

Natural dissemination of hypovirulent *Cryphonectria parasitica* strain used for biological control of chestnut blight

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Abstract: Chestnut blight, caused by *Cryphonectria parasitica* Murr. Barr, is the most serious disease of chestnut trees in Turkey on which several studies related to biological control were conducted. The aim of this study was to determine the cause of the occurrence of healing cankers on untreated chestnut trees and the role of carriers in the spread of hypovirulence by laboratory tests, and to discover the effect of some environmental factors (temperature and relative humidity) on the natural spreading of hypovirulence. The study was carried out in a chestnut grove where biological control studies of the disease had been performed in previous years. Bark samples were collected from healed cankers and 388 possibly hypovirulent isolates were obtained. Two of them that were white converted virulent isolates to hypovirulent ones, but others developed yellowish cream-colored mycelia and did not convert virulent isolates. dsRNA analysis was performed on randomly selected possible hypovirulent isolates collected from healed cankers. After the virus concentration had increased, dsRNA-positive reactions were obtained for 56.73% of the isolates. Thirty-one possible hypovirulent isolates from Formicidae and Gastropoda did not convert virulent isolates to hypovirulent ones. dsRNA analysis was performed on these isolates and 61.29% of them containing dsRNA were observed in the invertebrates collected from cankers that had healed from hypovirulence. It was also observed that there was no significant difference among the date of average temperatures and date of relative humidity from 3 different altitudes. It was determined that the reason for the occurrence of the healed cankers on untreated trees was the natural dissemination of hypovirulence, ants and snails might have played a role in the natural dissemination of hypovirulence, and healed cankers mostly occurred on the western sides of shaded slopes.

Key words: Hypovirulence, natural dissemination, relative humidity, temperature, carriers

1. Introduction

European chestnut (*Castanea sativa* Miller) is a native species of Turkey that grows in the coastal Black Sea, Marmara, and Aegean regions. Chestnut blight (*Cryphonectria parasitica* (Murr.) Barr.) is the most important disease of this valuable tree. It was first reported in Turkey in the late 1960s (Akdoğan and Erkan, 1968) in the Marmara region and it has spread to other growing areas in a short time, causing great damage to chestnut stands (Delen, 1979; Çeliker and Onoğur, 2001; Gürer et al., 2001b). Various sources in the literature indicate that chemical control of the pathogen is not feasible and cultural methods alone are not adequate to stop the disease in a given area. The most promising, long-term way to combat the pathogen seems to be biological control, which depends on the transfer of hypovirulence into virulent populations of *C. parasitica* (Heiniger and Rigling, 1994). For this reason, some in vitro (Çeliker and Onoğur, 1998,

2001; Coşkun et al., 1999; Açıkgöz et al., 2009; Akıllı et al., 2009; Döken et al., 2009; Gürer et al., 2001a) and in vivo (Çeliker and Onoğur, 2001, 2009, 2011; Aksoy et al., 2004; Akıllı et al., 2011) studies on the biological control of chestnut blight were conducted by different researchers in Turkey.

For this purpose, we conducted a study to evaluate the possibility of controlling chestnut blight with hypovirulent strains in Turkey between 1996 and 2000. During the study, a survey was performed in the chestnut-growing areas of the Aegean and Marmara regions. A total of 14 hypovirulent isolates were obtained from only the Marmara region (Çeliker and Onoğur, 1998). Inoculation tests with these hypovirulent isolates resulted in healing cankers. Thus, all hypovirulent isolates were approved to be suitable for biocontrol because they all prevented mortality and induced callus growth (Çeliker and Onoğur, 2001). As a second step, a new study was conducted in Manisa Province

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of the Aegean region between 2003 and 2005 on biological control of chestnut blight by using one of the hypovirulent isolates in a chestnut grove where hypovirulent strains were not found (Çeliker and Onoğur, 2009, 2011). After the determination of the canker dimensions and types on young chestnut trees, the introduced hypovirulent strain was inoculated into the holes around these cankers. As a result of this study, most of the treated cankers were healed and white isolates having the ability to convert the virulent ones were reisolated from the healed cankers in 2004 and 2005. However, during the study, natural dissemination of hypovirulence was not detected in the area. Thus, surveys were conducted in the study area between 2007 and 2009 and revealed new 'healed cankers' on untreated trees (Çeliker and Onoğur, 2009, 2011). Therefore, a new study was carried out in the same area by us between 2010 and 2014. The objectives of this study were to determine the reason why healed cankers occurred and to research the effects of carriers and some environmental factors on natural dissemination of hypovirulence.

2. Materials and methods

2.1. Study site

The study was carried out in a chestnut grove located in an approximately bowl-shaped area of 0.2 ha in Turgutlu (Hacısalar), Manisa Province (38°21'55"N, 27°49'19"E), in the Aegean region. The flat part of the area was at the height of 678 m above sea level, one of the slopes (inclination 60%) was exposed to the west at a height of 692 m, and the other slope (inclination 45%) was exposed to the east at a height of 700 m. The plot area included 96 chestnut trees (13–20 years old) and some young oak trees.

2.2. Collection of bark and invertebrate samples from healed cankers

To determine the presence of hypovirulent isolates in the healed cankers, bark samples were removed with a cork borer (diameter of 4 mm) from the margins of each healed canker (Bissegger et al., 1997), which had occurred in the same years between 2010 and 2012. According to the dimensions of each healed canker area, 10 to 20 bark samples were collected.

Invertebrate samples from different groups and families were collected from the area every week during the vegetation period by hand or with a weasel brush from each healed canker in 2011 and 2012.

2.3. Isolation and characterization of *C. parasitica*

All bark samples were dipped in 1% calcium hypochlorite, immediately flamed, and placed on 39 g/L of potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA).

Small insects and large insects, which were cut into small pieces, were placed on a medium containing a mixture of 0.5% tannic acid (Sigma-Aldrich, St. Louis,

MO, USA), 1.5% Difco malt extract, and 2% Difco Bacto agar (Rigling et al., 1989; Daniel Rigling, personal communication). To assess culture characteristics of the isolates, all *C. parasitica* isolates were grown on PDA plates at 25 °C for 12 h in the dark and 12 h in the light for 7 days. The isolates that formed white or yellowish-cream mycelia with sparse pycnidia on PDA medium were considered as possible hypovirulent isolates.

2.4. Hypovirulence conversion tests

Pairings were made by inoculating possible hypovirulent and virulent isolates 5 mm apart at the edge of a PDA plate (diameter of 9 cm). Cultures were incubated at 25 °C in the dark for 14 days. Conversion was considered successful if the orange strain developed white culture characteristics. Conversion tests were performed twice for each pair of isolates (Anagnostakis, 1988; Bissegger et al., 1997).

2.5. Hypovirus dsRNA isolation

For hypovirus isolation, virus-infected (reference culture) and possible hypovirulent *C. parasitica* cultures were grown on PDA covered with a sterile cellophane sheet (Celloclaire Inc., Liestal, Switzerland). After peeling off the entire *C. parasitica* culture from the cellophane, the mycelium was immersed in liquid nitrogen and ground to a fine powder using a mortar and pestle, and then approximately 300 mg of the mycelium powder for possible hypovirulent isolates and 40 mg for reference isolates was used for isolations. The dsRNA was extracted using CF-11 chromatography as described by Allemann et al. (1999). Two known dsRNA-containing isolates of *C. parasitica* (M 5582 and Sinop 50) were obtained from Daniel Rigling (Swiss Federal Institute for Forest, Snow, and Landscape Research WSL, Birmensdorf, Switzerland) and Seçil Akıllı (Çankırı Karatekin University, Department of Biology, Çankırı, Turkey) and a virulent isolate was included in each preparation. Analysis of the dsRNA preparations was done by 0.8% agarose gel electrophoresis.

2.6. Mapping of natural dissemination of hypovirulence

The map of the study area was updated in 2010. All surviving trees were numbered again, healed and active cankers on trees were marked on the map, and it was updated annually between 2010 and 2013. Cankers that obtained hypovirus-infected isolates were considered as a healing reason of the natural spread of hypovirulence. These trees were also marked on the map. Healed cankers that occurred in 2013 were only examined visually.

2.7. Effects of carriers and environmental factors (temperature and humidity) on natural dissemination of the hypovirus

To determine the presence of invertebrates carrying a hypovirus, or in other words the role of these carriers on the natural dissemination of hypovirulence, insects on the healed cankers were collected and then laboratory

tests including isolation (see Section 2.3) (Anagnostakis, 1988; Rigling et al., 1989; Allemann et al., 1999; Daniel Rigling, personal communication), characterization of *C. parasitica* isolates, conversion tests, and hypovirus dsRNA isolation (see Sections 2.4 and 2.5) were performed as described by Bissegger et al. (1997).

To determine the effect of environmental factors on the natural dissemination of the hypovirus, HOBO Onset Data Logger recorders were hung from the trees at a height of approximately 1.5 m above ground level at 3 different altitudes (flat middle part at a height of 685 m, eastern slopes part at 687 m, and western slopes at 689 m) of the area. The data of monthly average temperature and relative humidity were collected from these devices during the vegetation period for 3 years.

3. Results and discussion

3.1. Reason for the healing of the cankers

During the study, 104 healed cankers were determined on 47 chestnut trees. A total of 921 *C. parasitica* isolates were obtained from 1806 bark samples (Table 1), but 388 of them were different from the virulent ones with their orange colony morphology. Two of these 388 isolates developed a white mycelium and the other 386

isolates developed a yellowish-cream mycelium with sparse pycnidia. Thus, they were approved as possible hypovirulent isolates. White isolates could convert virulent isolates to hypovirulent ones by transferring the hypovirus during the conversion tests (Table 1). These white isolates were obtained from cankers inoculated with hypovirulent isolate during our previous study in the same area in 2003 (Çeliker and Onoğur, 2009). It was thought that hypovirulence was still present in the same area after 11 years because white isolates containing dsRNA were obtained from the treated cankers. While the other 386 possible hypovirulent isolates could not convert virulent isolates to hypovirulent ones, possible hypovirulent isolates were also collected from healed cankers and hypovirulent isolates were still present in the area. Thus, dsRNA assays were performed for 104 randomly selected isolates by using 2 reference hypovirulent isolates. First, the isolates produced dsRNA-negative reactions, except for 2 white isolates. After the mycelial amount was increased for dsRNA extraction, positive reaction was obtained for 59 of the possible hypovirulent isolates; in other words, 56.73% of them contained dsRNA (Table 2; Figure 1). However, the remaining 44 possible hypovirulent isolates (43.27%), although the mycelial amount was increased for dsRNA extraction, produced negative reactions. During the study

Table 1. Number of healed cankers, collected bark samples, *Cryphonectria parasitica* isolates, possible hypovirulent isolates, and converted isolates in research area.

Year	Number of trees with healed cankers	Number of healed cankers	Number of bark samples	Number of <i>C. parasitica</i> isolates	Number of possible hypovirulent isolates	Number of converted isolates
2010	32*	50*	1080	458	274	2**
2011	9	19	426	366	60	0
2012	4	21	300	97	54	0
2013	2	14	-	-	-	-
Total	47	104	1806	921	388	2

*Three of them were inoculated in 2003. **White isolate. 0: No conversion; -: bark samples not collected.

Table 2. Number of *Cryphonectria parasitica* isolates contained dsRNA among some possible hypovirulent isolates from healed cankers and potential vectors.

Possible hypovirulent isolates from bark samples			Possible hypovirulent isolates from potential vectors			
Years	Number of tested isolates	Number of isolates containing dsRNA	Year	Insects	Number of isolates	Number of dsRNA-carrying isolates
2010	38	36	2011	Formicidae	13	9
2011	42	16	2011	Gastropoda	4	4
2012	24	7	2012	Formicidae	14	6
Total	104	59	Total		31	19

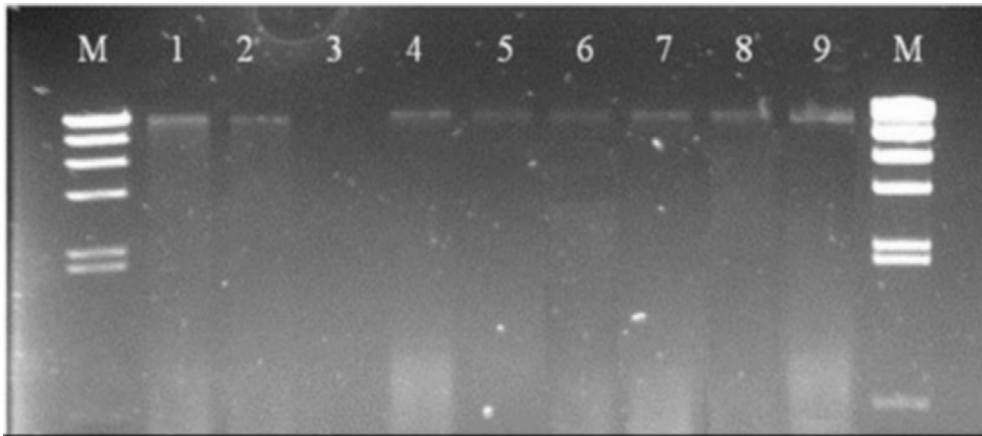


Figure 1. Double-stranded RNA profiles extracted from hypovirulent isolates of the chestnut blight fungus, *Cryphonectria parasitica*. M, DNA marker (Lambda DNA digested with HindIII); Lane 1 (M5582 from Switzerland) and Lane 2 (Sinop 50 from Turkey), reference hypovirulent isolates; Lane 3, virulent isolate; Lanes 4–9, possible hypovirulent isolates from bark.

we used the dsRNA extraction method to isolate dsRNA, but Akilli et al. (2013) tested both the virus-specific RT-PCR on total RNA extraction method and dsRNA extraction method to isolate dsRNA in their study. According to their test results, the virus-specific RT-PCR method required a small amount of mycelia and was a more sensitive and convenient test than the dsRNA extraction method to screen hypovirus-infected *C. parasitica* isolates.

When we evaluated our field observations and laboratory test results together, we thought that the other 327 possible isolates might also contain dsRNA and perhaps the virus concentrations of the isolates were below the detection limits.

Finally, it was thought that the reason for healed canker occurrence on untreated trees was the natural spread of hypovirulence. Bissegger et al. (1997) also indicated that the increase of nongrowing cankers could be the result of hypovirulence because orange strains in active cankers were converted or because new cankers were initiated by white, hypovirulent strains in chestnut groves.

3.2. Mapping of natural dissemination of hypovirus

The map was established according to the presence of the healed cankers and/or isolates that contained dsRNA from the cankers in the area. When the distribution of healed cankers on untreated trees was evaluated, it was determined that they generally occurred around the 3 trees inoculated with hypovirulent isolates in 2003 (Figure 2), but 11 trees with healed cankers were dead and 24 trees showed dieback symptoms because of the secondary infection of *C. parasitica* in the area between 2010 and 2014.

3.3. Effects of carriers and some environmental factors (temperature and humidity) on the natural dissemination of hypovirulence

To investigate the effects of carriers on the natural dissemination of hypovirulence, 1193 invertebrate samples belonging to different groups and families were collected from healed cankers in 2011 and 2012. Laboratory test results showed that 67 of the invertebrate samples were carrying *C. parasitica* isolates (Table 3). Thirty-one of them formed a yellowish-cream mycelium with sparse pycnidia on PDA medium. Therefore, the isolates were approved as possible hypovirulent isolates, but they could not convert virulent isolates to hypovirulent ones. However, the carriers were collected from cankers healed due to hypovirulence. Only one isolate belonging to the family Formicidae formed a whitish-cream mycelium. Although the carrier had been collected from a canker inoculated in 2003 (Çeliker and Onoğur, 2009), the isolate also could not convert virulent isolates to hypovirulent ones (Table 3). Despite these negative results, dsRNA assays were performed for 31 possible hypovirulent isolates, but they produced dsRNA-negative reactions, as well. After the virus concentration was increased, dsRNA-positive reactions were obtained for 19 possible hypovirulent isolates (Figure 3). Among these isolates, 15 of them belonged to Formicidae and 4 to Gastropoda (Table 2). This result showed that ants and snails might play a role in the natural dissemination of hypovirulence as potential vectors. Turchetti and Chelazzi (1984) also indicated that snails (*Lehmannia marginata* Müller) could be considered as a short-range vector for the natural spreading of hypovirulence. Among the ant

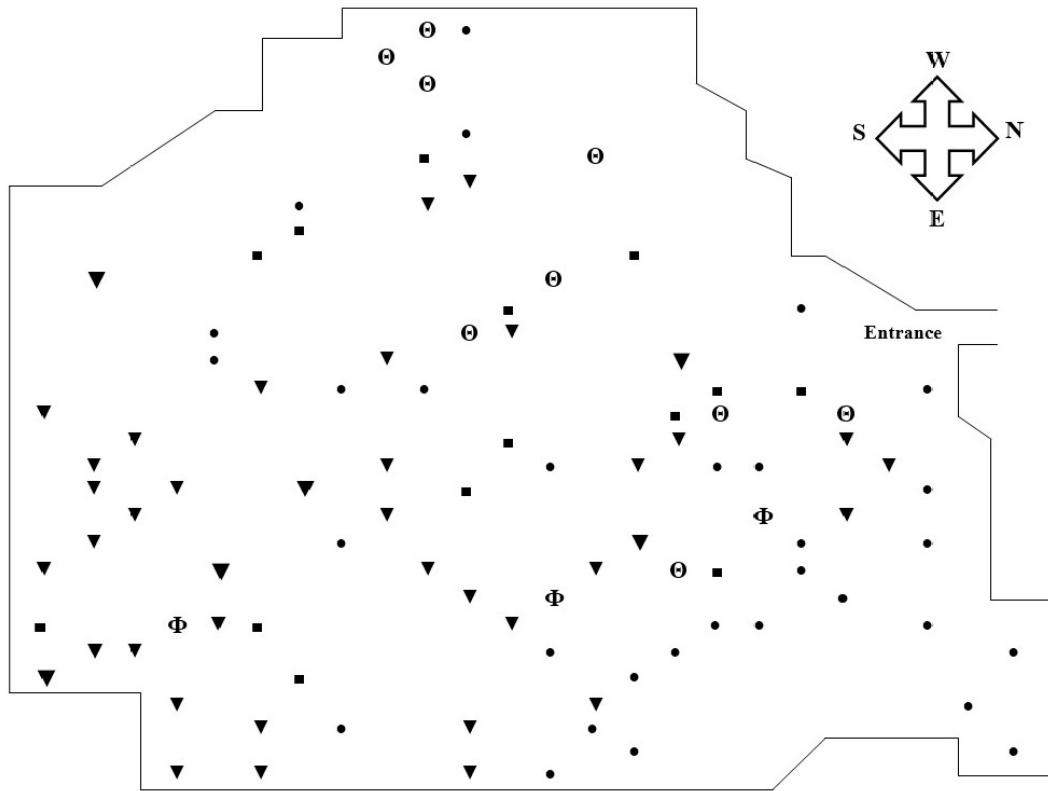


Figure 2. Distribution of trees with healed cankers on the map of natural dissemination of hypovirulence. Φ: Healing canker on treated (with hypovirulent isolate) tree in 2003, healthy tree; Θ: healing canker on treated (with hypovirulent isolate) tree in 2003, but the tree died over time; ▼: healing canker occurred on nontreated tree between 2010 and 2013, healthy tree; ●: nontreated healthy tree; ■: healing canker on nontreated trees, but the tree died over time between 2007 and 2009.

Table 3. Determined invertebrate species on the healed cankers, number of *Cryphonectria parasitica* isolates from possible carriers, and the isolates that converted virulent ones.

Number of trees with collected insects	Invertebrate species	Number of invertebrates	Number of insects with isolated <i>C. parasitica</i>	Number of isolates that converted virulent ones
43	<i>Crematogaster scutellaris</i> Olivier, <i>Prenolepis nitens</i> (Mayr)	1015	58	1*
	Gastropoda	5	2	
	<i>Forficula auricularia</i> L.	17	1	
	<i>Lachnus roboris</i> L.	86	0	
	<i>Lyristes plebejus</i> Scop. (molt)	51	1	
	<i>Dolycoris baccarum</i> L.	2	0	
	<i>Nezera viridula</i> L.	1	0	
	<i>Potosia</i> sp.	3	1	
	<i>Rhynocoris</i> sp.	2	0	
	<i>Poecilimon</i> sp.	2	0	
	<i>Anomola</i> sp. (larvae)	1	0	
	<i>Chrysopa</i> sp.	4	1	
	Araneidae	4	3	
	Total	1193	67	

*White *C. parasitica* isolates from ant could not convert virulent ones; 0: *C. parasitica* not isolated.

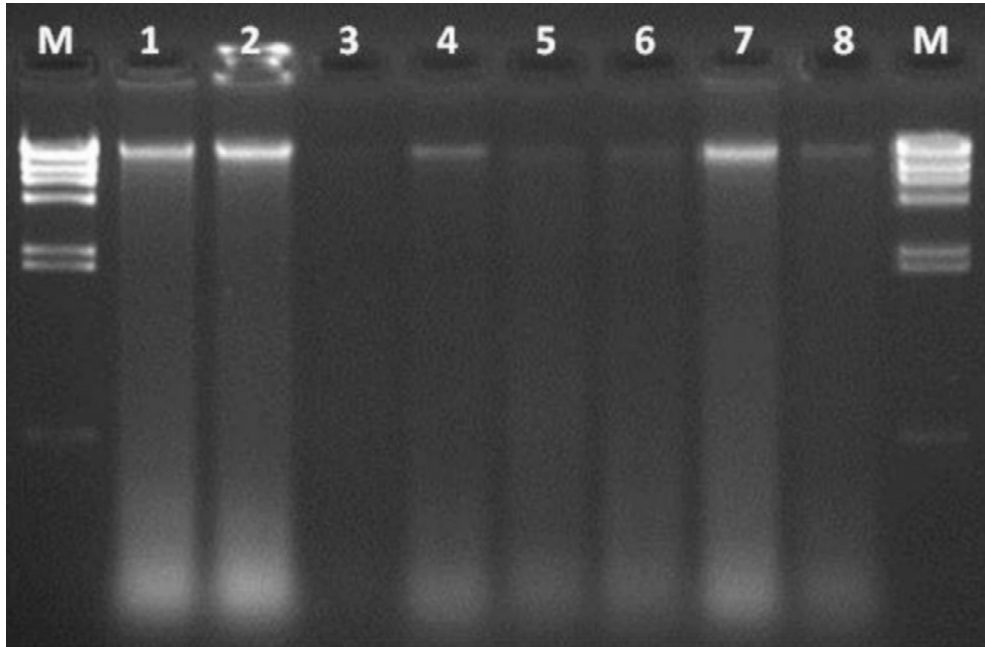


Figure 3. Double-stranded RNA profiles extracted from hypovirulent isolates of the chestnut blight fungus, *Cryphonectria parasitica*. M, DNA marker (Lambda DNA digested with HindIII); Lane 1 (M5582 from Switzerland) and Lane 2 (Sinop 50 from Turkey), reference hypovirulent isolates; Lane 3, virulent isolate; Lanes 4–8, possible hypovirulent isolates from potential vectors.

species, *Crematogaster scutellaris* (Olivier) and *Prenolepis nitens* (Mayr) (Hymenoptera: Formicidae) were the most common species (Table 3). Some researchers reported that carriers like insects and mites, which move between cankers, might be involved in the transport of dsRNA-containing conidia (Russin et al., 1984), and hypovirulent strains also could be spread in tiny chips of infected bark disseminated by wind, insects, birds, or mammals (Heiniger and Rigling, 1994).

To determine the effect of temperature and relative humidity on natural dissemination of hypovirulence, 2 different datasets were collected from HOBO Onset Data Logger recorders at 3 different altitudes and different directions of the area. When temperature and humidity data were evaluated separately, there were no significant differences among average temperature data or relative humidity data. These results showed that there were no effects of temperature and relative humidity on the natural dissemination of hypovirulence.

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We determined that healed cankers occurred most commonly on the slopes exposed to the west that were most shaded, but some healed cankers also occurred on the east slopes, which were exposed to the sun's rays for a long period of the day. A prior study indicated that the effect of direct sun rays on the growth of active cankers was the most important factor (Anagnostakis and Donald, 1984).

In conclusion, the study results showed that the reason for the occurrence of the healed cankers on nontreated trees was the natural spread of hypovirulence. Ants and snails might play a role in the natural dissemination of hypovirulence as potential carriers.

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