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Full Length Research Paper

Feasibility of biological control of chestnut blight caused by *Cryphonectria parasitica*, in Marmara region of Turkey

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Bursa and Yalova provinces are very important centers of chestnut production and processing. Chestnut blight is a serious disease of *Castanea* spp. and the only feasible method to control it is the use of hypovirulence. Hypovirulence and vegetative compatibility (vc) types of 198 isolates of *Cryphonectria parasitica* obtained from 15 different locations from Bursa and Yalova provinces in three years were determined and discussed. Vc types of *C. parasitica* isolates were evaluated by using six European vc types; EU–1, EU–2, EU–5, EU–6, EU–12 and EU–14. All strains were found to belong to EU-1 vc type. White mycelial growth which is typical for hypovirulence was observed in 12% of the isolates. All these white-growing isolates were found to contain ds-RNA. Presence of only one fungal vc type and one hypovirus subtype (Subtype I) which was determined by analyzing gene sequences of dsRNA of the hypovirus in our previous work suggests that a possible biological control of the disease might be applied in this region.

Key words: Chestnut blight, *Cryphonectria hypovirus,* vc types, hypovirulence.

INTRODUCTION

There are 200,400 ha chestnut forests in Turkey, pure and mixed with broad leaved and conifer trees. Of this area, 6,383; 52,174 and 141,841 ha are located in Aegean, Marmara and Black Sea regions, respectively. The Black Sea Region comprises about 70% of the total chestnut area, Marmara and Aegean regions about 26 and 3%, respectively. The majority of chestnut forests in the Marmara Region is located in Kocaeli and Sakarya provinces (53%), followed by Bursa and Yalova (17%), Balıkesir (15%) and İstanbul and Çanakkale (15%) provinces (Bucak, 2006).

Chestnut Blight caused by *C. parasitica* is present throughout chestnut growing regions in Turkey and threatens chestnut production (Delen, 1979; Çeliker and Onoğur, 2001; Erincik et al., 2007; Akıllı et al., 2009). The most feasible method to control the disease is using transmissible hypovirus. There are viruses infecting *C. parasitica* causing hypovirulence, which are named as *Cryphonectria hypovirus* (CHV) 1, 2, 3 and 4 (Hillman et al., 1995). When the fungus is infected by the virus, virulence is reduced and the plant forms callus on the edges of the cankers and they recover soon. In nature, viruses infecting *C. parasitica* can only be transmitted by hyphal conjugation (also called anastomosis) (Anagnostakis, 1977). This conjugation is dependent upon vegetative compatibility (vc); for this reason in order to transmit the virus, fungal strains should be vegetative compatible. Vc is a genetic-codified trait controlled by specific loci. Vc types show great diversity in nature and only one hypovirus (CHV1) and 72 vc types have been determined in Europe so far (Robin et al., 2000). This high level of diversity complicates biological control via hypovirulent strains. For biocontrol management of chestnut blight in an infested area, primarily hypoviral type (if present) and vc types of the fungal pathogen should be determined. The virus found in Europe (CHV1) has also 7 subtypes based on the RFLP patterns of dsRNA and these subtypes might affect biocontrol of Chestnut Blight (Sotirovski et al., 2006). When only one subtype is present in a locality sustainability of biocontrol

could last for a long time. In order to reach good results, hypovirulent isolates should be obtained and used in their own regions (Anagnostakis et al., 1986; MacDonald and Fulbright, 1991; Heiniger and Rigling, 1994; Cortesi et al., 1998).

Vc types and hypovirulent strains of *C. parasitica* have been studied in the Marmara region several times. Gürer et al. (2001a, b) using 89 isolates found only EU-1 vc type in Marmara region and obtained 18 hypovirulent strains. Çeliker and Onoğur (2001) collected 324 isolates from Aegean, Marmara and Black Sea regions and found two vc types, EU-1 from Marmara and Black Sea regions and EU-12 from the Aegean region. They also obtained few hypovirulent isolates. In another research, presence of vc types was investigated in the same area and in some parts of the Black Sea region but EU tester isolates were not used (Coskun et al., 1999). Beside the previous analysis of these regions, Bursa and Yalova provinces were not so deeply analyzed.

Some chestnut varieties reported to be resistant to chestnut blight and grafting in the forests of Bursa and Yalova provinces were practiced extensively (Soylu, 2004). Anyway, recent surveys performed in these locations revealed that the disease is present on grafted trees (TCP/TUR, 2009) probably caused by hypovirulent strains. Chestnut varieties coming from this area have not been tested with aggressive isolates of *C. parasitica* and, due to expected changes in the virulence of the pathogen; biological control using hypovirulent isolates would have a great importance in this area.

There are modern chestnut processing facilities in Bursa province and there is a great demand for good quality chestnuts, which should be large, tasty and easy to process, especially for chestnut candy production. In Bursa and Yalova provinces, along with the production of grafted chestnut saplings, grafting of wild chestnut trees with good quality varieties is a wide practice. Farmers and forestry staff encourage grafting in this area but this practice promotes disease spread. To prevent disease spread in these areas, biological control can be used.

In this research, isolates of *C. parasitica* obtained from various places and in different years were evaluated for biological control. For this aim, their vc types, hypovirulence and CHV subtypes were determined.

MATERIALS AND METHODS

Sampling and isolation of the fungus from bark

To obtain hypovirulent isolates, surveys were carried out under the direction of forestry staff and samples were collected especially from the healing cankers. Mostly pure chestnut areas were visited. The disease was surveyed in 9 localities (Table 1) in Bursa and Yalova provinces of Marmara region in 1999, 2009 and 2010 and 54, 70, and 74 samples were collected each year, respectively. Bark samples of 3 × 5 cm were removed from the edges and centers of the recovering cankers. Only one stem per stump was sampled. Fungal cultures were stored as mycelial disks having spores in 15% glycerol at -85°C.

Identification of vc types of *C. parasitica*

The vc type identification of fungal isolates was performed by using the technique described by Bissegger et al. (1997). Six European tester isolates (EU–1, EU–2, EU–5, EU–6, EU–12 and EU–14), which were already used in our previous work (Akıllı et al., 2009) and found to comprise all the vc types present in Turkey; (kindly provided by Dr. Çeliker, Plant Protection Research institute, Bornova, İzmir, Turkey) were tested with all the isolates. For vc type determination, conidial inocula from the isolates to be identified and from testers were put in pairs 2 mm apart on PDAmb (Difco PDA amended with methionine (100 mg/L) and Biotine (1 mg/L) (Anagnostakis and Day, 1979) in 90 mm Petri dishes at 5 mm away from the dish edge and observed for barrier formation. All tests were repeated twice. The cultures were maintained at 25°C in the dark for seven days and under natural light at room temperature for the same time. When the two isolates fell in different vc types, a barrier zone was formed between the two colonies with one or two lines of pycnidia. Inversely, when they belonged to the same vc type, there was hyphal anastomosis and the two colonies merged without any barrier zone or pycnidia formation.

Determination of hypovirulence

Determination of hypovirulence was initially based on the morphological features of the colonies grown on PDAmb after seven days of incubation at 25°C in the dark and five days under fluorescent light (Akıllı et al., 2009). Whitish growing, sparse sporulating colonies were assumed as hypovirulent. To verify hypovirulence dsRNA isolations were carried out using cellulose CF-11 chromatography as described by Allemann et al. (1999). An isolate of *C. parasitica* obtained from Tullio Turchetti (Institute of Forest Tree Pathology National Research Council Firenze, ITALY) was included as control in each preparation. The quantity and quality of the preparations were examined by agarose (0.8%) gel electrophoresis.

RESULTS AND DISCUSSION

Chestnut blight caused by *C. parasitica* has been found widespread in the entire Bursa and Yalova provinces in Turkey. Only one vc type corresponding to EU-1 was found in this region. This vc type was also reported to be dominant in the Marmara region previously (Gürer et al., 2001a, b; Çeliker and Onogur, 2001), but it was found at half ratio in Aydın and İzmir provinces of the Aegean region (Erincik et al., 2007). EU-1 has also been found dominant in the Black Sea region of Turkey (Akıllı et al., 2009). It seems that this vc type is present in the more humid and cool regions of Turkey.

The vc type EU-1 has also been reported in some European countries in varying rates. It was dominant in eastern France together with EU-5 (Robin and Heiniger, 2001) and in Spain together with vc type EU-66 (Montenegro et al., 2008). It is also present in Bosnia-Herzegovina (Trestic et al., 2001). The presence of vc type EU-1 at very low percentage (2%) in Greece is quite strange since the two countries are neighbors.

Table 1. Distribution of virulent and hypovirulent isolates of *Cryphonectria parasitica* in Bursa and Yalova provinces collected in the years 1999, 2009 and 2010.

Province	District	No. of isolates	No. of white growing isolates	No. of dsRNA positive isolates	VC type
Bursa 1999*	Malkara-Center	7	0	0	EU-1
	Uludağ	1	0	0	EU-1
	Yiğitali	11	1	0	EU-1
	Bayramdere	7	1	1	EU-1
	Kurşunlu	24	4	4	EU-1
Bursa 2009	Malkara-Elmalı	4	2	2	EU-1
	Malkara-Yedikovan	13	0	0	EU-1
	Malkara-Taşocağı	16	2	1	EU-1
	Kocatarla	2	1	1	EU-1
Bursa 2010	Kurşunlu-Yeniköy	44	9	8	EU-1
Yalova 1999*	Çınarcık	4	2	2	EU-1
Yalova 2009	Kurtköy	6	0	0	EU-1
	Güneyköy	5	0	0	EU-1
	Safran	16	0	0	EU-1
	Teşvikiye	7	1	1	EU-1
	Esenköy	1	1	1	EU-1
Yalova 2010	Çınarcık-Teşvikiye	30	10	9	EU-1
Total		198	34	30	

*, Isolates obtained from Dr. M. Gürer.

Chestnut blight is a highly serious disease responsible for considerable damage in chestnut ecosystems in several European countries (Trestic et al., 2001). The only efficient protection strategy applied in various places is biological control which is based on the transmission of hypoviruses (CHVs) into the virulent strains of the fungus converting them to hypovirulent isolates. In the field, the conversion is achieved by artificial inoculation of hypovirulent strains on the canker areas (to get hypovirulence, both fungal strains should belong to the same vc type). Therefore, the variability of vc types of *C. parasitica* in this field crucially affects the transmission of CHVs (Anagnostakis et al., 1986; MacDonald and Fulbright, 1991; Heiniger and Rigling, 1994). Biological control of the disease in France and Italy revealed the effectiveness of the technique (Bisiach et al., 1988; Heiniger and Rigling, 1994). For this reason, vc types of the pathogen has been investigated in many countries and various vc types have been found in different countries. This work showed that hypovirulence is well established in Marmara region since several hypovirulent strains were identified, therefore the data obtained can be directly used for disease management. Determination of hypovirulence based solely on phenotype is unreliable. For this reason, the presence of dsRNA was verified by isolation and analysis (Figure 1).

There is a great demand to graft the chestnut trees in the forest areas with varieties having bigger fruits and biological control on these grafted trees is necessary since grafting makes wounds on the trees and this increases disease spread. The dominance of EU-1 vc type of *C. parasitica* and the natural occurrence of hypovirulent isolates compatible with EU-1, will make feasible a local programme of introduction of hypoviruses in this region. Such programmes have been already applied in other countries with satisfactory results (Heiniger and Rigling, 1994). Chestnut blight is an important concern in Bursa and Yalova provinces since they have many facilities to process chestnut fruit. Due to the intensive grafting applications, biological control of the disease would be a good practice in this region to reduce the damage caused by *C. parasitica.* This can only be achieved by application of hypovirulent strains. There are effective hypovirulent strains collected from this region and they all belong to the same subtype which will provide long lasting biocontrol (Akıllı et al., 2012). This study provides insights into biological control of the disease, giving the locations where hypovirulent strains

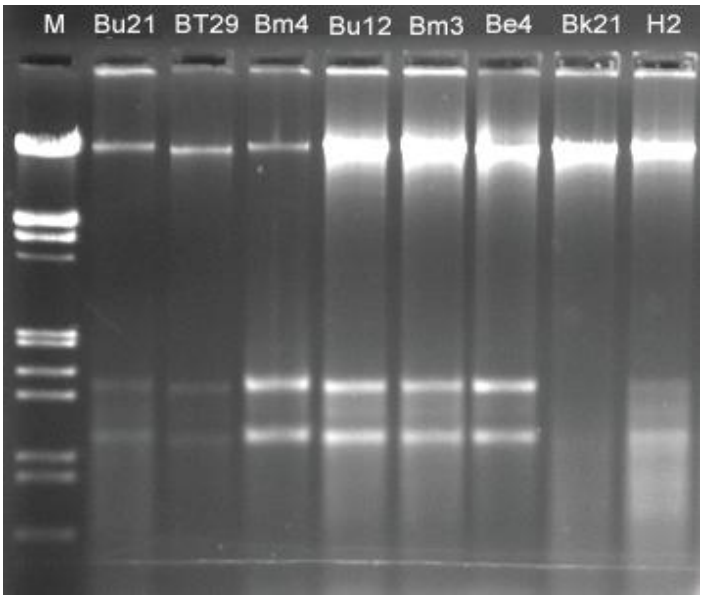

Figure 1. Electrophoretic pattern of dsRNA extracted from *Cryphonectria parasitica* infected mycelia. Picture shows several dsRNa bands of 7 *C. parasitica* isolates obtained from Bursa and Yalova provinces, ranging from aproximately 1.0 to aproximately 21.0 kb. Lane H2 is a positive control (CHV1-infected strain) provided by Dr. Tullio Turchetti; Institute of Forest Tree Pathology, National Research Council, Firenze, Italy. DNA molecular marker Lambda DNA EcoRı+HindIII in lane M.

can be collected for future possible disease management.

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