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Biological control of chestnut canker, caused by *Cryphonectria parasitica*, by antagonistic organisms and hypovirulent isolates

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Abstract: Biological control of chestnut blight was investigated by using 3 hypovirulent isolates of *Cryphonectria parasitica*, 5 *Trichoderma* sp., 4 *Penicillium* sp., and 4 *Bacillus* sp. isolates. Hypovirulent isolates and antagonistic organisms were obtained from samples collected from the Black Sea region of Turkey, in 2008 and 2009. Effectiveness of the hypovirulent isolates and antagonistic microorganisms was tested on 3-year-old chestnut saplings. In the tests, bark disks of 6 mm were removed from the stem bases and culture disks of the virulent isolate of *C. parasitica* were first placed into the hole and then the hypovirulent isolate or the antagonistic fungi. *Bacillus* strains were applied as bacterial suspensions of 10^{10} cell mL^{-1} to the holes; then the virulent isolate of *C. parasitica* was placed. Evaluations were made by measuring the canker lengths in 3 periods and effectiveness was given as percent inhibition (PI) of the treatments. Antagonistic microorganisms yielded varying PI values in 3 time periods and the highest rate of inhibition (68%) was obtained from the *Trichoderma* sp. isolate T - 2. A *Penicillium* sp. isolate (P - 3) and 2 *Bacillus* spp. isolates (B - z and B - b) also provided 30%, 40%, and 31% disease inhibition, respectively, 58 days after the inoculation. Effectiveness of the hypovirulent isolates varied depending on the virulent isolates and the hypovirulent isolate Z - 1 provided 59% inhibition against the most virulent isolate K - 19, while it gave 32% inhibition against the less aggressive isolate K - 44. The other hypovirulent isolate Ba - 6 also inhibited the canker development of the virulent isolate by 42%.

Key words: *Cryphonectria parasitica*, chestnut blight, biological control, hypovirulence, antagonists

Cryphonectria parasitica'nın neden olduğu kestane kanserinin hipovirülent izolatlar ve antagonistik mikroorganizmalarla biyolojik savaşımı

Özet: Kestane kanserinin biyolojik savaşımı Karadeniz bölgesinden 2008-2009 yıllarında toplanan örneklerden elde edilen *Cryphonectria parasitica*' nın iki virülent, üç hipovirülent izolatı, beş *Trichoderma*, dört *Penicillium* ve dört *Bacillus* izolatı kullanılarak araştırılmıştır. Hipovirülent izolatlar ve antagonistik mikroorganizmaların etkinliği üç yaşında kestane fidanları üzerinde araştırılmıştır. Denemelerde 6 mm'lik kabuk diskleri çıkarılmış ve açılan çukurlara önce virülent *C. parasitica* izolatı kültür diski sonra hipovirülent izolat veya antagonist fungusların diski yerleştirilmiştir. *Bacillus*' ların ise 10^{10} hücre mL^{-1} bakteri süspansiyonu önce çukura uygulanmış sonra virülent *C. parasitica* kültürü uygulanmıştır. Değerlendirmeler üç dönemde kanser uzunluklarının ölçülmesiyle yapılmış ve etkinlik uygulamaların

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yüzde engellemesi olarak verilmiştir. Antagonistik mikroorganizmalar üç dönemde değişen yüzdelerde engellemeler oluşturmuş ve en yüksek engelleme (%68) bir *Trichoderma* izolatından (T - 2) elde edilmiştir. Bir *Penicillium* sp. izolatı (P - 3) ve iki *Bacillus* sp. izolatı (B - z ve B - b) inokulasyondan 58 gün sonra sırasıyla %30, %40 ve %31 etki sağlamıştır. Hipovirülent izolatların etkinliği virülent izolata bağlı olarak değişmiş, hipovirülent izolat Z - 1 en virülent izolat K - 19' a karşı %59 engelleme sağlamış diğer yandan, aynı izolat daha az saldırgan olan izolat K - 44' e karşı %32 engelleme oluşturmuştur. Diğer hipovirülent izolat Ba - 6 virülent izolat K - 19' deki kanser gelişimini %42 oranında engellemiştir.

Anahtar sözcükler: *Cryphonectria parasitica*, kestane kanseri, biyolojik kontrol, hipovirulens, antagonistler

Introduction

Chestnut decline is a serious disease in all the chestnut growing areas not only in Turkey but in many other countries as well (Heiniger and Rigling 1994; Akıllı et al. 2009). The primary cause of this decline is chestnut blight caused by *Cryphonectria parasitica*. Many people who obtain their subsistence from chestnut fruit, wood, and honey are negatively affected by this disease since it causes serious damage to the trees and it is not controlled successfully. However, reduction in the virulence of the causal agent and recovery from the disease have been observed in many countries where the chestnut grows, including Greece, Italy, France, Spain, and Switzerland (Gobbin et al. 2003; Perlerou and Diamandis 2006; Turina and Rostagno 2007; Montenegro et al. 2008).

This recovery in nature was attributed to the presence of hypovirulent strains of *C. parasitica*. This fungus can be infected by a few viruses and it loses its virulence. These viruses contain dsRNA and are named *Cryphonectria Hypo Virus* (CHV) 1, 2, 3, and 4 (Hillman et al. 1992; Heiniger and Rigling 1994; Perlerou and Diamandis 2006).

New calli are formed around the cankers infected by the hypovirulent strains of *C. parasitica* and the infected trees recover slowly. Pigmentation and sporulation of these strains are weaker compared to the virulent ones. They exhibit a whitish growth on Potato Dextrose Agar (PDA). When they are inoculated to active cankers on the diseased trees, the pathogen loses its virulence and cankers heal gradually. Hypovirulent strains transfer their virus to the virulent ones by hyphal anastomosis and convert the virulent strains to hypovirulent ones, unless they are vegetative compatible (vc). When they are incompatible, anastomosis does not take place and the virus is not transmitted (Heiniger and Ringling 1994; MacDonald and Double 2006).

Biological control by hypovirulent strains is the most widely used control method for chestnut canker and it has been successfully applied in many countries as a curative measure. In France, biological control with hypovirulent isolates was carried out by inoculating the hypovirulent strains to 200 cankers in 20 ha area and healing of the cankers was observed after 4 years. Application of the hypovirulent strains on 233 cankers gave similar success in Italy (Heiniger and Rigling 1994).

In Turkey, various studies on the determination of the hypovirulent strains and their vc types have been carried out (Coşkun et al. 1999; Çeliker 2000; Gürer et al. 2001). Çeliker and Onoğur (1998) and Çeliker (2000) found 2 vc types, EU-1 and EU-12, from the 310 orange color isolates collected from Aegean and Marmara regions. Çeliker (2000) used 1 of the 7 hypovirulent strains for biological control of chestnut blight in a chestnut forest in Manisa Hacıisalar village and obtained sufficient control. Çeliker and Onoğur (2001, 2009) obtained effective results from the application of various hypovirulent isolates against chestnut canker under field conditions. Moreover, Akıllı et al. (2009) found 5 vc types, namely EU - 1, EU - 12, EU - 14, EU - 2, and EU - 5, among the 296 isolates obtained from the Black Sea region of Turkey, Eu-1 being the most widespread. They also detected hypovirulence in 9 of the 11 provinces surveyed.

The characteristics of the hypoviruses in terms of the conversion capacity to the hypovirulence are of crucial importance and need vegetative compatibility, which changes from place to place. This situation urged researchers to find some other biological agents against the disease. Treatment of the cankers by a *Bacillus subtilis* strain obtained from sap of the chestnut branches and by a *Bacillus megaterium* strain obtained from barks of chestnut coppices provided healing (Wilhelm et al. 1998; Groome et al. 2001). Wilhelm et al. (1998) concluded that an endophytic

B. subtilis yielded systemic acquires resistance against the pathogen on the treated plants. Accumulation of PR - proteins in the host provided this resistance effect.

During our previous study on vc types of *C. parasitica* in Black Sea region (Akıllı et al. 2009), antagonistic fungi like *Trichoderma* spp. and *Penicillium* spp. were especially isolated from the healing cankers. Chestnut blight is effectively controlled by the use of hypovirulent isolates but their use requires elaborate work and trained staff; especially in some places where there are many vc types it is difficult to apply it. The use of an antagonist does not have these drawbacks if it is effective. For this reason effects of the isolates obtained from the Black Sea region on canker development were investigated in this research. Additionally the effects of some *Bacillus* spp. isolated from soils and chestnut barks together with some hypovirulent *C. parasitica* isolates were also tested against chestnut canker on 3-year-old chestnut saplings.

Materials and methods

Plant material

Three-year-old chestnut saplings cv. Osmanoğlu obtained from a nursery in Bursa were used for the inoculation experiments. Stem diameter of the saplings was around 1 cm. Inoculations were made 10 cm above the soil level of the stems. Saplings were maintained in the greenhouse at 22 ± 5 °C. All the tests were carried out in the same conditions.

Isolation of *Cryphonectria parasitica* and antagonist microorganisms

During our previous study on vc types of *C. parasitica* in the Black Sea region (Akıllı et al. 2009), along with the virulent and hypovirulent isolates of *C. parasitica*, antagonistic *Trichoderma* spp. and *Penicillum* spp. were also frequently isolated and their single spore cultures were stored. Isolations were made as previously described (Akıllı et al. 2009).

In order to isolate antagonistic *Bacillus* spp., bark samples from both active and healing cankers and healthy looking branches of chestnut were collected. Approximately 20 g of these samples were boiled in 20 mL of water at 95 °C in a beaker for 5 min and two 25 mL suspensions were streaked on PDA from every sample (Sneath 1986). By using the methods described by Sneath (1986) and Wilhelm et al. (1998), *Bacillus* spp. isolates were obtained from the soils collected from chestnut forests of Düzce, Sinop, and Zonguldak provinces, which did not have any vegetation under the trees. Isolates of *Bacillus* spp. were also obtained from the samples having bacterial growth during the pathogenicity tests carried out on excised stem pieces. The origins of the *Bacillus* spp. and antagonists used in the experiments are given in Table 1.

Determination of virulence and conversion tests

Virulent and hypovirulent isolates of *C. parasitica* and their vc types were differentiated by their cultural aspects as reported previously (Akıllı et al. 2009). The virulence of the isolates was also tested on excised

Table 1. Antagonists used in the experiments and their origin.

Antagonists	The origin of the antagonists	No. of isolates	Tested isolates[1]
Penicillium spp.	Bartın, Düzce, Kastamonu, Sinop, and Zonguldak	15	P - 3, P - 4, P - 15, P - 26
Trichoderma spp.	Bartın, Düzce, Kastamonu, Sinop, and Giresun	8	T - 2, T - d, T - 25, T - 28, T - 35,
Bacillus spp.	Düzce and Sinop (from soils), Bartın, and Zonguldak (from excised branches)	5	B - d, B - s, B - b, B - z

[1]The isolates used in the experiments were selected from the ones having restriction on the growth of *C. parasitica* in culture or on excised stem tests.

branches as previously described (Fulbright 1984; Akıllı 2008). Two virulent (K - 19 and K - 44) and 3 vegetative compatible hypovirulent isolates (Z - 1, Ba - 6, K - 49) were selected for the in vivo tests based on the lesion lengths they formed on the excised chestnut branches. The virulent isolates formed the largest necrosis and hypovirulent ones the smallest. The hypovirulent isolates were also tested for their ability to convert the virulent isolates to hypovirulent by dual culture tests and the ones found effective were used in the experiments.

Hypovirulence tests

Effectiveness of hypovirulent isolates on canker development was studied on 3-year- old disease-free chestnut saplings. For this aim, barks of 6 mm in diameter were removed with a cork borer from the stems and culture disks of the virulent and hypovirulent compatible isolates (EU - 1) were inversely placed into the wells. After placing the culture disks, bark disks were placed over them and the inoculation points were covered by wet cotton and stretch film. Five saplings were used for each treatment and the tests were repeated twice.

In this work, 2 virulent isolates (K - 19 and K - 44) belonging EU - 1 vc type and 3 hypovirulent isolates (Z - 1, Ba - 6, K - 49) belonging to the same vc type were used. After inoculations, lesion lengths of each application were measured on days 28, 43, and 58, and percent inhibition values of the hypovirulent isolates were calculated by comparing with the virulent controls.

Tests with antagonist microorganisms

Effectiveness of *Trichoderma* spp. and *Penicillium* spp. was determined by the same method as described above for the hypovirulent isolate applications. For this test 5 *Trichoderma* spp. (isolates T - 2, T - d, T - 25, T - 28, and T - 35) and 4 *Penicillium* spp. (isolates P - 3, P - 4, P - 15, and P - 26) were used. On the other hand, 4 *Bacillus* isolates (B-d, B-s, B-b, B-z) were grown on PDA and suspensions of 10^{10} cell mL^{-1} were prepared from the young growths and 10 µL from this suspension was placed in each well on the saplings. In these tests, only the most aggressive virulent isolate was used since there were not sufficient saplings to use 2 virulent isolates. The virulent isolate was applied after 10 min. The other treatments were the same as described in the hypovirulence tests**.** The tests of the antagonists were carried out in 2 groups at different periods as the hypovirulent applications because of the insufficiency in the greenhouse. Effectiveness of the antagonists was determined by comparing the lesion lengths of the pathogen and antagonists - pathogen co-inoculations.

Statistical analyses

In all the experiments, the values of canker lengths of each treatment were subjected to analyses of variance and the differences were compared by Duncan's multiple range test (P = 0.05) using SPSS.

Results

Effects of hypovirulent isolates on canker development

Effectiveness of the hypovirulent isolates on canker development varied based on the pathogenic isolates (Table 2). For instance, the hypovirulent isolate K - 49 when applied together with the virulent isolate K - 19 produced the largest cankers (2.7 cm) in the first evaluation, but this was not significantly different from that of the virulent control (K - 19) (2.5 cm). K - 49, as a hypovirulent control, produced the smallest cankers in this period. The hypovirulent isolate Z - 1 produced small cankers (1.5 cm) when applied together with the pathogenic isolates K - 19 and K - 44. The other hypovirulent strain Ba - 6 provided a reasonable disease control on day 28 (about 30%) when applied together with K - 19 and it maintained this effectiveness in the final period. The hypovirulent isolate K - 49 produced the largest cankers in the final evaluation (7.8 cm) like the combination of it with K - 19 (6.7 cm) and K - 44 (5.6 cm).

Effects of the antagonists on canker development

Results of the effectiveness of the antagonists are summarized in Tables 3 and 4.

***Trichoderma* spp.**

Trichoderma isolate T - 25 gave the highest rate of inhibition after 28 days from inoculation but its effect decreased with time (41.8% in the first evaluation and 1.5% in the final) (Table 3). Inoculation of *Trichoderma* isolate T - 28 together with the virulent isolate K - 19 produced longer cankers than the virulent isolate K - 19. This also applies to some

Table 2. Percent inhibition values and lesion lengths formed by hypovirulent isolates, applied together with the pathogens and controls.

Treatments	Percent inhibition and lesion lengths (cm)[1] at 3 periods		
	day 28	day 43	day 58
K - 44 + Z - 1	42.3 a[2] (1.5 a)	37.5 a (2.0 a)	32.6 a (3.3 a)
K - 19 + Z - 1	40.0 a (1.5 a)	48.7 a (2.0 a)	59.4 a (2.8 a)
K - 44 + Ba - 6	26.9 abc (1.9 abc)	0.0 abc (3.2 abc)	18.3 abcd (4.3 abcd)
K - 19 + Ba - 6	32.0 ab (1.7 ab)	12.8 bc (3.4 bc)	42.0 abc (4.0 abc)
K - 44 + K - 49	19.2 abc (2.1 abc)	-21.8 abc (3.9 bc)	-14.3 bc (5.6 bcd)
K - 19 + K - 49	-08.0 d (2.7 d)	-12.82 bc (4.4 c)	2.8 bcd (6.7 d)
Z - 1[3]	2.0 abc	2.0 a	4.5 ab
Ba - 6[3]	2.4 bcd	4.2 c	5.8 bcd
K - 49[3]	1.4 a	2.8 ab	7.8 cd
K - 44[4]	2.6 cd	3.2 abc	4.9 abcd
K - 19[4]	2.5 cd	3.9 bc	6.9 cd

[1]The numbers are means of 5 replicates.
[2]Numbers having the same letters are not significant according to Duncan's multiple range test (P = 0.05).
[3] Hypovirulent isolates
[4] Virulent isolates

Table 3. Percent inhibition of some antagonists against chestnut canker development on 3-year-old saplings.

Treatments[2]	Percent inhibition and lesion lengths (cm)[1] at 3 periods		
	day 28	day 43	day 58
K - 19 + T - 25	41.8 a[3] (1.8 a)	13.2 a (3.0 a)	01.5 a (3.3 a)
K - 19 + T - 35	08.8 ab (2.9 ab)	-10.1 ab (3.8 ab)	02.7 a (3.3 a)
K - 19 + T - 28	18.5 ab (2.6 ab)	05.7 ab (3.3 ab)	-4.2 abc (3.5 abc)
K - 19 + P - 4	15.1 ab (2.7 ab)	-21.5 b (4.2 b)	-35.9 bcd (4.5 bcd)
K - 19 + P - 15	27.3 ab (2.3ab)	-00.6 ab (3.5 ab)	-58.1 d (5.3 d)
K - 19 + B - d	14.5ab (2.7 ab)	-12.9 ab (3.9 ab)	-38.3 cd (4.6 cd)
K - 19 + B - s	03.5 b (3.1 b)	04.0 ab (3.3 ab)	-34.1 abcd (4.5 abcd)
K - 19	(3.2 b)	(3.5ab)	(3.3 ab)

[1]Numbers in brackets show mean canker lengths in cm
[2]K - 19 is the virulent strain of *C. parasitica*; T - 25, T - 28 and T- 35 are *Trichoderma*; P - 4 and P - 15 are *Penicillium* and B - d and B - s are *Bacillus* isolates.
[3]Numbers having the same letters are not significant according to Duncan's multiple range test (P = 0.05).

other antagonists. In the second group experiment, *Trichoderma* isolate T - 2 gave an increasing rate of canker inhibition at every evaluation period and reached 68.2% in the final evaluation on day 58 (Table 4). This rate was significantly different from that of the other treatments.

Table 4. Percent inhibition of some antagonists against chestnut canker development on 3-year-old saplings.

Treatments[2]	Percent inhibition and lesion lengths (cm)[1] at 3 periods		
	day 28	day 43	day 58
K - 19 + T - 2	36.1 a[3] (1.6 a)	58.9 a (1.6 a)	68.2 a (1.8 a)
K - 19 + T - d	07.2 bc (2.3bc)	13.4 b (3.4 b)	30.5 b (3.9 b)
K - 19 + P -26	23.3 abc (1.9abc)	08.3 b (3.6 b)	02.2 c (5.7 c)
K - 19 + P - 3	15.3 abc (2.1abc)	06.8 b (3.7 b)	30.4 c (5.1 c)
K - 19 + B - z	25.7 ab (1.8 ab)	25.6 b (2.9 b)	40.9 b (3.4 b)
K - 19 + B - b	20.9 abc (2.0 abc)	11.6 b (3.5 b)	31.4 b (3.9 b)
K - 19	(2.5 c)	(3.9 b)	(5.7 c)

[1]Numbers in brackets show mean canker lengths in cm.
[2]K - 19 is the virulent strain of *C. parasitica*; T - 2 and T - d are *Trichoderma*; P - 26 and P - 3 are *Penicillium* and B - z and B - b are *Bacillus* isolates.
[3]Numbers having the same letters are not significant according to Duncan's multiple range test (P = 0.05).

***Bacillus* spp.**

No *Bacillus* isolates were detected from the intact chestnut branches or from the healing cankers. For this reason *Bacillus* isolates obtained from other sources (Table 1) were used in the control experiments.

Bacillus spp. did not provide any inhibition in the first group of tests (Table 3). *Bacillus* isolates used in the second group tests, which were obtained from the growths during the pathogenicity tests on excised branches, provided reasonable rates of control. One *Bacillus* isolate (B - z) gave up to 41% control in the second group tests and formed another significant group together with the isolates T - d and B - b (Table 4).

***Penicillium* spp.**

Penicillium spp. did not prevent canker development very much except for the isolate P - 3. This isolate provided some control, varying with time, and it was 30.4% in the control on day 58. The other *Penicillium* isolate (P - 26) was effective at the beginning of the experiment but the effect dropped to 2% in the final evaluation (Tables 3 and 4).

Comparison of the antagonists with hypovirulent isolates

Comparison of the necrotic areas obtained by co-inoculations of the pathogenic isolate K-19 plus hypovirulent isolates and antagonists was made only between the isolates found effective after the second group of experiments. On day 28, the hypovirulent isolate Z - 1 and *Trichoderma* isolate T - 2 produced the smallest cankers; in other words they were highly effective and they formed a statistically significant group while on days 43 and 58 only the *Trichoderma* isolate T - 2 retained its high effectiveness and the hypovirulent isolate Z - 1 together with some other isolates was rated as less effective than T-2 (Table 5).

Discussion

Very few studies have been done to control chestnut canker by antagonists. Wilhelm et al. (1998) found that inoculation of a *B. subtilis* strain to stems of chestnut saplings 2 weeks before the pathogen inoculation provided complete control against the disease. In another study carried out in the USA, it was stated that *B. megaterium* strains isolated from chestnut barks from the trees having no vegetation under them prevented in vitro mycelial growth of *C. parasitica* and when these strains were applied to trees the rates of recovery obtained from the 3 strains after 11 months were 44%, 24%, and 26%, respectively (Groome et al. 2001).

Groome et al. (2001) suggested that the bacterium could suppress the disease in nature since the

Table 5. Comparison of the lesion lenghts on chestnut saplings, caused by the pathogen (K - 19) plus hypovirulent isolates and antagonists.

Lesion lengths (cm)[1] at 3 periods					
day 28		day 43		day 58	
Treatments	Lesion length	Treatments	Lesion length	Treatments	Lesion length
K - 19 + Z - 1	1.52 a[2]	K - 19 + T - 2	1.61 a	K - 19 + T - 2	1.83 a
K - 19 + T - 2	1.59 a	K - 19 + Z - 1	1.70 ab	K - 19 + Z - 1	2.64 ab
K - 19 + Ba - 6	1.77 ab	K - 19 + B - b	2.39 abc	K - 19 + B - b	3.40 bc
K - 19 + B - z	1.85 abc	K - 19 + T - d	2.42 abc	K - 19 + Ba - 6	3.74 bc
K - 19 + P - 26	1.91 abc	K - 19 + B - z	2.56 c	K - 19 + B - z	3.95 c
K - 19 + B - b	1.97 abc	K - 19 + Ba - 6	2.65 c	K -19 + T - d	4.00 c
K - 19 + P - 3	2.11 abc	K - 19 + P - 3	3.02 cd	K - 19 + P - 3	4.01 c
K - 19 + T - d	2.31 bcd	K - 19	3.10 cd	K - 19 + K - 49	5.34 d
K - 19	2.49 cd	K - 19 + K - 49	3.56 d	K - 19 + P - 26	5.63 d
K - 19 + K - 49	2.78 d	K - 19 + P - 26	3.58 d	K - 19	5.76 d

The numbers are means of 5 replicates
[2]Numbers having the same letters are not significant according to Duncan's multiple range test (P = 0.05)

bacterium prevented in vitro mycelial growth of the pathogen and could colonize the chestnut barks. In the present study, one of our *Bacillus* isolates (B - z) provided 41% disease control; therefore, this value should not be underestimated. Some *Bacillus* strains obtained from the branches and soils also restricted canker development, while others produced the opposite effect.

There is not much information on the effects of *Bacillus* sp. on living tissues as tested here. In a study conducted by Wilhelm et al. (1998), it was stated that 12 *Bacillus* isolates obtained from xylem sap of chestnut prevented canker development at varying degrees and the most effective isolate was a strain, L - 25, of *B. subtilis*. *Bacillus* treatment 3 days before the pathogen inoculation resulted in 71% control but did not cause any preventive effect when applied simultaneously. In the present study, isolate B - z when applied at the same time as the pathogen could have been more effective if applied before the inoculation but this situation cannot be mimicked in nature.

Occurrence of *Trichoderma* species together with *C. parasitica* was also observed by Robbins (1997) but their effectiveness against chestnut canker has not been tested. There is no information about the effectiveness of *Trichoderma* spp. on the prevention of canker development caused by *C. parasitica* and effectiveness of the isolate T - 2 (68%) should not be underestimated since this isolate prevented canker development very effectively. This isolate could be applied in the same way as the hypovirulent isolates in nature. In our experiments, *Trichoderma* sp. was found more promising than *Penicillium* spp. In addition, a *Trichoderma* sp. strain, T - 2, was as effective as the most effective hypovirulent isolate.

Use of hypovirulent isolates in biological control of chestnut blight in Turkey has not been investigated extensively. Çeliker (2000) inoculated a hypovirulent strain above and below the growing cankers after 1 week from the inoculation of the virulent strain and measured the canker area at various periods and 2 months later found that the canker sizes on the

saplings treated by hypovirulent + virulent strains showed limited growth and the area became smaller with callus formation while the sizes of the cankers of the virulent strain increased gradually. Later Çeliker and Onoğur (2001, 2009) tested various hypovirulent isolates in field conditions and found those isolates effective against chestnut canker.

However, we found that hypovirulent isolates might produce diverse effects on the virulent ones and before giving a decision on an isolate it should be tested against various pathogenic isolates. Our hypovirulent isolate Z - 1 provided a stable disease control against chestnut blight. This implies that Z - 1 could be an effective biocontrol agent. The other hypovirulent isolate, K - 49, however, did not produce a stable positive effect and did not appear to be promising for biocontrol. These findings show that before starting a biocontrol application with hypovirulent isolates, their performance has to be tested against various pathogenic isolates in the field.

For the biological control of chestnut canker with hypovirulent isolates, vegetative compatibility of the isolates has to be known. Only by this way can the transfer of hypoviruses to the virulent isolates occur and so disease suppression. For this reason, the use of hypovirulent isolates is not applicable in places where more than one vc type is present. Biological control with antagonistic organisms, however, is rather easy in this respect since they were found as effective as the hypovirulent isolates, but they have to be tested extensively in the field.

Conclusions

Biological control by hypovirulent isolates is the most common approach to control chestnut blight caused by *C. parasitica*. In this approach, every region has to use its own isolates and, for this application, hypovirulent isolates that are compatible with the virulent ones have to be found. In the present study, hypovirulent isolates obtained from the Black Sea region were effective against this disease. In addition, among the antagonists tested, 1 *Trichoderma* and 1 *Bacillus* isolate were also effective. Vegetative compatibility restricts the use of hypovirulent isolates in biological control but there is not such a problem in the use of antagonistic organisms.

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