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Pine Wilt Disease in Portugal

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6.1 Introduction

In Europe, species of the nematode genus *Bursaphelenchus* have been known and studied for a long time (Fuchs 1937; Ruhm 1956). Earlier, except from a purely biological or ecological point of view, no particular interest was paid to this group of mycophagous nematodes. In 1979, however, a study conducted in southwestern France showed that the nematode *Bursaphelenchus lignicolous* was associated with declining pines (Baujard et al. 1979). This report caused alarm in Europe, since *B. lignicolous* is a synonym *of B. xylophilus*; the nematode in question was later identified as *B. mucronatus* (De Guiran et al. 1986), which had been described as a new species that year. In 1984, a shipment of wood from North America to Finland was found to carry the pine wood nematode (PWN), *B. xylophilus* (Rautapaa 1986). This important interception prompted European authorities to develop more rigorous inspections at sea ports, and in particular of wood products coming from North America. However, no equivalent emphasis was placed on such products coming from East Asia. Between 1996 and 1999, an EU-funded project (RISKBURS) resulted in an updated survey of the *Bursaphelenchus* species in Europe (European Communities 2003). For an updated situation on the species distribution in the EU, see Braasch (2001). In 1999, the PWN, the causal agent of pine wilt disease, was first detected in the European Union (EU), in Portugal (Mota 2004; Mota et al. 1999), and this immediately prompted several national and EU governments to assess the extent of the nematode's distribution, and to restrict *B. xylophilus* and its insect vector (*Monochamus galloprovincialis*) to an area with a 30-km radius in the Setúbal Peninsula, 20 km south of Lisbon (Rodrigues 2007).

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The origin of the population of PWN found in Portugal remains unknown, although recent research indicates that it originated from Eastern Asia (Vieira et al. 2007). Several hypotheses have been suggested on how it entered the country, namely from North America or from Japan or China. World trade of wood products such as timber, wooden crates, and palettes play an important role in the potential dissemination of the PWN (Evans et al. 1996). In fact, human activities involving the movement of wood products may be the single most important factor in PWN spread. Despite the dedicated and concerted actions of government agencies, both the PWN and pine wilt disease continue to spread. In 2006 in Portugal, forestry and plant quarantine authorities (DGRF and DGPC) announced a new strategy for managing the problem. The plan is to establish a phytosanitary strip, 3-km wide, devoid of *Pinus pinaster*, surrounding the affected area, for the control and ultimately the eradication of the nematode, under the coordination of the national program for the control of the PWN (DGRF 2006). Research on the bioecology of the nematode and its insect vector, new detection methods, for example, involving real-time PCR, tree ecology and pathology, and control methods, has been underway since 1999. As well there are two major ongoing projects for the European Union (EU): PHRAME (http://www.forestresearch.gov.uk/website/forestresearch.nsf/ByUnique/INFD-63KGEF) and PortCheck (http://www.portcheck.eu.com/index.cfm). This research has been instrumental in helping to understand the scientific aspects of pine wilt disease. The objective of the present paper is to highlight the progress made in Portugal and the EU. International agreements (GATT, WTO) and sharing of scientific information is of paramount importance for achieving effective control of the nematode and its vector, and in turn protection of our forest ecosystems and forest economies.

6.2 General Surveys

With the discovery of PWN in Portugal in 1999, all EU governments were prompted to conduct general surveys for the nematode in their pine forests. Although some surveys had been conducted prior to 1999 (Braasch et al. 2000), it was not until then that new records of *Bursaphelenchus* species plus descriptions of new species began to appear (Braasch 2001; Escuer et al. 2004; Kulinich 2004; Magnusson et al. 2004; Michalopolous-Skarmoutsos et al. 2004; Penas et al. 2004, 2006a, b). These activities, funded mainly by national governments and the European Community, have been extremely useful in monitoring for the presence of PWN and also in gaining a better understanding of the diversity of the genus *Bursaphelenchus*. The results of these surveys are assessable on the EU web page on the PWN. In Portugal, such surveys have been undertaken in the affected area on the Setúbal Peninsula, near Lisbon and outside the affected area (Rodrigues 2007; Mota and Vieira 2004). These results are available on the PROLUNP web site (DGRF 2006). To date the PWN has only been found within a well-defined area in Portugal, but not elsewhere in the EU.

6.3 PWN Taxonomy and Biology

The genus *Bursaphelenchus* was established in 1937 by Fuchs, with *B. piniperdae* Fuchs, 1937 as the type species. To date, more then 80 valid *Bursaphelenchus* species have been described worldwide (Vieira et al. 2006; Ryss et al. 2005), mainly in the northern hemisphere. However, after 1999 26 new species have been described, frequently from Asia. Although 28 *Bursaphelenchus* species are known from Europe (Braasch 2001), no research had been done on this genus in Portugal before detection of the PWN in 1999. Since then, several studies have been made to determine the species' diversity and distribution within Portugal. Although the survey has focused mainly on maritime pine (*P. pinaster*), nine other *Bursaphelenchus* species, besides *B. xylophilus*, have been identified and characterized based on morphological characters and by using molecular biology tools (ITS-RFLP, 18S and 28S rDNA D2/D3 domain sequences, RAPD-PCR) (Vieira et al. 2007; Penas et al. 2004, 2006a; Mota et al. 1999). The *Bursaphelenchus* species, namely *B. hellenicus*, *B. leoni*, *B. mucronatus*, *B. pinasteri*, *B. sexdentati*, *B. terastospicularis*, *B. tusciae* (Penas et al. 2004) mainly occur in north and central Portugal, coinciding with the occurrence of the major pine forests. Recently, a new *Bursaphelenchus* species, *B. antoniae*, has been described (Penas et al. 2006). To determine the insect vectors of *Bursaphelenchus* spp. associated with *P. pinaster*, bark- and wood-boring insects belonging mainly to the families Cerambycidae, Scolytidae, Buprestidae and Curculionidae (Coleoptera) were captured from specific locations in Portugal, and their associated nematodes identified using ITS-RFLP analysis of dauer juveniles and morphological identification of the adults (Penas et al. 2006). Several insect-nematode associations have beed identified such as *B. teratospicularis* and *B. sexdentati* associated with *Orthotomicus erosus*; *B. tusciae*, *B. sexdentati* and/or *B. pinophilus* with *H. ligniperda* and *B. hellenicus* with *T. piniperda*, *I. sexdentatus* and *H. ligniperda*. Other nematode genera besides *Bursaphelenchus*, which were found associated with the insects included two species of *Ektaphelenchus*, and one species each of the genera *Parasitorhabditis*, *Parasitaphelenchus*, *Contortylenchus*, and other unidentified nematodes. Of major interest is the molecular characterization of the PWN isolates within the affected area, using rDNA genes, and in particular the ITS regions (ITS-1 and ITS-2) of rDNA. Intra-specific variability using RAPD–PCR techniques has proven very useful for evaluating genetic distances and for helping develop phylogenies and pathway analysis of world populations of the PWN (Burgermeister et al. 2005; Metge and Burgermeister 2007; Ye et al. 2007). Recent studies (Vieira et al. 2007) have focused on the genetic diversity of *B. xylophilus* in Portugal using RAPD–PCR techniques for evaluating intra-specific variation. The results show that there is no significant genetic variation among PWN isolates from the pine wilt disease affected area. Also, the data seems to suggest a recent and single PWN introduction into Portugal; however, since no correlation could be made between the genetic and geographic matrices, it seems that this technique is not useful in such studies. Nonetheless, the studies have shown that the Portuguese isolates display a much lower genetic distance to Asian isolates, especially from China as compared to North American isolates (Vieira et al. 2007). Recently the use of satDNA as a marker for *B. xylophilus* has been developed in the EU (François et al. 2007; Castagnone-Sereno et al. 2007); and it may be a useful tool for intra-specific analysis of the PWN and, together with mtDNA, provide additional information on the geographic spread of he nematode. Such information would supply important information for pathway analysis (P. Vieira, personal communication). Also, PCR using a "real-time" technique (Leal et al. 2007) has been developed and may be very useful when field equipment becomes available for use by quarantine authorities. This is a main goal of the EU "PortCheck" project mentioned above; initial ring-testing, utilizing a species-specific probe based on the MspI satellite DNA family, has already been done in Portugal. The results have demonstrated great precision in discriminating *B. xylophilus*, however the issue of a reliable sampling method from the tree remains to be solved. Regarding PWN biology, and specifically cytogenetics, progress has been made in clarifying the basic chromosome number of the species ($n = 6$) and also the possible sex determination mechanism (XX–XY) (Hasegawa et al. 2006). Ongoing research should hopefully provide more detailed information on PWN cell division and developmental biology processes, and also the possibility of triploid populations (K. Hasegawa, personal communication).

6.4 Insect Vector, *Monochamus* spp.

Initial surveys done in Portugal (Sousa et al. 2001, 2002) demonstrated that the cerambycid beetle *Monochamus galloprovincialis* was the only insect vector for the PWN. Elsewhere, studies have focused on surveying insects for the presence of nematodes, tree-insect acoustics, and molecular taxonomy (Garcia-Alvarez et al. 2007; C. Tomiczek, personal communication; Vincent et al. 2007). Research on the bioecology of the insect vector in Portugal has provided much data such as how the nematode enters the vector, PWN population dynamics inside the vector (Naves et al. 2006a), feeding and oviposition (Naves et al. 2007, 2006b), flight patterns, effectiveness of lures and traps, reproduction (Naves et al. 2006c), and also on natural enemies such as parasitoids (Naves et al. 2005). The latter paper includes a review of parasitoids from East Asia and North America. The reproductive potential of *M. galloprovincialis* in Portugal seems to be less than elsewhere (Naves et al. 2006c). Studies on feeding and oviposition demonstrate that in Portugal *Pinus sylvestris* and *P. halepensis* are also good hosts for the PWN (Naves et al. 2006b). Laboratory studies on PWN transmission to *P. pinaster* by *M. galloprovincialis*, have provided valuable information including the fact that the critical period for nematode infection to the tree is within the first few weeks of beetle emergence. This type of information is particularly useful for control measures (Naves et al. 2007). Sousa et al. (2007) provide an overview of recent studies on insect vector biology and ecology.

6.5 Disease Modeling and Climate

Earlier, a pest risk analysis (PRA) had been done regarding EU concerns about wood shipments arriving in Europe (Evans 1996). However, this PRA was done without PWN actually being present in Europe. After 1999 the need arose for a new PRA with the inclusion of new perspectives and parameters. The EU funded an eight partner (six nation) consortium, in 2002, designated "PHRAME" (Development of improved pest risk analysis techniques for quarantine pests, using the PWN in Portugal as a model system) which was supposed to, among other things, establish a predictive model and sub-models, based on numerous parameters related to climate, the nematode, the soil, the insect vector, for risk evaluation in the EU. One goal within this project was "process-based modeling" (PBM) on the plant system and which considered a mechanistic approach to analysing the disease progress, based on water potential and carbon availability (Evans et al. 2007). Another approach has been to use spatial modeling, based on a set number of eco-climatic variables (Pereira and Roque 2007). The results from these models are still being tested, both in the laboratory and the field, and will hopefully continue during the coming years. The recent concerns over global warming and climatic changes may constitute an increased threat to pine forests in Portugal (J. Corte-Real, personal communication).

6.6 Pathogenicity

In Portugal, *Pinus pinaster* is most likely the only known host as it is the preferred pine upon, which the insect vector does maturation feeding. However, *P. sylvestris* and *P. halepensis*, are also fed upon by *M. galloprovincialis* (Naves et al. 2006b), but their abundance and distribution is relatively limited in Portugal. In nature, *P. pinea* is not affected by PWN since the vector, *M. galloprovincialis*, does not feed upon or colonize the tree. Recently, preliminary results (M.M. Mota et al., unpublished data) have demonstrated that PWN can invade, multiply in, infect and kill *P. pinea*, but less quickly than for *P. pinaster*. Inoculation studies using Portuguese and Japanese PWN isolates on *P. thunbergii* (Mota et al. 2006) show more virulence (i.e., greater mortality) for one Portuguese isolate (T) to Japanese black pine. Other recent studies have dealt with PWN distribution and migration behavior when inoculated into a susceptible host (Daub et al. 2007). Until now, and although studies have been made on the development of disease and of resistance factors, no single report has been made on the plant expression (mRNA, proteins) when in the presence of the PWN. This information is essential for understanding the pathogenicity of PWN on pine species and also for making decisions on reforestion practices. In 2007–2008, under a COST program (COST 872: http://www.cost.esf.org/index.php?id=181&action_number=872), it is expected that some progress may be made in this area, in Portugal and elsewhere in the EU.

6.7 Other Pine Wilt Disease Research

A deluge of information is a common feature of research in any scientific area. Today it is extremely difficult to keep abreast of all the relevant literature in a particular field. Also, it is important to establish new taxonomic keys, based mainly on computer technology and using specific algorithms. Regarding taxonomical databases, much effort has been put forward during the past few years to access all published descriptions of all *Bursaphelenchus* species, in pdf format, making it less time consuming to access such documents (Eisenback et al. 2007; Vieira et al. 2006, 2004). Also, programs such as EndNote or RefManager have become increasingly helpful in organizing bibliography information. Regarding taxonomical keys, a new system, based on the simultaneous use of several morphological and morphometric characters (polytomous key) has been developed for the identification of *Bursaphelenchus* species (Ryss et al. 2004).

Another important issue concerns quarantine and international borders (Braasch and Enzian 2004). Joint collaborations between the EU and neighboring countries such as Turkey and Russia have been instrumental in helping prevent the spread of the PWN and its insect vectors (Akbulut et al. 2006; Kulinich 2004).

6.8 Future Outlook and Conclusions

1. The pinewood nematode continues to pose a threat to forests worldwide (Vieira and Mota 2007). International trade and globalization, despite its great economic benefits, carries a certain amount of risk in introducing new pathogens and pests to countries and continents.
2. In recent years, the EU has particularly felt this risk with the introduction of the PWN into Portugal. We still do not know exactly where the nematode originated, although preliminary scientific results point to an East Asian source.
3. It is only through the mutual cooperation and exchange of scientific and technical information that these issues can be minimized or resolved. Also, the basis for decision-making must always be based on scientific information.