PINE WILT DISEASE: A THREAT TO PINE FORESTS IN TURKEY?

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ABSTRACT

The pinewood nematode, is the causal agent of pine wilt disease, a serious threat to native pine forest in eastern Asia (Japan, Korea, China and Taiwan) and some parts of North America (USA, Canada and Mexico). In 1999, this nematode was found and identified for the first time in Portugal and in Europe. The detection of this quarantine pest in Portugal has indicated the need to know more about the distribution of *Bursaphelenchus spp*. in coniferous trees in Europe in order to describe the geographic range of the species and to act quickly in case of the nematode's unwanted introduction into other European regions. Pine forest has a wide distribution in Turkey that increases the number of susceptible host trees for pinewood nematode. Because of these resaons, some regions of Turkey were surveyed for the presence of the nematode. Three different species of *Bursaphelenchus* were found. However, *B. xylophilus* was not detected. The detection of *B. mucronatus*, very similar to *B. xylophilus* biologically and morphologically, is very important. The presence of this species indicates that *B. xylophilus* could spread easly in conifer forests of Turkey. A study was conducted to determine the pathogenicity of *B. mucronatus* and 80% of seedlings of *P. sylvestris* were wilted. Biological characteristics of *M. galloprovincialis* were compared with *M. carolinensis*, Nort American vector, and some of them were found to be similar.

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 through Taiwan and Korea as well (Mamiya, 1999). Recently, the nematode was isolated from *Pinus pinaster* (maritime pine) wood in Portugal for the first time in Europe (Mota et al., 1999). The detection of this A1 quarantine organism (EPPO) in one of the member states of the European Union (EU) forced the implementation of specific measures to control and eradicate this nematode and its vector from the affected area and to conduct surveys to confirm the absence of the pinewood nematode in pine forests of all EU member countries. Turkey as a member of the EPPO follows the phytosanitary regulations for quarantine organisms in Europe.

Turkey is in an important transitional geographic area between Europe and Asia, which may increase the possibility of inadvertent introduction of the pinewood nematode from infested regions like Asia. Because of increasing global trade, exportation and importation of wood products among countries increases the threat of the pine wilt disease in the uninfested regions of the world. Turkey imports wood products from different countries every year. Consequently, the possible introduction of the pinewood nematode and other exotic pests is a constant threat in Turkey.

The presence and distribution of *Bursaphelenchus* species in Turkey is poorly known, and limited to the first report of the genus by Vieira *et al.* (2003), and by the occurrence of *B. mucronatus* by Akbulut *et al.* (2006). There are several resons to conduct and develop studies related to the potential of pine wilt disease establishment in Turkey. One is geographic location: a transitional area between Europe and Asia. Most of the trade routes between Asia and Europe pass through Turkey which may increase a possible introduction of any non indigenous pests.

Secondly, the total forest area covers about 27% of the country's total land (21 million ha) (Figure 1) (Anonymous, 2006a). Conifer species (pure stands) cover 54% of this total land (Figure 2). In Turkey, there are three widely distributed native pine species; *Pinus brutia* Ten., *P. nigra* Arnold and *P. sylvestris* L. They cover an area of almost 10.9 million ha of country's land. This wide distribution of pine species in Turkey increases the number of susceptible host trees for the pinewood nematode.

Despite the richness of the forest area, Turkey has to import industrial wood from different countries every year to cover the gap between supply and demand (Figure 3).

The third reason for survey is the presence of at least one *Monochamus* species (*Monochamus galloprovincialis* (Olivier)) in Turkey. It has been previously reported from a number of different sites in Turkey (Çanakçıoğlu and Mol, 1998, Özdikmen *et al.* 2005). These studies give only general distribution and some morphological characteristics of the betle. The pine sawyer beetle, *M. galloprovincialis* was found to be the vector of the pinewood nematode in Portugal (Sousa et al.,2001). According to Hellrigl (1971), five species of *Monochamus* occur in Europe; *M. galloprovincialis, M. sartor* (Fabricius), *M. sutor* (Linnaeus) *M. urussovi* (Fischer) and *M. saltuarius* (Gebler). Other *Monochamus* species reported in Europe (Hellrigl, 1971) have not been found in Turkey yet.

Detailed biology and ecology of *M. galloprovincialis* were not known in Turkey. Recently, several studies on biology and ecology of *M. galloprovincialis* have been conducted. These studies suggested that both *P. sylvestris* and *P. nigra* are suitable hosts for the development of the beetles (unpublished data). This result indicates that *P. sylvestris* and *P. nigra* stands located at different regions of Turkey may increase the chance of rapid growth of the pinewood nematode’s populations in case of introduction.

The other reason to study pine wilt disease in Turkey is the presence of highly suitable climatic conditions for the development of the pinewood nematode in Turkey. Distribution of annual mean temperature values is presented in Figure 4. Annual mean temperature was over 12^{o}C between 1928 and 2005. The temperature is over 20^{o}C between June and September in most parts of Turkey particularly in south and west Anatolia (Anonymous, 2006b).

High temperature (over 20^{o}C) is important for the development of pine wilt disease. The absence of disease in Europe (except recent introduction to Portugal) may be related to the relative absence of large forest areas located at hot regions (Webster, 2004). Most of the *P. sylvestris* forests are distributed at cooler northern part of Europe. Braasch and Enzian (2004) stated that the temperature more than 20^{o}C for at least 8 week period increase the number of vulnerable pine trees to the pine wood nematode. These climatic conditions are always present in most parts of Turkey.

A survey of *B. xylophilus* was initiated in 2003 in Turkey, in view of the considerations presented. The survey has not been completed yet. In addition to survey, the potential insect vector species of the nematode have also been investigated.

STUDIES CONDUCTED BETWEEN 2003 AND 2006 in TURKEY

The pine wilt disease complex involves three organisms: the nematode (*B. xylophilus*); the vector insect, in general a *Monochamus* species; and a tree host, mostly a pine species (genus *Pinus*). Therefore, these three organisms and their interactions must be studied. In Turkey, the first step was to conduct a survey for the presence of the pinewood nematode in pine forests. The second step was to find potential insect vectors and to investigate their biological and ecological characteristics. The third step was to carry out different experiments based on the results of the nematode survey and insect vector studies.

SURVEY OF *BURSAPHELENCHUS* SPECIES

MATERIALS AND METHODS

A survey was conducted between 2003 and 2006 in 11 different Regional Forestry Directorates (Ankara, Artvin, Bolu, Istanbul, Amasya, Trabzon, Çanakkale, Bursa, Balıkesir, İzmir and Mersin) between 2003 and 2006. Selection of these sites was decided according to the following criteria: 1. forests adjacent to harbors, 2. forests adjacent to wood industry centers, and 3. forests in which wilting of trees was observed. Wood samples (40-80g each) were collected from pine trees displaying declining symptoms, at 1.5m of the trunk level (DBH), using a Pressler borer, from both sides of each tree, and stored in polythene bags. Nematodes were extracted using a modified Baermann funnel technique, and processed within 48 h. The collected nematodes were inoculated on *Botrytis cinerea* Pars., growing in malt agar, and incubated for 2 weeks at 25º C.

For nematode identification, special attention was given to the group of species closely similar to *B. xylophilus* (*xylophilus*-group *sensu* Ryss *et al.* 2005). Identification was performed to species level, using morphological and molecular methodologies. For optical microscopic studies (Olympus BX50), nematodes were fixed with hot formalin (4%), processed to anhydrous glycerin and mounted in permanent slides according to the "express technique" described by Ryss (2003). The molecular analysis was performed following the methodology described in Cenis (1993) for DNA extraction, and the ITS-RFLP profiles were obtained following the methodology previously described (Hoyer *et al.*, 1998; Mota *et al.*, 1999).

RESULTS AND DISCUSSIONS

A total of 1254 samples were collected from the study sites. Over 400 samples have been processed and the nematodes identified. The remaining samples have been checked for the presence of *Bursaphelenchus* species. Some of them revealed *Bursaphelenchus* species (Table 1). Eighteen of 400 samples contained *Bursaphelenchus* species. Three species of *Bursaphelenchus* were identified: *B.*

mucronatus Mamiya & Enda, 1979 from *Pinus nigra*, *P. sylvestris*, *B. pinophilus* Brzeski & Baujard, 1997 from *P. nigra* and *B. sexdentati* Rühm, 1960 from *P. pinaster*. *B. mucronatus* was found in the areas of Artvin and Düzce, *B. sexdentati* was recorded from Düzce and Ankara, and *B. pinophilus* distributed only in Ankara. The identification of *Bursaphelenchus* species found in other cities has not yet been completed.

This survey is important to provide information on *Bursaphelenchus* species from Turkey. The presence of *B. mucronatus*, a closely related species to *B. xylophilus*, is very important. This indicates that Turkey has suitable conditions for the establishment and development of *B. xylophilus* populations. The survey of the pinewood nematode has not been completed yet. The remaining regions, located in the south and western parts of Turkey will be surveyed in the coming years.

PATHOGENICITY OF *BURSAPHELENCHUS MUCRONATUS* MAMIYA & ENDA, 1979

MATERIALS AND METHODS

Inoculation tests were carried out between May 5th and August 10th of 2005. Three widely distributed native pine species, *Pinus brutia*, *P. nigra*, *P. sylvestris*, were selected for inoculation tests. In the experiment, 3 year-old pine seedlings were used. *P. brutia* were obtained from Balıkesir, *P. nigra* and *P. sylvestris* seedlings from Bolu forest nurseries. During the experiment 15 seedlings of each species were inoculated with *B. mucronatus* and 10 seedlings of each served as controls. The *B. mucronatus* population was 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 inoculation, the cultures of

B. mucronatus were maintained on *Botrytis cinerea* Pers. cultures on malt agar at 25^{o}C for 7-10 days. When the nematodes appeared to reach sufficient numbers, they were extracted using the Baermann funnell technique (Southey, 1986). The inoculum used for each seedling contained approximately 1000-1100 nematodes of all life stages in 0.5 ml of distilled water. The inoculation point on the seedlings was located just above the lowest shoots (5-10cm above the soil surface). The needles around the site were removed and a half T slit was made by using a scalpel. The bark was gently pulled and a small piece of sterile cotton was inserted into slit. The nematode suspension (0.5ml) was introduced slowly by using a syringe. After inoculation, the area was wrapped with a parafilm strip. The seedlings used as controls were prepared the same way and the inoculation procedure was as described above except that instead of a nematode suspension, distilled water (0.5 ml) was used.

The inoculated plants and controls were kept under greenhouse conditions (26-28^{o}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 after 3 months after the inoculation date. Wilted seedlings were cut off at 1cm above soil level, the needles and short shoots removed. The main stem of the seedlings was weighted divided into small pieces and put into a Baermann funnell for 24 hours for nematode extraction. The collected nematodes were counted under a stereomicroscope. The number of nematodes for wilted and healthy seedlings was determined. Control seedlings were not cut for counting the nematodes unless they wilted.

RESULTS AND DISCUSSIONS

In this study, a total of 45 seedlings were inoculated with *B. mucronatus*. The first wilting symptoms were observed 10 days after inoculation for both *P. nigra* and *P.*

sylvestris. The cumulative mortality rates during a 13-week period are shown in Figure 1. The first total wilting case was observed at the 5th week of the experiment. There was a big increase in the number of wilted seedlings of *P. sylvestris* at the 7th week (Figure 5). The highest mortality was observed in *P. sylvestris* seedlings followed by *P. nigra* (Table 2). Only 1 seedling of *P. brutia* wilted near the end of the experiment (Figure 5) and the remaining 14 seedlings displayed no sign of wilting.

The average number of nematodes re-isolated from inoculated seedlings was the highest for *P. sylvestris* seedlings followed by *P. nigra* (Table 2). The lowest average number of nematodes was recovered from *P. brutia* seedlings (Table 2). The highest number of nematodes counted was 11 980 for *P.sylvestris*. We obtained more nematodes from dead seedlings than from living ones. The control seedlings showed no mortality indicating that the inoculation techniques had no negative effect on seedlings.

This is the first study on the pathogenicity of *B. mucronatus* on pine species in Turkey. The highest mortality was observed on *P. sylvestris* seedlings followed by *P. nigra*. Only one *P. brutia* seedling wilted . These results suggest that *B. mucronatus* may be highly pathogenic to *P. sylvestris*. The results on *P. nigra* also revealed serious pathogenicity with percentages of seedling mortality of 47%. *B. mucronatus* seems to be non pathogenic to *P. brutia* seedlings. These differences in mortalities suggest that the *B. mucronatus* population was not able to develop successfully in *P. brutia* seedlings and to cause substantial mortality. It may be related to a defense mechanism within *P. brutia*. No information is available on the defense mechanism of *P. brutia* against *B. mucronatus*.

Several studies have been carried out to determine the pathogenicity level of *Bursaphelenchus* species on 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 displayed different levels of pathogenicity (30-100% of inoculated plants) against *P. sylvestris*, *P. pinaster*, and *Larix decidua* Mill. in a climatic chamber. The highest mortality rate was 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. Mamiya (1998) stated that the occurrence of *B. mucronatus* has never been related to epidemic disease spread throughout its distributed area. But there are several reports indicating the potential pathogenicity of *B. mucronatus* (Kulinich et al., 1994, Kishi, 1995). Pathogenicity tests with *B. mucronatus* 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).

The results of greenhouse experiments may not present the real situation in natural forest stands but may provide some idea about the pathogenic potential of the nematode species. It is difficult to compare the results of this study with previous reports, mainly, because, there are differences in methods, inoculum densities, host ages, study conditions and other factors. It is important to carry out new studies under different conditions such as, different inoculum densities, different nematode species, different host ages, and different ecological conditions.

There are some criticisms related to pathogenicity tests on young plants. Forty-one species of *Pinus* species were killed in the pathogenicity tests by using *B. xylophilus* but only seven of them are known to be killed in the field (McNamara, 2004). McNamara (2004) also suggested that in some situations inoculation experiments conducted on older trees or plants in the field may not also give the real situation in the field. He suggested that the is a need for detailed studies considering various factors such as age of host plant, inoculation method, state of sterility of nematode suspension, nematode life stages in suspension, environmental conditions and other related factors (McNamara, 2004).

COMPARİSON OF SOME BİOLOGİCAL CHARACTERİSTİCS OF *M. CAROLİNENSİS* AND *M. GALLOPROVİNCİALİS*

MATERİALS AND METHODS

In this study, colony data of *M. carolinensis* reared on *P. banksiana* Lamb. and colony data of *M. galloprovincialis* reared on *P. sylvestris* L. were used. Data collections were made at different time periods. Comparisons were made between the number of eggs laid, the number of larval entry holes, the number of adults emerged, generation survivorship, survivorship from egg to larva, and survivorship from larva to adult.

RESULTS AND DİSCUSSİONS

A total of 27 trees from *P. banksiana* and 23 from *P. sylvestris* were used to compare the beetles. Log surface area and log volume averaged approximately 2000cm^2 and 7100cm^3 respectively for *P. sylvestris* and *P. banksiana* had a lower values for both variables (Table 3). *M. carolinensis* laid significantly more eggs and produced significantly more larval entry holes than *M. galloprovincialis* (p=0.0011, p=0.0001). On the other hand, apparent survivorship from larva to adult was significantly greater for *M. galloprovincialis* than for *M. carolinensis* (p=0.0026), and the number of adults emerged and generation survivorship did not differ significantly between the two beetle species (p=0.4558, p=0.8264).

In this study, we wanted to examine and compare the life histories of the two vector beetles of the pinewood nematode. Although the two species were reared on different host trees, there are remarkable similarities in some of biological characteristics of both vectors which suggest that *M. galloprovincialis* would have a similar vector pattern as *M. carolinensis*. This is important to note, considering the potential threat of pine wilt disease to native European pine forests, and the rich body of knowledge already gathered on *M. carolinensis*.

Since the first report of the pinewood nematode from Portugal (Mota et al., 1999) studies on biological and ecological characteristics of *M. galloprovincialis* have been undertaking more intensely in several European countries, such as Portugal, Spain and recently Turkey (Naves et al., 2006a, b, Naves et al., 2007).

CONCLUSIONS

Pine wilt disease is an important threat to susceptible pine forests of the world. In this study, several *Bursaphelenchus* species were found in Turkey. Fortunately, *B. xylophilus* has not been detected yet. The presence of *B. mucronatus* indicates that the conditions are suitable for possible introduction and establishment of *B. xylophilus* in pine forests of Turkey. The pathogenicity study showed that *B. mucronatus* has a high pathogenic potential on 3-year old seedlings of *P. sylvestris* and *P. nigra*. Detailed and large scale pathogenicity studies should be carried out to reveal the susceptibility of pine species and the pathogenic potential of *Bursaphelenchus* species found in pine forests of Turkey.

Potential vector beetle species of *B. xylophilus* and other *Bursaphelenchus* species are not known well in Turkey. The pine sawyer beetle, *M. galloprovincialis* (Olivier), was found to be the vector of the pinewood nematode in Portugal (Sousa et al., 2001). The presence of *M. galloprovincialis* was previously reported from a number of different sites in Turkey (Özdikmen et al., 2005). The biology and ecology of *M.*

galloprovincialis have been studied under laboratory conditions since 2002 but the data have not been published yet. Investigation of the biology and ecology of *M. galloprovincialis* is critical to the potential management of pine wilt in Turkey.

In conclusions, Turkey is located at a very important transitional area between Europe and Asia. The pinewood nematode is present in both Europe (Portugal) and Asia, which increases the possible introduction of the nematode into Turkey. Two necessary components for the spread of pine wilt disease, a vector beetle and suitable host trees, are already present. Climatic conditions for development of the pinewood nematode are very suitable in a large area of Turkey. Therefore, it is important to study the pine wilt disease complex for prevention of possible introduction of the pinewood nematode and to control and eradicate both the nematode and insect vector.

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Table 1. *Bursaphelenchus* survey results between 2003 and 2006 in Turkey

Name of Regional Forestry Directorate	**Tree Species**	**No. of samples collected**	**No. of samples with *Bursaphelenchus* species**
Artvin	*P.sylvestris*	66	6
Trabzon	*P. sylvestris, Picea orientalis*	80	1
Amasya (Samsun)	*P. sylvestris, P. nigra, P. brutia*	71	-
Ankara	*P.nigra*	30	3
İstanbul	*P. nigra, P. sylvestris*	46	-
Bolu (Düzce)	*P. sylvestris, P. nigra, P. pinaster*	74	8
Bursa	*P. brutia, P. nigra, P. pinaster*	200	-
Balıkesir	*P. brutia, P. nigra*	213	?
Çanakkale	*P. brutia, P. nigra, P. pinaster*	166	-
İzmir	*P. brutia, P. nigra, P. pinea*	177	?
Mersin	*P. brutia*	131	?

Table 2. Number of wilted plants and average number of re-isolated nematodes

Plant species	No. of seedlings	Health conditions		Mortality of plants (%)	Average no. of re-isolated nematodes	
		Wilted	Live		Wilted	Live
P. sylvestris	15	12	3	80	3221	9
P. nigra	15	7	8	47	2143	42
P. brutia	15	1	14	6.7	971	8

Table 3. Summary statistics of holding time, log metrics, numbers of eggs laid, laval entry holes, adult emerged and survivorship percentages for *M. galloprovincialis* and *M. carolinensis*.

Variable	N	Mean	
	M.g. *M.c.*	*M. galloprovincialis*	*M. carolinensis* (Akbulut et al., 2004)
Holding time (d)	23 27	10±5	11±5
Log area (cm^2)	23 27	2046.2±618.8	1504.0±152.2
Log volume (cm^3)	23 27	7109.7±4476.4	4548.0±905.5
No. eggs laid	23 27	74±38	127±61
No. larval entry holes	23 27	13±3	19±6
No. adult emerged	23 27	7±2	8±4
Generation survivorship (%)	23 27	13±5	12±15
Apparent survivorship (egg to larvae %)	23 27	22±7	21±6
Apparent survivorship (Larvae to adult %)	23 27	56±14	41±19
Adult density (Adults/dm^3)	23 27	1±1	2±1