, G4, G6, G7, G10, G20, G24, Kf3, Kf40) producing larger cankers, from 12.0 to 18.5 cm^2, not significantly different from those produced by the virulent control. After 28 days, 22 isolates had also produced few large pycnidia. Only one isolate (G2) did not produce pycnidia on infected bark (Table 4).

The virulence of the isolates inoculated on coppice sprouts was variable. Lesions were moistened for more accurate measurement in accordance with Dunn and Boland (1993).

In general, fifteen isolates (A17, B3, B6, B43, C6, G2, G7, G9, G10, G15, Kf19, Kf20, Ks2, T3, T9) produced cankers smaller than 20 cm^2 (Fig. 1). Isolates B3, B6, B43, G2, G7, G9, G10, G15, Kf19, and Ks2 produced swollen split (healing) infections that no longer grew or formed pycnidia after two months. Isolates C6 and T3 like the uninoculated controls, failed to produce mycelial fans under chestnut bark tissue.

Cankers formed by isolates C12, G4, G6, G16, G20, G24, Kf3 and Kf40 ranged in area from 20 to 60 cm^2. These isolates consistently produced healing cankers and formed pycnidia in the six months after inoculation. The virulent reference strain TVM1 produced normal cankers that killed the sprouts after one year.

Table 4. Canker area (cm^2) on excised stems produced by twenty-three Turkish hypovirulent strains and the virulent control (TVM1) after four weeks.

Isolate No.	Canker area (cm^2) [a]
A17	9.0 [P] a
B3	6.0 [P] a
B6	6.0 [P] a
B43	6.0 [P] a
C6	6.0 [P] a
C12	15.0 [P] b
G2	10.0 a
G4	12.0 [P] b
G6	16.5 [P] b
G7	16.0 [P] b
G9	4.0 [P] a
G10	17.5 [P] b
G15	8.0 [P] a
G16	11.0 [P] a
G20	18.5 [P] b
G24	13.0 [P] b
Kf3	16.0 [P] b
Kf19	6.0 [P] a
Kf20	9.0 [P] a
Kf40	18.0 [P] b
Ks2	3.0 [P] a
T3	2.0 [P] a
T9	5.5 [P] a
TVM1	18.5 [P] b

[a] Means followed by the same letter are not significantly different according to cluster analysis (P= 0.05).
P, pycnidia presence.

None of the H isolates produced pycnidia after one year, and all their cankers were healed by then. The results are summarised in Fig. 6.

Discussion

The present investigation continues that initiated by Coskun *et al*. (1999) on chestnut blight in some regions of Turkey (Marmara, Black Sea). Hypovirulence was detected in both new and previously surveyed (Bursa and Kesap) districts. Abnormal cankers and related H isolates were recognised in the surveyed chestnut stands, suggesting that hypovirulence in chestnut blight can spread in Turkey, as it has done in other European countries such as Italy, France, Spain.

The spread of hypovirulence is influenced by different factors: environmental conditions, host- resistance to the parasite and differences in the virulence of isolates could all play an important role.

Turkey is one of the centres where *C. sativa* originates (Zohary and Hopf, 1988) and chestnut trees form extensive forests in the country. Chestnut is optimally suited for the Turkish environment and recent studies on the genetic structure of chestnut populations in this country have shown that there are two genetically distinct populations of *C. sativa* (Villani *et al*., 1992): a western and an eastern one with a high degree of genetic differentiation, and a hybrid zone between them, located in Bithynia region. The highest level of polymorphism was detected in Bithynia, with conspicuous morphological and physiological traits (Villani *et al*., 1999). These differences and their interactions could influence the level of host-resistance to the parasite, the epidemiological progression, and the development of the disease. The occurrence of healing and intermediate cankers in all the districts examined, and especially in the hybrid zone, reveals an interesting trend in the evolution of the disease. Further field investigations are needed to assess the real impact of blight in this context.

Two Turkish H isolates were found to be dsRNA-free. These isolates confirm reports of dsRNA-free H isolates in Greece and Italy (Xenopulos, 1982; Maresi *et al*., 1995). The results also show that some dsRNA-free isolates (classed as virulent) are only weakly pathogenic so that V isolates show different levels of pathogenicity. Furthermore, abnormal morphology and variations in pathogenicity are not associated with the presence/absence of dsRNA, as was stated by Fulbright (1985) and Dunn and Boland (1993).

At least twenty-one of the isolates from Turkey had reduced virulence and possessed dsRNA, a presence that is commonly associated with hypovirulence. The dsRNA in these H isolates had the same molecular weight as that of H isolates from Italy and previously tested Turkish H isolates. The conversion ability of Turkish H isolates varied and also dsRNA-free isolates were able to transmit hypovirulence. The capacity of isolates to produce hyphal anastomosis and transmit hypovirulence was therefore independent of dsRNA occurrence in those isolates.

Differences in cultural morphology, pigmentation, conidiation and virulence were numerous in

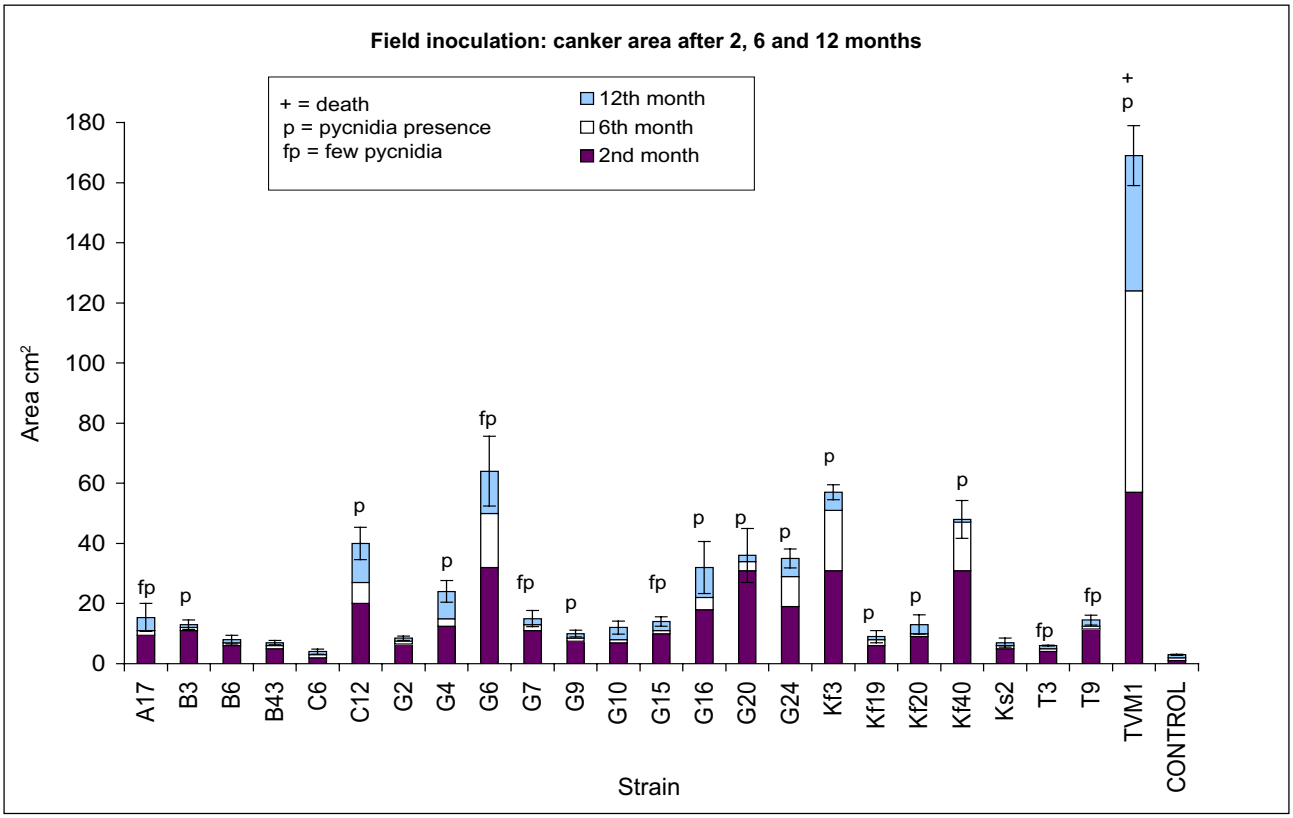

Fig. 6. Pathogenicity tests on chestnut stem segments inoculated with hypovirulent isolates.

the twenty-three isolates tested as previously reported for European and American *C. parasitica* strains by Elliston (1978), Anagnostakis (1984) and Dunn and Boland (1993).

Elliston (1985) suggested that several factors indicate hypovirulence in *C. parasitica* strains, and reduced laccase and phenol oxidase activity in dsRNA-containing H strains was reported by Rigling *et al*. (1989). On the basis of these results, and considering the wide variations in the morphological and physiological features of the isolates, a protocol for the characterisation of H isolates of *C. parasitica* can be drawn up. A lack of correspondence between the Bavendam test, the virulence assays on excised stems, and the inoculation test on living sprouts was found only with three isolates (G7, G10, G16). Artificial inoculations on chestnut sprouts on the same stump are the only way to ascertain the pathogenicity of *C. parasitica* and they supplement the laboratory tests, which only detect some morphological and physiological characters but do not identify this pathogenicity securely.

The percentage of H conidia produced by the selected Turkish H isolates tended to be high (52-75%). The proportion of H conidia found by Elliston (1978) varied from 2 to 18%, while Garbelotto *et al*. (1992) reported that 65% of conidia from H isolates were hypovirulent. The proportion of H conidia that an isolate produces is another important criterion to consider when selecting H strains that can be released in the field, and is basic to promote the natural spread of hypovirulence.

Pycnidia were produced on cankers caused by most Turkish H isolates even if their persistence on artificially infected bark was limited to a few months. Canker growth and healing processes differed between isolates. The H phenotype was transmitted by the Turkish H isolates, but only isolates A17, C12, G6, G7, G24 and Kf3 had a broad conversion capacity and converted the three testers, representing the three VC groups. These results underline the importance of using different H isolates for biological control: a single strain can fail in one of the main characters, H conidia produc-

tion, establishment of healing cankers or conversion. On this basis, C12, G6, G24 and Kf3 are suitable for biological control.

The identification of these Turkish H *C. parasitica* isolates is a potential step forward in the control of chestnut blight achieved with indigenous selected H isolates in combined inoculations carried out as described in Turchetti and Maresi (1991). The present study confirms the importance of dsRNA in controlling chestnut blight, but also suggests that other factors can be relevant as genetic and morphological characters of the fungal parasite, genetic characters of the host and ecological interactions, (Griffin, 2000). In conclusion, the occurrence of H isolates of *C. parasitica* in Turkey suggests that the biological control of blight in this country is a real possibility.

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