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Pathogenicity of *Bursaphelenchus mucronatus* in Pine Seedlings under Greenhouse Conditions

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Abstract: *Bursaphelenchus mucronatus*, a species closely related to the pinewood nematode (*Bursaphelenchus xylophilus*, a quarantine pest), was isolated from several wilted pine trees in different provinces of Turkey. To understand the role of *B. mucronatus* in these wilting cases, the pathogenicity of *B. mucronatus* on 3-year-old seedlings of the 3 pine species most widespread in Turkey was investigated under greenhouse conditions. In all, 30 seedlings of each tree species were used for nematode inoculation. For inoculation, 1000-1100 nematodes in 0.5 ml of distilled water were used per seedling. An additional 20 seedlings of each tree species were inoculated with an equal amount of distilled water and served as controls. The first complete wilting case was observed in the fifth week of the experiment. The highest rate of mortality occurred in *Pinus sylvestris* seedlings (83%), followed by *P. nigra* (47%). Only 2 seedlings of *P. brutia* wilted (7%). These results suggested that *B. mucronatus* was highly virulent to *P. sylvestris* seedlings.

Key Words: *Pinus sylvestris*, *Pinus nigra, Pinus brutia*, *Bursaphelenchus* sp., pathogenicity, Turkey

Bursaphelenchus mucronatus'un Sera Koşulları Altında Çam Fidanlarındaki Patojenliği

Özet: Çam odun nematoduyla (*Bursaphelenchus xylophilus*, karantina listesindeki bir zararlı) benzerlikleri çok olan *Bursaphelenchus mucronatus* türü Türkiye'nin farklı illerinde kurumuş olan bazı çam ağaçlarından elde edilmiştir. *B. mucronatus*'un bu kurumalardaki rolünün belirlenmesi için en çok yayılışa sahip üç çam türünün fidanları kullanılarak sera koşullarında patojenlik testi yapılmıştır. Her ağaç türünden 30 adet fidan kullanılmıştır. Aşılamalarda her fidan için 0.5 ml distile edilmiş su içersinde 1000-1100 adet nematod kullanılmıştır. Her ağaç türünden 20 adet fidana kontrol amaçlı olarak 0.5 ml distile edilmiş su inoküle edilmiştir. İlk tam kuruma, denemenin 5. haftasında gerçekleşmiştir. En yüksek ölüm yüzdesi (% 83) *Pinus sylvestris* fidanlarında ve takiben *P. nigra* fidanlarında (% 47) görülmüştür. *P. brutia* fidanlarından sadece iki tanesi ölmüştür (% 7). Bu sonuçlar, *B. mucronatus*'un *P. sylvestris* fidanlarında yüksek oranda öldürücü olduğunu göstermektedir.

Anahtar Sözcükler: *Pinus sylvestris*, *Pinus nigra, Pinus brutia*, *Bursaphelenchus* türü, patojenlik, Türkiye

Introduction

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934), Nickle, 1970, causes serious damage to pine forests in Japan and China (Mamiya, 1988). It has spread throughout Taiwan and Korea as well (Mamiya, 1998). Recently, the nematode was isolated for the first time in Europe from the wood of *Pinus pinaster* Ait. in Portugal (Mota et al., 1999).

The pathogenicity of *B. xylophilus* has been investigated and well documented, in both field and laboratory conditions in different countries. It has caused high mortality in pine species (Mamiya, 1983; Bedker et al., 1987; Linit and Tamura, 1987; Bedker and Blanchette, 1988; Braasch, 1997).

Bursaphelenchus mucronatus Mamiya and Enda, 1979 was found during surveys of the pinewood nematode in different European countries. It is widely distributed in Europe (Evans et al., 1996). The occurrence of *B. mucronatus* has never been related to epidemic diseases in its distributed areas (Mamiya,

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1998). Because of morphological and biological similarities between *B. mucronatus* and *B. xylophilus*, scientists are interested in studying the pathogenicity of *B. mucronatus* in East Asia, Europe, and North America (Mamiya, 1998). *B. mucronatus* is considered nonpathogenic, or to have very low virulence, in comparison to *B. xylophilus* (Mamiya and Enda, 1979; Cheng et al., 1986; Wang and Shi, 1986; Bakke et al., 1991; Tomminen, 1993; Braasch, 1996; Braasch et al., 1998). Additionally, several reports indicate the potential pathogenicity of *B. mucronatus* (Kulinich et al., 1994; Kishi, 1995). There are some differences among the pathogenicity results of these studies because of differences in tree species, age of tested host plants, climatic conditions, and inoculation techniques.

In Turkey, during a survey of *B. xylophilus* in 2003 and 2004, wilting of different pine species due to unknown causes was observed. *B. mucronatus* was isolated from several of these wilted pine trees in different regions, such as Düzce and Artvin (Akbulut et al., 2006). It is crucial to show the role of *B. mucronatus* in pine wilting cases. For this reason, the current study was conducted to determine the pathogenicity of *B. mucronatus* on the 3 native pine species most widely distributed in Turkey.

Materials and Methods

The inoculation tests were carried out during the summer months of 2005. The 3 most widely distributed native pine species in Turkey, *Pinus brutia* Tenn., *P. nigra* Arnold, and *P. sylvestris* L., were selected for inoculation tests. In the experiment, 3-year-old pine seedlings were used. *P. brutia* were provided from Balıkesir, and *P. nigra* and *P. sylvestris* seedlings from Bolu Forest Nurseries. During the experiment 30 seedlings of each species were inoculated with *B. mucronatus* and another 20 seedlings of each species served as controls. The *B. mucronatus* population originated from a wilted *P. nigra* tree in Kurugöl, Düzce.

The pathogenicity experiment was conducted in a greenhouse located at the Western Black Sea Forest Research Institute in Bolu. Before the inoculation test, cultures of *B. mucronatus* were multiplied on *Botrytis cinerea* Pers. cultures on malt agar at 25 °C for 7-10 days. When the nematodes appeared to reach sufficient numbers, they were extracted using the Baermann funnel technique (Southey, 1986). The inoculum used for each seedling contained approximately 1000-1100 nematodes (all life stages) in 0.5 ml of distilled water. The number of nematodes in the initial suspension was determined by taking 5 samples of 0.1 ml. Nematodes in each sample were counted and the mean calculated. The desired concentration was then adjusted by either adding or removing water.

The inoculation point on the seedlings was located just above of the lowest shoots (5-10 cm above of soil surface). The needles around the site were removed and a slit-like half T was made using a scalpel. The bark was gently pulled and a small piece of sterile cotton was inserted into the slit. Then nematode suspension (0.5 ml) was dropped slowly by a syringe. After inoculation, the inoculation point was wrapped with a Parafilm strip. The seedlings used as controls were prepared in the same manner and the inoculation procedure was exactly the same as described above, except that instead of nematode suspension only distilled water (0.5 ml) was used.

The inoculated plants and controls were kept under greenhouse conditions (26-28 °C, 70%-80% RH). All seedlings were irrigated twice a week. The inoculated plants were observed on a daily basis and were cut when wilted or 3 months after inoculation. Wilted seedlings were cut off 1 cm above the soil level, and the needles and short shoots removed. The main stems of the seedlings were weighed, divided into small pieces, and put into a Baermann funnel for extraction of nematodes for 24 h. The collected nematodes were counted under a microscope. The number of nematodes for wilted and healthy seedlings was determined. Control seedlings were not cut for nematode counting unless wilting was observed.

A t-test was used to compare the pine seedling mortality rates of both groups.

Results

In this study, 90 seedlings were inoculated with *B. mucronatus*. The first wilting symptoms were observed 10 days after inoculation, both for *P. nigra* and *P. sylvestris*. The cumulative mortality rates during the 13-week test period are given in Figure 1. The first complete wilting case was observed during the fifth week of the experiment. There was a big increase in the number of wilted *P. sylvestris* seedlings in the seventh week (Figure 1). Cumulatively, 20 of 30 *P. sylvestris*

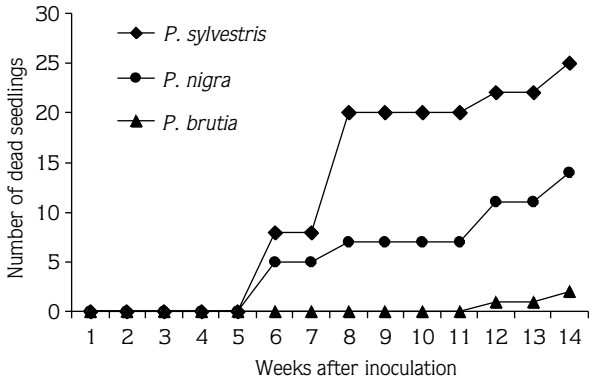

Figure 1. Cumulative mortality rate of 3-year-old seedlings of *Pinus sylvestris*, *P. nigra*, and *P. brutia* after inoculation with *Bursaphelenchus mucronatus* under greenhouse conditions.

seedlings wilted in the eighth week of the experiment. A sudden increase in the number of wilted seedlings was not observed in *P. nigra* (Figure 1). Wilting of seedlings was almost equally observed in *P. nigra* during the experiment. Only 2 seedlings of *P. brutia* wilted near to the end of the experiment (Figure 1) and the remaining 28 seedlings showed no signs of wilting. The highest rate of mortality occurred in *P. sylvestris* seedlings (25 seedlings, 83%), followed by *P. nigra* (14 seedlings, 47%) and *P. brutia* (2 seedlings, 7%). According to pairwise comparisons (t-test), mortality rates differed significantly between the following pairs: *P. sylvestris* and *P. nigra* ($t = 2.98$; $df = 58$; $P = 0.05$), *P. sylvestris* and *P. brutia* ($t = 5.96$; $df = 58$; $P = 0.05$), and *P. nigra* and *P. brutia* ($t = 3.50$; $df = 58$; $P = 0.05$).

The average number of nematodes re-isolated was the highest in *P. sylvestris* seedlings, followed by *P. nigra* (Table 1). The lowest average number of nematodes was recovered from *P. brutia* seedlings (150 nematodes per seedling). The highest number of nematodes recovered from a single seedling was 11,980 in a *P. sylvestris* seedling. The average number of nematodes re-isolated was higher in dead (wilted) seedlings than in healthy seedlings for all 3 species (Table 1). The average number of nematodes re-isolated from the wilted seedlings of all 3 pine species was almost identical, but a similar trend was not observed among the healthy seedlings (Table 1). The highest number of nematodes recovered from a healthy seedling was 804 from a *P. sylvestris* seedling. Nematodes were not recovered from 2 seedlings of *P. sylvestris*, 4 seedlings of *P. nigra*, and 19 seedlings of *P. brutia*. Control seedlings showed no mortality, indicating that inoculation techniques did not have a negative effect on seedlings. Therefore, control seedlings were not cut for nematode counting.

Discussion

Results of this study demonstrated that *Bursaphelenchus mucronatus* was highly and significantly pathogenic to *P. sylvestris* seedlings, causing a mortality rate that exceeded 83%, and causing a 47% mortality rate among *P. nigra* seedlings. There was a significant difference between the mortality rates of *P. nigra* and *P. brutia* seedlings. *B. mucronatus* seems to be nonpathogenic to *P. brutia* seedlings. Only 2 seedlings were wilted and the other seedlings had no nematodes or

Table 1. Pathogenicity test results of *Bursaphelenchus mucronatus* on 3-year-old seedlings of *P. sylvestris* (*P. s.*), *P. nigra* (*P. n.*), and *P. brutia* (*P. b.*) under greenhouse conditions.

Tree species	Seedling type	N	Mortality (%)	Average no. of re-isolated nematodes per seedling	Average no. of re-isolated nematodes per seedling	
					Wilted	Healthy
P. s.	Inoculated	30	83.33	1937.09	2268.69	279.10
	Control	20	0.00			
P. n.	Inoculated	30	46.67	1067.61	2203.13	74.03
	Control	20	0.00			
P. b.	Inoculated	30	6.67	150.11	2175.15	5.47
	Control	20	0.00			

a very low number of nematodes. This result suggests that the population of *B. mucronatus* did not develop well in *P. brutia* seedlings. On the other hand, the average number of nematodes recovered from 2 wilted seedlings of *P. brutia* was similar to those recovered from *P. sylvestris* and *P. nigra* seedlings. It does not prove that *B. mucronatus* can reach the same population level in *P. brutia* as it does in *P. sylvestris* and *P. nigra* because the average number of nematodes re-isolated from 30 inoculated seedlings and 28 healthy seedlings of *P. brutia* were very low compared to *P. sylvestris* and *P. nigra*. These differences in numbers suggest that the *B. mucronatus* population was not able to develop well enough on inoculated *P. brutia* seedlings to produce substantial mortality. The death of 2 seedlings may have been related to other factors, which may have promoted the development of the *B. mucronatus* population, such as individual characteristics of seedlings, mishandling during transportation, or the inoculation process

It is well known that *P. brutia* is considered a fast growing pine species in Turkey. Although all seedlings were the same age, seedlings of *P. brutia* were longer and wider than those of *P. nigra* and *P. sylvestris*. This might explain why a large number of *P. brutia* seedlings did not wilt in response to *B. mucronatus*. It may also be related to the defense mechanism of *P. brutia* seedlings. No information is available on the defense mechanism of *P. brutia* against *B. mucronatus* attack.

Several studies have been conducted to determine the level of pathogenicity of *Bursaphelenchus* species to pine species in Europe. Caroppo et al. (2000) used 3 different *Bursaphelenchus* species on conifer seedlings under controlled and open air conditions. They found that *B. mucronatus* isolates showed different levels of pathogenicity (30%-100% of inoculated plants) to *P. sylvestris*, *P. pinaster*, and *Larix decidua* Mill. in a climatic chamber. The highest mortality rate is recorded for *P. sylvestris* seedlings, which is the most widely spread conifer species in central and northern Europe (Caroppo et al., 2000). A similar result was reported in the current study for *P. sylvestris* seedlings. In Greece, *B. sexdentati* Rühm, *B. leoni* Baujard, and *B. hellenicus* Skarmoutsos, Braasch, Michalopoulo, were tested for pathogenicity to European pine seedlings (Skarmoutsos and Michalopoulos-Skarmoutsos, 2000). It was suggested that *B. sexdentati* was the most pathogenic species among the 3 *Bursaphelenchus* species (Skarmoutsos and Michalopoulos-Skarmoutsos, 2000).

Mamiya (1998) stated that to date the occurrence of *B. mucronatus* has never been related to epidemic disease anywhere in its distributed area. Nonetheless, there are several reports indicating the potential pathogenicity of *B. mucronatus* (Kulinich et al., 1994; Kishi, 1995). Damage caused by *B. mucronatus* may be related to special environmental conditions of susceptible pine species (Braasch et al., 1998). In Japan, inoculation tests with *B. mucronatus* were carried out using 26-year-old *Pinus densiflora* Sieb and Zucc. and it was found to be nonpathogenic (Mamiya, 1998). Inoculation of *B. mucronatus* resulted in severe destruction of parenchyma cells and death of 6-month-old *P. thunbergii* seedlings. This result indicated that *B. mucronatus* can cause the death of seedlings less than 1 year old (Mamiya, 1998). *B. mucronatus* pathogenicity tests were also carried out in Germany. It was found that the German isolate of *B. mucronatus* caused wilting symptoms on the apex of 60% of inoculated *P. sylvestris* seedlings (Braasch, 1996). In Finland, both seedlings and field-grown *P. sylvestris* trees were inoculated with *B. mucronatus* and the health of seedlings and young trees was not affected (Tomminen, 1993). Under greenhouse conditions, Norwegian isolates of *B. mucronatus* induced pine wilt, to some extend, on 3-year-old *P. sylvestris* seedlings (Schauer-Blume, 1990; Bakke et al., 1991).

In the current study the results were similar to those of previous studies. The seedlings of *P. sylvestris* were the most susceptible to *B. mucronatus*, followed by *P. nigra*. Although the results of greenhouse experiments may not be representative of the situation in natural forest stands, they do provide some ideas about the pathogenic potential of the nematode species. In Turkey, studies related to the pathogenicity of nematode species on forest trees are very limited or nonexistent. It is especially important to study *Bursaphelenchus* species, which mostly inhabit coniferous trees. *B. xylophilus*, a quarantine pest in Europe and Asia, which causes enormous damage in pine forests in several East Asian countries, is also a member of this genus. Therefore, studies should be conducted by inoculating different tree species with different *Bursaphelenchus* species, under both greenhouse and natural stand conditions.

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