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PINE WILT DISEASE AND THE PINEWOOD NEMATODE, *BURSAPHELENCHUS XYLOPHILUS*

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Abstract. Pine wilt disease (PWD) is one of the most damaging events affecting conifer forests (in particular *Pinus* spp.), in the Far East (Japan, China and Korea), North America (USA and Canada) and, more recently, in the European Union (Portugal). In Japan it became catastrophic, damaging native pine species (*Pinus thunbergii* and *P. densiflora*), and becoming the main forest problem, forcing some areas to be totally replaced by other tree species. The pine wilt nematode (PWN) *Bursaphelenchus xylophilus*, endemic, with minor damage, to North America, was introduced in Japan in the early XX century and then spread to Asia (China and Korea) in the 1980s. In 1999 it was detected for the first time in Portugal, where, due to timely detection and immediate government action, it was initially (1999-2008) contained to a small area 30 km SE of Lisbon. In 2008, the PWN spread again to central Portugal, the entire country now being classified as "affected area". Being an A1 quarantine pest, the EU acted to avoid further PWN spreading and to eradicate it, by actions including financial support for surveyes and eradication, annual inspections and research programs. Experience from control actions in Japan included aerial spraying of insecticides to control the insect vector (the Cerambycid beetle *Monochamus alternatus*), injection of nematicides to the trunk of infected trees, slashing and burning of large areas out of control, beetle traps, biological control and tree breeding programs. These actions allowed some positive results, but also unsuccessful cases due to the PWN spread and virulence. Other Asian countries also followed similar strategies, but the nematode is still spreading in many regions. In Portugal, despite lower damage than Asia, PWD is still significant with high losses to the forestry industry. New ways of containing PWD include preventing movement of contaminated wood, cutting symptomatic trees and monitoring. Despite a national and EU legislative body, no successful strategy to control and eventually eradicate the nematode and the disease will prevail without sound scientific studies regarding the nematode and vector(s) bioecology and genetics, the ecology and ecophysiology of the pine tree species, *P. pinaster* and *P. pinea* , as well as the genomics and proteomics of pathogenicity (resistance/ susceptibility).

1. INTRODUCTION

For millions of years the distribution of the world's biota has been constrained by natural barriers. However, with increasing globalization and the breaking down of

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geographical boundaries, new biological invasions by non-indigenous species have become a global environmental issue, often causing severe outbreaks with economic and ecological disruption in various ecosystems (Liebhold et al., 1995; Sakai et al., 2001).

In forest ecosystems the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, is considered one of the most important pests and pathogens in the world. The general fear of establishment of the PWN, the causal agent of the pine wilt disease (PWD), into countries where conifer forests assume great importance, stems from the devastating damage caused by this nematode to pine forests (Mamiya, 2004; Mota & Vieira, 2008; Shin & Han, 2006). The introduction of the PWN into non-native areas (outside of North America) is primarily associated with trade and the global flow of forest products (Bergdahl, 1999; Webster, 2004).

Unmanufactured wood, especially in raw log form, has been identified as one of the most high-risk pathways of movement of forest insects and pathogens into new environments, between continents (Evans et al., 1996; Tkacz, 2002). Many of the *Bursaphelenchus* species, including the PWN, have been routinely intercepted in packaging and wood products in several countries, e.g. Austria (Tomiczek et al., 2003), China (Gu et al., 2006), Finland (Tomminen, 1991) and Germany (Braasch et al., 2001). Furthermore, the recent detections of the PWN in packaging wood imported from countries considered free of this pest, due to the repeated use and circulation of this type of wood material, e.g. Brazil, Belgium, Italy and Spain, (Gu et al., 2006), undoubtedly stresses the importance of trade globalization for the potential entry/establishment of this pathogen into endemic forests worldwide.

The damage by this invasive species is clearly demonstrated by the devastation caused in non-native regions where the disease became established, e.g. Japan and China (Yang, 2004; Shimazu, 2006). The introduction of this nematode into non-native areas has resulted in huge annual losses due to the effects on increased mortality and growth loss of the pine forest (26 million m^3 of timber lost since 1945 in Japan), and by the increased costs in management procedures and disease control (Mamiya, 2004; Mota & Vieira, 2008; Shimazu, 2006). In addition, the introduction of this pest has resulted in vast and irreversible changes to the native forest ecosystems including tree species conversions, wildlife habitat destruction, soil and water conservation and loss of biodiversity (Kiyohara & Bolla, 1990; Suzuki, 2002).

The PWN is already established for more then 100 years in Japan (Yano, 1913), and in the past two decades the new reports of pine wilt disease came mainly from East Asia (Cheng et al., 1983; Yi et al., 1989). However, in 1999 the PWN was reported for the first time in Portugal and in Europe (Mota et al., 1999). Following this finding, there has been considerable activity in both delineating the extent of the infested area and preventing the spread to the remainder of the country and the European Union (EU) (EC, directive 2001/218/EC). The potential threat of the PWN to coniferous forests is real and the most effective way of reducing this threat is to be more restrictive to the importation of wood products, and to carry a rigorous inspection system for wood material (Evans et al., 1996; Bergdahl, 1999; Gu et al., 2006). Therefore, specific measures have been applied in Portugal in order to control the PWN and its insect vector, and in each EU member country, national surveys were performed to determine whether the nematode is present in other territories beside Portugal (directive 2001/218/EC).

The current situation in Portugal assumes great importance not only because of the economic implications, but also through the destruction of the pine forest in the area where the PWN became established (Setúbal Península). On the other hand, pine forests occupy a huge area of the continental territory ($1.25 \cdot 10^6$ ha) representing one of the greatest natural resources of the country, namely in the form of timber (*Pinus pinaster*), wood products and pine nuts (*Pinus pinea*). Consequently, strict requirements have been imposed on all wood movements from the affected area to other regions in Portugal, as well as to other EU member states. These measures have had serious implications for the timber industry within the affected area, creating a significant impact on the national economy and markets of wood industries (Rodrigues, 2008) (Fig. 1). Unfortunately, these measures have not been successful in preventing the spread of the PWN in Portugal.

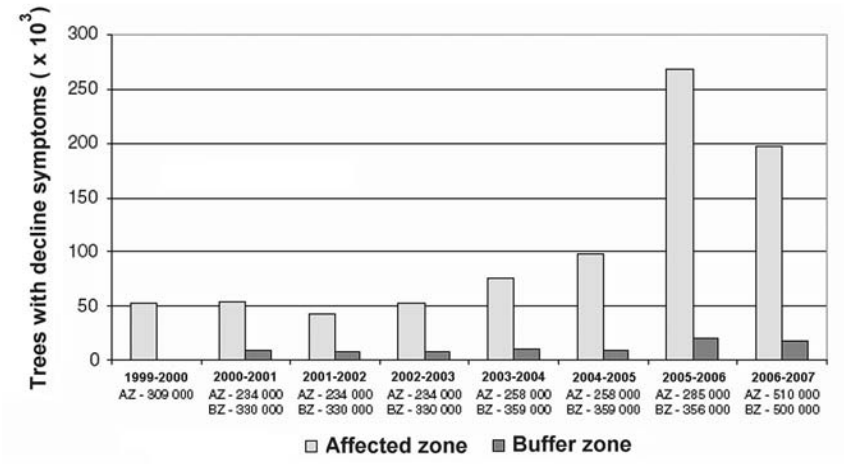

*Figure 1. Evolution of declining maritime pine (*Pinus pinaster*) trees in the 1999-2007 demarcated area in Portugal (Setúbal Peninsula) (from Rodrigues, 2008).*

The occurrence of pine wilt disease in Portugal was initially (1999-2008) limited to a relatively small area (ca. 500 000 ha). Nevertheless, the danger of spread of this disease assumes a high phytosanitary risk because of the wide distribution of both the insect vector (*Monochamus galloprovincialis* Oliv.) and the known susceptible host (*Pinus pinaster* Ait.) in Portugal (Rodrigues, 2008). Until recently, no consensus has emerged on the possible pathway of the PWN introduction in Portugal. This is partly due to a scarceness of studies using different sources of isolates from the affected area in the country.

Several hypotheses have been put forward to explain this introduction, such as from endemic areas where the nematode naturally occurs (North America), or non-endemic areas where the nematode behaves as an exotic pest (Asia) (Iwahori et al., 2004; Mota et al., 2004). They were recently tested, suggesting a possible double introduction of the PWN in Portugal (Metge & Burgermeister, 2006), both from East Asian countries. Although this study incorporates a large number of different isolates from different regions of the world, concerning Portugal it is restricted to the use of three isolates only, and representative of a small area of the full affected area. Recently, a more complete genetic analysis has been made using 24 isolates from the original demarcated area (Setúbal Peninsula) (Fig. 2) and the results clearly indicate a lack of genetic diversity among isolates as well as a confirmation of the proximity with East Asian populations of the PWN (Vieira et al., 2007).

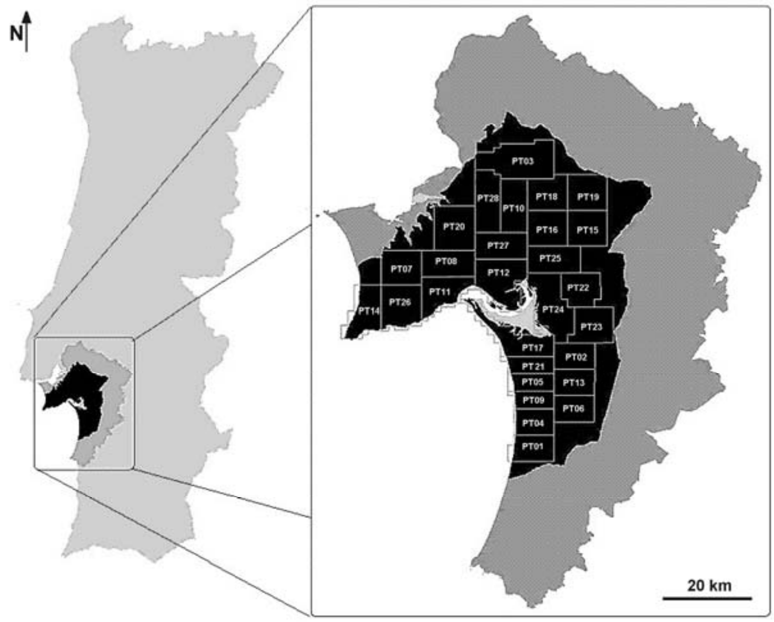

Figure 2. Portugal (continental, left) and location of the 1999-2008 quarantine area. Location of Bursaphelenchus xylophilus *isolates (right) from different blocks within the affected area. Black: the area affected by the PWN; dark grey: the buffer area, established in 1999 for safety reasons (free of PWN) (from Vieira* et al., *2007).*

2. PWN DISTRIBUTION AND DISEASE DISSEMINATION

PWN is considered a native species from North America, where it is distributed throughout Canada and USA (Robbins, 1982; Bowers et al., 1992; Sutherland & Peterson, 1999), and also with a single report from Mexico (Dwinell, 1993). In these regions, the PWN has been associated with several conifer species: blue spruce and white spruce (*Picea* spp.), atlas cedar and deodara cedar (*Cedrus* spp.), eastern larch and european larch (*Larix* spp.), balsam fir (*Abies* spp.) and Douglas fir (*Pseudotsuga* spp.), however, it is mainly found in pine species (*Pinus* spp.) (Robins, 1982; Bowers et al., 1992).

Bursaphelenchus xylophilus has both phytophagous (transmission by feeding) and mycophagous (transmission by oviposition) phases of development (Fig. 3). The nematode is carried by *Monochamus* beetles that feed on twigs in the crowns of healthy trees (known as “maturation feeding”). Later, the female beetles lay their eggs in damaged or dying trees as well as in freshly cut stems with bark. Fourth-stage (J_{IV}) dispersal juveniles (“dauer” larvae,) of *B. xylophilus* are carried under the elytra (wing cases) and in the tracheae (breathing tubes) of the beetles and migrate into the tree through the wounds caused by feeding or ovipositing beetles.

Transmission during maturation feeding is the initiation of the phytophagous phase of the nematode, which has the greatest importance for the potential development of pine wilt disease. In a suitable tree species and under favorable climatic conditions, the nematodes multiply rapidly in susceptible trees, feed on plant tissues and move from the cambium into the xylem. Their generation time is 6 days at 20°C and 3 days at 30°C. The nematodes contribute to plant death by blocking water conductance (cavitation) through the xylem. The damaged trees become available for oviposition by *Monochamus* spp. females; therefore, nematodes also enter the tree through the oviposition slits in the bark. In dead trees, the nematodes feed on fungi, in particular on blue stain fungi (*Ceratocystis*, *Gliocladium*). *Monochamus* larvae develop initially in the cambium and then burrow into the wood, where the nematodes congregate in the vicinity of the pupal chambers formed by the mature beetle larvae. When the new beetle emerges, the nematodes migrate into the tracheae and to the area beneath the elytra of the beetles. The presence of suitable fungi in the trees encourages nematode reproduction and survival and, consequently, increases the number of nematodes carried by the emerging beetles (Mamiya, 1984; Linit, 1988; Evans et al., 1996).

The introduction and spread of this species into new areas has also been aided by the high phenotypic plasticity of the nematode, including excellent adaptation for resistance in the host tree (i.e. long periods of starvation) and dispersion (ectophoretic insect association) (Mamiya, 1984). In the native host species of North America, the nematode does not cause disease, since both plant and nematode have co-evolved for a very long time and thus the trees have become resistant/tolerant to its presence (Kiyohara & Bolla, 1990), except in some exotic *Pinus* spp. plantations (Evans et al., 1996). On the other hand, this scenario changes drastically when this organism reaches non-native habitats.

It is assumed that the presence of the PWN in Japan is the result of an accidental introduction by means of contaminated wood products from the USA (California) to the southern Japanese island of Kyushu, in the beginning of the 20^{th} century (Yano, 1913). However, only in 1971 was the PWN associated with the high mortality of pine trees and identified as the causal agent of PWD, mainly of Japanese black pine (*P. thunbergii*) and Japanese red pine (*P. densiflora*) (Kiyohara & Tokushige, 1971). In spite of the numerous efforts to control the nematode and the insect vector (*M. alternatus*), the disease spread throughout the entire country, with the exception of the most Northern prefectures of Aomori and Hokkaido, occupying nowadays 28% of the total pine forest area (580 000 ha) (Mamiya, 2004; Shimazu, 2006).

During the eighties, the PWN was reported in other east Asia countries as well. In 1983 it was found for the first time in mainland China, associated with dead and dying Japanese black pine, in Nanjing (Jiangsu Province) (Cheng et al., 1983). The situation in China assumes great importance firstly by the continuous spreading of the disease (up to date affecting 75000 ha, and more then 20 million pine trees destroyed) among different regions of the country (Jiangsu Province, Anhui Province, Guangdong Province, Zhejiang Province, Shandong Province and Hubei Province) mainly due to human factors, and secondly by the potential threat to other areas where all the conditions that determine the establishment of the disease are present, and which are still free of the PWN (Yang, 2004).

In Taiwan the first report of the PWN occurred in 1985, identified from a luchu pine (*P. luchuensis*) stand displaying 50% mortality, in the Taipei prefecture (Tjean & Jan, 1985a). It has also been reported from Japanese black pine in Taoyeun prefecture (Tjean & Jan, 1985b).

In 1989, the PWN was detected in South Korea, in Pusan (the largest harbor city located in the extreme southern part of the country), associated with the Japanese black pine and Japanese red pine (Yi et al., 1989). Although the area of distribution of the disease was controlled until 1997, and limited in relatively small areas in the southern part of the country (La et al., 1999), in the last years a continuous spread of the disease has been observed, and more recently it has been reported simultaneously from new different areas (Mokpo, Sinan, Yeongam, Daegu, Gumi, Andong, Gyeongbuk, Gangneung and Donghae), constituting today the major forest pest in the country (Shin & Han, 2006).

In 1999, the PWN was reported for the first time in Portugal, and in Europe, associated with maritime pine (*P. pinaster*) (Mota et al., 1999), and with a single species as the insect vector (*M. galloprovincialis*) (Sousa et al., 2001). After the initial detection, a national survey was carried out along the pine forests, and a quarantine area was established where the nematode occurred, in the Peninsula of Setúbal (ca. 30 km SE of Lisbon).

The initial PWN affected area covered 510,000 ha, surrounded by a buffer zone of 500,000 ha more, for safety reasons. Although the initial affected area persisted as almost identical from 1999 ro 2007, in the last survey/eradication campaign the number of declining trees in the demarcated area increased significantly within the affected zone (Rodrigues, 2008), followed by an expansion of the demarcated area, particularly to the south of the country (Sines, corresponding to the south point), and very recently to the central areas of Arganil and Lousã. As a result of this trend, in 2007 prevention measures were established by the EU, i.e., the implementation of a 3 km phytosanitary strip surrounding the initial quarantine area, where all pine trees were cut and removed until the end of 2007 (Rodrigues, 2008). The effectiveness of this strip was questioned at the time and now with the new areas of implantation of the nematode (ca. 200 km North of the initial affected area) has become useless.

3. PINEWOOD NEMATODE TAXONOMY

3.1. Morphological Approaches

The genus *Bursaphelenchus* was established by Fuchs (1937) and includes nematodes that are associated with insects and dead or dying trees, mainly conifers, and which have an ectophoretic stage. Most species are fungal feeders and are either transmitted to dead or dying trees during oviposition by insect vectors, or to healthy trees during maturation feeding of their insect vectors (Hunt, 1993). The genus is mainly distributed in the northern hemisphere, however a few number of species have been reported outside of this geographical range (South Africa), associated with plantations of pine species (for a detailed information see Ryss et al., 2005).

The current concern on the introduction of the PWN into new areas has increased the interest and the knowledge of this genus and the number of species recorded worldwide. Up to date, the genus comprises nearly 100 described species, 10 of which where described in the last two years, mainly from east Asia (Hunt, 2008; Ryss et al., 2005). In Portugal, until the report of the PWN in 1999, no knowledge of this genus was available. At the moment, 10 species have been reported for the country, associated with maritime pine trees (Penas et al., 2004), including the description of a new species to science, *B. antoniae* Penas, Metge, Mota and Valadas, 2006 (Penas et al., 2006).

The economic importance posed by the PWN clearly reinforced the need for an accurate diagnosis of the species, where morphological studies remain the standard method for routine identification. Different criteria may be used to divide the large number of nominal species of the genus *Bursaphelenchus*, into smaller and more convenient species groupings. Tarjan and Baéza-Aragon (1982) were the first to attempt the assembly of morphological identification keys for this genus, providing a detailed classification of the spicule characters and other useful morphological diagnostic data. Braasch (2001), and for the species associated with conifer trees in Europe (28 at that time), proposed the establishment of the species groups based on the number of lateral lines (nine different groups), followed by the distribution of the male papillae, spicule shape, presence and size of the female vulval flap and the shape of female tail.

Yet, an integrated morphological identification system to all the species of the genus has been lacking. Furthermore, the fact that more then 70% of these species occur in pine trees makes the identification even more uncertain. Therefore, Ryss et al. (2005) ellaborated a synopsis of the genus in order to provide an identification system to all the nominal species, where the spicule structure is the main diagnostic character to separate the species into groups. The six species groups (*aberrans*-group, *borealis*-group, *eidmanni*-group, *hunti*-group, *piniperdae*-group and *xylophilus*-group) are merely recognized as identification units in order to facilitate species identification. However, some of these groups could be considered as natural, i.e. phylogenetically related (e.g. the *xylophilus*-group) (Ryss et al., 2005).

Despite the clear separation of the members of the *xylophilus*-group (*B. baujardi*; *B. conicaudatus*; *B. doui*; *B. fraudulentus*; *B. kolymensis*; *B. luxuriosae*; *B. mucronatus*; *B. singaporensis*; *B. xylophilus*) from other groups based solely on the male spicule shape, the variability and overlapping in range of several other taxonomic characters within some species of this group is such that their accurate identification is difficult.

One of the major characters used for distinguishing the PWN from all other members is the shape of the female tail, i.e. rounded, and lacking a distinct mucron. However, specimens of *B. xylophilus* from North America show a wide variation in female tail shape, showing variations from rounded to a mucronated form, similar to the female tail of *B. mucronatus* (Wingfield et al., 1983). In addition to the morphological similarities between *B. xylophilus* and *B. mucronatus*, these two species are capable of genetic exchange, either directly or via intermediate forms (De Guiran & Bruguier, 1989), which clearly compromise the identification at the species level using morphological data only. Furthermore, the presence of males or juvenile stages alone deemed to be an unreliable method in the identification at the species level within the *xylophilus*-group, as well as for the differentiation of geographic isolates.

3.2. Molecular Approaches

Due to the difficult identification and constrains of morphological observations between *Bursaphelenchus* species, alternative molecular tools have become a valuable instrument for species and sub-specific separation. Initially these molecular tools were mainly developed for the differentiation of some species of the *xylophilus*-group, such as *B. xylophilus* and *B. mucronatus*, in order to achieve a better understanding of the relationships, and the clear identification of the *B. xylophilus* isolates.

The first methods used for the *Bursaphelenchus* species identification and isolates separation were based on protein profiles (Hotchkin & Giblin, 1984) and enzyme electrophoresis (De Guiran et al., 1985). However, the value of these methods was limited by differential gene expression during the life cycle of the nematode or by the response to external environmental influences (Harmey and Harmey, 1993). Immunological approaches have also been used for species-specific identification, using polyclonal antibodies that could differentiate specific antigens of certain *B. xylophilus* isolates (Lawler & Harmey, 1993), as well as monoclonal phage antibodies (Fonseca et al., 2006).

With the expansion of DNA-based methodologies, new alternatives, independent of the development stage and phenotypic variation due to external influences (Harmey & Harmey, 1993), have been able to detect genetic variation that can be exploited or adapted for taxonomic and diagnostic purposes. Bolla et al. (1988) differentiated *B. xylophilus* pathotypes using restriction enzyme analyses and hybridization with total genomic DNA. Others have used cloned DNA hybridization probes from *C. elegans* (Abad et al., 1991), or *Bursaphelenchus*, based on ribosomal probes (Webster et al., 1990), DNA probes (Abad et al., 1991; Tàres et al., 1992) and satellite DNA (Tàres et al., 1994), for a more reliable characterization of the species, and for the differentiation of specific and intraspecific groups.

The development of the polymerase chain reaction (PCR) promoted the improvement of some of the previous methods, and the establishment of new methods where only small amounts of DNA are required. The amplification of specific genomic regions is a highly effective methodology to detect inter- and intra-specific variations among taxa. Species-specific DNA fragments have been amplified using primers derived from a cloned repetitive DNA sequence (Harmey & Harmey, 1993). ITS-RFLP has been used mainly for *Bursaphelenchus* species identification (Burgermeister et al., 2005; Metge et al., 2008), while other methods have been carried out for the specific-species detection of *B. xylophilus*, namely PCR-based diagnostics with species-specific primers (Kang et al., 2004; Matsunaga & Togashi, 2004; Li et al., 2004; Leal et al., 2005; Leal et al., 2008), real-time PCR assay (Cao et al., 2005), and PCR amplification using satellite DNA-based primers (Castagnone et al., 2005; Castagnone-Sereno et al., 2008).

Concerning the assessment of the relationships among isolates with different geographical origins the following molecular methods have been applied: sequencing of heat shock protein genes, hsp70 (Beckenbach et al, 1992), sequence of rDNA ITS regions (Iwahori et al., 1998; Beckenbach et al., 1999; Zhang et al., 2001; Kanzaki & Futai, 2002; Megte et al., 2008), sequence of D2 and D3 of the 28S gene (Zheng et al., 2003; Metge et al., 2008). The random amplified polymorphic DNA technique (RAPD) has also been used for the study of intra-specific variation of PWN isolates from China (Zheng et al., 1998; Zhang et al., 1999), Japan (Kusano et al., 1999), and a mixture of different geographical isolates (Braasch et al., 1995; Irdani et al., 1995a, 1995b; Wang et al., 2001; Zhang et al., 2002). Recently, a more integrated study has been conducted using several isolates each from the native regions (Canada and USA) and non-indigenous areas (China, Japan, Korea and Portugal) (Metge & Burgermeister, 2006).

4. PWN INTRODUCTION IN PORTUGAL AND THE EU

The way of introduction of the PWN to non-endemic areas has been primarily attributed to several hypotheses related with human activities, especially by the movement of infected wood products, between long (among continents and countries) and short (within a country) levels of distance. However, the short distance level of the disease spreading is attributed to the biological development of the insect vector as well. The genetic diversity of an exotic species in a new established area is always dependent on the diversity of the initial colonizers. An understanding of the role played in the Portuguese situation has been hindered by the lack of detailed studies from the isolates distributed in this region (Vieira et al., 2007).

The native forms of an organism are the major source of genetic variation, regularly displaying a higher level of genetic diversity when compared with those populations found in non-native areas and due to its artificial establishment. The effect of human activities on spreading the PWN into new areas is well documented, and variation on the PWN, at different levels, can explain a substantial part of the within-isolate variation observed from different geographical areas. Genetic variation among the PWN isolates is certainly not new. According to previous studies, the isolates collected from the USA and Canada exhibit a high level of diversity, the greatest level of diversity being reached among isolates collected in some areas of Canada (Iwahori et al., 1998). On the other hand, isolates found in the non-endemic areas express a low level of genetic diversity. Indeed, even in some of the non-native areas the genetic variation reaches some heterogeneity among some of the PWN isolates. Nevertheless, the degree of this variation could be limited by several hypotheses, i.e. the origin of the isolate (endemic area vs. non-endemic area), or by the number of introduced isolates. Furthermore, the number of individuals present in the infected wood products that reach the new site of infection could also limit the genetic variation of the initial introduction.

In Portugal, the extension of this genetic variation has not been clear. Recently, the origin of the PWN in Portugal was stated as being from an Asia region, and by a possible double introduction. If the introduction of this pathogen occured at least twice (even from non-native regions), different levels of genetic variability among the affected area in Portugal are to be expected, since a relative degree of variability in the Portuguese isolates was shown (Metge & Burgermeister, 2006). Still, this result might be due to a genetic shift of one of the isolates kept in fungal culture for a long period of time (Chapter II). The fact that the Portuguese *B. xylophilus* isolates show a high genetic similarity, using RAPD-PCR and satellite DNA clearly exclude the idea of a possible double introduction in Portugal (Vieira et al., 2007). Furthermore, the Portuguese isolates display a close genetic similarity with the East Asia isolate, confirming the results previously obtained by other authors (Metge & Burgermeister, 2006).

4.1. Dispersal of the PWN Within the Affected Area in Portugal

According to the data generated from other countries, the detection of the PWN is consistently coincident with port areas, associated with the trade of goods between countries. Initially the main concern came from those countries where the PWN was already naturally or artificially established. However, the report of several detections of PWN in wood products originating from PWN-free countries, increased the unpredictable introduction of this pathogen into new areas. It has been shown (Vieira et al., 2007) that the lack of genetic diversity among the PWN isolates in Portugal reflect a single introduction. Furthermore, the proximity of the international sea harbor in the Setúbal Península could determine the initial point of introduction.

The evolution of a forest disease within a country is guided by a widely studied framework involving two main processes: *i)* transport of contaminated wood by human activities and *ii)* biological development of the insect vector. In Portugal, the PWN distribution is limited to a relatively small area and no other detection has been reported outside this area. In addition, the insect vector species occurs throughout the affected area (Sousa et al., 2001; 2002). Such overlapping distribution of the insect vector coupled with human activity in moving wood may provide the main source of spreading of the pine wilt disease in Portugal.

5. CONTROL MEASURES FOR PWD

Controlling PWD and the PWN is not an easy task. The complex biological system (Fig. 3) involves knowledge concerning many aspects of the bioecology of both the nematode and the insect vector, coupled with knowledge regarding the degree of susceptibility/ resistance of the tree, as well as the environmental factors (climate and soil) that play a pivotal role in the development of the disease.

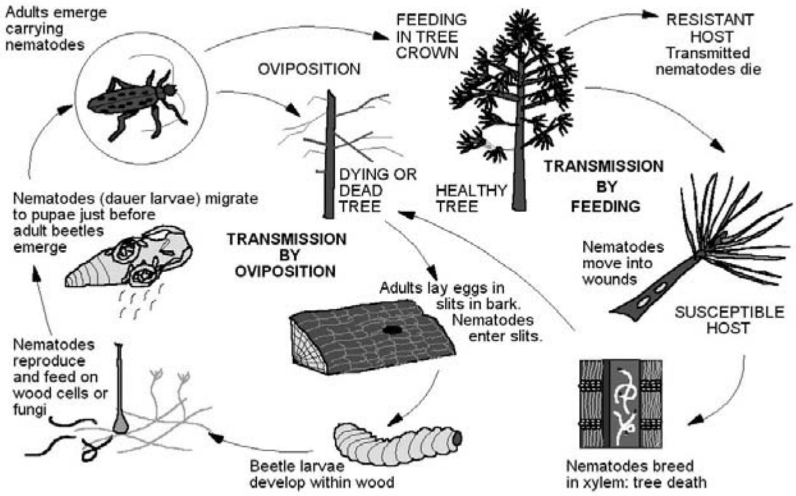

Figure 3. Schematic representation of the inter-relationships between the pinewood nematode, Bursaphelenchus xylophilus, *and its insect vector (*Monochamus *spp.) (adapted from* Evans, 1996*).*

Most of the knowledge and success regarding the control of PWD has stemmed from the dramatic Japanese experience during the XXth century, followed by the more recent experiments and results obtained from China and Korea. For details on the speific actions taken in these countries, see Mota and Vieira (2008). Europe, and namely Portugal, has limited experience concerning tactics and strategy for an effective and sustainable control of PWD, which is easily understandable due to the relatively recent (1999) detection of the PWN and the need to take immediate actions for prompt containment of the disease (Rodrigues, 2008). However, an urgent coordinated effort between research and forest authorities is badly needed in order to stop the spread of the nematode beyond the borders of Portugal. The European Union (EU) should also contribute to this effort, as a pre-emptive action, in order to avoid the appearance of the nematode in other Southern, or even Central European countries where climatic conditions, the presence of the insect vector and of several highly-susceptible pine species would be catastrophic for EU forestry.

5.1. Control Measures Before the Discovery of PWN as the Causal Agent

When the first outbreak of pine wilt disease occurred at Nagasaki in Kyushu Island in 1905, local people in Japan made considerable efforts to eradicate the epidemic forest disease, though they did not recognize PWD as an epidemic. The dead trees were felled down and debarked completely to stamp out the first incidence of the PWD by 1915. Pine wilt disease, however, recurred at a harbor town in Hyogo prefecture, in the western part of the mainain in 1921, and also at another harbor town, northern part of Kyushu Island in 1925 (Fig. 4). Then, PWD gradually spread surrounding regions year by year. In 1940s PWD remarkably expanded its distribution not only into surrounding regions but also to remote regions such as Shikoku Island and Kanto districts, eastern part of Mainland.

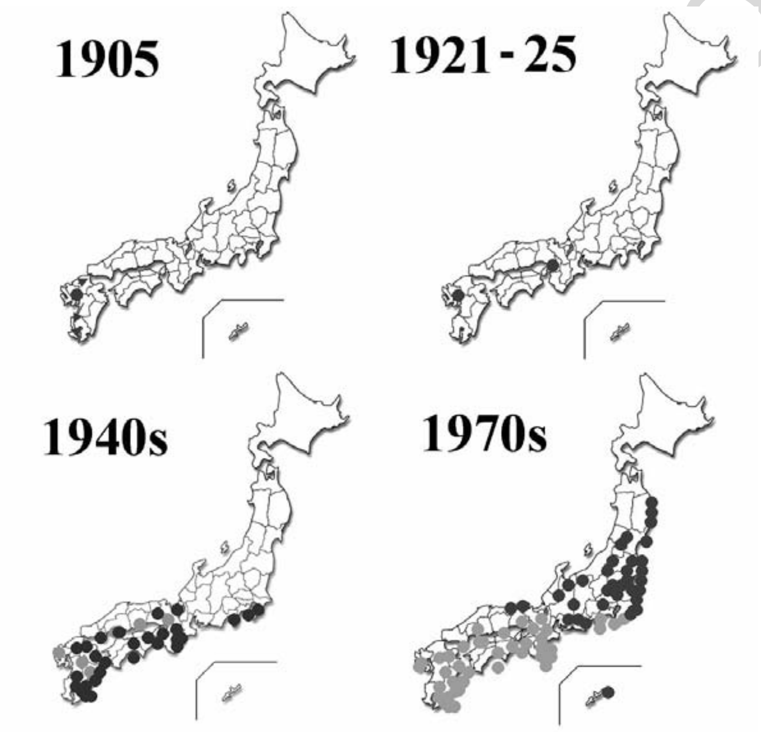

Figure 4. Spread of pine wilt disease (PWD) in Japan, during the XXth century. 1905: the first outbreak of PWD was reported from Nagasaki prefecture in Kyushu island. 1921-1925: in mainland Japan (Honshu island), the first occurence of PWD was reported from Hyogo prefecture. In1925, PWD recurred in a harbor town, 50 km apart from the first recorded place, and spread into the surrounding regions (grey dots). In 1940s: PWD spread over a wide area in southeastern Japan, and then moved to eastern Japan. In 1970s: PWD spread to a wide area of northeastern Japan.

Because of World War II, the Japanese people had to live very harsh times in the 1940s and therefore dead pine trees were apt to be left in stands. Furthermore, it became difficult to eradicate dead Japanese black pine, *Pinus thunbergii*, during wartime because the harbor area where black pines were dominating became restricted area, for military reasons. This background facilitated the vector beetle *Monochamus alternatus* to build up their population, and thereby remarkably increased the damage.

Before the discovery of PWN as the causal agent of PWD, most Japanese scientists had attributed the massive loss of pine trees to pine bark and wood borers.

So several measures such as felling and burning, immersion in water, and spraying insecticides were recommended to control PWD. The insecticides used in this period were carbon disulfide and chloropicrin.

After World War II, General Headquarters (GHQ) of the Allied occupation military was seriously concerned about the devastated pine forests, and charged a forest entomologist, Dr. R. L. Furniss, to inspect pine forests damaged by PWD. After intensive field survey and discussion with Japanese experts he submitted two reports indicating seven issues to be revised: *i)* to establish a special organization that would be in charge of controlling forest insect pests, *ii)* as a part of the organization, special survey crews should be involved in evaluating the exact status of the infestation so that control projects could be properly planned, *iii)* of several control measures adopted till then, the best available method under the conditions in Japan was felling, peeling and burning dead pine trees.

Other methods used so far were of no use, but immersing infested logs for several weeks was effective, *iv)* governmental subsidization should be limited to epidemic outbreak, *v)* to carry out the recommended control methods effectively, relevant statute should be modified, *vi)* to keep the population of forest insect pests under control, appropriate silvicultural treatments were needed, *vii)* more experts trained in forest management and protection were especially needed (Furniss 1950; 1951).

The GHQ adopted these recommendations and urged the Japanese government to implement the control measures recommended by Furniss. The extensive control efforts following Furniss's recommendations, together with plentiful labor available then, succeeded in reducing the damage. Thus, the annual loss of pine trees due to PWD was reduced in the 1950s and until early 1960s. The life style of public people in Japan, however, changed remarkably in this period and pine needles and fallen twigs that had been used as fuel and/or fertilizer became abandoned and thus accumulated, which contributed to eutrophication of the forest soil. Soil eutrophication damaged the mycorrhizal relationship of pine trees, and thereby imposed serious stress on pine trees. Annual loss of pine trees increased again in the middle to the later half of the 1960s.

To establish a control method for PWD, a new national project was organized (1968–1971). This project team found that the insect pests that had supposedly been the causal agent of pine death could not lay their eggs on healthy trees, and the trees had reduced resin exudation as an early wilting symptom before the attack of insects (Nitto et al., 1966; 1967). Therefore, the national project had to change the study target from insect pests to other unknown factors such as microorganisms, edaphic factors, meteorological factors, and so on. In 1968, Tokushige, a tree pathologist of the project team found *Bursaphelenchus* nematodes and confirmed its pathogenicity against pine trees by a well-designed series of inoculation tests (Tokushige & Kiyohara, 1969; Kiyohara & Tokushige, 1971).

After a massive search for vector insects, the Japanese pine sawyer, *Monochamus alternatus* was found to be the sole vector of the nematode, one which transfered pathogenic nematodes from dead to healthy pine trees (Mamiya & Enda, 1972; Iwasaki & Morimoto, 1972). When the complete infection cycle of PWD was thus clarified, traditional control measures were abandoned and new ones, which set the vector beetle as a target, were applied.

5.2. Control Measures After the Discovery of the PWN and its Vector, Monochamus alternatus

After the discovery of PWN and its vector beetle, various control efforts were focused mainly on the vector beetle, *Monochamus alternatus*.

5.2.1. Physical Control

Among physical control measures, "felling, debarking and burning" which are rather traditional control methods, are still effective to eradicate vector beetles. This method, however, is laborious, and entails danger of forest fires and may facilitate some thermophilic pathogens such as *Rhizina undulata* (Sato, 1974). To avoid danger of forest fires, dead trees felled down were also burried under soil or submerged in water, though either of these measures was more laborious than burning.

5.2.2. Chemical Control

Before the discovery of the PWN, control measures had been targeted at larvae of bark or wood borers inhabiting in dead pine trees. For control purposes, therefore, the most predominant chemical measure was sanitation spraying with such insecticides as BHC and DDT on the bark of felled pine trees. These, however, were banned for use against forest pests in 1971 because of its residual toxicity to mammals. To control the newly-found vector of PWD, various insecticides were examined, and organophosphate insecticides such as fenitrothion and fenthion seemed to be the most effective and were applied instead of BHC and DDT.

Based on the information of the infection cycle of PWD (Fig. 3), scientists recommended the use of insecticides preventively to living trees; when *Monochamus* beetles emerge from dead pine trees their reproductive organs are not yet matured (Katsuyama et al. 1989), and they therefore move to surrounding healthy pine trees to feed on the bark of young shoots and thereby they become reproductively active ("maturation feeding"). Meanwhile, pathogenic PWNs enter pine trees via feeding wounds made by *Monoachamus* beetles, and the trees ultimately become diseased. Insecticides such as fenitrothion and fenthion may be sprayed over the crown of pine trees. This measure does not kill vector beetles directly but protect living trees from feeding of *Monochamus* beetles, and so has been called "preventive spraying". When this new measure was applied by aerial spraying, however, public people, some scientists and some media opposed the application for fear that these insecticides would harm the environment.

When this preventive spray was applied to forests when and where PWD was rampant, healthy living trees would be protected from PWN infection, while trees that were asymptomatic carriers and those that have been infected beforehand in the season could become diseased and then be killed even after preventive spraying. These actions seemed to fail in controling PWD, and gave people and the media arguments against the government. So the national and local governments became very cautious in applying aerial spraying with insecticides, and carried out these actions just in limited areas and/or in limited periods with as little amount of insecticides as possible. The *Monochamus* beetle, however, could often fly a few kilometers or more. When the insecticide lost its toxicity, the beetles could visit the area from untreated surroundings and kill pine trees that had received insecticide beforehand. Thus cautious application made the measure more ineffective.

To reduce environmental damage by insecticides, fumigation with methyl bromide, EDB, NCS and so on was applied after dead pine trees were felled down, cut into small-sized logs, and piled up. This method is apparently laborious, and time-consuming. Discarding the vinyl sheets used for covering the pile of dead pine logs is another problem after fumigation.

Prophylactic trunk injection of a nematicide is alternative method to control PWD. A company has applied a vermicide (morantel tartarate) to living pine trees, and succeeded in protecting them against PWD infection. Some other chemicals such as levamisol hydrochloride, methyl phenphos, emamectin benzoate, milbemectine have also been used as candidates for trunk injection nematicides. Among them, emamectin benzoate, milbemectine and nemadectin are antagonists of gamma-aminobutyric acid (GABA)-receptor, morantel tartarate and levamisol hydrochloride are muscle activity blockers, and mesulfenfos is an acetylcholine esterase inhibitor. Thus, these chemicals used for trunk injection were not necessary to kill nematodes in pine tissues, but may disturb nematode activity and/or reproduction, thereby facilitating host resistance against PWN. This measure (trunk injection) is very effective to control PWN, but the cost of the chemicals and that for manpower are so expensive that most owners of forests hesitate to use this measure.

5.2.3. Biological Control

To reduce application of insecticide for PWD control, some natural enemies have been examined as biological control agents against the vector beetle (further indicated as M), and PWN (further indicated as N). Among them were woodpeckers (M), predaceous insects (M) such as *Trogossita japonica*, *Dastarcus longulus*, and parasitoid insects (M) such as *Sclerodermus* spp.

Entomoparasitic fungi (M) such as *Beauveria* spp. have been examined their effects in control *Monochamus* beetles (Fig. 5). These fungi seem to be effective, but it is often difficult to apply in the field because of indirect contamination of other useful insects such as silk worm and honey bee. Trapping fungi (N) and entomopathogenic nematodes (M) have also been examined for their ability to control PWN and the vector beetle, respectively. These biological control measures have not yet been practiced because these require more cost and labor than chemical ones. Exception is the case of *Sclerodermus* species in China, which have been reproduced in bulk and applied to pine forest with successful result (Fig. 5).

5.2.4. Breeding of Resistant Hosts

Trees of the genus *Pinus* propagate predominantly by sexual reproduction, so genes are mingled by pollination every year. Thus genetic diversity is very high among progenies. Host resistance against PWD takes advantage of genetic diversity, various among individual pine trees. Host resistance against PWD seems to be determined not by a single gene but by multi genes, though the host resistance mechanism has not yet been elucidated.

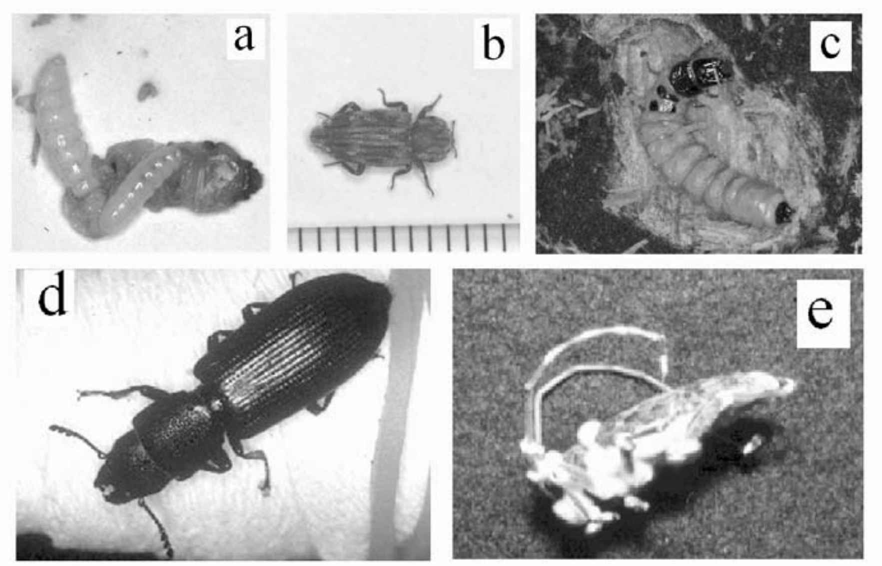

Fig. 5. Natural enemies of the Monochamus *vector:* Dastarcus longulus *(a, b) ;* Trogossita japonica *(c, d);* Monochamus *beetle infected with* Beauveria *sp. (e).*

When PWD rages fiercely through a pine stand, several surviving trees may remain due to a somewhat higher resistance. From such remaining pine trees, scientists have collected scions or seeds to breed resistant clones. When these candidate plants (grafts or seedlings) grow enough to serve for inoculation tests, they are inoculated once with the PWN, then the surviving plants receive another inoculation.

Pine grafts or seedling surviving two inoculation tests are regarded as resistant clones. Since the beginning of this project in 1978, 135 and 41 plants have been selected so far as resistant clones against PWD for *P. densiflora* (Japanese red pine) and *P. thunbergii* (Japanese black pine), respectively. These resistant clones have been propagated by grafting and cutting, and the resulting seedlings are being distributed over various regions of Japan. This tactic seems to be a reasonable way to make Japanese pine forests more resistant, but can not protect pine trees being exposed to PWD at present. As in the case of Dutch elm disease, and plant parasitism by *Meloidogyne* spp., once resistance-breaking individuals develop within the PWN population, resistant clones obtained after long selection procedure may be easily defeated.

6. A BLIND SPOT IN PWD CONTROL STRATEGY: THE ASYMPTOMATIC CARRIER AND ITS SOPHISTICATED DETECTION METHOD

To prevent pine wilt disease (PWD) from spreading over pine forests, elimination of pine trees killed by PWN is desirable, although this method is very laborious and time-consuming. If such dead trees are left in the field, pathogenic nematodes and their vector, *Monochamus* beetles, could spread from tree to tree without any difficulty. In the Kyoto University arboretum, where many precious foreign pine species are planted in the field, all pine trees killed by PWD have been eradicated thoroughly before the next pine wilt season.

Despite intensive efforts in removing dead trees from the stands, new dead trees tend to appear in the vicinity of the stumps of trees killed in the previous year, and wilting recurs in the same pine stand every year. To understand the reason why PWD recurs at the same stand even after thorough eradication of dead pine trees, a long-term survey at a stand of Korean pine, *Pinus koraiensis*, has been undertaken, and thus revealed the important role of asymptomatic carriers in spreading PWD to surrounding pine trees. When PWD-infected pine trees survive asymptomatically, and begin the symptom appearance far later than usual and overlapped with the following season of the beetles' activity, such trees could play a role as strong attractants to the vector beetles, posing a danger to pine stands (Futai, 2003).

To remove asymptomatic carriers from pine forests, a rapid and accurate detection of the PWN is needed. The population of PWN in asymptomatic trees is generally too low to be detected by traditional methods such as the Baerman funnel method. To detect low densities of PWN from living pine trees, a new diagnostic method based on a simple DNA extraction and nested-PCR has been developed (Takeuchi et al., 2005). This new method has been applied to two natural stands (Japanese black pine and a Japanese red pine) and found that many trees of either pine species contained PWN, though some of them displayed no external and/or internal symptoms (Takeuchi & Futai 2007). Thus some trees of Japanese black and red pine survived for one or more years after PWN infection without any symptoms, suggesting that they may have been overlooked during eradication, and may play a role in initiating new PWD occurrences.

7. CONCLUSIONS

Pine wilt disease constitutes a major threat to forest ecosystems worldwide, both from the economical point of view as well as from the environmental (landscape) perspective. In countries, such as Japan, China and Korea, where the disease is present and the pinewood nematode well established, forest authorities have undertaken extensive and very costly efforts to contain the disease, and to prevent further spread. In many cases, these actions have not been successful due to the high susceptibility of the tree species and the agressive virulence of the nematode. In

Kyoto, Japan, for example, some large areas of local pine species have simply been replaced by other tree species such as oaks. In other more localized situations, such as religious temples or national scenic sites (e.g., Amanohashidate, Kyoto), PWD control programs using various approaches (resistance varieties, chemical control, etc..) have been successful, albeit at a high economical cost, but defrayed by the high cultural and environmental value.

The relatively recent detection of the nematode in the EU (Mota et al., 1999), poses a serious threat and challenge to European forestry officials and national plant protection authorities. Although the nematode is present, for the time being, in Portugal, the EU must maintain a continuing effort in: *1)* supporting surveying and control measures in Portugal; *2)* increasing the level of inspections at ports of entry, namely sea ports, in order to guarantee a rigorous interception of potential sources of PWN from non-EU countries; *3)* establishing a European network of diagnostic labs; *4)* establishing a EU-level research network involving the major scientific centers, to study the bio-ecology of the nematode and insect vectors, as well as the natural conditions that may enable the establsihment of the PWN in other areas of the EU.

The issue of PWD is one that constitutes a good example of the urgent need for a concerted action, not only at the EU level, but also worldwide due to the important economical sector of wood trade.

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