QLK5-CT-2002-00672: Development of improved pest risk analysis techniques for quarantine pests, using pinewood nematode, *Bursaphelenchus xylophilus*, in Portugal as a model system

PHRAME – Plant Health Risk And Monitoring Evaluation

FINAL REPORT, JULY 2007

Chapter 1 Membership of the PHRAME Consortium and authorship of Chapters and Sections

The staff members for each organisation in the PHRAME Consortium contributed to data gathering, experimentation and to analysis and writing up of results. The Principal Scientist for each organisation coordinated the activities of the research teams and provided continuity and linkage with the Coordinator and other participants. The membership and authorship of chapters and sections in the Final Report is shown in Table 1.

Table 1: Participants and Chapter/Section contributions to the Final Report of PHRAME

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Chapter 2 Introduction

2.1 *Background and context*

Increased volumes of traded goods consequent on rapid transport systems by air, land and sea have brought with them associated risks from the transportation of pests between countries and ecosystems. Some of these pests are carried on plants or plant products and range from those that are visible on the substrate and others that are cryptic and well hidden from cursory inspection. Although essential in making decisions to safeguard against international movement of plant pests, development of Pest Risk Analysis (PRA) techniques is still in its infancy, despite international collaboration within the context of the International Plant Protection Convention (IPPC) and Regional Plant Protection Organisations (RPPOs) world-wide. This uncertainty is emphasised further when dealing with new pest infestations where knowledge of the risk factors may be limited and, hence, decisions on pest management are constrained and may not be the most appropriate.

This is the case in Portugal where the discovery of pinewood nematode, *Bursaphelenchus xylophilus*, has led to development of pest management strategies that have, of necessity, been based on limited information and which make the task of managing the threat very difficult. Refinements in the PRA process are required to aid this management requirement and increase confidence in assessing further risks and consequences of management actions applicable to Portugal and the rest of Europe.

This report summarises the main findings, partially funded under European Union Framework 5 research initiatives, of a collaborative research programme under the acronym PHRAME (Plant Health Risk And Management Evaluation). The main purpose of the research programme was to develop scientifically verifiable methods to enable the current high levels of uncertainty in Pest Risk Analysis to be reduced, thus giving confidence in proposing quarantine and phytosanitary measures that are justifiable and minimise recourse to the Precautionary Principle.

The research programme has addressed the twin objectives of improving the process of Pest Risk Analysis within the Plant Health arena for the EU as a whole and of developing knowledge-based pest management strategies consequent on improved PRA methodology. The combined experimental and observational approach employed by the participants in this project have utilised the unique opportunity that has arisen with the discovery, in 1999, of *B. xylophilus* in Portugal. It has enabled the research group to address known weaknesses in PRA technology arising from the lack of direct knowledge of the ecological and socio-economic aspects of the introduction of PWN to Europe. Integration of more traditional techniques of survey and gathering of biological information with new techniques such as molecular identification, Geographic Information Systems and use of ecological and process-based models have advanced knowledge of this important pest with both European and global contexts.

2.2 *The pest problem*

Pinewood nematode [PWN] (*Bursaphelenchus xylophilus*) is a saprophytic organism, in the Parasitaphelenchidae family, usually exploiting dead or dying coniferous tree species. It is native to North America, where it is widespread, but it has been distributed internationally through trade and its present geographical distribution ranges from Japan, Korea, Taiwan to China and also, since 1999, Portugal[1]. A key element of its life history, which is the initial determinant of its ultimate impact on living potential host trees, is maturation feeding by adults of its principal distribution vector, longhorn beetles of the genus *Monochamus*, (Coleoptera: Cerambycidae). During the feeding phase in the crowns of living trees, the vectors can introduce *B. xylophilus* into healthy

[1] http://www.eppo.org/QUARANTINE/nematodes/Bursaphelenchus_xylophilus/BURSXY_map.htm

trees, which in its native range has no apparent effect on plant activity (Futai and Furuno 1979; Rutherford and Webster 1987). Unusually, and in a number of geographical regions, PWN has been identified as the causal agent for the death of mature, healthy host plants within a short time after introduction to the living tree following maturation feeding by adult vectors (Mamiya and Enda 1972; Linit 1989).

The symptoms of rapid wilting of trees have led to the description of the syndrome as Pine Wilt Disease [PWD] (Kiyohara and Tokushige 1971; Mamiya and Kiyohara 1972; Kishi 1995). The nematode is now known to be responsible for large-scale tree death in Japan, China, Korea and, since 1999, Portugal, and is identified as a major driver to successional processes in semi-natural stands (Fujihara et al. 2002), and a corresponding source of considerable financial loss to the timber industry as well as imposing restrictions on movement of conifer wood in international trade (EPPO 1986; EPPO 1989).

2.3 *Approach adopted*

Critical to current and future management of this important pest, is improved understanding of the components of the system in the context of the current, ongoing, strategy to locate and, ideally, eradicate the pest from Portugal. The Portuguese strategy commenced with very little knowledge, including limited information on the identity of both host trees and of vectors of the nematode. It has, therefore, been essential to gather knowledge quickly and to integrate the research programme with the evolving strategy of the Portuguese Authorities. After initial intensive surveys which covered not only the area where *B. xylophilus* was detected, but the rest of the country, the affected area was found to be concentrated in the Setúbal region, SE of Lisbon. Management of the PWN threat is coordinated through the National Program of Pinewood Nematode Control (PROLUNP, “Programa Nacional de Luta contra o Nemátode do Pinheiro”). The affected zone has been delimited by annual surveys and by a buffer zone of about 20 Km width which completely surrounds it, the whole being subject to a survey, fell and destroy strategy to remove symptomatic trees and reduce the incidence of both PWN and potential vectors. Of necessity, the identification of PWN-infested trees has been based on ground-based visual surveys for the presence of putative symptoms, which then need to be destroyed before emergence of the next generation of nematode carrying vectors. Information on the scale and extent of the management strategy is available on-line

The research reported here includes the latest information on the vectors of PWN in Portugal and their interactions with the nematode in the pine systems in the country. Detailed studies of the biology and interactions of the nematode, vectors and host trees have been carried out using a range of experimental and observational techniques.

The key purposes of the study have been to carry out research and report on the:

- Identification and distribution of nematodes in the genus *Bursaphelenchus* in Europe
- Identification and biology of vectors of *Bursaphelenchus xylophilus* in Europe, particularly the Iberian Peninsula
- Biology and pathogenesis of *Bursaphelenchus xylophilus*
- Pinewood Nematode, its vectors and host trees
- *Bursaphelenchus xylophilus* dynamics in Portugal and development of control methods
- Molecular identification of *Bursaphelenchus* species and pathway analysis of *B. xylophilus* introduction to Portugal
- Ecoclimatic risk factors and modelling of the likelihood of wilt expression

These major research areas are described in individual chapters, each providing a self-contained presentation and analysis of the main topics studied. Although, they are presented as separate chapters, the topics are inter-related and provide supporting data for integration within the overall predictive and modelling approach. These jointly contribute to a new process of risk analysis that is process based and should be employable for pinewood nematode and other pests.

Chapter 3 Identification and distribution of nematodes in the genus *Bursaphelenchus* in Europe

3.1 *Introduction*

After the detection of the pine wood nematode in Portugal in 1999 the official authorities implemented an intensive survey which covered not only the region where *B. xylophilus* was detected but also other areas potentially at risk in the country, integrated within the National Program of Pinewood Nematode Control (PROLUNP, "Programa Nacional de Luta contra o Nemátode do Pinheiro"). Later this quarantine organism was confirmed to be restricted within an area (affected zone) in the Setúbal region, SE of Lisbon. The affected zone has been delimited by annual surveys and by a buffer zone of about 20 Km width which completely surrounds it, being also included in the Demarcated Region which is subject to restrictive quarantine measures.

During the annual nematode surveys conducted both in and outside the affected zone several other nematodes of the genus *Bursaphelenchus* have been found. Some of these species are quite difficult to differentiate, as they display similar morphological characters which confound precise identification, which is a crucial step in establishing quarantine measures for the different species. The purpose of this research was to contribute to the survey and identification of the *Bursaphelenchus* species associated with maritime pine in Portugal, by the use of classic morphological and biometric methodologies, along with molecular analysis (PCR-ITS RFLPs). Furthermore, the interactions between nematodes of the *Bursaphelenchus* genus and bark and wood-boring beetles found on maritime pines have also been studied.

Since it was not certain that *B. xylophilus* was restricted only to Portugal, nematode surveys were carried out in other countries, particularly Austria, France and Spain that were closest to the affected zone. Although not part of the current study, all EU Member States have also been carrying out surveys for PWN and, so far, there has been no evidence of the presence of *B. xylophilus*.

3.2 *Materials and Methods*

The methods used, especially for extraction of nematodes from wood samples, are common to all participants in the research programme, unless specifically indicated otherwise.

3.2.1 Nematode sampling and isolation

An extensive nematode national survey was carried out annually in continental Portugal, made in accordance with the official responsible for the PROLUNP programme in order to determine any possible infestation by the pine wood nematode outside the demarcated area (the area where nematode presence is confirmed). All wood samples were examined in order to determine the occurrence of any species of the genus *Bursaphelenchus.*

The samples were collected according to the following plan:

- Forty four "risk areas" were pre-defined according to the occurrence and accumulation of wood risk material in forest areas or storage places. These areas were delimited by a 5 Km radius circle, having as centre the site of wood concentration and/or circulation of wood material;
- The survey was carried out in a fixed number of 32 forest plots within each risk area, to concentrate 16 plots in a circle with radius of 1,5 Km and the rest dispersed over the remaining area;

- Within each plot the samples were collected from a maximum of 50 trees with decline symptoms, using a drill at DBH level, and enclosed in plastic bags, individually or in mixed sample (maximum of 5 sampled trees in close proximity);
- A total of 5587 samples were collected, 3914 from the Demarcated zone and 1673 from the remaining area.
- The samples were processed by Baermann technique for 48 hours and the *Bursaphelenchus* species identified under a microscope.
- From each sample, the nematodes extracted were observed using a stereoscopic microscope and *Bursaphelenchus* species were isolated for detailed characterisation and identification.

To support the morphological and molecular identification, cultures of the different nematode populations were initiated and maintained in non sporulated *Botrytis cinerea* and *Monilinia fructicola* fungi. The following nematode species found in Portugal, *B. antoniae*, *B. hellenicus*, *B. pinasteri*, *B. sexdentati*, *B. tusciae* and *B. xylophilus* were maintained in Petri dishes cultures of these two fungi at room temperature.

In Austria, during the period 2004 to 2006, similar surveys were carried out but using slightly different selection criteria for sampling of trees. Samples of freshly dead or still living coniferous trees with decline symptoms were taken from *Pinus sylvestris*, *Pinus nigra*, *Abies alba* and *Larix decidua*. The sampling took place in forest stands with dying conifers with no identifiable cause, in forest stands with dying conifers with decline symptoms of beetle attack (longhorn beetles, bark beetles, jewel beetles, weevils) and blue stain fungi within the sapwood, at locations of high risk, and at campsites with round wood present.

During the monitoring period of February to October 2004, 42 wood samples were taken in the whole federal territory of Austria. The samples comprised 24 from *Pinus sylvestris*, 9 from *Pinus nigra*, 9 from *Abies alba*, and 1 from *Larix decidua*. During the monitoring in 2005, 23 wood samples were collected using the same criteria as in the year before. Eighteen samples were taken from *Pinus sylvestris* and five from *Pinus nigra*. In 2006, fourteen samples were collected using the same criteria as in the years before. Eight samples were taken from *Pinus sylvestris* and six from *Pinus nigra*.

In Spain, the guidelines established in the EC protocol were followed for sampling, in compliance with the 2000/58/EC Decision of January 11, 2000. Sawmills and timber factories, stands near points of timber handling, conifer stands near ports and national borders (PIFs) as well as entry points and high-risk lines of transportation were chosen as high risk areas.

Samples were taken from factories, transformation industries and sawmills whenever possible, differentiating between national and imported timber. Declining trees were sampled that presented bluestain symptoms and/or emergence holes. Five samples per tree were taken giving a total of 150 g (30 g per sample). Samples were taken with a 2 cm diameter drill. Samples of shavings and sawdust were also taken from various points from the stacks (150 g total). At the same time, samples of the nearest stands around the factories, sawmills, etc. were taken in a radius of 5 km, sampling 5 trees with or without symptoms. A drill was also used for taking samples, gathering 30g per tree, for a total of 150 g.

On highway routes leaving high risk areas, stands of *Pinus* spp. were examined (in an approximate radius of 5 km) and samples were taken in the same way as in previous cases from 5 trees where symptoms were observed. In areas that presented symptoms and which were not placed in previous sections, samples were taken from 5 trees using the same methodology.

The wood samples were collected in plastic bags, properly identified to be sent to the laboratory where they were incubated at 25ºC for two weeks before extraction. Nematodes were extracted through the modified Baermann Funnel Technique. Once collected, the nematodes were heat

killed at 65°C and fixed in Formalin: Acetic acid. Nematodes were mounted in Amman lactophenol or in anhydrous glycerin to be studied.

3.2.2 Morphological characterisation

In all cases, nematode identification was based on observations of the main morphological characters, particularly vulval flap, spicules shape, female tail, number and disposition of caudal papillae, number of incisures in the lateral field and head shape (Braasch, 2001) using light (LM) and scanning electron microscopy (SEM).

Precise equipment and methodology varied in each country. In Portugal, nematodes were observed and identified in temporary mounts using an Olympus BX51 light microscope. For a better spicules observation at LM, a new staining method was tested with the use of Rotring® Brilliant Ultramarine Blue ink (solution 1 = one 0.5 ml cartridge in 20 ml of acetic acid; prepare a final solution by diluting 1 ml of solution 1 in 10 ml of lactofenol). Nematodes were transferred live to a drop of this final solution and heated briefly over an alcohol lamp. This staining method was applied on *B. hellenicus* and *B. sexdentati* which have inconspicuous spicules. In Spain, mounted material was observed using a Dialux microscope with Nomarsky interference optics.

For SEM, nematodes were fixed in a mixture of 4% gluteraldehyde/2% formaldehyde for several days, post-fixed in 2% OsO_4 overnight, dehydrated in an ethanol series, critical point dried and sputter coated with gold (Eisenback, 1985). Observations were made with a Jeol 35 SEM.

Bursaphelenchus spicules were excised for more detailed SEM observations. The method used was modified after Eisenback (1985). Live males were transferred to a drop of a solution composed of lactic acid (45%) + acetic acid (45%) + Rotring® Brilliant Ultramarine Blue Ink (120:4:0.1), briefly heated over an alcohol lamp and left for one to two hours in the solution. Under a stereo microscope (Olympus SZX12) the spicules were carefully cleaned and separated from the attached tissues using a cactus thorn and transferred to a drop of 2% formalin on a cover slip. The formalin was removed with a fine pipette and the cover slip with the spicule was attached to a stub with double-sided tape. After coating with gold, the spicules were observed and photographed using a JEOL 35 scanning electron microscope.

3.2.3 Biometric characterisation

After extraction from wood, specimens were fixed in hot F.A. (4:1) solution for at least 48 hours, processed into glycerine using the Seinhorst (1959) method, mounted on permanent slides and measured. The nematodes were measured and drawn using a *camera lucida* attached to an Olympus BX-51 microscope.

3.2.4 Molecular characterisation

In Portugal, after extraction from wood, aliquots of 1-10 nematodes were stored for DNA extraction. When the populations were successfully maintained in cultures, DNA isolation was carried out using nematodes collected from the fungal cultures. Nematodes were heated at 95ºC for five min, homogenised on a glass slide with a micro pestle (Eppendorf®) and DNA obtained using the DNeasy Tissue Kit (Quiagen®). This procedure was applied to different nematode life stages, namely adult, *dauer* juvenile, propagative juvenile and resistant juvenile.

DNA amplification for ITS-RFLP profiles was conducted using a Biometra Thermocycler, following the method of Braasch *et al*. (1999). After PCR, 5 µl of the amplified product was analysed in a 1% agarose gel. DNA fragments were visualised by staining in 1 µg/ml ethidium bromide solution and data analysis was performed using the Versa doc analysis system. Amplified DNA was digested for at least 3 hours at 37ºC using 10 U of each of the five enzymes (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I, and *Alu*I) (Amersham BioSiences®) following the manufacturer's instructions. Species-specific ITS-RFLP profiles for *Bursaphelenchus* were generated using these five restriction enzymes (Burgermeister

et al., 2005). The products of digestion were resolved in a 2% agarose gel, stained with 1µg/ml ethidium bromide solution and analysed as described above.

In Spain, for PCR / RFLP analysis the following procedure was followed: For DNA extraction 20 nematodes were placed in nematode lysis buffer (1X PCR buffer, 10 mg/ml protease K) heated to 60^{o}C for 1 hour and boiled for 15 mins. The nematodes were centrifuged and 5µl of superman was used for PCR amplification. The forward primer 5´-CGTAACAAGGTAGCTGTAG-3´ (Ferris et al., 1993) and the reverse primer 5´-TTTCACTCGCCGGTTACTAAGG -3´ (Vrain, 1993) were used in reactions containing, 10mM Tris-HCl (pH8.3), 1.5mM MgCl2, 50 mM KCl, 200mM each of dATP, dCTP, dGTP and dTTP and one unit of DNA polymerase (Biotools S.L). Initial denaturalization was carried out for a period of 2 mins at 94^{o}C followed by 35 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 1 min, followed by a final 5 min extension period at 72^{o}C. Amplified DNA fragments were digested with the restriction enzymes Alu I, Mnl I, Hha I, Hinf I, Rsa I, Hae III, Dra I, EcoRI and Hinc II (New England Biolabs) following manufacturers instructions. Restriction fragments were separated by electrophoresis in a TRIS-Borate-EDTA (TBE) buffered 2.5 % high resolution agarose gel, stained with ethidium bromide and visualised with UV illumination. RFLP banding patterns were compared to previously published banding patterns.

3.3 Results

3.3.1 Presence of Bursaphelenchus *species in Europe*

3.3.1.1 *Portugal*

Several species of nematode were isolated from wood samples during the surveys carried out in Portugal (Penas *et al*., 2004). The species present in the samples are listed in Table 2.

Table 2: Number of wood samples with *Bursaphelenchus* species and total numbers of samples observed in Portugal.

Species	Demarcated region*		Remaining area	
	1999-2000 Trunk traps	1999-2003 Trees samples	1999-2002* Trees samples	2003-2004 Trees samples
B. xylophilus	4	1422	0	0
B. pinasteri	-	134	3	2
B. leoni	47	15	30	13
B. sexdentati/ B. pinophilus	12	17	29	15
B. tusciae	4	10	7	1
B. hellenicus	7	4	1	0
B. teratospicularis	5	11	4	9
B. mucronatus	0	0	1	0
Bursaphelenchus spp[a]	5	18	8	0
Total	233	3681	1124	549

* The results also incorporate other studies conducted before the beginning of the PHRAME project

[a]Not identified but definitely not *B. xylophilus*

In addition, *B. antoniae* (Penas, *et al*, 2006) has been found in Portugal.

Bursaphelenchus xylophilus was found to be restricted to the infested region (Table 2, Figure 1). Apart from this species, all the other species were usually found in low densities dispersed throughout the country (Figure 1); *B. pinasteri* was the most frequent in the demarcated region, followed by *B. leoni* and *B. pinophilus/ B. sexdentati.* The latter species were also the most frequent in the remaining area of Portugal, where curiously *B. pinasteri* is almost absent. *B. mucronatus* was found in only one location, outside the demarcated region. Moreover, “mucronate forms” of *B. xylophilus* are often found in Portuguese populations within the infested area (these identifications were corroborated by molecular analysis integrated in other projects).

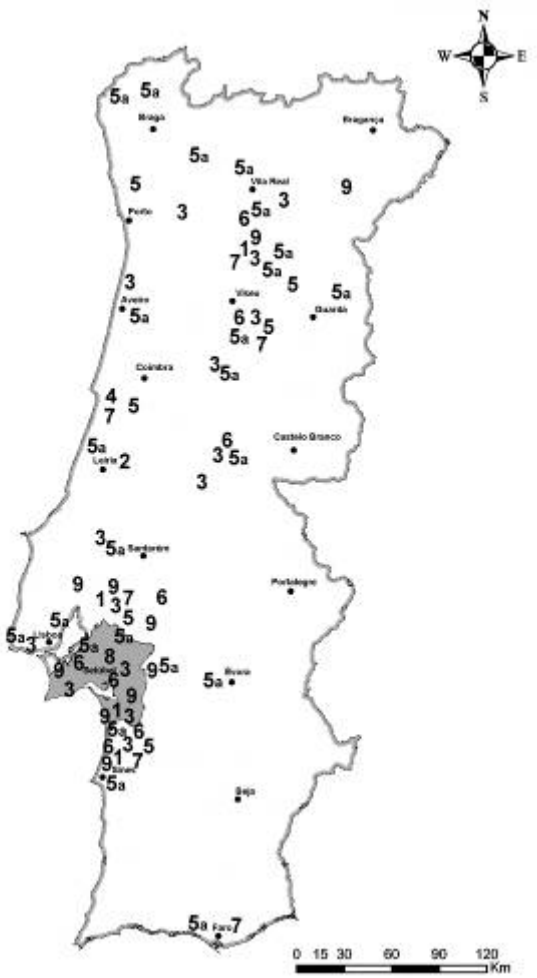


Figure 1: Occurrence of *Bursaphelenchus* species in continental Portugal: 1 - *B. hellenicus*; 2 - *B. antoniae*; 3 - *B. leoni*; 4 - *B. mucronatus*; 5 - *B. sexdentati*; 5a – *B. sexdentati/B. pinophilus.*; 6 - *B. teratospicularis*; 7 - *B. tusciae*; 8 - *B. xylophilus* (inside the grey region); 9 - *B. pinasteri*. (From Penas, *et al*, 2004)

3.3.1.2 Spain

Some 3000 wood samples from pine forests, most of them from *Pinus halepensis* (31.5%) and *P. pinaster* (30.2 %), but also from *Abies alba* Miller (0.2%), *P. nigra* (0.8%), *P. pinea* (0.9%), *P. radiata* (0.5%), *P. sylvestris* (0.5%) and other *Pinus* spp. (35.6%) were sampled. Nematodes were found in approximately 15% of the samples and were mainly Aphelenchids (68%) from the genera *Cryptaphelenchus* Fuchs (20%), *Bursaphelenchus* Fuchs (19%), *Laimaphelenchus* Fuchs (6%), *Ektaphelenchus* Fuchs (Skrjabin, Shikhobalova, Sobolev, Paramonov y Sudarikov); 30% were rhabditids and the rest tylenchid predators from the genus *Seinura* Fuchs, as well as *Aphelenchus* Bastian and *Paraphelenchus* Mickoletzy.

Aphelenchidae nematodes appeared in 52% of the samples, 1.4% from them belonging to the genus *Bursaphelenchus* Fuchs, 1937, in which the following species were found, *B. eggersi* Rhüm, 1956 (J. B. Goodey, 1960); *B. fungivorous* Franklin & Hooper, 1962; *B. hylobianum* (Korenchenko, 1980) Hunt, 1993; *B. leoni* Baujard, 1980; *B. mucronatus* Mamiya & Enda, 1979; *B. pinasteri* Baujard, 1980; *B. sexdentati* Rühm, 1960, and *B. teratospicularis* Kakuliya & Devdariani, 1965.

B. eggersi, B. mucronatus and *B. sexdentati*. Their distribution in Spain suggests the existence of three faunistic groups. The first one represented by *B. eggersi*, *B. hylobianum* and *B. mucronatus,*

that have been found in the Eusiberian region of the Iberian Peninsula, under an Atlantic climate, according to their distribution in Northern and Central Europe. A second group is constituted by *B. fungivorous*, *B. leoni*, *B. pinasteri* and *B. teratospicularis*, which are species of Mediterranean environments. Finally, *B. sexdentati* is a widespread species in Europe, not only in the Mediterranean environments but also in Central European regions (Figure 2, Figure 3).

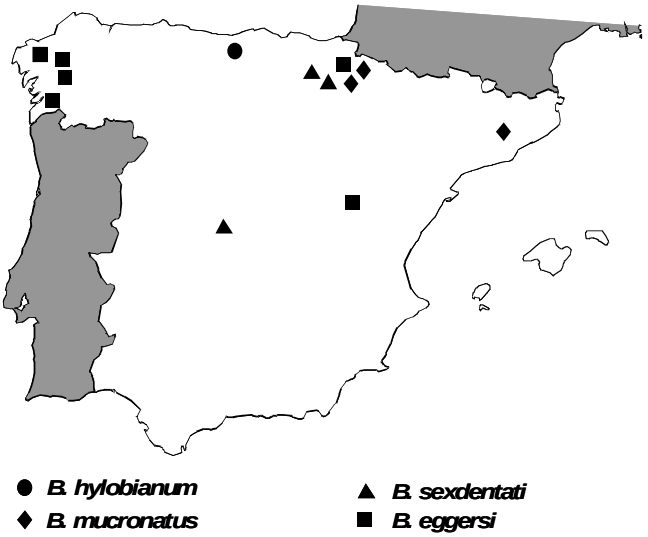


Figure 2: Distribution of *B. hylobianum*, *B. sexdentati*, *B. mucronatus* and *B. eggersi*

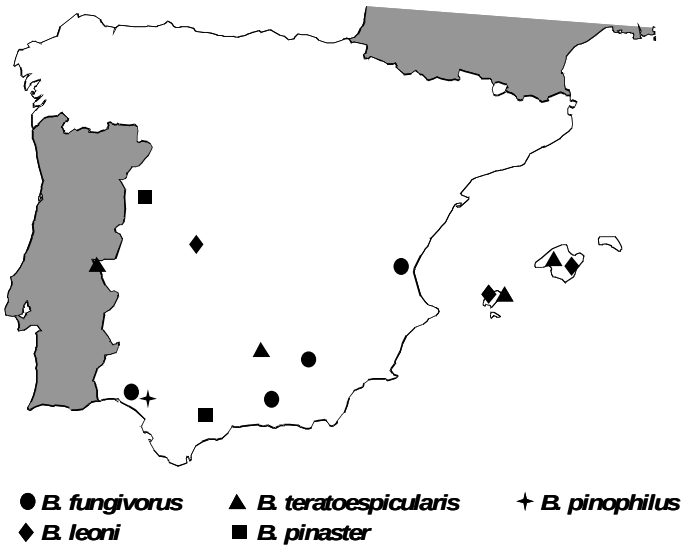


Figure 3: Distribution of *B. fungivorus*, *B. leoni*, *B. teratospicularis, B. pinaster* and *B. pinophilus*

3.3.2 Morphological characterisation

Morphological examination is usually reliable to differentiate most *Bursaphelenchus* species. However, for some of the species (*B. xylophilus*, *B. mucronatus*, *B. sexdentati* and *B. pinophilus*) the morphological characterisation did not lead to a clear identification. The identification of *B. xylophilus* based on morphological characters was only possible when the females had rounded

tails; when mucronated tails were observed a molecular analysis was carried out for precise identification.

The differentiation of *B. pinophilus* and *B. sexdentati* was not possible based only on morphological characters, due to the great similarity between these two species and some confusion concerning the presence or absence of a cucullus in the latter species. Furthermore, the small number of specimens usually found did not allow clarification of their identification. However, the spicules of males had a cucullus in most of the samples.

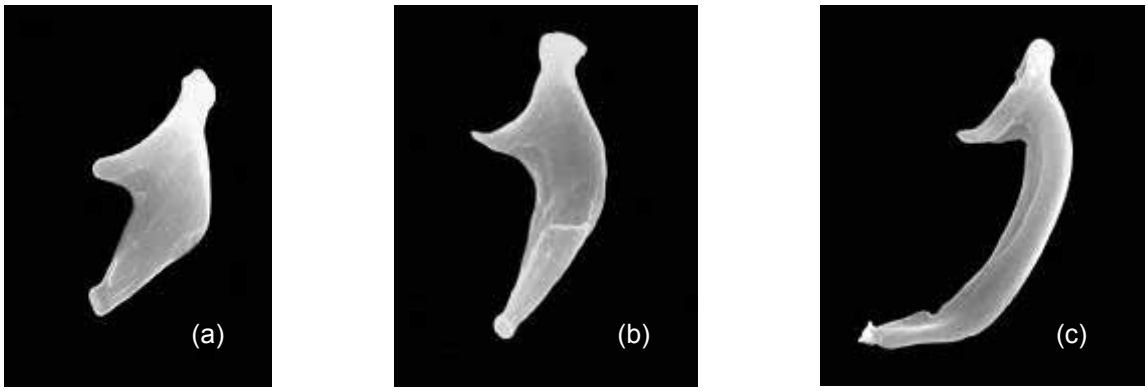


Figure 4: SEM photographs of excised spicules of *Bursaphelenchus* species: (a) *B. hellenicus*; (b) *B. sexdentati*/*B. pinophilus*; (c) *B. xylophilus*. Scale bar: ——— = 5 µm. (From Penas *et al*, 2004)

3.3.3 Biometric characterisation

Populations of the different *Bursaphelenchus* species were measured. The characters considered and their respective values are present in Table 3.

Table 3 Morphometrics of Portuguese *Bursaphelenchus* spp.

	n	L	a	c	V (%)	Stylet	Tail	Spicule
B.hellenicus	13 ♀♀	800.7±70.9 (607-908)	40.2±4.0 (30.4-46.1)	18.76±1.61 (14.80-21.12)	73.83±3.27 (71.90-84.51)	13.7±0.9 (12-15)	42.7±1.8 (41-47)	-
	14 ♂♂	713.7±55.2 (637-802)	39.8±2.2 (36.6-43.9)	19.25±1.04 (17.44-21.19)	-	13.5±0.9 (12-15)	37.1±2.7 (33-41)	12±0.5 (11-13)
B.pinasteri	20 ♀♀	637.9±54.7 (572-745)	37.9±2.5 (33.6-43.8)	24.13±2.17 (20.96-31.04)	72.44±1.11 (71.09-75.43)	12.4±1.4 (10.5-15)	26.5±1.8 (24-29)	-
	23♂♂	550.9±37.5 (464-732)	39.2±2.7 (33.1-43.9)	19.26±1.72 (15.47-22.92)	-	11.8±0.8 (11-13)	28.7±1.9 (26-33)	12.0±0.6 (11-13)
B.antoniae	20 ♀♀	584±54 (512-709)	36.6±2.6 (32.0-42.1)	14.6±0.9 (12.8-16.3)	69.96±1.37 (65.90-72.01)	12.0±1.0 (10-14)	40.1±2.9 (34-46)	-
	20♂♂	578±64 (476-660)	39.8±3.1 (34.2-44.0)	18.7±1.6 (15.5-21.3)	-	12.1±1.0 (11-14)	30.9±2.6 (27-34)	17.9±1.4 (15-20)
B. leoni	17 ♀♀	747.7±52.7 (679-846)	40.5±2.7 (36.4-45.6)	12.30±1.21 (9.86-14.59)	69.32±0.68 (68.14-70.17)	13.3±1.1 (12-15)	61.1±5.2 (53-71)	-
	18♂♂	690.9±47.1 (640-839)	38.3±2.4 (32.5-42.0)	18.30±1.14 (16.79-20.69)	-	12.6±0.9 (11-14)	37.8±2.5 (33-43)	15.9±1.0 (14.5-18)
B.teratospicularis	21 ♀♀	688.8±66.8 (597-931)	38.8±1.5 (34.4-40.9)	15.61±1.23 (14.04-18.75)	78.74±0.66 (77.59-79.94)	18.5±1.2 (17-21)	45.3±5.5 (36-58)	-
	15♂♂	571.1±38.4 (497-648)	35.6±2.6 (29.2-39.4)	18.09±1.57 (13.81-20.74)	-	18.1±2.1 (15-22)	31.7±2.7 (27-38)	14.3±1.2 (13-16.5)

	n	L	a	c	V (%)	Stylet	Tail	Spicule
B. tusciae	2 ♀♀	631±2.8 (629-633)	41.4±1.1 (40.6-42.2)	18.47±2.57 (16.66-20.29)	73.30±0.66 (72.83-73.77)	11.8±0.4 (11.5-12)	34.5±4.9 (31-38)	-
	6 ♂♂	654.8±66.4 (594-773)	43.9±3.0 (39.6-48.3)	24.31±1.56 (22.61-26.50)	-	12.8±0.8 (12-14)	26.9±1.8 (25-30)	18.2±1.8 (16-20)

3.3.4 Molecular characterisation

Molecular characterisation of *B. xylophilus*, *B. mucronatus*, *B. tusciae*, *B. sexdentati*, *B. pinasteri*, *B. antoniae* and *B. hellenicus* was carried out (Penas *et al*., 2004, Penas *et al*., 2006a, 2006b) (Figure 5).

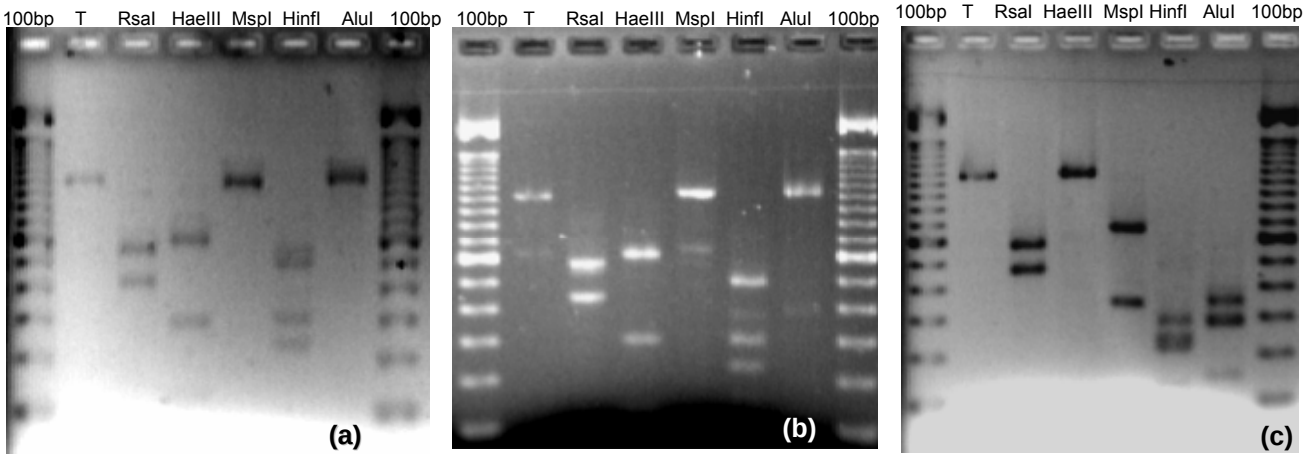


Figure 5: ITS-RFLP patterns of *Bursaphelenchus* isolates. (a) *B. sexdentati* pattern (adults found on pine wood from the survey); (b) *B. sexdentati* pattern (dauer juveniles found under the wings of *Orthotomicus erosus*); (c) *B. pinasteri* (adults found on pine wood from the survey).

3.4 *Discussion*

The high number of *Bursaphelenchus*–infested samples in north and central Portugal reflects the higher density of maritime pine forests from those regions. The higher diversity of species within the Demarcated Zone simply reflects a more intensive survey.

Bursaphelenchus xylophilus has not been found out of the affected zone. The closest species to *B. xylophilus* found in Portugal was *B. mucronatus*, but this species was found only once, outside the affected zone, in the north of Portugal (Penas *et al.*, 2004). Although this species is reported in many countries around Europe its predominance is in the north of Europe, in regions with colder weather like France (Baujard *et al*., 1979), Finland (Tomminen *et al*., 1989), Sweden (Magnusson & Schroeder, 1989), Germany (Braasch, 1991) and Russia (Kulinich *et al*., 1994).

Other species, like *B. leoni*, *B. tusciae* and *B. teratospicularis* are found commonly in Mediterranean regions. The presence of *B. pinasteri* in Portugal is easily explained since this species is commonly associated with *P. pinaster* (Baujard, 1980), a widespread and abundant pine in this country.

Tail and head shape, appearance of the vulval region and particularly the shapes and comparative sizes of the spicules were sufficient for the separation of most species. The presence of round-tailed females, with a well developed vulval flap and males with a typical spicule shape provided a

definitive morphological identification of *B. xylophilus*. Differentiation, based solely on morphological characters, is not reliable for some species. Some Portuguese *B. xylophilus* populations have mucronate females, which may confuse this species with *B. mucronatus*. ITS-RFLP analysis, however, distinguishes the mucronate form of *B. xylophilus* from *B. mucronatus* (Tarès *et al*., 1992). Concerning differentiation between *B. sexdentati* and *B. pinophilus*, in the *B. sexdentati* original description (Rühm, 1960), no cucullus was mentioned; Brezski and Baujard (1997) did not diagnose *B. pinophilus* in comparison to *B. sexdentati,* and Braasch (2001) distinguished these species on the basis of the presence of a cucullus in *B. pinophilus* whereas Ambrogioni and Caroppo (1998), using SEM techniques, illustrated *B. sexdentati* with a distinct cucullus (knob-like appendage). Although, most of the populations studied exhibited spicules with a distinct cucullus which seems to indicate that they belong to *B. pinophilus*, ITS-RFLPs analysis of these same populations produced the characteristic pattern for *B. sexdentati*.

The high variability observed in many of the biometric characters for *Bursaphelenchus* species between populations makes it difficult to use them for specific differentiation.

Chapter 4 Identification and biology of vectors of *Bursaphelenchus xylophilus* in Europe, particularly the Iberian Peninsula

4.1 *Introduction*

As a new arrival to Europe, the information on potential and actual vectors of *Bursaphelenchus xylophilus* was initially scant. However, early observations in Portugal indicated that the cerambycid *Monochamus galloprovincialis* was acting as the vector in the affected zone of the country. This confirms the finding, from South-east Asia, that the local species of *Monochamus* takes on the role of vector when *B. xylophilus* establishes in a new location (Mamiya 1988; Rutherford, Mamiya & Webster 1990). Surveys for the presence of potential vectors of *B. xylophilus* were, therefore, carried out in several countries involved in the PHRAME consortium, but with particular focus on the Iberian Peninsula.

4.2 *Insects associated with Bursaphelenchus species in Portugal*

4.2.1 Materials and methods

Bark and wood boring insects belonging mainly to the families Cerambycidae, Scolytidae, Buprestidae and Curculionidae (Coleoptera) were captured in 1999 to 2004 from six different localities in Portugal (Figure 7) and screened for the presence of *Bursaphelenchus* species.

Three methods were used to obtain adult insects: collecting insects from *P. pinaster* trees displaying symptoms of decline (between 1999 and 2004), mainly from the affected zone, but also from North-western Portugal. The trees were cut and divided into logs, some of which were debarked and all the adult insects collected while the remaining logs were kept in boxes at room temperature to allow insect emergence. Due to the presence of *B. xylophilus* a more intensive survey was required in the area affected by this species and two additional methods were used for insect collection: trap trees and flight traps. Trap trees (2003-2004) consisted of healthy trees that were felled and divided into logs, sprayed with 70% ethanol to attract insects and left for one month in the field. After one month, some of the logs were caged and left at room temperature to allow insect emergence while the remainder were debarked to collect the insects. Flight traps (2003-2004) were installed on *P. pinaster* trees and baited with turpentine and ethanol.

All the adult insects collected were identified in the laboratory to species or genus level. To determine the presence of nematodes, insects were placed individually in a Syracuse dish in a small amount of water. First, the elytra and wings were opened and observed and then the insect was crushed and left in water for few hours at room temperature. All the nematodes resembling *Bursaphelenchus* dauer juveniles were collected for species identification.

The molecular identification of dauer juveniles was performed as described above. When the dauer molecular identification was not possible, the dauer were developed to adult specimens, using three different techniques: inoculation and incubation of juveniles in branches of *P. pinaster* and/or in fungal cultures (*Monilinia fructicola* or non sporulating *Botrytis cinerea*) for three weeks at 26ºC and incubation of dauer juveniles in water at 26ºC. The adults obtained were identified using the morphological methods described above.

4.3 *Insects associated with Bursaphelenchus species in Spain*

4.3.1 Materials and methods:

Selection of risk areas and insect trap sites: All trap sites were selected on the basis of aridity data and forest mass data along with climatic data. Sampling was performed in three campaigns in

2003, 2004 and 2005 (Figure 6). To capture the insects, double window traps were installed in *P. pinaster* forests using ethanol (98 %) and turpentine (1:1 v/v) as attractant, along the Portuguese border in June 2003, in the provinces of Pontevedra (1), Ourense (2), Zamora (3), Salamanca (4), Cáceres (5) and Huelva (6); light traps were installed in the provinces of Jaén and Teruel. They were checked every two weeks until their removal in late September.

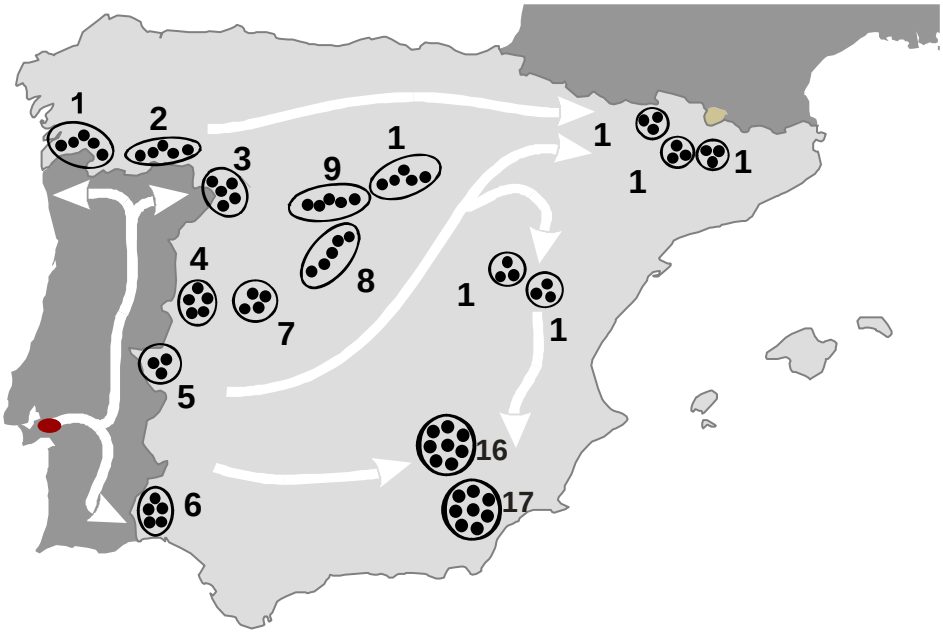


Figure 6: Location of insect traps in the Spanish Iberian peninsula during 2003-2005 and possible pathways of *Bursaphelenchus xylophilus* introduction.

In the second year nine new sites (7-15) were characterized taking into account their altitude, rainfall, annual temperature and conifer species, establishing the potential pathways of PWN entrance in Europe through Spain from the Portuguese border (Figure 6), 3 or 5 traps were located in each site, when possible, on *P. pinaster* stands. In early May of 2005 traps were located in *P. nigra* forests of Cazorla Natural Park in Jaén (16) and in the area of Almerian Sierra Nevada (17) (Table 4).

Table 4: Climatic data of the trap sites in Spain including pine species, altitude, average precipitation and temperature

Site	Province	Vegetation	Altitude (m)	Precipitation (mm)	Annual temperature (ºC)		
					Tmean	Tmin	Tmax
1	Pontevedra	*P. pinaster* / *P. radiata*	154-320	1434-1465	12.7-14.1	2.9-4.5	23.4-27.7
2	Orense	*P. pinaster*	462-824	842-1050	12.1-12.3	1.1-1.9	25.9-28.2
3	Zamora	*P. pinaster*	974	991	9.8	-2.0	26.6
4	Salamanca	*P. pinaster*	991	859	11.0	-1.4	28.7
5	Cáceres	*P. pinaster*	424	619	15.5	3.2	33.7
6	Huelva	*P. pinaster* / *P. pinea*	505	505	17.7	5.7	32.5
7	Ávila	*P. sylvestris* / *P.pinaster*	661-1447	1072-1139	10.7-14.2	0.2-0.7	29.6-33.4
8	Segovia I	*P. sylvestris*	1471	643	9.6	-2.2	27.4
9	Segovia II	*P. pinaster*	824	482	12.3	-0.6	31.0
10	Soria	*P. sylvestris* / *P.pinaster*	1153	691	9.3	-3.0	28.2
11	Teruel I	*P. sylvestris*	1545	618	7.8	-4.2	26.8

Site	Province	Vegetation	Altitude	Precipitation	Annual temperature (ºC)		
12	Teruel II	*P. sylvestris* / *P. nigra*	1159	489	11.2	-0.4	28.1
13	Huesca	*P. sylvestris*	1823	1073	6.7	-5.9	21.8
14	Lleida I	*P. nigra*	698	675	12.5	-1.3	26.9
15	Lleida II	*P. nigra*	860	779	11.6	-2.1	25.9
16	Cazorla	*P. nigra*	885-1290	976	12.7	0	32
17	Sierra Nevada	*P. sylvestris* / *P. nigra*	921-1800	606	12.7	1.7	28.4

Insect traps and identification

Double window traps were allocated at those sites described in Table 4, using ethanol and turpentine (1/1) as an attractant. The captured insects were recovered every fifteen days from May, when the traps were allocated, until their removal at the end of October. All insects were observed under a Zeiss Stemi 2000, (Jena, Germany) stereomicroscope for the presence of nematode dauer stage juveniles under their elytra. Specimen insects were preserved in alcohol for later identification by experts in forest entomology based at the phytopathological station in Pontevedra, Spain.

4.3.2 Insects associated with Bursaphelenchus species in Austria

In Austria beetle collecting was carried out in different Pine-stands (*Pinus sylvestris* and *P. nigra*) in a southern (Styria) and a northern province (Lower Austria), where pine decline has been observed. Three different methods for collecting *Monochamus* beetles were used:

- pheromone traps with different pheromones and different constructions,
- trap trees of various coniferous species,
- log traps with stem pieces and branches of *Pinus sylvestris*.

The pheromone traps used were multi funnel and cross vane traps containing different types of specific pheromones, mostly consisting of ethanol, α-pinene and terpinolene. The traps were placed on *Pinus* branches 5 metres above ground.

The log traps consisted of stem pieces and branches of *P. sylvestris* or of burned branches and stem-pieces and fresh twigs of *P. sylvestris*. These log traps were installed in different pine stands where pine decline was observed.

Culturing of stem pieces of *P. sylvestris* with symptoms of long horned beetle attack was another method of collecting *Monochamus* species. Therefore an 80-100 years old *P. sylvestris* which seemed to be attacked by *Monochamus* sp. was felled in spring in a southern province of Austria (Carinthia). The stem was cut into seven pieces of ~ 50 cm length and 40 – 50 cm diameter. The pieces were stored in cages in outdoor insectaries until June. Since mid-June the cages with the stem pieces were transferred indoors into the laboratory for better observation of beetle emergence. At another location in northern Austria (Lower Austria) within a pine stand in poor condition, *P. sylvestris* was felled during summer and thirteen stem pieces were taken to the laboratory and assessed for emergence of longhorn beetles. The wood was stored in the laboratory until November, in outdoor insectaries until the following May and then again in the laboratory over the emergence period in the summer.

4.4 Results

4.4.1 Insects associated with Bursaphelenchus species in Portugal

Several associations between *Bursaphelenchus* species and insects were found throughout Portugal and are described in Table 5 and Figure 7. A new *Bursaphelenchus* species *B. antoniae* was isolated from an insect species (*Hylobius* sp.) but was never found on wood samples (Penas *et al*., 2006a, 2006b).

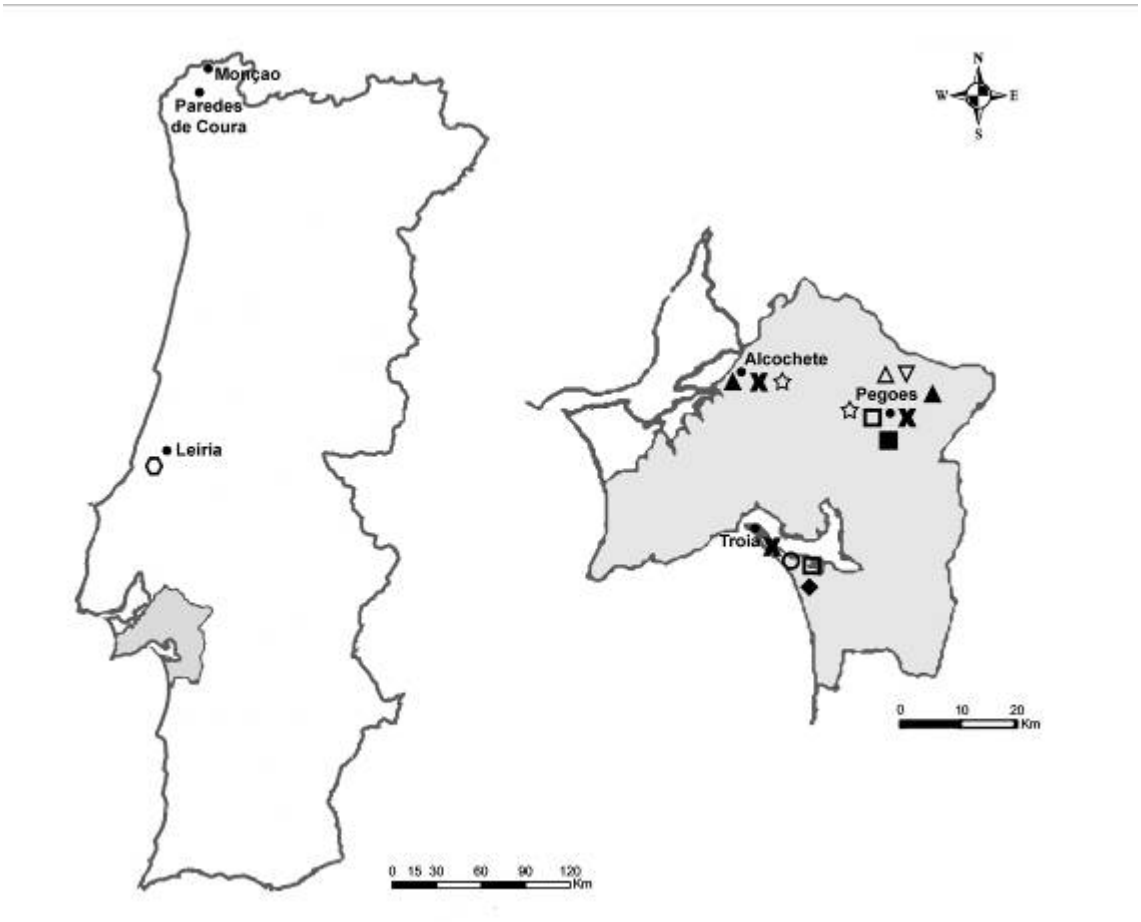


Figure 7: Sampling sites(●) and distribution of *Bursaphelenchus*-insect associations. ▭ - region affected by *B. xylophilus*. Associations: *Bursaphelenchus* sp.-*Hylobius* sp. (⬡); *B. xylophilus- M. galloprovincialis* (x); *B. teratospicularis - O. erosus* (O); *B. sexdentati* - O. erosus(☐); *B.sexdentati*/*B. pinophilus - H. ligniperda* (■); *B. hellenicus - T. piniperda* (Δ); *B. hellenicus - I. sexdentatus* (▽); *B. hellenicus - H. ligniperda* (▲); *B tusciae - H. ligniperda* (☆); *B. leoni - H. ligniperda* (◆).

Table 5: Insects in Portugal screened for the presence of *Bursaphelenchus* species between 1999 and 2004.

Family/Species	Observed insects (n)	Insects with *Bursaphelenchus* dauer juveniles (%)	*Bursaphelenchus* species associated
Coleoptera			
Cerambycidae			
*Monochamus galloprovincialis**	541	20	*Bursaphelenchus xylophilus*
Arhopalus ferus	26	0	-
Arhopalus syriacus	80	0	-
Pogonocherus perroudi	55	0	-
Acanthocinus griseus	78	0	-
Spondylis buprestoides	13	0	-
Rhagium inquisitor	3	0	-
Ergates faber	1	0	-
Scolytidae			
Orthotomicus erosus	899	19	*Bursaphelenchus teratospicularis* *Bursaphelenchus sexdentati*
Tomicus piniperda	168	24	*Bursaphelenchus hellenicus*
Ips sexdentatus	300	19	*Bursaphelenchus hellenicus*
Hylurgus ligniperda	557	5	*Bursaphelenchus tusciae* *Bursaphelenchus hellenicus* *Bursaphelenchus sexdentati* and/or *Bursaphelenchus pinophilus***
Pityogenes sp.	175	2	*Bursaphelenchus leoni****
Hylastes sp.	34	0	-
Buprestidae			
Crysobothris solieri	50	0	-
Calcophora mariana	11	0	-
Phaenops cyanea	3	0	-
Curculionidae			
Pissodes castaneus	177	2	-
Eremotes porcatus	50	2	-
Hylobius sp.	62	14	*Bursaphelenchus antoniae*
Elateridae			
Unidentified sp.	10	0	-
Hymenoptera			
Siricidae			
Sirex noctilio	1	0	-
Total	3294	13	-

* Collected only in the affected zone
** Only differentiated by molecular methods
***Association not definitively established

4.4.2 Insects associated with Bursaphelenchus species in Spain

Nematodes were found under the elytra in about 10 % of the following *Cerambycidae* insects: *Arhopalus ferus* from Navafría (Segovia), As Neves (Pontevedra) and Navasfrías (Salamanca); *Rhagium bifasciatum* and *R. inquisitor* , *Spondylis buprestoides* from As Neves and O Rosal (Pontevedra), Verín (Ourense), Santa Ana (Zamora) and several different sites in the area of Navasfrías (Salamanca) (Fig. 4); in the *Scolytidae*: *Hylastes ater* and *Hylurgus ligniperda* from Puerto del Pico and San Esteban del Valle (Avila), *Ips sexdentatus* and *Orthotomicus erosus* from Cabecerán (Cáceres), Las Cumbres, Villablanca y El Granado (Huelva), El Payo and Navasfrías (Salamanca), Cuéllar (Segovia) and Cabrejas de Pinar (Soria), *Pityogenes bidentatus* from Las Cumbres (Huelva), and El Payo (Salamanca) and Cuéllar (Segovia) and *Tomicus piniperda* from Navasfrías area (Salamanca), and in the Curculionidae *Hylobius abietis* from Navasfrías (Salamanca) and Cabrejas del Pinar (Soria) and *Pissodes validirostris* from El Payo (Salamanca) (Tab. 2).

Table 6: Species found in traps after the 2003, 2004, and 2005 campaigns

Family /Subfamily	Species	Frequency*	Nematodes
Cerambycidae			
	Acanthocinus hispanicus Sama & Schurmann 1980	1	-
	Anaesthetis testacea (Fabricius 1781)	3	-
	Anoplodera sexguttata (Fabricius 1775)	3	-
	Arhopalus ferus (Mulsant 1839)	1	+
	A. rusticus (Linnaeus 1758)	3	-
	A. syriacus (Reitter 1895)	3	-
	Clytus arietis (Linnaeus 1758)	3	-
	Gracilia minuta (Fabricius 1781)	3	-
	Monochamus galloprovincialis (Olivier 1795)	1	-
	Nustera distigma (Charpentier 1825)	3	-
	Prionus (Prionus) coriarius (Linnaeus 1758)	3	-
	Paracorymbia fulva (DeGeer 1775)	2	-
	Phoracantha semipunctata (Fabricius 1775)	3	-
	Plagionotus arcuatus (Linnaeus 1758)	3	-
	Rhagium (Hagrium) bifasciatum (Fabricius 1775)	2	-
	R. (Rhagium) inquisitor (Linnaeus 1758)	3	-
	Spondylis buprestoides (Linnaeus 1758)	1	+
	Stictoleptura rubra (Linnaeus 1758)	1	-
Scolytinae			
	Hylastes ater (Paykull 1800)	2	+
	H. attenuatus (Erichson 1836)	3	-
	Hylurgus ligniperda (Fabricius 1787)	2	+
	Ips acuminatus (Gyllenhal 1827)	1	+
	I. mannsfeldi (Wachtl 1879)	1	+
	I. sexdentatus (Börner 1776)	3	-
	Orthotomicus erosus (Wollaston 1857)	1	+
	Pityogenes bidentatus (Herbst 1784)	1	+
	P. chalcographus (Linnaeus 1761)	1	-
	Pityokteines vorontzowi (Jakobson 1895)	3	-
	Tomicus piniperda (Linnaeus 1758)	1	+
	Xyleborinus saxesenii (Ratzeburg 1837)	3	-
Other Curculionidae			
	Brachyderes (Brachyderes) confusus Viedma 1967	3	-
	B. (Brachylophus) lusitanicus (Fabricius 1781)	3	-
	B. (Brachyderes) suturalis Graells 1851	3	-
	Curculio salicivorus Paykull 1792	3	-
	Hylobius (Callirus) abietis (Linnaeus 1758)	2	-
	Magdalis (Magdalis) memnonia (Gyllenhal 1837)	2	-
	Pissodes (Pissodes) castaneus (De Geer 1775)	3	-
	P. (Pissodes) validirostris (C.R. Sahlberg 1834)	2	+
	Polydrusus (Polydrusus) tereticollis (De Geer 1775)	3	-

Family /Subfamily	Species	Frequency*	Nematodes
	Rhynchites bicolor (Fabricius 1775)	3	-
Buprestidae			
	Acmaeoderella (Carininota) flavofasciata (Piller & Mitterpacher 1783)	3	-
	Anthaxia (Haplanthaxia) confusa (Gory 1841)	3	-
	A. (Melanthaxia) quadripunctata (Linnaeus 1758)	3	-
	A.(Melanthaxia) rugicollis Lucas 1849	3	-
	Buprestis (Buprestis) novemmaculata Linnaeus 1758	2	-
	B. (Buprestis) octoguttata Linnaeus 1758	3	-
	Chalcophora mariana (Linnaeus 1758)	3	-
	Coraebus florentinus (Herbst 1801)	3	-
	Phaenops cyanea (Fabricius 1775)	3	-

* 1: Frequent; 2: Infrequent; 3: Occasional (Species in grey box are vectors of *Bursaphelenchus* spp.)

Among the Cerambycidae (Figure 8) *Monochamus galloprovincialis,* the only European insect captured known to vector PWN, appears widespread in the Peninsula, especially along the Portuguese border, reaching the forests in Andalusia, the central region and even in the North-western and East towards the Pyrenees. None of the captured specimens carried nematodes under their elytra. *Arhopalus ferus* and *Stictoleptura rubra* have been found commonly in the Peninsula, from Navafría (Segovia), O Rosal (Pontevedra) and Navasfrias (Salamanca) carrying nematodes other than *Bursaphelenchus* spp. On the contrary, *A. rusticus* has only been found at Las Cumbres, Villa Blanca (Huelva). Also specimens of *Spondylis buprestoides*, mainly captured along the Portuguese border but widespread in the Northern half of Spain, were found in the North-western regions (Pontevedra, Orense, Zamora and Salamanca provinces) carrying nematodes other than *Bursaphelenchus spp.* Other captured *Cerambycidae* are *Rhagium bifasciatum* in the Northwest (Pontevedra province) and at Lugar Nuevo (Jaén) or Laujar (Almería), in the Southeast*, Rhagium inquisitor* at Quintos de Mora (Toledo) and El Payo (Salamanca) on *P. pinaster* forests*.*
Among the *Scolytinae* (Figure 9, A) *Hylastes ater* appeared widespread in Andalusia and Extremadura, in the South-West, spreading towards Avila, Salamanca, and Soria and in the Northern plateau, in *P. pinaster* forests.

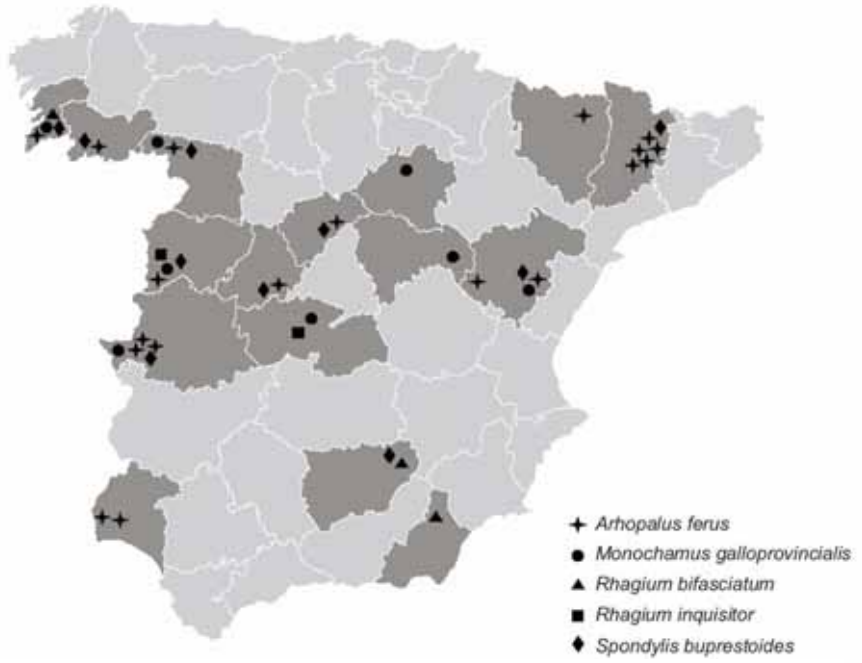


Figure 8: Distribution of Cerambycidae captured in Spain

Specimens from Puerto del Pico, San Esteban del Valle carried nematodes. *Hylurgus ligniperda* have been trapped in the West, along the Portuguese border, carrying nematodes. The specimens from Puerto del Pico, San Esteban del Valle (Avila), as well as *Orthotomicus erosus* which is

widespread in the Spanish Peninsula, associated with damage in *P. pinaster* and frequently carrying nematodes, among them *Bursaphelenchus fungivorus* (ARIAS et al., 2005). Also *Tomicus piniperda* has been found commonly in *P. pinaster* forests with the specimens from Avila, Cáceres, Huelva, Jaén, Lérida, Salamanca, Soria, Teruel and Toledo provinces carrying nematodes. Other Scolytinae found were *Ips sexdentatus* in three points at the Southeast and Southwest of Spain, *Pityogenes bidentatus* and *P. chalcographus* on *P. pinaster*.

Other Curculionidae (Figure 9, B) found were *Hylobius abietis* trapped in *P. pinaster* forest from the North-West, carrying nematodes, some specimens from Navasfrías (Salamanca) and Cabrejas de Pinar (Soria), and *Pissodes validirostris* also trapped on *P. pinaster* along the Northern part of the Portuguese border (Orense, Pontevedra and Zamora provinces).

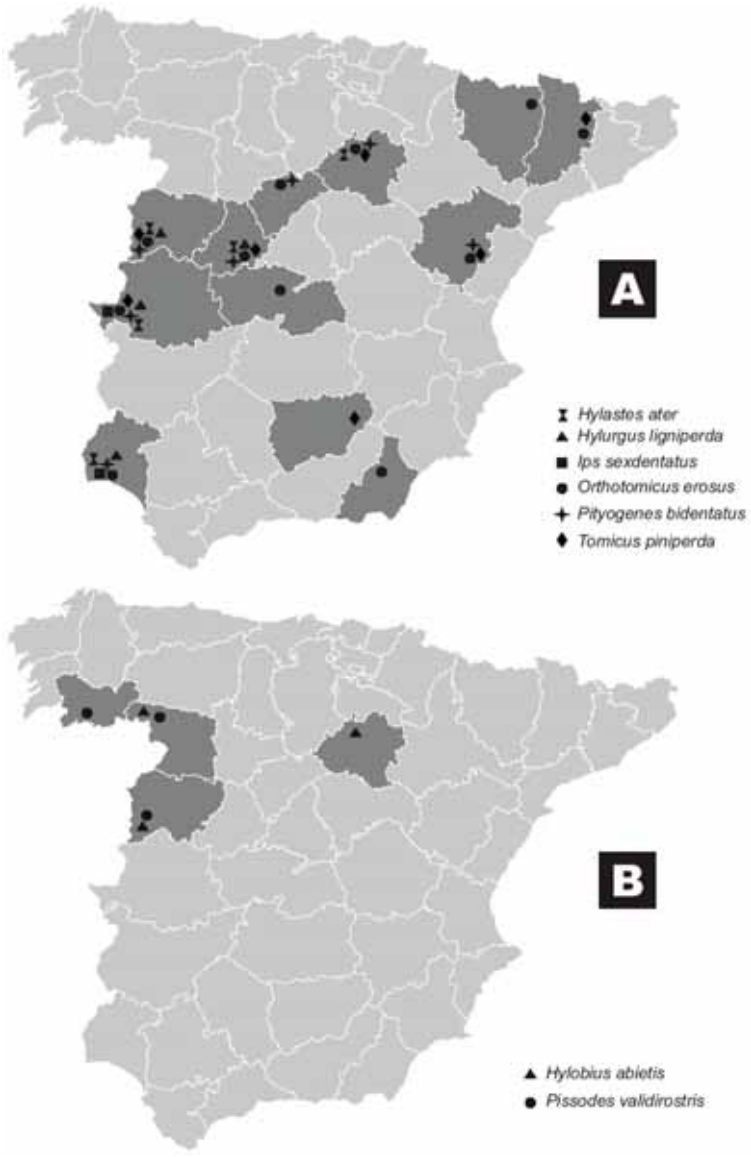


Figure 9: Distribution of Scolytinae (A) and Curculionidae (B) captured in Spain.

4.4.3 Insects associated with Bursaphelenchus species in Austria

Within the first two years of the project, no *Monochamus* beetles were captured with the pheromone "Monowit" of the Austrian company "Witasek", consisting of 96% α-pinene, and different log traps during the monitoring periods of 2004 and 2005 (June - October). The only collected beetles in the traps which could be classified as potential pine pests were bark beetles (*Pityogenes* spp.) and *Spondylus buprestoides*. Other beetles captured were click beetles, stag-beetles, *Scarabaeoidea* and *Cerambycini*.

In the third monitoring year, 2006, other pheromones were used due to good experiences with one pheromone in Croatia and the development of new pheromones by the Austrian company. The one pheromone, also used by colleagues in Croatia, is produced in the USA and consists of 99.99% α-pinene, according to a chemical analysis by Portuguese colleagues. The new pheromone, containing α-pinene in an unknown concentration, and the additive of the Austrian company "Witasek" called "gallowit" and "hostowit" were used separately and in combination with each other. These three pheromones were installed with cross vane traps at two locations in Lower Austria where the occurrence of different *Monochamus* species was known: in Sollenau (25 km south of Vienna in Lower Austria), within the pine stand in poor condition near to a sawmill with known occurrence of *M. galloprovincialis pistor*, and in Nasswald, a mountain region in Lower Austria at an altitude of 600-1200 m, where three *Monochamus* species (*M. sartor*, *M. sutor*, *M. galloprovincialis pistor*) occur.

In Nasswald only six *Monochamus* specimens were caught: one *M. sartor* and two *M. sutor* with the combination "gallowit/hostowit" and three *M. sutor* with the US-pheromone, none with the separately used pheromone "gallowit". *M. sartor* was captured in Mid-July, *M. sutor* in the first three weeks of August. Additionally, other long horned beetles like *Spondylis buprestoides*, *Rhagium* spp. were found. Due to the bark beetle component of the pheromones used, large numbers of bark beetles (*Ips typographus*, *Pityogenes chalcographus*) and predatory ant beetles (*Thanasimus formicarius*) were also caught.

In Sollenau the capture rate was greater. Altogether, 25 *M. galloprovincialis pistor* beetles were caught. The most *M. galloprovincialis pistor* (16 = 5 male + 11 female) were caught with the pheromone combination "gallowit/hostowit" in the period of Mid-June to Mid-July, mainly between 5th and 14th of July. The pheromone "gallowit" used separately collected five, and the US-pheromone only four *M. galloprovincialis pistor*. Besides large numbers of the ant beetle (*Thanasimus formicarius*) bark beetles like *Ips typographus*, *Ips acuminatus*, *Ips sexdentatus*, *Orthotomicus* spec., *Pityogenes bidentatus*, and *Rhagium* spec. were also caught in the pheromone traps.

Culturing of stem pieces of *Pinus sylvestris* with long horned beetle attack was another way of collecting *Monochamus* species. The 80-100 years old *Pinus sylvestris* infested by *Monochamus* sp., which was felled in March 2004 in Carinthia (a southern province of Austria), was cut into pieces and stored in outdoor insectaries until June and afterwards indoors for observation of the emergence period. Long horned beetles emerged from two of the seven stem pieces during the time period 12.07.-26.07.2004 (7 males: 7 females = 1:1 ratio). The beetles were determined as *M. galloprovincialis pistor*. The stem wood of this *P. sylvestris* tree was investigated to assess for the occurrence of *Bursaphelenchus* ssp., but none were detected.

Some *P. sylvestris* from a pine stand in Sollenau/Lower Austria (25 km south of Vienna), being in poor condition, had been felled in summer 2005. Larval activity in the thirteen stem pieces was very intensive over several months. From seven of the thirteen stem pieces, 16 *Monochamus galloprovincialis pistor* adults (3 males:13 females ≈ 1:4 ratio) emerged, mainly in June. Compared with the activity of larvae the numbers of emergent beetles was surprisingly low. One explanation could be the very severe winter of 2005/2006 with very low temperatures over a long time period. The final analysis of these *P. sylvestris* stem pieces by chopping into small pieces and strips resulted in further four living and five dead *Monochamus* larvae.

Collecting of *Monochamus*–species by trap trees or during flight was very successful in a mountain region in Nasswald/Lower Austria at an altitude of 600-1200 m within the time period July to September over four years. The following *Monochamus* specimens were caught:

Monochamus sutor	*Monochamus sartor*	*M. galloprovincialis pistor*
2003: 5 ♂ + 3 ♀ = 8	2003: 1 ♂ + 2 ♀ = 3	
2004: 1 ♂ + 8 ♀ = 9	2004: 2 ♂ + 1 ♀ = 3	
2005: 4 ♂ + 3 ♀ = 7	2005: 6 ♂ + 5 ♀ = 11	
2006: 14 ♂ + 5 ♀ = 19	2006: 7 ♂ + 2 ♀ = 9	2006: 1 ♂ + 0 ♀ = 1

With regard to the study of the biology of the different *Monochamus* species, the emerged and collected *Monochamus* beetles were not investigated for occurrence of *Bursaphelenchus* species, but some of the wood samples were. No *Bursaphelenchus* spp. were found.

4.5 *Discussion*

Concerning the associations of *Bursaphelenchus* species with insect vectors, the studies reported here confirm that one insect species can vector several *Bursaphelenchus* species (e.g. *O. erosus* and *H. ligniperda*). It was also shown that the same *Bursaphelenchus* species can have different insect vectors (e.g. *B. hellenicus*, Table III), confirming previous observations for this genus (Braasch, 2001, Ryss *et al*., 2005). This suggests a non-specialised relationship between *Bursaphelenchus* spp. and their vectors. However, in Portugal, *B. xylophilus* may have a close and specialised relationship with *M. galloprovincialis,* as this nematode species was not found in any other insect and *M. galloprovincialis* was not associated with any nematode species other than *B. xylophilus*. A new *Bursaphelenchus* species, *B. antoniae,* was isolated from an insect species (*Hylobius* sp.) but was never found associated with wood samples (Penas *et al*., 2004; Penas *et al*., 2006a).

Some of the associations found have already been previously reported in other countries. The association between *B. teratospicularis* and insects belonging to the genus *Orthotomicus* has been reported previously (Kakuliya & Devdariani, 1965); *B. hellenicus* has formerly been associated with *T. piniperda* (Braasch *et al*., 2000). Nevertheless, the phoretic associations between *B. hellenicus*-*I. sexdentatus*, *B. hellenicus-H. ligniperda*, *B. sexdentati-O. erosus*, *B. sexdentati-H. ligniperda* and *B. tusciae-H. ligniperda* have never been previously reported to our knowledge. The confirmation that *Pityogenes* sp. is a vector of *B. leoni* requires confirmation as only one male was observed in the sawdust attached to the insect. *Bursaphelenchus leoni* is difficult to multiply and maintain under controlled conditions and even if *B. leoni* dauer juveniles were obtained it would be difficult to rear adults for precise identification. This nematode may therefore be under-represented in our analysis.

The ITS-RFLP patterns of nematode dauer juveniles were difficult to obtain. It is possible that the high lipid content of this specialised survival stage difficult the recovery of DNA from the few nematodes available.

Observations from Portuguese studies suggest that *Bursaphelenchus teratospicularis* has some affinities with nematodes belonging to the genus *Ektaphelenchus*. Adults of this species were found in a cocoon-like structure under the elytra of *O. erosus*, and some *Ektaphelenchus* species are known to be transported in similar structures under the insect elytra (Thorne, 1935; Rhüm, 1956; Massey, 1974). Morphologically, *B. teratospicularis* shares many characteristics with *Ektaphelenchus*. Both are characterised by a slender, medium sized, ventrally arcuate body, a coarsely annulated cuticle, flattened cephalic region, wide and distinctly offset stylet with a long wide lumen, cylindrical procorpus joining a large prominent rounded-rectangular median bulb, vulva with lips not protuberant, intestine ending in a blind sac and an anus that is very difficult to discern (Hunt, 1993). Furthermore, *Ektaphelenchus* cocoons are described as usually containing only immature females, and sporadically males (Massey, 1974). Of the *B. teratospicularis* adults found in *O. erosus* 22 were females and three males. The presence of a terminal bursa on the male tail tip of these nematodes, absent in the genus *Ektaphelenchus* (Hunt, 1993), was the specific and diagnostic character used to include it in the genus *Bursaphelenchus*. Because of the similarities between these species to nematodes in the genus *Ektaphelenchus* in morphology and cocoon-forming habit, a more detailed and precise study will be required to clarify their taxonomic status.

Overall, the studies conducted, based both on the morphological and molecular characterisation of the nematodes, confirmed the identity of several Bursaphelenchus species detected in Portugal and proved their associations with different insect species belonging primarily to the Scolytinae sub-family. A more detailed characterisation of all Bursaphelenchus species found in Portugal, including morphology, morphometrics and DNA analysis, is being developed and will be published in the future.

Although no *B. xylophilus* were found in Spain, the results on studies of potential vectors provide useful information for assessing future potential risk should pinewood nematode establish there. Some of the trapped insects such as *Orthotomicus erosus* can themselves produce damage and even death to different pine species when in large numbers, but more important could be their capability to be PWN vectors. It seems that the role of these vector insect do not imply any specificity in their relationships with the nematodes they carry, but they are a mere vehicle due to both organisms having coincident development in the same ecological conditions. Technically, therefore, any insect able to carried nematodes, especially if they belong to the genus *Bursaphelenchus,* could be considered as potential vector.

The most important insect is the cerambycid *Monochamus galloprovincialis*, the vector of PWN *Bursaphelenchus xylophilus* in Portugal (Sousa et al. 2001), which has also been found associated to *B. mucronatus* in Finland, Germany, Italy and Portugal (Magnuson and Schroeder 1989; Tomminen et al. 1989; Palmisano et al. 1992; Ambrogioni et al. 1994; Braasch et al.1999). It appears widespread in the Peninsula, especially along the Portuguese border, reaching the pine forests in the central region, although no nematodes were found under the elytra of any of the captured specimens. *Monochamus galloprovincialis* was reported in Spain on the branches of dead *Pinus* spp., *Abies* spp., *Picea* spp. and *Larix* spp. (Verdugo, 2004; Vives, 2000). It represents a risk of PWN introduction in Europe through the Peninsula (Figure 8), and its establishment in the southern half of Spain as there are long periods during spring and summer of temperatures above 25º C, low rainfall rates and, consequently, a higher probability of trees with drought stress.

Other Cerambycidae that must be considered because they have been found carrying nematodes under their elytra are *Arhopalus ferus* (Figure 8), which is widespread in the Peninsula and was reported in Japan as carrying *B. xylophilus*, as well as on dying shoots of *P. pinaster* in Piedmont, Italy and on *P. sylvestris* in Missouri, USA (Mamiya and Endo 1972; Kondo et al. 1982; Caroppo et al. 1998). *Spondylis buprestoides*, is a very common species in the Iberian Peninsula on recently felled *Pinus* spp. and *Abies* spp. trees (Vives, 2000), which was also reported in Japan as carrying *B. xylophilus* (Kobayashi, Yamane and Ikeda 1984).

Many Scolytinae have pest significance to many pine species, besides being potential nematode vectors of *Bursaphelenchus* spp. as they carry nematodes under their elytra. *Hylastes ater*, which lives under the bark of *P. sylvestris*, *P. nigra* and *P. pinaster*, can cause the death of young plantations in nurseries (Browne, 1968; Gil and Pajares 1986). *Hylurgus ligniperda* often only localized in the basal part and on the roots of declined or dead trees, becoming a pest in weak reforestations of many pine forests (Browne, 1986, Wiedma, 1964). Besides it was reported in Greece associated with *Bursaphelenchus sexdentati* and in Portugal carrying *B. leoni*, *B. sexdentati* and *B.teratospicularis* (Skarmoutsos and Skarmoutsos, 1999; Penas et al., in press).

Ips sexdentatus is included in the quarantine list of the EU directive 2000/29/EC. This species is of no significance as a primary pest in northern and central Europe, where it breeds only in fresh logs or in weakened or dying trees. It has caused death of *P. sylvestris* and *P. radiata* suffering from drought stress in the south, central and southern France, north of Portugal and Spain, often in association with other pests such as *Ips acuminatus* or *Tomicus piniperda*. It is not considered to be a quarantine pest by EPPO or any other regional plant protection organization, because it is not generally a primary pest and is only capable of attacking trees already suffering stress, either environmental or from other pests. It is unlikely to spread naturally, so that phytosanitary measures could be justified (CABI and EPPO 2003). It reduces tree growth and the weakened trees often die, but in high population levels can also cause death to healthy trees. It has been reported in Portugal carrying dauer larvae of *Bursaphelenchus* spp and *B. hellenicus* (Sousa et al. 2002; Penas et al., 2005).

Orthotomicus erosus has been found in several sites in the Central System and in Southern Spain associated with pine trees in decline. This fact must be taken into account, because the beetle although it appears to be a primary pathogen, infests recently fallen trees, wounded, and stressed living trees that often die and, at high population levels, massive attacks can lead to the death of healthy trees. It has been found attacking *P. pinaster* and *P. halepensis* from Tuscany (Italy), in

Greece appeared on the same trees as *B. leoni* and *B. sexdentati*, in Portugal was reported to be carrying dauer larvae of *Bursaphelenchus* spp, and recently *B. sexdentati* and *B. teratospicularis* and *B. fungivorus* in Spain (Arias et. al. 2005; Caroppo et al. 1998; Penas et al, 2006; Skarmoutsos and Skarmoutsos, 1999; Sousa et al., 2002). High numbers of *O. erosus* were found at several sites in the Central System and Southern Spain, associated with pine trees in decline, though the beetle seems not to be a primary pathogen; it infests recently fallen trees, wounded, and stressed living trees. Stressed trees are more prone to attacks. Weakened trees often die, and at high population levels, the attack can lead to the death of healthy trees.

Tomicus piniperda, a specific insect of pine trees particularly *P. sylvestris*, *P. Halepensis*, *P. brutia* and *P.nigra* has been reported in Cyprus causing malformations and loss of growth (Grüne, 1979). It is widespread in the Iberian Peninsula, usually associated with the bluestain fungi; it represents a problem in young reforestation and in established pine forest under adverse environmental conditions (Gil and Pajares 1986). It appeared in Greece associated with *B. eggersi*, *B. leoni*, *B. hellenicus*, *B. sexdentati* and *B. teratospicularis*, to *B. sexdentati* in Germany and, in Portugal carrying dauer larvae of *Bursaphelenchus* spp. and *B. hellenicus* (Braasch et al., 1999; Penas et al. 2006; Skarmoutsos and Skarmoutsos, 1999; Sousa et al. 2002). In the Spanish study it appeared to be widespread, associated mainly with *P. pinaster*. *Hylobius abietis* (Curculionidae) must be also highlighted because some specimens have been found carrying nematodes under their elytra and in high populations could cause damage to young pines.

Finally, it must also be remarked the potential risk of these insects as vectors of PWN or other *Bursaphelenchus* spp. which represent a pathological risk by themselves, as is the case for *B. sexdentati* which can cause damage to *Pinus nigra*, *P. pinaster* and *P. sylvestris* plantlets (Skarmoutsos and Michaloppoulo-Skarmoutsos, 2000), *B. fungivorus* to *P. sylvestris* (Caroppo et al., 2000) and *B. leoni* to *P. brutia* (Philis, 1996; Skarmoutsos and Michaloppoulo-Skarmoutsos, 2000).

By contrast with the situation in the Iberian Peninsula, it could be confirmed that the population density of *Monochamus* beetles is in general very low in Austria due to intensive forest protection measures in most of the stands. *M. sutor* and *M. sartor* are more frequent in the colder and more northern parts of Austria, whereas *M. galloprovincialis pistor* can be found mainly in southern warmer parts. A higher density of *M. galloprovincialis pistor* could be observed only in forests in bad condition.

Chapter 5 Biology and pathogenesis of *Bursaphelenchus xylophilus*

5.1 *Introduction*

The presence of *B. xylophilus*, the pinewood nematode (PWN), an extremely damaging organism and a threat to the territories of the EU, was first reported in Portugal and the EU in May 1999 (Mota et al., 1999). *B. xylophilus* is native to North America, occurring in Canada, the USA and Mexico. It was introduced into Japan, probably by way of timber trade from North America, where it has spread into China, Taiwan and South Korea (Mota & Vieira, 2004). PWN has become the number one pine pest in those countries and causes huge damage in Japan and China (Mamiya, 2004). *B. xylophilus* is now well established throughout the survey area in the Setubal region and constitutes a main concern for national (DGRF, 2006; DGRF-PROLUNP, 2006; Vieira & Mota, 2006) and EU government decision bodies.

Reflecting its partial saprophytic nature in the field, *B. xylophilus* can be easily maintained in the laboratory through its feeding on plant calli or fungi (Iwahori & Futai, 1990). Several fungi have in fact been studied to assess nematode reproduction (Ikonen, 2001). The PWN has a large brood size with a short generation time (Mamiya, 1975) and is suitable for microscopy because of its simplicity and transparent body and embryo (Hasegawa *et al*., 2004).

Knowledge about the biology and pathogenesis of the Pine Wood Nematode (PWN) was early reported and reviewed by Mamiya (1983, 1976), who provided the basis for the evaluation of the pathogenicity of *Bursaphelenchus xylophilus*. He perceived the relation between rapid wilting and a disorder of conifer water regulation as well as the role of the nematodes rapid population build up and migration into the tree in determining the severity of the Pine Wilt Disease (PWD). The evaluation of pathogenicity and susceptibility of the PWN – conifer pathosystem to date has been carried out in the classical pathological context of Koch's postulates. As a rule evaluation of susceptibility is based upon wilting and finally mortality of the tree, whereas pathogenicity is determined by the number of nematodes extracted form the tree *post mortem* (Bakke et al. 1991; Bedker et al. 1987; Braasch 1996; Braasch 1997b; Braasch 1997a; Schauerblume 1989; Schauer-Blume 1990; Panesar and Sutherland 1989; Skarmoutsos and Michalopoulos-Skarmoutsos 2000).

While the onset of discolouration in needles appears days to weeks after infestation with *B. xylophilus*, early wilt symptoms occur in trees several hours up to days, soon after nematode entry to the host tree. In general, wilting is a result of a disturbed sap ascent in infested trees that comes along with cavitations of the xylem vessels which is also called embolism (Kuroda and Kuroda 2004). Direct and indirect involvement of *B. xylophilus* in this process was confirmed histopathologically using dye solutions and microscopic examination of microtome sections from trees where PWN was previously inoculated (Fukuda et al. 1992). Histopathological reactions of the tree could be observed primarily in sections of the plant, where *B. xylophilus* was present (Ichihara et al. 2000). In particular early and rapid migration of nematodes is assumed to be a key factor for the pathogenicity of *B. xylophilus* after conduction of comparative inoculation trials and also *in vitro* using virulent and avirulent *B. xylophilus* and *B mucronatus* isolates (Iwahori and Futai 1995; Iwahori and Futai 1996; Odani et al. 1985). The ability of *B. xylophilus* to reproduce in a short time at a high multiplication rate is the other key factor of pathogenicity. Population dynamics of *B. xylophilus* has been studied during the development of the PWD in stem and root parts of highly susceptible host trees like *P. thunbergii*, *P. densiflora* (Futai 1980) and *P. sylvestris* (Melakeberhan and Webster 1990) as well as various North American conifer species (Forge and Sutherland 1996). Susceptible, tolerant and resistant conifer species are known by observation or inoculation trials from North America (Bolla and Wood 2004), Japan (Kishi 1995) and China (Yang 2004). Information about possible hosts for *B. xylophilus* other than conifer species like *P. sylvestris* and *P. pinaster* for Europe is restricted (Mota et al. 2004). Hence a first advance to fill this gab was achieved during the present study in testing nematode–host compatibility of 13 European conifer species (9 *Pinea* species, 2 *Larix* species, 1 *Picea* and 1 *Abies* species) and three known pathogenic provenances of *B. xylophilus* (Portugal, China and North America).

The development of early wilt symptoms of the PWD and migration as well as population build up proceed rapidly. Accordingly it is difficult to relate these causes to the expression of late symptoms like mortality. Mortality is regarded as the physiological “long term” reaction of the tree which in fact explains concerns about the effects of *B. xylophilus* on potential host trees. In bringing early development of the nematode population together with early and late reactions of the tree, this study aimed at developing a basis for the evaluation of pathogenicity of the PWN. Therefore two highly sensitive conifer species *P. sylvestris* and *L. decidua* and a tolerant conifer *P. abies*, all having a wide distribution in central and western Europe were part of this study as an exemplary approach. This study was the first approach to investigate the biology of the Portuguese isolate of *B. xylophilus* and its relevance for the pathogenicity and vulnerability of endogenous European conifers.

Despite several studies on the pathogenicity of different *B. xylophilus* isolates (mainly for *P. sylvestris*), no information is yet available about the relative virulence of the pine wilt nematode population established in Portugal, particularly under field conditions. Thus, the objective of this part of the overall pathogenicity study carried out by scientists at INIA, Portugal was to determine, via seedling inoculations, i) the pathogenicity of the Portuguese isolate of *B. xylophilus* for *P. pinaster* and *P. sylvestris* under Portuguese outdoors conditions; and ii) *B. xylophilus* population density and distribution in the early stages of pine wilt expression in *P. sylvestris*. We discuss the development of pine wilt expression in relation with the density and distribution of *B. xylophilus* within the seedlings.

In Portugal, no studies have yet been published on the pathogenicity of the isolates collected from the affected area. Under natural conditions, *P. pinea* does not appear to a susceptible host since the vector, *M. galloprovincialis*, seems to be unable to feed and reproduce in this tree species. Recently, preliminary results (Mota et al., unpublished) have demonstrated that PWN is capable of invading, multiplying and infesting *P. pinea* and causing plant mortality, although at a lesser rate than on *P. pinaster*. This, however, does not represent, at the present moment a significant risk.

Twenty-four isolates of *B. xylophilus* from nearly 100 collected so far have now been studied for their genetic diversity using RAPD-PCR analysis (Vieira et al., 2007). The degree of variability seems to be very low. There is a need to understand the pathogenicity of these isolates and to compare them with isolates from other geographical regions, such as Japan.

Another important issue, which needs to be addressed, is the reproductive performance of Portuguese *B. xylophilus* isolates when grown on different fungi associated with *P. pinaster* (maritime pine). The main fungal group that seems to colonise and dominate pine trees, from the affected area, is *Ophiostoma* spp. (A.C. Rodrigues, unpublished).

The purpose of the research carried out by scientists at the University of Evora was to investigate the biology and pathogenicity of certain Portuguese isolates and compare them with two Japanese isolates of the PWN. The main comparative aspects investigated were: (a) reproduction, sexual compatibility and cytogenetics; (b) culturing and reproduction of *B. xylophilus* in various fungi isolated from pine trees; (c) comparative pathogenicity (seedling mortality ratio) with Japanese isolates and also pathogenicity on 2 Portuguese pine species.

5.2 *Materials and Methods general*

Various sections describe materials and methods which were used in several different investigations while those used only in specific tests are mentioned in the appropriate sections.

5.2.1 Nematodes

The main focus of the investigations was on the causal agent for Pine Wilt Disease, the Pine Wood Nematode *Bursaphelenchus xylophilus*. For comparing tests *B. mucronatus*, the closest related species in the genus *Bursaphelenchus*, was also used.

5.3 *Portugal UEVORA: Nematode isolates, pine species and fungal isolation*

Bursaphelenchus xylophilus isolates were used from Portugal and Japan; two isolates from Portugal, “Tróia” (T) and “Herdade da Ferraria” (HF), were collected from within the affected zone, an area in the Setúbal Peninsula, of approx. 30 km radius centred in Pegões, 35 km SE of Lisbon. Nematodes were cultured on *Botrytis cinerea*, in Petri dishes with MEA medium, and kept in the laboratory, with frequent re-culturing. Four PWN isolates from Japan, “S10” and “T4” (virulent) and C14-5 and OKD-1 (avirulent), were used for comparative studies with the Portuguese isolates. One hundred and sixty 4-yr-old Japanese *Pinus thunbergii*, Japanese black pine, seedlings were used for inoculation and pathogenicity tests.

In Portugal, 50 *Pinus pinaster* and 50 *P. pinea* (with the respective controls) were used for the pathogenicity tests. Two PWN isolates (T and Bx) were used in the tests. Plants were kept inside a greenhouse, in Évora, following proper authorisation from DGPC. The laboratory is many km outside the affected area, in an olive and cork oak growing region, with no pine trees in proximity. Daily monitoring was implemented during the testing period (July-October 2006) to check for any possible insect vectors (*Monochamus* spp.) and at the end of the experiments all plants and soil were incinerated.

For fungal isolations, mainly for collection of *Ophiostoma* spp., 8-cm diameter sections of *Pinus pinaster* trees were cut, surface sterilised and maintained inside humid Petri dishes (with cotton and sterilised water). After 3 weeks, perithecae were collected and cultured in an appropriate *Ophiostoma* medium (Wingfield, 1993). The isolated fungal species (6) were cultured and used for media for nematode multiplication rate studies (Ikonen, 2001).

5.3.1 Developmental biology and cytogenetics

Detailed procedures are described in Hasegawa et al., (2006). Methods for culturing, handling, and observing *B. xylophilus* were as described by Hasegawa et al. (2004). We examined the chromosome behaviour in *B. xylophilus* during gametogenesis and early embryogenesis from fertilisation to the 2-cell stage embryo, without squashing, by DAPI staining and confocal laser-scanning microscopy. The coding regions of ribosomal RNA on the chromosomes were visualised by fluorescence *in situ* hybridisation (FISH), distinguishing this pair of chromosomes from the others.

5.3.2 Hybridisation experiments

To evaluate the sexual compatibility of the isolates, three males of one isolate and a single female of another isolate were placed in a plastic Petri dish (30 mm diam.) together with mycelia of *Botrytis cinerea* growing on 0.05% malt extract agar. Compatibility of all combinations among the four isolates tested (T, HF, S10, and C14-5) is shown in Table 7. After 5 days, the progeny were extracted with a Baermann funnel and counted. The data, minimum two and maximum 11 replicates were log-transformed and analyzed.

Table 7: Replication number for each combination of mating between four PWN isolates

	Male			
Female	**HF**	**T**	**S10**	**C14-5**
HF	4	11	4	5
T	9	11	6	7
S10	6	5	5	4
C14-5	6	2	5	3

5.3.3 Pathogenicity experiments

On 8 August 2005, each 4-yr-old pine seedling was inoculated at the base with approx. 5,000 nematodes of one of the four isolates (T, HF, S10, and C14-5) and embedded in cotton to assure nematode survivability during the entry and infestation process. Forty-five seedlings were used for each nematode isolate. After inoculation, the bark and cotton were covered with parafilm to prevent them from desiccating. The seedlings were outdoors during the experiment. The maximum and minimum temperatures during the experimental period averaged 31.7°C and 24.2°C, respectively. Symptoms were examined 21, 25, 29, 33, and 37 d after inoculation. Symptoms appearing in each seedling were categorised into three stages: *Stage 1* - needle necrosis, discolouration, or drooping of some 1-yr-old needles; *stage 2* - almost all the 1-yr-old needles drooped, displaying necrosis or discolouration; and *stage 3* - wilting symptoms extending over 50% of current-year needles. Mortality ratio was estimated for each nematode isolate by the number of seedlings reaching stage 3. After 4 weeks, the seedlings were cut at the base, and 50-cm shoot segments were weighed (wet weight), cut into small pieces, and extracted on a Baermann funnel. After 24 hr, nematodes were collected and counted. Nematode density (number per gram dry weight) was log-transformed, and the transformed data subjected to analysis of variance (ANOVA) and statistically compared using the Tukey-Kramer post-hoc analysis.

5.4 *Germany: Isolates of Bursaphelenchus xylophilus and Bursaphelenchus mucronatus used for inoculations trials*

All *B. xylophilus* isolates, which were used for the present inoculation trials were reared as part of the reference collection of life *Bursaphelenchus* species of the Department for national and international Plant Health, Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany (Table 8).

Table 8: Bursaphelenchus isolates used in the investigations and origin. Isolate code according to the collection of BBA, Germany

Isolate	Geograph. origin	Isolated from	Date of Isolation
Bursaphelenchus xylophilus			
US DE 2 (w)	USA	Wood package	2002
Ne 12/02	Nanjing / China	*P. thunbergii*	2002
PT 3 (w)	Lezirias / Portugal	*P. pinaster*	2003
PT 3.1 (w)	re-isolate from PT 3(w) after first test year	*P. sylvestris* test tree	2004
Bursaphelenchus mucronatus			
DE 30 (w)	Germany	Wood package	2005

According to the objectives of the project, the main focus of this study was on the Portuguese provenance of *Bursaphelenchus xylophilus* and its interrelation with European conifers. The *B. xylophilus* isolate PT 3 (w) was provided by Dr E Sousa and was used as a reference isolate. Morphological identification was conducted with the identification keys and descriptions by Hunt (1993), Mamiya and Kiyohara (1972) and Nickle *et al* (1981).

Molecular identification was carried out by Dr. W. Burgermeister and co-workers (BBA) using the ITS RFLP technique as described in Burgermeister *et al* (2005) and was applied to all isolates of *B. xylophilus* before inoculation and after re-isolation from host trees.

5.4.1 Baermann-extraction of nematodes

A modified Baermann funnel technique was used in this study to extract *B. xylophilus* from wood and root samples. The method was modified as follows: Plastic (PVC) funnels had different

diameters to fit the variety of sample sizes (∅ 100 mm, 120 mm and 150 mm). The outlet consisted of a silicon pipe and a clip acting as a valve. The samples were placed on two layers of a commercial cotton milk-filter (∅ 140 mm – 200 mm). The funnel was then filled with water to cover the entire sample. Wood and root samples were left for 48 hours in water. After this time, nematodes were collected by opening the clip and letting off a 10 ml extract for further investigations.

5.4.2 Rearing and multiplication of nematodes on artificial medium

All isolates of *Bursaphelenchus xylophilus* were reared in the reference collection using a sporulating form of the grey mould rot fungus *Botrytis cinerea* as feeding source. The fungus was cultured on a 1.5 % malt extract Agar medium (MEA: 15 g of Agar, 15 g of malt extract, 750 ml distilled water, 7.0 pH) in sterile Petri-dishes. When nematodes were multiplied for inoculation they were cultured exclusively on the "non-sporulating" form of *B. cinerea*. The "non- sporulating" form does not produce spores and survives exclusively by vegetative growth of the mycelium The "non sporulating" form mutated from the original "sporulating" form and first was used by Dr. M.A. Harmey (Formerly Department of Botany, University College Dublin. Ireland). The main reason to use this type for multiplication of nematodes was to prevent contact between nematodes and spores of the fungus *Botrytis cinerea* in its sporulating form, which is known as a pathogen on many hosts. The population for inoculation trials was built up for a period of about 8 weeks. To inoculate predominantly vital and active individuals, nematodes were extracted using the modified Baermann funnel technique with collection of nematodes 24 hours later. Nematodes were selected for general vitality (movement).

5.4.3 Preparation of nematodes for inoculation

Depending on the particular inoculation trial, nematodes were inoculated at different densities per tree in a suspension of 300 µl with distilled water. To achieve the target inoculum, nematode extracts from all funnels were combined into one 500 ml beaker and concentrated by discarding the supernatant after deposition of nematodes. The beaker was placed on a magnetic stirrer to mix the suspension. Concentration of nematodes was then determined by taking two 100 µl samples of the homogenised nematode suspension and counting the number of individuals. This procedure was repeated until the final concentration was obtained. Finalised concentrations were portioned out in Eppendorf tubes (Eppendorf, Hamburg, Germany) containing the 300µl nematode suspension and used on the same day. Table 9 provides the numbers of nematodes inoculated per tree for all trials.

Table 9: Number of inoculated nematodes per trial.

Isolate	Number of nematodes inoculated per tree	Trial code	Trial	Year
Bursaphelenchus xylophilus				
US DE 2 (w)	4000	P 1	Pathogenicity of *B. xylophilus* isolates against 13 conifer spec.	2003
Ne 12/02	4000			
PT 3 (w)	4000			
	100,300,800,2400, 4000,6000,10000	P 2	Effect of inoculated population on mortality	2004
	2400	P 3	Migration and distribution of *B. xylophilus*	
	4000	P 4	Effect of temperature on *B. xylophilus*-host interaction	
	4000	P 5	Development of a technique to measure transpiration of inoculated trees	2005
	4000	P 6	Measuring physiological parameters of inoculated trees	

PT 3.1 (w)	2400	P 7	Development of *B. xylophilus* before and after dormancy	
Bursaphelenchus mucronatus				
DE 30 (w)	4000	P 8	Migration and distribution of *B. mucronatus*	2005

5.4.4 Fixation and counting

The processing of nematodes relates to extraction, fixation and counting. After extraction, by the modified Baermann funnel technique, the 10 ml suspension was left for about five hours at room temperature to let nematodes deposit at the bottom of the vessel. The supernatant then was carefully removed by using a water jet filter pump. Then a 80 C hot solution for fixation (TAF concentrated) was added to the vessels to kill and preserve the nematodes. Depending on the density of nematodes this suspension (approximately 5 ml) was either completely counted for *B. xylophilus* or an equivalent of 50 µl to 1 ml was transferred to a counting plate after homogenising the suspension. The counting plates were provided with a 5 by 5 mm grid to prevent double counting. Whenever an equivalent was withdrawn, there were two parallels being counted to control possible variations.

5.5 *Host trees*

To study the pathogenicity, population dynamics and invasion of *B. xylophilus* on the one hand and the susceptibility of host trees on the other hand, conifer species were selected according to their occurrence, distribution and importance in Europe. Due to the constraints of a restricted space in greenhouses and climate chamber facilities on the one side and the requirement of a reliable number of replications on the other side, only small trees could be used for inoculation trials. The host trees in general, therefore, were not older than 4-5 years old, when being inoculated.

5.5.1 Tree species and stage of development

Overall, 13 different Conifer species were used in the trials. *Pinus sylvestris* (L.), *Larix decidua* (Mill.) and *Picea abies* (Karst.) were investigated to a more specific degree. The major host tree species was *Pinus sylvestris* (L.) as it fulfilled two essential functions for studying the nematode behaviour to a relevant degree. First it was known to be highly susceptible to *B. xylophilus* (Bakke et al. 1991; Bergdahl and Halik 1999b; Braasch 2000b; Schauer-Blume 1990b). Second it is broadly distributed in Germany and northern central Europe with expansions from Scandinavia to the Mediterranean (Kindel 1995). All other conifer species were mainly selected due to their distribution in Europe and *P. abies* as well as *L. decidua* in particular for their known association with *B. xylophilus.* An overview about host tree species and respective number of trees involved in all inoculation trials of this study, as well as age classes and geographic distribution is provided in Table 10.

Table 10: Tree species with geographic distribution, age, height, stem diameter and number of used trees. *trees provided by a nursery in Belgium, **provided by an Italian nursery.

Year	Tree species	Geogr. Distribution	Age years	Height cm	Stem Ø mm	Number of trees	Trial
2003	*Pinus sylvestris*	north eastern Europe	2-3	61.9	9.9	140	P1
	P. nigra austrica	southern Europe	3-4	56.5	11.7	140	
	P. mugo	central (mountinious) Europe	3-4	24.4	7.9	140	
	P. cembra	central (mountinious) Europe	3-4	15.1	9.6	140	
	P. halepensis *	western Mediterranean	2-3	54.1	6.1	140	
	P. strobus	north America	3-4	34.5	7.6	140	
	P. pinaster **	Mediterrenean	2	48.1	5.7	140	
	P. radiata	south-west USA	2-3	41	6.1	140	
	P. pinea *	Mediterrenean	3-4	84.4	9.9	140	
	Picea abies	north east central Europe	3-4	38.3	6.1	140	
	Abies alba	south east (mountinious) Europe	3-4	38.3	11.7	140	
	Larix decidua	central (mountinious) Europe	2-3	56.7	6.4	140	
	L. kaempferi	northern coastal Europe	3-4	81.1	9.1	140	
2004	*P. sylvestris*	north eastern Europe	3-4	58.4	8.7	160	P2
						110	P3
						360	P4
	L. decidua	central (mountinious) Europe	3-4	64.9	7.7	360	P4
	Picea abies	north east central Europe	3-4	39.6	6.2	360	P4
2005	*P. sylvestris*	north eastern Europe	3-4	not measured		100	P5
			3-4			30	P6
			4-5			90	P7

5.5.2 Segmentation of trees

Trees were segmented in small sections to track nematodes inside the trees. This particular method was applied in three trials of this study. The basic segmentation that first was used consisted of 17 segments that were selected according to the morphological characteristics of the trees (Figure 10).

After this detailed segmentation another division consisting of 11 segments but basing on this first segmentation was applied to trees of the two other trials. One focused on the migration of *B. mucronatus* and the other one on the distribution of *B. xylophilus* during the detection of physiological parameters of the Pine Wilt Disease. These 11 segments were found to give an evident resolution of the tree in order to detect the nematodes distribution (Fig 2.3a -2.3b). There was a minor but important difference concerning the positions of some segments between the two trials with 11 segments. Whereas the root parts of trees inoculated with *B. mucronatus* were divided into roots and root collar this was not divided in trees carrying *B. xylophilus*. Vice versa the main stem below the inoculation point was divided into the segments Sb 1 and Sb 2 in trees with *B. xylophilus* but there was only one segment called Sb1 in trees with *B. mucronatus*. The reason for this method was that main interest for segmentation of roots with *B. mucronatus* inoculated trees was, if they could enter all parts of the roots. In contrast segmentation of trees inoculated with *B. xylophilus* was carried out to find out, if nematodes could leave the inoculation side.

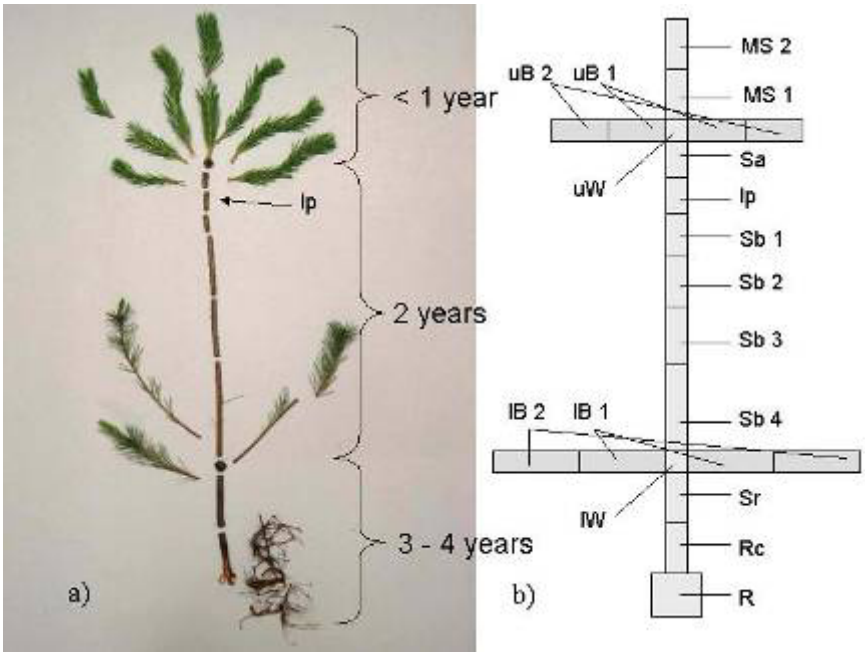


Figure 10: Segmentation (11 segments) of *P. sylvestris* saplings modified according to the basic segmentation (17 segments) applied in the inoculation trial a) Distribution and migration of *Bursaphelenchus mucronatu*s in young *Pinus sylvestris* at controlled optimum temperature b) development of *Bursaphelenchus xylophilus* before and after dormancy of *P. sylvestris* at late seasonal infestation; Grey segments differ from the basic segmentation; Encircled segments differ between the two modifications a) and b); MS (main shoot), uB (upper branches), uW (upper whorl), Sa (Stem above inoculation point), IP (inoculation point), Sb (1+2) (Stem below inoculation point), lB (lower branches), lW (lower whorl), Sr (Stem above root collar), RC (root collar), R (roots)

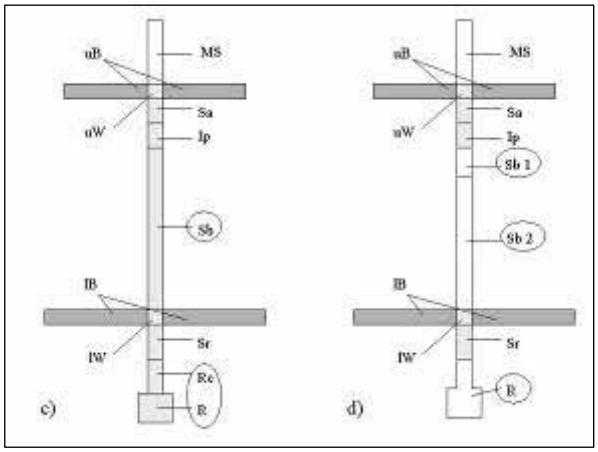


Figure 11: Processing of wood (W), root (R) samples and needles (N) from conifers for the determination of nematode density, fresh and dry weight of samples; Samples were processed in the order: 1. Separation of wood /needles and roots, 2. determination of fresh weight (W, R and N), 3. Baermann Extraction of nematodes (W,R), 4. Drying at 105 °C for 48 h and 5. determination of dry weight (W, R, N)

5.5.3 Bark-inoculation technique

The aim of the inoculation technique was to enable *B. xylophilus* to enter its host to a most natural degree. Comparing 10 different techniques of inoculating *B. xylophilus* into host trees Kishi (1995a) found the bark inoculation technique to be most efficient in relation to mortality of the host trees. The bark inoculation is basically a longitudinal L-shaped slit of approximately 1 cm that is cut into the bark of test trees. In this cut a stripe of cotton is inserted, wrapped with foil and sealed at the bottom. The nematode suspension was inserted with a pipette and the foil was sealed at the top to prevent desiccation (Figure 12). This technique was applied in several studies (Bakke et al. 1991; Braasch 2000b; Melakeberhan et al. 1992; Schauer-Blume 1990b) and modified to the situations of the host trees used in this study. Because *Monochamus* beetles in nature approach the crown of pines the bark inoculation focused on the previous year's shoot of the tree. The youngest shoot was avoided due the fact that these shoots had not yet differentiated a wood part.

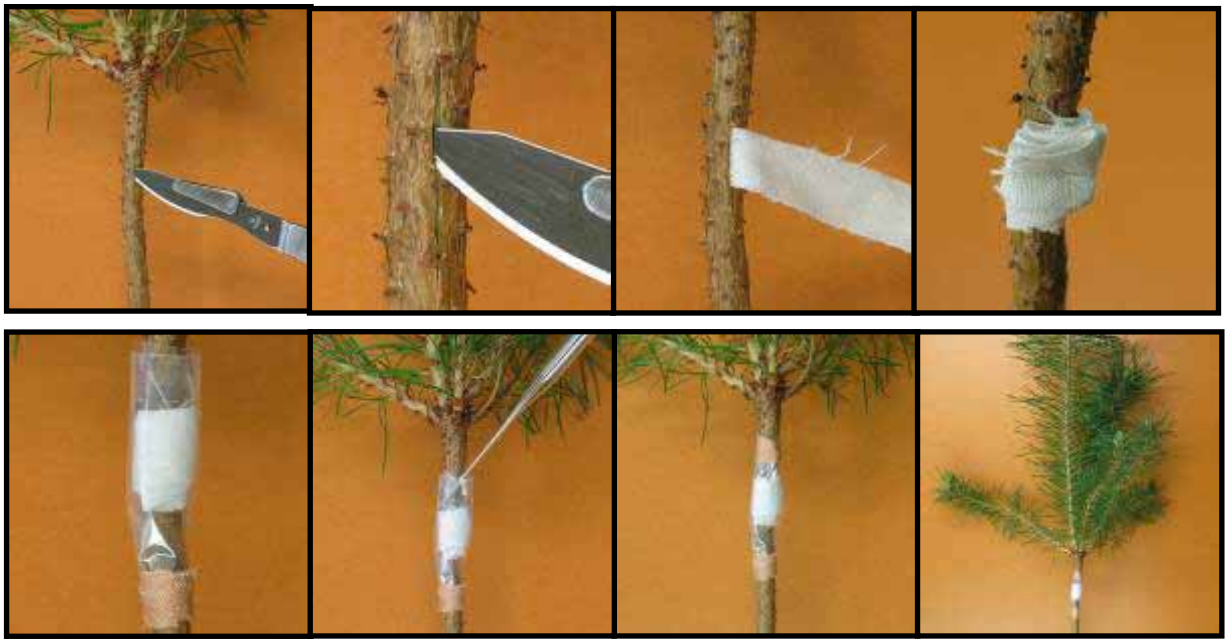


Figure 12: Technique to inoculate B. xylophilus suspension into young trees using the example of Pinus sylvestris

Taking *Pinus sylvestris* as the reference host tree, inoculation of this species was performed on the previous years shoot below the youngest whorl.

5.5.4 Assessment of wilt symptoms

The characteristic symptom of the Pine Wilt Disease (PWD) appears as wilting of needles that implies an optical and a physical change of needles. The visual evaluation of wilt symptoms based upon the assessment of the symptom coverage and its progression during the induced PWD. Altogether 6 wilting classes were defined that covered a range from: No visual symptom "0 %" to completely wilted and dead "100 %" (Table 11). The assessment of wilt symptoms was applied similarly to all different conifer species in this study.

Table 11: Classification of wilt according to expression of needle discoloration and relation to development of Pine Wilt Disease

Wilt class	Tree coverage of needle discoloration in %	Physiological condition
0	0	alive
1	1 - 25	Pathogenesis of PWD
2	26 - 50	
3	51 - 75	
4	76 - 99	
5	100	dead

5.5.5 Sampling and handling of samples

The sampling of trees was carried out in a similar way between the trials in this study. In general above ground plant parts and root parts were sampled and prepared separately (Figure 13).

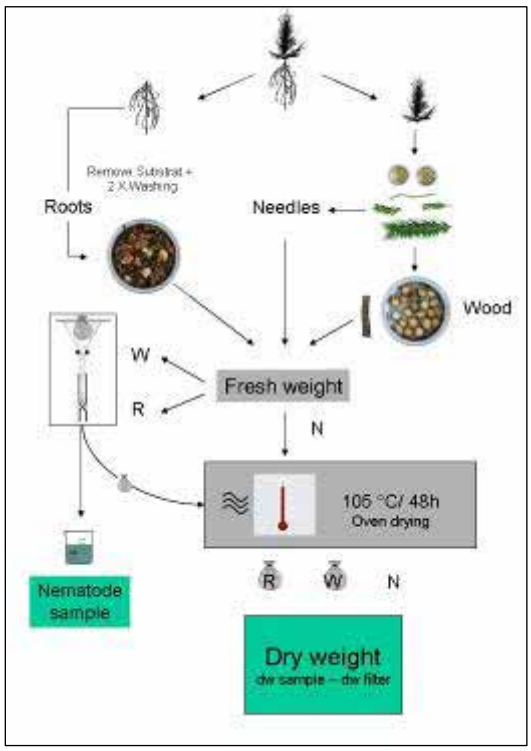


Figure 13: The process of sample treatment; W = wood, R = roots, N = needles, dw = dry weight

Above ground parts of the trees were separated from roots. The cutting site always was the intersection between the root collar and first stem part. Depending on the particular experiments, above ground plant parts were cut into further segments. The whole plant (without roots) or the segments were labelled according to respective tree number and if needed to the segment. All plant parts then were cut separately into 5 to 10 mm pieces. The resulting samples were weighed to obtain the fresh weight. Then samples were submitted to the Baermann extraction procedure for 48 hours. Before this step, root parts were separated from the substrate and substrate dwelling nematodes by three consecutive steps: 1. removing the main part of the substrate mechanically, 2. pre-washing roots and 3. washing roots again to remove adherent material from the pre-wash. Afterwards root parts were cut into 5 to 10 mm sections. Fresh weight of roots was not recorded, due to the disturbance of either remaining substrate particles or washing. To detect the relative water contents, old and young needles were dried separately at 105 °C for 24 hours immediately after sampling the trees. This step was applied to all wood and root samples, after nematode extracts were collected from Baermann funnels. All wood and root samples including the milk filters were oven dried at 105 C for 48 hours. It was necessary to take the dry weight of filters prior to extraction, because samples were dried together with the milk filter at the end of the extraction in order to prevent loss of material. Dry and fresh weight of wood, root samples and needles were determined by the method described in chapter 5.5.6.

5.5.6 Determination of fresh, dry weight and relative water contents

Relative water contents were determined in accordance with the method for the determination of dry weight and water contents of woody plants consistent with DIN ISO 11465 described in the handbook on analytics in forestry (BMELV 2006). Samples basically were oven dried at 105°C until consistency of weight, which was usually reached after 48 hours. The relative water contents were only taken for wood and needles, as the fresh weight of roots could not determined to a reliable

scale. It was impossible to remove the substrate totally from the root surface except by washing it off. The water content was calculated from the formula:

$$H_2O\,[\%] = \frac{fres\ weight - dry\ weight}{fresh\ weight} * 100$$

5.6 *Influence of nematode-density in the inoculum on the development of wilt and population after inoculation*

The range of the number of nematodes inoculated in young pine trees to investigate pathogenicity varies greatly among numerous studies depending on host tree species, age class of trees, *Bursaphelenchus* species and isolates used (Bergdahl and Halik 1999a; Braasch 2000a; Kishi 1995b; Kawaguchi et al. 1999; Melakeberhan and Webster 1990; Riga et al. 1991; Schauer-Blume 1990a). A population that is inoculated enters the host in parts due to callus formation of the tree. Therefore the inoculum needs to contain a sufficient number of nematodes that is capable to sustain and to build the initial population (Braasch 1997).

5.6.1 Materials and Methods

To assess the effect of the inoculum level of *B. xylophilus* on susceptibility of *P. sylvestris*, wilt development, tree mortality and population dynamics seven different densities of nematodes were inoculated into trees: 100, 300, 800, 2400, 4000, 6000 and 10000 nematodes / tree. The range was expected to give an adequate base line on the inoculation densities to utilise for further trials with this pathosystem.

Altogether 140 *Pinus sylvestris* trees were inoculated with nematodes of the *B. xylophilus* isolate "PT 3 (w)". Twenty trees were inoculated with distilled water as control variant. All trees were placed in a climate chamber controlled at 25°C and 50 % rH. Light duration was adjusted to 12 h. Each inoculum density level was inoculated into 20 *P. sylvestris* trees, which were aged three to four years. These trees were divided into two groups. The first group comprised ten trees, which were sampled four weeks after inoculation and submitted to Baermann funnel extraction of nematodes to investigate the population levels of *B. xylophilus* per tree achieved over time. The second group (also 10 trees), were left until death. When trees were dying, they were sampled individually to determine the nematode densities at this stage. As water contents of wood were thought to be differently altered depending on the particular tree, nematode density was related to one gram of dry matter. Additionally trees of the second group were segmented into two parts: The above soil plant organs, which will be further referred as to "plants" and the roots. Trees of both groups and the control plants were assessed for wilting symptoms according to the assessment scheme described in section 5.5.4. Relative water contents of wood was determined for trees of the second group in accordance with the description in section 5.5.6 when they reached wilting class five (death of the trees).

5.7 *INIA Portugal: Plant material and nematode inoculation*

5.7.1 Materials and methods

Four-year-old *P. pinaster* and *P. sylvestris* were obtained from Portuguese nurseries and placed in field conditions inside the Portuguese affected area (Tróia, Portugal) two months before the inoculations. Throughout the experiment, all the seedlings had a regular water supply. *Pinus pinaster* seedlings were 130,9±2,5 cm (mean ± SE) in height and 317,4±23,0 g in weight and *P. sylvestris* 60,1±1,2 cm and 43,2±3,3 g, respectively.

The Portuguese isolate of *B. xylophilus* PT-3 (W) was obtained from a *P. pinaster* tree in the Setúbal peninsula and reared on non-sporulated *Botrytis cinerea* Pars. growing in PDA. The nematodes were extracted a day before the seedling inoculations using the modified Baermann

funnel technique. On 1 July 2004, seedlings of both species were inoculated with 4000 *B. xylophilus* in water suspension and the control seedlings were inoculated with wash water of Petri dishes with non-sporulated *B. cinerea*. To inoculate the nematodes a slight cut was made in the main stem of the seedlings and a piece of cotton tissue was introduced inside the wound. The suspension was then injected within the cut and the wounded area was wrapped and sealed with a plastic stripe.

5.8 *INIA Portugal: Symptom development and nematode detection*

5.8.1 Materials and methods

Thirty *P. pinaster* and thirty *P. sylvestris* seedlings were inoculated with the nematode and 10 seedlings of each species were used as controls to assess the pathogenicity of *B. xylophilus.* The 50 *P. sylvestris* used in the *B. xylophilus* early stages of wilt expression experiment were followed over a period of 52 days. In a first phase 5 specimens were randomly selected and collected 2 times a week. After 18 days the effort was reduced to a weekly collection.

The disease development of inoculated seedlings was examined weekly by observing the percentage of needles showing chlorosis. The wilt level was classified as follows: null= 0%; weak = 1-49%; moderate = 50-74%; strong = 75-99%; dead tree = 100%. The dead seedlings were collected and the nematodes extracted. After four months all the remaining inoculated trees and the control trees were also collected.

After the needles were removed, each tree was divided in four vertical segments: the apical end (small branches included), the inoculation segment, the basal segment and the root system. The segments were then cut into small fragments and the nematodes extracted using the modified Baermann funnel technique over a 48h period for the determination of *B. xylophilus* numbers. The nematodes in each segment were counted in three replicate aliquots. After nematode extraction the dry weight of each segment was determined after drying at 80 ºC for 48h and the number of *B. xylophilus* per dry weight calculated.
All statistical analyses were conducted using SPSS 12.0®. Data dispersion is given by range or standard error. Comparisons between means were used to determine differences in days of treatment and nematode numbers. Nematode density within the pine segments was log transformed ($\log_{10}$ (x+1)) to meet the assumption of normality. This data were submitted to analysis of variance and Student-Newman-Keul's multiple-range test (SNK) was used to test the significance of differences among means.

5.9 *UEVORA, Portugal Results*

5.9.1 Developmental biology and cytogenetics

Results are described in detail in the “Results” section of Hasegawa et al. (2006) and in Figure 14.

Chromosome structure and behaviour in both meiosis of the germ cells and mitosis of the embryo from fertilisation to the 2-cell stage in *Bursaphelenchus xylophilus* were examined by DAPI staining and three-dimensional reconstruction of serial-section images from confocal laser-scanning microscopy. By this method, each chromosome’s shape and behaviour were clearly visible in early embryogenesis from fertilisation through the formation and fusion of the male and female pronuclei to the first mitotic division. The male pronucleus was bigger than that of the female, although the oocyte is larger and richer in nutrients than the sperm. From the shape of the separating chromosomes at anaphase, the mitotic chromosomes appeared to be polycentric or holocentric rather than monocentric. Each chromosome was clearly distinguishable in the male and female germ cells, pronuclei of the one-cell stage embryo, and the early embryonic nuclei. The haploid number of chromosomes (N) was six (2N=12), and all chromosomes appeared similar. The chromosome pair containing the ribosomal RNA-coding site was visualised by fluorescence *in situ* hybridisation. Unlike the sex determination system in *Caenorhabditis elegans* (XX in hermaphrodite and XO in male), the system for *B. xylophilus* may consist of an XX female and an XY male.

5.9.2 Hybridisation experiments

The numbers of progeny that resulted from the mating among isolates are presented in Figure 15; results that yielded less than 5 nematodes were disregarded, thus the figure displays trimmed data. All crossings (male x female, presented in this order) produced viable progeny, with higher numbers from C14-5 x HF, C14-5 x T, S10 x HF, and S10 x T. The lowest values of progeny resulted from C14-5 x C14-5 and S10 x C14-5. A sex effect may be involved since the numbers of progeny produced from the crossing between male C14-5 x female HF were much higher than those produced from the reciprocal crossing between female C14-5 x male HF. The same phenomenon was observed with male S10 x female C14-5 and the reciprocal female S10 x male C14-5 cross.

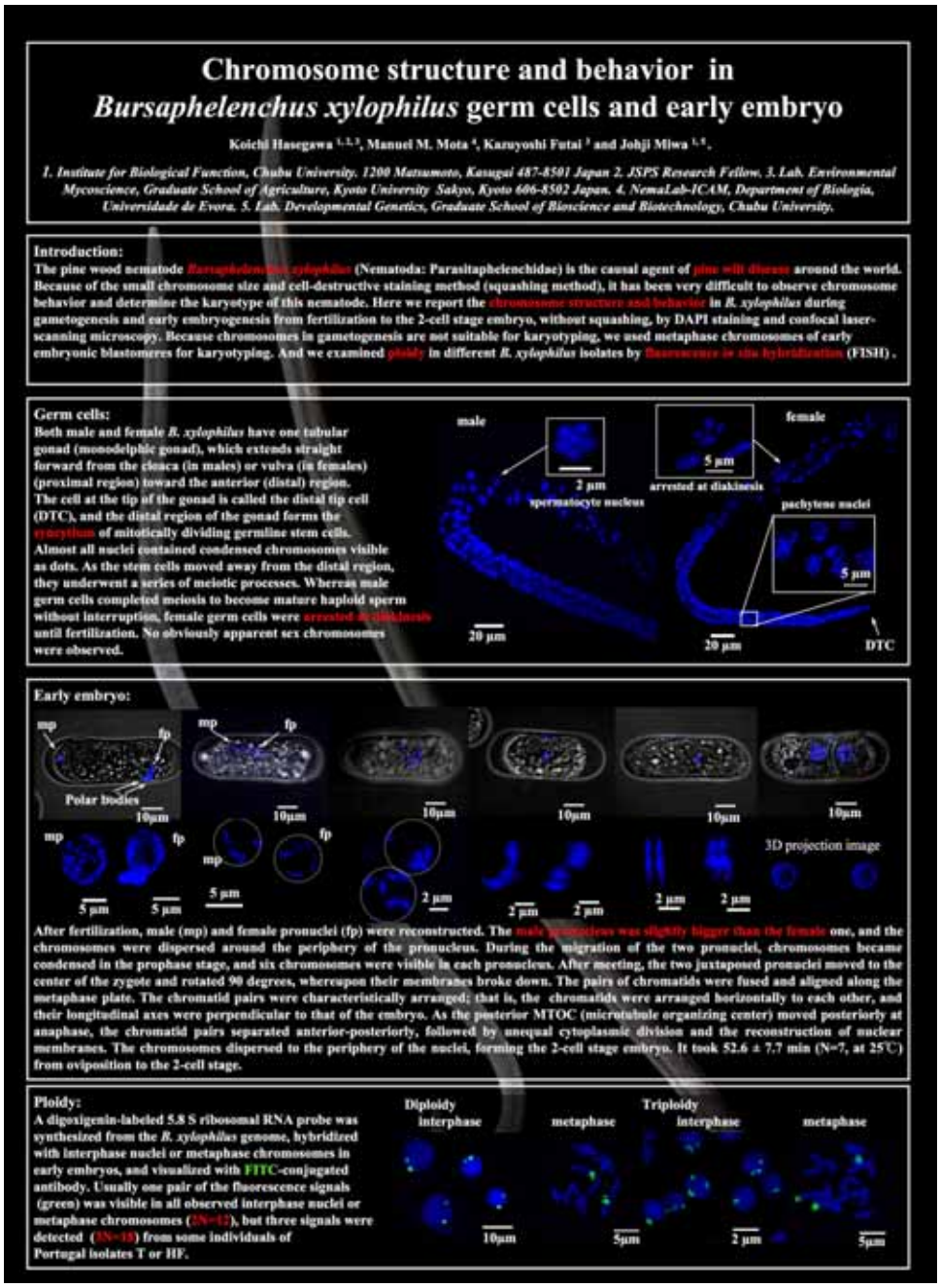


Figure 14: Summary of chromosome structure and behaviour of *Bursaphelenchus xylophilus* from Hasegawa *et al* (2006)

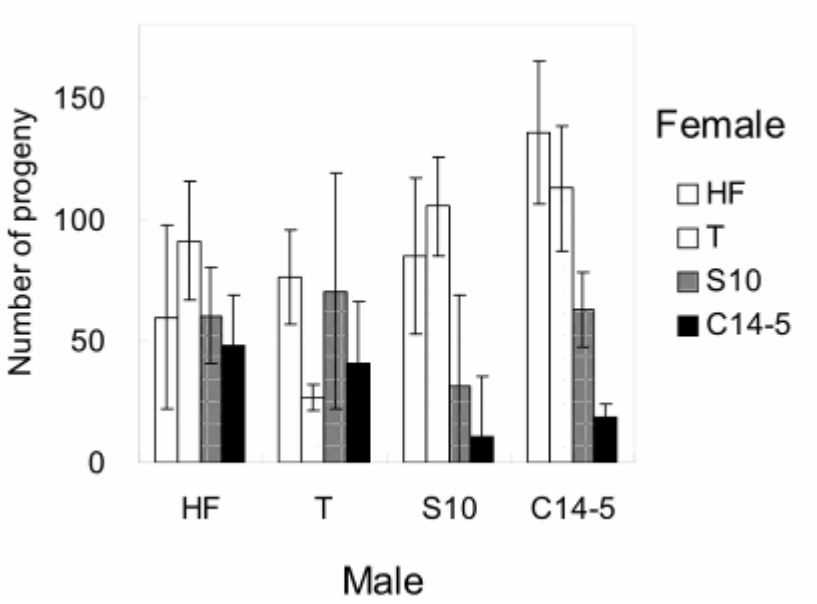


Figure 15: Number of progeny yielded from each combination of crosses between *Bursaphelenchus xylophilus* isolates

5.9.3 Nematode reproduction on different fungi

Very preliminary observations on this aspect have been made so far, and which have not yet been published. One fungus (F8) seems to allow for a greater reproduction of the nematode, greater than in *B. cinerea*, however it is still too early to allow for solid data and conclusions.

5.9.4 Pathogenicity experiments

In comparing mortality ratios (rate of seedlings reaching stage-3 symptoms), isolate T was significantly more pathogenic than S10 (Tukey's WSD method, α = 0.05), reaching 90% mortality ratio 37 days after inoculation, and also by reaching a greater speed in causing mortality (Figure 16, Figure 17).

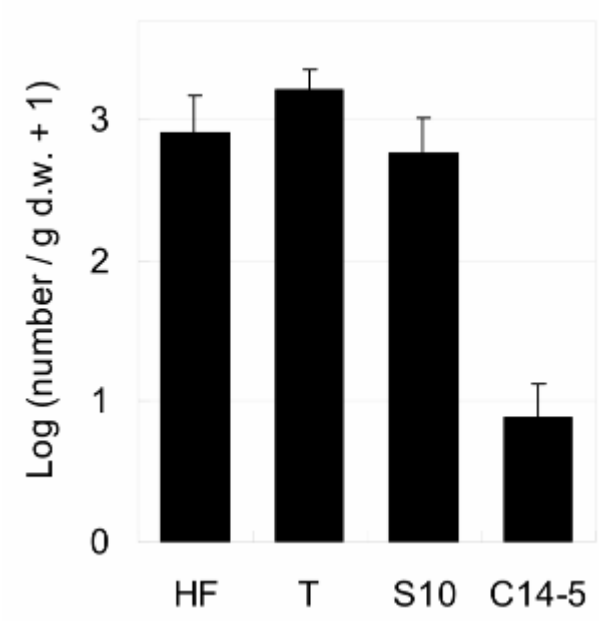


Figure 16: Propagation of four *Bursaphelenchus xylophilus* isolates in *Pinus thunbergii seedlings*

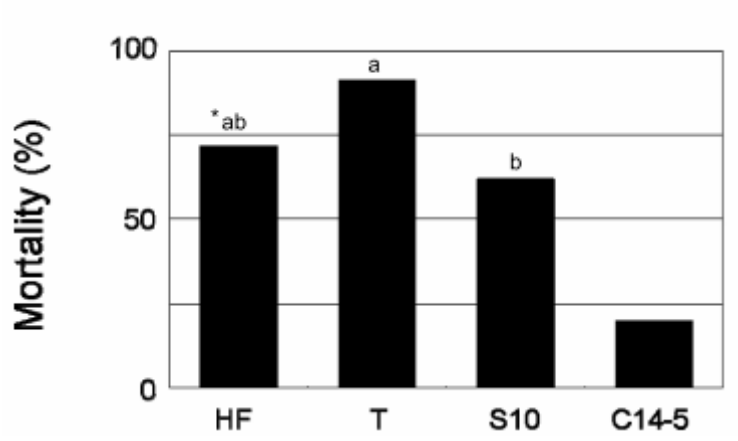


Figure 17: Mortality of Japanese black pines five weeks after inoculation with *Bursaphelenchus xylophilus*

HF and S-10 isolates performed in a very similar fashion, causing 60-70% mortality rate at the same time (37 days). The Portuguese isolates (T and HF) were capable of causing a 100% value of visible symptom development (stage 1 - 3) whereas the Japanese virulent isolate (S10) caused a 90% value (Figure 18).

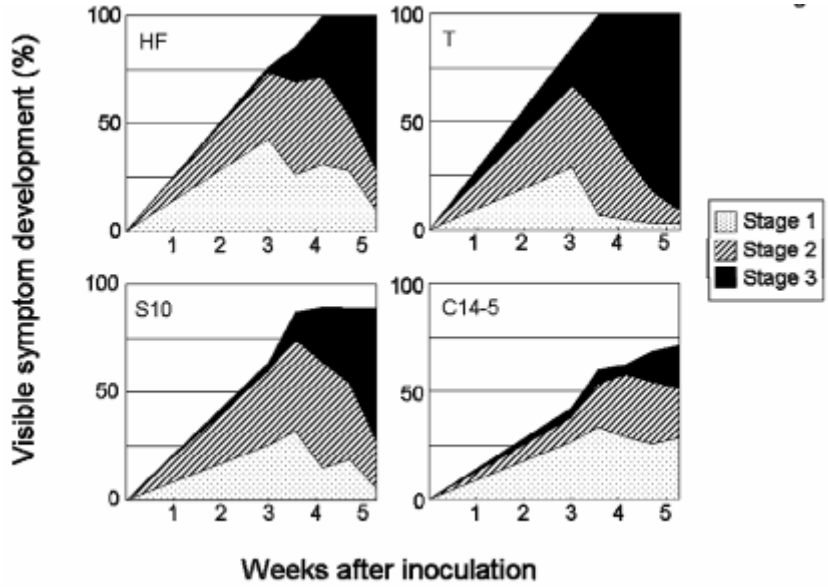


Figure 18: Development of visible symptoms on host pine trees inoculated with *Bursaphelenchus xylophilus*. Stage 1: needle necrosis, discolouration or drooping on year 1 needles; Stage 2: drooping of almost all year 1 needles – necrosis or discolouration; Stage 3: wilting of over 50% of current year needles

All virulent isolates reached the maximum values of mortality approximately at the same time (29 days). Regarding nematode propagation in pine seedlings, both Portuguese isolates yielded higher density (800-1700 nematodes/g drw weight (dw) on average) than the Japanese virulent isolate S10 (585 nematodes/g dw). As expected, the avirulent isolate C14-5 yielded significantly lower density (6 nematodes/g dw. on average) than the others ($p < 0.05$, Tukey-Kramer method). In all experiments, the Portuguese isolates, T and HF, produced a higher number of nematodes propagating within the seedlings as compared to the Japanese isolate S10, with a slightly higher value of T (Figure 18) Isolates HF and S10 generated similar values for nematode propagation.

As to pathogenicity of *B. xylophilus* to Portuguese pine species, results show a very fast infestation and mortality rate of *P. pinaster*, when compared to *P. pinea*, which was to be expected (Figure 19) Only after many weeks did *P. pinea* display some symptoms of possible susceptibility. These are still preliminary results and new experiments will take place in the Summer of 2007.

Figure 19: Stages in pathogenesis of *Bursaphelenchus xylophilus* on *Pinus pinaster* and *P. pinea* seedlings

5.10 *Germany Results*

The aim of the current investigation was to determine the influence of different nematode inoculum levels on symptom development, tree mortality and population dynamics of *B. xylophilus* in *Pinus sylvestris* trees. Symptom development of trees in the different inoculation levels (100 – 10.000 nematodes) expressed as the median of wilting classes are given in Figure 20.

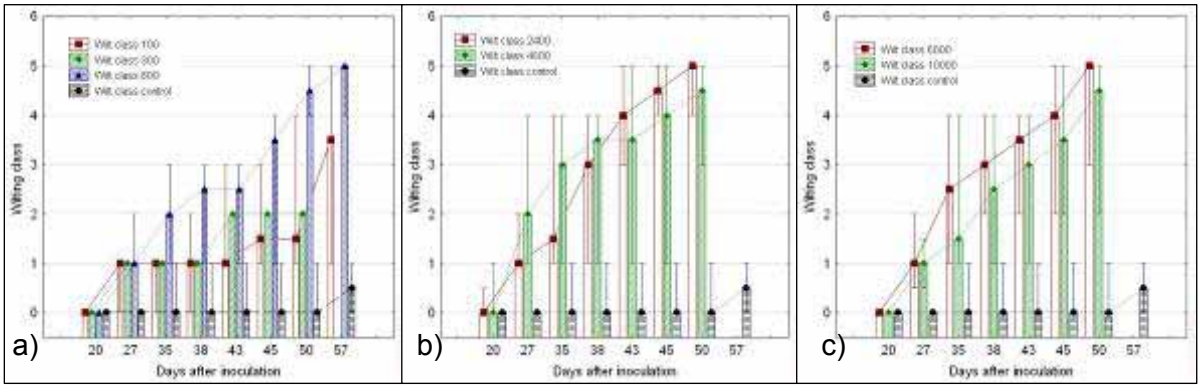


Figure 20: Median of symptom development in Pinus sylvestris trees after inoculation with *Bursaphelenchus xylophilus* at different inoculation densities: a) 100, 300 and 800; b) 2400and 4800; c) 6000 and 10000 nematodes per tree (n = 10).

Development of symptoms appeared to be similar between the inoculation levels 2400, 4000, 6000 and 10000. The majority of trees belonging to these levels showed a wilting class of three within the same period, 35 to 43 days after inoculation. The transmission from class two to three and four in the levels 2400 and 4000 nematodes per tree appeared slightly ahead of the same development in levels 6000 and 10000 nematodes per tree. Lower inoculation levels resulted in a delay of symptom development, more obviously in the inoculation levels of 100 and 300 nematodes per tree than in 800. As a whole, the majority of trees that were inoculated with any numbers of nematodes of the applied levels had approached the most severe wilting class five at the end of the experiment, 72 days after inoculation. The maximum wilt class that was assessed with the control variation was one but the majority remained healthy and so clearly below this class for the whole period. Consequently mortality of inoculated trees in general was high, regardless of the number of nematodes inoculated. A slightly lower mortality of 70 % was achieved when inoculating 100 or 300 nematodes per trees compared to higher levels, where 80 % to 90 % of trees died within 72 days after inoculation. When inoculating 100 nematodes per trees the rate of trees where *B. xylophilus* could be extracted was low (60 %).

The relative water content of wood of healthy trees in the control variation in average was above 70 %, whereas infested trees showed reduced relative water content (Table 12).

Table 12: left - Mortality of *Pinus sylvestris* trees after inoculation with different numbers (variation) of *Bursaphelenchus xylophilus*; right - Average relative water content of *Pinus sylvestris* trees four weeks after inoculation with different densities of B. xylophilus (n = 10)

Variation	Mortality in %	Inoculation success in %
100	70	60
300	70	85
800	80	90
2400	90	95
4000	80	90
6000	90	95
10000	90	95
control	0	--

	H_2O in %	
Variation	mean	SD
100	48.2	18.2
300	45.8	17.7
800	44.6	12.9
2400	30.2	11.8
4000	38.5	14.6
6000	43.3	17.2
10000	39.3	13.1
control	72.8	6.9

The inoculation of either 2400 or 4000 nematodes per trees affected the average reduction to the most intensive degree, compared with all other inoculation levels. As a result, the nematode densities used in this experiment evidently altered the development of wilting and relative water contents in wood differently. When using 2400 and 4000 nematodes per tree the development was quicker and the reduction of relative water contents in wood was higher compared to any other level. In contrast trees appeared to be highly susceptible independent of how many nematodes were used for inoculation.

The development of the *B. xylophilus* populations in *P. sylvestris* showed a contrary situation when taking trees of the first group (4 weeks after inoculation). The total number of reisolated *B. xylophilus* four weeks after inoculation is presented in Figure 21, a.

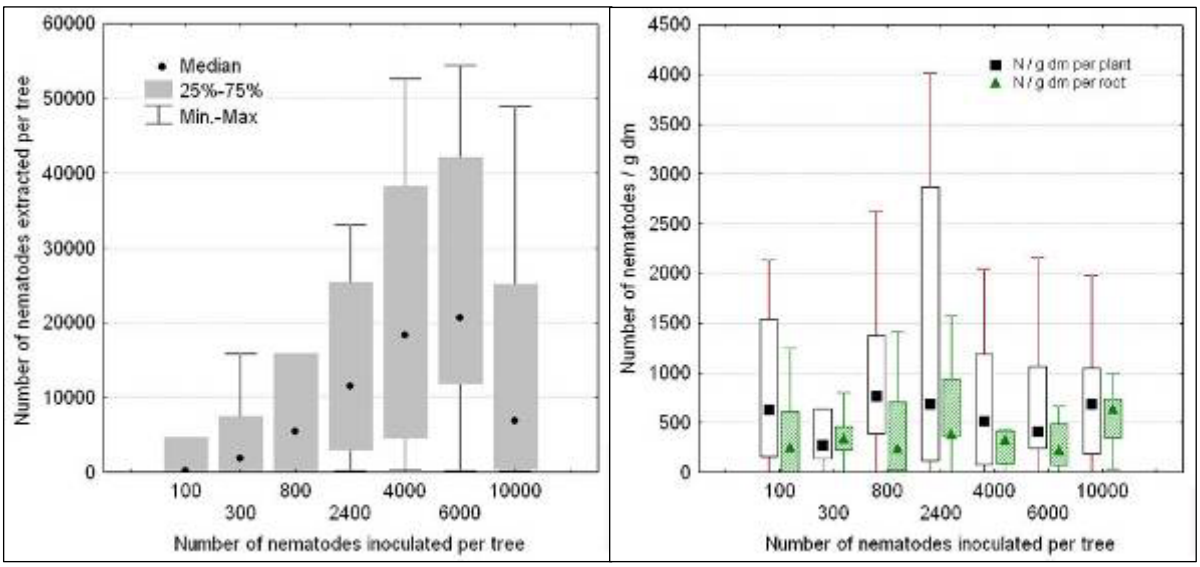


Figure 21 a) Median of total number of *B. xylophilus* reisolated 4 weeks after inoculation from roots and above ground plant parts (n = 10); b) median of number of *B. xylophilus* extracted from roots and above ground plant parts (plant) when trees reached wilting class 5 (dead).

Comparing all levels of nematodes initially inoculated into trees, the majority (median) of these trees are described by a saturation curve. The number of nematodes that were extracted from the entire tree (roots and plants together) after 4 weeks increased with the number of nematodes inoculated until 6000 nematodes per tree. This capacity resulted in a density of about 20000 nematodes extracted per tree. At the level of 10000 nematodes this interrelation disappeared and the population density of nematodes extracted was in the same range as that achieved when inoculating 800 nematodes per tree. Thus a possible capacity of three to four year old *P. sylvestris* trees apparently was reached between inoculation densities of 6000 and 10000 nematodes per tree.

Figure 21, b shows the median of population densities of *B. xylophilus* per one gram of dry matter (divided into plant and root) in those trees that reached wilting class five. The overall population densities of plant and roots compared between all levels of inoculation densities were in the same range. The respective level remained clearly below 1000 nematodes /g dm. Population densities either in plants and roots followed a negative trend with increasing levels of inoculation densities, when comparing levels 800 to 6000. The lowest densities were detected in plants and roots of the level of 300 nematodes per tree.

The conclusion of the current test was that an inoculation density of 2400 to 4000 nematodes per 3-4 year old conifer saplings is suitable to cause Pine Wilt Disease including the development of symptoms during an appropriate time scale and provides the possibility to assess population dynamics of the nematodes in the tree.

5.11 *Susceptibility of European conifers against the Portuguese isolate of* B. xylophilus *in comparison to isolates from China and North America*

5.11.1 Introduction

As PWN in Portugal was the first occurrence in Europe and the origin of this introduction was not known an obvious question was whether the pathogenicity of the Portuguese PWN-isolate was comparable to strains from known infestations elsewhere in the world. So the first step of a series of pathogenicity tests was a general review on 13 European conifers in relation to the pathogenicity of the Portuguese strain in comparison to isolates from known infestations in both the native range of *B. xylophilus* and in areas where PWN was also introduced.

5.11.2 Materials and methods

In total 13 conifer species were used for the test: nine *Pinus* and two *Larix* species, *Abies alba* and *Picea abies* (Table 10). Each tree species was subject to inoculation with three different origins of *B. xylophilus*. From each tree species / nematode combination 4 replicates of 10 trees each were inoculated with nematodes, 20 plants (4 replicates with 5 trees each) served as control group and were inoculated with distilled water. In total from each tree species, 140 trees were used.

Inoculation was carried out according to the method described in chapter 0 using 4000 nematodes per tree. Three different *Bursaphelenchus xylophilus* strains with originating in Portugal (PT 3 (w)), China (Ne 12/02) and the USA (US DE 2 (w)) were used for inoculation.

The trees were grouped per replicate within each species in a random block design in a greenhouse at a temperature of 25°C and 80 % relative humidity (rh). Four weeks after the inoculation five plants of each replicate (= 20 plants) were used to determine the number of nematodes colonising the above ground plant parts. Isolation of the nematodes for all five trees together was carried out at the day of cutting the trees according to the scheme explained in sections 5.4.1 and 5.5.5.

The tests were run for three months in total. Each week the vitality class was determined according to section 5.5.5. At the end of the test all trees were cut and the above ground plant parts were

analysed for the presence of nematodes (sections 5.4.1 and 5.5.5). This investigation was carried out for 5 trees of one replicate together. The aim of this was to amalgamate the number of re-isolated nematodes because it is known that their number greatly differs from tree to tree.

5.11.3 Results

The investigations were carried out to evaluate whether *B. xylophilus* with different origins causes the same rate of symptomatic or dead trees of 13 conifer tree species grown in Europe. The results concerning trees death presented in Figure 22 show that three groups of trees with different ranges of mortality can be distinguished. The trees in the first group (*P. nigra*, *P. sylvestris*, *P. cembra* and *Larix decidua*) died all within three months after inoculation. The mortality rate in the second group (*P. strobus*, *P. pinaster*, *P. radiata*, *P. mugo* and *Larix kaempferi*) was also high but did no reach 100 %. In the third group (*P. pinea*, *P. halepensis*, *Picea abies* and *Abies alba*) the trees showed a high tolerance against *B. xylophilus*, except *Pinus pine*a inoculated with the Portuguese strain.

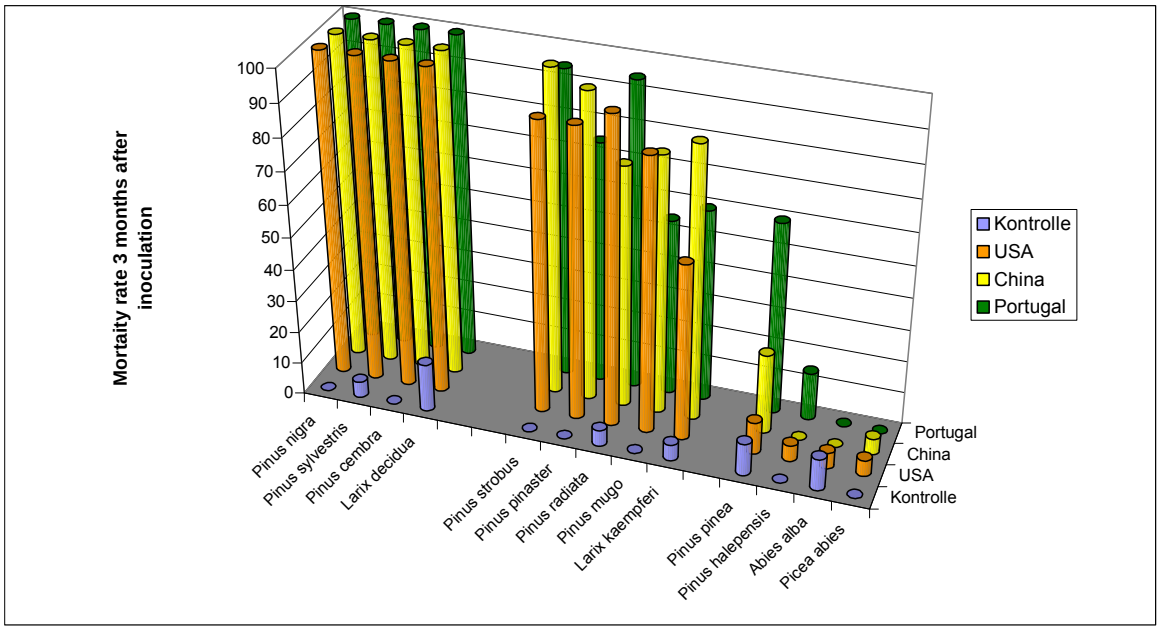


Figure 22: Percentage of 13 conifer tree species three months after inoculation with *B. xylophilus* (n = 20).

A more detailed assessment of the development of dying trees is given in Figure 23 as a sum curve. The data for *Abies alba* are not mentioned because in the whole period only one inoculated and two untreated trees died whereas all the other trees stayed healthy.

Figure 24 to Figure 27 give an overview on the development of the vitality (described by 6 wilting classes) of the test trees over the three months test period. As examples the species *P. sylvestris*, *P. pinaster*, *P. pinea* and *Larix decidua* were chosen.

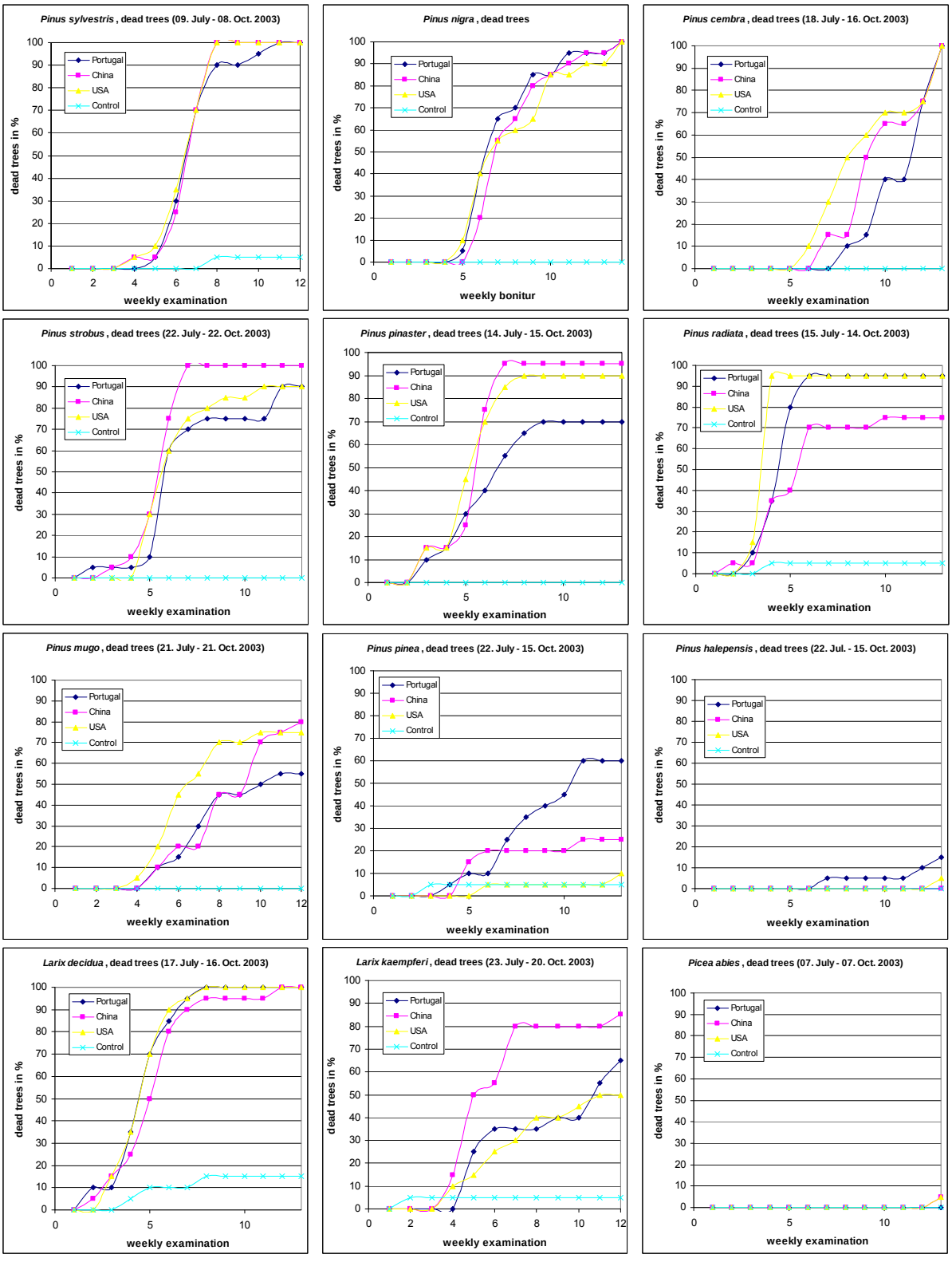


Figure 23: Summarised curve of dead trees, 3 to 4 years old, following inoculation with 4000 *B. xylophilus* each within 12 to 13 weeks. Trees were inoculated with three different strains of *B. xylophilus*: Portugal, China, USA (n = 20).

The development of the wilting symptoms at *P. sylvestris* saplings, resulting in the described wilting classes shown in Figure 24, was quite similar between the tested *B. xylophilus* strains. This is true for the classes one to four as well as for trees death (wilting class 5).

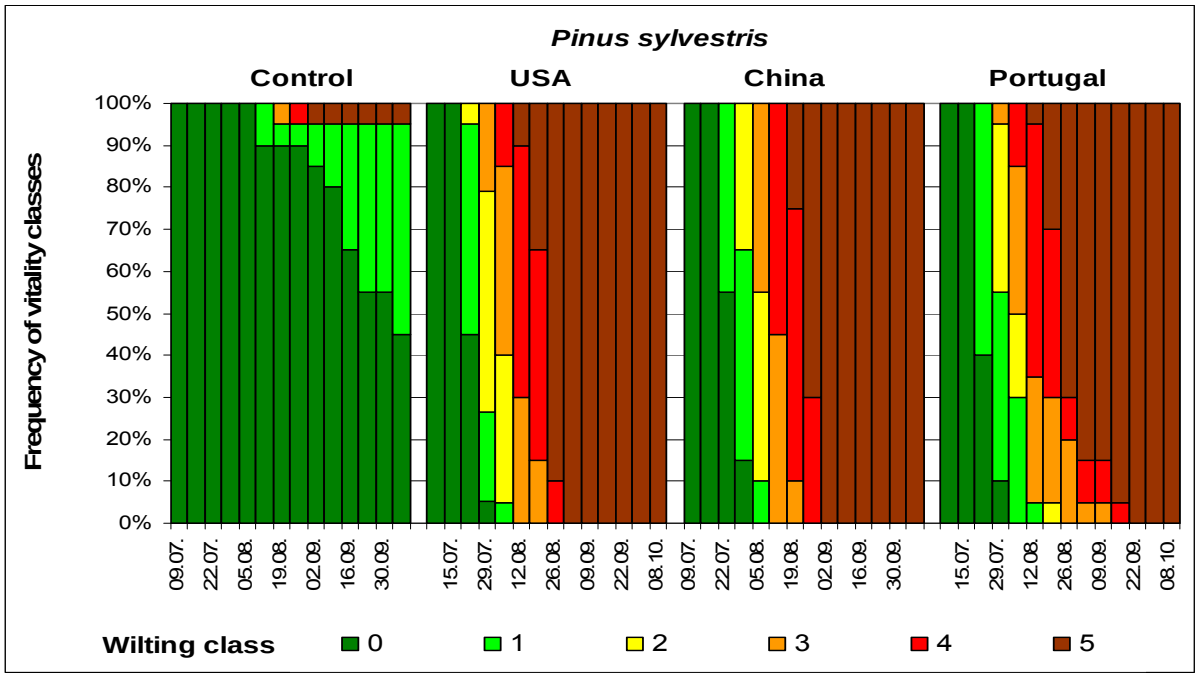


Figure 24: *Pinus sylvestris*: development of vitality (wilting) classes in the period from 09.07.2003 (date of inoculation) to 08.10.2003 (end of test) after inoculation with *B. xylophilus* from the USA, China and Portugal as well as with water (control), weekly assessment (n = 20)

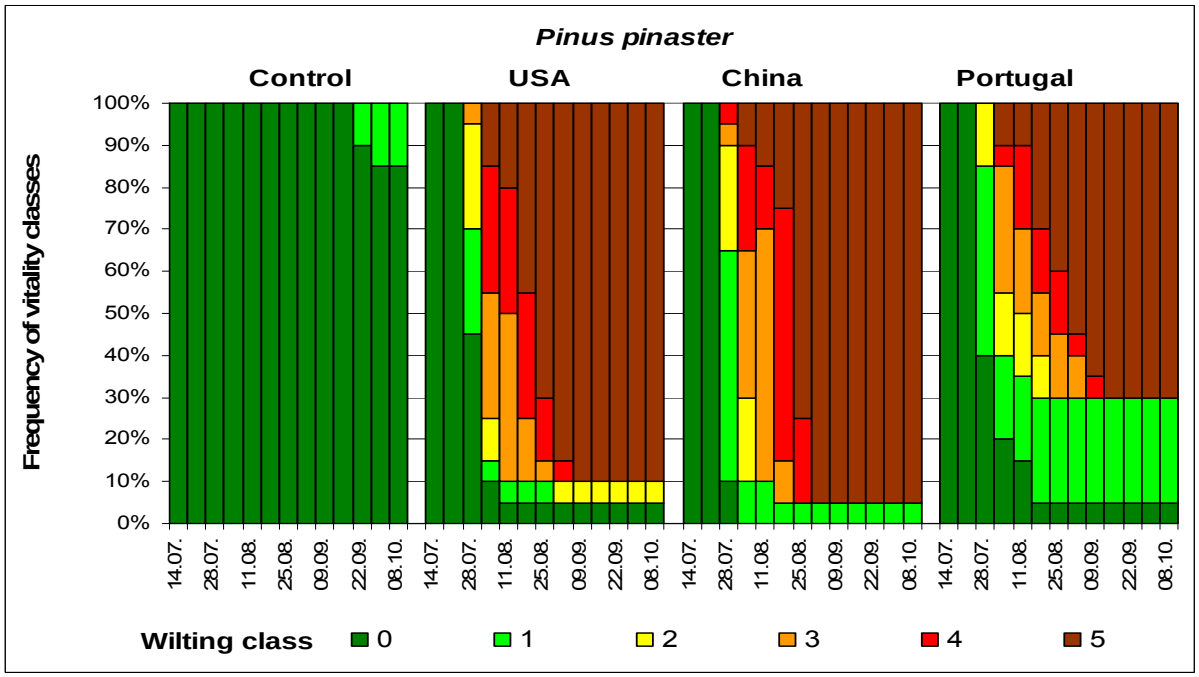


Figure 25: *Pinus pinaster*: development of vitality (wilting) classes in the period from 09.07.2003 (date of inoculation) to 08.10.2003 (end of test) after inoculation with *B. xylophilus* from the USA, China and Portugal as well as with water (control), weekly assessment (n = 20).

The strain from Portugal used in the test on *Pinus pinaster* resulted in less dead trees in comparison to the strain from China and the USA (Figure 25).

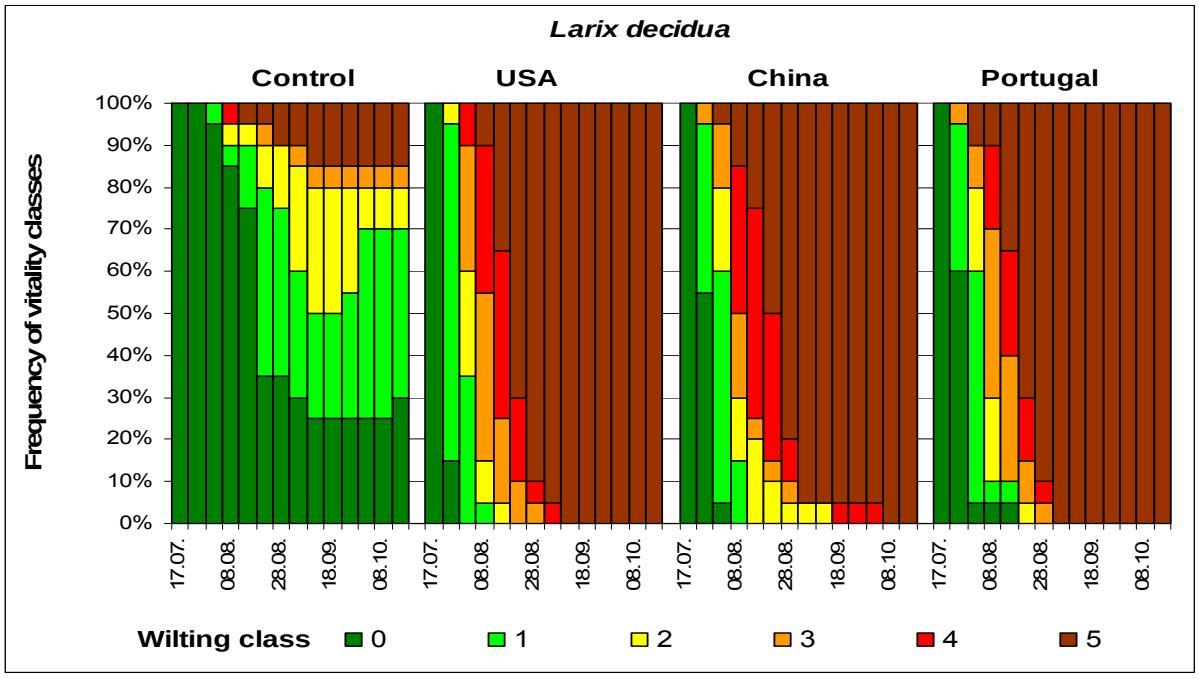


Figure 26: *Pinus pinea*: development of vitality (wilting) classes in the period from 09.07.2003 (date of inoculation) to 08.10.2003 (end of test) after inoculation with *B. xylophilus* from the USA, China and Portugal as well as with water (control), weekly assessment (n = 20

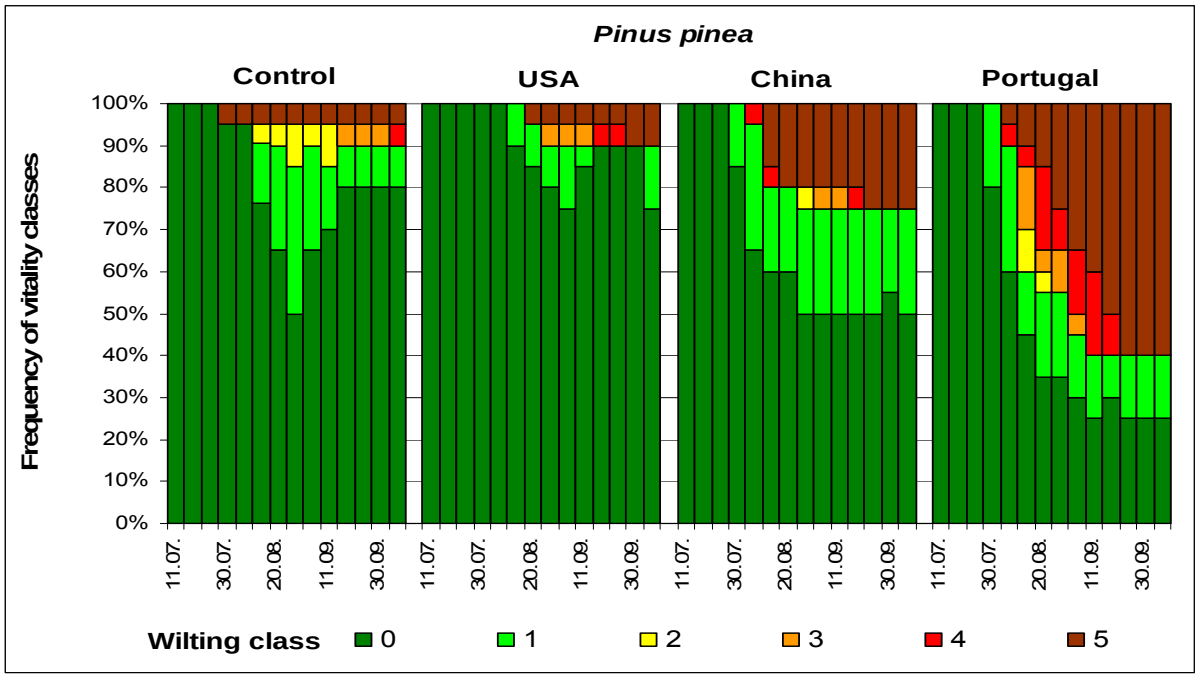


Figure 27: *Larix decidua*: development of vitality (wilting) classes in the period from 09.07.2003 (date of inoculation) to 08.10.2003 (end of test) after inoculation with *B. xylophilus* from the USA, China and Portugal as well as with water (control), weekly assessment (n = 20)

In contrast to *P. sylvestris* and *P. pinaster* during the test, some trees which showed wilting symptoms and were classified as “1” or “2” recovered within the weeks and appeared to be healthy (control and USA group). While in *P. pinaster* (Figure 25) the Portuguese *B. xylophilus* strain resulted in lower tree mortality, in *P. pinea* this strain caused tree mortality which was two times that of the Chinese and four times that of the USA isolate (Figure 26).

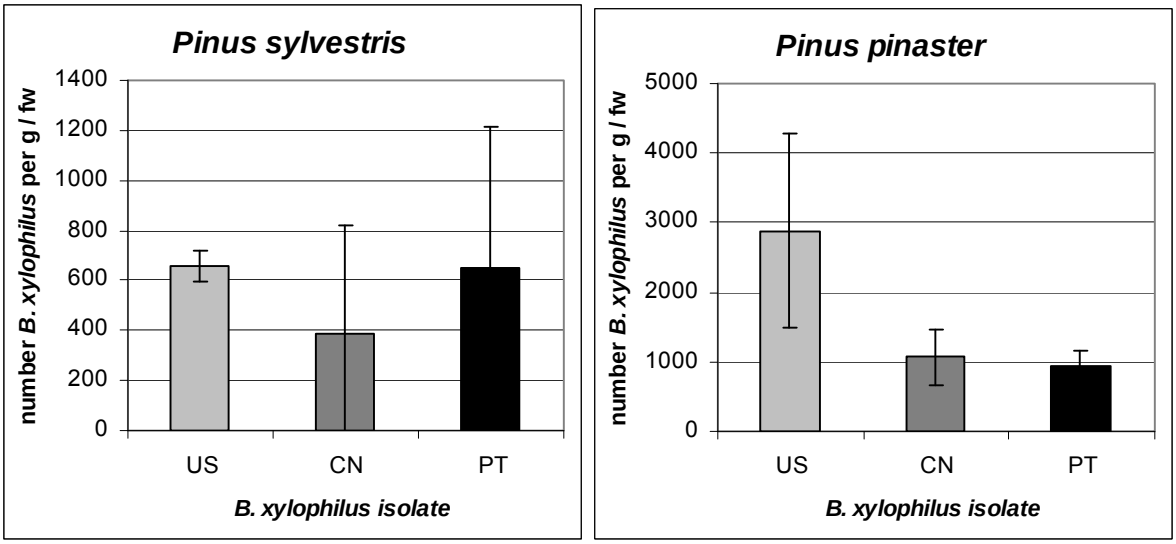


Figure 28: Median value of reisolated nematodes per gram fresh weight (fw) from *Pinus sylvestris* (left) and *Pinus pinaster* (right) trees four weeks after inoculation with three different *B. xylophilus* strains (US = USA, CN = China, PT = Portugal) (n = 4 x 5 trees).

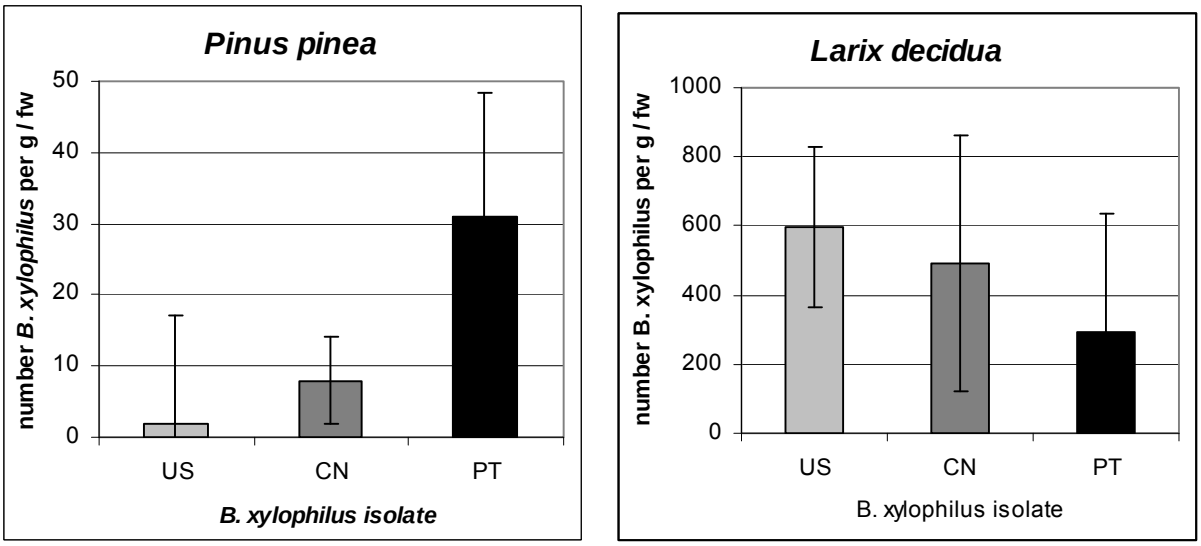


Figure 29 Median value of reisolated nematodes per gram fresh weight (fw) from *Pinus pinea* (left) and *Larix decidua* (right) trees four weeks after inoculation with three different *B. xylophilus* strains (US = USA, CN = China, PT = Portugal) (n = 4 x 5 trees).

The trees of *Larix decidua* seemed to be highly susceptible for all *B. xylophilus* strains tested. More than 50 % of all trees were dead five weeks after inoculation with the nematodes and after eight weeks there was only one living tree left, which died later on also (Figure 27).

Looking at the nematode density four weeks after inoculation their number differed greatly even between the replications within one tree species. To be able to compare these data the number of nematodes were calculated per one gram fresh weight (fw). For example, the results of the same 4 tree species as presented above are described in the following.

Between the three variants of inoculated *B. xylophilus* strains in *P. sylvestris* trees there seems to be no statistical difference regarding the number of reisolated nematodes four weeks after inoculation (Figure 28). In comparison, the number of reisolated nematodes from *Pinus pinea* trees was much higher especially for the strain from the US (Figure 29).

From *Pinus pinea* only very few *B. xylophilus* could be reisolated after four weeks. Nevertheless from the Portuguese strain significantly more nematodes were reisolated in comparison to the US and Chinese strain (Figure 29). Concerning the number of reisolated *B. xylophilus* of the three tested strains from *Larix decidua* there was no significant difference.

5.11.4 Discussion

The inoculation trial on 13 conifer species showed a clear pathogenicity of all *B. xylophilus* isolates for all Pine species. *Abies alba* was resistant to *B. xylophilus*. It was not a suitable host for any isolate tested, as there was neither a symptom reaction nor nematodes that could be extracted from this species. *Picea abies* was found to be a tolerant species, as they carried a negligible number of *B. xylophilus* but mostly until 4 weeks post inoculation. Thus the very low susceptibility of these conifers confirmed results of other studies (Braasch 1997a; Futai and Sutherland 1989; Sutherland et al. 1991).

All pine species were susceptible to all *B. xylophilus* isolates inoculated. Three groups of pines could be categorised. Both tested *Larix* species showed an unexpectedly high mortality, *L. decidua* (100 %) and *L. kaempferi* exceeding 50 % mortality. However several *Larix* species were already described as highly susceptible (Sutherland et al. 1991). Both *Larix* species contained distinctively lower population densities than the Pine species. In consideration of the high sensitivity of the seedlings, *Larix* appeared more susceptible towards all isolates of *B. xylophilus* than *Pinus*. Hence this observation confirmed the same experience made by Braasch (1997a). Differences in mortality of similar tree species but different provenances of *B. xylophilus* could be observed occasionally among isolates from North America (Bolla, *et al,* 1986) in particular in tree species from which they were isolated (Wingfield *et al,* 1983). Other studies showed that different provenances of the same tree species and different times when nematodes were inoculated in trees contributed more to mortality of hosts than did provenances of *B. xylophilus* (Panesar and Sutherland 1989).

Mortality is a difficult parameter for the evaluation of susceptibility of host trees. This problem was discussed for wilt symptoms too, as wilt and death of trees are not specific reactions of the tree that can be related to *B. xylophilus* in accordance with Koch's postulates (McNamara 2004). Another method was used by (Braasch 1997b), who computed a relative host susceptibility index (RHS), which related the population densities of nematodes detected in trees when they were assigned to be dead to the number of successfully inoculated trees. Applied to the situation in the present study, RHS would indicate a different susceptibility between the isolate from USA and both the other isolates in *P. pinaster*, which would match the observations in the present study (after 4 weeks). Applied to the differences between the isolate from Portugal and USA or China in *P. nigra*, RHS would detect a higher pathogenicity for the Portuguese isolate, which is not consistent with the observation made in this study. Again, taking the difference between *P. cembra* and *P. mugo,* RHS would detect *P. mugo* as more susceptible than *P. cembra*, as the population density in *P. mugo* was distinctively higher in dead trees. There is an unpredictable factor that is the population density which can be detected in dead trees at the end of the inoculation trial, as it typically was applied in many studies (Bakke et al. 1991; Braasch 2000; Braasch 1996; Bakke et al. 1991; Caroppo et al. 2000; Skarmoutsos and Michalopoulos-Skarmoutsos 2000). For example, comparing the population densities between the isolates in dead *P. mugo* seedlings, the Portuguese isolate actually achieved the highest population density which was related to the lowest mortality of 45% in comparison to 80-85% with both other isolates.

There is no obvious correlation that can be detected between the population density of a certain isolate and mortality of a host (Melakeberhan and Webster 1990; Bolla, I et al. 1986; Riga et al. 1991). Under ideal conditions at 25^{o}C, *B. xylophilus* was shown to achieve generation cycles of 4 days, when feeding on *Botrytis cinerea* (Wang et al. 2005). In pathogenicity trials the common

period of investigation extends over several weeks and populations were often extracted at the end of a preset period or on death of the trees. In both cases, the PWN may have completed many generation cycles and it is impossible to predict the population dynamics from one or two extractions. Extracting nematodes even after 4 weeks provide an estimate of this population at its progression, capacity or regression of the population dynamics and, therefore, give different results at different times. This might explain the observed dissimilarities between population densities and mortality in the present study. Another unknown component is the lack of knowledge that arises from the mode of action, which changes in *B. xylophilus* in relation to its hosts. *B. xylophilus* shifts from a phytoparasitic behaviour to a mycophagous stage (Wingfield 1987), which primarily is part of the decomposition process in trees. Populations of *B. xylophilus* that are extracted from dead wood are hardly to relate to their pathogenicity towards their hosts (McNamara 2004).

Nevertheless, sensitivity of most *Pinus* and *Larix* saplings could be clearly detected. It is evident that all *B. xylophilus* isolates could achieve extremely high populations among the pine species and in *L. kaempferii*. In principle, tree species, like *P. halepensis,* that were known from the literature as being moderately resistant clearly could be separated from highly susceptible pine species in this study. The Portuguese isolate of PWN showed a tendency for extreme deviations and extraordinarily high population densities compared to both other isolates. A recent preliminary study on the Portuguese isolate indicated a distinctive variability of this isolate in its cytogenetic characteristics and pathogenicity (Mota et al. 2006).

5.12 *Distribution, migration behaviour and population dynamics of Bursaphelenchus xylophilus in young Pinus sylvestris (L.) trees at controlled optimum temperature*

5.12.1 Materials and methods

5.12.1.1 Experimental set up and processing

This trial focused on the Portuguese isolate PT 3 (w). *Pinus sylvestris* was taken as a representative and susceptible host tree of *B. xylophilus*. The 3-4 year old trees were selected according to morphological characteristics, which was the crucial factor for the segmentation of trees (Figure 10). In Table 13 the type of segmentation and the position of each segment in the tree is described.

Table 13: Segmentation and coding of morphological tree parts of P. sylvestris; Adjacent segments in larger parts of the tree having the same code but different numbers; Upper and lower branches represent aggregated samples of all respective branches.

Segment number	Segment	Morpholical part of the tree	Aggregated	Position
1	**MS 1**	Main Shoot		Basis
2	**MS 2**			Terminal
3	**uB 1**	Upper Branches	x	Basis
4	**uB 2**		x	Terminal
5	**uW**	Upper Whorl		
6	**Sa**	Stem above Inoculation Point		
7	**Ip**	Inoculation Point		
8	**Sb 1**	Stem below Inoculation Point		
9	**Sb 2**			
10	**Sb 3**			
11	**Sb 4**			
12	**lB 1**	Lower Branches	x	Basis
13	**lB 2**		x	Terminal
14	**lW**	Lower Whorl		
15	**Sr**	Stem above Root Collar		
16	**RC**	Root Collar		
17	**R**	Root		

Altogether 120 trees (90 regular and 30 backup trees) were compiled having a similar length of stem parts, a comparable crown circumference and positions of the old and young whorls. All selected trees were taken from outdoors and placed inside a climate chamber that was already adjusted to the correct environmental settings (25^{o}C, 50% rh, 12 h light) for the adjacent trial. Trees were kept there for two days before inoculation. Altogether 4000 nematodes were inoculated per tree on 23rd of June 2004.

The trees were divided into 17 separate segments in order to track nematodes inside the hosts to a precise degree. The dissection of the plant aimed at morphologically different parts. The upper and lower branches were aggregated to one shoot respectively. These shoots then once again were divided into a base (pointing at intersection) and a terminal part that each gave one individual segment. The same division was applied to the main (leader) shoot.

The nature of this trial was to observe a population (*B. xylophilus*) inside a closed system (*P. sylvestris*) within a defined observation period (pathogenesis of the PWD). To cover the four growth phases that were assumed for *B. xylophilus*, nematodes were extracted at nine sampling dates in three different intervals during a period of 27 days. The end of the experimental observation period was determined by the death of trees. The first three samples were taken every 2nd, next three samples every 3rd and the last three every 4th day. Each sampling date totalled 10 trees that together yielded 170 samples. Trees were arranged in blocks of 10 plants that were placed in one tray each. Altogether 12 trays were placed on a table inside the climate chamber. Each tree was labelled with an individual code and randomly placed at its position.

During the experiment all trees were watered according to their demand. The bark of the inoculation part and the neighbouring segments were washed before the extraction immediately after fresh weight was determined. This was a necessary step to exclude nematodes, which were part of the original inoculum, from extraction,. The majority of segments were extracted by the Baermann funnel method. Exceptionally, nematodes were extracted from very small segments (upper whorl, stem above inoculation point, inoculation point and stem below inoculation point) using a modified method. After cutting and taking the fresh weight, samples were enclosed in a piece of commercial cotton filter (4 x 5 cm), then placed inside a 10 ml vessel, which was filled with H_2O. After 48 hours the sample and filter was removed from the vessel, which contained the nematodes at the bottom. Dry and fresh weight of wood, root samples and needles were determined corresponding to the method described in Chapter 0

5.12.2 Analysis of data and statistics

5.12.2.1 Change of relative water contents of wood and needles during PWD

Each segment, except root collars and roots delivered ten values for the relative water contents at nine sampling dates for the 27 day period. To calculate a representative value for the relative water contents of wood and the respective dates, values of the following segments were aggregated to one value: MS 1-2, uB 1-2, Sb 2-4, lW and lB 1-2. Very small segments namely uW, Sa, lp, Sb 1 featured disproportionate minor fresh and dry weights than bigger segments. There was a high probability of losing material between weighings. As a result, these small segments produced measurement artefacts during the determination of relative water contents of wood and therefore were excluded from the aggregated values (for wood). All values for relative water contents were displayed as means and standard deviation. Descriptive statistics were applied to fresh and dry weights as well as relative water contents of all segments. The relative water content for each date was tested statistically between the aggregated “wood” sample and the segments “Sr” by means of Chi^2 test.

5.12.2.2 Distribution and migration of *B. xylophilus* in Scots Pines

Each segment provided the estimated numbers of adults and larvae of *B. xylophilus*. The number of nematodes therefore refers to adults and larvae together. These data were connected with the dry weight of each segment and related to 1 g of dry weight. This value represents the **population density**. When different segments were aggregated, the parameters, dry weight and numbers of nematodes first were summed separately before totalling the population density of the new aggregated segment. Population densities were calculated (1) for each single segment, (2) for aggregated segments that represented certain areas of the tree, (3) for the aggregated segments that made up the entire tree, except root parts and (4) root parts. To display the distribution of nematodes inside the trees, population densities were depicted in a special tree diagram. The median was used as the representative statistical parameter for the population density that was presented together with minimum (min) and maximum (max) span of data. The percentage distribution of nematodes per tree in the comparison made between two major tree parts was based upon absolute numbers of nematodes. These numbers were summed from all segments that were apportioned to the respective tree parts and its proportion was related to the total

nematode count per tree. Differences between the two tree parts (R, Rc , Sr vs. rest of the tree) were tested statistically using the Wilcoxon test.

5.12.2.3 Population dynamics of *B. xylophilus* in Scots pines

The population dynamics of *B. xylophilus* was presented as a Box Whisker plot using the median of population densities for each consecutive sampling date. Minimum maximum- span of data and if necessary the 25 % and 75 % quartiles were accessible. Population dynamics were presented for the single segments and their aggregated products, which already were described. Population dynamics represents patterns of population densities in time, therefore calculations made on densities were relevant for population dynamics, too.

5.13 *Results*

5.13.1 Distribution and migration of B. xylophilus in Scots pines

Population densities of *B. xylophilus* determined for the segments of. *P. sylvestris* were calculated per g of dry matter. The dry weight of the individual segment was established at each sampling day for 10 segments. The average dry weights of segments therefore was calculated from 10 values at 9 sampling dates.

The distribution of *B. xylophilus* in *P. sylvestris* was gained from the population densities determined for each of the 17 segments per tree. The change in the population densities of segments were assessed over 9 sampling dates. Figure 30 displays the distribution of nematodes in trees, utilising tree-diagrams that stand for the arrangement of segments for 6 sampling dates.

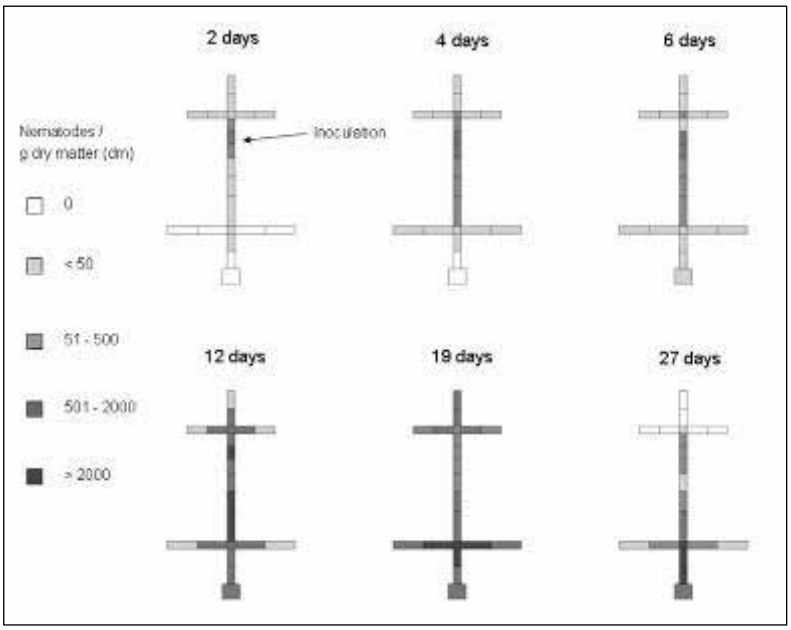


Figure 30: Distribution of *B. xylophilus* in 3-4 year old *P. sylvestris* saplings presented for 6 dates at a 27 day period post inoculation at 25°C; Each tree diagram resembles the true position of segments in 10 trees each; 5 grey scales represent classes of population densities computed as median of nematodes / g dry matter (0, < 50, 51 - 500, 501 - 2000, > 2000 .

Each diagram comprises all segments of 10 trees. Population densities were expressed as median values. For comparison reasons the representative population density of the entire tree is given in Table 14.

Table 14: Population densities of *B .xylophilus* / g dry matter in tree segments of 3-4 year old *P. sylvestris*, n= 10; Values are expressed as median of the densities detected at 9 sampling dates during 27 day period after inoculation; Population densities of (1) the merged area Plant refers to segments: MS 1 +2 (main shoot), uB 1+2 (upper branches), uW (upper whorl), Sa (Stem above inoculation point), IP (inoculation point), Sb 1-4 (Stem below inoculation point), lB 1+2 (lower branches), lW (lower whorl), Sr (Stem above root collar); (2) of the merged area Roots to the segments: RC (root collar), R (roots).

	Number of nematodes /g dm: median n = 10								
Days after inoculation	2 days	4 days	6 days	9 days	12 days	15 days	19 days	23 days	27 days
MS 1	8	20	10	20	1366	352	2003	70	0
MS 2	6	3	7	10	132	84	1016	29	0
uB 1	2	15	21	26	1374	62	1207	49	0
uB 2	2	3	5	12	133	30	459	25	0
uW	47	63	135	367	1979	199	139	27	14
Sa	130	280	545	882	1220	336	505	138	60
Ip	1502	1595	1755	1902	3773	1189	322	1140	68
Sb 1	281	699	264	1124	1474	353	468	111	71
Sb 2	46	95	64	164	1680	407	256	170	7
Sb 3	24	116	30	100	3104	363	915	517	90
Sb 4	25	129	27	63	2956	806	1288	538	1205
lB 1	0	21	0	9	351	108	2662	282	645
lB 2	0	5	1	25	108	70	706	96	30
lW	7	34	23	22	1407	299	2109	468	3044
Sr	9	20	14	39	1120	1148	3301	1795	5073
RC*	0	0	5	34	1041	1996	7177	1132	3353
R*	0	0	5	6	624	705	860	428	317
Plant	46	122	63	124	1840	406	2431	938	1569
Roots*	0	0	5	15	931	1416	4915	803	2093

Two days after inoculation *B. xylophilus* were found to be established in the host with 33 nematodes /g dm. At this date, most nematodes were concentrated at the inoculation point (ip) with 1500 nematodes /g dm and its neighbouring segments with 130 and 280 nematodes/g dm. The lower branches, ´lB1´ and ´lB2´, as well as root parts (´R´ and ´Rc´) were not yet invaded by PWN. During the first week, nematodes mainly migrated into neighbouring parts, starting from the inoculation point and dispersed further downwards in the trees. Population densities increased to 262 nematodes/g dm in segment ´Sb1´and to 545 nematodes/g dm in segment ´Sa´. Six days after inoculation nematodes were present throughout the entire tree including roots. After nematodes were passed barriers like the upper whorl ´uW´, nine days after inoculation, they could establish higher densities in the crown parts (´uW´, ´uB 1-2´ and ´MS 1-2´). Having invaded all segments nine days after inoculation, nematodes started to build up their population in all segments, which resulted in a density of 1820 nematodes/g dm for the whole tree. The increase in densities was not generated by certain segments, it moreover occurred simultaneously in all segments. After twelve days the population densities in most segments increased to clearly more than 600 to 1000 nematodes/g dm in all segments. This was the termination of population growth in the main stem part including inoculation point of the tree (segments: Sa, Ip, Sb 1-4), as it was the sampling date when these segments achieved their highest density. Termination of population growth in the segments of the crown parts (´MS 1-2´, ´uB1-2´) and the lower branches was reached on day 19 after inoculation. The highest nematode density at this date was achieved in the root collar with 7177 nematodes/g dm, followed by the stem above root collar ´Sr´ (3301 nematodes/g dm). Regarding the last sampling date, 27 days after inoculation, host trees showed a distinctive

polarisation in the distribution of nematodes. Whereas nematodes had vanished completely from the crown parts and only appeared at low densities around the inoculation point, they were mainly concentrated in the base of the tree. This was, primarily the root collar, stem above root collar and the lower whorl. The stem above root collar at this final date had the highest density with 5073 nematodes/g dm. Considering maximum values 2 days after inoculation there were 3 of 10 trees that contained nematodes in the terminal part of the main shoot ´MS 1´as well as in the root collar ´RC´. This means nematodes had invaded most parts of these particular trees four days before this fact was observed using the representative median values.

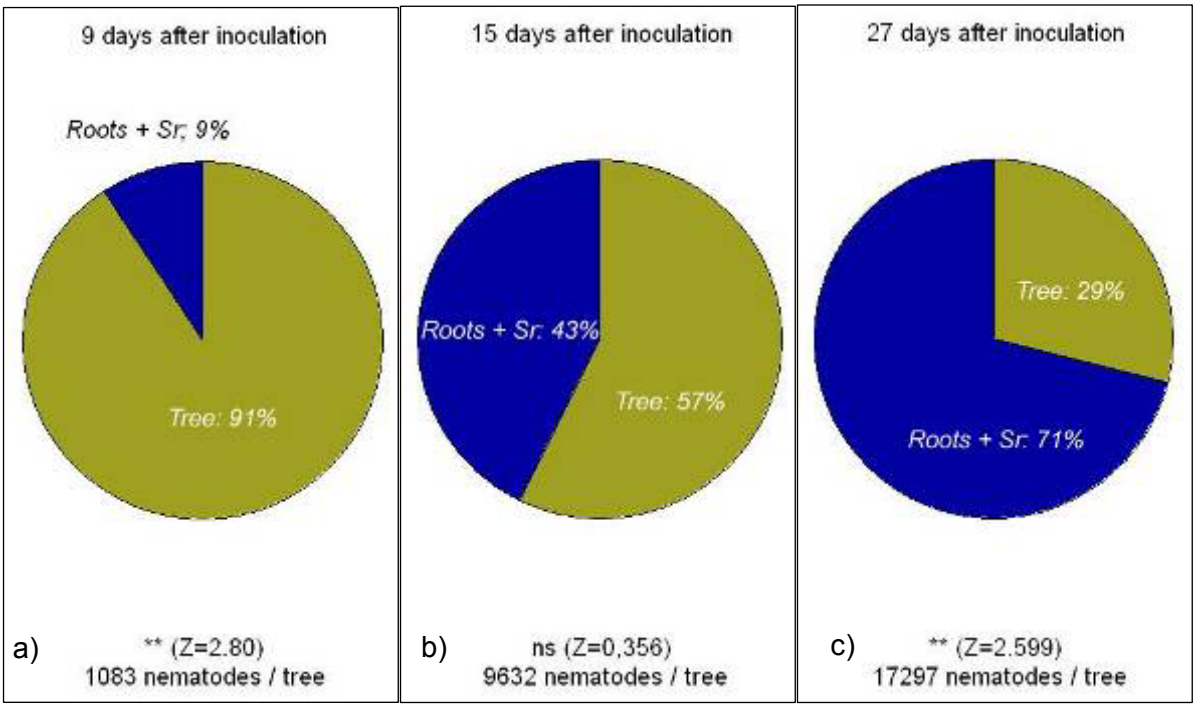


Figure 31: *P. sylvestris* - "Roots and Sr" = Nematodes in the segments Sr (Stem above root collar), RC (root collar) and roots in relation to the total number of nematodes in the tree. "Tree" = nematodes of all other parts of the tree in relation to the total number of nematodes in the tree. a) after 9, b) after 15 and c) after 27 days post inoculation; Wilcoxon-test relate to differences between both proportions, computed Z values were indicated, when significant * or insignificant 'ns'; (= 10, α = 0.05)

In general the span between minimum and maximum values of segments deviated extremely. Figure 31 a-c presents cake diagrams which displays two confined regions of the host trees and the proportion of *B. xylophilus* they contain in relation to the total numbers of nematodes per tree.

5.13.2 Population dynamics of B. xylophilus in different plant parts

The population dynamics of *B. xylophilus* in pines were related to certain parts of the host trees, appropriate to the different conditions, such as root parts or above ground plant parts, they could offer to the development of nematodes. Another factor for the focus on certain tree areas was the accordance in the dynamics of population growth between certain segments. Figure 33 and Figure 32 display the population dynamics of nematodes per gram dry matter in host trees without root parts respectively. The initial population after two days established in the trees (without roots) with 46 nematodes/g dm. 12 days after inoculation the population in above ground tree increased distinctly. The population in above ground tree parts showed three distinctive peaks at day twelve, nineteen and towards the end of the experiment.

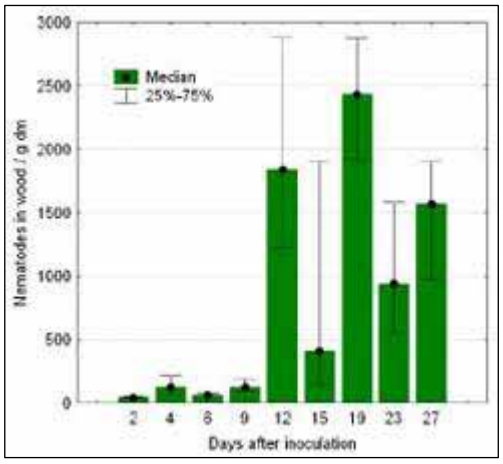


Figure 33: a) Population dynamics of *B. xylophilus* in above ground organs of 3-4 year old *P. sylvestris* saplings detected as median of number of nematodes / g dry matter (dm) at 9 sampling dates during a 27 day period after inoculation at 25 °C, n = 10; Box-whisker plot, Box (median), whisker (25 % - 75 % quartiles of values); b) Area of the sapling to which population dynamics is related

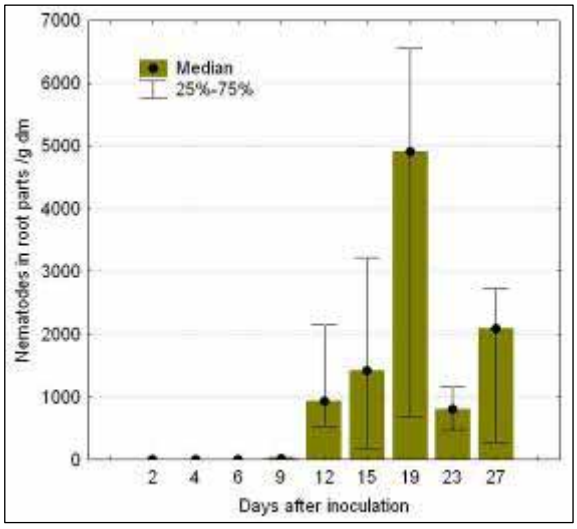


Figure 32: a) Population dynamics of *B. xylophilus* in roots of 3-4 year old *P. sylvestris* saplings detected as median of number of nematodes / g dry matter (dm) at 9 sampling dates during a 27 day period after inoculation at 25 °C, n = 10; Box-whisker plot, Box (median), whisker (25 % - 75 % quartiles of values), b) Area of the sapling to which population dynamics is related.

The population in roots grew continuously until nematode densities reached an outstanding peak with 4915 nematodes/g dm, 19 days after inoculation. This was roughly two weeks since they were observed in roots at day six. Analogous to the trend observed for the above ground tree parts, the population declined at day 23 but formed another final peak at day 27, remaining at 2093 nematodes/g dm, which was moderately higher than achieved in the above ground tree parts. Different segments of the tree independently showed similar population dynamics for *B. xylophilus* but at slightly deviating periods during the experiment. The computed nematode densities of segments that corresponded in their peaks of population dynamics were rearranged and aggregated to tree regions. Altogether three regions could be identified that distinctly showed matching dynamics of the PWN population. Figure 34, Figure 35 and Figure 36 show the three regions in a tree diagram and its corresponding nematode population dynamics.

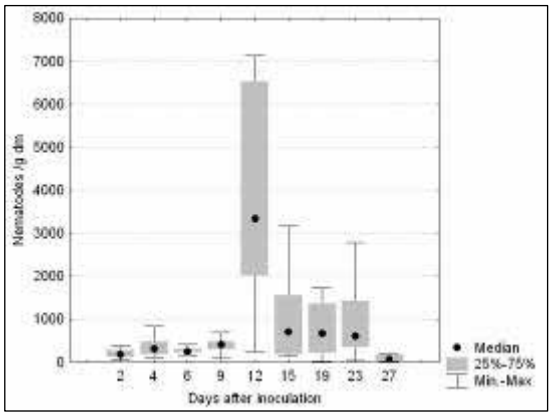


Figure 34 a) Population dynamics of *B. xylophilus*; b) in the area around the inoculation point of *P. sylvestris* saplings; Nematode densities (nematodes / g dry matter) detected at 9 sampling dates during a 27 day period after inoculation at 25 °C, n = 10; Box-whisker plot, Point (Median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

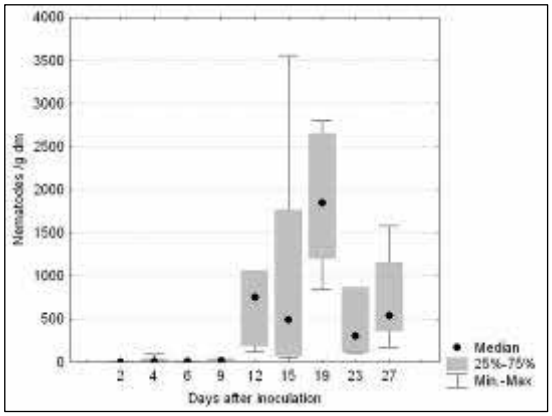


Figure 35: a) Population dynamics of *B. xylophilus* b) in the area of the crown and lower branches of *P. sylvestris* saplings; Nematode densities (nematodes / g dry matter) detected at 9 sampling dates during a 27 day period after inoculation at 25 °C, n = 10; Box-whisker plot: Point (Median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

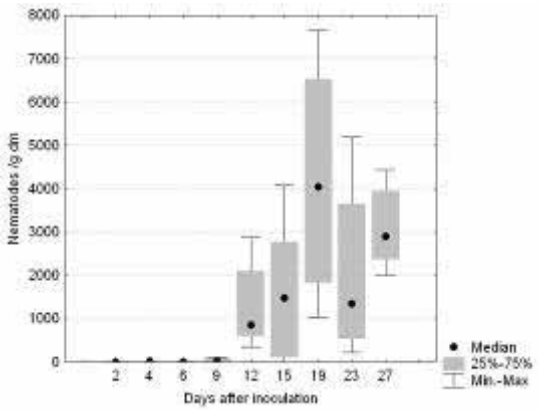


Figure 36 a) Population dynamics of *B. xylophilus* b) in the area of the roots and basis of the stem of *P. sylvestris saplings*; Nematode densities (nematodes / g dry matter) detected at 9 sampling dates during a 27 day period after inoculation at 25 °C, n = 10; Box-whisker plot: Point (Median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values).

There was a first population maximum at day twelve after inoculation that was clearly present in the stem segments around the inoculation point. This was the only distinct peak (3331 nematodes/g dm) that occurred in this region during the experiment. A second population maximum 19 days after inoculation could be spotted in the crown parts and the area around the lower branches. Here the nematode density achieved 1846 nematodes/g dm together in these segments. A slight rise in densities was also observed for day 12 (752 nematodes/g dm) and day 27 (541 nematodes/g dm) but not as clearly as on day 19. The final increase of nematode population was mainly represented by the region, which formed the base of the tree (root parts and stem above root collar). Unlike the other regions there were two distinct peaks here: The maximum at day 19 (4043 nematodes/g dm) and the last or third maximum at day 27 (2892 nematodes/g dm). However, none of the other segments or their aggregations exposed the third growth phase of the nematode population as clearly as this region. When comparing the bases of trees and the crown parts plus lower branches the second population maximum was more distinctive in the base in relation to mean population density. All three peaks occurred in accordance with the peaks at the same time detected for the above ground plant parts but differed among tree regions.

5.13.3 Change of relative water contents in wood and needles

To gain a representative value for the relative water contents of wood, all segments that were allocated to above ground tree parts were computed to one value (except segment ´Sr´). The Chi^2 test was applied to median values of the relative water contents of both fractions (all segments vs. segment Sr). Figure 37 presents the Box plot of relative water contents of wood and the stem above root collar ´Sr´.

Relative water contents in wood and ´Sr´ remained in between 60 % to 70 % for the first 12 days after inoculation. From day 15 onwards the relative water contents in wood decreased from 55 % (Table 15) continuously to 25 %. The respective segments experienced a similar reduction except ´Sr´. The relative water contents in the stem above root collar decreased hardly and remained at a level above 50 %. From day 19 to 27, water contents in the stem above root collar therefore was significantly higher than in wood.

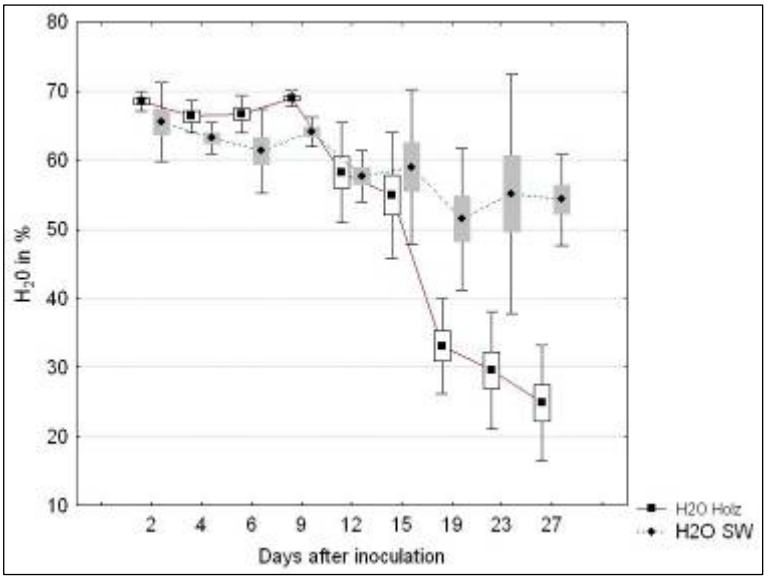


Figure 37: Relative water content of wood (above ground plant parts) and basis of stem (Sr) of *P. sylvestris* saplings at 9 sampling dates during a 27 day period after inoculation with *B. xylophilus* at 25 °C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

Table 15 Moisture content in different parts of *Pinus sylvestris* saplings following inoculation with *B. xylophilus* (n = 10).

	H_20 in (%)					
Days after inoculation	Wood Mean ± SD		SW Mean ± SD	*Chi²*	*FG*	*p*
2	68,58 ± 1,4		65,55 ± 5,74	5,41	8	0,73
4	66,34 ± 2,36		63,20 ± 2,28	1,6	7	0,97
6	66,70 ± 2,68		61,33 ± 6,03	8,48	9	0,49
9	68,98 ± 1,10		64,13 ± 2,15	3,82	9	0,92
12	58,20 ± 7,30		57,73 ± 3,79	7,12	9	0,62
15	55,00 ± 9,16		59,03 ± 11,18	18,24	9	0,33
19	33,11 ± 6,89	***	51,52 ± 10,33	153,38	9	0
23	29,57 ± 8,50	***	55,11 ± 17,39	407,62	9	0
27	24,82 ± 8,37	***	54,34 ± 6,70	441,58	9	0

In general the relative water content of young needles was about 10 % higher than in old needles, when focusing on the first period until day 12 after inoculation (Figure 38).

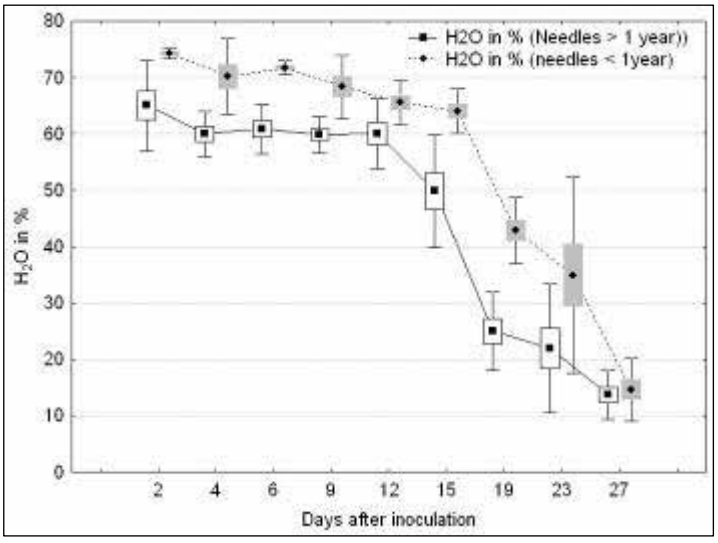


Figure 38 Relative water content of age classes of needles (< 1 year, > 1 year) of *P. sylvestris* saplings at 9 sampling dates during a 27 day period after inoculation with *B. xylophilus* at 25 °C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values).

The distance increased from day 12 onwards as relative water contents started to decrease stronger in old needles than in young ones. In fact the reduction of relative water contents in young needles appeared distinctively at day 19 which was four days after the onset of reduction in old needles. Considering the reduction of relative water contents as physiological wilting, the progression of wilting in young needles occurred to a smoother degree than it did in old needles. They regained this time when old needles already showed 22 % at day 12.

Old needles moreover followed a similar trend to wood. At the end of the experiment needles in general reached a relative water content of 14% to 15%. Within a period of 12 days (from day 15 to the end) young needles in the crown lost about 50% water and old needles roughly 40% in the same period. Although no assessment of wilt symptoms took place in this trial, it was observed as phenological change in coloration and shape of primarily young needles. Here the first appearance of yellowish needles was observed at day 12. In between day 19 and 23, wilting progressed rapidly and needles apparently dried out and turned completely to brownish colours.

5.14 *Discussion*

The investigations were made under the most optimal conditions for the development of *B. xylophilus* in a highly susceptible host like *P. sylvestris*. Few studies exist that have focused on population dynamics and migration of nematodes in this pathosystem, but mainly on *P. thunbergii*. This is the first investigations concentrated on the Portuguese isolate PT 3 (w).

Within 2 days after inoculation *B. xylophilus* could already be found in most segments of the pine saplings and had completely distributed in the sapling 6 days post inoculation. This was clearly observed before the nematode population started to increase. Similar observations were made by Iwahori and Futai (1996) with *B. xylophilus* in 2 year old *P. thunbergii* at 25°C, where the nematode was distributed 7-14 days after inoculation and then population increased until days 21. Likewise it was found that there was a systematic distribution of the PWN in 3 year old *P. thunbergii* reaching from roots to the terminal parts of the youngest shoots within 1 week, then populations started to increase (Fukuda et al. 1992). In the present study the nematode population density during this time remained at a level around 30 to 90 nematodes/g dm in wood but markedly showed a higher density around the inoculation site of the pine saplings. Nevertheless only 8 to 11% of inoculated nematodes could establish in pine saplings. This proportion was consistent among the 10 saplings after 2 days. This was the initial population density of *B. xylophilus*, which appeared as a specific host nematode relationship at the very beginning of the invasion. Consecutively nematodes reached higher population densities in segments adjacent to the inoculation site between day 4 and 6 and also in segments with greater distance downwards from day 12 on, after it had established within the segments. The establishment of *B. xylophilus* in these segments was observed at a population density of less then 50 nematode/g dm wood.

Considering the population dynamics of nematodes in selected areas of the tree it is highly evident that PWN built up its population after distribution and establishment in certain tree areas. First, it could be observed at the inoculation site and its neighbouring segments, where nematodes reached a peak after 12 days, then with increasing distance in lower and upper braches including the main shoot, as well as the bases of the pine saplings, where the peaks could be detected after 19 and 27 days. This behaviour is most likely a strategy of PWN to invade its host. Ichihara *et al* (2000) found a distinctive concentration of *B. xylophilus* (>100 nematodes) in radial and axial resin channels of the xylem after it was distributed in 2 year old *P. thunbergii* over a period of 3 to 7 days. In their study, they proved that histopathological key symptoms before the onset of physiological wilt were clearly observed only where nematodes were present. Thus the migration ability of the PWN in a very short time was determined to be a key factor in pathogenicity. Similar conclusions were drawn by Futai (1980b) and Odani et al. (1985) having found a restriction of the distribution of avirulent *B. xylophilus* and *B. mucronatus* in trees whereas virulent *B. xylophilus* moved freely inside inoculated pine seedlings and caused severe symptoms.

The second and highest peak of the population density in the tree and also root parts was achieved after 19 days. Taking this peak as the maximum capacity of the *B. xylophilus* population in *P. sylvestris* in this particular experiment, it provides a good reference base for the population dynamics of the PWN detected in other studies. Melakeberhan and Webster (1990) inoculated 2500 *B. xylophilus* into 7 month old Scots pines and trees were kept at 25°C to 30°°C. Nematodes achieved a capacity of 20,000 to 40,000 nematodes per plant after 28 days. In 6 year old *P. thunbergii* in a nursery, inoculated PWN reached a maximum density of 1800 nematodes/g dm within a 20 day period at about 25°C (Kiyohara and Suzuki 1978). Both studies pointed out that wilt

started by the time the nematode population had reached its capacity. This fact is also in accordance with several studies conducted in Japan, where PWN developed densities around 1000 nematodes/g dm wood (Kishi 1995). The present study clearly confirmed this experience with the Portuguese isolate of *B. xylophilus* in *P. sylvestris.* Physiological wilting was detected as the reduction of relative water contents of needles during the experiment. First colouration of needles could be observed 12 days post inoculation. A week later the water contents in needles was severely reduced. Onset of physiological wilt appeared synchronously with the highest reproduction of the nematode population. It is not clear whether wilt was already induced by the PWN during the 1st population peak, which particularly was observed in the stem parts adjacent to the inoculation site.

In this experiment the PWN apparently retreated from the above ground plant parts, when most trees showed severe wilt symptoms. The proportion of the whole population in this plant part started to increase continuously and at the end of the experiment was significantly higher, than in the rest of the tree. This retreat or concentration was accompanied by a reduction in the relative water content of the wood parts, except for significantly higher water content in the small stem above the roots. A similar retreat of the PWN was observed by Futai (1980b) at a relative water content in wood between 20 and 40 %. Accordingly Melakeberhan and Webster (1990) found a concentration of nematodes after wilt symptoms could be detected in the stem parts. As a result they hypothesised that nematodes start to invade roots in search of a toxic free environment or availability of food.

The population dynamic of *B. xylophilus* detected in above ground plant parts and partly in roots showed three distinctive peaks at day 12, 19 and 27 days post inoculation. This dynamic has not been reported previously for *B. xylophilus* populations inside trees. The reliability of the existence of three consecutive population peaks is given, as it simultaneously occurred in segments, which were sampled and submitted to Baermann extraction separately. It was always neighbouring segments at certain tree areas that showed similar dynamics. The short sampling interval of two, three and four days uncovered extraordinary dynamics of population development that otherwise would not have been observable. However, exponential growth models were assumed for PWN populations *in vivo* and *in vitro* (Iwahori and Futai 1995; Futai 1980b; Futai 1980a; Mamiya and Furukawa 1977; Melakeberhan and Webster 1990; Wang et al. 2005). Segmentation and short interval sampling was an essential method to identify these peaks as a result of an independent development of several populations inside *P. sylvestris* saplings. The results reflect the rapid life cycle of *B. xylophilus* on the one hand and its rapid invasion ability on the other hand. Both abilities were already discussed as possible key factors for the pathogenicity of *Bursaphelenchus xylophilus*.

5.15 *Influence of temperature on the population dynamics and pathogenicity of* Bursaphelenchus xylophilus *in* Pinus sylvestris *(L.),* Larix decidua *(Mill.) and* Picea abies *(Karst.)*

5.15.1 Materials and methods

5.15.2 Experimental set up and processing

To examine the population dynamics of *B. xylophilus* in conifers, the Portuguese isolate PT 3 (w) was chosen as a direct reference isolate, which was also investigated during the inoculation experiment on migration behaviour and distribution (chapter 0). The conifers were selected according to their presumed susceptibility against *B. xylophilus* which was partly experienced in previous trials (Chapter 0). *P. sylvestris* and *L. decidua* were taken as susceptible conifer species and *P. abies* as a tolerant representative. All trees were three to four years old, but held different dimensions (Table 10). The main factor of influence on the population dynamics of *B. xylophilus* in these conifer species was temperature. Three temperatures 25°C, 20°C and 15°C were found to represent the main August Isotherms that are known from central Europe as indicated by Evans et al. 1996.

Trees were inoculated with 2400 nematodes each. Control plants were inoculated with distilled water. Inoculation of all conifer species took place on consecutive days for each temperature regime starting from the 23rd of July in 2004. Within each temperature regime tree species were arranged together but the various samples within each conifer species were randomised.

There were three main parameters that were investigated during the examination: 1. the assessment of wilt symptoms, 2. the population densities of *B. xylophilus* in the two major tree parts and 3. the relative water contents in wood and needles. The assessment of wilting symptoms was relevant for a particular variation consisting of 20 individual trees that were inoculated and 20 control trees. This variation existed for each temperature regime and for each tree species. The trees were assessed on a weekly basis in concordance with the sampling dates of the other parameters. The actual assessment dates were: 7, 15, 21, 28, 32, 46 and 60 days after inoculation. The population density of *B. xylophilus* and the relative water contents of wood and needles were detected in accordance with the procedure described in the chapters 0 and 0. Consequently each conifer species was represented by 120 trees for each temperature. Notwithstanding a control variation at each sampling date, there was only one date when control trees were sampled at the end of the experiment. It was assumed that untreated trees would not change their relative water contents more distinctly in between the various sampling dates than at the end of the 61 day period. All trees were watered dependent on their demand.

5.15.3 Influence of temperature on the progression of PWD in P. sylvestris, L. decidua and P. abies

The assessment of wilt symptoms (chapter 0) provided a set of 20 ordinal values for treated and control trees at each date. These values were expressed as median that stood for a representative wilting class of the respective date. Medians were presented as modified box whisker plots for the whole period. The wilting classes of treated trees and control trees were tested statistically using the Chi ^{2} test on the distribution of classes. This was carried out for every tree species and temperature. Trees that were allocated to wilting class five were consequently detected as dead trees. Comparisons of mortalities between tree species in the within and between temperatures in the same tree species were determined, based on Chi ^{2} test. These comparisons were carried through for combinations at day 32, 46 and 61 days after inoculation but not between assessment dates. As physiological patterns, the relative water contents of wood and needles were determined for every sampling date, each temperature and all tree species. Exceptionally, the controls delivered data for the last sampling date only. Each sampling date gave 10 values for the relative water contents that were expressed as medians and arranged in modified box whisker plots. Comparisons of relative water contents between temperatures but within a certain tree species were tested using the Wilcoxon test. The comparison made between controls and treated trees for each species and temperatures based on the last sampling date 61 days after inoculation. Both were statistically tested by the Mann Whitney U-test.

5.15.4 Influence of temperature on the population dynamics of B. xylophilus

Population densities in this trial were expressed exclusively per gram of dry matter and referred to either “wood” (above ground plant part without roots) or “roots”. Consequently twenty samples were drawn from ten trees at one sampling date for each tree species and temperature. Population dynamics were presented as medians of ten trees for *B. xylophilus* in *P. sylvestris*, *L. decidua* and *P. abies* at 25°C, 20°C and 15°C. Two main comparisons were made to analyse the influence of temperature on the population dynamics of *B. xylophilus* in the two tree parts. Comparisons of population dynamics (population densities in time) between temperatures within the same trees species were tested statistically using the Wilcoxon test. Comparisons of the population dynamics or densities at particular sampling dates between tree species but within the same temperatures were tested by applying the median test.

5.16 *Results*

5.16.1 Influence of temperature on the progression of PWD

Comparing the distribution of wilting classes of nematode inoculated variations and controls 61 days after inoculation, only *P. sylvestris* and *L. decidua* showed significant differences in the two temperatures 25°C and 20°C (Table 16). Trees of both species at 15°C did not develop distinctively different symptoms than the control trees. This was also true for inoculated *P. abies* at all temperatures.

Table 16: Chi^2 test of differences between the distributions of wilt classes in the comparison between *P. sylvestris*, *L. decidua* and *P. abies* saplings inoculated with *B. xylophilus* and control at 61 days post inoculation , n = 20, FG = 19, α = 0.05, Significantly different Chi values are marked.

	Differences of distributions of wilt classes between **inoculated varaitions and control**		
	25 °C	20 °C	15 °C
	Chi ^{2}	Chi ^{2}	Chi ^{2}
P. sylvestris	112 *	84.25 *	32
L. decidua	51.42 *	203.1 *	7
P. abies	0	0	0

Figure 39 presents the median of wilting classes of *P. sylvestris* trees at the three temperatures over time. Trees at the 25°C variation developed symptoms earlier than at 20°C. Progression of wilt appeared similar between both temperatures, but trees at 20°C remained at a median of "class" four, for a period of two weeks. As a consequence, temperature had a clear effect on the development of wilting symptoms on *P. sylvestris* comparing 25°C and 20°C on the one hand and the 15°C variation on the other hand. Temperature did not distinctly alter wilting, when compared between 25°C and 20°C.

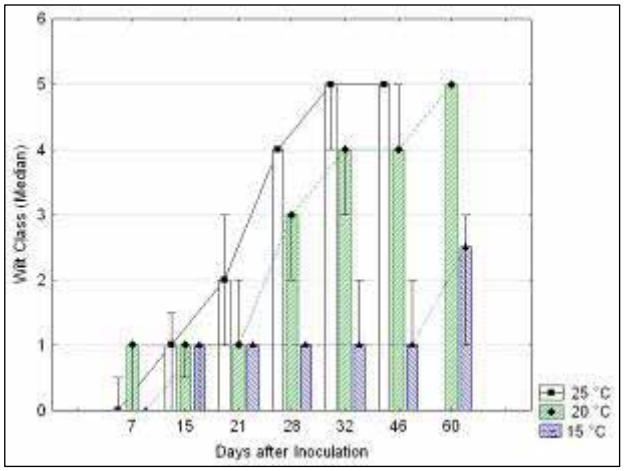


Figure 39: Median of wilt classes (0 - 5) of *P. sylvestris* saplings inoculated with B. xylophilus at 15°C, 20°C, 25°C assessed at 7 dates during a 60 day period, n = 20; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

Taking the development of wilting classes of *L. decidua* presented in Figure 40, the situation seems to resemble that of *P. sylvestris*. There is no obvious reaction in wilting of *L. decidua* at 15°C but severe wilting occurred at 25°C as well as at 20°C. Again, trees at 25°C reached a median of "class" four a week earlier then did trees at 20°C. Trees at this temperature rapidly developed symptoms to the degree where no differences could be detected to the 25°C variation from days 28 on. Temperatures above 20°C in *L. decidua* therefore had a distinct effect on wilting. Comparing *P. sylvestris* and *L. decidua* that were inoculated with *B. xylophilus*, there was a tendency for *L. decidua* to develop symptoms earlier at both 25°C and at 20°C than in pines. Furthermore *L. decidua* showed a remarkably similar development of symptoms between 25°C and 20°C whereas symptoms of *P. sylvestris* at 20°C had a deferred development to trees at 25°C.

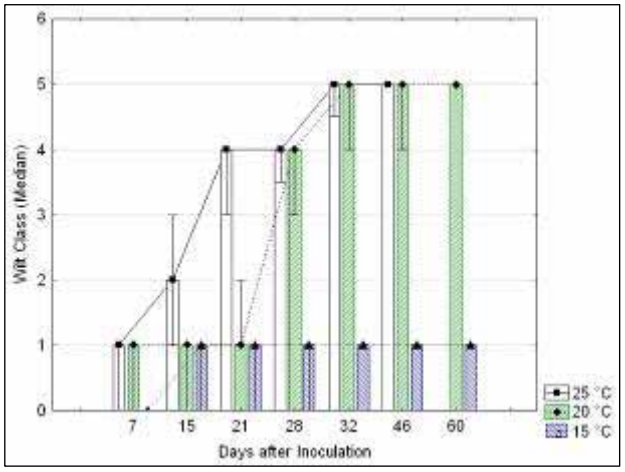


Figure 40: Median of wilt classes (0 - 5) of *L. decidua* saplings inoculated with *B. xylophilus* at 15°C, 20°C, 25°C assessed at 7 dates during a 60 day period, n = 20; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

Picea abies showed a slight but insignificant development of wilting symptoms towards the end of the experiment at 25°C. Temperature had no clear effect on wilting of *Picea abies*.

Figure 41 expresses the mortality as number of trees related to 20 reference trees that reached "class" five over time. If indicated by different letters comparisons between the two mortalities were statistically different at α = 0.05 % according to the 2*2 Chi^2 test. Comparisons only were made within dates, therefore letters a-c stand for 32 days after inoculation, letters d-f stand for 46 days after inoculation and letters g-h stand for 60 days after inoculation. In fact, no tree of any species died at 15°C within the period of investigation.

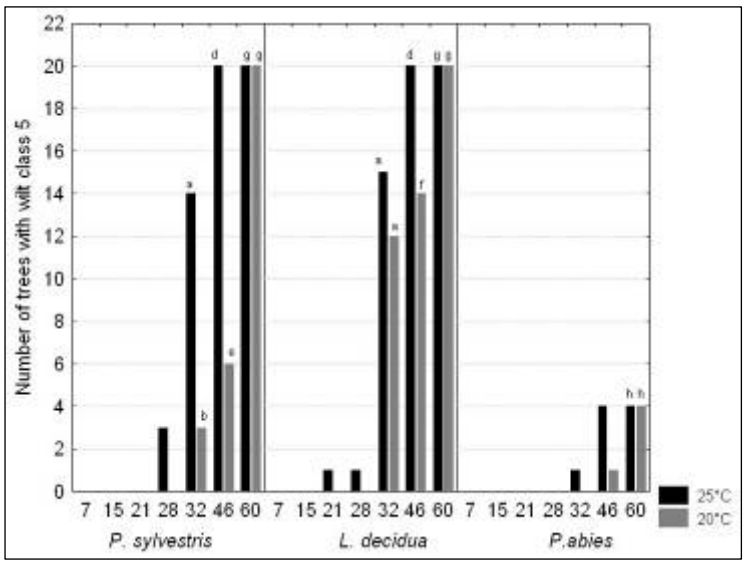


Figure 41: Number of dead trees of *P. sylvestris*-, *L. decidua*-, *Picea abies*-saplings (assessed with wilt class 5) at 20°C and 25°C during a 60 day period after inoculation with *B. xylophilus*, n = 20; Different letter indicate significant differences (Chi^2 –test, α = 0.05) within the same assessment dates post inoculation, a-b (32 days), d-f (46 days), g-h (60 days).

Comparing 25°C mortality between *P. sylvestris* and *L. decidua* there was no evident difference at any time. From day 32 to 46 after inoculation apparently more *L. decidua* were dead than *P. sylvestris,* but these comparisons were not significantly different at the respective dates. Temperature had a significant influence on mortality of *P. sylvestris* between 25°C and 20°C after 32 and also after 46 days, but not on mortality of *L. decidua* at 32 or 60 days at either temperature. Regardless of being subjected to 25°C or 20°C, *Bursaphelenchus xylophilus* produced a mortality of 100 % in *P. sylvestris* and *L. decidua*. Altogether 4 of 20 *P. abies* trees died after 61 days at 25°C and 20°C and evidently less than the two other tree species.

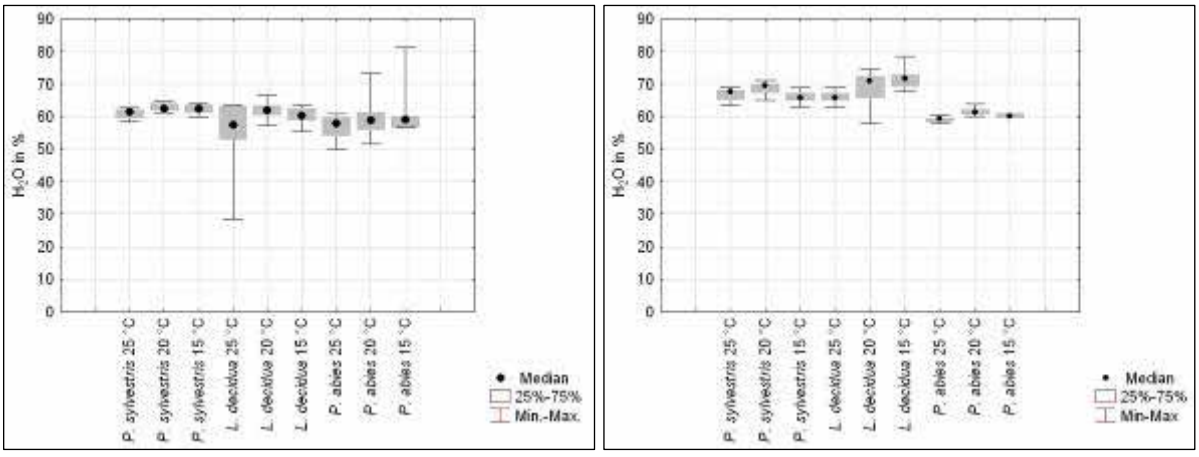


Figure 42: Relative water content of wood (left) and needles (right) of *P. sylvestris*-, *L. decidua*- and *Picea abies*- saplings in the control variation (inoculated with distilled water) at 15°C, 20°C and 25°C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

The relative water contents of wood and needles in the respective control variations are presented together in a Box Whisker plot (Figure 42), which is based on the median as representative of 10 trees.

At the end of the experiment water contents in wood of *P. sylvestris*, *L. decidua* and *P. abies* remained between 58 to 62 % in the controls irrespective of the temperature. Taking the relative water contents of needles in the controls, some slight deviations occurred within *L. decidua* and between the different temperatures. *P. abies* showed distinctly lower water contents in needles, which was based upon a replication of five trees only. *B. xylophilus* significantly altered the relative water contents of wood and needles of *P. sylvestris* at all temperatures compared to the controls, which were inoculated with water 61 days after inoculation.

L. decidua that carried nematodes appeared to show a significant difference of the relative water contents in wood relative to the control when kept at 20°C and in needles at 25°C as well as at 20°C. *B. xylophilus* also had a significant influence on the relative water contents of wood and also needles of *P. abies* at 25°C.

The relative water contents broken down by the sub-samples “wood” and “needles” of all tree species that were inoculated with *B. xylophilus* are assessable in Figure 43 and Figure 44. Taking the wood samples of *P. sylvestris*, the relative water contents gradually decreased as wilt progressed to the same extend in 25°C and 20°C but significantly different to 15°C.

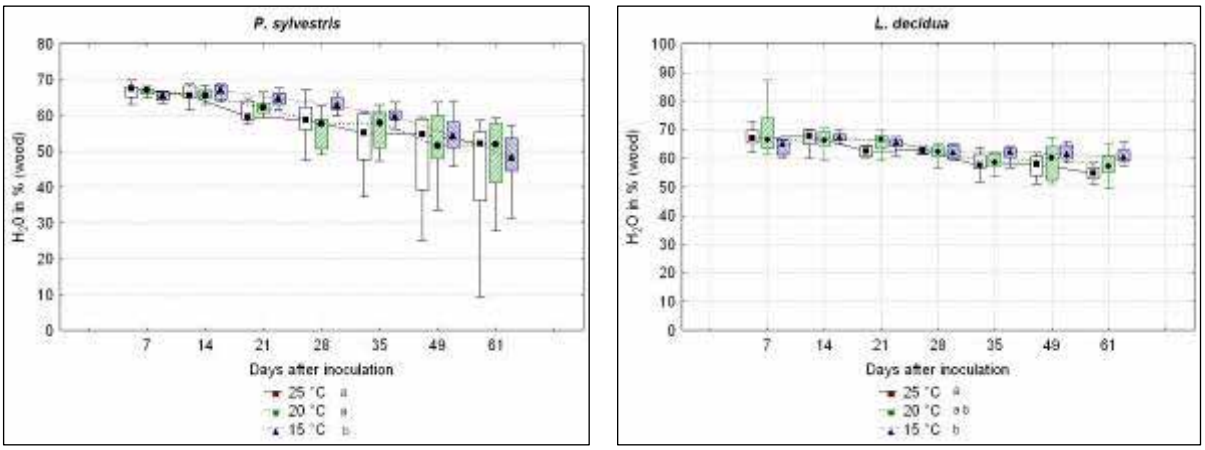


Figure 43: Relative water content of wood of *P. sylvestris* (left) and *Larix decidua* (right) saplings inoculated with *B. xylophilus* at 15°C, 20°C and 25°C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values). Different letters designated for the temperature variations at each diagram indicate significant differences (α = 0.05 %). Values were tested by Wilcoxon test between the temperatures for the entire period of investigation

Test plants at all temperatures finally reached 47 to 52 % water contents 61 days after inoculation. The depression in all temperatures appeared continuously. A different situation occurred when regarding the relative water contents of needles of *P. sylvestris*. All temperatures were related to significantly different water contents. Consequently the depression of relative water contents in needles appeared different between the temperatures. It started to decrease to a recognisable degree, 49 days after inoculation at 15°C. This decline in both other temperatures started after 21 to 28 days at 20°C and about a week earlier at 25°C. After 61 days, when the experiment was finalised, needles at all temperatures reached at their minimum water contents. The relative water contents in wood of *L. decidua* showed an overall decrease throughout time, similar to that on for *P. sylvestris*. This only came out significantly different when compared between 25°C and 15°C among all possible combinations. Corresponding to the relative water contents of wood above 20°C in *P. sylvestris,* those above 20°C of *L. decidua* finally reached 51 % to 52 %. The decrease of water content in needles at 25°C and 20°C with progression of wilt appeared as clear as in *P. sylvestris*. Despite distances between the water contents of needles at 25°C and 20°C

temperatures seemed to be smaller than observed in pines, the water contents were significantly different between all temperatures throughout the period of investigation.

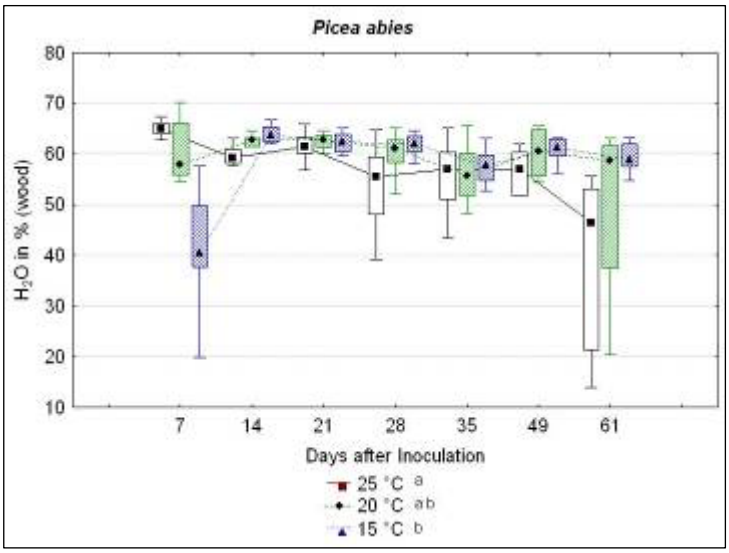


Figure 44: Relative water content of wood of *Picea abies* saplings inoculated with *B. xylophilus* at 15°C, 20°C and 25°C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values). Different letters designated for the temperature variations at each diagram indicate significant differences (α = 0.05 %). Values were tested by Wilcoxon test between the temperatures for the entire period of investigation.

There was no change in the relative water contents in needles of *L. decidua* when exposed to 15°C. Similar to *P. sylvestris,* the needles of *L. decidua* reached a minimum of 10 % to 15 % water contents when wilt was at its final stage at 25°C and 20°C after 61 days. The varying water contents of wood of *P. abies* at the beginning of the experiment is most likely an artefact, as water contents of all temperatures reached similarly high values at the following dates. Therefore the first date was excluded from further consideration. In doing so, *P. abies* did not show an alteration of relative water contents in wood at 20°C and 15°C. A significant difference is the result of a decline in the 25°C variation at the end of the experiment. This can be clearly attributed to the values of the four single trees that, at this temperature, finally died. This also caused a high deviation among water contents in needles of *P. abies* at this temperature. But in fact needles of this tree species did not show any difference throughout the period of investigation.

5.16.2 Influence of temperature on the population dynamics of B. xylophilus

Bursaphelenchus xylophilus could not been identified in any tree of the non-inoculated control variations of *P. sylvestris*, *L. decidua* or *P. abies*. Population dynamics were observed in two areas of the tree: The above ground plant parts and the below ground plant parts. To simplify matters, the trees above ground organs will be further referred to as “plant”, whereas below ground parts will be termed “roots”. Population dynamics of *B. xylophilus* are given as median and max-min spans of population densities per gram dry matter for plant and roots of all trees species, at all temperatures and for all sampling dates.

5.16.2.1 *P. sylvestris*

Population dynamics of *B. xylophilus* in *P. sylvestris* was strongly influenced by temperature in the plant (Figure 45 left). Population densities of nematodes were significantly higher at 25 °C, than at 20 °C and both higher, than at 15 °C.

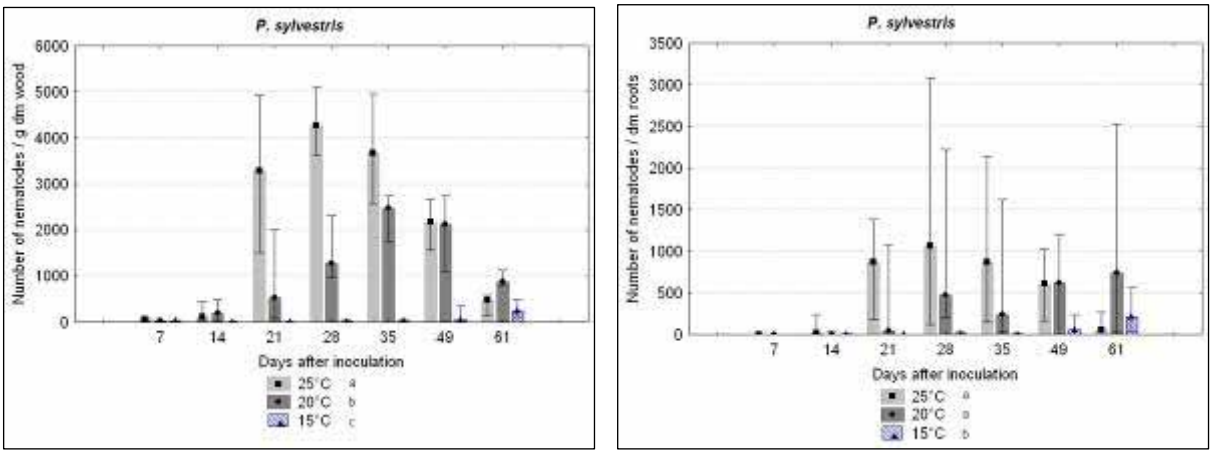


Figure 45: Population dynamics of *B. xylophilus* in above ground organs (left) and roots (right) of *P. sylvestris* saplings detected as median of number of nematodes/g dry matter (dm) at 7 sampling dates during a 60 day period after inoculation at 15°C, 20°C and 25°C, n = 10; Box-whisker plot, Box (median), whisker (25 % - 75 % quartiles of values), Different letters indicate significant differences between temperatures according to Wilcoxon-test (α = 0.05)

The population dynamics in roots of *P. sylvestris* in general was lower than detected in the plant at any date of investigation. Population dynamics of nematodes in roots at 25°C and 20°C were not significantly different. Populations in roots (Figure 45 right) at these temperatures reached a comparable high capacity of 1070 nematodes/g dm at 25°C and 745 nematodes/g dm at 20°C. At 25°C the growth phase, the peak and the regression of the two population dynamics in roots and plant overlaid narrowly in time. This was not the case at 20°C, where *B. xylophilus* tended to build up a population density towards the end of the experiment, which almost approximated the population density in the plant. The population in the plant at 20°C built up for a period of 35 days and achieved a capacity of 2480 nematodes/g dm, which was roughly half the capacity that *B. xylophilus* achieved after 28 days at 25°C. Generally the population dynamics of *B. xylophilus* at growth, capacity and regression in plants at 20°C appeared less than each of the phases at 25°C. Although not distinctive, there was a slight increase of the population densities in the plant as well as in roots simultaneously at 15°C towards the end of the experiment at 61 days after inoculation. *B. xylophilus* developed a density of more than 200 nematodes/g dm in both areas at the lowest temperature tested.

5.16.2.2 *L. decidua*

In both plants and roots (Figure 46), temperature did not induce a significant effect on the population densities between 25°C and 20°C throughout the period of investigation according to the Wilcoxon test. Though the population dynamics appeared retarded in growth, capacity and depression compared between these temperatures, *B. xylophilus* reached its maximum density of 2691 nematodes/g dm, 28 days after inoculation in the plant subjected to 25°C.

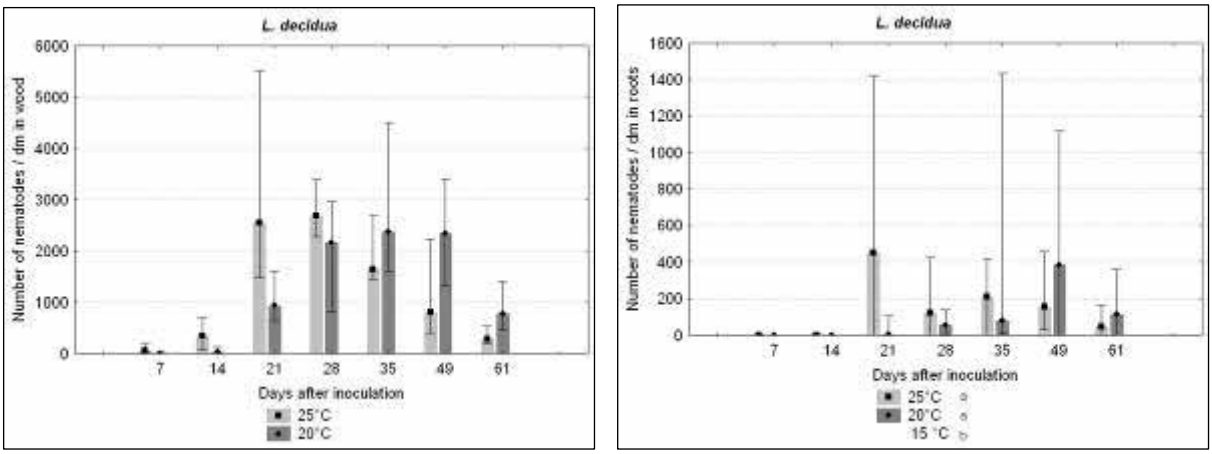


Figure 46: Population dynamics of *B. xylophilus* in above ground organs (left) and roots (right) of *L. decidua* saplings detected as median of number of nematodes/ g dry matter (dm) at 7 sampling dates during a 60 day period after inoculation at 15°C, 20°C and 25°C, n = 10; Box-whisker plot, Box (median), whisker (25 % - 75 % quartiles of values), Different letters indicate significant differences between temperatures according to Wilcoxon-test (α = 0.05)

The capacity of PWN to grown in *L. decidua* seedlings at 20°C was determined to be 2370 nematodes/g dm in plants 35 days after inoculation. Population densities in roots at 25°C and 20°C were lower than in plants. The maximum capacity of *B. xylophilus* in roots reached 450 nematodes/g dm at 25°C and 382 nematodes/g dm at 20°C. It is not possible to determine if the population dynamic of nematodes in roots overlap those of the plant. Taking the 25°C variant, there was a first peak of the population 21 days after inoculation and another 35 days after inoculation, where nematodes reached half the density they produced at the first peak. In the case of the 20°C variant, nematodes reached their capacity after 49 days. At this time the population in the plant part still remained at a comparatively high density of 2346 nematodes/g dm, which in fact was not different to that detected two weeks earlier. *B. xylophilus* could not establish in trees at 15°C. Nematodes were found apparently in very low numbers in plants and roots as well.

P. abies

In general the population density of *B. xylophilus* in *Picea abies* was very low at all temperatures (Figure 47). The nematode densities were significantly different among the temperatures in plants. In this respect temperature also influenced the population of *B. xylophilus* in *P. abies* in plants and roots. Nematodes reached a peak of 355 nematodes/g dm in plants 28 days after inoculation at 25°C, which was higher than in plants at 20°C. The initial population detected after 7 days was almost as high, but decreased before the next population peak. Nematode densities in roots were lower than in plants. Nevertheless the Wilcoxon test distinguished both populations to be significantly different, which is related to the occurrence of higher population densities in a minority of two to three trees (of ten) only.

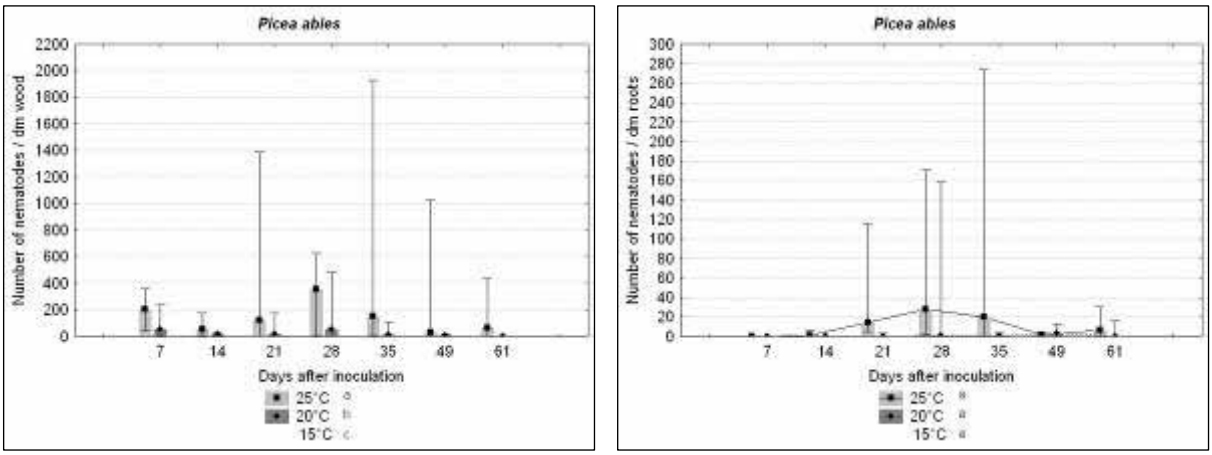


Figure 47: Population dynamics of *B. xylophilus* in above ground organs (left) and roots (right) of *Picea abies* saplings expressed as median of number of nematodes/ g dry matter (dm) at 7 sampling dates during a 60 day period after inoculation at 15°C, 20°C and 25°C, n = 10; Box-whisker plot, Box (median), whisker (25 % - 75 % quartiles of values), Different letters indicate significant differences between temperatures according to Wilcoxon-test (α = 0.05)

To compare population dynamics of *B. xylophilus* between the investigated tree species the median test was applied for general differences throughout time (Table 17). The nematode densities at their capacity appeared to be different between *P. sylvestris* and *L. decidua* at 25°C, whereas those at 20°C were at a similar level. Though the population densities in plants throughout the entire period were neither significantly different between *P. sylvestris* and *L. decidua* at 25°C, nor between both at 20°C.

Table 17: Median test of differences between the population dynamics of *B. xylophilus* (population densities at all sampling dates in above ground plant parts (left) and roots (right)) after inoculation in *P. sylvestris*, *L. decidua* and *P. abies* saplings. Comparisons were made within the temperature regimes used in climate chambers (15°C, 20°C and 25°C),(n = 70, α = 0.05; FG = 1)

Population dynamics in wood

Chi² values FG=1

		P. sylvestris			*P. abies*		
		25	20	15	25	20	15
P. sylvestris	25				22,4 *		
	20					45,71*	
	15						55,31*
L. decidua	25	1,03			22,4*		
	20		0,07			32,29*	
	15			77,14*			2,85

* sigificant

Population dynamics in roots

Chi² values FG=1

		P. sylvestris			*P. abies*		
		25	20	15	25	20	15
P. sylvestris	25				29,26*		
	20					31,57*	
	15						50,81*
L. decidua	25	11,43*			11,43*		
	20		5,54*			17,28*	
	15			38,53*			7*

* sigificant

In roots nematodes could build up significantly higher population densities in *P. sylvestris* than in *L. decidua* at 25°C. Irrespective of the tree species, *B. xylophilus* always reached its capacity in plants 28 days after inoculation when exposed to 25°C and after 35 days in *P. sylvestris* and *L. decidua* when subjected to 20°C.

As a result temperature had a significant effect within trees species at the observed range of 15°C to 25°C. *P. sylvestris* and *L. decidua*, which were susceptible to *B. xylophilus,* showed a

comparable population dynamics. In both species there appeared to be a temporary delay in the nematode growth phase, capacity and regression at 20°C compared with 25°C. In contrast to the compatibility between *B. xylophilus* and *L. decidua* at 25°C the PWN could achieve a higher capacity in *P. sylvestris*. Although this was not significantly different in plants, it was in roots between *P. sylvestris* and *L. decidua*. *B. xylophilus* could not establish in *L. decidua* at 15°C but showed a tendency to build up a population in *P. sylvestris*. *P. abies* was not susceptible to an invasion of *B. xylophilus* in general, but showed a tendency for a reduction of its resistance at the highest temperature tested. This was observed especially in some single trees, where maximum values could reach population densities above 1000 nematodes/g dm in wood irrespectively at 25°C or 20°C.

5.17 *Discussion*

The development of Pine Wilt Disease (PWD) in the tested species confirmed earlier results of the pathogenicity trial which was conducted in greenhouses. Both *P. sylvestris* and *L. decidua* appeared highly susceptible and *Picea abies* was tolerant against the Portuguese isolate of *B. xylophilus*. Irrespective of inoculating *B. xylophilus* in *P. sylvestris* and *L. decidua* at 20°C or 25°C, all trees died after a relatively short period of 60 days. However, no trees died at 15°C. Thus the results confirmed other studies that used the pathosystem *B. xylophilus* and *P. sylvestris* at these temperature levels (Melakeberhan et al. 1992). However, the development of PWD as well as mortality appeared accelerated by higher temperatures in both susceptible conifers, which was also observed by Sikora and Malek (1991). Accordingly, relative water contents of needles decreased earlier with rising temperatures in *L. decidua* and *P. sylvestris,* both reaching a similar low relative water content at the time that maximum mortality was detected. In this respect, relative water content of needles was a very useful predictor of PWD making a metric scaling of this phenomenon possible.

However, this was not the case for the relative water content of wood. Although it decreased in time and appeared significantly different between all temperatures in *P. sylvestris* and partly in both other species, these differences were not as distinct. Despite the very early reaction of *L. decidua,* the water content of wood of infested trees remained relatively stable, even though a majority of trees were already dead as early as 32 days post inoculation. Whereas *P. sylvestris* showed a wide distribution of values indicating that trees which were already died at this time had affected the water content of wood towards the end of the experiment. A reason for such differences between the two susceptible conifers most likely is a species specific physiological adaptation to the loss of water in leaves. Futai and Sutherland (1989) discussed a different and species specific pathological process between spruce and pines particularly in relation to the physiological reactions of the tree species.

Temperature had a significant effect on the population dynamics of *B. xylophilus* in above ground tree parts of *P. sylvestris*, but not on *L. decidua* when comparing 20°C and 25°C. This in fact has two implications:

(1) The population of nematodes in *L. decidua* has reached a capacity that determined the population density due to environmental conditions inside the tree. A higher temperature did not provoke a higher population capacity.

(2) The threshold population that induced PWD and finally death of *P. sylvestris* saplings is not affected by temperatures in the range from 20°C to 25°C. The existence of such a threshold population density was suggested by Braasch (1997). Also Melakeberhan *et al* (1992) suggested a threshold population density. The objective of their study was to determine how weather temperatures affect *B. xylophilus* populations, physiological responses or both, to demonstrate whether PWD is due to a direct influence of nematodes or to a physiological reaction by the tree. They suggested that (a) if temperature primarily alters reproduction, *B. xylophilus* would increase in number above the threshold temperature and infested pines should die, (b) if the effect of temperature is only on the host physiology, there should be no relationship between nematode numbers and pine death with increasing temperature, (c) if the effect of temperature is on both,

then a correlation between them could be expected with increasing temperature. Applying these hypothesis to the findings of the current study, hypothesis (c) becomes most evident for *P. sylvestris* and *L. decidua*. Population growth of *B. xylophilus* appeared clearly delayed at 20°C compared with 25°C. The same was true for wilt symptoms and also for the relative water contents of needles in both susceptible conifer species. Nevertheless, mortality of *L. decidua* saplings at 20°C was significantly higher at 32 days post inoculation then in *P. sylvestris*. Likewise, wilt symptoms reached a higher class earlier in *L. decidua* than in *P. sylvestris*. Therefore *L. decidua* appeared more susceptible then *P. sylvestris*. This situation is, in fact, well explained by an intermediate affect of temperature on both populations of the nematode and physiology of the trees (hypothesis c).

The reproduction of *B. xylophilus* between both tree species was similar, but nematodes reached a capacity in *L. decidua* which was lower then that in *P. sylvestris*. The reproduction compared between the temperatures showed a clear accelerating effect on reproduction with increasing temperature in both species. This was already observed on Agar plates using *B. cinerea* as food source (Futai 1980), and is mainly attributed to the embryonic development through which *B. xylophilus* passes swiftly (Wang et al. 2005).

Although there was no clear symptom development in pines at 15°C there was a tendency for initial population growth towards the end of the experiment in above ground plant parts as well as in roots. This was accompanied by a more severe reaction of the relative water content of needles. It is not clear whether nematode populations would have reproduced more and PWD would have proceeded further once the period of investigation was expiring 60 days after inoculation. Furthermore there was a clear reaction in some *Picea abies* saplings that altogether showed a maximum density of more then 1800 nematodes/ g dry matter in above ground plant parts. This is a high density that should be taken into consideration, as it does not reflect a high susceptibility of spruce but a possible higher risk being ignored under the monitoring regime of a pest risk policy.

The three major effects of temperature on the population dynamics of *B. xylophilus* in the present study were:

(1) on the capacity of the population (only in *P. sylvestris* and *P. abies*),
(2) on the retarded emergence of characteristic phases of the dynamic like growth, capacity and regression (all tree species). The major effect of temperature on the PWD was on the retarded emergence of wilt symptoms but not on mortality.

5.18 *Results – INIA Portugal: Pathogenicity of* Bursaphelenchus xylophilus

The inoculation of the Portuguese isolate of *B. xylophilus* in *P. pinaster* and *P. sylvestris* seedlings caused clear and distinct symptomatology when compared with the control plants. All inoculated seedlings that presented moderate or strong wilt symptoms died before the end of the experiment. No relation was found between the appearance of the first wilt symptoms and the final seedling mortality. After four months, 73,3% (n=22) of *P. pinaster* and 76,6% (n=23) of *P. sylvestris* seedlings were dead. Nevertheless, the two species showed some differences in the development of the wilt symptoms and in the timing of the occurrence of deaths (Figure 48). In *P. pinaster* the development of needle chlorosis was gradual but steady whereas in *P. sylvestris* it was more abrupt but later. The mean number of days (mean ± SE) to seedling death was 48,0±5,0 days in *P. pinaster* and 66,7±3,4 days in *P. sylvestris* (t-Student; t=3,12; p=0,03).

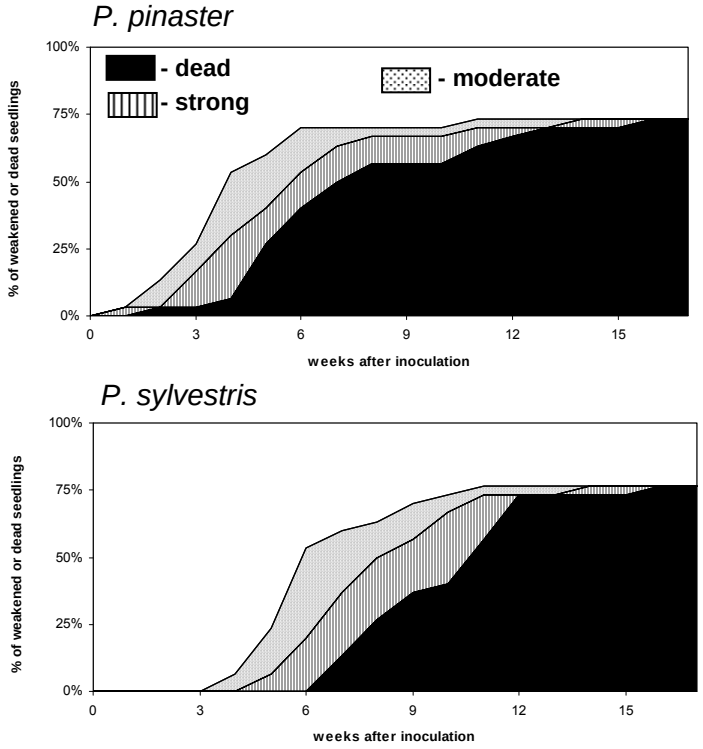


Figure 48: Changes in percentage of weakened or dead *P. pinaster* and *P. sylvestris* seedlings after inoculation with *B. xylophilus.* Dead (100% wilted needles); strong (75-99% wilted needles); moderate (50-74% wilted needles).

The first *P. pinaster* death occurred two weeks after the inoculation when 10% of the seedlings showed moderate wilt symptoms. The percentage of trees presenting moderate or strong wilt symptoms increased to 47% up to four weeks after the inoculation. In the following weeks the occurrence of new wilted seedlings decreased and a progressive increase of the percentage of deaths was observed, reaching 50% mortality after seven weeks. The percentage of *P. pinaster* with no noticeable wilt symptoms reached 27.3% after 11 weeks and remained constant until the end of the experiment.

In *P. sylvestris* moderate wilt symptoms only started to appear four weeks after the inoculation. The percentage of moderate or strongly wilted seedlings reached 53% after six weeks before any seedling death. First tree deaths occurred seven weeks after inoculation and 50% of seedling mortality was reached after 11 weeks. At the same time the percentage of trees with no noticeable wilt symptoms stabilized at 23.3%. After 12 weeks the percentage of dead *P. sylvestris* rose above the percentage of dead *P. pinaster*. No noticeable wilt symptoms were observed in the control

seedlings. In the 16th week a *P. pinaster* control tree suddenly died. No *Bursaphelenchus* sp. was found in this particular tree.

Bursaphelenchus xylophilus was found in all inoculated dead trees. The number and density of *B. xylophilus* in the inoculated seedlings are shown in Table 18. Pinewood nematodes were observed in higher numbers in dead *P. pinaster* than in *P. sylvestris* (t-Student; t=4,45; p=0,001), but both pine species had a similar nematode density *per* gram of dry weight (t-Student; t=1,17; p=0,250). Except for eight *P. sylvestris* seedlings, the number of the nematodes in dead seedlings was always higher than the initial *inoculum*. *Bursaphelenchus xylophilus* was absent or observed in low numbers in the surviving inoculated pines and always less than the initial inoculum level. It was observed only in four of the eight surviving *P. pinaster* seedlings and in two of the seven surviving *P. sylvestris*. No significant differences were found in nematode number (t-Student; t=0,73; p=0,478) and density (t-Student; t=0,02; p=0,989) between the surviving trees of both pine species. No *Bursaphelenchus* sp. was found in the control seedlings.

Table 18: Total numbers and density of nematodes recovered from the death and surviving pine seedlings inoculated with Portuguese strain of *B. xylophilus.*

		total number		nematodes / g dry weight	
Inoculated pine species		mean	Range	mean	range
P. pinaster	death (n=22)	100095	(4123-364643)	568	(29-1363)
	surviving (n=8)	158	(0-1248)	0,7	(0-5)
P. sylvestris	death (n=23)	6846	(236-26803)	420	(12-1410)
	surviving (n=7)	34	(0-230)	0,7	(0-4)

Bursaphelenchus xylophilus was found throughout the length of the dead seedlings, including in the small apical twigs and in the primary and secondary roots (Figure 49). In both pine species, *B. xylophilus* density was significantly different between the segments (ANOVA; F=7,98; p<0,001 and F=31,36; p<0,001, for *P. pinaster* and *P. sylvestris*, respectively). *Post hoc* SNK test showed that nematode density was higher in the area around the inoculation point and in the basal segment and lower in the apical and root extremes (Table 19).

Table 19: Mean number and range of *B. xylophilus per* g dry weight in the 4 vertical segments of inoculated pine seedlings.

Status	Pine species	Apical end	Inoculation segment	Basal segment	Roots
dead	*P. pinaster*	419 (0-2847)	1510 (18-6050)	630 (3-3329)	224 (0-899)
	P. sylvestris	152 (0-2977)	1164 (20-9373)	727 (31-3914)	213 (0-1270)
live	*P. pinaster*	0,03 (0-0,2)	0,11 (0-0,9)	1,25 (0-10,0)	1,42 (0-11,4)
	P. sylvestris	2,48 (0-17,4)	0,05 (0-0,2)	0,14 (0-1,0)	0

5.18.1 Nematode density and distribution in the early stages of pine wilt expression

None of the *P. sylvestris* seedlings inoculated with *B. xylophilus* showed strong wilt symptoms (<75% wilted needles) or died during the 52 days of the experiment. The trees sampled in the first 18 days presented null or weak wilt symptoms. Moderate wilt level in the trees started to appear after a month. After this date no seedling with null wilt level was sampled and the wilt level of the removed trees was in general increasing (Figure 50).

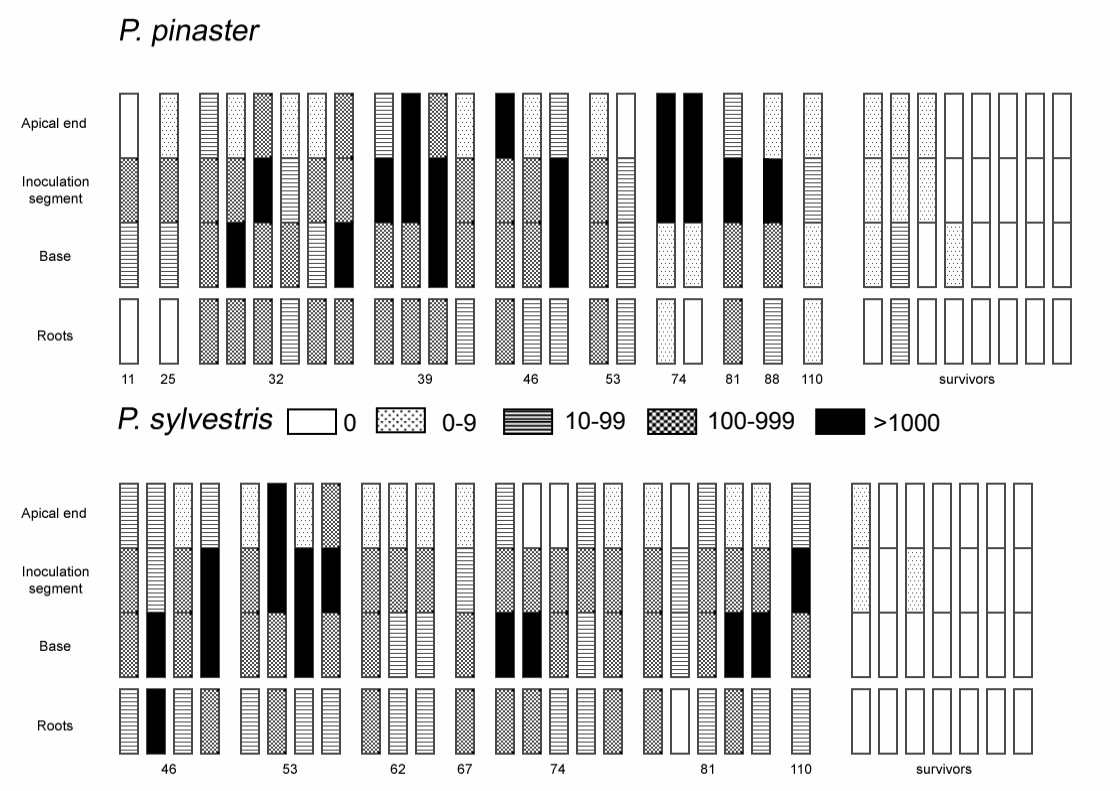


Figure 49: Diagram of density and distribution of *B. xylophilus* within the inoculated *P. pinaster* and *P. sylvestris* seedlings. Survivors are seedlings with the nematode that remain alive after the 4 months experiment. The bottom numbers indicate the days from inoculation until dead. *B. xylophilus* / g dry weight are indicated by the key boxes (0 to >1000)

B. xylophilus was found in all the *P. sylvestris* seedlings. Using the *inoculum* number as reference (4000 *B. xylophilus*), the mean number of *B. xylophilus* inside the seedlings decreased dramatically in the first days. The mean nematode population level reached a minimum at day 10 and in the posterior week the population level showed a slight increase. At day 25 the mean population level overcome the initial *inoculum* number and reached the maximum mean level at day 32. From this point further, the population levels decreased gradually, but always in numbers superior to the initial *inoculum* level. The relation between the days after inoculation and the mean density of *B. xylophilus per* gram of dry weight is identical to the described above for the mean number. The *B. xylophilus* mean density also reached a minimum at day 10 and a maximum at day 32 (Figure 50).

Three days after the inoculation, *B. xylophilus* was already found throughout the trees, including in the roots of one seedling. Later, as indicated by an accentuated decrease of the nematode numbers, the sampled seedlings showed a more restricted distribution, with many seedlings showing extreme areas without any *B. xylophilus.* In this phase, the main proportion of the *B. xylophilus* population within the seedlings seemed to be congregated near the inoculation point. When the population levels began to rise, from the 18th day, *B. xylophilus* distribution became broader and high densities were achieved in all locations of the seedlings. After this day, very high densities of *B. xylophilus* were observed in almost every seedling and a nematode-free root was observed in only one case.

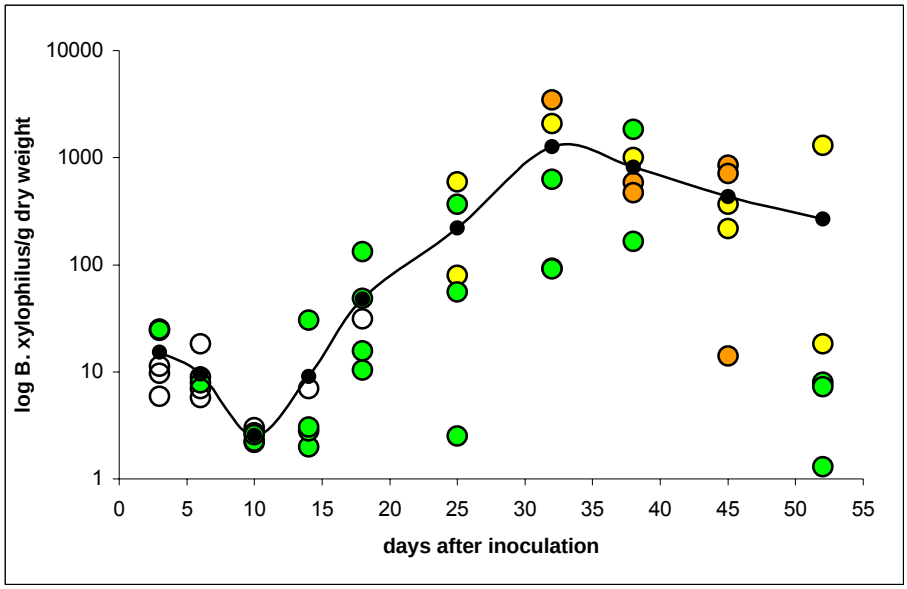


Figure 50: Density of *B. xylophilus* by dry weight of wood in *P. sylvestris* seedlings along 52 days after the inoculation. Each circle represents a seedling. Wilt level of the seedlings: ○- 0% of wilted needles; ●- 1-24% of wilted needles; ●- 25-49% of wilted needles; ●- 50-75% of wilted needles. The black circles are the mean values

There seemed to be a relation between the wilt level of the seedlings and the density of *B. xylophilus*. The seedlings collected with a null wilt level had a mean density of 10,7±9,4 nematodes; seedlings collected with wilt levels 1,2 and 3 had densities of 150,2±395,5, 639, 9±696,7 and 1013,5±1226,5, respectively. Unfortunately, since the variation within wilt levels was very high, no robust statistical evidence could be taken for this fact. Despite this, as shown in Figure 51, there was a clear tendency that the progression of the wilt level in seedlings was associated with the density of *B. xylophilus* within the tree.

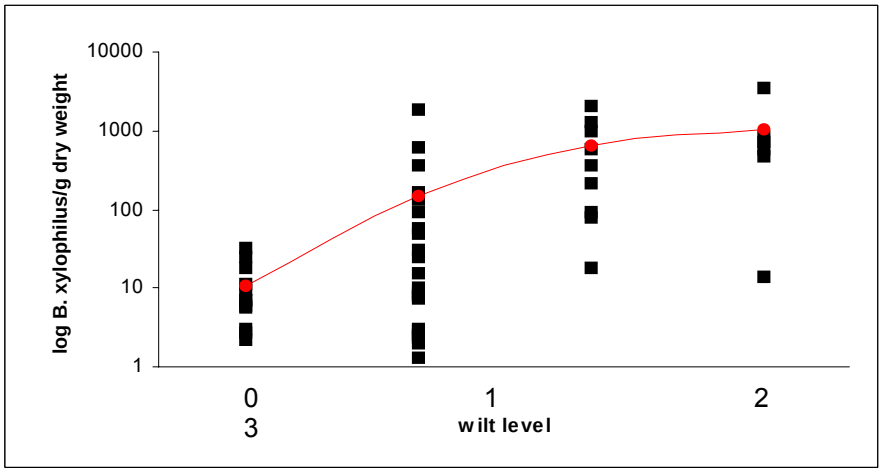


Figure 51: Density of *B. xylophilus* by dry weight of wood in *P. sylvestris* seedlings in relation to symptomatology level. Each black square represents a seedling. The red circles are the mean density values.

5.19 *Conclusions*

5.19.1 UEVORA Portuguese study

As expected, all crosses between Portuguese and Japanese isolates yielded viable F1 progeny (Figure 15), however, different combinations gave different results. Matings between Japanese and Portuguese isolates, overall, produced more progeny than did isolates from within each country, i.e., Japanese x Japanese or Portuguese x Portuguese. This may be the result of inbreeding depression, and/or hybrid vigour (heterosis), in which genetically more distant pairs within a species produce larger number of progeny. These results are similar to previous matings between Japanese isolates in which S10 x C14-5 yielded more progeny than S10 x S10 or C14-5 x C14-5 (Takemoto, unpublished data). Certain combinations yielded more progeny than the reciprocal crossings: for example, male C14-5 x female HF produced many more progeny than did the reciprocal crossing. Also the crossing between male S10 x female T produced more progeny than did the reciprocal crossing. In all combinations between Japanese x Portuguese isolates male x female combinations yielded more progeny than did the reciprocal mating. These results may indicate that maternal inheritance (mtDNA) may play a role in the number of offspring produced.

Although the morphology of all the *B. xylophilus* chromosomes was apparently identical, we could distinguish one pair of chromosomes from the others by using a ribosomal RNA probe (Figure 14). Similarly, we should be able to analyze the linkage groups of genes that have already been cloned, and distinguish individual chromosomes by amplifying resolution. And cloning of the male-specific chromosome (Y chromosome) sequence by using RAPD-PCR (Random amplified polymorphic DNA) and FISH methods should prove to be a useful tool for revealing the sex determination system in *B. xylophilus*.

This is the first comparison made between Portuguese and Japanese isolates of the PWN, and the first record of the pathogenicity of *B. xylophilus* from Portugal on Japanese black pine. It is unknown at this moment how pathogenicity varies among different isolates of *B. xylophilus* from Portugal. In particular, isolate T, and others originating from Portugal, could present a serious threat to Japanese black pine. The presence of the PWN in two very distant countries naturally raises questions regarding the possible origin and pathway analysis for each country. Iwahori et al. (1998) reported that three Japanese virulent isolates (S10, T4, S-6), one Chinese, and USA virulent isolates, BxC and MO, respectively, were identical to each other in the same rDNA regions as examined in this study; however the route for their worldwide distribution and the mechanisms involved in variation including speciation remain unsolved. These two Portuguese isolates may reflect natural variability in pathogenicity, or they may represent 2 separate introductions into the country which is difficult to explain considering the very short time of probable entry (5-10 years). More studies are needed using a wider range of PWN isolates from Portugal, as well as other isolates, including avirulent ones from Japan.

5.19.2 German study

The range of pine species from a wide geographical distribution was confirmed as highly susceptible host trees for *Bursaphelenchus xylophilus* provenances from North America, China and Portugal. These were *P. sylvestris*, *P. cembra*, *P. nigra, P. strobus*, *P. pinaster*, *P. radiata* and *P. mugo*. The pine species *P. halepensis* showed a low susceptibility. *P. pinea* and the Portuguese provenance of *B. xylophilus* was a compatible host nematode pathosystem under artificial conditions. Further investigations are needed as natural infestation of *P. pinea* by the Pine Wood Nematode (PWN) is not yet known.

The present study indicated that susceptibility and pathogenicity both determine the compatibility between the PWN and its hosts but needs careful consideration when being related to the pest risk of *B. xylophilus.* Tree species like *P. abies* are tolerant but, depending on stress factors, single trees could provide reservoirs for the PWN. In this respect the definition of tolerance, resistance and susceptibility of trees towards the PWN is urgently needed as a baseline for pest risk analysis. Accordingly a definition of what is the crucial host - nematode factor that determines a high risk,

should be a future goal. This study found mortality is not a good predictor for risk. It only relates to the reaction of host trees and not to the multiplication and latency of the PWN, which is still to an unknown extend related to its transferability to other hosts.

Inoculation trials in general produce artificial results and thus were believed to present a distorted account of the natural situation. In this study it was an essential method to confirm and predict a) the susceptibility of trees like *Larix* which is not known to be naturally infested by the PWN in the EU, b) the relation between population dynamics and migration of the PWN inside the tree on a systematic level, c) the joint influences of temperature, population dynamics and pathogenesis.

Some new advances for an invasion model of the PWN in highly susceptible trees like *P. sylvestris* could be drawn from this study. In contrast to the governing theory that the PWN builds up a population prior to its dispersal in trees this could not be observed in *P. sylvestris*. It was shown clearly that invasion in this host species by *B. xylophilus* was characterised by several systematic but divided steps:

(1) Formation of the initial population inside the host in a confined area around the side of entrance,
(2) Rapid migration and colonisation of all tree parts by a highly active part of the initial population of nematodes entering the tree,
(3) Establishment of the nematode inside the host through exponential growth,
(4) Retreat of the nematode into the basal parts of the tree.

Population growth inside areas of the tree appeared asynchronously and declined with distance from the site of inoculation. As a consequence, locally established populations of *B. xylophilus* developed dependent on their position in the tree (top, middle and base) and thus nematodes appeared to be distributed heterogeneously throughout the tree. Monitoring of PWN in potential host trees therefore should be based on taking samples from different parts of the tree.

The growth of the *B. xylophilus* population was confirmed significantly to be mediated by temperature but varied between highly susceptible host tree species like *P. sylvestris* and *L. decidua*. Pine Wilt Disease (PWD) in general occurred at temperatures above 20°C in these susceptible host trees. This observation indicates the existence of a threshold population density of *B. xylophilus* that induces irreversible wilt at 20°C. Considering the exponential growth of the PWN in susceptible hosts, small populations might use even very short periods with temperature above 20°C to successfully invade hosts that remain asymptomatic during cooler periods. There is some evidence that temperature controls both the nematode population and the physiological reaction of the tree leading to the pathogenesis of PWD. The interrelations between the two factors need further investigations. This should be undertaken with special reference to the induction of PWD through nematode activity. This would be useful in estimating the pathological importance of *B. xylophilus* in the context of a climate change for central and northern Europe, where *P. sylvestris* is the conifer of major economic importance.

5.19.3 INIA Portuguese study

This study substantiates the high susceptibility of *P. pinaster* to *B. xylophilus* in outdoor conditions (Yang et al., 1993; Linit and Tamura, 1987). Our results also show that the Portuguese isolate of *B. xylophilus* is highly pathogenic to *P. pinaster* and *P. sylvestris* seedlings. Summer mean temperature in the Setúbal peninsula rise above 20º C (National Institute of Meteorology and Geophysics: www.inmg.pt), a condition recognized as adequate to the development of PWD (Rutherford et al., 1990). *Pinus sylvestris* seedlings, although not a planted tree species in Portugal, are highly susceptible to *B. xylophilus* under high temperature regimes (Braasch, 2000). Due to the differential symptom development and mortality rates between the two pine species, different pathological responses of the trees to nematode infestation in the early stages of pathogenesis should be considered, although the pines showed similar wilt symptoms, percent mortality and after-death nematode density.

Bursaphelenchus xylophilus reproduced well in both pine species and were extracted from all wood parts of the dead seedlings. The numbers of nematodes recovered from dead *P. pinaster* seedlings were considerably greater than those from *P. sylvestris* because the latter seedlings were considerably smaller. The maximum number of nematodes *per* gram of dry wood extracted from dead seedlings was estimated at about 10,000 in *P. densiflora* and *P. thunbergii*. (Mamiya, 1981). Our results concerning the maximum nematode density in whole trees were much less, although some segments reached similar maximum density levels. This discrepancy was not a reflection of the time of sampling relative to nematode population growth (Mamiya, 1983), since the nematodes were extracted just after the death of trees and very few dispersal larvae were observed, but probably as consequence of several interacting climatic and chemo-physiological factors of tree species.

We found a consistent spatial pattern of distribution of *B. xylophilus* in dead pine seedlings, with higher nematode densities around the inoculation site, followed by the basal segment and with the lowest densities in the extreme parts of the tree. This distribution confirms the nematode behaviour associated with the disease development, with nematode migration and population increase within the host occurring shortly after the inoculation and reaching all wood tissues (Fukuda, 1997; Mamiya, 1983).

B. xylophilus showed a complex pattern of population dynamics inside the seedlings of *P. sylvestris.* This pattern could be described as an initial and accentuated decrease of the number of *B. xylophilus* in the 1^{st} – 2^{nd} week, followed by a sudden increase of the population levels in the 3^{rd} – 4^{th} week and a gradual decline of the population numbers in the following days. The high mortality observed in *B. xylophilus* populations during the first days could be the result of two factors: first, the seedlings were inoculated with adults and propagative juveniles and not *dauer larvae* as occur in natural conditions. These stages are less resistant to adverse conditions, including entry to the host. The second factor, which could be linked with the first, is tree defences. The inoculation wound and the entrance of a strange organism could provoke tree defences and, as above, the adult and propagative stages of *B. xylophilus* are less resistant to these defences (Mamiya, 1981). Although there was high mortality of *B. xylophilus* populations in the first few days, the few resistant nematodes multiplied very fast and established very dense populations a month after the inoculation.

Although reservations about the applicability of pathogenicity tests using seedlings have been expressed (McNamara, 2003), if the results were applied carefully, they could provide relevant information about the pathogenic potential of a nematode species or provenance and which factors influence development and action of the nematodes in the field (Braasch, 2000). Future pathogenicity studies using the Portuguese *B. xylophilus* isolate in the other pine species common in Portugal, *P. pinea* and *P. halepensis*, and in adult trees within the Portuguese affected area should be conducted to provide complementary data on factors influencing PWD occurrence and development in southern Europe, and particularly in Portugal.

Chapter 6 Pinewood Nematode, its vectors and host trees

6.1 *Life History and Seasonal Development of Monochamus galloprovincialis in the pine wilt disease affected zone, Portugal*

Insects in the genus *Monochamus* are known worldwide because of the association of some of the species with the pine wood nematode (PWN) *Bursaphelenchus xylophilus*, which is a quarantine organism in the European Union and constitutes a restriction to the international trade of some forest products (Evans et al., 1996). In 1999, PWN was detected in dead Maritime Pine (*Pinus pinaster* Aiton) for the first time in Europe, near Lisbon, Portugal (Mota et al., 1999), and soon after the endemic cerambycid beetle *M. galloprovincialis* was found to be the nematode's vector (Sousa et al., 2001). Before the introduction of PWN, this insect was considered to be a secondary forest agent in Portugal, associated with over-mature forests and burned stands. Little was known about the biology of *M. galloprovincialis* both in Portugal and in Europe, except for the classic paper of Hellrigl (1971) on the bionomics of European *Monochamus*, which is largely a literature review paper. Since the introduction of PWN into Europe, studies about the biology and seasonal development of *M. galloprovincialis* are necessary in order to understand the insect's population dynamics, predict population growth and integrate this knowledge with the epidemiological cycle of wilt disease in Portugal.

The main objectives of this study were:

- to determinate the number of species of the genus occurring in Portugal, their distribution and hosts;
- to study the seasonal development of the immature stages and the emergence pattern and flight curve of the adult *M. galloprovincialis* beetles;
- to investigate the mortality of the immature beetle stages;
- the feeding and oviposition preference of the adult beetles;
- to characterise the most important reproductive and longevity parameters of *M. galloprovincialis* populations obtained from the nematode affected zone.

6.2 *Materials and Methods*

6.2.1 Survey of insects of the genus Monochamus

A national *Monochamus* spp. survey was conducted in regions with conifer forests in continental Portugal from North to South. Selected declining conifer forests were visited periodically along the boundaries of fire-burned stands, where the presence of bark and wood boring insects was thought to be maximised. Dead or declining suspected trees were felled and carefully examined for the presence of *Monochamus* spp., collecting wood material with suspected *Monochamus* presence whenever possible, to later be identified in the laboratory. Selected anatomical features of adult *M. galloprovincialis* were observed with the help of a Scanning Electronic Microscope (SEM) to investigate distinctive anatomical features that would help morphological identification of the adults.

6.2.2 Seasonal development of the pine sawyer

On five distinct occasions (23 May, 18 June, 13 July, 13 August and 13 September 2001) 12 recently-cut maritime pine logs approximately 30cm long were placed on a large wooden box containing adult *M. galloprovincialis* couples. Female beetles were allowed to breed freely on the logs which were removed after two days exposure to the insects and kept in a shaded place with ambient temperature and humidity. One log was sampled randomly from each week's exposure and analysed after 1, 2, 3, 4, 6, 8, 12, 16, 20, 24 and 28 weeks. Sampled logs were debarked, assessed for *Monochamus* developmental stages and, when galleries penetrated into the xylem

were opened with the help of a vertical electric saw, collecting the larvae present. One log from each date was also maintained at ambient temperature to study adult emergence, which was checked every two days from April to October 2002. After the emergence period (October 2002) the final log was also analysed and dissected.

6.2.3 Mortality of developmental stages

To study mortality of eggs and young larvae, six adult *P. pinaster* trees were cut on June 2004 at Comporta (38º 22'N; 8º 46'W) and divided into 28 similar logs each approximately 50cm long. The logs were placed in large wooden boxes containing between three and four adult *M. galloprovincialis* couples and, after three days with the insects, they were removed on the 9th of July taken to an adult *P. pinaster* stand at Comporta, where they were attached with ropes in the lower canopies of three adjacent healthy maritime pine trees. After 1, 2, 3, 4, 6, 8 and 10 weeks at ambient conditions, four of the logs were randomly selected on each occasion and taken to the laboratory, where they were debarked to observe the frequency and health condition of the insect's developmental stages. The ambient temperature (hourly records) and precipitation were recorded locally from July 9 to September 17th 2004 using a portable weather station.

The mortality of the mature larvae, pupae and pre-emerging adults was studied by dissecting the pine bolts. Larval galleries with absence of emergence holes were dissected with an axe and an electrical vertical saw to determinate the fate of the larvae. Dead larvae were observed under a binocular microscope to determinate the cause of mortality, and when fungi were present they were collected to be later identified. Parasitized larvae were kept isolated in a plastic Petri dish at ambient temperature until emergence of the responsible agent.

6.2.4 Emergence pattern of M. galloprovincialis in the pine wilt affected zone

Between four and nine dead maritime pine trees with *M. galloprovincialis* were cut every year during January/February from 2001 to 2004 at Tróia peninsula (38º29' N; 8º53' W) and Companhia das Lezírias (38º 47'N; 8º 49'W), inside the pine wilt disease affected region. The trunk and branches were carefully analyzed and all segments thought to contain *M. galloprovincialis* larvae were divided into approximately 50cm-long bolts, placed in large round plastic containers and covered with semi-transparent cloth mesh, after which were kept in a shaded place at ambient temperature at Tróia and Lezírias. Each year from April to October all the containers were observed, at least twice a week (although usually between three and four times per week), collecting all the emerging beetles which were sexed, weighed and measured (length of the right elytra). The sex ratio of emerged beetles was calculated as ♀/(♂ + ♀).

6.2.5 Flight curve of adult Monochamus galloprovincialis

The flight curves of the adult beetles were studied by placing traps in the forest environments (on Tróia and Lezírias) from May to October 2001 to 2004. The captured beetles were recorded weekly, along with a change of trap attractants. Three types of trap were used: multi-funnel Lindgren traps baited with ethanol and alpha-pinene; basket traps with fresh *P. pinaster* logs and wood debris; and transparent interception traps baited with ethanol and turpentine. Traps were placed at four different locations on the same tree (upper crown, lower crown, middle trunk and lower trunk) to compare insect captures at different heights.

6.2.6 Host preference of Monochamus galloprovincialis

Adult insects used in the experiments emerged in June 2003 from *P. pinaster* logs kept in wooden boxes in Tróia. Only insects from PWN-free pines were used. The trees used in the experiments were cut in the last week of June from three locations: *P. pinaster* and *P. pinea* from Tróia peninsula; *P. halepensis* and *Cupressus lusitanica* from Monsanto Park, Lisbon (38º43' N; 9º11' W); and *P. sylvestris, P. radiata,* and *Pseudotsuga menziesii* from V.N. de Cerveira, Minho province (42º0' N; 8º35' W). Branches were used in experiments 3-4 days after cutting and bolts 20 days after cutting. Three experiments were conducted: the evaluation of the feeding preference of

adult *M. galloprovincialis* among five pine species, a non-choice oviposition test with seven conifer species, and a choice test to evaluate oviposition preferences between *P. pinaster* (the beetle's host) and other pine species.

6.2.7 Feeding preference of Monochamus galloprovincialis

Thirty recently emerged (max. 24 h) adult insects (30 of each sex) were randomly selected and placed individually in a transparent acrylic box containing five similar pine branches (15 cm long x 1.5 cm diam.) glued vertically to the sides. Branches of five pine species (*P. pinaster, P. pinea, P. sylvestris, P. halepensis,* and *P. radiata*) were placed randomly in the box for each replication. After 36 h the insects were removed and measured (length of right elytron) and each branch was carefully analysed to detect feeding wounds on the bark, which were individually photographed using a Leica DC 300 digital camera attached to a microscope. The feeding areas on the photographs were measured with the "Measure" module of the QW in software programme (Leica Imaging Systems, Wetzlar, Germany). The relative amount of bark fed upon by the insects (in mm^2) was considered an indication of host preference.

6.2.8 Oviposition confinement

Three adult insect couples (ca. 25 days old) that had not previously reproduced were randomly chosen and placed in a screened wooden box with a single bolt (60 cm long, 6-12 cm diam.) of one of the seven conifers tested (*P. pinaster, P. pinea, P. halepensis, P. sylvestris, P. radiata, Pseudotsuga menziesii,* and *C. lusitanica*). The experiment was replicated five times for each treatment, always with different insects and bolts. Any dead insect was immediately replaced by another one of the same sex and age. After 5 days, oviposition slits were counted, and the bolts kept at adequate temperature regimes to allow emergence of brood adults. The number of days until emergence (counted from the return to 25 ºC), size (length of right elytron), and sex of the emergent adults was recorded. After 120 days at 25 ºC, the bolts were debarked and dissected, and the dead immatures counted.

6.2.9 Oviposition preferences between P. pinaster and other pine species

Recently emerged *M. galloprovincialis* adults were kept for maturation feeding in ventilated acrylic boxes with one *P. pinaster* branch and one branch of either *P. pinea, P. sylvestris, P. halepensis,* or *P. radiata.* After 25 days, four couples were randomly selected and placed in screened wooden boxes containing two pine bolts (60 cm long x 6-12 diam.), *P. pinaster* and the other species on which maturation feeding was allowed. Each of the four paired-bolt treatments was replicated five times, using different insects and bolts. If possible, bolts of similar diameter and bark thickness were paired, and placed in opposite corners of the box. Any dead insect was immediately replaced by another one of the same sex and age. After 72 h, the bolts were debarked and the number of oviposition slits with eggs counted.

6.2.10 Reproductive biology and longevity

Three dead *P. pinaster* trees colonized by *M. galloprovincialis* were cut in Tróia in November 2001 and divided into 50cm logs which were kept in containers under natural temperature and humidity conditions. During the peak period of adult emergence 74 adult beetles (*B. xylophilus* free) were randomly collected and sexed, weighed, measured and paired (37 pairs, using insects of similar size) in transparent plastic containers at 25ºC 12-12 LD. Each couple was given a Maritime pine bolt (cut five days before) for 24 hours, after which it was replaced by a fresh one. The bolt was examined under a binocular microscope and all oviposition slits were dissected to detect eggs. After the detection of the first egg produced by each couple, the bolts were replaced every 3-4 days, after which they were kept for 15 days at 25ºC to allow the eggs to hatch, and debarked. If the male of the pair died before the female, it was replaced immediately by a similar one. Substituted males were excluded from the longevity analysis. If a female died, it was dissected and the number of eggs remaining in its ovaries was recorded.

6.2.11 Statistical Analysis

Parametric and non-parametric statistical analysis was performed as considered adequate.

6.3 *Results*

6.3.1 Survey of insects of the Monochamus Genus

Monochamus galloprovincialis was the only species of *Monochamus* detected and, although normally in low numbers, its presence was reported in almost all the survey plots all over continental Portugal. More than eleven conifer species were surveyed for the insects (*Pinus pinaster, P. sylvestris, P. halepensis, P. pinea, P. nigra laricio, P. radiata, Larix decidua, Chamaecyparis lawsoniana, Pseudotsuga menziesi, Cupressus sempervirens* and *Cupressus lusitanica*), although *M. galloprovincialis* was frequently detected on *P. pinaster* and, only once, on *P. halepensis*. Maritime pine undoubtedly appears to be the insect's main host in Portugal.

Several anatomical features of the adult's head, elytra and thorax were observed using a scanning electronic microscope (SEM), although no distinctive characteristic was detected that could be used to further help the morphological identification of the adults of this Genus, whose separation into species is not particularly difficult (Figure 52).

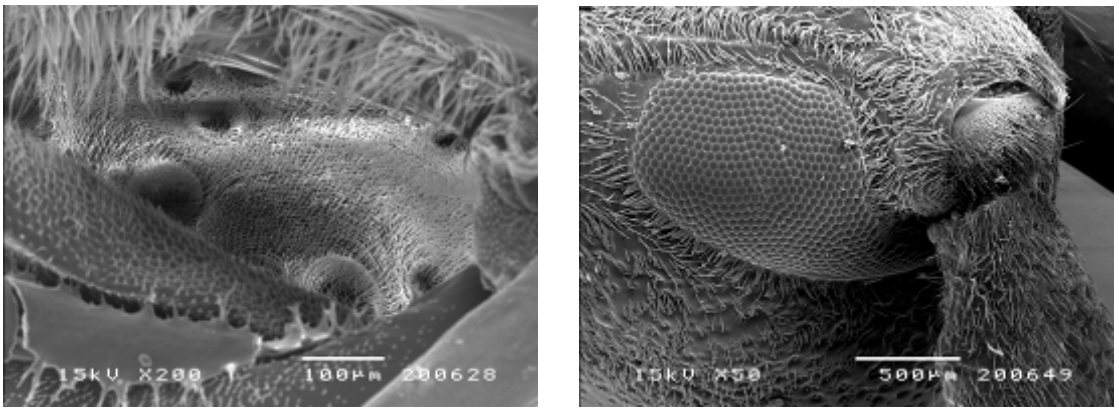


Figure 52: Photographs of adult *M. galloprovincialis* beetles using a SEM; on the left detail of an open trachea, right detail of eye and insertion of antennae.

6.3.2 Seasonal development

No adult beetles emerged in the same year as oviposition took place. The sampled logs contained a mean of 4.1±0.4 eggs each, which hatched in less than two weeks at ambient temperature on the five sampling dates. Development of the young individuals was very rapid and the larvae actively fed beneath the pine bark usually for one month, after which they built tunnels penetrating into the wood (Table 20). Some of the larvae were even more precocious and began tunnelling into the xylem in just two or three weeks after hatching during the summer months.

The larvae from the first oviposition date (late May) were fully developed and settled in galleries inside the wood by the end of August/beginning of September, although they did not emerge during that year. All the later stages of eggs laid from May to August over-wintered as mature larvae inside the wood and, as the individuals moulted only once in spring (when turning into pupae), the large majority of the population thus survived winter as last-instar larvae. Only the insects from the mid-September logs had a different seasonal development pattern, as the young larvae developed more slowly during autumn and winter and tunnelled into the wood only from January onwards.

Table 20 Developmental instars of *M. galloprovincialis* on pine logs kept at ambient temperature and periodically sampled from June 2001 to April 2002. scl – larvae in galleries beneath the bark (sub-cortical); xl – larvae in galleries inside the wood (xylem).

Sampling month	Oviposition date				
	25 May	20 June	15 July	15 August	15 Sept.
June	eggs/scl	eggs			
July	scl	scl	eggs		
August	scl/xl	scl/xl	scl	eggs	
September	xl	scl/xl	scl	scl	eggs/scl
October	xl	scl/xl	scl/xl	scl/xl	scl
November	xl	xl	xl	scl/xl	scl
December	xl	xl	xl	xl	scl
January		xl	xl	xl	scl/xl
February			xl	xl	scl/xl
March				xl	xl
April					xl

None of the eggs or larvae was found dead through the experiment and after a year a total of nineteen adult insects (five males and 14 females) emerged from the logs exposed to the parental insects. Emergence was concentrated during July and August 2002 for the five logs, with no apparent influence of the different oviposition dates.

6.3.3 Mortality of developmental stages

During the two-month period 16% of the 44 *M. galloprovincialis* eggs did not hatch, due either to failure to hatch/desiccation and resinosis. The mortality of the sub-cortical larvae was even lower and affected 11% of the 80 individuals, being caused by resinosis, fungi (*Beauveria bassiana*) and unknown factors (empty galleries without larvae).

Due to the very low number of dead larvae detected (only nine) no relation could be established between larval mortality, competition with other insects on the logs or climatic conditions. The pine logs were colonised by four other insect species, namely the small scolytid *Orthotomicus erosus* (by far the most abundant) along with *Hylurgus ligniperda*, the cerambycid *Arhopalus syriacus* and one unidentified buprestid. No predators or parasitoids were observed on the logs or on the dead larvae. The weather conditions were normal for the season with a daily mean temperature of 23ºC for the 70-day period, with maximum values of 41ºC observed on August and September, while rainfall totalled 16.2mm.

Regarding the larvae that tunnelled into the wood, its density (per m^2 of wood) was different between tree sections ($F= 4.61$; $df= 4$; $P= 0.001$), being higher in the upper trunk and lower in the lower crown (Table 21). Larval mortality also differed between tree segments ($F= 11.31$; $df= 4$; $P\leq0.001$) and affected mainly the upper crown branches, resulting in larger numbers of adult *M. galloprovincialis* emerging per m^2 of wood on the larger trunk bolts while the lowest values were found on the upper crown branches ($F= 6.36$; $df= 4$; $P\leq0.001$).

Table 21 Comparison of tree sections (in parenthesis number of bolts analysed) for bolt diameter (in mm), number of larvae that penetrated the wood (per m^2), adult emergencies (per m^2) and larval mortality (%)

	Tree section	Diameter	Larvae in wood	Emergences	Mortality
Crown	Upper (75)	34 ± 1.4 a	52 ± 3.2 ab	24 ± 3.3 a	44 ± 5.1 a
	Mid (158)	52 ± 1.3 b	45 ± 2.7 a	29 ± 2.8 ab	27 ± 3.5 b
	Lower (150)	60 ± 1.8 c	43 ± 2.4 a	25 ± 2.3 a	26 ± 3.7 b
Trunk	Upper (93)	70 ± 1.9 d	60 ± 4.2 b	39 ± 4.0 bc	22 ± 4.3 b
	Lower (83)	120 ± 2.6 e	49 ± 3.0 ab	42 ± 3.0 c	11 ± 2.6 c

[1] Means within each column followed by the same letter do not differ, Tukey HSD test P≤0.05.

Overall, mortality affected 26% of the mature larvae inside the wood. The dissection of 579 larval galleries without adult emergence found that more than half of the mortality was either due to unknown causes (a dead larva was found with no mortality factor detected) and/or missing larvae (galleries inside the wood contained no larvae) (Figure 53). Parasitism accounted for almost one-quarter of the mortality, with three Braconidae detected: *Cyanopterus flavator* Fabricius was the most numerous species, whereas *Iphiaulax impostor* (Scopoli) and *Coeloides sordidator* Ratzeburg were the other species found. Fungi were found on the body of 23% of the dead larvae, with *B. bassiana* as the dominant species. Predation by woodpeckers was minor and affected only 2% of the mature larvae.

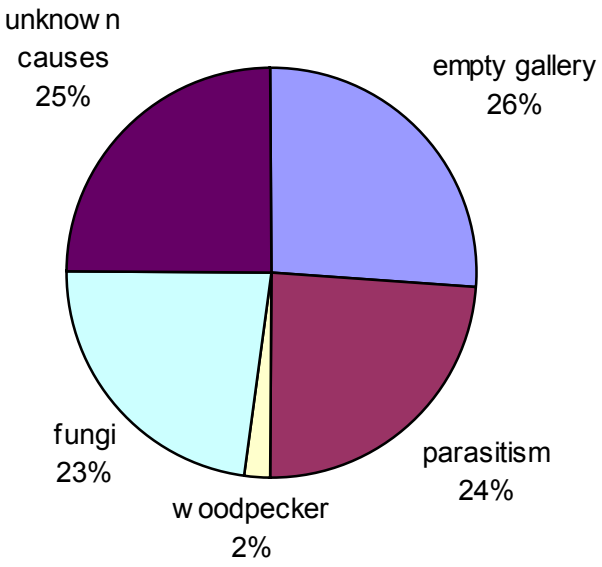


Figure 53 Mortality factors (in %) of *M. galloprovincialis* mature larvae in galleries inside the wood

Only 0.8% of the pupae were found dead inside the pupal chamber without any apparent mortality cause, while 2% of the adults dyed in the course of boring the exit holes from the pupal chambers to the exterior.

6.3.4 Emergence pattern of M. galloprovincialis in the pine wilt affected zone

The emergence of *M. galloprovincialis* occurred during a defined period in late spring/early summer each year and usually took place during a period of two or three months. Of the 2470 insects that emerged during the four-year study, the earliest individuals appeared in mid-May and the latest

ones in early September, although the emergence peak was generally in July during all years (Figure 54; Table 22).

Female beetles were significantly bigger than males (F= 26.3; df= 1; P≤0.005) although the weight did not differ between the two sexes (F= 1.89; df= 1; P=0.170). The sex ratio of the adult beetles recorded at emergence was slightly biased towards males, resulting in a 0.48 value.

Table 22 Dates of emergence for both sexes of *M. galloprovincialis* adults, duration of emergence (in days) and number of emerged insects from 2001 to 2004

Year:	**2001**		**2002**				**2003**				**2004**			
Place:	**Tróia**		**Tróia**		**Lezírias**		**Tróia**		**Lezírias**		**Tróia**		**Lezírias**	
Sex:	**M**	**f**	**m**	**f**	**M**	**f**	**m**	**f**	**m**	**F**	**M**	**f**	**M**	**f**
First emergence	17 May	14 May	18 Jun	14 Jun	27 Jun	1 Jul	4 Jun	4 Jun	23 Jun	3 Jul	25 May	25 May	25 May	27 May
10% emergence	1 Jul	4 Jul	11 Jul	14 Jul	11 Jul	15 Jul	16 Jun	18 Jun	8 Jul	12 Jul	29 Jun	25 Jun	6 Jul	7 Jul
50% emergence	14 Jul	20 Jul	25 Jul	27 Jul	25 Jul	27 Jul	8 Jul	10 Jul	16 Jul	20 Jul	9 Jul	9 Jul	17 Jul	17 Jul
90% emergence	30 July	1 Aug	15 Aug	15 Aug	15 Aug	15 Aug	20 Jul	23 Jul	30 Jul	31 Jul	24 Jul	25 Jul	27 Jul	2 Aug
Last emergence	14 Aug	14 Aug	5 Sep	2 Sep	29 Aug	29 Aug	1 Aug	11 Aug	11 Aug	11 Aug	4 Aug	8 Aug	11 Aug	11 Aug
Emergence period (d)	89	92	79	80	63	60	58	68	49	39	71	75	78	76
Emerged insects	301	255	351	320	181	155	135	128	128	106	86	79	92	102

When dissecting the pine bolts after the emergence period to investigate larval mortality, an additional 64 *M. galloprovincialis* larvae were found alive in their galleries inside the wood, although unfortunately when taken to the laboratory and kept at 23ºC all the collected larvae died in a few weeks.

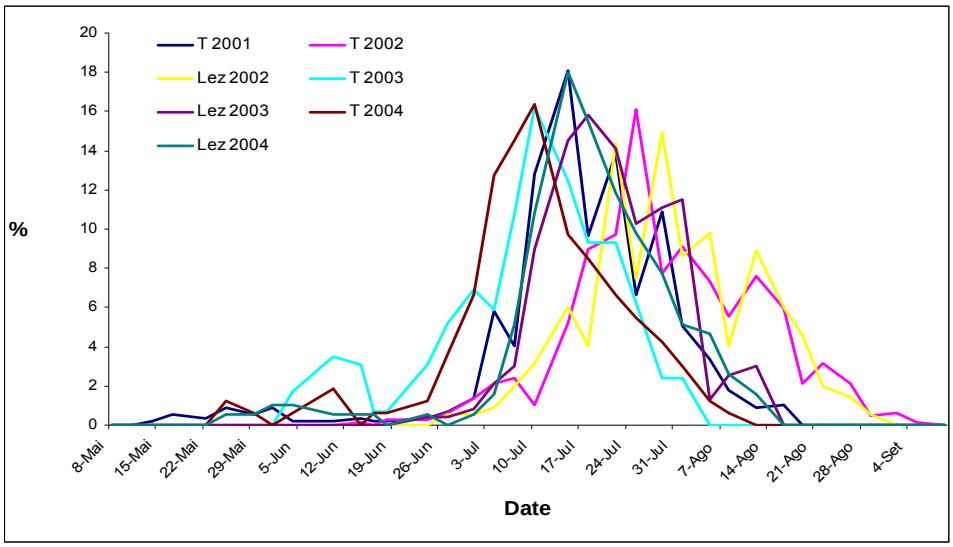


Figure 54: Emergence pattern (%) of *M. galloprovincialis* from 2001 to 2004 in Tróia (T) and Lezírias (Lez), inside the pine wilt affected zone, Portugal

6.3.5 Flight curve of M. Galloprovincialis adults

Overall, the captures during the four year period were not very high, at least considering the high number of *M. galloprovincialis* beetles usually found in pine wilt affected areas. The results from Tróia and Lezírias are presented jointly in Figure 55.

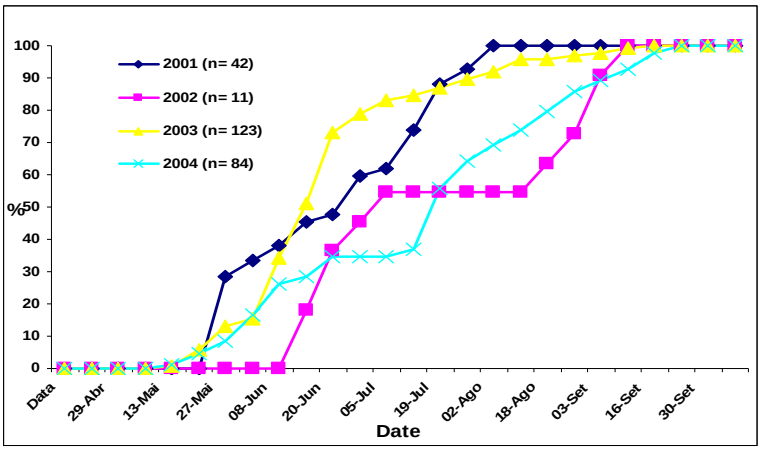


Figure 55: Captures of *M. galloprovincialis* (accumulated %) from 2001 to 2004

The earliest insects were captured in May, while the latest ones were caught in late September. The majority of the beetles were caught at the canopy level while the lowest captures were on the traps at the base of the tree (Figure 56). The overall sex-ratio for the 230 beetles captured during the four years was 0.44.

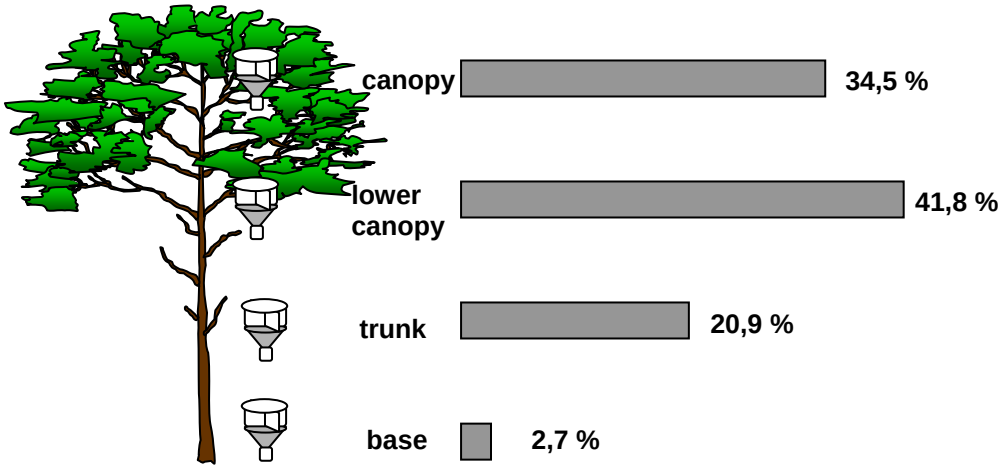


Figure 56: *M. galloprovincialis* captures (in %) at four positions within a tree

6.4 *Host preferences*

6.4.1 Feeding preference

Two females that did not feed were excluded from the analysis, and all the remaining beetles fed on one or more branches. Most chose only one (47% of the individuals) or two (33%) pines to feed on, although some beetles fed on three (17%) or four (3%) of the species. *Pinus sylvestris* was selected by 34 insects, followed by 24 on *P. pinaster* (Table 23). The amount of bark consumed by

the beetles varied among pines (F=8.95, df=4, P<0.001), with *P. sylvestris* again having the highest values, while radiata pine was the pine least chosen and fed upon. There were no differences in the bark area consumed by both sexes, neither in the total amount (males: 240.3 ± 22.0 mm^2, females: 232.0 ± 29.5 mm^2; F= 0.02; df= 1; P=0.878) nor between treatments (F= 0.58; df= 4; P=0.677).

Table 23 Number of adult *M. galloprovincialis* which fed and mean bark area consumed per sex on branches of five *Pinus* spp. (n = 28 females, 30 males)

	No. of insects that fed		Mean area (mm^2) of bark consumed [1]	
Species	Male	Female	Male	Female
P. halepensis	10	11	55.5 ± 19.2 bc	59.3 ± 26.3 ab
P. pinaster	15	9	44.6 ± 14.0 cd	28.7 ± 13.2 c
P. pinea	8	11	21.7 ± 7.7 cd	46.9 ± 15.4 abc
P. radiata	2	2	9.7 ± 7.1 d	6.5 ± 5.8 c
P. sylvestris	17	17	108.8 ± 22.5 a	90.5 ± 18.7 a

[1] Means within each column followed by the same letter do not differ, Fisher LSD test P≤0.05.

6.4.2 Oviposition confinement

Eggs were laid on six of the seven conifers tested, with the highest number on *P. sylvestris*. All five bolts of *P. sylvestris*, *P. halepensis* and *P. pinaster* contained eggs. Eggs were also present on three bolts of *P. radiata*, two *P. pinea,* and one *Pseudotsuga menziesii* (two eggs). No eggs were found on *C. lusitanica*. Adult insects emerged only from *P. halepensis, P. pinaster, P. radiata,* and *P. sylvestris*. No differences were found among these pine species in the number of days to emergence (F= 1.72; df= 3; P= 0.167), emergence rate (Kruskal-Wallis test: χ^2 = 0.9, d.f. = 3, P = 0.817), or beetle sex (F= 0.68; df= 3; P=0.568). No *M. galloprovincialis* emerged from *P. pinea* or *Pseudotsuga menziesii* bolts, and dissections revealed dead larvae in galleries in the phloem tissue of these species.

6.4.3 Oviposition preferences between P. pinaster and other pines

The number of eggs laid differed among treatments (Kruskal-Wallis test: χ^2 = 8.69, d.f. = 3, P = 0.034), existing a clear preference for oviposition on *P. pinaster* over *P. pinea* and *P. radiata* (Wilcoxon signed rank test, Z = 2.0, P = 0.043, for both), but no preference was recorded between *P. pinaster* and *P. sylvestris* (Wilcoxon signed rank test, Z = 1.6, P = 0.106) or *P. halepensis* (Wilcoxon signed rank test, Z = 0.8, P = 0.423).

6.4.4 Reproductive biology and longevity

The longevity of adults from both sexes was similar (F= 0.0952; df= 1; P= 0.759), with an average lifespan of two months, although some of the insects lived for more than four months. Sixteen insects (eight males and eight females, corresponding to 22% of the population) died within 20 days and before the onset of egg-laying (Figure 57). No eggs were found in the dissected ovaries of these females, and they were excluded from the reproductive analyses. The insects with early mortality had a significantly (F= 6.3102; df= 1; P= 0.0142) smaller weight (0.2257 ± 0.0289g) than those that survived to reproduce (0.3071 ± 0.0150g) (mean ± SE).

After the initial mortality during the first 20 days the mortality lowered for both sexes in the following weeks and reached the 50% level by the 58th day for males and 67th day for females.

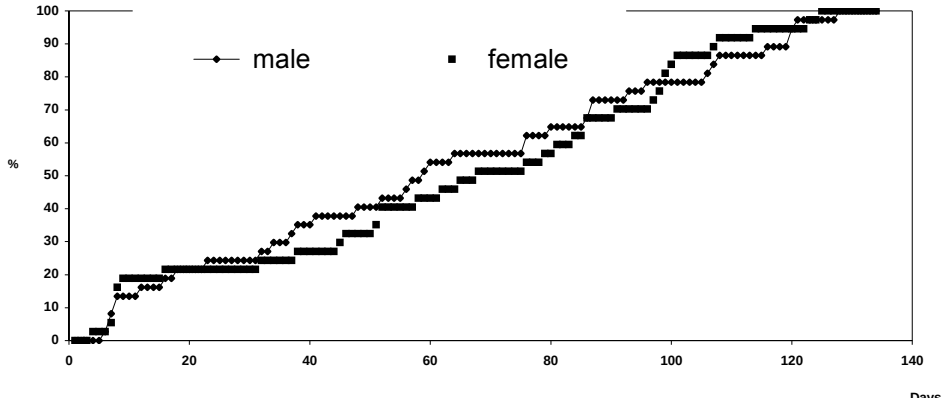


Figure 57 Accumulated mortality curves (in %) for *M. galloprovincialis* males and females

The mean time to construction of the first egg pits was 11 days, although most of the early egg pits contained no eggs. The first eggs were deposited only 20 days after emergence (mean) (Table 24).

Table 24 Fecundity parameters of *M. galloprovincialis* reproductive females (n= 29 beetles; mean ± SE; minimum-maximum values on brackets)

first egg pit (days)	first egg (days)	oviposition period (days)	eggs laid (n)	oviposition rate	eggs in the body (n)
11.5 ± 1.1 (5 – 25)	20.4 ± 0.7 (11 – 29)	54.0 ± 4.2 (7 – 98)	67.0 ± 6.0 (11 – 112)	1.3 ± 0.1 (0.2 – 1.6)	4.0 ± 0.7 (0 – 12)

Each female laid, on average, 67.0 ± 5.96 (mean ± SE) eggs throughout its life. The oviposition rate increased very quickly during the first weeks after emergence, peaking to almost two eggs per day during days 30-44 after emergence, and gradually dropped in the following weeks (Figure 58). Throughout their reproductive lives, females also continued to make empty egg pits, although with a more uniform pattern. There were no external visual differences between empty and egg-containing pits, and it required dissection to determine that, on average, only 73% of egg pits contained eggs.

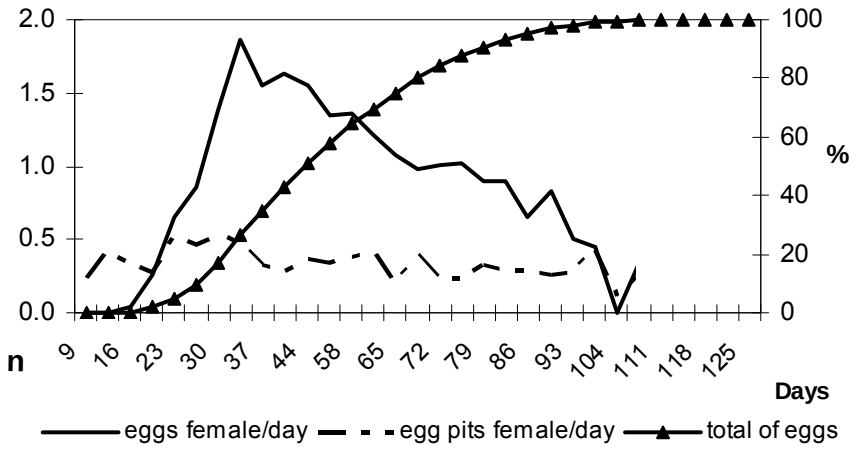


Figure 58: Mean number of egg pits and eggs per female per day and cumulative number of eggs laid (%)

Fifteen females had a period of three or more days without laying eggs before their death, while the remaining 14 females died less than three days after laying their last egg. Dissection indicated that 72% of females (21 insects) still had 4.0 ± 0.7 (mean ± SE) eggs in their ovaries.

Of the 1928 egg pits analysed, 99.2% contained one egg, while 13 (0.7%) contained two eggs, and only one had three eggs (0.1%). The hatch rate was 92.6 ± 1.0% (mean ± SE).

6.5 *Interactions between the pine sawyer Monochamus galloprovincialis and the pine wood nematode in the pine wilt disease affected region, Portugal.*

The pinewood nematode (PWN) *Bursaphelenchus xylophilus* is transported as fourth-stage dispersal juveniles (JIV) by cerambycid beetles of the genus *Monochamus*, and in Portugal the species is associated with the endemic *M. galloprovincialis* (Sousa *et al*., 2001). After the insect's emergence the fourth-stage dispersal larvae are inoculated into a new host through the feeding wounds made by the adult insects or the female's oviposition activity (Mamiya, 1984).

Over the years, the most important aspects of the interaction between the pinewood nematode and its North American and Asian vectors have been intensively studied by several authors (e.g., Mamiya 1984; Linit, 1989; Togashi, 1985). Due to the recent detection of the *B. xylophilus*-*M. galloprovincialis* association occurring in Portugal, no similar studies had been conducted so far for these two organisms. The purpose of this research was to initiate the characterisation of the nematode-vector association occurring in Portugal, studying the temporal timing and life stage of *M. galloprovincialis* infested by the nematode, the PWN abundance and distribution on body segments of the vector, and the patterns and frequency of *B. xylophilus* transmission through maturation feeding and female oviposition.

6.6 *Material and Methods*

6.6.1 Association of the pine wood nematode with the immature stages of Monochamus galloprovincialis

Six adult maritime pine trees (*P. pinaster*) infested with *B. xylophilus* (which had previously been found to be the only *Bursaphelenchus* present on the wood) and with *M. galloprovincialis* presence were cut, divided in one meter logs and kept under ambient conditions (temperature and humidity) on January 2003 in Troía, Portugal. Between January and July 2003 a random section of approximately 30cm of wood containing *M. galloprovincialis* galleries was collected monthly and divided on a vertical saw in thin sections, collecting all *M. galloprovincialis* individuals detected (larvae, pupae and callow adults). The insects were crushed individually in a Petri dish with water, counting and identifying the nematodes associated with them. A sample of wood with 5-7mm diameter was collected with a knife from the gallery or chamber where the immature insect was found, extracting the nematodes using the modified Baermann technique (Southey, 1986). The xylem galleries or chambers containing *M. galloprovincialis* mature larvae were denominated "larval galleries", while galleries or chambers containing either pupae or pre-emergent callow adult beetles were referred as "pupal chambers".

6.6.2 Pine wood nematode distribution on M. galloprovincialis body

Recently emerged *M. galloprovincialis* beetles originating from *B. xylophilus*-infested maritime pine logs were kept in large netted wooden boxes jointly with small *P. pinaster* branches for feeding. Two and 30 days after emergence, twenty beetles (ten males and ten females per date) were randomly selected, measured (length of the right elytra) and divided with a sharp knife into nine distinct body segments: antenna, head, elytra, wings, legs, pro-thorax, meso-thorax, meta-thorax and abdomen. Each segment was individually macerated in a plastic Petri dish with water, kept at

ambient temperature for 24 hours and observed under a binocular microscope, identifying and counting the nematodes present.

6.6.3 Transmission of B. xylophilus through the maturation feeding of M. galloprovincialis

Ten adult maritime pine trees (*P. pinaster*) infested with *B. xylophilus* and *M. galloprovincialis* were cut near Comporta, divided in 70cm logs and kept under ambient temperature in netted boxes. Upon emergence, adult *M. galloprovincialis* were checked for the presence of *B. xylophilus* on their bodies using the method of Zhang *et al*. (1995). A total of 28-PWN infested insects (14 males and 14 females) were placed individually in transparent acrylic boxes along with a single recently-cut (maximum 48 hours old) maritime pine branch for feeding. Branches were replaced weekly or until the death of the insect, and stored in closed plastic bags at 25ºC ± 1ºC and 75% relative humidity for two weeks, to allow the reproduction of the nematodes introduced into the wood, thus increasing the probability of extracting them. The modified Baermann technique (Southey 1986) was used to extract *B. xylophilus* from the wood. The bark feeding area on the branches was measured weekly with a digital planimeter PLANIX 7P (Tamaya Digital Planimeter) for half of the insects (seven males and seven females).

Four dead *P. pinaster* trees from the same location which were found to be *B. xylophilus*-free were cut and divided into small logs, which were kept under ambient temperature in netted boxes. Emerging *M. galloprovincialis* (14 males and 14 females, totalling 28) were maintained individually in acrylic boxes with pine branches as described above, acting as a control group. All the adult insects were measured (length of the right elytra) and weighed upon emergence, being macerated in a Petri dish with water after their natural death in order to count the nematodes remaining in their bodies.

6.6.4 Transmission of B. xylophilus during oviposition by M. galloprovincialis

6.6.4.1 Nematode transmission under laboratory conditions

Adult maritime pine trees infested with *B. xylophilus* and *M. galloprovincialis* were cut in January 2004 near Comporta. Recently-emerged beetles were immediately checked for the presence of *B. xylophilus* using the methodology described by Zhang et al. (1995). Fifteen female beetles found to be infested with PWN were placed individually in transparent acrylic boxes to which were added two adult males of similar size and age. Every two days, the insects were given fresh maritime pine branches for feeding (15cm long, 0.5-1cm diameter) and some pine bolts (10cm long, 4-5cm diameter) for oviposition. When they reached 30 days of age the males were removed from the boxes and each female was given a single *P. pinaster* bolt (10cm long, 4-5cm diameter) for oviposition. Each bolt remained with the female for 24h, after which it was replaced by a similar one, totalling four replicates (bolts) per beetle. After the removal of the last bolt, the females were measured (length of the right elytra), weighed and macerated in a Petri dish with water, counting the number of nematodes present in the water after 12 hours.

Oviposition wounds on bolts were counted and debarked, to determinate if they contained eggs. If present, feeding scars on the bark were counted and removed with a vertical electric saw, along with a piece (2-3cm) of adjacent wood, in order to prevent the contamination of the wood with nematodes potentially introduced during the female's feeding activity. The pine bolts were then stored in closed plastic bags at 25ºC ± 1ºC and 75% RH for two weeks, to allow the reproduction of the nematodes introduced in the wood and to increase the probability of extracting them, which was made using the modified Baermann technique (Southey 1986). Direct counts were made for samples lower that 500 nematodes, while for higher nematode values a counting grid with 1ml of the sample was used, replicating the count five times and extrapolating the mean of the five replicas to the initial sample volume.

6.6.4.2 Nematode transmission under field conditions

Maritime pine trap-trees were set inside Tróia and Comp. das Lezírias inside the PWN affected zone. Each trap consisted of an apparently healthy adult *P. pinaster* tree (mean 18cm breast height diameter) debarked at waist height, removing part of the cambium and xylem wood to completely stop the resin flow. The objective was to slowly debilitate and eventually kill the tree, thus turning it into a highly attractive host for oviposition by the local *Monochamus* population. An initial sample of wood from each tree was taken at breast height and analysed in the laboratory using the modified Baermann technique to ensure that the pines were nematode-free. The trap-trees were created between May and September of 2001 (two trap-trees for each month and location) and 2002 (three trap-trees for each month and location), in a total of 50 trees for both years. In November of each year the trees were cut, divided in one meter logs and taken to the laboratory, where they were analysed (trunk and branches) for the presence of *M. galloprovincialis* oviposition and feeding scars. Two wood discs were collected at breast height and mid-crown from each tree and analysed for the presence of *B. xylophilus* using the modified Baermann technique. Parametric and non-parametric statistical analysis was performed as considered adequate.

6.7 *Results*

6.7.1 Association of the pine wood nematode with the immature stages of Monochamus galloprovincialis

Pinewood nematodes were recovered from 21 out of 124 (11%) *M. galloprovincialis* larvae, with a mean of only 2.0 ± 0.7 (mean ± SD) individuals per larva. Practically all the nematodes were of the JIII stage, the majority of which were dead. Live nematodes were found associated with the insect's pupae (17% infested, mainly with JIV stage) and, much more frequently, with callow adults (91% infested, mainly with JIV stage) (Table 25). Like the frequency of infestation, the mean number of nematodes was also significantly higher on callow adults than on larvae or pupae (χ^2 test).

Table 25: Number (represented as "n") of *M. galloprovincialis* larvae, pupae and callow adults observed, infested with pinewood nematode (PWN), number of PWN present on the insect's body and dominant PWN life-stage. The May, June and July observations consist on the aggregation of two samples from each month. When applicable, values are presented as means ± standard deviation

	***M. galloprovincialis* life-stage in galleries/chambers**											
	Larvae				**Pupae**				**Callow adults**			
Month	n	infested with PWN	PWN counted	PWN life-stage	n	infested with PWN	PWN counted	PWN life-stage	n	infested with PWN	PWN counted	PWN life-stage
Jan.	37	1	3	JIII	-	-	-	-	-	-	-	-
Feb.	51	9	1.3±0.7	JIII	-	-	-	-	-	-	-	-
March	27	5	1.6±0.5	JIII	-	-	-	-	-	-	-	-
April	47	6	2±1.5	JIII	-	-	-	-	-	-	-	-
May	21	0	-	-	4	1	2	JIV	-	-	-	-
June	5	0	-	-	15	0	-	-	10	10	1951±3812	JIV
July	1	0	-	-	16	5	6.8±9.2	JIV	12	10	227±252	JIV
Total (%)	194	21 (11)	2±0.7	JIII	35	6 (17)	4.4±3.4	JIV	22	20 (91)	1089±1219	JIV

6.7.2 Pinewood nematode distribution within M. galloprovincialis body

All the 40 beetles analysed carried pinewood nematodes within their bodies, with no correlation between body size and nematode load (r^2 = 0.16, P = 0.335). The number of nematodes carried by the insects varied between a minimum of 12 and a maximum of 32 8, with a mean of 3980 ± 1055 (mean ± SE), without sex difference (F = 0.346, d.f. = 1, p= 0.560) (Table 26).

Table 26: Nematode load of two and 30-day-old male and female *M. galloprovincialis* beetles. Mean ± SE; n = 10 insects of each sex and age class

2-day old insects		30-day old insects	
m	f	m	f
7889 ± 3386	4830 ± 2062	1324 ± 370	1878 ± 634

All the insect body sections were found to carry pinewood nematodes. The vast majority were isolated from the thoracic segments of both sexes, with significantly higher abundance on the meta-thorax, both for the two-day (F = 7.33, d.f. = 8, p= 0.001) and 30-day-old insects (F = 11.71, d.f. = 8, p= 0.001). Conversely, the body segments containing less nematodes were the antennae, legs, wings and elytra (Table 27).

Table 27: Number of *B. xylophilus* found on nine body segments of two and 30-day-old adult *M. galloprovincialis*. Mean ± SE; n = 20 insects of each age class

		number of days post-emergence	
	Body segment	Two days [a]	30 days [a]
Thorax	Antenna	61 ± 41 a	6 ± 3 a
	Head	136 ± 67 a	36 ± 21 a
	Elytra	160 ± 142 a	4 ± 3 a
	Wings	68 ± 63 a	14 ± 9 a
	Legs	173 ± 82 a	29 ± 20 a
	Pro-thorax	208 ± 80 a	32 ± 12 a
	Meso-thorax	882 ± 293 a	373 ± 155 a
	Meta-thorax	4540 ± 1573 b	1070 ± 267 b
	Abdomen	131 ± 99 a	62 ± 14 a

[a] Means within each column followed by the same letter do not differ, Tukey HSD test, P ≤ 0.05.

Although a higher number of *B. xylophilus* were found on the recently-emerged two-day-old insects rather than on the 30-day-old beetles (F = 5.695, d.f. = 1, p= 0.022), nematode abundance among body segments did not differ between the two age classes studied (Mann-Whitney *U* tests, $p \leq 0.05$).

6.7.3 Transmission of B. xylophilus through the maturation feeding of M. galloprovincialis

Females consumed a larger amount of branch bark than males (F = 25.11, df= 1, p<0.001), with no significant differences among over time (F= 0.86, df= 23, p= 0.649). No unfertilized eggs were laid by the females, and as so, for these females, all nematode transmission is assumed to have occurred via feeding activity, and not oviposition. No correlation was found between feeding intensity (area of bark fed) and the effective transmission of *B. xylophilus* to the branches (r = -0.12, $p \leq 0.05$) and number of nematodes recovered from the branches (r = 0.06, $p \leq 0.05$).

All the 28 *B. xylophilus* infested beetles transmitted nematodes to branches on which they fed, while no nematodes were recovered from the branches fed by the PWN-free insects (control group). Each insect infested between two and 12 branches with the nematode, with a mean of five (Figure 59, Table 30), independently of the beetle's sex (Mann-Whitney *U* test, p= 0.562) or longevity (Mann-Whitney *U* test, p= 0.782).

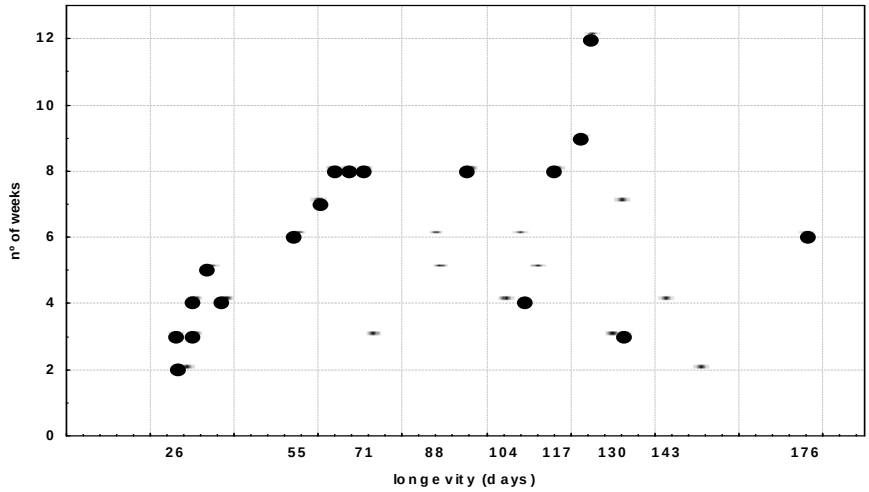


Figure 59: Number of weeks adult *M. galloprovincialis* (n= 28) successfully transmitted *B. xylophilus* to *P. pinaster* branches and insect longevity (days). Closed circles represent insects found to carry *B. xylophilus* after natural death

Table 28: *M. galloprovincialis* size, weight and longevity, weekly bark area consumed, number of weeks with successful *B. xylophilus* (PWN) transmission, number of PWN on branches and PWN remaining on the bodies of both sexes of infested (n=28) and non-infested (n=28) insects

Insects	sex	Size (mm) †	weight (g) †	Longevity (days) †	bark area eaten per week (cm2) ††	weeks with successful PWN transmission †††	No. of PWN recovered from branches ††	No. of PWN remaining on beetles †††
PWN infested	m	13.7±0.3ab	0.33±0.03a	92±12a	17.1±1.0 a	5.3±0.7a	22352 ±6979a	136±74a
	f	15.7±0.4c	0.43±0.03b	84±12a	26.5±1.4 b	5.9±0.7a	28766 ±7956a	486±349a
PWN non infested	m	12.8±0.5a	0.27±0.03a	103±10a	-	-	-	-
	f	14.5±0.2b	0.32±0.02a	105±11a	-	-	-	-

Means within each column followed by the same letter did not differ ($p \leq 0.05$) according to:
† Post-hoc Pairwise means comparison after Kruskal-Wallis analysis of variance test.
†† Fisher LSD test after ANOVA.
††† Mann-Whitney *U* test.

The transmission frequency changed over time (F = 1.90, df= 27, p= 0.009), being more frequent during the first six weeks after emergence, when two transmission peaks occurred (Figure 60). After that initial period the nematode transmission quickly diminished, practically ceasing after the ninth week, even in the insects which were found later to still carry *B. xylophilus* in their bodies. In fact, after the natural death of the insects, it was found that 64% of them (18 out of 28) still contained nematodes (mean of 311 ±178 PWN), without differences between male and female beetles (Mann-Whitney *U* test, p= 0.783).

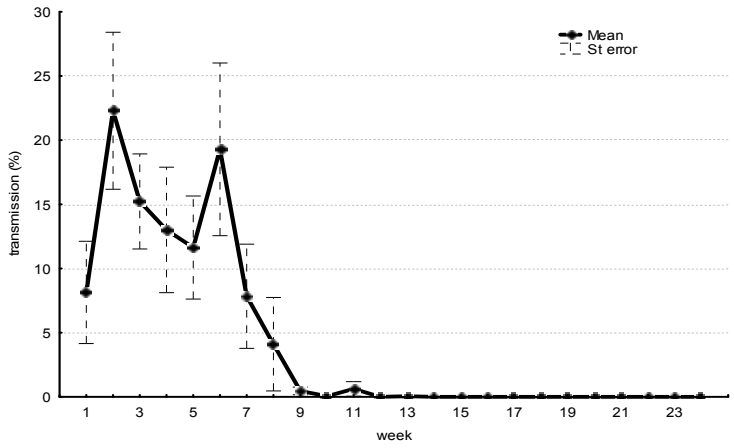


Figure 60: Weekly transmission of *B. xylophilus* to *P. pinaster* branches (% of the total infested branches) by *M. galloprovincialis*

The number of nematodes recovered from the infested branches varied between 1 and 324,300, with a mean of 25,813 ± 5,349 nematodes/branch, without differences between branches fed upon by both sexes (F= 0.36, df.=1, p= 0.552). The longevity of the nematode-free insects (control group) was slightly higher than of the *B. xylophilus* infested group (Table 4), although the difference was not significant (Kruskal-Wallis test, χ^2=2.50, df=3, p=0.475).

6.7.4 Transmission of B. xylophilus through the oviposition of M. galloprovincialis

6.7.4.1 Nematode transmission under laboratory conditions:

Of the 60 bolts analysed, 46 (77% of the total) contained a total of 124 *M. galloprovincialis* oviposition wounds, of which 66% had eggs. Despite the presence of small pine branches adequate for maturation feeding, 38 % of the bolts intended for oviposition also had maturation feeding scars (Table 29). Only 37% of the bolts contained nematodes and usually with low values, as the mean was lower than 300 individuals per bolt (Table 29). *B. xylophilus* was the only nematode present on the wood.

Table 29: Mean number of *M. galloprovincialis*' feeding and oviposition wounds per bolt and mean number of *B. xylophilus* recovered from the bolts. n = 60 bolts. Mean values ± S.E.

feeding wounds	oviposition wounds		number of PWN
	without eggs	with eggs	
0.78 ± 0.15	0.70 ± 0.19	1.37 ± 0.22	290 ± 70

No relation was found between *B. xylophilus* abundance in wood and the number of insect feeding wounds (r = 0.208, p = 0.110) or oviposition wounds without eggs (r = -0.118, p = 0.371). There was a modest (but significant) correlation between the number of oviposition wounds with eggs and nematode abundance (r = 0.575, p<0.001).

All the dissected *Monochamus* females were found to carry *B. xylophilus* within their bodies, with a mean value of 1684 ± 516 (mean ± *S.E.*) nematodes per insect. The mean length of the 15 female beetles was 15.2 ± 0.4mm, with a weight of 0.3868 ± 0.023g (mean values ± *S.E.*), and it was found that the smaller beetles carried a larger number of nematodes than the larger ones, as modest negative correlations were found between the nematode load and beetle size (r = -0.432, p = 0.108) and weight (r = -0.566, p = 0.028), being the second significant correlation. No relation

was found between the insect's nematode load and PWN abundance on wood (r = 0.079, p = 0.547).

6.7.4.2 Nematode transmission under field conditions

All the wood samples collected initially from the pines tested negative, and thus the selected trees were considered to be nematode-free at the date of trap-tree creation. Over the two years, 29 of the 50 pines (58%) were colonised by *M. galloprovincialis*, with a total of 439 oviposition wounds detected (Table 30).

Despite the abundant presence of *Monochamus* reproductive activity on the majority of the pines, only four of the trees with sawyer presence were found containing *B. xylophilus*, with a mean of 371 nematodes per 100g of wood. The pine wood nematode was the only species of the *Bursaphelenchus* genus present on the wood, although other Aphelenchoididae were also found on some trees.

Table 30: Number of trap-trees colonised by *M. galloprovincialis* (*M. g.*) at two locations (Lezírias and Tróia), total number of *M. g.* oviposition wounds on the trees, number of trees infested with *B. xylophilus* and mean number of PWN per 100g of wood

date (month/ year)	Lezírias Trees colonised by *M. g.*	*M. g.* oviposition wounds	Trees with PWN	PWN /100*g* wood	Tróia Trees colonised by *M. g.*	*M. g.* oviposition wounds	Trees with PWN	PWN /100*g* wood
May/01†	2	41	0	-	2	120	0	-
May/02††	1	27	0	-	1	5	1	116
June/01	2	54	1	833	1	8	1	477
June/02	2	6	0	-	2	11	0	-
July/01	1	31	0	-	1	9	0	-
July/02	3	19	0	-	2	2	0	-
Aug/01	2	10	0	-	2	40	0	-
Aug/02	0	-	0	-	0	-	0	-
Sept/01	2	22	0	-	0	-	0	-
Sept/02	2	32	0	-	1	2	1	56
Total:	17	242	1	-	12	197	3	-

† two trap-trees on each month and location during 2001;
†† three trap-trees on each month and location during 2002.

6.8 *PWN and Insect Vector Host Distribution in Host Trees*

Although *Monochamus galloprovincialis* is the only vector of *Bursaphelenchus xylophilus* identified in Portugal, other possible infestation pathways must be assessed (i.e. pine roots contact and older pine branches natural decay) to exclude the possibility of overlooking an important reservoir of beetles that is not recognised within the eradication procedures carried out every year. Outside the affected zone and before the arrival of the pinewood nematode, *B. xylophilus,* the cerambycid *Monochamus galloprovincialis* was present, but at low densities, throughout Portugal, mainly on dying pines and decaying branches due to windstorms or natural branch feeling.

6.9 *Material and Methods*

6.9.1 Development of pine wood nematode in wood

Six adult maritime pine trees (*Pinus pinaster*) infested with *B. xylophilus* (the only *Bursaphelenchus* present on the wood) and with life stages of *M. galloprovincialis* present were felled, divided in one metre logs, and kept under ambient conditions (temperature and humidity) from January 2003 in Troía, Portugal. From January to July a random section of approximately 10cm of wood was cut

from each of the six trees and taken to the EFN laboratories at Oeiras, where it was divided in small pieces to extract nematodes using the modified Baermann technique (Southey, 1986). The number of nematodes was counted and the life-stages present identified morphologically.

6.9.2 PWN infested and non-infested trees and insect colonization

All dead pines from two Experimental Plots at Tróia Peninsula and Companhia das Lezírias, in areas bounded by a 55 m square shape, each containing about 200 pine trees were felled for assessment of mortality factors and insect colonization. After recording the spatial position of the trees, all wood from the selected trees were assessed for vector and nematode populations and a series of dendrometric measures were taken.

During the PWN eradication campaign at Tróia, several dead trees were surveyed and trunks and branches colonized by *M. galloprovincialis* were stored in plastic baskets covered by strong cloth to avoid adult escapes. After each emergence season, the logs that had *M. galloprovincialis* adult emergence were analysed and measured (size and diameter).

6.9.3 PWN pine root infection and insect colonization

To assess PWN presence and distribution in maritime pines stumps and roots, a recently dead infested pine was selected at the Tróia pine site and the root was completely dug out and surveyed. Root depth, length and thickness was measured and about 60 root samples were taken and nematodes extracted by the Baermann Funnel method. Insect colonized roots were marked and larvae collected and brought to the laboratory to complete their development and emergent adults were identified to species. The health of all nearby pines were monitored and at the season end wood samples were collected from dying pines for nematode survey.

6.9.4 Dead branches colonization by PWN vector

To assess the potential of *M. galloprovincialis* completing its life cycle on dying branches *in situ* on pine trees, three surveys were established at Tróia and Comporta pine stands where PWD is well established and under eradication procedures. Each survey consisted on a 50 metre straight walk inside the pine stands followed by a U turn and a parallel 50 metre straight return, 50 metres apart. Using binoculars, dying branches of all pines along the straight line and within 10 metres side to side distance were carefully surveyed for holes. All branches with holes were cut and opened to identify the causal agent involved.

6.10 *Results*

6.10.1 Development of pine wood nematode in wood

Pine wood nematodes were present in all wood samples taken periodically from the six trees, with the exception of three July samples, which contained no nematodes. Mean nematode abundance was slightly higher during the first three months of the year, but it did not differ between months, except for July (Kruskal-Wallis test $\chi^2 = 18.89$, df = 6, p = 0.004).

Throughout the months, practically all nematodes found on wood were third stage dispersal juveniles (JIII), which were also the dominant life stage on the wood surrounding the insect larval galleries (January-July) and pupal chambers with pupae present (May-July). By contrast, JIV larvae were the most abundant life stages on the wood surrounding the pupal chambers with callow adult insects (June and July).

6.10.2 Insect colonization of PWN infested and non-infested trees

From the 48 dead trees surveyed, 7 bark beetle species, 3 cerambycid species and one curculionid (*Pissodes castaneus*) were found. Some buprestid larvae were also found but the

species were not identified. The bark beetle *Orthotomicus erosus* was the most abundant and frequent, both in branches (54%) and under the bark of the trunks (96%). This was the case and with equal frequencies at both experimental plots – Tróia and Companhia das Lezírias. Another bark beetle, *Pityogenes bidentatus* was also very frequent, but only attacking the branches, and was found in half the surveyed trees. In the trunks of attacked trees, 3 cerambycid species were found in most of the dead trees at both plots:

Lower trunk: *Arhopalus syriacus* (83% of the trees)
Middle height trunk: *Acanthocinus griseus* (81%)
Upper trunk: *Monochamus galloprovincialis* (58%).

The PWN vector *M. galloprovincialis* was also found in the branches in 19% of the trees, mainly in infested trees at Tróia (29%), probably reflecting the fact that they were on the larger trees killed that had thicker branches.

All beetle species were found in PWN-infested and non-infested dead trees, but since the infested tree were the larger trees (with one exception at Tróia) the differences found were related to bark characteristics and tree height. Consequently, cerambycids were less abundant in the non-infested trees and the most interesting difference was the abundance of the aggressive bark beetle (*Tomicus* spp.) in the non-infested trees (29% against 10% on PWN trees), and this species could be a contributor to the trees' death.

From sampled trees, only the upper part of the tree revealed the presence of the known nematode vector *M. galloprovincialis*, with major occurrences on the trunk at the level of the commencement of the lower canopy (present in almost every infested dead tree trunk section) (Figure 61). The trunk diameter at the *M. galloprovincialis* colonization varied between 15 cm, with around 1 cm bark thickness, and less than 5 cm with bark thinner than 0,5 cm. These results agree with surveys results previously described.

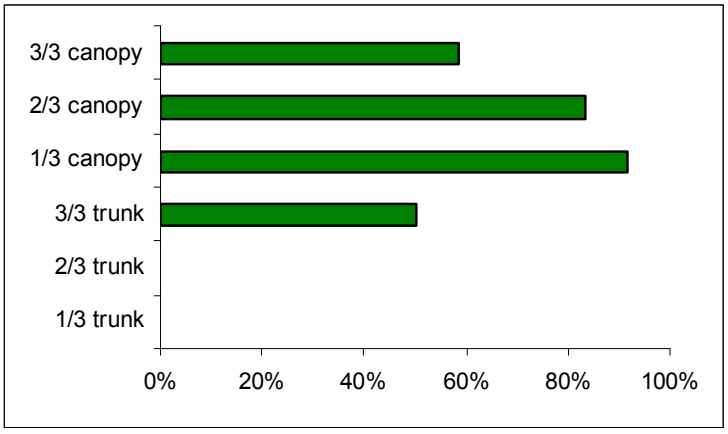


Figure 61: Vertical distribution of *M. galloprovincialis* along the height of infested trees

6.10.3 PWN pine root infection and insect colonization

The stumps and the thicker parts of the main roots were heavily colonized by *Arhopalus syriacus* and *Spondylis buprestoides* (L.), two cerambycid species captured very commonly in the traps set for *M. galloprovincialis.*

The roots of the pine trees spread through the stand, crossing other trees' roots reaching an overall length 122 metres, having the longer root 21 metres (root H) and 43 metres (root E) (Figure 62). Sampling for *B. xylophilus* confirmed its presence in all roots, in patches along the roots and in variable densities reaching a maximum of 282 PWN/10 g of wood in the middle portion of root A.

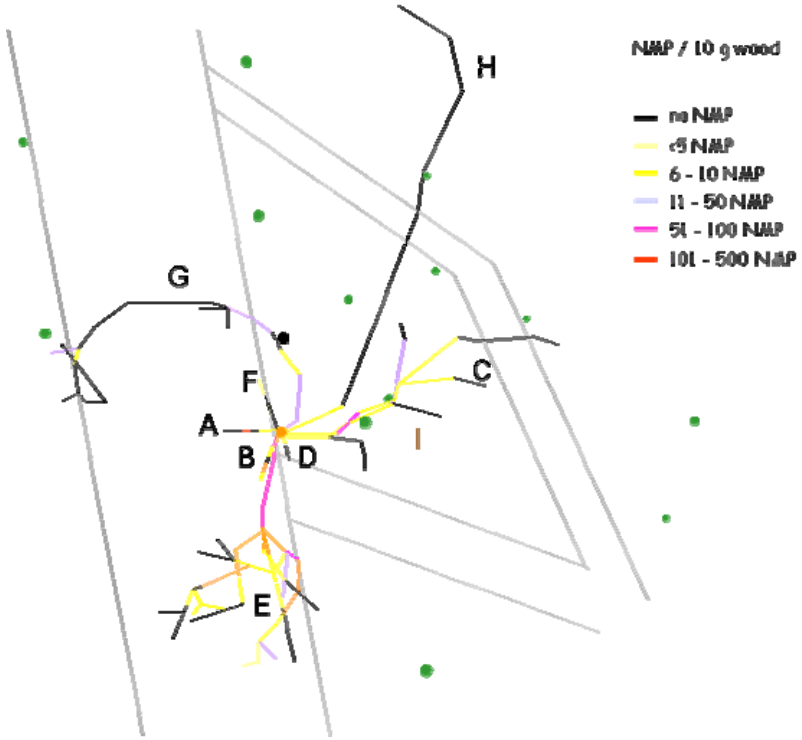


Figure 62: Pine root surveyed. Colours are related to quantity of *Bursaphelenchus xylophilus* infestation. Green circles are other pine trees.

The trees in the surroundings were all healthy (all produced a normal resin flow and had no visual symptoms) when the roots were extracted. During the winter of 2004 one tree was dead but the wood samples taken were all free of PWN. The tree died probably because the roots might had been too disturbed, although the sand was replaced after the surveyed root was removed, or as a result of processionary moth defoliation and *O. erosus* attack, since it was a rather small tree.

3.4 – Natural pine dead branches colonization by PWN vector

A total of 883 branches from 277 trees were observed, of which 26 (3%) contained round shaped similar to those made by emerging *Monochamus* adults. The analysis of the branches with holes indicated that, except for one hole, made by *Monochamus*, all the others had been made by carpenter bees of the *Xylocopa* genus (Hym.; Anthophoridae) Figure 63).

The carpenter bees entrance hole is approximately 15 mm in diameter, being perfectly circular and very similar to the *Monochamus* emergence hole. The bees dig tunnels inside the wood that extend for 30 cm or more, being divided into a series of cells by partitions which are made from a mixture of saliva and sawdust.

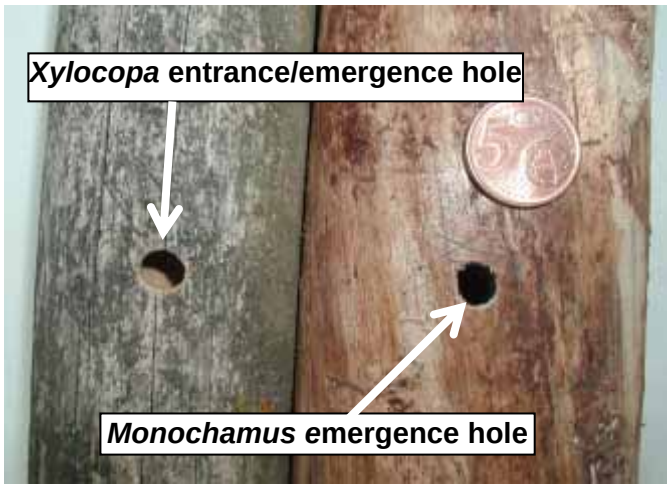


Figure 63: Carpenter bee (*Xylocopa* spp.) entrance hole and *Monochamus galloprovincialis* emergence hole

6.11 *Discussion*

The pine sawyer *M. galloprovincialis* was found to have a univoltine life cycle in Portugal as no adults emerged during the same year of oviposition. The newly-hatched young larvae had an extremely fast development during the summer months, although when they reached maturity and tunnelled into the wood their development greatly diminished or even ceased, resulting in the majority of the population over-wintering as last-instar larvae in galleries inside the wood. The eggs laid in September were the exception and the insects over-wintered as young larvae in sub-cortical galleries, with their development probably delayed due to lowering autumn temperatures.

Adult emergences occurred eleven to fourteen months after oviposition in the field. Even with the yearly natural fluctuations caused by temperature differences, the general emergence pattern was very regular during the four years and consistently more intense in July, despite the long duration (of up to three months) of the emergence period. The sex ratio of 0.48 is similar to values reported by Francardi & Pennacchio (1996) for the same species (0.50), while for *M. alternatus* a ratio of 0.49 has been reported by Togashi & Magira (1981).

A minor proportion of the insect population, corresponding to 4-5% of the individuals did not emerge after one year as most insects did, and apparently would develop through a two-year life cycle. A two-year life cycle is a normal development pattern for *M. galloprovincialis* populations from cooler regions of central and northern Europe and according to Tomminen (1993) the pattern observed in southern Finland is almost the inverse of Portugal, with 90% of the insects taking two years to develop and only 10% of the beetles having a one-year generation.

Mortality was generally low for all developmental instars, ranging from a maximum of 26% for the mature larvae inside the wood to a minimum of 0.8% for the pupae. Overall, the most important mortality-inducing agents identified were fungi, namely *B. bassiana* and parasitism by three generalist hymenopteran species. The implementation of a biological control programme focused on the vector would be most welcome, but the possibility of using local parasitoids may be difficult as the three species found in the region seem to cause limited mortality among beetle populations, and there is no obvious method to enhance their action.

Mortality due to interspecific competition was not observed and is probably negligible to the pine sawyer as its larvae are always bigger and more aggressive than neighbouring bark-beetle and buprestid larvae, while the similar-sized and equally aggressive *A. syriacus* offspring are usually found lower on the tree as they colonise trunk sections with larger diameters (Sousa et al., 2000). Furthermore, the *M. galloprovincialis* young larvae appear to be quite resistant to high temperatures, as ambient temperatures of 38ºC (which could have been higher on the sun-exposed logs) apparently did not harm them.

More than half of the larval mortality was due to unidentified causes and/or missing larvae. Other non-surveyed pathogenic micro-organisms like yeasts, bacteria and nematodes might be responsible for the unexplained larval mortality detected, while intraspecific cannibalism and unidentified predators could justify the empty larval galleries. By combining the mortality of the various developmental instars a within log generation survivorship from egg to adult of 53% was calculated, which can be considered a high value and may be one of the reasons why pine wilt disease easily reaches epidemic levels if uncontrolled and helps to explain the high impact of the disease inside the nematode affected zone in Portugal.

Regarding the host preference of the pine sawyer, four of the pines studied (*Pinus sylvestris, P. halepensis, P. pinaster* and *P. pinea*) were all suitable for maturation feeding by *M. galloprovincialis* in the laboratory, and as so may be considered to have some susceptibility to became infected with *B. xylophilus* in the presence of nematode-carrying beetles. Of the four species, only *P. sylvestris* does not occur in the pine wilt disease affected area, but nevertheless until now maritime pine has been the only tree ever found infected with *B. xylophilus* in the terrain. This suggests that other factors, like the resistance of the tree to the nematode, may be

responsible, besides vector preference, for the success of *B. xylophilus* inoculation and development in new hosts.

The low number of eggs laid and the absence of beetle emergence from *P. pinea* and *Pseudotsuga menziesii* indicates that these species are not adequate hosts for *M. galloprovincialis*, similarly to *C. lusitanica* on which no egg was laid. The absence of differences in adult emergence from *P. halepensis, P. pinaster, P. radiata,* and *P. sylvestris* demonstrates that all can be adequate hosts. Overall, *P. sylvestris* had systematically the highest values for feeding and oviposition preference. Scots pine is considered highly susceptible to *B. xylophilus* (Evans et al., 1996) but is absent from the PWN affected zone in Portugal. The nearest plantations are more than 200 km away, so the experimental beetle population has never been in contact with *P. sylvestris*. The characteristic thinner bark of this pine (compared to *P. pinaster*, *P. pinea,* and *P. halepensis*) may have favoured its acceptance and colonisation by the insects, as there is a negative correlation between the bark thickness and the density of *M. galloprovincialis* (Francardi et al*.*, 1998; Sousa et al., 2000). Future studies should identify the volatile semiochemicals that mediate host selection by *M. galloprovincialis*, as those attractants could be used to improve catches in flight traps employed to monitor and suppress *M. galloprovincialis* populations.

Regarding the reproductive behaviour of the insects, almost one quarter of the adults died before starting to reproduce. The high initial mortality could be related to the effort necessary for emergence, as the mortality affected mainly smaller insects, which may have been less vigorous, and in weaker condition following the exertion of emergence. Due to this significant early mortality, the average longevity of the population was 63 days, whereas if these are excluded, average longevity increases to 78 days. Both these values are close to the extremes of the range of 65-80 days described by Hellrigl (1971) for this same species of *Monochamus.*

The mean number of eggs laid by the females (67) is within the 45-87 range reported by Hellrigl (1971), being much greater than the 37 eggs reported by Francardi and Pennacchio (1996), although these authors only studied 10 couples. Overall, the *M. galloprovincialis* population studied exhibited high reproductive diversity, with different groups of either small-sized insects that died before starting to reproduce (22 % of the total population) and other groups of reproducing insects separated by fecundity, longevity and morphological parameters. The large individual variation in the longevity and fecundity of *M. galloprovincialis* seems also to occur in other *Monochamus* species, namely *M. saltuarius* (Jikumaru *et al*., 1994) and *M. carolinensis* (Walsh & Linit, 1985).

Overall, 60% of the population belonged to one of two very distinct groups: either small sized, low-longevity, non-reproducing beetles or large-sized, high longevity and high fecundity insects. The extrapolation of these results to field conditions should be done with some caution, as the beetles were kept under controlled laboratory conditions in low densities (grouped by pairs only), with regular food supply and a constant and adequate environment without predators or other natural enemies, a combination of factors which rarely, if ever, are met under natural conditions. The presence of *B. xylophilus* in the insect's body, for instance, is a factor that can negatively influence *Monochamus* spp. population parameters, namely longevity and/or fecundity (Togashi & Sekizuka, 1982; Akbulut & Linit, 1999), and so is the ambient temperature (Jikumaru & Togashi, 2000). The same applies to fecundity and oviposition parameters of *M. saltuarius,* which are significantly higher at 25ºC than at 20ºC (Nakayama *et al.*, 1998). Similar future studies should be done using beetles infected with different *B. xylophilus* loads and at different ambient temperatures, in order to evaluate the influence of the pine wood nematode on *M. galloprovincialis* biological parameters.

Although *B. xylophilus* was recovered from all immature stages of *M. galloprovincialis* surveyed, their association with larvae and pupae of the beetle appears to be irregular and incidental, as its scarcity and low frequency confirm. These results agree with Bolla et al. (1989), which states that *M. carolinensis* larvae contain protein extracts exerting a repellent nature against PWN, a mechanism to prevent premature invasion by the nematode. In contrast, high nematode loads and infestation frequencies were found on the callow adults. The association of the JIV *B. xylophilus* with the callow stage of the *Monochamus* beetles has also been reported from North America and Japan (e.g., Mamiya, 1984; Warren & Linit, 1993).

The distribution of the nematodes in the body of the beetle vector appears not to be conditioned by its sex or age. Also, *B. xylophilus* did not made significant movements between body segments of the adult beetle, as there were no differences in the distribution patterns between the two and 30-day-old insects. The lower nematode load found on the 30-day-old beetles could be caused by the departure of nematodes that occurred through the feeding activity of the insects, as PWN transmission into pine branches reached its peak during the second week after emergence, continuing to be frequent in the following weeks, which could explain the decrease through time in the number of nematodes carried by the beetles.

B. xylophilus aggregated mainly in the thoracic segments of both sexes of *M. galloprovincialis*, especially on the meta-thorax, where large respiratory spiracles exist. These results corroborate similar studies performed on other *Monochamus* vectors, like *M. carolinensis* (North America) and *M. alternatus* (Japan), where the PWN was also found most abundantly in the thoracic region, mainly in the tracheae arising from the metathoracic spiracles (Mamiya, 1972; Wingfield & Blanchette, 1983; Linit, 1989; Linit et al., 1983).

After becoming associated with their vector insect, the nematodes need to be transmitted to suitable hosts in order to continue with their life cycle. Nematode transmission through the feeding activity of the insects was proved under laboratory conditions, as all the individuals infested with *B. xylophilus* successfully transmitted nematodes to pine branches, the majority for several consecutive weeks. The infested branches were found to contain very high numbers of nematodes (mean higher than 20,000 per branch), a clear indication of the success of *B. xylophilus* in colonising and reproducing inside the *P. pinaster* branches. The maintenance of the branches at very favourable conditions in order to maximize the probability of *B. xylophilus* to establish and reproduce in the wood (25ºC for 15 days) did not allow the determination of the exact number of nematodes transmitted by the vector insects.

Although there was large variation in the number of branches that each insect infested with *B. xylophilus* (between two and 12), nematode transmission was clearly more intense during the insect's early adult life, reaching its higher peak during the second post-emergence week. The occurrence of a second transmission peak during the sixth week may reflect individual differences in transmission for beetles with different nematode loads. The overall temporal transmission pattern observed in this study (low immediately after beetle emergence, peaking rapidly during the second week and declining gradually after), is extremely similar to the patterns described for other important PWN vectors, namely *M. alternatus* in Japan (Togashi 1985; Shibata and Okuda 1989) and *M. carolinensis* in the United States (Linit 1989, 1990).

The diminishing and eventual cessation of nematode transmission six weeks after emergence, found even in the beetles still infested by *B. xylophilus*, may be caused by the incapacity of nematodes older than five weeks to leave their vector, because of body desiccation or depletion of energy reserves (Stamps and Linit 1998).

The larger amount of pine bark consumed by the female insects is probably a result of their larger size and weight and/or higher nutritional needs arising from the onset of vitellogenesis and the investment the females make in eggs and egg laying. Nevertheless, the beetle's sex (and feeding area) did not affect the nematode transmission efficiency.

The absence of significant differences between the longevity of the infested and non-infested beetles contrast with the clear deleterious effect that PWN exerts on the longevity of *M. alternatus* (Togashi & Sekizuka, 1982). The absence of a negative influence of PWN on *M. galloprovincialis* longevity may be a consequence of low beetle nematode load upon emergence, as the deleterious effect of *B. xylophilus* on *Monochamus* longevity is related to high nematode numbers (Togashi & Sekizuka, 1982).

Transmission of the PWN by the oviposition activity of the female beetles was also demonstrated under laboratory conditions. Some of the oviposition bolts also contained additional feeding

wounds and oviposition wounds without eggs, but bolt nematode abundance appears to be related to the existence of oviposition wounds with eggs. However, the transmission efficiency was rather low, as only 37% of the laboratory bolts and 14% of the field trap-trees became PWN infested, and usually with low nematode numbers (less than 100 nematodes per bolt for the laboratory experiment, despite the bolts having been kept under favourable conditions for *B. xylophilus* reproduction over 15 consecutive days). Concerning the field experiment, the percentage of trap-pines with *M. galloprovincialis* oviposition wounds (58%) and the total number of oviposition wounds detected (439) can be considered relatively low, and reflect a low abundance of *Monochamus* females reproducing in the two areas selected and/or an abundance of oviposition-suitable pine hosts, which spread the reproductive activity of the field insects. This might also explain the very low number of eggs laid in the September trap-trees, which probably reflect a decrease in the insect population during late summer in parallel with a significant increase in the number of naturally occurring decaying and dead pine trees suitable for oviposition.

It is not possible to determinate the number of insects that visited the trap-trees to lay eggs, or know their nematode infestation rate, but analysis of field collected beetles captured at both locations showed that usually about 70% of the field-captured insects were infested with *B. xylophilus* (Sousa, unpublished). As such, it is reasonable to assume that a significant proportion of the insects that visited the pine trees were infested by *B. xylophilus*, despite the fact that only four of the trees became contaminated by PWN. The possibility of additional nematode transmission occurring through the feeding activity of the beetles cannot be excluded, but it was impossible to evaluate as the trees had multiple bark-feeding scars made by different insect species (Cerambycidae, Curculionidae and Scolytinae), which cannot be discriminated to species.

As the *M. galloprovincialis* females only lay eggs on already dead or dying conifers, a large proportion of the *Monochamus* females from the *B. xylophilus* infested region in Setúbal peninsula laid their eggs on pines already contaminated by PWN. Even if the female insect lays eggs on nematode-free pines the transmission efficiency appears to be rather low, as demonstrated above. Our results suggest that in Portugal, as is the case in other areas of the world where *B. xylophilus* was introduced and is causing significant wilt expression (*e.g.* Mamiya 1984), and unlike PWN transmission through feeding activity, transmission of *B. xylophilus* through oviposition wounds is an infrequent and secondary inoculation pathway and probably constitutes a minor component of the pine wilt disease epidemiology. This contrasts with the situation in the natural range of *B. xylophilus* where wilt expression is rare and transmission by oviposition is the normal pattern (Wingfield *et al.* 1982).

Overall, the results reported in this chapter emphasize the urgency and necessity to study and develop efficient insect control procedures (traps, attractants, natural enemies, etc.) aimed at capturing and destroying the insect's immature stages or the recently-emerged adult beetles, before they initiate transmission of nematodes to healthy pine trees in the short period of a few weeks immediately after emergence. The results of this study also demonstrate that the *B. xylophilus-M. galloprovincialis* association occurring in Portugal is, in general, extremely similar to other well-studied *B. xylophilus-Monochamus* spp. interactions from North America and East Asia. Apparently, the abiotic and biotic stimuli that regulate and synchronize the nematode-vector phoresis maintain their efficiency and successfully exert their effect independently of the *Monochamus* vectors and pine hosts, in all regions where the two organisms are associated. As the eventual dispersal of this pathogen into new geographical areas in Europe cannot be excluded, the establishment of new functional nematode-vector interactions in other locations in Europe is possible based on the results of the successful *M. galloprovincialis-B. xylophilus* association occurring in Portugal and described in this study.

The dominant presence of the dispersal third-stage nematode juveniles on wood during the winter months reflects their adaptability to survive in adverse ambient conditions (Wingfield *et al.*, 1982; Mamiya, 1983; Evans *et al.*, 1996). In contrast the JIV larvae appeared late in the season and reached their highest abundance to coincide with the presence of callow insects, a prelude to the forthcoming association between the two organisms.

Although *B. xylophilus* were recovered from all immature stages of the pine sawyer surveyed, their association with *M. galloprovincialis*' larvae and pupae appears to be irregular and incidental, as its scarcity and low frequency confirm. These results agree with Bolla et al. (1989), which state that *M. carolinensis* larvae contain protein extracts exerting a repellent nature against PWN, a mechanism to prevent premature invasion by the nematode. In contrast, high nematode loads and infection frequencies were found on the callow adult. The association of the JIV *B. xylophilus* with the callow stage of the *Monochamus* beetles has also been reported from North America and Japan (e.g., Mamiya, 1984; Warren & Linit, 1993).

Observed cluster effects of the disease spreading could easily be explained if the new infection source remains inside the affected stands. This could happen due to inefficient removal of infested dead trees and branches during eradication procedures or by other ways of nematode entry to healthy trees.

The root survey showed nematode presence and insect larval activity by a range of insects. From insects found in the roots, *A. syriacus* is also very common in the lower part of the pine trunks, but *S. buprestoides* is never present in the main tree trunk which confirms its exclusivity for the stumps and roots reported by other authors (Martinelli, 1996). However, many specimens from these species were surveyed for nematodes and none had any *Bursaphelenchus*.

Since no root grafting was found and apparently is not possible for maritime pines (Mauge, 1987) and no insect vector was found on the roots, nematode exit and survival is very unlikely to happen. Roots and stumps may be considered as dead ends for spreading of pine wilt disease that could have arisen if nematode movement from tree to tree was demonstrated.

With the thorough eradication procedures that took place at the experimental plots, no infested beetles emerged within them, indicating that new infestations where caused by new immigrant vector adults that carried out maturation feeding in the crowns of some trees. Host selection at the edges of the clearing is related to differential susceptibility, with apparent preference for taller trees, either because those trees have larger visual stimuli or release stronger chemical clues.

These findings are of great importance in optimising eradication procedures because any infested material left behind will endanger a larger area than previously thought. Eradication near the border of an affected area must be very effective to prevent migratory flights that might spread the disease to adjacent non-infested areas, such as the buffer zone set up within the Prolunp eradication campaign in Portugal.

Chapter 7 *Bursaphelenchus xylophilus* dynamics in Portugal and development of control methods

7.1 Bursaphelenchus xylophilus *Dynamics in Portugal*

The behaviour of the nematode-carrying cerambycid beetle *Monochamus galloprovincialis* in pines stands, in relation to adult flight, host selection for maturation feeding and, later, for egg laying, drives the dynamics of nematode transmission leading to a typical disease cluster pattern.

Eradication procedures, concerned mainly with reducing or eliminating vector transmission, begins by marking dead and symptomatic trees during late autumn. Reliability on visual symptoms is sometimes influenced by subjective criteria. To improve eradication it is important to develop direct quantitative early detection methods, based on tree physiology changes soon after nematode infestation. Eradication success in avoiding the spread of pine wilt disease depends on understanding the interactions between the disease components: host tree, insect vector and nematode. The present study contributes to this in the context of the situation in Portugal.

7.2 *Materials and Methods*

7.2.1 Population estimates at Tróia

Adult *Monochamus* population estimates were gathered every year for the Tróia Peninsula Experimental Station, within the PWN affected zone. The estimates were made by the Mark-Release-Recapture techniques. Nematode-free insects produced under laboratory controlled conditions and a selection with different ages (from 4 days to 1 month) had their elytra marked with ink. Each year over 100 marked insects (107, 121 and 118) were released in August at the centre of different radius circles forming a grid of insect traps. Weekly captures were collected and marked and unmarked (wild) beetles counted.

Each test lasted 1 month (15 days less than the average beetle life span) to avoid overestimations due to death of marked beetles and the continuous arrival of newly emerged beetles from the wild population. Population estimates were made using the Petersen model or Lincoln Index (Southwood, 1978), adjusted as proposed by Fletcher *et al.* (1981) and Bailey (1951 *in* Begon, 1979) for low recaptures.

7.2.2 Temporal and spatial distribution

To follow the disease dynamics and to assess several early detection methods, two Experimental Plots with 55 m square shape, each containing about 200 pine trees, were established at Tróia Peninsula and Companhia das Lezírias in January 2001. The relative locations of all trees were established and dendrometric variables were measured. Early detection methods were tested during the flight period of the vector insect and yearly mortality was surveyed during the winter and early spring. Dead trees were felled for mortality factor assessment; wood was sampled for nematode detection and insect colonization surveyed.

7.3 *Early Detection Methods for presence of Bursaphelenchus xylophilus*

Several methods were tested to assess tree physiological and functional status alteration, resulting from *Bursaphelenchus xylophilus* nematode infestation.

7.3.1 Oleoresin flow observation with punch hole wound method

This study took place in 2001, from April to September on trees thicker than 15 cm DBH, making a 14 mm diameter punch hole through the bark to induce oleoresin flow. A plastic 10 ml tube was placed under the hole to collect oleoresin over a 24 hour period and trees were ranked on a four-point scale:
1 – Full flow (more than 10 ml);
2 – Half flow (less than 10 but more than 5 ml);
3 – Quarter flow (less than 5 ml);
4 – No flow.

7.3.2 Electric resistance measurements

This trial took place at the experimental plot in Tróia peninsula, during 2004, using electric conductivity Conditiometer (Mervit). measurements taken on all 300 trees of the experimental plots (160 trees at Companhia das Lezírias and 140 at Tróia Plot) twice a month, comprising 4 measurements per tree, according to the main cardinal directions and average values per tree were considered for statistical analysis.

Average electric conductivities of healthy trees were grouped in 3 categories:
Healthy trees under 15 cm DBH (Thin trees);
Healthy trees over 15 cm DBH;
Diseased trees during the whole flight season (1 at Tróia and 4 at Lezírias Plot).
Non-parametric statistics were used.

7.3.3 Sap flow measurements

In order to assess tree water status during the *Monochamus* flight period, which was equivalent to the potential nematode infestation period, sap flow (tree transpiration) was continuously monitored in 6 trees of Tróia experimental plot, surrounding killed trees from previous years.

Tree transpiration was evaluated by continuously measuring sap flow density (Granier thermal dissipation method) in a sample of trees surrounding infested trees. Sensors were inserted radially into the xylem (avoiding resin concentrations) and each sensor consisted of two probes. The upper probe was heated to constant power, using solar panels and regulating power supply units. The lower reference probe remained at trunk temperature. The copper-constantan thermocouples of both probes were connected together by the constantan wires in order to give their temperature difference directly. Sapflow density was calculated using the thermal difference between probes occurring at times of positive (ΔT) and zero flow ($\Delta Tmax$). Sensors were connected to a CR10X Campbell Scientific data logger and data recorded every 10 minutes.

Sound methods for cavitations detection

The electronic equipment from Fa LAAR consisted of a very sensitive microphone which was placed in a previously drilled hole in the stem of some nematode infested trees in the Tróia Plot and a digital receiver was used to record the noise signal produced by the beetle-larvae or by the breaking of the vessels in the wood because of PWN attack (cavitation). The method was tested and later applied to the same sample trees of the electric resistance measurements.

7.4 *Dispersal of* Monochamus galloprovincialis

An experimental square of 7 year old maritime pines was delimited, covering an area of 0.3127 ha including 237 maritime pines, at Companhia das Lezírias, where 178 nematode free marked *M. galloprovincialis* were released, with the aim of understanding its dispersal behaviour (Figure 64). The beetles were released in the middle of the plot in order to maximize the available distances for dispersal. The dimensions of the trees allowed visual surveys of the canopy and collection of beetles.

The trial took place on two different occasions:
1 – With recently formed adult beetles (up to 5 days old); 105 insects, on June 14th.
2 – With older adult beetles (9 to 12 days); 73 insects, on June 28 th.

After releasing the marked beetles the plot was visited several times (1, 3, 7, 14, 21 and 28 days) and all trees were carefully surveyed and shaken to dislodge and collect adult beetles. The marking codes of recaptured beetles were identified and they were replaced on the upper trunk of the same trees on which they were detected.

7.5 *Results*

7.5.1 Population estimates at Tróia

Marked beetles were recaptured only in 2001 and 2003 (2 in each year) and in both years 3 wild beetles were also captured, allowing the use of Petersen population estimation formulae. Based on this, *M. galloprovincialis* population density at the Tróia pine stands were 23 and 28 insects per hectare.

The very low recapture rates (1.9 and 1.7%), are quite normal for cerambycid tests (Loyttyniemi, 1980; Bonifácio, 1996), and the resulting estimates may be biased. The estimate should be considered for a narrow window of time to disregard migrations that may induce false results, mainly if recapture rates are over 20% (Wileyto, 1995).

7.5.2 Temporal and spatial distribution

The maritime pines inside the experimental plots at Tróia Peninsula and Companhia das Lezírias had different mortality patterns. At the Tróia Plot, after the first years with high PWN incidence, the last two years killed trees were smaller and no nematodes were found. The most probable reasons for these deaths were intense defoliation by the pine processionary moth (*Thaumetopoea pityocampa*) and scolytid, *Orthotomicus erosus* (in all trees at high densities) and *Tomicus* sp. (present in 4 trees), attacks (Table 31, Figure 64).

Table 31: Overall annual mortality rates at the Tróia experimental plot and association with the Pine Wood Nematode

	2000	**2001**	**2002**	**2003**	**2004**	**2005**
Mortality	5,7% (11)	8,2% (15)	6,5% (11)	8,3% (13)	0,7% (1)	3,5% (5)
PWN	4,1% (8)	5,5% (10)	4,8% (8)	3,8% (6)	0	0

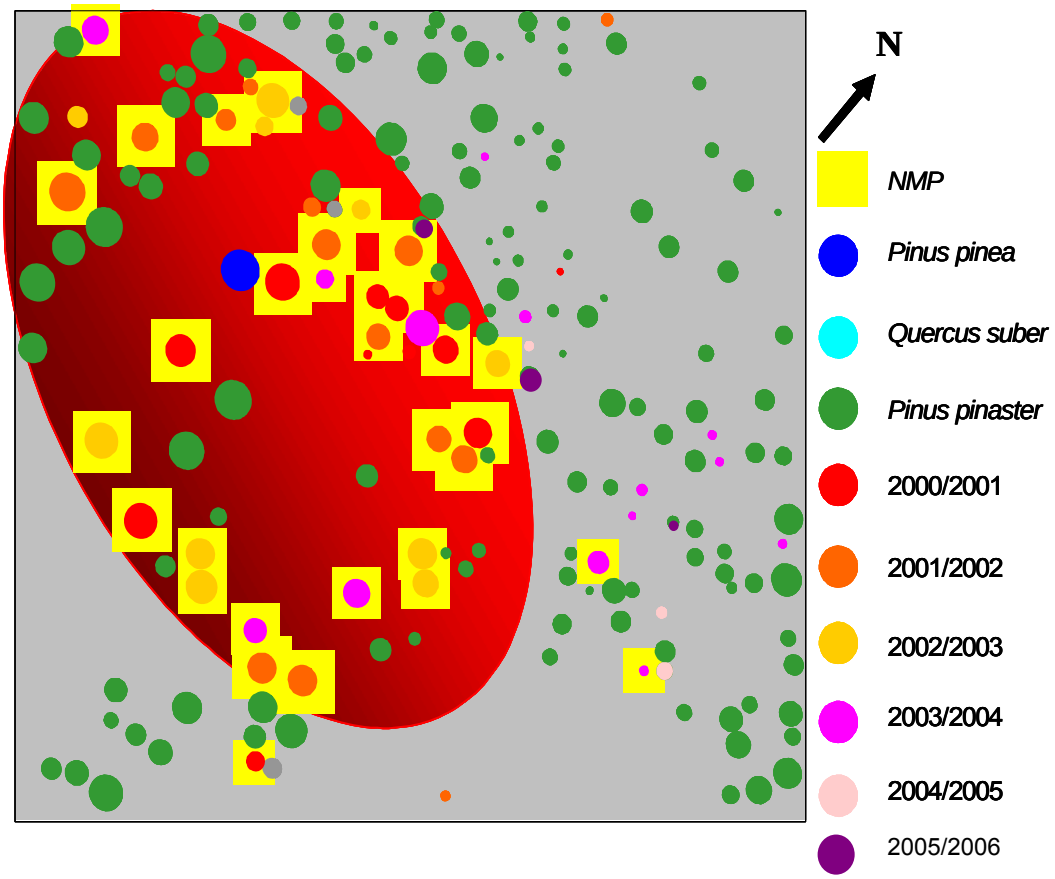


Figure 64: Pine mortality at Tróia plot. Yellow background indicates PWN infested trees

At Companhia das Lezírias Plot, tree death was very stable along all the years and always related with PWN (Table 32, Figure 65).

Table 32: Overall annual mortality evolution at Companhia das Lezírias experimental plot and associated with the Pine Wood Nematode

	2000	2001	2002	2003	2004	2005
Mortality	4,0% (8)	1,0% (2)	1,6% (3)	2,2% (4)	2,2% (4)	1,7% (3)
PWN	2,5% (5)	1,0% (2)	1,6% (3)	2,2% (4)	2,2% (4)	1,7% (3)

On the other hand, the dead trees at Tróia Plot were located near previous year's mortalities, extending the clearing in the middle of the plot in a south-easterly direction. At the Lezírias Plot many trees died around the main northwest edge but, since 2002, near the southern border of the plot a second mortality cluster began to appear.

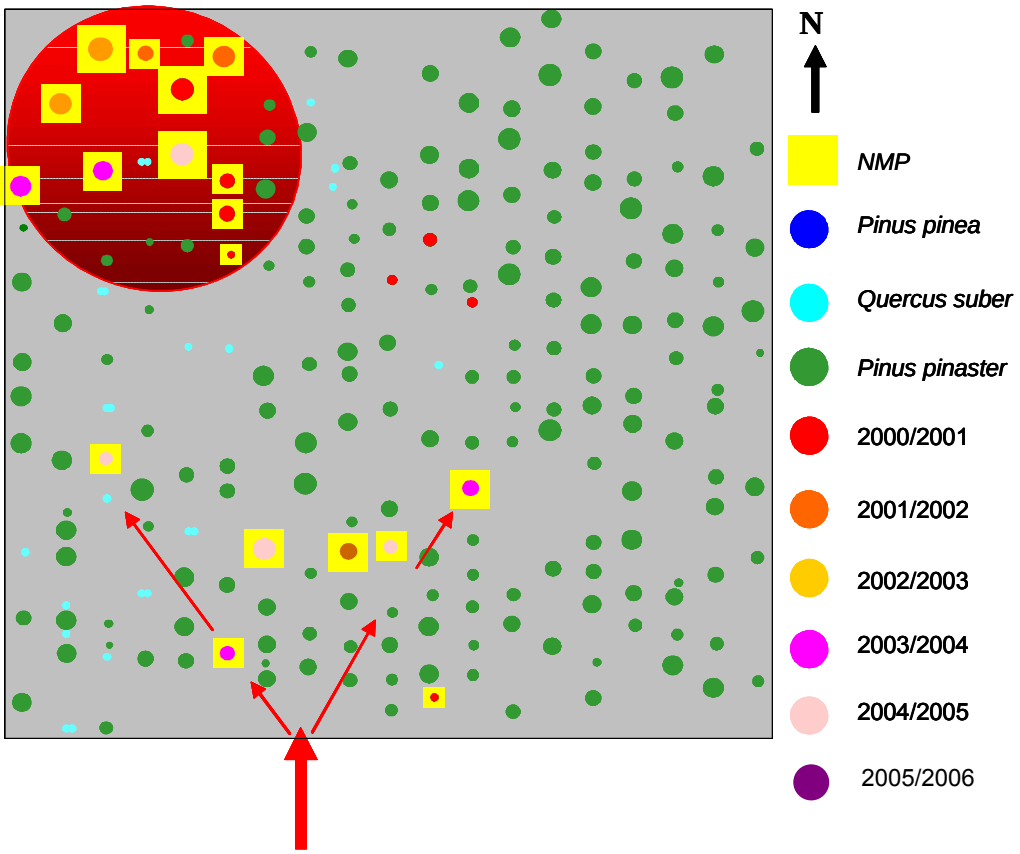


Figure 65: Pine mortality at Companhia das Lezírias plot. Yellow background indicates PWN infested trees

7.5.3 Early Detection Methods

7.5.3.1 Oleoresin flow observation with punch hole wound method

During the years of the trials some trees in both experimental plots failed to produce oleoresin and later died, some infested by PWN. Sometimes the weak oleoresin flow arose because of poor technique application, especially in smaller trees (less than 15 cm DBH) where 57% of the 102 punch holes attempted were not accurate. But in bigger trees the accuracy improved significantly, producing a good reliable method (Table 33).

Table 33: Reliability data for the punch hole technique for oleoresin measurement

DBH	Punch holes	Reliable flow data
10-15 cm	177	45%
15-20 cm	751	61%
20-35 cm	1446	71%
>35 cm	331	74%

When the failure in oleoresin production was complete all trees, with the exception of one individual, died in the following years, and therefore the trees were decaying.

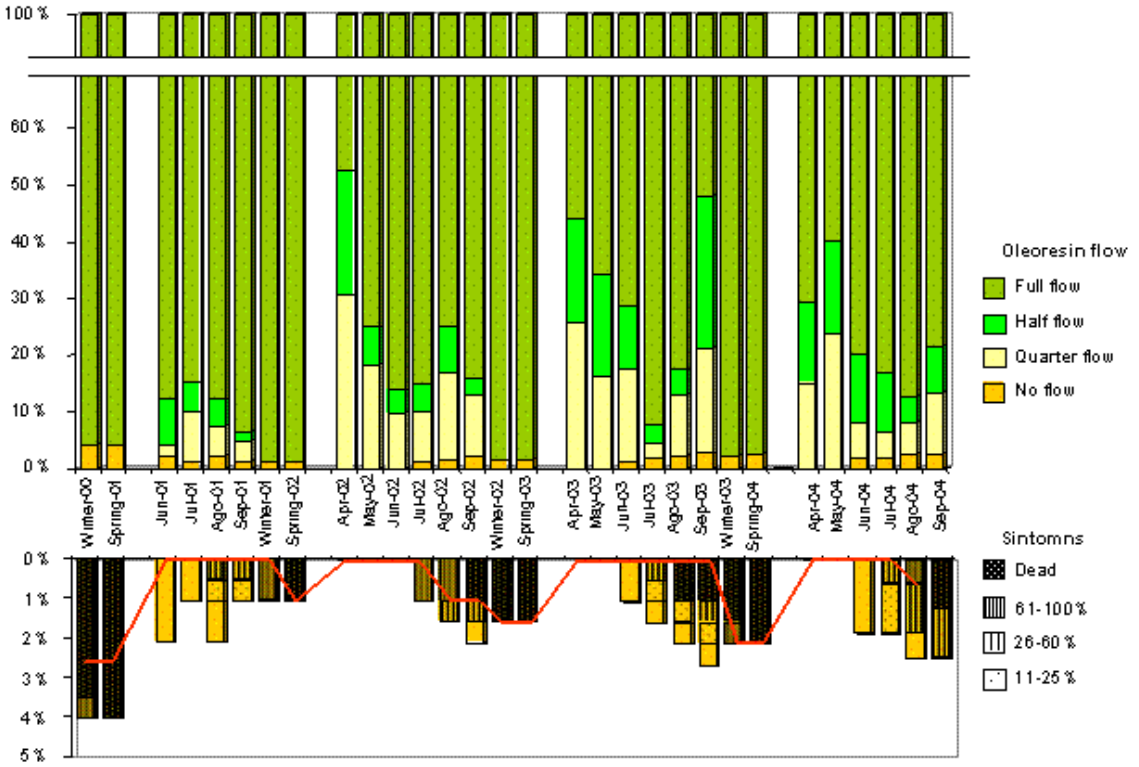


Figure 66: Evolution of oleoresin flow and monthly evaluation of tree symptoms, within the Companhia das Lezírias experimental plot

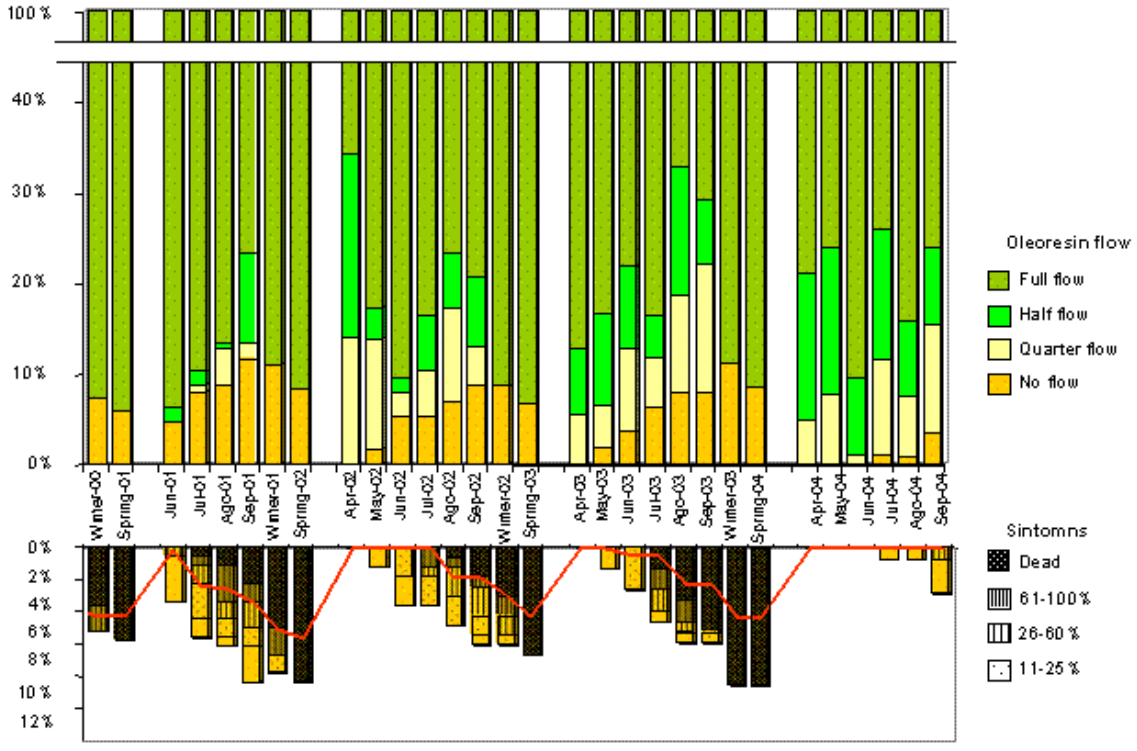


Figure 67: Evolution of oleoresin flow and monthly evaluation of tree symptoms, within the Tróia Experimental plot.

In Figure 66 and Figure 67 the results of the three year study show that the trees that did not produce any oleoresin died in the same year, although in Tróia not all were infested by the pinewood nematode. The visual symptoms were delayed by one to 2 months.

7.5.3.2 Electric resistance measurements

Significant differences were found between Healthy Thin trees and all the others, which is probably due to physiological differences; in smaller pine trees, active xylem is distributed throughout the inner wood, resulting in higher electric conductivity values.

The presence of only one diseased tree at the Tróia Plot makes it impossible to compare results statistically, but differences are obvious at the October measurement. By then the tree was already dead. The graphics in Figure 68 and Figure 69 show the fluctuations of the measurements through the tree decay cycle.

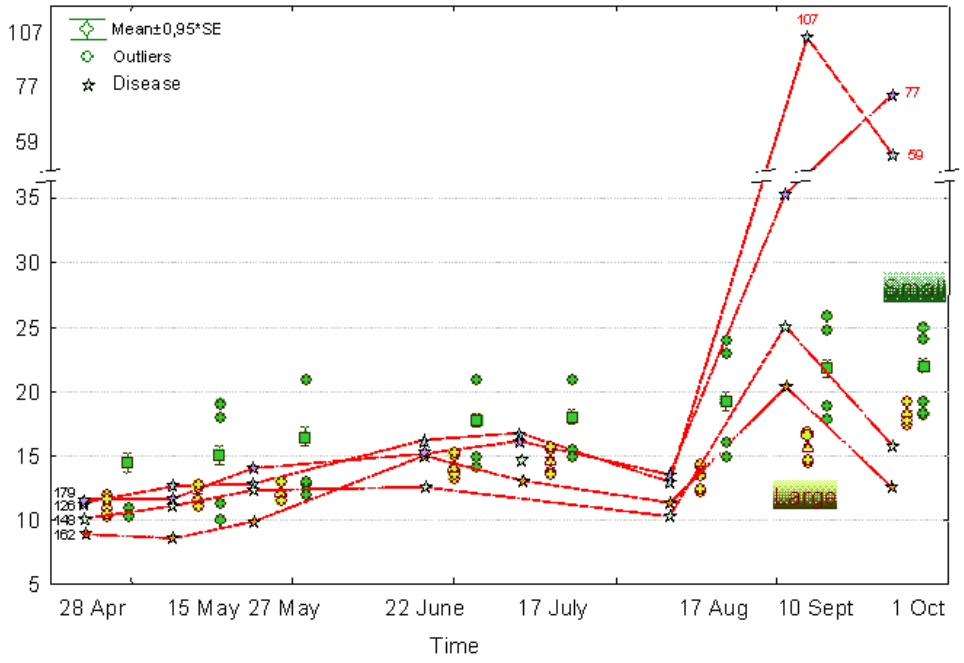


Figure 68: Electric conductivity of pine trees in the Companhia das Lezírias experimental plot

The measurements from Disease trees did not differ from the Healthy trees until the visual symptoms were very strong and the trees were undoubtedly decaying or already dead, in September (Table 34) or October (Table 35).

These results show that this method is only accurate after visual symptoms and can not be used as an early wilt symptomatic technique.

Table 34: Average electric conductivity measurements at C. Lezírias Plot (Unit – Ohm) and statistical results of Kolmogorov-Smirnov tests

	Max Neg Diff	Max Pos Diff	p-level	Mean disease	Mean healthy	SD disease	SD healthy	Valid N disease	Valid N healthy	Sig
Diseased tree vs. Healthy trees										
28 Apr	-0.264	0.128	p>.10	10.75	11.18	1.399	1.608	4	129	NS
15 May	-0.341	0.112	p>.10	11.00	11.82	1.947	1.679	4	129	NS
27 May	-0.227	0.331	p>.10	12.31	12.25	4.014	1.556	4	129	NS
22 Jun	-0.155	0.471	p>.10	15.00	14.19	1.061	1.880	4	129	NS
17 Jul	-0.094	0.311	p>.10	15.25	14.62	1.958	2.071	4	127	NS
17 Aug	-0.465	0.031	p>.10	12.13	13.45	1.479	2.027	4	127	NS
10 Sep	0.000	0.750	p<.05	45.94	15.71	41.539	2.111	4	129	**
1 Oct	-0.242	0.500	p>.10	41.00	16.06	32.242	2.022	4	128	NS
Diseased tree vs. Small diameter trees										
	Max Neg Diff	Max Pos Diff	p-level	Mean disease	Mean small	SD disease	SD small	Valid N disease	Valid N small	Sig
28 Apr	-0.741	0.000	p<.05	10.75	14.43	1.399	4.386	4	27	**
15 May	-0.852	0.000	p<.025	11.00	14.98	1.947	3.898	4	27	**
27 May	-0.565	0.000	p>.10	12.31	16.47	2.014	3.836	4	27	NS
22 Jun	-0.630	0.000	p>.10	15.00	17.72	1.061	3.014	4	27	NS
17 Jul	-0.454	0.000	p>.10	15.25	18.04	1.958	2.862	4	27	NS
17 Aug	-1.000	0.000	p<.005	12.13	19.26	1.479	4.129	4	27	**
10 Sep	-0.250	0.500	p>.10	45.94	21.89	42.539	3.620	4	27	NS
1 Oct	-0.426	0.500	p>.10	41.00	20.41	32.242	3.285	4	27	NS
Healthy tree vs. Small diameter trees										
	Max Neg Diff	Max Pos Diff	p-level	Mean healthy	Mean small	SD healthy	SD small	Valid N healthy	Valid N small	Sig
28 Apr	-0.512	0.000	p<.001	11.18	14.43	1.608	4.386	129	27	***
15 May	-0.550	0.000	p<.001	11.82	14.98	1.679	3.898	129	27	***
27 May	-0.652	0.000	p<.001	12.25	16.47	1.556	3.836	129	27	***
22 Jun	-0.613	0.000	p<.001	14.19	17.72	1.880	3.014	129	27	***
17 Jul	-0.639	0.008	p<.001	14.62	18.04	2.071	2.862	127	27	***
17 Aug	-0.764	0.000	p<.001	13.45	19.26	2.027	4.129	127	27	***
10 Sep	-0.755	0.000	p<.001	15.71	21.89	2.111	3.620	129	27	***
1 Oct	-0.629	0.000	p<.001	16.06	21.41	2.022	3.285	128	27	***

Table 35: Average electric conductivity from measurements at Tróia Plot (Unit – Ohm) and statistical results

	Max Neg Diff	Max Pos Diff	p-level	Mean disease	Mean healthy	SD disease	SD healthy	Valid N disease	Valid N healthy
Diseased tree vs. Healthy trees									
30 Apr			---	17.00	15.14	0.00	3.892	1	105
14 May			---	19.25	14.81	0.00	3.888	1	103
26 May			---	19.00	16.50	0.00	4.090	1	104
22 Jun			---	18.00	17.51	0.00	4.219	1	107
17 Jul			---	17.75	18.36	0.00	4.325	1	106
28 Jul			---	20.50	18.99	0.00	4.549	1	104
17 Aug			---	12.00	15.22	0.00	4.260	1	107
9 Sep			---	15.50	16.85	0.00	3.758	1	103
1 Oct			---	69.75	16.61	0.00	3.573	1	103
Diseased tree vs. Small diameter trees									
	Max Neg Diff	Max Pos Diff	p-level	Mean disease	Mean small	SD disease	SD small	Valid N disease	Valid N small
30 Apr			---	17.00	21.54	0.00	5.148	1	29
14 May			---	19.25	20.73	0.00	4.447	1	27
26 May			---	19.00	22.48	0.00	6.015	1	27
22 Jun			---	18.00	23.95	0.00	5.455	1	29
17 Jul			---	17.75	24.00	0.00	5.018	1	28
28 Jul				20.50	23.89	0.00	3.985	1	28
17 Aug			---	12.00	21.18	0.00	5.403	1	28
9 Sep			---	15.50	23.00	0.00	5.524	1	28
1 Oct			---	69.75	23.53	0.00	6.180	1	32

Healthy tree vs. Small diameter trees									
	Max Neg Diff	Max Pos Diff	p-level	Mean healthy	Mean small	SD healthy	SD small	Valid N healthy	Valid N small
30 Apr	-0.550	0.000	p<.001	15.14	21.54	3.892	5.148	105	29
14 May	-0.547	0.000	p<.001	14.81	20.73	3.888	4.447	103	27
26 May	-0.466	0.000	p<.001	16.50	22.48	4.090	6.015	104	27
22 Jun	-0.541	0.000	p<.001	17.51	23.95	4.219	5.455	107	29
17 Jul	-0.526	0.000	p<.001	18.36	24.00	4.325	5.018	106	28
28 Jul	-0.545	0.010	p<.001	18.99	23.89	4.549	3.985	104	28
17 Aug	-0.608	0.000	p<.001	15.22	21.18	4.260	5.403	107	28
9 Sep	-0.527	0.000	p<.001	16.85	23.00	3.758	5.524	103	28
1 Oct	-0.509	0.000	p<.001	16.61	23.53	3.573	6.160	103	32

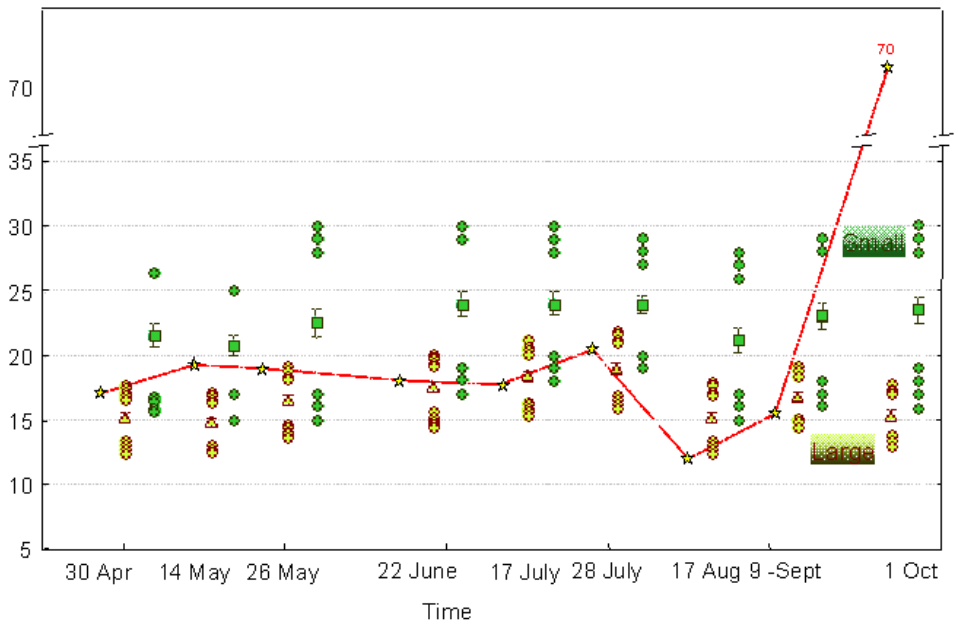


Figure 69: Electric conductivity of pine trees in the Tróia experimental plot

7.5.3.3 Sap flow measurements

In the graphics in Figure 70 are shown as an example the sap flow daily fluctuation pattern in 3 of the assessed trees. There is an increase of the sap flow during day and a decrease in response to the sunlight reduction. The overall sap flow are quite low because both year were very dry and also because the trees don't have large canopies. The highest values were observed in the month of May in response to the high soil humidity levels associated with the favourable conditions for high evaporative potential. The decrease observed along the time of the experience is only due to usual summer drought.

Unfortunately for the main goals of this assay none of the trees selected were neither infested by the nematode nor died resulting by the attack by any other pest or disease during the experiment. Nevertheless the data base created for the Spring/Summer/Autumn will be very important for comparative studies in future attempts.

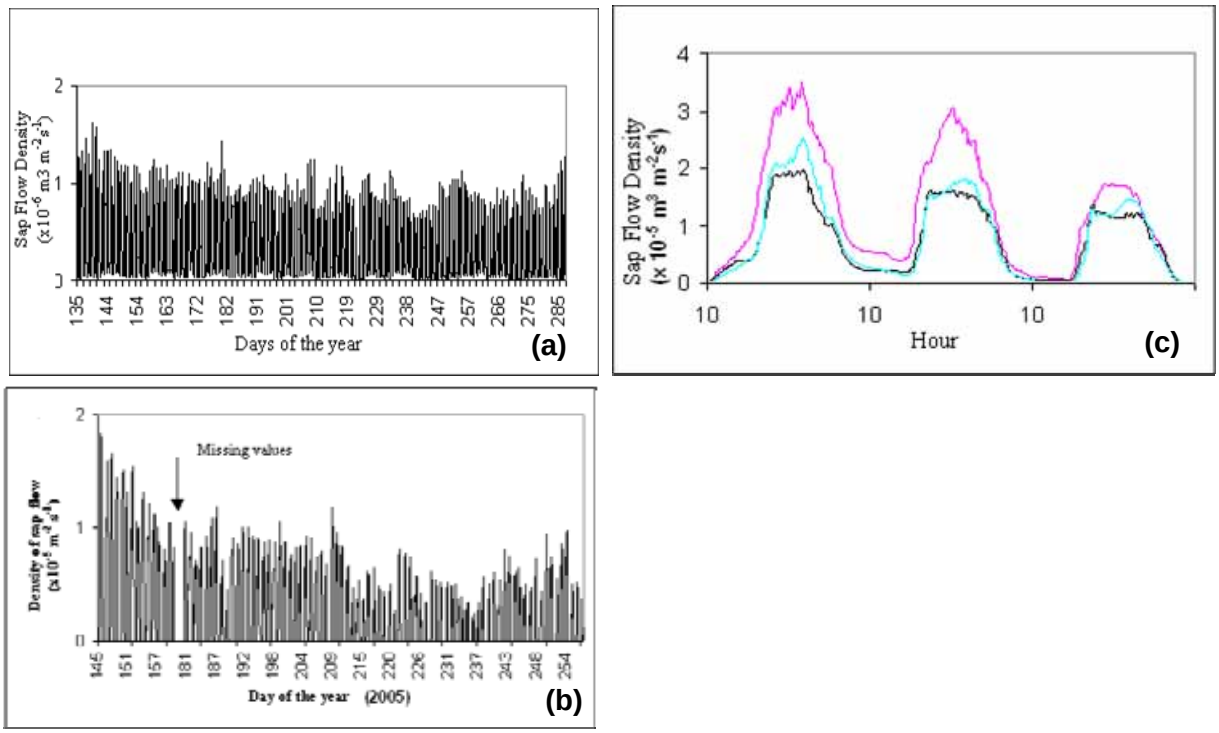


Figure 70: Overall variation of the sap flow of the assessed trees in Tróia Plot in both years of **(a)**2004 and **(b)** 2005; **(c)** Daily sap flow fluctuation in 3 assessed trees of Tróia Plot.

7.5.3.4 Sound methods for cavitations detection

The preliminary test performed by the Austrian team was not conclusive and, therefore, no further tests were carried out.

7.5.4 Dispersal of Monochamus galloprovincialis

In the first test, with recently formed beetles, the first survey was made on the 3rd day after release only allowed the recapture of one female that had flown over 15 metres, while in the second test, with older beetles, at the same time 57 different beetles were recaptured (78% recaptures) giving an average flight distance of 5.22 m, and many (23 insects) remained on the same tree between release and 1st day after release survey or between 1st and 3rd day survey (Figure 71).

At the end of the test (after no more beetles were found in the plot), 13 cross-vane traps baited with ethanol and turpentine were placed in the nearest adult maritime pine stand (over 200 metres away) which allowed the recapture of 3 marked beetles (one from the first test and the 2 others from the second test), after flying 237.14±15.30 meters and ageing 78 and 66 days, respectively.

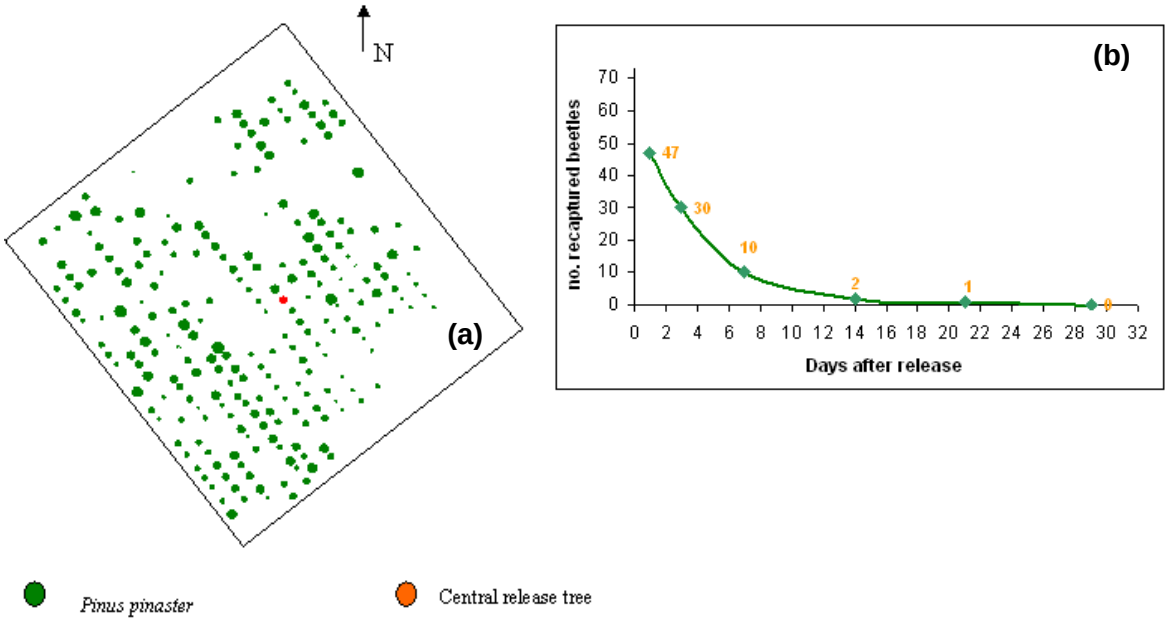


Figure 71: **(a)** Plot used for study on *M. galloprovincialis* dispersal movements. **(b)** Recaptures of marked *M. galloprovincialis* on surveys made after release.

7.6 *Control methods to reduce the incidence of Pine Wilt Disease*

The control of the spread of the Pine Wilt Disease can be partially achieved taking actions against one or all disease vectors: namely the Host tree, Insect vector or Nematode. Within Portugal the main efforts of the national programme Prolunp concentrate on identification of affected trees followed by their removal and destruction during the period of insect vector larval development inside the wood, thus decreasing the flying population during the next season. Tests were carried out to improve control measures and techniques relevant to nematode and vector flight trapping.

Since the pine wilt nematode vector in Portugal was found to be *Monochamus galloprovincialis*, each year experiments were made using several types of trap and lures, aiming to improve trapping capability as part of monitoring and control of vector populations.

In the first year (2003), 4 different traps and 7 sets of chemical lures were tested; in 2004 again 4 traps and 4 combinations of attractants were tested in separate tests; Finally, in 2005, 4 different traps used internationally to catch *Monochamus* species and 2 bark beetles pheromones were tested separately for *M. galloprovincialis*.

7.7 *Materials and Methods*

7.7.1 Insecticides and Nematicides

A preliminary experiment to test the injection of insecticides and nematicides into healthy pine trees was conducted inside the PWN affected zone in Tróia, where healthy maritime pines were randomly selected in Spring 2004 and inoculated with one of the following combinations, according to the product instructions:

- 5 trees inoculated with a nematicide solution – active substance: Lamdazyotrin;
- 5 trees inoculated with an insecticide solution (Carate Ceon) – active substance: Imidacloprid
- 5 trees inoculated with equal doses of both products;

The adult trees were inoculated at their base, by drilling the thick bark and injecting the product capsule directly into the phloem, allowing the product to be disseminated along with the sap flow. Tree condition was assessed monthly until the end of the inoculation year.

To test the efficiency of the products inoculated, two months after inoculation some lower branches were collected from all the trees injected with insecticides and, under laboratory conditions, offered to adult *M. galloprovincialis* to determinate if they were suitable for maturation feeding and oviposition. Similarly to the previous experiment, *B. xylophilus* was also inoculated under laboratory conditions to recently-collected pine branches obtained from both nematicide-injected trees and healthy trees (control).

7.7.2 Traps and Lures

Traps tested:

Year 1
Multi-funnel (based on Lindgren, 1983);
Black Stove pipe with glue (based on Groot & Not, 2001; 2003;);
Cross-vane interception (based on Nakamura *et al.*, 1999);
Wood logs inside plastic basket nailed to a plate with glue.
Year 2
Multi-funnel trap (same as in 2002);
Transparent cross-vane interception (best in 2002);
Black cross-vane interception;
Cross-vane interception with vertical black profile of 1/3 width.
Year 3
Multi-funnel trap (Pherotech);
Black cross-vane interception (sent by Nakamura, K.);
Transparent cross-vane interception (best in previous years);
Long black cross-vane interception (based on Pherotech Colossus).

Chemical attractants tested:

Year 1
(+) α pinene;
Turpentine;
Resin;
Ethanol;
(+) α pinene + ethanol;
Turpentine + ethanol;
Resin + ethanol;
Control (no lure).

Year 2
Turpentine and Ethanol in separate vials (best in 2002);
Turpentine and Ethanol mixed in 1:1 proportions;
Turpentine and Ethanol mixed in 1:2 proportions;
Turpentine and Ethanol mixed in 2:1 proportions;

Year 3
Turpentine and Ethanol (best in 2002);
Turpentine and Ethanol plus Ipsenol;
Turpentine and Ethanol plus Ipsdienol;
Turpentine and Ethanol plus Ipsenol and Ipsdienol;

In 2003 there were 3 replications of 8 plots, each plot with 4 traps (one of each type) placed in a 50 meters square (distance considered to reduce overlapping of trap attractive areas), and 100 m apart from the closest plot. Traps in each plot received the same lure. Every week, all wood boring insects from Cerambycidae, Buprestidae and Curculionidae families present in the traps were counted and removed for species' identification. Lures were changed from plot to plot in clockwise rotation (to diminish site effects). The test lasted 8 weeks (from mid July to September 2002), when all lures had been tested in all plots.

In 2004 and 2005 tests the Latin Square experimental design 4x4 was used. To assure normality and heteroscedacity insect capture data were transformed (log+1) (Zar, 1984) before performing statistical variance analysis ANOVA and LSD means comparison test.

7.8 *Results*

7.8.1 Insecticides and Nematicides

The inoculation of the chemical products apparently did not affect the trees, as no change in the sanitary condition or canopy defoliation was observed during the following months. The adult insects fed and oviposited normally on the branches in the laboratory and the eggs laid by the female beetles hatched and developed normally, apparently indifferent to the chemical products inoculated.

The pine wood nematode inoculated into the nematicide-injected trees and control trees developed in both situations, apparently not affected by the product. The application of nematicide and insecticide apparently had no negative effects on the trees, as their sanitary condition was good for the rest of the year. The products might have prevented attacks by the pine sawyer or the PWN, although under laboratory conditions neither the insects nor the nematodes were affected by the substances inoculated.

7.8.2 Traps and Lures

For the 2002 test, both traps ($F_{(3,752)}$= 6,93; p=0,001) and lures ($F_{(7,748)}$= 5,10; p=0,0001) showed highly significant statistical differences in catching *M. galloprovincialis*. The multi-funnel trap did not catch any beetles while the other three types captured identical numbers of insects (Figure 72). The most effective lure was turpentine and ethanol, but also resin and pinene when combined with ethanol were better than the control blank trap, therefore ethanol has a strong synergistic effect with host volatiles (Figure 73).

In the 2004 tests, the Multi-funnel trap was again tested against the other traps and, although it caught 3 pine sawyers it was still the poorest, with statistically very significant differences to the 3 cross-vane traps ($F_{(3,252)}$=3,3983; p=0,0184) which were of identical trapping efficiency for *M. galloprovincialis* (Figure 74). The comparison between the three cross-vanes revealed that apparently the visual clues are less important than chemical, since the best results were obtained by transparent traps.

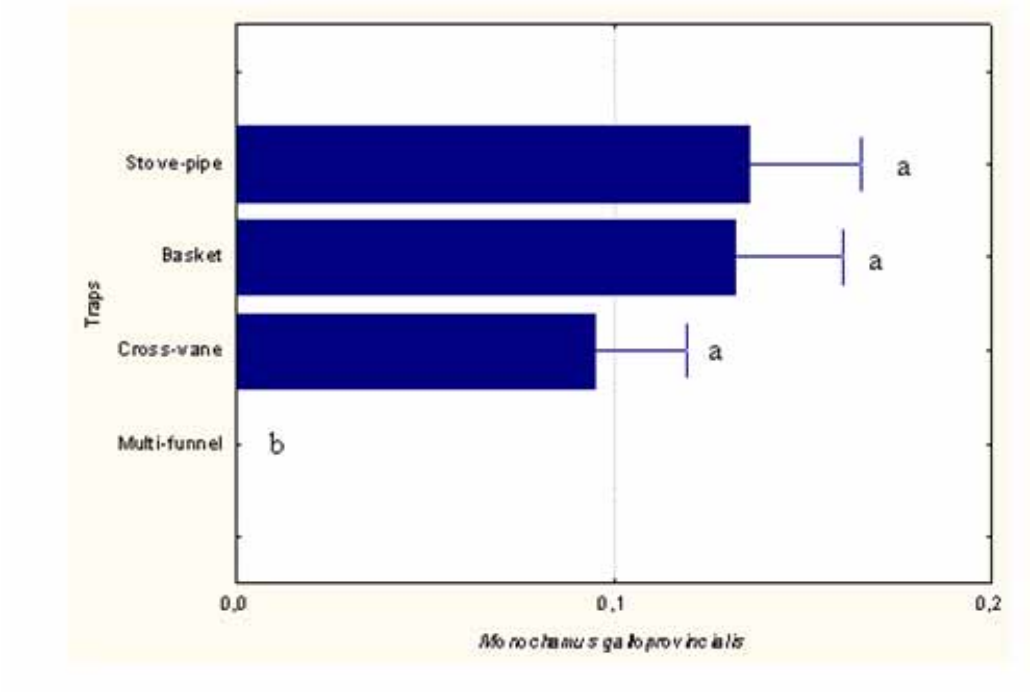


Figure 72: Performance of Traps to catch *M. galloprovincialis* (avg±SE*0,95), in 2002. Same letters refer to same LSD means comparison homogeneous group.

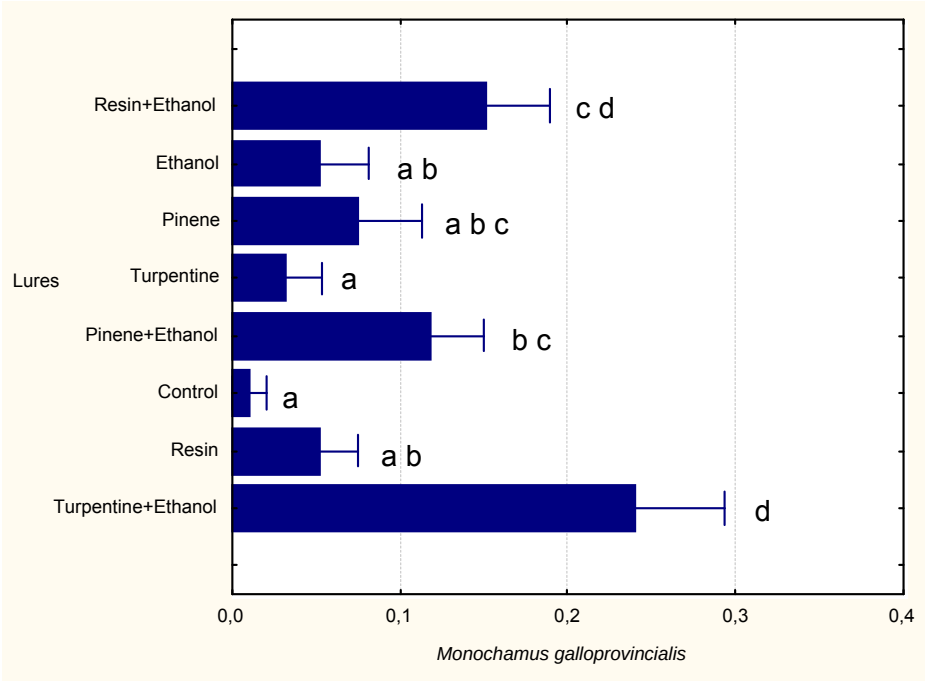


Figure 73: Performance of lures to catch *M. galloprovincialis* (avg±SE*0,95), in 2002. Same letters refer to same LSD means comparison homogeneous group.

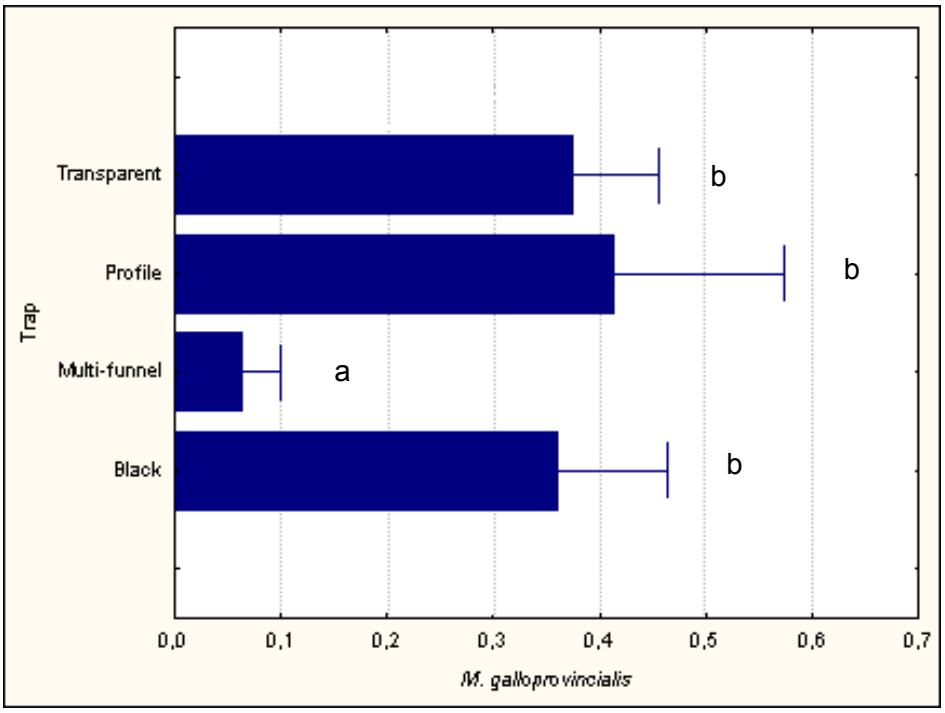


Figure 74: Performance of Traps to catch *M. galloprovincialis* (avg±SE*0,95), in 2004. Same letters refer to same LSD means comparison homogeneous group.

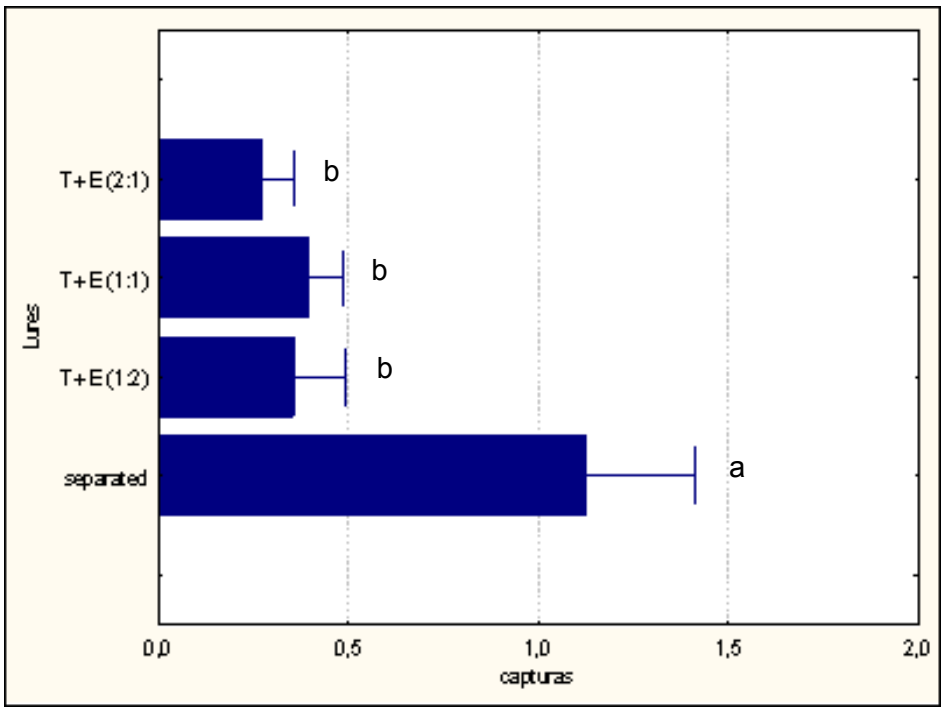


Figure 75 Performance of lures to catch *M. galloprovincialis* (avg±SE*0,95), in 2004. Same letters refer to same LSD means comparison homogeneous group

Tests carried out in the following year employed different combinations of the previous best lure – turpentine with ethanol - and it was evident that pine turpentine and ethanol must be presented independently ($F_{(3,188)}$=4,129; p=0,0073). Irrespective of the proportions in mixtures, all were worse than the two chemicals presented separately (Figure 75).

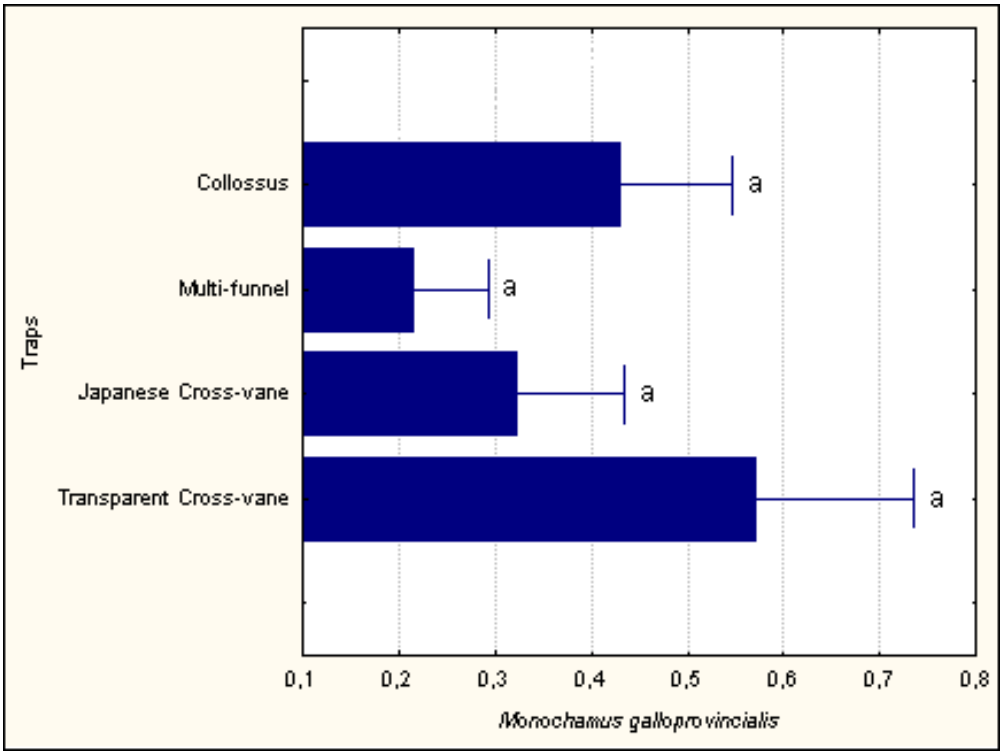


Figure 76: Performance of Traps to catch *M. galloprovincialis* (avg±SE*0,95), in 2005. Same letters refer to same LSD means comparison homogeneous group

The above tests enabled a comparison to be carried out with the previous years' best results: Transparent Cross-vane trap with worldwide commercial available pine sawyer traps, such as the Canadian Pherotech Multi-funnel and Colossus and Japanese Black cross-vane traps. All traps showed identical capacity to capture *M. galloprovincialis* ($F_{(3,220)}$=1,5443; p=0,2040), but again, the transparent cross-vane trap caught more beetles than the others (Figure 76).

The trials of the lures showed some evidence that scolytid aggregation pheromones have kairomonal effects on *M. galloprovincialis* because both *Ips* pheromones attracted more beetles than the pine volatiles with ethanol, although without statistical differences ($F_{(3,172)}$=0,4169; p=0,7411) (Figure 77).

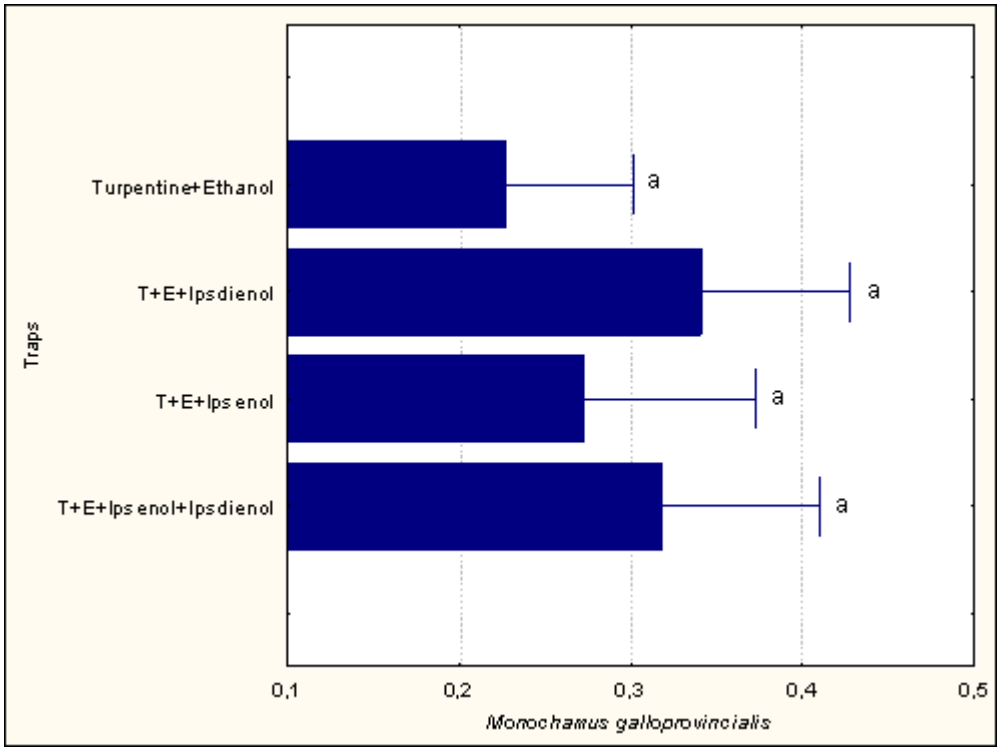


Figure 77: Performance of lures to catch *M. galloprovincialis* (avg±SE*0,95), in 2005. Same letters refer to same LSD means comparison homogeneous group

7.9 *Discussion*

Comparing the decreasing number of dead trees removed from Tróia during the three year eradication programme, the estimates may be biased by the use of traps and lures with limited power to attract *Monochamus* beetles, but these low densities were expected considering the Tróia eradication results.

From the early detection methods tested, only oleoresin flows survey is reliable. Electric resistance measurements only gave differences when trees were already showing visual symptoms of pine wilt expression and sound measurements only registered larval feeding activity as described by Izumi *et al*. (1990).

Therefore, all dying pines were found to cease oleoresin production but not all deaths were related to the effects of PWN infestation. But if a pine is infested with the nematode then oleoresin will stop two months before visual symptoms are observed, when needles begin to turn a yellowish colour. A similar pattern was described for *M. alternatus* infested pine, by Mamiya (1988)

During the first week after release in the mark-recapture experiment, the recaptured beetles had flown an overall distance between trees of 12.72 meters which is within the weekly distance describe for *M. alternatus*, in Japan (10 – 20 m) (Shibata, 1986; Togashi, 1990). The Japanese pine sawyer tends to remain throughout its life cycle within young pine stands, feeding (and transmitting PWN to healthy trees), mating and egg-laying and there is no published reference to long-distance migratory flights. Previously referred Japanese papers described the recapture of a beetle 9 weeks after release inside the same young pine stand coincident with the appearance of several dead trees. It is known that *M. alternatus* prefers to colonise the lower trunk of the host tree and transmits the nematode to young trees, quite the opposite preference to *M. galloprovincialis*.

The recapture in a trap of marked beetles proves that they had flown out of the plot to the adult pine stands, immediately after release (first test) and after sexual maturation (second test). The behaviour of the beetles in the second test is explained by the search of suitable hosts for egg laying (trees inside the plot were too small for *M. galloprovincialis*), but in the first test the beetles were too young and immature to be induced by reproductive stimuli. Therefore, we must conclude that *M. galloprovincialis* beetles have a requirement for a migratory flight after emerging, potentially covering long distances, and only after they will establish in the stand making short range flights for feeding, sexual mating and egg-laying.

Insect communication is based either on specific pheromones (e.g. Pine Processionary Moth) or depends on visual (Vasechko, 1978; Niemeyer, 1985) and tree host chemicals (Chénier & Philogène, 1989; Allison *et al.*, 2004).

The synergistic effect of ethanol when combined with host volatiles was previously noted for other *Monochamus* and wood borer species (Fatzinger, 1985; Ikeda *et al.*, 1986; Chénier & Philogénè, 1989). The results from the several years of experiments in the current programme have led to great improvements in the efficiency of *M. galloprovincialis* trapping. All available traps were tested and the cross vane design gave better results as with other *Monochamus* species (McIntosh *et al.*, 2001), but visual stimuli (black silhouette) was not important for trapping as referred by some authors (McIntosh *et al.*, 2001; Morewood *et al.*, 2002; Groot & Not, 2003).

The introduction of scolytid pheromones apparently played a major role in enhancing the ability of the traps to attract beetles, despite background levels of host volatiles from standing trees, and reveals a possible co-evolution between the nematode vector and the scolytids. This would apply particularly in the main source of weakened trees (along with forest fires) outside the affected zone (Billings & Cameron, 1984; Billings, 1985; Allison *et al.*, 2001; Allison *et al.*, 2003), although bark beetle pheromones by themselves may be an incomplete signal to *Monochamus* spp. (Groot & Nott, 2004).

The reason for the failure of tree injections might be because the products tested were not suitable for controlling these particular species, and so were not effective against them specifically (the products are generalists, not specific to *Monochamus* or *Bursaphelenchus*), or maybe the inoculation dosage was too low for the sizes of the adult trees tested and the products could not disperse adequately throughout the whole tree, as these substances had never been tested on maritime pine. Additional tests, with other products and different substance combinations are needed to determinate the potential effectiveness of using these methods to prevent infestation of trees or as curative methods for already infested trees.

Chapter 8 Molecular identification of *Bursaphelenchus* species and pathway analysis of *B. xylophilus* introduction to Portugal

8.1 *Introduction*

The genus *Bursaphelenchus* was established by Fuchs (1937) and includes nematodes that are associated with insects and dead or dying, mainly coniferous, trees 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.

Species of the genus *Bursaphelenchus* are widely distributed, occurring predominantly in Europe, Asia and North America. Most of them live in conifer trees and are associated with wood-inhabiting beetles. The pinewood nematode (PWN), *Bursaphelenchus xylophilus* Steiner & Buhrer 1934, Nickle, 1970 is native to North America. Presumably in the late 19th century the nematode was introduced to Japan. It was identified by Kiyohara & Togashige 1971 as the causal agent of pine wilt disease and thereafter as an important pest of many pine species. More recently, *B. xylophilus* has spread to China, Taiwan, South Korea and Portugal, where it was detected in 1999 in an area around Setúbal (Evans et al. 1996; Mota et al. 1999). *B. xylophilus* is associated with longhorn beetles of the genus *Monochamus* (Cerambycidae, Coleoptera) as principal vectors.

The current concern on the introduction of the PWN into new areas has increased the interest and the knowledge of this genus and has led to the discovery of a number of new species. To date the genus comprises 85 species, of which 10 were described since 2004, mainly from Asian regions (Ryss et al., 2005). The importance assumed by *Bursaphelenchus xylophilus* clearly reinforced the need for accurate identification of species. Morphology remains the standard, traditional method for routine identification of nematode species. In the case of the *Bursaphelenchus* species associated with pines, several characteristics have been used such as the male spicule shape, the female vulva, presence or absence of a mucron in the female tail, etc. These light microscopy observations have been improved with the use of scanning electron microscopy (SEM) (Eisenback, 1985), however clear identification and separation of the species belonging to the pinewood nematode species complex (PWNSC) remain a difficult task, only possible by a trained nematologist.

Different criteria may be used to divide the large number of nominal species of the genus *Bursaphelenchus* into smaller and more convenient species groups. Tarjan and Baéza-Aragon (1982) were the first to attempt the assembly of identification keys for this group, given a detailed classification of the spicule characters and other morphological diagnostic data. Braasch (2001), used the number of lateral lines (nine different groups), followed by the distribution of the male papillae, spicule shape, presence and size of the female vulva flap and the shape of female tails, for the purpose of establishing species groups - for species associated with conifer trees in Europe, 28 at that time. Yet, an integrated morphological identification system to all the species of the genus was lacking. Ryss et al. (2005) produced a synopsis of the genus *Bursaphelenchus* in order to provide an identification system of all the nominal species, where the spicule structure is the main diagnostic character to separate the species. The six species groups (*aberrans*-group, *borealis*-group, *eidmanni*-group, *hunti*-group, *piniperdae*-group and *xylophilus*-group) are intended purely as identification units in order to facilitate species identification. However, some of these groups may be considered as natural ones (*i.e.* phylogenetically based), as the case for the *xylophilus*-group, which includes PWN.

Despite the clear separation of the members of the *xylophilus*-group (*B. baujardi* Walia, Negi, Bajaj & Kalia, 2003; *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000; *B. doui* Braasch, Gu, Burgermeister & Zhang, 2004; *B. fraudulentus* Rhüm, 1956; *B. kolymensis* Korentchenko, 1980; *B. luxuriosae* Kanzaki & Futai, 2003; *B. mucronatus* Mamiya & Enda, 1979; *B. singaporensis* Gu, Zhang, Braasch & Burgermeister 2005; *B. xylophilus*) from all others groups (distinctive angular shape of

spicules, presence of four lateral lines and the large vulval flap in females), the species identification level within this group requires a high level of expertise, due to the morphological similarities among these species. One of the major characters used for the distinguishing of PWN from all other members is the shape of the female tail, *i. e.*, rounded tail, with the lack of a distinct mucron. In addition to the morphological similarities between *B. xylophilus* and *B. mucronatus*, these two species may be capable of genetic exchange, either directly or via intermediate forms, which clearly compromises the identification based at species level using morphological data only.

Several molecular biological techniques have been used for the study of genetic variability among different geographical isolates of *Bursaphelenchus xylophilus*. Differences in nucleic acid sequences, revealed by means of molecular biology techniques, such as ITS-RFLP (Hoyer *et al.,* 1998; Braasch *et al*., 1999), may help to characterise each species and complement its morphological description. Initially, the genetic differentiation of some populations was achieved by the use of restriction analyses and hybridisation with total genomic DNA (Bolla et al., 1988), or applying DNA probes (Webster et al., 1990; Abad et al., 1991; Tàres et al., 1993). Other studies using the heat shock protein *Hsp70* gene (Webster et al., 1990; Beckenbach et al., 1992; Leal, 2006), PCR-RFLP and rDNA sequencing (Iwahori et al., 1998; Beckenbach et al., 1999), demonstrated some genetic differences among different isolates.

The RAPD-PCR technique 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; Irdani et al., 1995b; Vieira *et al*, 2007, Wang et al., 2001; Zhang et al., 2002). Recently, a more integrated study has been conducted using several isolates from the native regions (Canada, USA) and non-indigenous areas (China, Japan, Korea and Portugal) (Metge and Burgermeister, 2006). PWN rapid identification methods utilising real-time PCR are also being developed (Castagnone et al., 2006).

The introduction of a species into a new area can be used as a natural case study, where the species must be able to cope with a range of new environmental pressures. The genetic diversity among the Portuguese isolates of *B. xylophilus* is not known since available information is only restricted to 3 isolates, from adjacent blocks of the affected area (Metge and Burgermeister, 2006). Significant degrees of differentiation have been observed among different isolates from countries where PWN has become established (Zheng et al., 1998; Metge and Burgermeister, 2006).

Neighbouring countries with large forest areas, such as Turkey have recently established major surveying actions, in order to search for the possible presence of PWN (Akbulut et al, 2006; Vieira et al., 2006). These surveys provide important information regarding the EU and risk of pest introduction.

Earlier molecular diagnostic work has concentrated on differentiation of *B. xylophilus*, *B. mucronatus* and *B. fraudulentus* with respect to the close morphological similarity of these species and the economic importance of *B. xylophilus*. Two species-specific DNA probes (pBx6 and pBm4) derived from the non-transcribed spacer region of ribosomal RNA genes were used in dot blot hybridisation and RFLP analysis (Webster et al., 1990). Species-specific RFLP patterns were also obtained using a heterologous unc-22 DNA probe from *Caenorhabditis elegans* (Abad et al., 1991). A repetitive DNA fragment cloned from *B. xylophilus* (X14) was used to obtain isolate-specific DNA fingerprints upon RFLP analysis (Harmey & Harmey, 1993). A cloned satellite DNA from *B. xylophilus* formed the basis for highly sensitive and specific detection of the pinewood nematode in dot blot hybridisation and PCR (Tares et al., 1993; 1994).

Attempts were also made to extend molecular diagnosis to a larger number of *Bursaphelenchus* species. Analysis of restriction fragment length polymorphism of internal transcribed spacer regions of rDNA (ITS-RFLP) has been used as a valuable tool for species differentiation of several nematode genera (Vrain, 1993; Ferris et al., 1993; Zijlstra et al., 1995) including *Bursaphelenchus* (Burgermeister et al., 2005). Most new *Bursaphelenchus* species described in recent years were

identified using the combination of morphological features, ITS-RFLP patterns and ITS1/2 sequences.

For population analysis, polymerase chain reactions (PCR) with non species-specific primers have become popular because no sequence information from the target species is required. Additionally, an unlimited number of potential markers for different isolates can be produced. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) techniques are commonly used for identification of DNA markers and genetic comparison of isolates. RAPD was first described in 1990 (Williams et al. 1990; Welsh and McClelland 1990) It was first applied to nematodes in 1992 (Caswell-Chen et al. 1992) and to the genus *Bursaphelenchus* in 1995 (Braasch et al. 1995; Irdani et al. 1995). ISSR was introduced in 1994 (Zietkiewicz et al. 1994) and was predominantly applied to plants (Reddy et al. 2002). For similar studies in animals, ISSR polymorphisms were demonstrated for corals (Snell & Coffroth, 1999), silkworm (*Bombyx mori*) (Reddy et al. 1999), Artiodactyla (Kostia et al., 2000), aphids (*Acyrthosiphon pisum*, *Pemphigus obesinymphae*), mosquito (*Aedes aegypti*), rotifer (*Philodina* spp.) (Abbot, 2001) and honey bee (*Apis mellifera*) (Sylvester, 2003).

In order to trace the origin of the Portuguese PWN isolate, ISSR- and RAPD-polymorphisms were examined to determine genetic relationships among indigenous isolates from North America and introduced isolates from various countries. Introducing species by global trade contributes directly to mixing of fauna from geographically separated regions. Package wood and dunnage wood are of importance as transport material for the worldwide spread of economically important invasive species like *B. xylophilus* (Pfeilstetter, 2003). These low-quality wood materials were suspected to have transported the initial colonists of the Portuguese PWN population during the last decades of the 20th century from an unknown area of distribution (Tomiczek et al. 2003). In spite of extension of EU quarantine regulations to phytosanitary treatments of package wood in 2001 (2001/219/EC), living PWN have been intercepted in Europe throughout the years up to now.

8.2 *Materials and Methods: UEVORA, Portugal*

Methodology concerning the survey and characterization of Bursaphelenchus species in Portugal are described in Penas et al., 2004, 2006a and 2006b; methodology regarding other aspects of taxonomy are described in Ryss et al., 2005, Akbulut et al, 2006 and Vieira et al., 2006)

8.2.1 Nematode isolates

Between 2003 and 2005, during the annual survey for the PWN carried out by PROLUNP (http://www.dgrf.min-agricultura.pt/prolunp), a total of 1050 pine wood samples was collected from *P. pinaster* (maritime pine) trees displaying symptoms of pine wilt disease, from the 28 blocks that compose the affected area in Portugal (Figure 78, Table 36).

Wood samples - 40-80g each - were collected from pine trees at a height of 1.5m on the trunk (DBH), using a low-speed drill of 1.2 cm diameter, and stored in small plastic bags. Nematodes were extracted using a modified Baermann funnel technique, and processed within 48 h.

Table 36: Geographic origin of 26 isolates of *B. xylophilus*, and 1 isolate of *B. mucronatus*, used in this study

Species	Isolate code	Location	Year of isolation	Source
Bursaphelenchus xylophilus	PT1	B16	2005	*Pinus pinaster*
"	PT2	B25	"	"
"	PT3	B49	"	"
"	PT4	B19	"	"

Figure 78: Location of the *Bursaphelenchus xylophilus* isolates obtained from 28 different blocks of the affected area in Portugal

"	PT5	B23	"	"
"	PT6	B22	"	"
"	PT7	B35	"	"
"	PT8	B36	"	"
"	PT9	B18	"	"
"	PT10	B78	"	"
"	PT11	B83	"	"
"	PT12	B82	"	"
"	PT13	B21	"	"
"	PT14	B85	"	"
"	PT15	B73	"	"
"	PT16	B79	"	"
"	PT17	B27	"	"
"	PT18	B77	"	"
"	PT19	B72	"	"
"	PT20	B31	"	"
"	PT21	B26	"	"
"	PT22	B75	"	"
"	PT23	B76	"	"
"	PT24	B80	"	"
"	CH	Nanjing/China	2002	*Pinus thunbergii*
"	US	Missouri/USA	2000	unknown
B. mucronatus	BM	Brendeberg/ Germany	1996	*Pinus sylvestris*

8.2.2 Culturing geographic isolates

Nematodes were collected and cultured on *Botrytis cinerea* Pars., grown on potato dextrose agar (PDA), and incubated at 25ºC for 2 weeks. After successful rearing, 24 isolates of *B. xylophilus* were selected, representing 24 different blocks (the exclusion of four of the remaining blocks was made due to the unsuccessful rearing of the culture and limited number of sample slots in the electrophoresis apparatus). From each isolate 100-200 nematodes (without separation according to sex or developmental stage) were collected and washed several times in distilled water, transferred to a 1.5 ml Eppendorf tube with distilled water, and stored at -80ºC until use. All isolates were confirmed as *B. xylophilus* by ITS-RFLP (data not shown). The additional isolates used were *B. xylophilus* from Nanjing (China) and Missouri (USA), as well as *B. mucronatus* Brandenburg (Germany), as an outgroup.

8.2.3 Morphology

Nematode identification was based on observations of morphological characters, particularly vulval flap, shape of spicules and female tail, using light and scanning electron microscopy (SEM). Whenever possible, nematodes were observed by simply heat-relaxing and mounting in a drop of water, or placed in a small slab of 4% agar (Hasegawa et al., 2004). For SEM, nematodes were fixed in a mixture of 4% gluteraldehyde/2% formaldehyde for several days, post-fixed in 2% OsO_4, overnight, dehydrated in an ethanol series, critical point dried and sputter coated with gold (Eisenback, 1985). Observations were made with a Jeol 35 SEM.

8.2.4 DNA extraction

DNA extraction was performed using the QIAmp DNA Micro Kit (Qiagen, Germany). The nematodes were placed in 1.5 ml microcentrifuge tubes and pelleted by centrifugation at 9000 *g* for 2 minutes, and the supernatant discarded. To the pellet 30 µl of ATL buffer was added, and the nematodes were homogenised using Eppendorf micro pestles (Eppendorf, Hamburg, Germany). The homogenate was mixed with additional 150 µl of the ATL buffer, and further processed according to the manufacturer's instructions. DNA concentrations were measured fluorimetrically using the fluorescent dye Hoechst 33258 and a DyNa Quant 200 fluorimeter (Pharmacia Biotech, Germany).

8.2.5 ITS-RFLP

ITS regions of rDNA were amplified using primers F194 and P5368 described by Ferris *et al*. (1993) and Vrain (1993), respectively. All polymerase chain reactions were performed in a final volume of 50 µl, using 10 ng/µl of template DNA, 1 µM of each primer, 0.2 µM of dNTPs (Invitrogene®), 2 U of *Taq* DNA polymerase (Invitrogene®), 1x Reaction Buffer (Invitrogene®) and 1.25 mM of MgCl2 (Invitrogene®). The reaction mixture was overlaid with sterile mineral oil to prevent evaporation during PCR cycling. A Stratagene® Robocycler was used for amplification and the reaction consisted of one denaturation step at 94ºC for 1 min, 35cycles with denaturation at 94ºC for 1 min, annealing at 51ºC for 1 min, polymerization at 72ºC for 2 min and a final extension step at 72ºC for 5 min. After PCR, 5 µl of amplified product were analysed by electrophoresis in a 1% agarose gel. Data analysis was performed using the Kodak® 1D 2.0 system and 100 bp DNA Ladder (Invitrogene®) as a molecular size marker.

Restriction analysis of ITS regions was performed with *Alu*I, *Hae*III and *Rsa*I restriction endonucleases (Invitrogene®), using an aliquot of 4 µl of the PCR product and 10 U of each enzyme, according to the manufacturer's instructions. Fragments were resolved by electrophoresis in a 2% agarose gel and data were analysed as described above.

8.2.6 RAPD-PCR procedure

For this study 30 oligonucleotide decamer primers were used (Table 39). These primers were selected because they gave suitable results for the comparison of *Bursaphelenchus xylophilus* isolates in previous studies (Braasch et al., 1995; Metge & Burgermeister, 2006; Gonçalo Silva, *person. communication*). All RAPD reactions were performed as described by Schmitz et al. (1998), with slight modifications. Each PCR reaction (25 µl) contained Stoffel buffer (10 mM Tris pH 8.3, 10 mM KCl), 4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of primer, 5 units of AmpliTaq DNA Polymerase Stoffel fragment (Applied Biosystems, Germany) and 4 ng of DNA template. Amplification was performed in a Perkin Elmer 9600 thermocycler (Applied Biosystems). The PCR was started by an initial denaturation step at 94ºC for 2.5 minutes, followed by 40 cycles of 20 seconds at 92ºC, 15 seconds at 38ºC, 1 minute at 72ºC, and a final extension at 72ºC for 7 minutes. The rate of heating from 38°C to 72°C was regulated to 0.3°C/second. After amplification, 10 µl aliquots of the reaction mixture were loaded onto a 2% agarose gel in TAE running buffer and electrophoresed for approximately 4 hours at 80 volts. The gel was stained in a 1 µg/ml ethidium bromide-water solution for 30 minutes, and photographed with a UV system (Gel Jet Imager 2005, Intas, Germany).

8.2.7 Data collection and analyses

The RAPD fingerprint patterns obtained were converted into binary data matrices, scoring the presence of a band as 1 and its absence as 0. Only clearly visible bands were scored. The binary matrix was subjected to the MSVP ver. 3.12d software, using the Nei & Li coefficient (Nei & Li, 1979) to generate a matrix of genetic distances. The cluster analyses of genetic distances were performed with the unweighted pair-group method using arithmetic averages (UPGMA) in the module SAHN (sequential, agglomerative, hierarchical and nested clustering method) of NTSYS-PC ver. 2.1 (Rholf, 2000). The dendrograms were constructed with the TREE option of NTSYS-PC. For providing statistical support for the dendrograms obtained, the cophenetic correlation coefficient was calculated, and Mantel's test was performed to check the goodness-of-fit of the cluster analysis to the matrix on which it was based. To evaluate the robustness of dendrograms, bootstrap values (1000 replications) were calculated using the software TREECON ver. 1.3b (Van de Peer, 1997). The relationships between the Nei & Li genetic distance matrix and the geographic distance matrix was assessed using Mantel's test. In this case, the geographic distance between two isolates (only for the Portuguese isolates) was defined as the linear distance between the sites.

8.3 *Materials and Methods: BBA, Germany*

8.3.1 Nematode material

Nematode samples for *Bursaphelenchus* species determination were obtained from PHRAME project partners, plant protection services and quarantine control stations. A total of 314 samples was examined, as listed in Table 45.

For *B. xylophilus* pathway analysis, 30 *B. xylophilus* isolates from different geographic origins were obtained from the *Bursaphelenchus* culture collection at PHRAME partner 2. The cultures represent populations from the USA, Canada, Japan, China, South Korea and Portugal (Table 37). *B. mucronatus* and *B. fraudulentus* from Germany were used as outgroup (
Table **38**).

Table 37: Geographic origin of 30 reference strains of *B. xylophilus* used for pathway analysis.

Phrame code	code BBA-AG	country of origin	locality	culture BBA-AG
Phrame1	Ne4/99	Portugal	Marateca, Pegoes	1999
Phrame2	Ne5/02	USA	unknown	2002
Phrame3	Ne11/02	USA	unknown	2002
Phrame4	Ne 5/00	USA	Missouri	2000
Phrame5	Ne4b/00	USA	Missouri	2000
Phrame6	US2	USA	Burlington, Vermont	1994
Phrame7	US9	USA	Tucson, Arizona	1994
Phrame8	US10	USA	Cloquet Forestry Center, Minnesota	1993
Phrame9	US11	USA	Vermont, New Jersey	1994
Phrame10	US15	USA	Cook County, Illinois	1993
Phrame11	Bx Alta	Canada	Smokey Lake, Alberta	1994
Phrame12	Cmz1	Canada	unknown	1994
Phrame13	Q52A	Canada	Quebec	1993
Phrame14	BxBC	Canada	British Columbia	1993
Phrame15	Q1426	Canada	Quebec	1993
Phrame16	St.John	Canada	New Brunswick	1993
Phrame17	Ne3/02	Japan	Tottori City, Admin. Division Tottori	2002
Phrame18	J2	Japan	Izuhara, Nagasaki	1994
Phrame19	J3	Japan	Ueki, Admin. Division Kumamoto	1994
Phrame20	J10	Japan	Nishiaizu, Admin. Division Fukushima	1994
Phrame21	BxJP	Japan	Mito, Admin. Division Ibaraki	1991
Phrame22	C-14-5	Japan	Chiba	1993
Phrame23	Ne28/01	China	unknown	2001

Phrame code	code BBA-AG	country of origin	locality	culture BBA-AG
Phrame24	Ne34/01	China	unknown	2001
Phrame25	Ne12/02	China	Nanjing City forest	2002
Phrame26	Bx China	China	unknown	1993?
Phrame27	Ne5/03	Portugal	Lezirias	2003
Phrame28	Ne4/03	Portugal	Troia	2003
Phrame29	KR1w	South Korea	Jinju, Gyeongsangnam-Province	2003
Phrame30	KR3w	South Korea	Mokpo, Jeollanam-Province	2003

Table 38: Geographic origin of 2 reference strains of *Bursaphelenchus* species used as outgroup for cluster analysis.

PHRAME code	code BBA-AG	country of origin	locality	culture BBA-AG
B. mucronatus	DE4w	Germany	Templin, Brandenburg	1996
B. fraudulentus	DE10w	Germany	Zusmarshausen, Bavaria	1997

The cultures were maintained in Petri dishes on *Botrytis cinerea*/malt agar prior to DNA extraction. Of these 30 cultures, 13 were isolated since 1999. The remaining 17 cultures were older. Most of them had been cultured at BBA or at other institutions since 1993. The oldest culture, Japan18 originating from Izuhara was isolated in 1970. Nineteen cultures were obtained from infested trees of the genera *Pinus*, *Picea* and *Abies*, five from package wood, one from dunnage wood, three from woodchips and the remaining five cultures from hosts or products, where information was no longer available. However, no culture had been isolated from an insect.

8.3.2 ITS-RFLP analysis and ITS1/2 sequencing

For ITS-RFLP or ITS1/2 sequencing, one or a few nematodes were used. Nematode pellets were frozen in liquid nitrogen and crushed with an Eppendorf micro pestle. The homogenate was treated according to the protocol of the DynalBeads genomic DNA Blood Kit (Dynal Biotech, Germany), with omission of the blood-specific steps.

ITS-PCR was carried out employing a 50 µl reaction volume and a Biometra T1 thermocycler. The reaction mixture contained 2 units Taq DNA polymerase (Fermentas, Germany), 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.01% Tween 20 (PCR buffer, Fermentas), 0.1 mM each dNTP, 0.6 µM forward and reverse primer (Table 39) (Roth, Germany) and template DNA. In RFLP analysis, only primers F194/5368 were used. The PCR program consisted of an initial denaturation for 2 minutes, 30 seconds at 96°C, 35 cycles with 1 minute denaturation at 94°C, 1 minute annealing at 55°C, 2 minutes extension at 72°C and a final extension for 6 minutes at 72°C. After completion of the PCR, small aliquots of the samples were separated electrophoretically using a 1.8%-agarose gel and 0.5xTBE buffer. The PCR products were visualised as described above.

Table 39: Primers (f: forward; r: reverse) for amplification and sequencing of ITS rDNA.

Primer	Sequence	origin
K1r	TTACCTACGGCTACCTTGTTACGACT	18S rDNA
K3f	CCCGGGACTGAGTTACTTCGAGA	18S rDNA
F194	CGTAACAAGGTAGCTGTAG	18S rDNA
5.8L f	GTCGATGAAGAACGCAGTGAATTGCG	5.8S rDNA
5.8r	CGCAATTCACTGCGTTCTTCATCGAC	5.8S rDNA
5368 r	TTTCACTCGCCGTTACTAAGG	28S rDNA
D2Ar	ACTTTCCCTCACGGTACTTGT	28S rDNA
D2Ap21r	GGTTTCACGTTCTCTTGCACT	28S rDNA

For ITS-RFLP analysis, suitable aliquots of the amplified DNA were digested with 3 units of restriction endonucleases *Alu*I, *Hae*III, *Hin*fI, *Msp*I and *Rsa*I, following the manufacturer's

instructions. Restriction fragments were resolved by electrophoresis in a 2.5% agarose gel and visualised as described above.

For sequencing, the PCR products were concentrated and desalted using Microcon YM-100 centrifugal filter devices (Amicon Bioseparations). Working steps were performed following the manufacturer‘s instructions. Additionally, the membrane was washed with 50µl ddH_2O. Small aliquots of the each final sample were applied to 1.8%-agarose gel and 0.5xTBE buffer to estimate the DNA concentration.

According to the instructions from the sequencing company (MWG Biotech AG, Germany), 20 ng/100bp of a PCR fragment were air-dried and sent to the company together with the appropriate primer to use their Value Read Service. The fragments were sequenced using primers listed in Table 39.

8.3.3 RAPD and ISSR analysis

Nematodes were extracted from culture medium using the Baermann funnel technique and washed twice with deionised water. For ISSR- and RAPD-PCR, genomic DNA was obtained from bulks of 2000 – 10000 animals of each culture without prior separation according to sex or developmental stage to obtain a representative sample of each isolate. They were transferred in water to an Eppendorf tube and sedimented for 2 minutes at 9000 x g. The supernatant was discarded. Nematode pellets were frozen in liquid nitrogen and crushed with an Eppendorf micro pestle. The homogenate was then treated according to the protocol of the High Pure PCR Template Preparation Kit (Roche, Germany). The yield of extracted genomic DNA was determined using the fluorescent dye, Hoechst 33258 and a DyNA Quant 200 fluorometer (Pharmacia, Germany). DNA extracts were stored at 4°C.

ISSR-PCR was carried out employing a 25 µl reaction volume and a Biometra T1 thermocycler. The reaction mixture contained 2 units Taq DNA polymerase (PeqLab, Germany), 20 mM Tris-HCl (pH 8.5), 16 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.01% Tween 20 (Y-PCR buffer, PeqLab), 0.2 mM each dNTP, 0.8 µM primer (Roth, Germany) and 4 ng template DNA. The PCR program consisted of an initial denaturation for 2 minutes, 30 seconds at 96°C, 35 cycles with 20 seconds denaturation at 94°C, 45 seconds annealing at 42 – 55°C, depending on the primer used (Table 40), 2 minutes extension at 72°C and a final extension for 6 minutes at 72°C. After completion of the PCR, aliquots of the samples were separated electrophoretically using a 1.8%-agarose gel and 0.5xTBE buffer. Gels were stained with ethidium bromide (1 µg/ml) and visualised with a UV transilluminator.

RAPD-PCR was carried out employing a 25 µl reaction volume and a Perkin Elmer 9600 thermocycler. The reaction mixture contained Stoffel buffer (10 mM Tris, pH 8.3, 10 mM KCl), 5 U AmpliTaq DNA Polymerase Stoffel fragment (Applied Biosystems, Germany), 4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM primer (Roth, Germany; Genosys Biotechnologies, USA) and 4 ng template DNA (Tab. 3). The PCR program consisted of an initial denaturation for 2 minutes, 30 seconds at 94°C, 40 cycles with 20 seconds denaturation step at 92°C, 15 seconds annealing at 38°C, 1 minute extension at 72°C and a final extension for 7 minutes at 72°C. The rate of heating from 38°C to 72°C was regulated to 0.3°C/second. The PCR products were visualised as described above.

8.3.4 Cluster analysis of genetic distances of B. xylophilus isolates and statistical treatment of data

Isolate relationships were assessed from bands scored from the fingerprint profiles, evaluated as presence or absence of bands. Distance data matrices were calculated using Nei & Li coefficient (Nei & Li, 1979) and dendrograms were constructed using the Neighbor joining (NJ) method (Saitou & Nei, 1987) and the Unweighted Pair Group Method with Arithmetic Means (UPGMA) (Sneath & Sokal, 1973). Bootstrap values of trees were calculated using PAUP*4.0 (Swofford, 1998).

Table 40**:** Primer sequences, annealing temperatures, number of markers and resolving power (Rp) generated in ISSR-PCR and RAPD-PCR for 30 *B. xylophilus* populations and an outgroup of one *B. mucronatus* and one *B. fraudulentus* population. Wobbles B: C, G or T; D: A, G or T; H: A, C or T; R: A or G; V: A, C or G; Y: C or T.

				30 *B. xylophilus* populations		
		Annealing		with outgroup	without outgroup	
Primer	Primer sequence	temperature [°C]	Primer type	marker [n]	marker [n]	Rp
11	$[GA]_9$-CCA	50	3'-ISSR	43	31	9.00
25	$[AC]_9$-TG	55	3'-ISSR	31	25	10.00
26	$[AC]_9$-GA	55	3'-ISSR	46	37	13.27
54	$[TC]_9$-CG	50	3'-ISSR	26	16	4.73
188	CGT-$[CA]_8$	55	5'-ISSR	37	33	9.40
190	CAG-$[GT]_9$	55	5'-ISSR	32	28	10.00
841	$[GA]_8$-YC	50	3'-ISSR	43	30	12.40
848	$[CA]_8$-RG	42	3'-ISSR	47	40	11.27
857	$[AC]_8$-YG	50	3'-ISSR	45	38	12.60
888	BDB-$[CA]_7$	55	5'-ISSR	39	31	8.87
890	VHV-$[GT]_7$	55	5'-ISSR	42	35	9.00
1423	HVH-$[TGT]_5$	50	5'-ISSR	42	34	13.93
1424	BDB-$[CAC]_5$	50	5'-ISSR	30	26	5.80
1425	BDV-$[CAG]_5$	50	5'-ISSR	27	18	2.27
Total				**530**	**422**	**132.54**
B07	GGT GAC GCA G	38	RAPD	49	40	14.20
Y01	GTG GCA TCT C	38	RAPD	29	23	5.80
Y04	GGC TGC AAT G	38	RAPD	33	28	7.93
Y08	AGGCAG AGC A	38	RAPD	49	45	16.20
Z01	TCT GTG CCA C	38	RAPD	34	32	10.13
Z04	AGG CTG TGC T	38	RAPD	43	38	11.27
Z06	GTG CCG TTC A	38	RAPD	36	29	8.40
Z07	CCA GGA GGA C	38	RAPD	51	39	13.20
Z08	GGG TGG GTA A	38	RAPD	53	42	14.40
Z11	CTC AGT CGC A	38	RAPD	51	40	14.47
Re08	CGA TCG ATG C	38	RAPD	62	50	16.47
Re09	GGA AGC TTC G	38	RAPD	63	55	18.27
Re10	CCC TGC AGG C	38	RAPD	58	48	16.27
Total				**611**	**509**	**167.01**

Resolving power (Rp) of primers (Table 40) was calculated according to Prevost & Wilkinson (1999). The resolving power is useful for estimating the power of primers to distinguish between examined objects like genotypes or isolates. Theoretically, for distinguishing all examined isolates, the Rp-value of a primer must be half of the number of isolates tested.

For testing the hypothesis that fingerprints obtained by RAPD markers and ISSR markers resulted in equal cluster analysis results, correlation between two genetic distance matrices was estimated by means of the Mantel test (Mantel, 1967), implemented in PopTools 2.6.6. (Hood, 2005).

The sequence data were aligned with ClustalW implemented in the computer program Bioedit 7.0.5.2 (Hall, 1999) or Mega 3.1 (Kumar et al., 2004). Phylogenetic trees were generated with Mega 3.1 by neighbour-joining (NJ) and maximum parsimony (MP) algorithms.

8.4 *Materials and methods: INRA, France*

8.4.1 Nematode isolates

The *Bursaphelenchus* isolates used in this study for the development of the diagnostic assay, with their original host and geographic origin, when available, are listed in Table 41. Isolates were cultured monoxenically on the fungus *Botrytis cinerea* in Petri dish on Potato Dextrose Agar medium at 25°C.

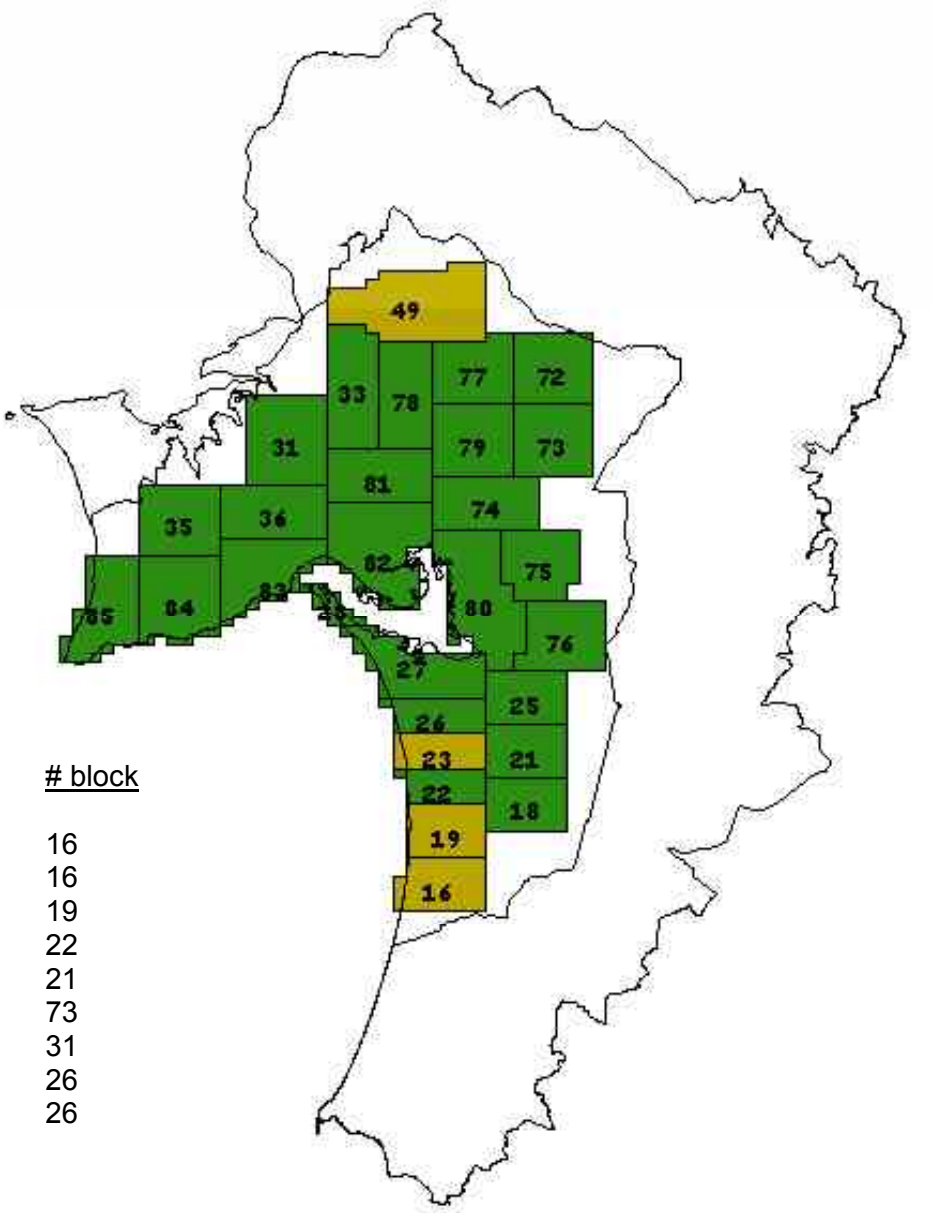
Table 41: Isolates of the *Bursaphelenchus* species tested in this study for the development of the diagnostic assay.

Nematode species	Isolate	Host	Origin
B. xylophilus	J10	*Pinus densiflora*	Japan
B. xylophilus	US9	*P. halepensis*	USA
B. xylophilus	US10	*Abies balsamea*	USA
B. xylophilus	01.667.1	packaging wood	Canada
B. xylophilus	PT-3	*P. pinaster*	Portugal
B. xylophilus	Nanjing 1	*P. thumbergii*	China
B. xylophilus	Tt 6	?	Japan
B. xylophilus	US-DE-3	*P. taeda*	USA
B. xylophilus	US2	*P. strobus*	USA
B. xylophilus	US15	*P. sylvestris*	USA
B. xylophilus	J2	*P. thumbergii*	Japan
B. xylophilus	Japon	*P. densiflora*	Japan
B. xylophilus	Chine	*Pinus* sp.	China
B. xylophilus	01-601-01	packaging wood	Canada
B. xylophilus	Alta	*P. banksiana*	Canada
B. xylophilus	BC	*Pinus* sp.	Canada
B. mucronatus	J13	*P. thumbergii*	Japan
B. mucronatus	BmF	*P. pinaster*	France
B. mucronatus	BmN	*P. sylvestris*	Norway
B. mucronatus	03.397.1	*P. nigra*	France
B. mucronatus	04.907.1	*P. pinaster*	France

Moreover, nine additional *B. xylophilus* isolates from Portugal were provided by Nemalab, University of Evora to perform the local scale diversity analysis. Their distribution in the Setubal infested area is shown in Figure 79.

8.4.2 DNA preparation

Genomic DNA was extracted from pooled nematodes of each isolate tested using the phenol/chloroform method (Sambrook *et al.*, 1989).



Isolate	# block
Bx 18.10	16
Bx 18.13	16
Bx 18.20	19
Bx 22.58	22
Bx 33.29	21
Bx 33.60	73
Bx 37.05	31
Bx 37.20	26
Bx 37.14	26

Figure 79: Spatial distribution of the *Bursaphelenchus xylophilus* isolates sampled in the Setubal infested area in Portugal.

Nematode DNA was also prepared on individual nematodes, according to a simple procedure modified from Williams *et al.* (1992). Briefly, single nematodes were transferred to a dry thin walled PCR tube, covered with 2.5 µl lysis buffer (50 mM KCl, 10 mM tris pH 8.2, 2.5 mM $MgCl_2$, 60 mg/ml proteinase K, 0.45% NP40, 0.45 % tween 20, 0.01% gelatin) and overlaid with mineral oil. Tubes were put at –80 °C for 45 min, and immediately transferred to 60 °C for 60 min and then 95 °C for 15 min in the thermal cycler.

8.4.3 PCR amplification

PCR primers were designed close to both ends of the sequence of the 160-bp monomer of the satellite DNA family previously characterized in *B. xylophilus* (Tarès *et al.*, 1993; GenBank accession L09652); J10-1, 5'-GGTGTCTAGTATAATATCAGAG-3' and J10-2Rc, 5'-GTGAATTAGTGACGACGGAGTG-3'. PCR was carried out in 25 µl reaction mixtures containing 10 ng of template DNA, 50 mM KCl, 10 mM Tris (pH 8.2), 2.5 mM $MgCl_2$, 200 mM dNTP, 250 ng of each of the primers and 1 unit of *Taq* DNA polymerase (Q-Biogene) using a TRIO-Thermoblock thermal cycler (Biometra). After denaturation at 94 °C for 5 min, cycling was performed for 25 cycles of 30 s at 94 °C, 1 min at 64 °C and 1 min at 72 °C, with a postcycling extension at 72 °C for 5 min.

8.4.4 Cloning, sequencing and sequence analysis

The selected products amplified by PCR with primers J10-1 and J10-2Rc were ligated into a pGEM T-vector System I (Promega) and transformed in *Escherichia coli* DH5α-competent cells. Recombinant clones were sequenced by Genome Express (France).

Multiple sequence alignments were done using CLUSTAL X. Phylogenetic analyses were performed using either the parsimony or distance criterion in PAUP* (Swofford, 1998). All nucleotide positions were considered equivalent and weighted equally, using gaps as missing data. Bootstrap values were calculated on 1000 replicates.

8.5 *Results: UEVORA, Portugal*

Results (and Discussion) regarding the morphological and molecular characterization of Bursaphelenchus species from Portugal and their insect associates are described in Penas et al. (2004), Penas et al. (2006a; 2006b). Results (and Discussion) regarding the general synopsis of the genus Bursaphelenchus are described in Ryss et al., 2005. Results (and Discussion) regarding surveys in Turkey and comparative studies with Portuguese species are described in Akbulut et al., 2006 and Vieira et al., 2007) .

With the exception of primers Z9 and Z17, which amplified a wide number of products, causing difficulties for a reliable band scoring, all remaining 28 primers were used for evaluation of amplification products and construction of the binary matrix. A total of 471 RAPD markers were scored for the isolates of *B. xylophilus* These included 24 Portuguese isolates and a duplicate sample of isolate 9 for control of reproducibility, and one isolate each from Asia (Nanjing, China) and North America (Missouri, USA). A total of 290 RAPD markers were scored for the out-group species, *B. mucronatus* (Brandenburg, Germany) (Table 42). The RAPD profiles were different with each of the primers. The variation in the number of fragments was higher among the different primers than among the different isolates, and, depending on the primer, the number of bands observed among the isolates ranged from 7 to 37 (Table 43). Figure 78 presents the RAPD profiles obtained from two of the 28 different primers used in order to illustrate the banding patterns observed. Within the Portuguese isolates, the banding patterns revealed a large number of monomorphic genetic markers in comparison to polymorphic genetic markers; however, intraspecific polymorphism was revealed at a low frequency in some isolates (Table 43).

Table 42: Primer sequences and number of random amplified polymorphic DNA-PCR bands produced by each primer, applied to 27 *B. xylophilus* isolates and 1 *B. mucronatus* isolates.

Primer	Sequence	Marker (Bx)	Marker Out-group (Bm)	Sum of all markers (Bx + Bm)
Z01	TCT GTG CCA C	12	7	16
Z02	CCT ACG GGG A	22	3	24
Z03	CAG CAC CGC A	16	9	23
Z04	AGG CTG TGC T	7	4	9
Z05	TCC CAT GCT G	20	10	28
Z06	GTG CCG TTC A	19	9	25
Z07	CCA GGA GGA C	12	6	18
Z08	GGG TGG GTA A	21	10	31
Z10	CCG ACA AAC C	23	13	29
Z11	CTC AGT CGC A	18	7	25
Z12	TCA ACG GGA C	10	10	19
Z13	GAC TAA GCC C	11	8	17
Z14	TCG GAG GTT C	13	8	19
Z15	CAG GGC TTT C	10	7	16
Z16	TCC CCA TCA C	12	4	15
Z18	AGG GTC TGT G	18	6	23
Z19	GTG CGA GCA A	27	6	32
Z20	ACT TTG GAG G	13	5	15

B07	GGT GAC GCA G	19	5	24
Re6	CGG AAT TCG C	14	8	20
Re8	CGA TCG ATG C	18	6	23
Re9	GGA AGC TTC G	17	7	23
Re10	CCC TGC AGG C	18	10	23
Y01	GTG GCA TCT C	11	8	16
Y04	GGC TGC AAT G	19	11	27
Y06	AAG GCT CAC C	26	12	37
Y08	AGG CAG AGC A	23	12	33
Y16	GGG CCA ATG T	22	11	30
Total	28	471	222	640

The genetic similarity matrix based on the Nei & Li coefficient is presented in Table 44. The lowest similarity (approximately 50%) was reached between the American isolate and all the other *B. xylophilus* isolates. A high genetic similarity was observed between the Portuguese isolates and the isolate from China, ranging from 84% to 94%. Within the Portuguese isolates the genetic distances reached very low values for all combinations of isolates. More than 90% of the pair-wise combinations had more than 95% genetic similarity, and the remaining pair-wise combinations were still above 90% similarity (Table 44). The pair-wise combination between isolate 9 and its duplicate sample (isolate 9') expectedly showed an extremely high genetic similarity (99%), thus illustrating the reproducibility of RAPD profiles obtained with each primer.

Table 43: Number of RAPD-PCR markers produced for the Portuguese *B. xylophilus* isolates.

	RAPD fragment score		
Primer	**Total bands**	**Polymorphic bands**	**Polymorphism (%)**
Z01	7	3	42.9
Z02	16	6	37.5
Z03	13	5	38.5
Z04	6	2	33.3
Z05	15	10	66.7
Z06	15	1	6.7
Z07	10	2	20.0
Z08	16	4	25.0
Z10	17	3	17.6
Z11	15	6	40.0
Z12	6	1	16.7
Z13	9	3	33.3
Z14	8	2	25.0
Z15	8	3	37.5
Z16	8	3	37.5
Z18	13	2	15.4
Z19	22	6	27.3
Z20	9	1	11.1
B07	10	4	40.0
Re6	10	1	10.0
Re8	16	6	37.5

Primer	RAPD fragment score		Polymorphism (%)
	Total bands	Polymorphic bands	
Re9	11	5	45.5
Re10	13	7	53.8
Y01	10	1	10.0
Y04	14	4	28.6
Y06	18	9	50.0
Y08	19	2	10.5
Y16	16	4	25.0
Total	350	106	

Cluster analysis of the genetic distances using the UPGMA algorithm, based upon Nei and Li's similarity, was conducted to generate a dendrogram indicating the relationships among the *B. xylophilus* isolates used in this study (Figure 81). The cophenetic correlation coefficient between the dendrogram and the original distance matrix of the RAPD profiles was significant, with a high correlation value r= 0.99 (1=best possible fit). The dendrogram obtained clearly illustrated the large intraspecific distances between the isolate from the USA and the other isolates from China and Portugal, supported by a high bootstrap value. The position of the Chinese isolate was found to be close to the group of the Portuguese isolates, with strong support by a high bootstrap interaction node value. Within the Portuguese isolates, a remarkable degree of similarity was obtained for all the 24 isolates representing the entire affected area in Portugal. Although some primers revealed a different number of polymorphic bands for some isolates (e.g. PT17 and PT24), all isolates were positioned together in the same and unique cluster (Table 44, Figure 81).

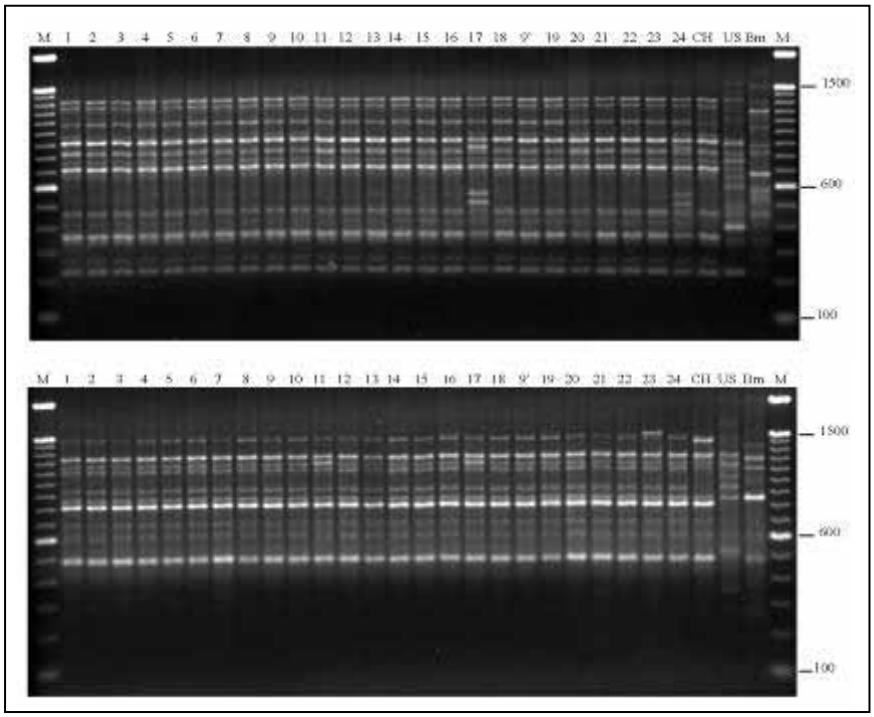


Figure 80: Banding profiles of amplified DNA from 26 isolates of *B. xylophilus*, and 1 isolate of *B. mucronatus* as outgroup, obtained using primer y16 (above) and primer re6 (below). M: marker; *B. xylophilus* isolates: 1-24: Portuguese, 9' as control isolate; CH: Chinese (Nanjing); US: American (Missouri); *B. mucronatus* isolates: Bm: German (Brandenburg).

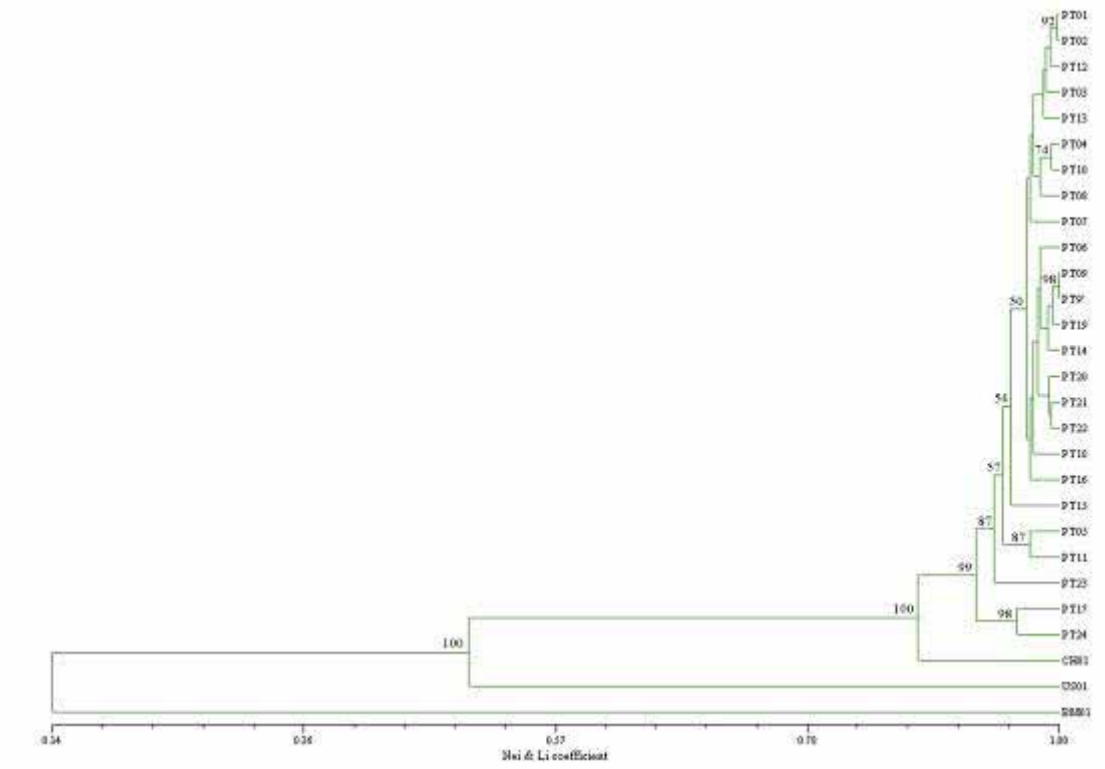


Figure 81: UPGMA tree inferred from 640 RAPD markers for 27 *B. xylophilus* isolates and 1 *B. mucronatus* isolate as the out-group.

UPGMA dendrograms were also constructed (based on Pearson product-moment correlation coefficient, using the software package Gel Compare vs. 4.1), for each single primer, using the profile intensity generated for the 28 isolates, and similar results were obtained, i.e. the USA

isolate was always clearly separated from the other *B. xylophilus* isolates, and the Portuguese isolates were very close to each other and close to the Chinese isolate (data not shown).

Results regarding joint surveys in Russia and in Turkey are shown in Figure 82. Results from the work developed jointly with Turkish colleagues, but combining information on the taxonomy and identification of Bursaphelenchus species from Portugal are now in print (Akbulut et al, 2006, Vieira et al, 2006).

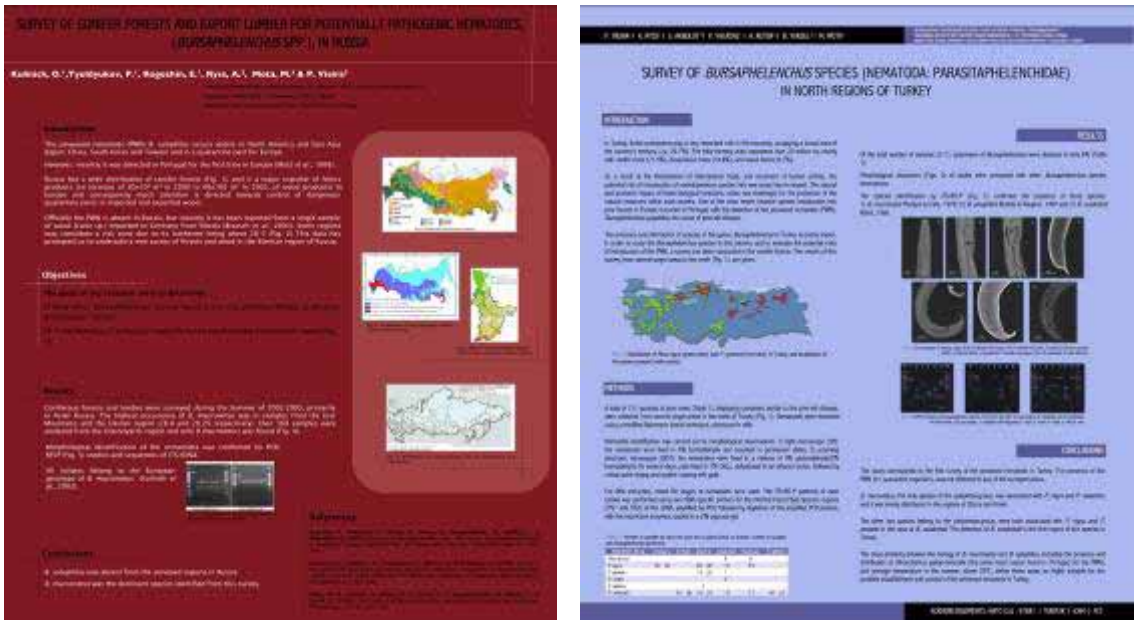


Figure 82: Poster outputs from joint surveys in Russia and Turkey

Table 44: Nei and Li's similarity matrix of 28 isolates

	PT01	PT02	PT03	PT04	PT05	PT06	PT07	PT08	PT09	PT10	PT11	PT12	PT13	PT14	PT15	PT16	PT17	PT18	PT9'	PT19	PT20	PT21	PT22	PT23	PT24	CH01	US01	BmDE
PT01	1.00																											
PT02	1.00	1.00																										
PT03	0.99	0.99	1.00																									
PT04	0.98	0.98	0.98	1.00																								
PT05	0.96	0.96	0.96	0.97	1.00																							
PT06	0.97	0.97	0.97	0.98	0.96	1.00																						
PT07	0.97	0.97	0.98	0.98	0.96	0.97	1.00																					
PT08	0.97	0.97	0.97	0.98	0.96	0.97	0.97	1.00																				
PT09	0.97	0.97	0.97	0.97	0.95	0.98	0.97	0.97	1.00																			
PT10	0.98	0.98	0.98	0.99	0.97	0.98	0.98	0.99	0.98	1.00																		
PT11	0.97	0.97	0.96	0.96	0.97	0.94	0.94	0.95	0.94	0.95	1.00																	
PT12	0.99	0.99	0.99	0.98	0.96	0.98	0.97	0.97	0.97	0.98	0.96	1.00																
PT13	0.98	0.98	0.98	0.98	0.96	0.98	0.97	0.97	0.97	0.98	0.96	0.99	1.00															
PT14	0.97	0.97	0.98	0.98	0.96	0.98	0.98	0.97	0.99	0.99	0.94	0.98	0.98	1.00														
PT15	0.95	0.95	0.95	0.96	0.94	0.97	0.95	0.95	0.96	0.96	0.92	0.96	0.96	0.97	1.00													
PT16	0.96	0.96	0.97	0.96	0.94	0.97	0.96	0.95	0.98	0.97	0.93	0.97	0.97	0.98	0.95	1.00												
PT17	0.92	0.93	0.93	0.94	0.92	0.93	0.94	0.93	0.93	0.94	0.91	0.93	0.93	0.93	0.91	0.92	1.00											
PT18	0.96	0.96	0.97	0.97	0.95	0.97	0.96	0.96	0.98	0.97	0.94	0.97	0.97	0.98	0.96	0.97	0.92	1.00										
PT9'	0.97	0.97	0.97	0.97	0.95	0.98	0.97	0.97	1.00	0.97	0.93	0.97	0.97	0.99	0.96	0.98	0.93	0.98	1.00									
PT19	0.97	0.97	0.98	0.98	0.96	0.98	0.97	0.97	0.99	0.98	0.94	0.98	0.98	0.99	0.97	0.98	0.93	0.99	0.99	1.00								
PT20	0.98	0.98	0.98	0.97	0.94	0.97	0.96	0.96	0.98	0.97	0.95	0.98	0.98	0.98	0.95	0.97	0.92	0.97	0.98	0.98	1.00							
PT21	0.98	0.98	0.98	0.97	0.95	0.97	0.96	0.96	0.98	0.97	0.95	0.99	0.98	0.98	0.96	0.97	0.92	0.98	0.98	0.99	0.99	1.00						
PT22	0.98	0.98	0.98	0.97	0.95	0.98	0.97	0.96	0.98	0.97	0.95	0.99	0.98	0.99	0.96	0.98	0.93	0.97	0.98	0.99	0.99	0.99	1.00					
PT23	0.94	0.94	0.95	0.94	0.93	0.95	0.94	0.93	0.95	0.94	0.92	0.95	0.95	0.95	0.94	0.96	0.90	0.94	0.95	0.95	0.95	0.95	0.95	1.00				
PT24	0.92	0.93	0.94	0.93	0.92	0.93	0.95	0.93	0.95	0.94	0.90	0.93	0.94	0.94	0.92	0.94	0.96	0.94	0.95	0.95	0.93	0.94	0.94	0.91	1.00			
CH01	0.88	0.87	0.88	0.88	0.86	0.89	0.88	0.89	0.89	0.89	0.85	0.88	0.88	0.90	0.88	0.88	0.85	0.88	0.90	0.89	0.89	0.88	0.89	0.86	0.87	1.00		
US01	0.50	0.50	0.50	0.49	0.49	0.50	0.49	0.49	0.50	0.49	0.50	0.50	0.50	0.50	0.50	0.49	0.49	0.50	0.50	0.50	0.51	0.51	0.51	0.49	0.49	0.49	1.00	
BmDE	0.15	0.15	0.15	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.15	0.15	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14	0.15	0.15	0.14	0.14	0.14	0.16	0.14	1.00

8.6 *Results: BBA, Germany*

8.6.1 Species identification of nematode samples

Out of 314 nematode samples obtained for *Bursaphelenchus* species identification by ITS-RFLP analysis, 119 samples could not be identified conclusively, because either no PCR amplicons were obtained or no species affiliation was available from morphology or ITS-RFLP reference patterns. In total, 195 samples were identified at species level and 26 *Bursaphelenchus* species were recorded. Table 45 shows the origin and number of samples and the species identified.

Table 45: Nematodes identified by ITS-RFLP analysis during the PHRAME project. E: Europe type; EA: East Asia type

PHRAME year	country of origin	Number of samples 1y	2y	3y	4y	ITS-RFLP result
1	Austria	1				*Aphelenchoides spec.*
1	Austria	1				*Aphelenchoides stammeri*
1	Austria	1				*B. eggersi*
1	Austria	3				*B. leoni (?)*
1	Austria	3				*B. mucronatus E*
3	Austria			1		*B. pinasteri*
1	Austria	8				*undetermined species*
1	Austria	12				*without PCR product*
4	China				1	*Aphelenchus sp.*
2	China		1			*B. aberrans?*
3	China			1		*B. arthuri sp. n.*
3	China			1		*B. doui*
2	China		2			*B. fungivorus*
2	China		1			*B. leoni type 2*
3	China			4		*B. lini*
2	China		3			*B. mucronatus EA*
3	China			3		*B. mucronatus EA*
4	China				2	*B. mucronatus EA*
2	China		1			*B. new sp.*
2	China		1			*B. pinasteri*
2	China		1			*B. rainulfi*
2	China		1			*B. rainulfi*
3	China			3		*B. rainulfi*
2	China		4			*B. thailandae*
3	China			1		*B. thailandae*
3	China			2		*B. yongensis sp. n.*
2	China		11			*not identified*
4	China				1	*Ruehmaphelenchus asiaticus sp. n.*
2	China		4			*undetermined species*
3	China			4		*undetermined species*
3	Germany			2		*Aphelenchoid species*
1	Germany	1				*Aphelenchoides spec.*
3	Germany			1		*Aphelenchoides stammeri*
4	Germany				1	*B. borealis*
3	Germany			1		*B. eggersi*
3	Germany			10		*B. eremus*
2	Germany		1			*B. fungivorus*
3	Germany			3		*B. fungivorus*
4	Germany				1	*B. fungivorus*
2	Germany		1			*B. leoni type 1*

		Number of samples				
PHRAME year	country of origin	1y	2y	3y	4y	ITS-RFLP result
2	Germany		3			*B. leoni type 2*
2	Germany		5			*B. mucronatus*
1	Germany	1				*B. mucronatus E*
2	Germany		1			*B. mucronatus E*
3	Germany			1		*B. mucronatus E*
4	Germany				1	*B. mucronatus E*
1	Germany	1				*B. mucronatus E, EA*
4	Germany				1	*B. mucronatus E, EA*
2	Germany		1			*B. mucronatus EA*
2	Germany		1			*B. pinasteri*
3	Germany			1		*B. pinasteri*
2	Germany		1			*B. poligraphi*
1	Germany	19				*B. sexdentati*
2	Germany		3			*B. sexdentati*
2	Germany		1			*B. sexdentati*
2	Germany		1			*B. sexdentati*
3	Germany			5		*B. sexdentati*
4	Germany				2	*B. sexdentati*
2	Germany		2			*B. silvestris*
1	Germany	1				*B. teratospicularis*
2	Germany		1			*B. thailandae*
2	Germany		4			*B. vallesianus*
3	Germany			2		*B. vallesianus*
4	Germany				8	*B. vallesianus*
3	Germany			1		*B. willibaldi*
2	Germany		2			*not identified*
3	Germany			25		*not identified*
2	Germany		4			*undetermined species*
1	Germany	8				*without PCR product*
3	India			2		*undetermined species*
2	Italy		1			*B. pinasteri*
3	Japan			2		*B. xylophilus*
2	Japan		2			*not identified*
3	Malaysia			2		*B. singaporensis*
2	Russia		3			*Aphelenchoides sp.*
2	Russia		1			*B. leoni type 1*
2	Russia		1			*B. leoni type 2*
2	Russia		7			*B. mucronatus*
2	Russia		1			*B. mucronatus E*
2	Singapore		1			*B. luxuriosae*
2	Singapore		1			*undetermined species*
4	South Africa				4	*undetermined B. species*
4	South Korea				1	*B. doui*
2	South Korea		3			*undetermined species*
2	Switzerland		5			*B. mucronatus*
1	Switzerland	18				*B. mucronatus E, EA*
1	Switzerland	3				*B. sexdentati*
2	Switzerland		2			*B. vallesianus*
3	Switzerland			10		*B. vallesianus*
2	Switzerland		4			*undetermined species*
1	Switzerland	21				*without PCR product*
2	Taiwan		1			*B. conicaudatus*
4	Taiwan				1	*B. corneolus*
2	Taiwan		1			*B. hylobianum*

PHRAME year	country of origin	Number of samples 1y	2y	3y	4y	ITS-RFLP result
2	Taiwan		1			*undetermined species*
4	Tansania				2	*undetermined species*
4	Turkey				1	*undetermined B. species*
Sum of isolates per year		102	97	88	27	314

ITS-RFLP patterns of five *Bursaphelenchus* species including the European and East Asian types of *B. mucronatus* are shown as examples in Figure 83. A compilation of ITS-RFLP patterns of 26 *Bursaphelenchus* species was published by Burgermeister et al. (2005).

Starting with the first year, 102 samples of at least five *Bursaphelenchus* and two *Aphelenchoides* species were obtained from Germany (31), Austria (29) and Switzerland (42). In the second year, 97 samples of at least one *Aphelenchoides* species and 15 *Bursaphelenchus* species were obtained from Germany (32), Italy (1), Switzerland (11), China (30), Singapore (2), Japan (2), South Korea (3), Taiwan (3) and Russia (13). During the third year, 88 samples were obtained from Germany (52), Austria (1), China (19), India (2), Japan (2), Malaysia (2) and Switzerland (10). Twenty-seven samples of at least two unknown species, one *Aphelenchus* species, one new *Ruehmaphelenchus* species and nine *Bursaphelenchus* species were obtained during the final period from China (4), Germany (14), South Korea (1), South Africa (4), Tansania (2), Taiwan (1) and Turkey (1).

8.6.2 Sequencing of the ITS1/2 region of rDNA

Several new nematode species were identified during the last 3 years (*B. antoniae, B. arthuri, B. doui, B. lini, B. singaporensis, B. willibaldi, B. vallesianus* and *B. yongensis*). Some of these species are very similar to known species of the *xylophilus* group. Therefore, molecular characterisation by sequencing of the ITS1/2 region was done to determine genetic relationships among the new species (Figure 84) and to verify their ITS-RFLP patterns. Table 46 shows the sequence sizes of ITS-PCR amplicons and RFLP fragment sizes of 31 *Bursaphelenchus* species.

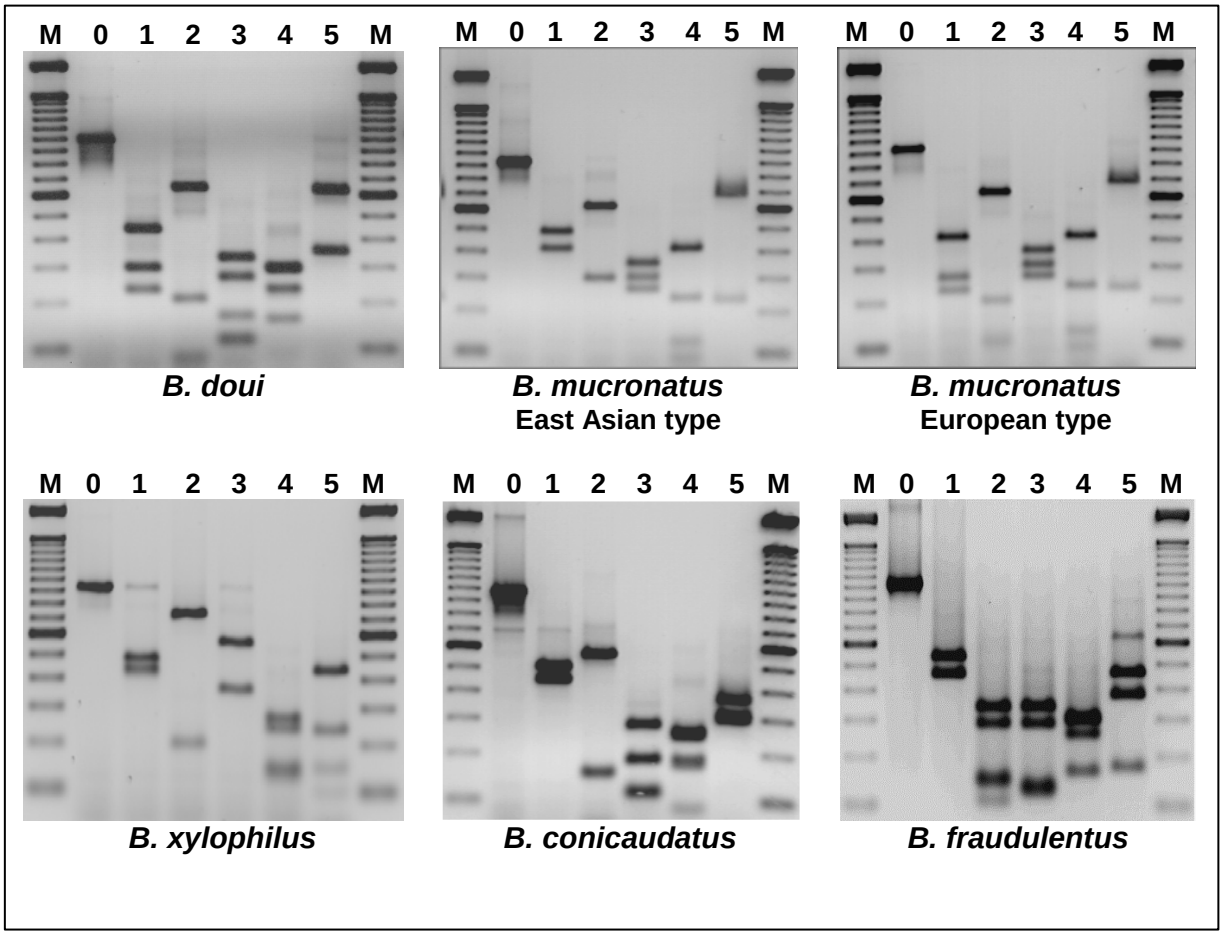


Figure 83: ITS-RFLP patterns of five *Bursaphelenchus* species including two types of *B. mucronatus*. Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with Rsa I (1), Hae III (2), Msp I (3), Hinf I (4) and Alu I (5). M: DNA marker (100 bp ladder, Invitrogen Life Technologies).

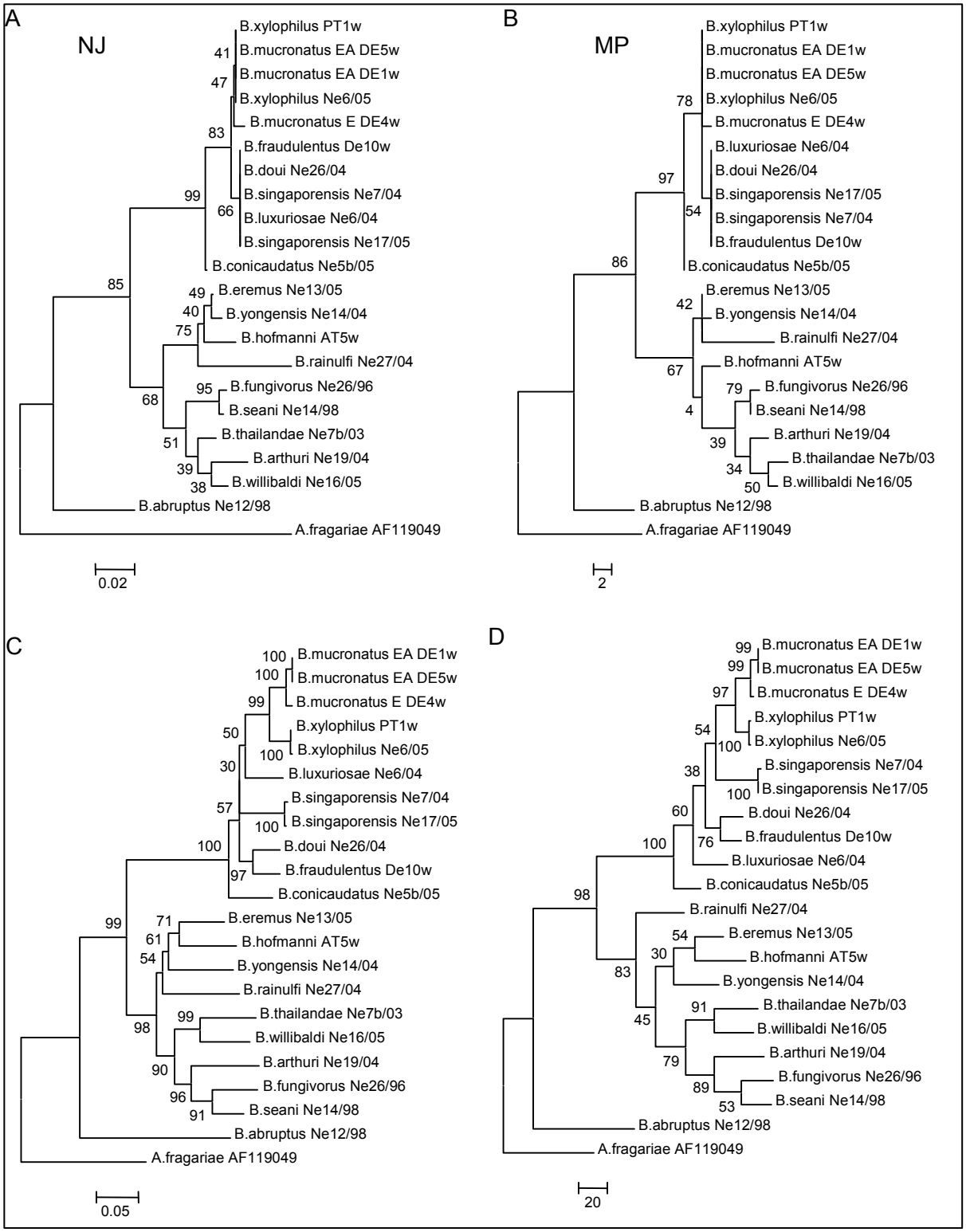


Figure 84: Phylogenetic relationships of *Bursaphelenchus* species. The global sequence alignments for tree constructions were calculated for 5.8S (A, B) and combined sequences (18S partial, ITS1, ITS2, 5.8S and 28S partial) (C, D) by neighbour-joining (NJ) and maximum parsimonious (MP) algorithms. Bootstrap values (%) are given for each node.

Table 46: Sequence sizes of ITS1/2 PCR amplicons obtained with primer combination F194/5368r and RFLP fragment sizes obtained with 5 restriction enzymes for 31 *Bursaphelenchus* species.

species	isolate	ITS	RsaI	HaeIII	MspI	HinfI	AluI
B. abruptus	Ne12/98	1252	717 154 117 111 85 46 22	844 123 103 76 63 43	820 137 127 104 36 28	484 309 274 77 65 24 19	679 227 220 126
B. antoniae	Portugal	1114	582 270 214 26 22	770 321 23	453 343 318	308 260 219 193 110 24	769 345
B. arthuri	Ne19/04	943	271 255 251 166	943	943	219 209 179 170 88 46 32	388 175 173 100 96 11
B. borealis	DE8w	978	286 278 198 128 66 22	529 333 116	978	443 278 212 24 21	964 14
B. conicaudatus	Ne5b/05	971	506 443 22	565 156 149 60 41	274 182 184 112 110 109	253 238 183 166 79 28 24	373 307 291
B. doui	Ne21/04	981	435 296 228 22	640 205 83 53	328 264 165 114 110	283 228 209 154 83 24	616 365
B. eggersi	DE32w	920	371 367 160 22	920	610 310	453 443 24	362 352 192 14
B. eremus	Ne13/05	933	557 354 22	933	636 297	479 219 211 24	261 265 213 194
B. fraudulentus	DE10w	1019	536 461 22	331 288 150 144 106	334 285 135 128 115 18 4	299 284 253 159 24	458 391 170
B. fungivorus	NE26/96	1058	406 364 175 113	854 204	792 203 63	323 297 210 149	652 314 92

species	isolate	ITS	RsaI	HaeIII	MspI	HinfI	AluI
						46 33	
B. gerberi	Ne11/98	1128	558 548 22	1128	509 491 128	480 269 215 140 24	451 310 201 93 37 36
B. hellenicus	GR2w	1038	577 279 160 22	511 379 148	656 382	499 306 201 24 8	319 281 176 117 87 43 15
B. hildegardae	DE24w	922	529 371 22	600 322	608 314	451 447 24	360 354 208
B. hofmanni	AT5w	1034	537 475 22	897 137	379 284 134 125 112	342 333 218 117 24	338 306 263 78 49
B. hylobianum	RUDE16w	1110	567 268 253 22	764 346	345 301 301 163	459 250 216 111 27 24 23	1110
B. luxuriosae	Ne6/04	912	486 404 22	708 143 61	450 238 115 109	255 251 229 153 24	578 334
B. mucronatus E	De4w	925	413 263 227 22	625 195 105	356 303 266	412 232 121 87 49 24	678 247
B. mucronatus EA	De1w	920	486 412 22	621 299	355 302 263	408 232 121 86 49 24	674 246
	De5w		486 412 22	621 299	355 302 263	408 232 121 86 49 24	674 246
B. paracorneolus	DE14w	1007	298 277 156 129 125 22	1007	637 257 113	212 206 199 170 121 48 27 24	744 263

species	isolate	ITS	RsaI	HaeIII	MspI	HinfI	AluI
B. pinasteri	DE32w	1019	549 448 22	1019	628 324 67	266 217 205 194 113 24	332 278 274 135
B. pinophilus	Ne5/04	983	416 346 199 22	586 278 119	983	381 280 212 86 24	983
B. poligraphi	DE17w	972	414 338 198 22	523 333 116	972	437 278 212 24 21	972
B. rainulfi	Ne27/04	1029	264 252 172 147 117 36 22 19	1029	662 367	505 201 190 101 24 8	340 328 194 95 72
B. seani	Ne14/98	924	499 425	924	924	501 423	570 265 89
B. sexdentati	DE29w	981	543 416 22	585 277 119	981	465 280 212 24	967 14
B. singaporensis	Ne7/04	914	474 418 22	800 532 268 114	299 254 237 124	494 261 135 24	357 209 195 153
B. thailandae	Kr2w	880	482 398 333 65	880	880	382 226 202 46 24	555 273 52
B. tusciae	IT14w	920	367 215 160 156 22	596 324	491 310 119	453 443 24	314 236 206 164
B. vallesianus	GR7w	981	543 416 22	862 119	981	465 280 212 24	967 14
B. willibaldi	Ne16/05	1132	543 301 288	1132	731 401	488 359 215 46 24	534 379 126 93
B. yongensis	Ne14/04	905	531 352 22	905	610 295	228 225 218 210 24	390 301 214
B. xylophilus	Ne6/05	925	483 420	728 197	562 363	263 232	433 254

species	isolate	ITS	RsaI	HaeIII	MspI	HinfI	AluI
			22			142	142
						139	96
						125	
						24	

Cluster analysis of ISSR markers from *B. xylophilus* DNA of the same laboratory culture isolated in 1994 and in 2003

DNA samples of six *B. xylophilus* isolates which had been kept frozen since the beginning of 1994, were analysed by ISSR and compared to DNA samples extracted from the same cultured isolates in 2003, to examine genetic stability of *Bursaphelenchus* isolates throughout nine years of culturing. Figure 85a shows an ISSR patterns of stored and fresh DNA extracts side-by-side for each *Bursaphelenchus* isolate.

Some differences in banding patterns have been circled red in the picture of ISSR patterns (Figure 85a). A total of 157 DNA markers obtained with six different ISSR primers were used for genetic distance calculation and construction of a dendrogram. As shown in Figure 85b, pairs of stored and freshly extracted DNA samples from the same isolate were still found in the same cluster. The bootstrap values were high for pairs of samples of Phrame7, Phrame8, Phrame11 (100%) and Phrame9 (88%), Phrame10 (77%), respectively. Only Phrame6 was not well supported (60%).

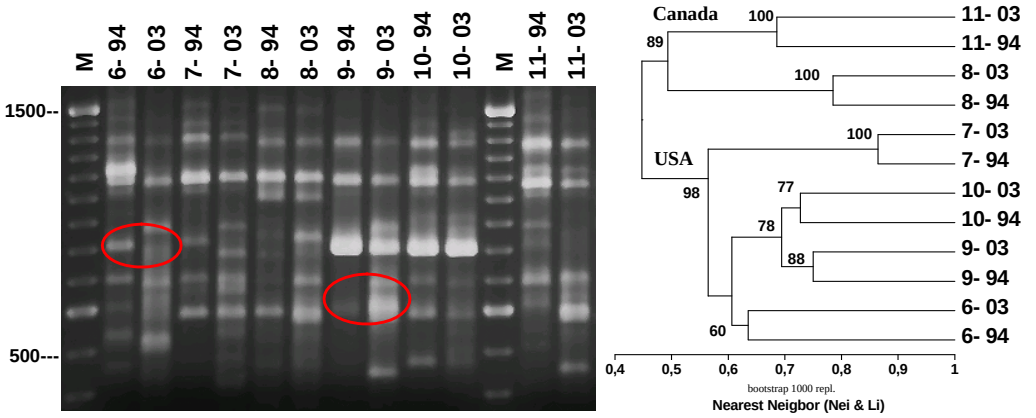


Figure 85 a. Banding pattern of ISSR primer 11. b. NJ dendrogram and bootstrap values obtained from 1000 replicates

In conclusion, cultures of *B. xylophilus* nematodes did change their genetic properties slightly during long culturing periods, but they remained useful to represent their original provenance in our studies. It is certainly preferable to use freshly isolated reference material in pathway analyses, because closely related isolates might otherwise not be distinguishable.

8.6.3 Cluster analyses of ISSR- and RAPD- fingerprints of 30 B. xylophilus isolates

For comparison of genetic relationships, 14 ISSR primers and 13 RAPD decamer primers were used in this study. Examples of banding patterns obtained with an ISSR and a RAPD primer are shown in Figure 86. Amplification of genomic DNA from 32 *Bursaphelenchus* isolates yielded 530 ISSR markers and 611 RAPD markers in total. Of these, 108 ISSR and 102 RAPD markers were contributed by the outgroup of *B. mucronatus* and *B. fraudulentus*. Thus, 422 ISSR and 509 RAPD markers were derived from the 30 *B. xylophilus* isolates. Among these, only eight monomorphic RAPD and five monomorphic ISSR markers were amplified. An average of 47 RAPD markers and 40 ISSR markers were found per primer (Table 40).

When increasing numbers of bands were scored, distance values became constant above a range of 200 to 250 ISSR or RAPD markers. Data evaluation for 530 ISSR markers and 611 RAPD markers resulted in two distance matrices. Both distance matrices were compared using Mantel`s test. The test gave a high correlation for both matrices with r = 0.953 and p = 0.99.

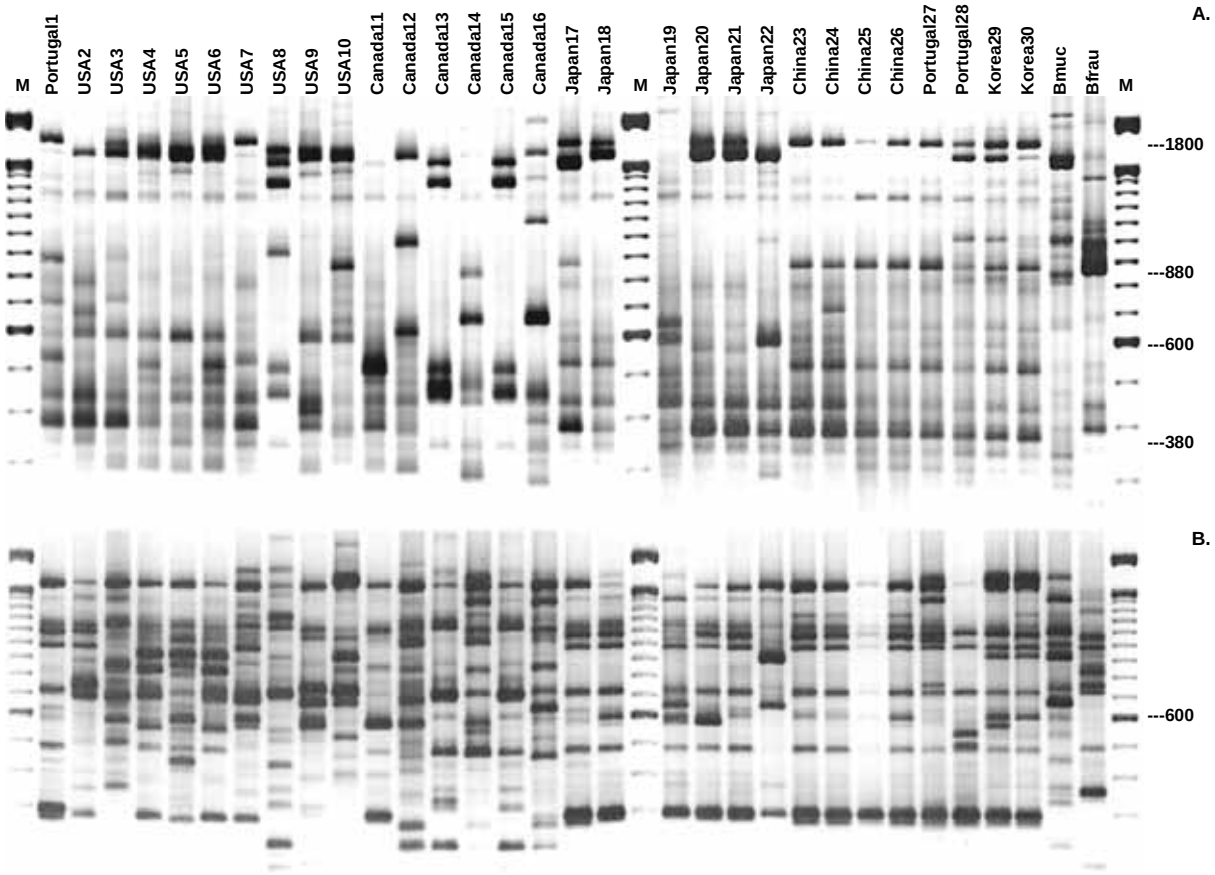


Figure 86: Banding profiles of amplified DNA from 32 *Bursaphelenchus* isolates using ISSR primer 26 (A) and RAPD primer Re09 (B). M: 100 bp ladder (Invitrogen, Germany)

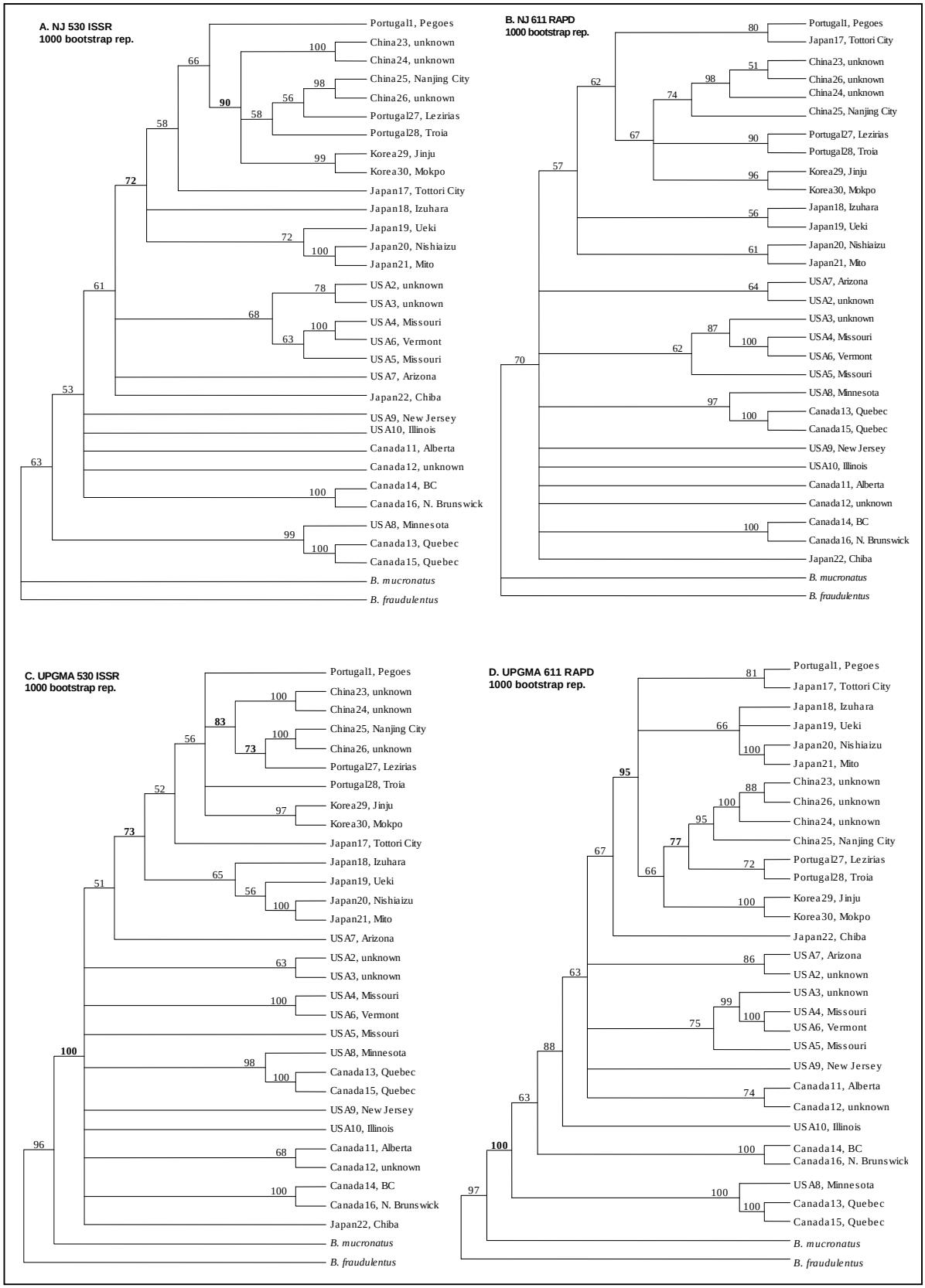


Figure 87: Neighbour joining and UPGMA trees inferred from 530 ISSR (A, C) and 611 RAPD markers (B, D) for 30 *B. xylophilus* isolates, with *B. mucronatus* and *B. fraudulentus* isolates as outgroup. Numbers at the branches represent > 50% values of 1000 bootstrap replicates supporting the indicated subbranches and groups.

Distance values were used for construction of dendrograms, employing the NJ and UPGMA clustering methods (Figure 87). *B. mucronatus* and *B. fraudulentus* were always positioned as outgroup. Both the NJ and the UPGMA trees were almost congruent using ISSR- and RAPD-based distances. With the UPGMA method bootstrap values of 100% support all 30 *B. xylophilus* isolates as one group, the species group. An Asian/Portuguese branch including the two South Korean, four Chinese, five Japanese and three Portuguese isolates forms a separate group within the species, which is sufficiently supported by RAPD (95%) and ISSR (73%). The nine US American isolates and the six Canadian isolates are more or less supported by bootstrap values between those of the outgroup and the Asian/Portuguese cluster. Using the NJ method, the Asian/Portuguese branch is less strongly supported by bootstrap values of 72% (ISSR) and 57% (RAPD).The five isolates USA2, USA3, Canada12, China23 and China24, which had been intercepted from package wood, could clearly be assigned to the countries as indicated in their respective accompanying documents.

Among the North American isolates, only the position of USA7 was less supported by bootstrap values of ISSR-based distances (51%), whereas iteration of RAPD-based distances did not support the position of USA9 from New Jersey and Japan22 from Chiba. In all dendrograms, Japan22 was separated from other Japanese populations. This isolate was grouped more or less closely to the North American branch, depending on the tree constructing method used.

In general, the isolates derived from native populations of North America were genetically more diverse than isolates derived from introduced populations found in the Asian/Portuguese branch. This was demonstrated by the significantly higher number of markers of the group of 15 North American isolates as compared to the group of 15 Asian/Portuguese isolates (Figure 88).

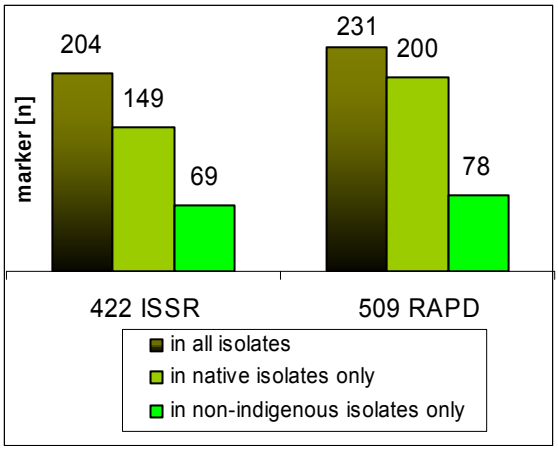


Figure 88: Number of genetic markers found for ISSR and RAPD fingerprints with 15 native and 15 introduced isolates of *Bursaphelenchus xylophilus.*

A total of 149 ISSR and 200 RAPD markers were found in native isolates only and not in isolates derived from introduced populations. On the other hand, 69 ISSR and 78 RAPD markers were detected as new markers in introduced populations.

The Asian/Portuguese branch was separated in a subcluster populated by Chinese, South Korean and Japanese isolates. The three Portuguese isolates were not grouped together. Instead they were distributed at different locations within the Asian/Portuguese branch. Portugal1 from Marateca/Pegoes and Japan17 from Tottori City built a pair when RAPD-based distances were evaluated. However, this result was not supported by ISSR dendrograms. Bootstrap values of 77% (RAPD, UPGMA), 56% (ISSR, UPGMA), 67% (RAPD, NJ) as well as 58% (ISSR, NJ) supported weakly a closer genetic relationship of Portugal27 from Lezirias and Portugal28 from Troia to Chinese isolates. Japan18 from Izuhara, Japan19 from Ueki, Japan20 from Nishiaizu and Japan21

from Mito built a Japanese-specific subcluster which was found in four trees with a bootstrap value of 66% (UPGMA, RAPD). Both isolates from South Korea were paired in a separate branch which was clustered in the Chinese subbranch and not in the Japanese one.

8.7 *Results: INRA, France*

8.7.1 Development of a molecular assay for B. xylophilus identification

Since the satellite DNA family had been shown to be constituted of repeats organized in tandem arrays (Tarès *et al.*, 1993), the amplification of a ladder of multimers of the160-bp monomer was expected in a PCR experiment using the selected primers. Indeed, with primers J10-1 and J10-2Rc, ladder patterns of monomers and multimers of the expected size were amplified from genomic DNA of the *B. xylophilus* isolates only, while no amplification was detected with the other *Bursaphelenchus* species tested (Figure 89). This result confirmed the specific distribution of the satellite DNA family in the genome of B. xylophilus only.

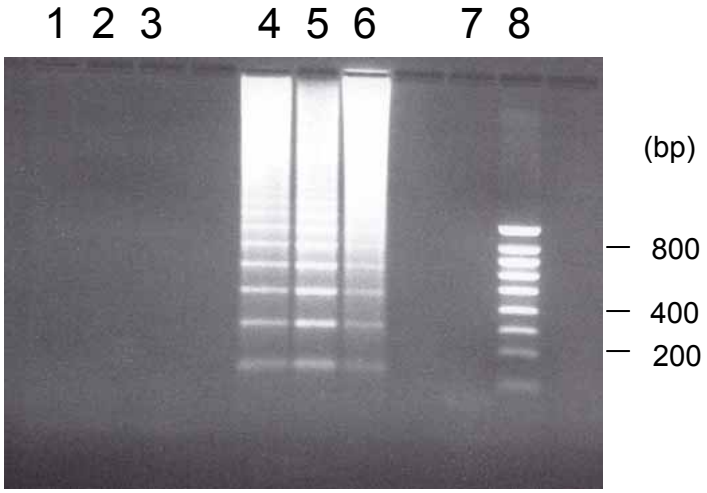


Figure 89: PCR amplification from purified genomic DNA using the satellite DNA-based primer set specific for *Bursaphelenchus xylophilus.* Lanes 1, 2, 3: *B. mucronatus* isolates BmN, BmF and J13, respectively; lanes 4, 5, 6: *B. xylophilus* isolates US9, US10 and J10, respectively; lane 7: water; lane 8: molecular weight marker.

To test the sensitivity of this procedure, amplification of DNA from single nematodes was considered. Use of proteinase K, in combination with the alternation of high and low temperatures, proved to be efficient to make the genomic DNA of a single individual suitable as a template for PCR. Amplification products from *B. xylophilus* single nematodes obtained this way were thus compared to those obtained from *B. xylophilus* phenol/chloroform purified genomic DNA. As shown in Figure 90, individual amplification patterns were identical to the one obtained from *B. xylophilus* genomic DNA, although some differences in banding intensity could occur. To confirm that amplification using J10-1 and J10-2Rc as primers was *B. xylophilus*-specific, PCR was carried with single nematodes from different *Bursaphelenchus* species and isolates. In Figure 90, one *B. xylophilus* isolate from Japan (J10) and two from Canada (01-667-1 and 01-602-1) were used, respectively. As expected, amplification was detected only in lanes corresponding to the three *B. xylophilus* isolates, and a regular ladder pattern was obtained. In contrast, no amplification occurred in samples belonging to other *Bursaphelenchus* species prepared in the same manner. The same results were obtained with the other *B. xylophilus* isolates tested (data not shown). These results have been published recently (Castagnone *et al.*, 2005).

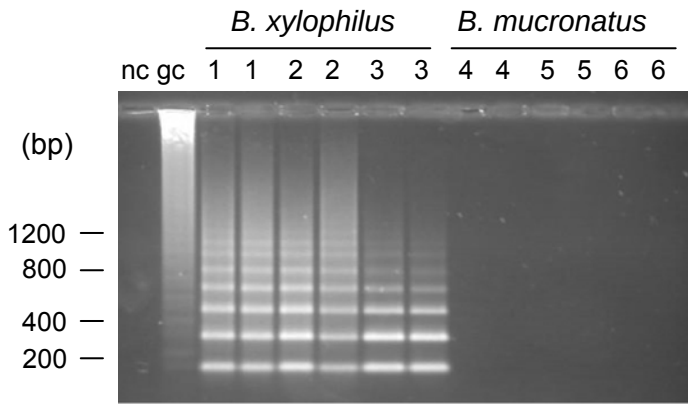


Figure 90: PCR amplification from single nematodes using the satellite DNA-based primer set specific for *Bursaphelenchus xylophilus.* nc, negative control; gc, genomic DNA purified from pooled *B. xylophilus* individuals. Lanes 1, *B. xylophilus* (isolate J10); lanes 2, *B. xylophilus* (isolate 01-667-1); lanes 3, *B. xylophilus* (isolate 01-602-1); lanes 4, *B. mucronatus* (isolate J13); lanes 5, *B. mucronatus* (isolate BmF); lanes 6, *B. mucronatus* (isolate BmN).

8.7.2 Sequence analysis

In order to assess the genetic distances and relationships among the *B. xylophilus* isolates from various geographic origins available from the PHRAME consortium, satellite DNA was used as the molecular target for a phylogenetic comparative sequence analysis. For that purpose, we initiated the cloning and sequencing of monomeric units from *B. xylophilus* isolates from various geographic origins, including Portugal, in order to study the distribution of mutations within and among isolates.

Using the primers designed previously (see section 8.4.3), PCR amplification of satellite DNA repeats was performed for each isolate, and 10 to 15 monomers selected at random were cloned in the plasmid vector pGEM-T. After discarding false positives, a total of 215 clones, representing up to 31,390 nucleotides, was analyzed (Table 47).

For each isolate, a consensus sequence was deduced from the alignment of the sequenced monomers (data not shown). Different levels of variability were detected within and among isolates, along with a differential structuring of this variability, which strongly suggested that these satellite DNA families exhibit differential steps of molecular evolution. In addition, alignment of consensus sequences for the 21 isolates showed alternance of highly conserved and variable domains within the monomeric sequences, which indicated that functional constraints are putatively actively acting upon them. An example of alignment and the presence of conserved domains is shown in Figure 91. These data highlighted the potential of satellite DNA monomer sequence analysis to reveal infraspecific genetic variability in *B. xylophilus.*

Table 47: Global results of the sequence analysis of satellite DNA monomers in *Bursaphelenchus xylophilus.*

Isolate	0rigin	# clones sequenced	total sequence length (nt)
US9	USA	7	1022
US10	USA	6	876
US2	USA	10	1460
US15	USA	10	1460
J10	Japan	12	1752
J2	Japan	10	1460
Japon	Japan	11	1606
Chine	China	11	1606
01-601-1	Canada	12	1752
01-667-1	Canada	11	1606
Alta	Canada	11	1606
BC	Canada	6	876
18.10	Portugal	11	1606
18.13	Portugal	12	1752
18.20	Portugal	12	1752
22.58	Portugal	10	1460
33.29	Portugal	12	1752
33.60	Portugal	8	1168
37.05	Portugal	11	1606
37.14	Portugal	11	1606
37.20	Portugal	11	1606
(21)		(215)	(31,390)

8.7.3 Comparative analysis of B. xylophilus isolates from worldwide origin

The consensus sequences obtained previously were further used in phylogenetic analyses. In the tree shown in Figure 92, no correlation could be found between the position in the tree and the geographic origin of the isolates, except for the Portuguese isolates. Moreover, the comparative analysis of the consensus sequences deduced from the clones from each isolate revealed that the isolates from Portugal are less genetically polymorphic compared to the isolates from North America or East Asia (Figure 92). However, an unexpected polymorphism was nevertheless noticed between the *B. xylophilus* strains originating from the infested area in Portugal.

8.7.4 Comparative analysis of B. xylophilus isolates from local origin

Moreover, a more detailed analysis of the sequences from the Portuguese isolates, in parallel with the exact location where each isolate was sampled, tends to indicate that a significant correlation exists between the genetic distance (based on satellite DNA sequences) and the location of the isolate in the infested region (Figure 93). If confirmed, this result would mean that the progression of the infestation could be followed in space, based on sequence analysis. Further experiments, conducted on a more representative number of Portuguese *B. xylophilus* isolates, are planned in the coming months, in collaboration with University of Evora, to validate this hypothesis.

```
cons667          GGTGTCTAGTATAATATCAGAGTGTTTAGCCTGGTGGGGGCGTAATTT
cons601          GGTGTCTAGTATAATATCAGAGTGTTTRGCCTGGTGSGGGCGTAATTT
consChine        GGTGTCTAGTATAATATCAGAGTTTTTCGCCCAGTGTGGGCGTAATTT
consUS15         GGTGTCTAGTATAATATCAGAGTTTTTCGCCCAGTGTGGGCGTAATTT
consJ2           GGTGTCTAGTATAATATCAGAGTGTTTCGCCCAGTGTGGGCGTAGTTT
consUS2          GGTGTCTAGTATAATATCAGAGTGTTTCGCCCAGTGTGGGCGTAATTT
consAlta         GGTGTCTAGTATAATATCAGAGTGTTTCGCCCAGTGTGGGCGTAATTT
consBC           GGTGTCTAGTATAATATCAGAGTGTTTCGCCTAGTGTGGGCGTAATTT
consJapon        GGTGTCTAGTATAATATCAGAGTGTTTCGCCCAGTGTGGGCGTAATTT
consJ10          GGTGTCTAGTATAATATCAGAGTGTTCGGCCTGGTRCGGGCGCARTTT
consUS9          GGTGTCTAGTATAATATCAGAGTGTTCGGHCCGGTACGGGCGCAGTTT
consUS10         GGTGTCTAGTATAATATCAGAGTGTTCGGCCTGATACGGGCGCARTTT
                 ********************** **  * *   *  ***** * ***

cons667          GACTCCAAAGAAGCTGAGACTTGCCACTCTGAAATCTCATTCAATTAC
cons601          GACTCCAAAARAGCTGAGACTTGCCACKCTGAAATCTCATTCARTTAC
consChine        GATTCCAAAAAAGCTGAAACTTGCCATGCTAAAATCTCAGGCGATTAC
consUS15         GATTCCAAAARAGCTGAAACTTGCCATGCTAAAATCTCATGCGATTAC
consJ2           GATTCCAAAAATGCTGAAACTTGCCATGCTAAAATCTCAGGCGATTAG
consUS2          GACTCCAAAAAAGCTGAAACTTGCCATGCTAAAATCTCAGGCGRTTAC
consAlta         GATTGCAAAAAAGCTGAAACTTGCCGTGCTGAAATCTTACGAGGTTAC
consBC           GATTGCAAAAAAGCTGAAACTTGCCGTGCTRAAATCTTACGAGGTTAY
consJapon        GATTGCAAAAAAGCTGAAACTTGCCGTGCTGAAATCTTACGAGGTTAC
consJ10          DACTCCAAAAAAGGCGAGACTTGCGGTGTTRAAATYTCATTCAGTDAC
consUS9          AVCTCCAAACAAGGCGAAACTTGCGGTGTTAAAATTTCATTCGGTAAC
consUS10         TGCTCCAAAAAAGGCGAGACTTGCGGTGTTAAAATTTCATTCTGTGTT
                    * ****   *  ** ******     * **** * *     *

cons667          GGTTCGAATGGTGTATGTCTTGTCTATTCACTCCGTCGTCACTAATTCAC
cons601          GKTTCGAATGGTGTATGTCTTGTCTATTCACTCCGTCGTCACTAATTCAC
consChine        CTTTCGAATGGTGTATGTCTTGTCAATTCACTCCGTCGTCACTAATTCAC
consUS15         CTTTCGAATGGTGTATGTCTTGTCAATTCACTCCGTCGTCACTAATTCAC
consJ2           CTTTCGAATGGTGTATGTCTTGTCAATTCACTCCGTCGTCACTAATTCAC
consUS2          CTTTCGAATGGTGTATGTCTTGTCAATTCACTCCGTCGTCACTAATTCAC
consAlta         CTTTCGAATGGTATTAGTCCTGTCAATTCACTCCGTCGTCACTAATTCAC
consBC           STTTCGAATGGTRYAAGTCCTGTCAATTCACTCCGTCGTCACTAATTCAC
consJapon        CTTTCGAATGGTATATGTCTTGTCAATTCACTCCGTCGTCACTAATTCAC
consJ10          GTTTCGAATGGTATAGGTCTYGTCTATTCACTCCGTCGTCACTAATTCAC
consUS9          GTTTCGAATGGTATAGGTCACGTCTATTCACTCCGTCGTCACTAATTCAC
consUS10         GTTTCCAATGGTATAGGTCGCGTCTATTCACTCCGTCGTCACTAATTCAC
                   *** ******    ***  *** *************************
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Figure 91: Alignement of consensus sequences of satellite DNA monomers from *Bursaphelenchus xylophilus* isolates from worlwide origin (see Table 41 for isolate codes). Stars indicate positions conserved between monomers.

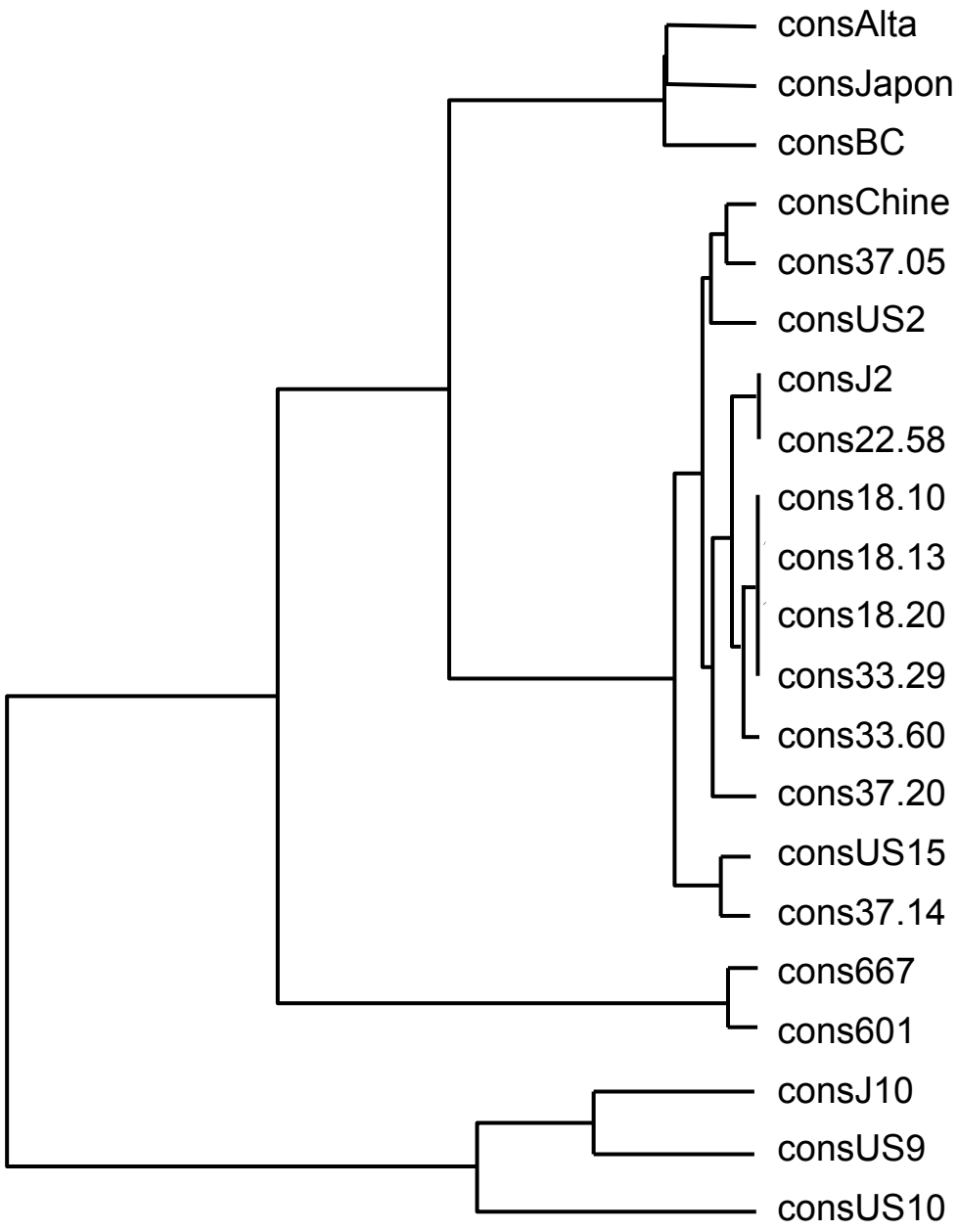

Figure 92: UPGMA tree showing the genetic relationships between *Bursaphelenchus xylophilus* isolates from worldwide origin, including Portugal.

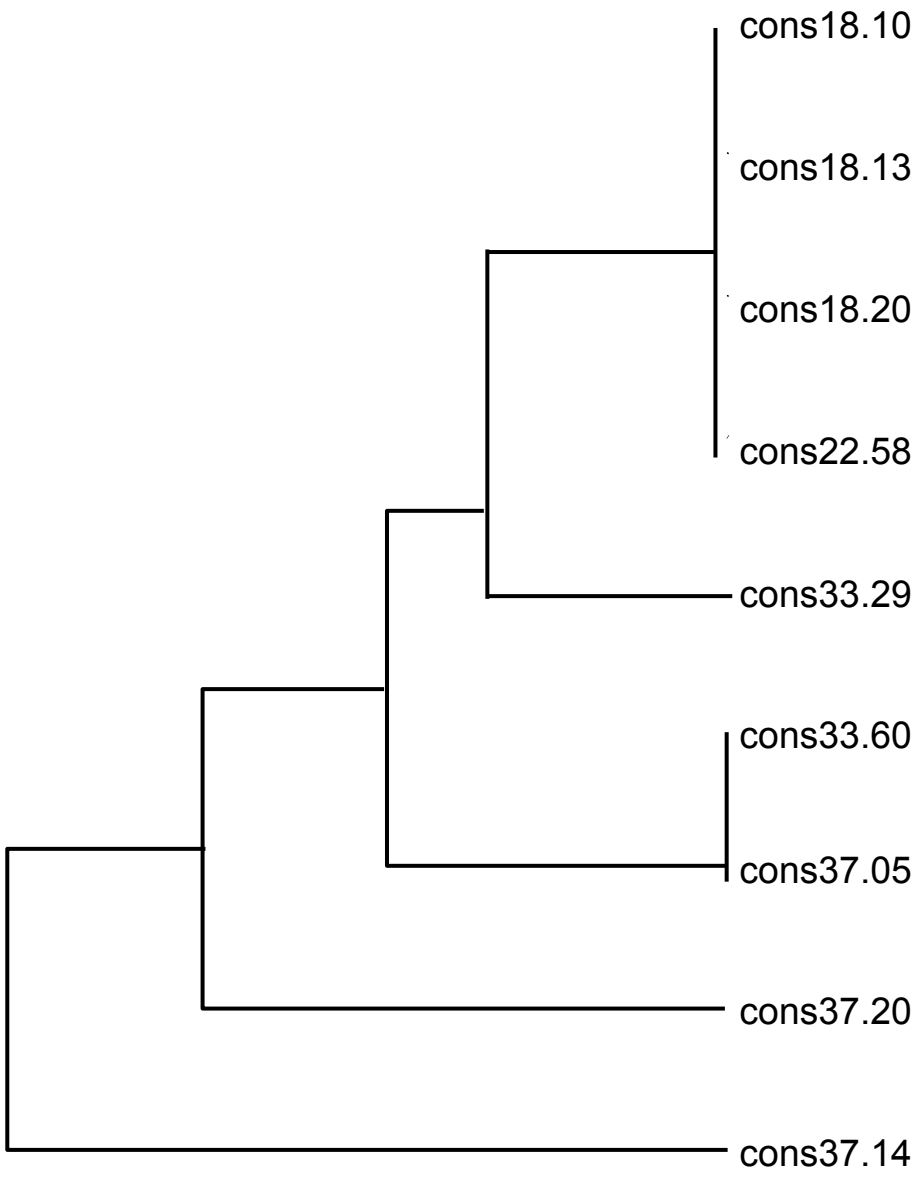

Figure 93: UPGMA tree showing the genetic relationships between *Bursaphelenchus xylophilus* isolates from the Setubal infested area in Portugal.

8.8 *Evaluation of pathways and construction of pathway-submodel*

The results obtained by cluster analyses, especially the clustering of isolates which were taken from populations of widely separated provenances of *B. xylophilus* in East Asia and Portugal, indicate that *B. xylophilus* was translocated to Portugal from its recently colonized sites in East Asia but not from its native habitat in North America (Figure 94).

Figure 94: Possible pathway of introduction of *B. xylophilus* to Portugal.

8.9 *Discussion*

The introduction of the PWN nematode into non-native areas (outside of North America) is related mainly to human factors (Webster, 2004; Yang, 2004). The detection of *B. xylophilus* and other *Bursaphelenchus* species on packaging and wood products found in several countries, clearly demonstrate the importance of trade globalisation and the potential entry/establishment of this organism into endemic forest ecosystems worldwide (Tomiczek et al. 2003; Gu et al., 2006). Since the detection of the PWN in Portugal since 1999, several surveys have been conducted throughout the country; however, *B. xylophilus* has not been detected outside the affected area (Mota et al., 1999; Penas et al., 2004; Rodrigues, 2006). The presence of an international seaport contiguous to the affected area suggests a strong probability of entry with wood packaging of the PWN in Portugal.

B. xylophilus isolates that differ in morphology, pathogenicity, host range and molecular markers have been reported in several studies (De Guiran and Brugier, 1989; Sutherland et al., 1991; Evans et al., 1996; Metge and Burgermeister, 2006). RAPD markers appear to be a useful molecular tool in resolving genetic variation among *Bursaphelenchus* species and populations (Braasch et al., 1995; Irdani et al., 1995; Irdani, 2000; Metge & Burgermeister, 2006), as well as in other nematode species/populations (Caswell-Chen et al., 1992; Schmitz et al., 1998; Syracuse et al., 2004).

The percentage of the DNA markers shared between different populations is expected to be correlated with the degree of genetic divergence of DNA, this proportion declining with the increase of the DNA sequence divergence (Nei & Li, 1979). The number of polymorphisms detected with the RAPD-PCR among the Portuguese isolates was very low, reflecting a high homogeneity in the different primers used. Our data demonstrate low levels of genetic divergence among the Portuguese isolates, sustained by the high similarity among all the pairwise combinations between the 24 isolates (Table 44), and strongly supported by the UPGMA dendrogram (Figure 81).

Previous studies demonstrate a significant degree of genetic divergence among different isolates of *B. xylophilus*, the highest level of intraspecific variability being obtained in the North American isolates when compared with isolates from the non-native areas; and less variability among the isolates of each non-native region. Metge and Burgermeister (2006) (see section 8.6) in a more extensive study applying RAPD-PCR and ISSR for a wide number of isolates (15 North America, plus 12 from Asia and 3 from Portugal) demonstrated two major clusters: one from North America (Canada and US) displaying a high level of genetic diversity, and a second cluster including all isolates from non-native areas (China, Korea, Japan and Portugal), with less genetic diversity.

In the case of the Portuguese isolates, Metge and Burgermeister (2006) suggest the possibility of a double introduction into Portugal from East-Asian areas, based on three different isolates from adjacent blocks of the affected area (Figure 94). In our study, such variation within the Portuguese isolates was not observed, the level of genetic diversity undoubtedly harbouring very limited genetic variations among these, strongly suggest that they may have been dispersed recently from a single source rather than representing different sources of introduction.

To avoid possible genetic shift during fungal culture, all the Portuguese isolates used were collected from pine trees, just before the establishment and execution of these experiments. Although each year, since 1999, all the symptomatic trees from this area have been felled and destroyed (resulting in a hypothetical bottleneck effect), the existence of two different introductions, even from non-native areas, should promote a higher genetic divergence comparing with the results obtained (and comparing with Metge and Burgermeister results). Consequently, conclusions should be drawn carefully relatively to the possible number of the PWN introduction in Portugal.

In Portugal, the affected area occupying 510,000 ha has been stable since 1999, with a slight increase to the south in 2005 (Rodrigues, 2006). The lack of information concerning the occurrence of the PWN, or report of the disease symptoms before 1999, does not clarify the initial point of PWN introduction in Portugal. Under the assumption that the first area to be infested by the PWN was the closest to the seaport, the two isolates at the root of the dendrogram (PT24 and PT17) could be assigned to that probable initial point. Therefore, due to these analyses, and mainly to the high similarity among the Portuguese isolates obtained by the RAPD-PCR, it was not possible to draw a clear correlation between the genetic distances and the geographic isolates of the affected area in Portugal.

Since the introduction of PWN to Portugal is very recent, the use of the RAPD-PCR was not sensitive enough to find such geographic correlations at a small scale, as was demonstrated by others, at a large geographical scale (Braasch et al., 1995; Zheng et al., 1998; Metge and Burgermeister, 2006). In a parallel study within the PHRAME project, a geographic correlation has been established among the Portuguese isolates using satellite DNA (Castagnone, 2006) and section 8.7. This study concluded that polymorphism between the *B. xylophilus* strains originating from the infested area in Portugal, suggests either multiple contaminations or contamination with a mixture of nematodes from diverse origins. New comparative studies using satellite DNA are in progress, in order to provide more information about the genetic structure of the Portuguese isolates, and possible correlation with the geographical distribution in the affected area in Portugal.

More extensive sampling in the native regions will help to identify the source population for the introduced nematodes and determine the history of introduced populations; the efficient assessment in natural populations is a research priority. Information about the genetic diversity of *B. xylophilus* associated with geographic distribution, especially at the country scale, will be very useful to the forest authorities in designing and implementing prevention strategies based on this information.

The sequence analyses of the ITS-PCR amplicons yielded exact sequence lengths of ITS amplicons and RFLP fragments, thus confirming the results of ITS-RFLP analysis of known and newly described species. However, some of the isolates examined revealed ITS rDNA sequence microheterogeneity. A cause of microheterogeneity within an individual nematode is the presence of repeated ITS rDNA sequences and point mutations in some of them. Sometimes, these microheterogeneities are at restriction sites and can be detected by digestion with corresponding restriction enzymes. With *B. singaporensis*, *B. lini* and *B. yongensis*, microheterogeneity was already suspected from extra bands in their ITS-RFLP patterns, but with *B. yongensis* it was not detectable by ITS-RFLP analysis. The base variations were only detected by sequencing. Nevertheless, ITS-RFLP analysis of nematode samples is an essential step for identification and diagnostic verification of *Bursaphelenchus* species.

The sequence alignments of rDNA regions demonstrated that the very high sequence polymorphism of the ITS2 loci limits phylogenetic studies based on ITS2 on broad relationships in the genus *Bursaphelenchus*, but it is useful to distinguish species on a closely related interspecific level (Metge et al. 2006). Both ITS loci separate groups within the genus (*xylophilus/ fungivorus*/ other groups such as *hofmanni*) and very closely related species such as *B. xylophilus/ B. mucronatus/ B. singaporensis* and *B. fraudulentus/ B. doui* as well as *B. fungivorus/ B. seani*. The 5.8S rDNA region alignment and the combined sequence alignment of partial 18S, ITS1, 5.8S, ITS2 and partial 28S suggest two co-evolutionary dependent branches in the phylogenetic tree: the first one includes species of the *xylophilus* group; they are phoretically associated with longhorn beetles. Species of the second branch belong to the *hofmanni* group and *fungivorus* group. As far as it is known from species of these groups, they are phoretically associated with bark beetles. *B. abruptus*, which is associated with bees, is separated from both branches at high genetic distance (Figure 84).

The results obtained in our pathway analyses illustrate the applicability of ISSR and RAPD techniques for genetic differentiation of *B. xylophilus* isolates from various countries worldwide. In general, ISSR markers were more reliable and robust than RAPD markers, mainly due to the fact

that ISSR primers are relatively long, permitting more stringent PCR conditions and fewer primer/template mismatches (Abbot 2001). Comparing RAPD and ISSR distance matrices, Mantel's test revealed a high correlation. This supports our hypothesis that both methods are equally suited in phylogenetic studies of *Bursaphelenchus* isolates. ISSR- and RAPD-derived fingerprints have not been compared before in genetic analysis of nematodes. The cluster analyses resolved thirty *B. xylophilus* isolates into an Asian/Portuguese branch and a North American group. Concerning the Canadian isolates, the close vicinity of Canada13 and Canada15 was as expected, since both originate from Quebec. On the other hand, the subcluster calculated for Canada14 from British Columbia and Canada16 from New Brunswick was surprising concerning the large geographic distance between them. These two isolates were isolated before 1992 and 1993, respectively and maintained in different laboratories successively. Therefore an error in designation might have occurred.

Low genetic distance values were found within the subclusters of the isolates from China, South Korea and Japan. In contrast, the Portuguese isolates were assigned to two different branches separated at higher genetic distances. This suggests that the founders of the Portuguese reference populations could have been introduced at least twice to Portugal from different sites. If the three Portuguese isolates were descendants of a single introduction or the same site, they would be expected to cluster in one distinct branch as seen with the Korean isolates. Genetic shift during laboratory culturing may also influence the pattern of detectable markers of *B. xylophilus* reference isolates. DNA samples of the same *B. xylophilus* isolates, obtained from cultures in 1994 and 2003, were examined by ISSR- and RAPD-PCR and cluster analysis of genetic distances (Metge et al. 2004). Slight differences between the 1994 and 2003 DNA extracts of the same culture were noted by direct comparison of electrophoretic fingerprints. Nevertheless, clustering of the 1994 DNA extracts corresponded to the 2003 DNA extracts of the same populations. The isolates examined (USA6, USA7, USA8, USA9, USA10 and Canada11) were also included in this study and clustered together with additional isolates originating from North America (Figure 87).

Although it was only found in 1999, it is certain that *B. xylophilus* was introduced to Portugal before 1999. It is hypothesised that the founders of this population were translocated by means of package wood. While the number of founder individuals translocated by package wood may have been small, they may not represent the whole genetic diversity of the species. If they were carried by their vectors, up to ten-thousands of nematodes originating from one tree could be translocated by one beetle (Sousa et al. 2001). Nevertheless, the variation of SSR- and RAPD-elements in the newly established population may be reduced. This effect will be strong when all founders were descendants from the same isolate, or weak when several colonisation events occurred in the same new area (Sakai et al. 2001). A lower number of ISSR and RAPD fragments could be expected for recently introduced *B. xylophilus* populations. We confirmed a reduced genetic variation of RAPD and ISSR markers in 15 introduced populations as compared to 15 native populations (Figure 88). A number of markers were found within isolates derived from native populations only and absent in isolates derived from introduced populations. On the other hand, a lower number of new markers were also detected in isolates derived from introduced populations. In general, the DNA fingerprints within the East Asian and Portuguese isolates appeared more homogeneous compared to the patterns obtained from North American isolates. This is most likely due to a higher genetic variability within native as compared to introduced populations.

Molecular diagnostics of *B. xylophilus*

The results obtained in the INRA study illustrate the specific distribution of satellite DNA sequences already documented between closely related taxa of nematodes of agronomic interest (Grenier *et al.*, 1997). Moreover, because of the repetitive nature of this satellite DNA family, positive amplification was achieved from single nematodes. Such high sensitivity of the technique is due, at least in part, to the presence of a large number of monomers of the *Msp*I satellite in each cell of the pinewood nematode. Indeed, this repetitive family was estimated to constitute up to 30% of the genome of the nematode (Tarès *et al.*, 1993). Taking into account the genome size, estimated to be about 30.10^6 bp for the closely related species *B. mucronatus* (Leroy *et al.*, 2003), this indicates an approximate copy number of 56.10^3 per haploid genome, which clearly designates satellite DNA

as a very suitable target for diagnostics.

In wood samples, the pinewood nematode is often associated with the closely related non-pathogenic species *B. mucronatus*, which constitutes the most prevalent *Bursaphelenchus* species found in European coniferous forests (Braasch, 2001; Lee Robertson, pers. com.). In this context, detection of the pinewood nematode in samples containing both species appears essential for diagnostic purposes. The procedure presented here meets such a requirement, since *B. xylophilus* and *B. mucronatus* were always clearly separated in our tests. From that point of view, it should be considered as a candidate methodology for diagnosing pinewood nematode infection in epidemiological surveys as well as in regulatory testing.

Because the result of the assay is very simple (a 'yes-no' answer), the primer set developed here will be useful in positively identifying *B. xylophilus* in samples collected in the wild, and could contribute to the development of a simple diagnostic procedure for this quarantine pest. However, notwithstanding the accuracy of this assay, it is dependent on how representative the original wood fragment is of the sample being assessed (e.g., coniferous tree during a field survey, packaging wood during a quarantine inspection) and how consistently the nematodes were extracted from that wood fragment. Currently, the Baermann funnel technique is universally used to recover nematodes from pine wood (Mamiya, 1975), although it may not allow collection of nematodes from samples containing few or weakened nematodes. Clearly, detection of the pinewood nematode directly from wood samples rather than from recovered nematodes would be a further step towards routine testing of a large number of samples in a reduced time, and will constitute the prolongation of this study. The need for such a test is especially pressing in light of the increasing impact of this invasive pest on international trade regulation.

Sequence variability of satellite DNA monomers

The variability in sequence monomers observed both within and between isolates is quite exclusively due to point mutations, a feature common to this kind of repetitive genetic elements. Because satellite DNAs are non coding sequences, they are thought to diverge rapidly during evolution. However, the observation that nucleotide changes are shared among monomers supports the hypothesis that some highly effective homogenization mechanism acts upon them (such as gene conversion or unequal crossing-over; Dover, 1986), in contrast to the accumulation of independent mutational events.

Alignment of all the consensus sequences from all the isolates tested showed domains with different levels of variability preserved across the set of sequences. Polymorphism observed recently in satDNA from the root-knot nematodes *Meloidogyne* spp. was interpreted as an indication of constraints imposed on particular segments of the monomer sequence (Mestrovic *et al.*, 2006). It was suggested that the effect may be caused by functional interactions between the satellite DNA and various protein components in heterochromatin and/or in the functional centromere, although direct experimental evidence for this hypothesis is still lacking. However, the same observation performed here on *B. xylophilus* sequences reinforces the hypothesis that non-random accumulation of mutations due to selective pressure on particular segments of the monomers directs the evolution of satellite DNA.

Relationships between *B. xylophilus* isolates at a regional scale: the case of the Setubal infested area in Portugal

No correlation between geographic origin and sequence variability was observed when comparing the satellite DNA sequences from *B. xylophilus* isolates sampled at a worldwide scale, except for the Portuguese isolates that appeared clustered on the phylogenetic trees (Figure 92). This is probably linked to the ancient origin of the North American and Asian isolates compared to the Portuguese isolates, and the accumulation of mutations in the genome of these isolates. On the contrary, the lower level of polymorphism and the closer relationships between the Portuguese isolates probably reflects the recent colonisation of Portugal by the nematode. However, an

unexpected polymorphism was noticed between the *B. xylophilus* strains originating from the infested area in Portugal, which suggests either multiple contaminations or contamination with a mixture of nematodes from diverse origins.

Moreover, a more detailed analysis of the sequences from the Portuguese isolates, in parallel with the exact location where each isolate was sampled, tends to indicate that a significant correlation exists between the genetic distance (based on satellite DNA sequences) and the location of the isolate in the infested region. If confirmed, this result would mean that the progression of the infestation could be followed in space based on sequence analysis. Further experiments, conducted on a more representative number of Portuguese *B. xylophilus* isolates, are planned in the coming months, in collaboration with University of Evora, to validate this hypothesis.

8.9.1 Conclusions

Species-specific ITS-RFLP reference patterns for more than 30 *Bursaphelenchus* species have been established within recent years. Identifications can be carried out on single animals and any developmental stage present in a nematode sample and do not require the presence of both sexes for species determination. ITS-RFLP analysis has become a valuable tool for identification of PWN and for investigations on the distribution of various *Bursaphelenchus* species. ITS-RFLP patterns have also been used as additional identification criteria in the description of several new *Bursaphelenchus* species.

Sequence analysis of the ITS1/2 region of rDNA has provided detailed information on genetic relationships between many different *Bursaphelenchus* species. Alignment studies using ITS1/2 sequences have been used to confirm and sometimes correct the affiliation of *Bursaphelenchus* species to species groups based on morphological features.

ISSR and RAPD polymorphisms permitted conclusions to be drawn on the origin of the Portuguese population. Clustering of biogeographically widely separated isolates from Japan, China and Portugal is an indication that PWN introduction was probably from East Asia to Portugal. The association of the Portuguese PWN isolates in two different subgroups within the Asian cluster indicates that founders of the Portuguese population have been translocated to Portugal from their recently colonised sites in East Asia and not from their native habitats in North America.

Translocation of *B. xylophilus* over large distances may easily be explained when the scale of international trade involving timber and package wood is taken into consideration. Four isolates (USA2, USA3, China23, China24) had been isolated from package wood. Their affiliation to the respective countries is only based on trading documents and has been confirmed by the results of our cluster analysis. This is not trivial since package wood is often used repeatedly and transported to third party countries. Until 2001 package wood was not examined officially for *B. xylophilus*. This has most probably facilitated its introduction to Portugal before 1999. Since 2001 the European Commission has ordered phytosanitary treatment of all susceptible packaging wood originating from PWN-infested countries or regions (Commission Decision 2001/219/EC). In spite of EU quarantine regulations, living PWN have been recorded in 63 samples of conifer wood (mostly package wood) imported to EU countries from 2000 to 2006 (Metge & Burgermeister 2005). Molecular phylogenetic approaches are useful tools to trace the origin of PWN found in package wood if reference PWN material from different provenances is available.

Chapter 9 Ecoclimatic risk factors and modelling of the likelihood of wilt expression

9.1 *Statistical and correlation modelling of Bursaphelenchus xylophilus dispersal and risk profiling within Portugal*

Pine Wilt Disease (PWD) could be a serious threat to pinewood forests in Europe, seriously affecting Scots pine (*Pinus sylvestris*) and maritime pine (*P. pinaster*) (Suzuki, 2002). The nematode causing the disease, also known as the pinewood nematode (PWN), is *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (Kiyohara and Tokushige, 1971) and its vector is usually a *Monochamus* sp. (Coleoptera, Cerambycidae) (Mamiya and Enda, 1972; Linit, 1988). The nematode is believed to have been introduced to Japan from the USA, and than spread to Taiwan, Korea and China (Kishi, 1995); more recently the PWN was identified in Europe (Mota *et al*., 1999). This section of the Report deals with the threat of the PWN occurrence outside the European affected zone, the Setubal Peninsula, in Southern Portugal, by modelling its potential distribution through different approaches.

We first focused on the nematode (the pest): we produced a model of the present distribution of the PWN on the Setubal Peninsula, using real data from the PROLUNP series, expecting to use this model to further extrapolate for the whole Portuguese territory and finally to produce a model of the PWN potential distribution over the Iberian Peninsula. Of all environmental data, research in Japan has already shown that the PWN distribution especially correlates with climatological variables (Rutherford and Webster, 1987; Rutherford *et al.,* 1990; Suzuki, 2002). A GIS (Geographic Information System) was built to store, integrate and model environmental data and data on the presence of the PWN in the Setubal Peninsula. This technology associated with statistical modelling is a powerful tool for extrapolating species distribution (Austin 1998). Unfortunately, different series of data in the model (PROLUNP includes three years of surveys) predicted quite different patterns of distribution for the Iberian Peninsula, making it clear that it was inappropriate to proceed with any kind of extrapolation using this model.

Yet also it is well-established that drought increases the susceptibility of pines to secondary pathogens and that warm spring and summer temperatures increase the reproductive rate of bark beetles (Wermelinger and Seifert, 1998, 1999) and pine wood nematodes (Rutherford and Webster, 1987; Bakke *et al*., 1991). Since the disease is favoured by hot summers (Rutherford and Webster, 1987), Southern Europe is especially at risk, but Japanese experience suggests that an epidemic could spread from such areas to climatically less favourable areas provided susceptible tree species are present. Also the presence of PWN in Europe and the on going climatic change may increase the threat of the PWD spreading North. Actually, climatic change may already be affecting pine decline in central Europe, where the number of warm days (mean>20 °C, maximum>25 °C) and potential evapotranspiration have significantly increased over the last 20 years (Rebetez and Dobbertin, 2004). At present, when the infestation focus is as yet geographically localized, it is of utmost importance to explore susceptibilities of pine forests in the surrounding regions, in order to eventually prevent mortality or to act as corridors for the disease. A second approach was thus tried, focusing on the pine trees (the host): this time we looked at the probability of a tree dying from the PWD in Portugal, independently of the nematode having been recorded or not in the region where the tree was located.

This second approach centred on the susceptibility of the tree to infestation, taking into consideration the literature that shows death to prevail in trees that were already under physiological stress (Rutherford and Webster, 1987; Suzuki, 2002, PHRAME findings). Since drought, in particular, is known to reduce tree resistance against pathogens (Rebetez and Dobbertin, 2004), our models assumed that if water stress reduces tree resistance to infestation, trees located in areas of high water stress will be especially prone to die from PWD; in contrast, trees that growth on sites of high suitability are expected to resist infestation. Given that also environmental stress hampers tree growth, we proceeded with the modelling of *P. pinaster* growth

conditions throughout the country. By overlaying the present distribution of the Atlantic pine with site suitability it was possible to identify areas of especially high risk to infestation by the PWN: those of high pine density and low suitability.

9.2 *Materials and methods*

9.2.1 Study area

Under study was the Setubal Peninsula, the whole of continental Portugal, and the Iberian Peninsula (Figure 95).

9.2.2 Variables

The data integrated into the PHRAME GIS can be grouped as: (1) Administrative information; (2) Forestry data; (3) Climatological data; (4) Bioclimatic data; (5) Digital Terrain Elevation Models (DTEM) data, (6) Lithological data and (7) PWN data. Data were considered separately for Portugal and for the Iberian Peninsula (Portugal and Spain).

9.2.2.1 Administrative data

In spite of being a continuous piece of land, there are many differences in the way geographical information is processed in the two countries that make up the Iberian Peninsula. Thus, if the information used in the models produced for Portugal was straightforward, in the case of the cartographic grid for the Iberian Peninsula it was necessary to make compatible information produced under different geographic metrics. Administrative data include boundaries and road network (Portugal - VPoly[2] - 1:25.000; Iberia - 1:100.000; Figure 97).

[2] Vector Polygon Data (ESRI Shapefile)

Figure 95: Study area: (a) Setúbal Peninsula *(*green dots: *Pinus pinaster* presence; lines represent affected areas in different years); (b) Portugal and (c) Iberian Peninsula

9.2.2.2 Forestry data

Pinus pinaster distribution data were obtained from photo-interpretation of 129 019 data points used by the National Forest Service (DGRF) for forests' monitoring (DGF, 2001) (see Figure 96); of these, 645 stands were checked in the field for data on tree age and biomass, data that was later used to calibrate the growth productivity model for *P. pinaster*.

Figure 97: Administrative Boundaries for Portugal [VPoly - 1:25.000 scale] and the Iberian Peninsula [VPoly - 1:100.000 scale]

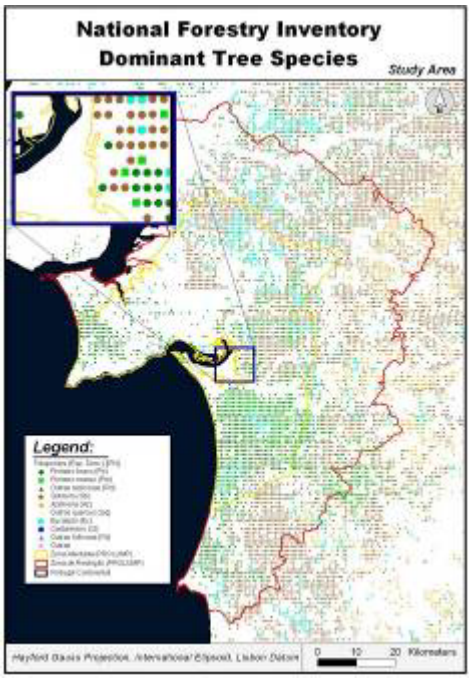

Figure 96: Point and Polygon Data from the National Forestry Inventory with information on main land use and dominant tree species (DGF, 2001)

9.2.2.3 Climatological Data

Unfortunately, climatological data for Portugal were not as yet available as continuous variables. Thus, our first task was to apply co-kriging to the 30-years data series of monthly climatological data to produce suitable variables for the modelling.

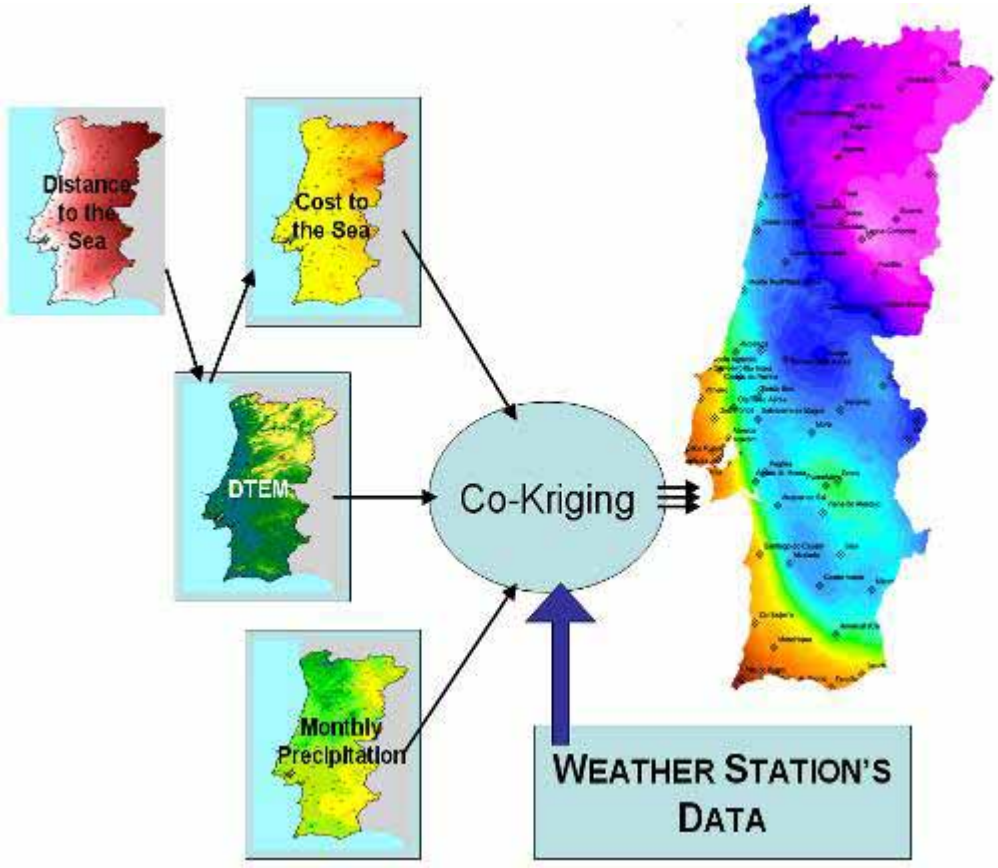

Figure 98: Environmental variables for Portugal, interpolated by co-kriging using data from 79 weather stations (30 years dataset), a Digital Terrain Elevation Model and a Monthly Precipitation Model

The data available were collected at 79 weather stations over a period of 30 years: (1) Frost (Number of Days), (2) Humidity (%), (3) Insolation, (4) Average Precipitation, (5) Number of days with precipitation over 0,1mm, 1mm and 10mm, (6) Average Daily Temperature, (7) Average Daily Temperature at 9am, (8) Maximum and Minimum Absolute Temperature, (9) Average Maximum and Minimum Temperature, and (10) Average Wind Speed. Using a co-kriging methodology, with the DTEM and the variable "*distance to the coast"* as co-variables, it was then possible to produce layers of climatological information to cover the whole territory (Figure 98).

For the Iberian Peninsula, we used a 1Km resolution model in RASTER format [RST – 1km] from the WORLDCLIM Project (http://www.worldclim.org/) that was georeferenced to a system compatible with the remaining data used in the PHRAME Project. Variables used included: (1) Monthly minimum, maximum and average temperatures (36 datasets – see Figure 99 for an example), (2) Monthly number of days with minimum temperature below 0 ºC, above 20º and above 25º – (36 datasets – see in Figure 100 an example for Portugal), (3) Mean Monthly Evapotranspiration (mm) – 12 datasets, and (4) Monthly Sunshine Duration. The latter was calculated for Portugal [250m resolution] and the Iberian Peninsula [1Km resolution] from the average number of hours of Sunshine per day, derived from Digital Terrain Elevation Model – 12 datasets, and (5) Monthly Precipitation Model for Portugal and IP - 15 datasets.

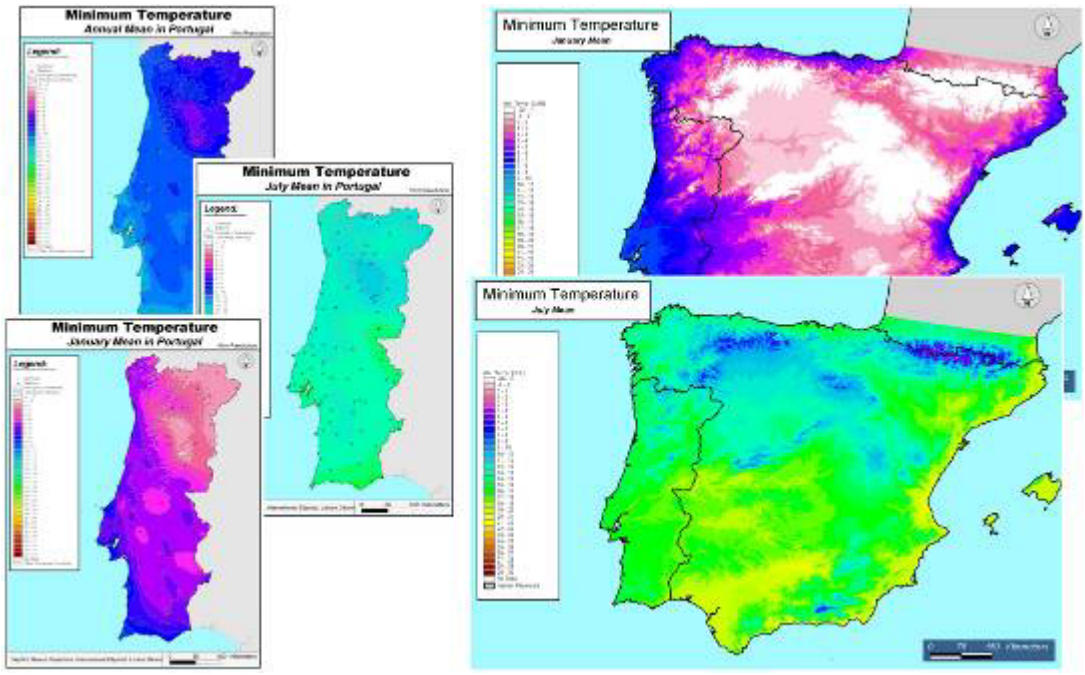

Figure 99: Monthly minimum temperatures (ºC) for Portugal and for the Iberian Peninsula – 12 datasets [RST – 1Km]

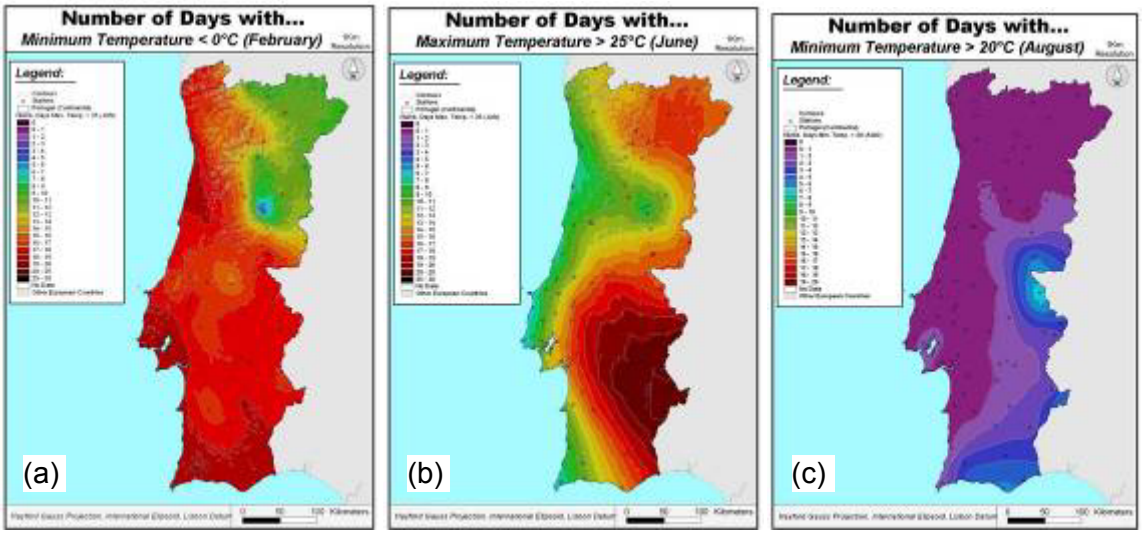

Figure 100: Monthly number of days with minimum temperature (a) below 0 ºC, (b) above 20º and (c) above 25º – 36 datasets [RST – 1Km]

9.2.2.4 Bioclimatic data

Bioclimatic data are variables, derived from climatological data, known to be ecologically meaningful for a given organism. Usually, bioclimatic variables represent extreme environmental conditions that hamper growth or threaten survival of the study organism (Busby, 1992; Austin, 2002). In the case of the Atlantic pine, temperature and precipitation are the main factors that explain pine distribution (Sabate *et al*, 2002; Manzaneque *et al*. 2005).

For Portugal, 19 bioclimatic variables were derived from the previously described monthly temperature and precipitation models, as described in the WORLDCLIM Project [RST – 1Km]

BIO1 = Annual Mean Temperature
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3 = Isothermality ((BIO2 / BIO7) x 100)
BIO4 = Temperature Seasonality (standard deviation *100)
BIO5 = Max Temperature of Warmest Month
BIO6 = Min Temperature of Coldest Month
BIO7 = Temperature Annual Range (BIO5 - BIO6)
BIO8 = Mean Temperature of Wettest Quarter
BIO9 = Mean Temperature of Driest Quarter
BIO10 = Mean Temperature of Warmest Quarter
BIO11 = Mean Temperature of Coldest Quarter
BIO12 = Annual Precipitation
BIO13 = Precipitation of Wettest Month
BIO14 = Precipitation of Driest Month
BIO15 = Precipitation Seasonality (Coefficient of Variation)
BIO16 = Precipitation of Wettest Quarter
BIO17 = Precipitation of Driest Quarter
BIO18 = Precipitation of Warmest Quarter
BIO19 = Precipitation of Coldest Quarter

Also the environmental variables for the Iberian Peninsula were extracted from the WORLDCLIM Project: 19 Bioclimatic variables derived from monthly temperature and precipitation models, as described in the ANUCLIM Project (see an example in Figure 101).

9.2.2.5 Digital Terrain Elevation Models (DTEM)

Three DTEM were generated in RASTER format, one for the Setubal Peninsula, a second one for Portugal [RST – 25m], and a third one for the Iberian Peninsula [RST – 1km] (Figure 102). All originated from the WORLDCLIM Project (http://www.worldclim.org/) and were georeferenced to a system compatible with all other data from the PHRAME Project. The following layers were derived from these DTEM: (1) Surface Slope, (2) Aspect (Terrain Orientation), and (3) Surface roughness (calculated using the difference between 2 different spatial averages of the Slope model).

9.2.2.6 Lithology

Data on lithology available from the literature was re-coded into three variables only: Lito (19 classes), Lito 2 (10 classes), and Lito 3 (4 classes) (Table 48).

9.2.2.7 PWN data

The modelling used the PROLUNP data series on the presence of the PWN on the Setubal Peninsula. Data include trees with symptoms, nematode positive and negative analyses, and number of vector insects captured within the study area, from 2002 and 2004 (Figure 103).

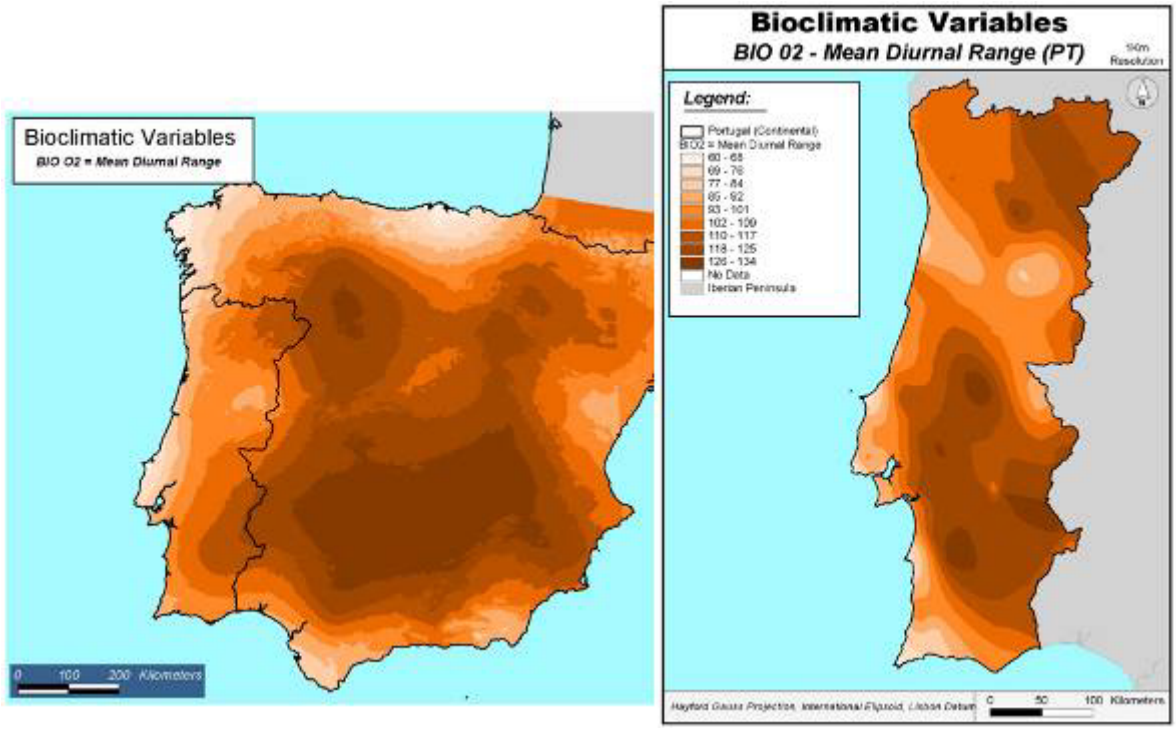

Figure 101: Bioclimatic variables for Portugal and the Iberian Peninsula: mean temperature diurnal range

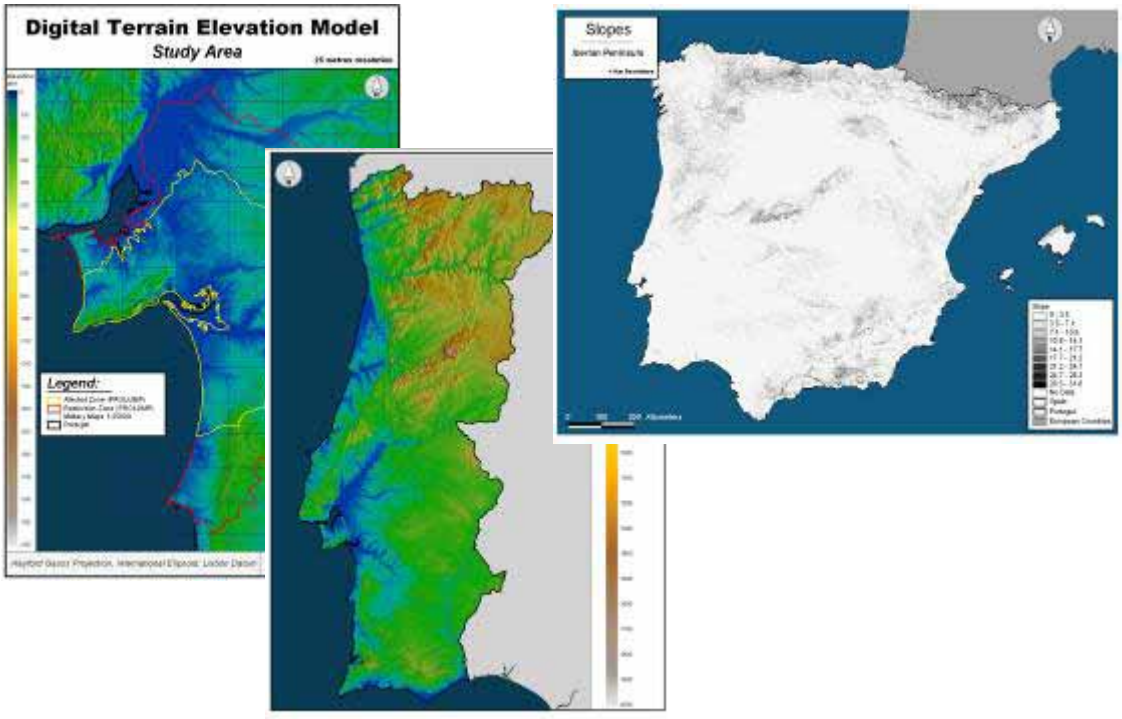

Figure 102: Digital Terrain Elevation Model for the Setubal Peninsula, Portugal and Iberian Peninsula

Table 48: Lithological variables

Description	Lito	Lito2	Lito3
Rochas plutónicas (granitos e afins)	A	G	G
Areias arenitos e argilas	B	D	A
Rochas metamórficas (complexos xisto-grauvaquicos)	C	X	X
Areias aluvionares	D	A	A
Granitos e afins	E	G	G
Quartzitos	F	Q	G
Peridotitos piroxenitos hornoblenditos	G	P	G
Areias aluvionares eólicas	H	A	A
Cascalheiras	I	L	A
Rochas carbonatadas	J	C	C
Conglomerados, xistos carbonosos e xistos argilosos	K	X	X
Complexos de arenitos, conglomerados, calcários e margas	L	R	A
Calcários	M	C	C
Depositos glaciários	N	A	A
Basaltos	O	B	G
Depósitos de vertente areias superficiais e de terraço	P	A	A
Conglomerados	Q	X	X
Tufos calcários	R	C	C
Andesitos	S	G	G

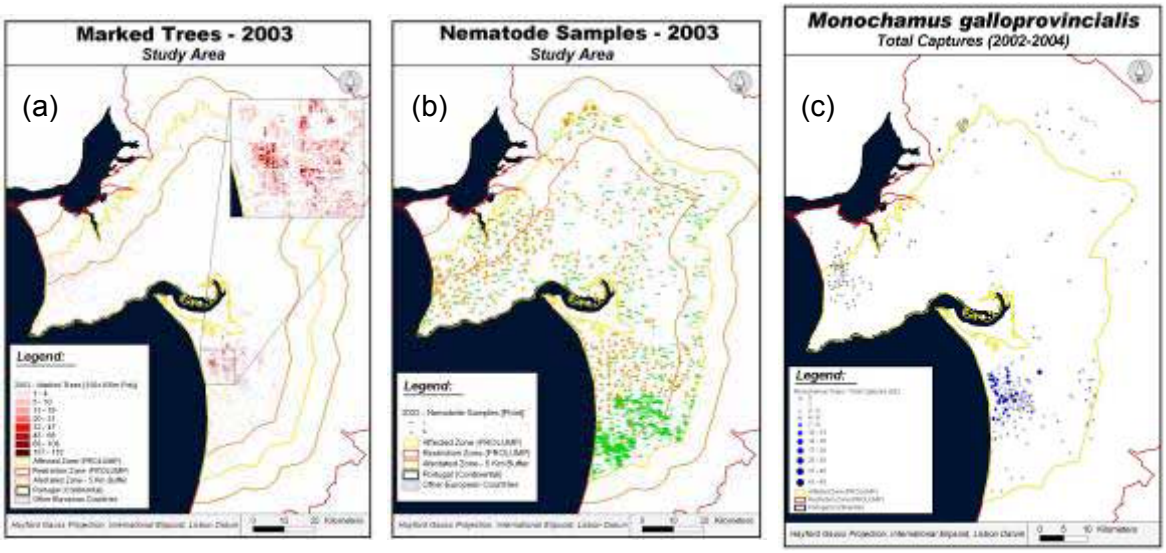

Figure 103: Biological data for a single year (2003): (a) distribution of trees with symptoms (these trees are taken out of the area and burned every year); (b) presence (orange) and absence (green) of the PWD (positive and negative samples of *Bursaphelenchus xylophilus* from trees chopped down in 2003); (c) vector density (from *Monochamus galloprovincialis* captures in the study area).

9.2.3 Statistical modelling of the potential spreading of the PWN out of the affected zone

9.2.3.1 The Pest approach

In the first modelling attempt we concentrated on the existing focus of PWN at the Setubal Peninsula, and used a Regression Tree methodology to estimate model parameters from climatological data within the affected zone. Regression Trees are especially suitable to deal with complex situations that involve many independent and dependent variables (Breiman *et al*, 1984). Unlike linear methodologies, regression trees may reach the same result using different sets of variables: they allow for a simple and intuitive interpretation of the results obtained, thus best reflecting real relationships (Clark and Pregibon, 1990). For this first model we used a PWN data series from a single survey-year (2002, PROLUNP: PWN abundance on a 5km grid), in order to be able to use the remaining surveys to test the model. We then predicted the PWN distribution over the whole of the Iberian Peninsula using this initial model and environmental variables with 1 Km resolution. We finally tested the model by using the two remaining PWN data series (2003 and 2004, PROLUNP: idem) as dependent variables.

9.2.3.2 The Host approach

A host approach demanded that first we investigated whether there was a case for spatial variability in productivity for *P. pinaster* in Portugal (the Iberian Peninsula was left aside because of the low resolution of its available environmental variables, 1 Km, which did not match the needs of fine resolution models). In order to predict differences in pine susceptibility to the PWN throughout the country, (using both heuristic and empirical models) we further assumed that:

- Tree death is a combination of infestation and low tree resistance
- Dry summers and low annual precipitation represent high environmental stress for *P. pinaster*
- *P. pinaster* under environmental stress show low productivity

9.2.4 P. pinaster potential productivity

The potential productivity model for *P. pinaster* was obtained following a stepwise methodology. First, we produced an ecological envelope for the species using variables referenced in the literature as ecologically meaningful for *P. pinaster* (Table 49). An ecological envelope is obtained through a unique combination of variables that ought to be adjusted until 75-95% of the distribution data is included within the envelope. This technique allows the stratification of the null values included in the model, avoiding bias due to incorrectly labelled zeros (an absence of trees where there was a potential for tree growth). We used data from the National Forestry Inventory (DGF, 2001), where trees are classified (according to age and DBH determined in the field) in five classes, labelled 1 (low productivity) to 5 (high productivity).

We then proceeded with the statistical modelling of the *P. pinaster* productivity potential, again adopting a regression trees methodology: the data on pines' distribution was split into two groups (the algorithm chooses the split that partitions the data into two parts, such that it minimises the sum of the squared deviations from the mean in the separate parts), and the variable that best explained the obtained partition was identified. The process was repeated iteratively, branching off the dependent variable until each node reached a user-specified minimum node size and became a terminal node. In our case, the pruning of the tree was performed automatically (S-Plus R-part extension, Clark and Pregibon, 1990) and the groups obtained labelled as Suitability Classes, from Marginal (low site suitability) to Excellent (high site suitability).

Table 49: Variables used to produce a potential productivity model for *P. pinaster*

Model code		Description	Variable	Extension	
TMAX_AGO	TMA	August maximum temperature	Continuous	[24 - 33]	º C
TMIN_AGO	TmA	August minimum temperature	Continuous	[11 - 18]	º C
TMIN_JAN	TmJ	January minimum temperature	Continuous	[-1 - 9]	º C
TMAX_JAN	TMJ	January maximum temperature	Continuous	[5 - 16]	º C
MAR_CUSTO	MC	Distance to the coast (Altitude as *cost*)	Continuous	[0 - 35000]	m x m
TMA_MJ	Tcont	Continentality: TMAX_AGO - TMIN_JAN	Continuous	[17 - 29]	º C
PH_S	PHS	Difference in precipitation between a dry year and a wet year	Continuous	[100 - 1125]	Mm
P_JFD	PInv	Winter precipitation	Continuous	[150 - 1380]	Mm
P_JJA	PVer	Summer precipitation	Continuous	[10 - 250]	Mm
P_TOTIL	P	Total precipitation	Continuous	[350 - 3400]	Mm
MDT_expC	MExp	Aspect (Cos)	Continuous	[-1 - 1]	-
MDT_INCL	Minc	Slope	Continuous	[0 - 100]	%
MDT_ALT	Malt	Altitude	Continuous	[0 - 1980]	M
I_ARI	Iari	Índice de aridez	Continuous	[0 - 4]	-
I_RI	Iri	Índice de risco de incêndio	Ordinal	[1 - 5]	-
I_RA	Ira	Índice de região de arborização	Nominal	16 classes	-
I_PPF	Ippf	Productivity index	Ordinal	[1 - 7]	-
I_PPC	Ippc	Potential productivity index	Ordinal	[1 - 5]	-
L_DUR	Ldur	Lithologia: dureza do solo	Continuous	[1 - 3]	-
LITO	L	Lithologia (17 classes)	Nominal	[A a Q]	-
LITO2	L2	Lithologia (9 classes)	Nominal	[1 - 9]	-
LITO3	L3	Lithologia (5 classes)	Nominal	[1 - 5]	-
Oc1	Oc1	Pine as the dominant species	Dicotomic	[0 ou 1]	-
Oc2	Oc2	Pine as a non-dominant species	Dicotomic	[0 ou 1]	-
IQ	IQ	Quality index	Ordinal	[1 - 4]	-
P	P	Presence/absence	Dicotomic	[0 ou 1]	-

Each of the five suitability classes obtained resulted from a unique combination of variables that was identified following its “branch” back to the original undivided group. The model was checked out using real presence/absence data. Zeros (the species absence) were randomly selected from the National Forestry Inventory (DGF, 2001): half of these were located throughout the study area, whereas the other half represented locations specifically identified by the envelope as pine free.

9.2.5 P. pinaster site suitability (heuristic models)

The heuristic models were produced by overlaying the new suitability classes with *P. pinaster* species actual distribution in Portugal. When *P. pinaster* is present in great numbers (high cover values), but does not grow properly (low productivity potential), we assumed trees were under stress and therefore had a high probability of dying from a PWN infestation. Whenever *P. pinaster* is present at sites of high productivity potential, usually associated with enough water availability, we considered that there was a low probability that the tree could die from PWD. A pine cover above 80% and a marginal productivity potential (marginal = 1; excellent = 5) represented the highest risk of disease spreading, whereas a low pine cover (e.g. < 5%) and excellent productivity potential (=5), represented the lowest risk. Of course, where there were no pine trees, the risk was considered non existent, zero.

9.2.6 P. pinaster site suitability (empirical models)

The empirical model was intended to look for any combination of climatic variables that could explain the previous model results. By performing a regression tree analysis of those results, indeed we obtained a model where potential infestation by the PWN was expressed as a function of a combination of variables. The dependent variable was given a score of:

(1) 2 to 3 for areas with a large potential to be infested
(2) 1 for healthy growing Pinus forest
(3) 0 for Pinus forest with no potential to be infested or non existent

9.2.7 Final mapping

For the final mapping, the ecological envelope was overlaid with the models, all of which were reclassified in order to produce a single layer displaying the five suitability classes. This map was then compared to the original *P. pinaster* distribution obtained straight from the data of the National Forestry Inventory (DGF, 2001).

9.2.8 Dispersal routes

Although beetles have been recorded as able of flying for up to 3.3 km, in most cases they do not exceed a few hundred metres in their dispersal (Kobayashi *et al*., 1984). However, also infested wood can be an effective way of spreading the PWN, and the species has been intercepted on a number of occasions on sawn wood, round wood and wood ships (CABI, and EPPO. 1996): chance does play an enormous role here. Yet we thought we should explore the part played by the host: although the risk for PWD is associated with high pine cover density, a low pine density may be enough to act as a dispersal corridor. In order to explore potential corridors of dispersal from the already affected zone, a mask of 2% pine cover (enough trees to allow the insect vector to move from one area to another) was applied to the previous models.

9.3 *Results*

9.3.1 The Pest approach

The predicted potential PWN distribution over the Iberian Peninsula using the three PWN data series (2002, 2003, 2004; PROLUNP) were inconsistent, each model pointing to different explanatory variables (Figure 104): depending on the year of sampling, the resulting model varied considerably, showing the impossibility of using the available surveys on PWN to produce accurate pest risk maps.

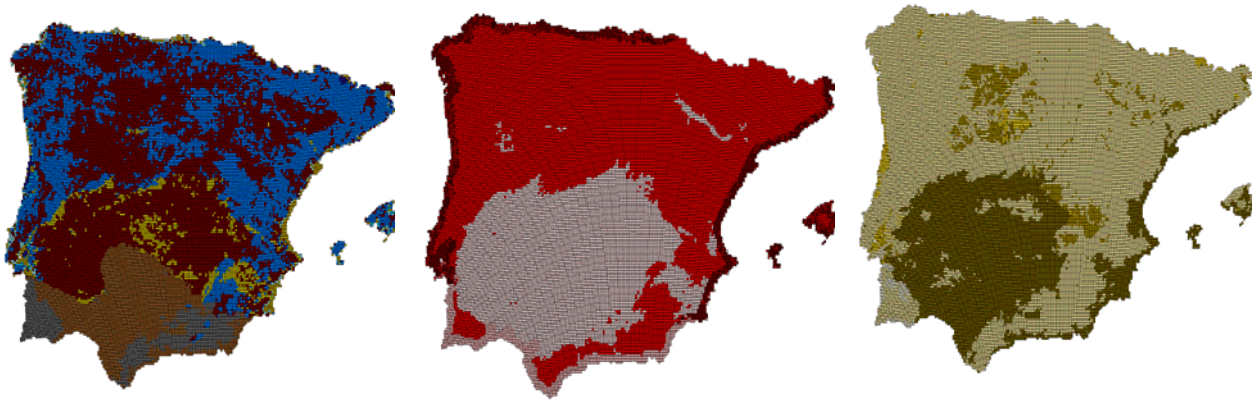

Figure 104: Prediction of PWD patterns of distribution over the Iberian Peninsula using data from three different datasets (2002, 2003, 2004, PROLUNP).

9.3.2 The Host approach

9.3.2.1 *P. pinaster* potential productivity: the ecological envelope

The ecological envelope for *P. pinaster* is shown in Figure 105. It resulted from a unique combination of variables that can be described as:
Difference between August maximum daily temperature and January minimum daily temperature < 26ºC
August maximum daily temperature < 29.9 ºC
Total precipitation > 850mm
Altitude < 800m
Lithology - other than limestone

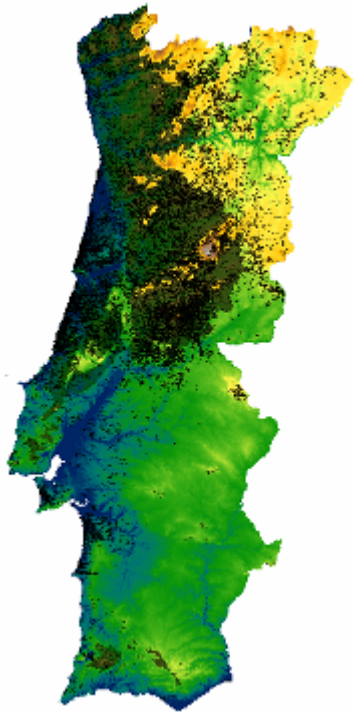

Figure 105: Ecological envelope for *Pinus pinaster* (in brown the ecological optimum for pine predicted by the model; black dots represent actual data (pine occurrence) from the Pine National Forests Inventory (DGF, 2001))

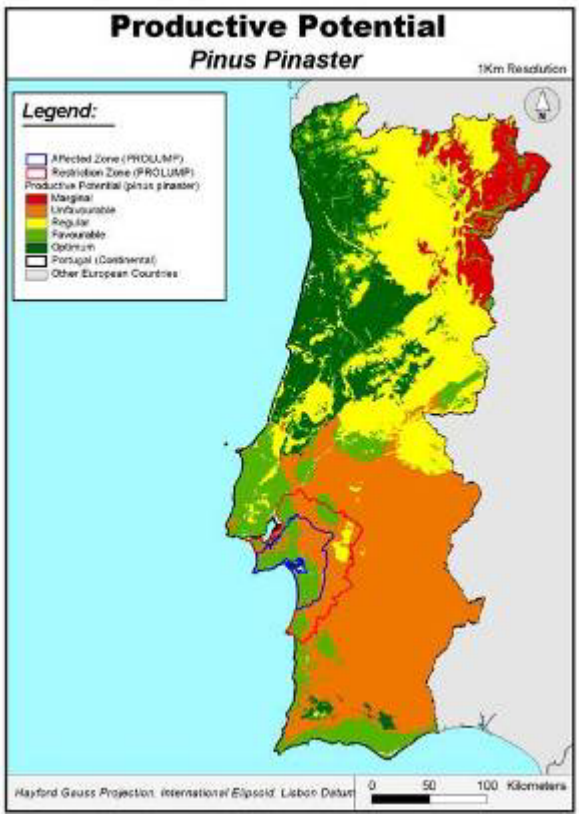

Figure 106: Potential productivity model for *Pinus pinaster*

Over 75% of the photo points referenced by the National Forest Service to be *P. pinaster* dominated were caught inside the produced ecological envelope. The results of the regression tree of *P. pinaster* distribution data are shown in Figure 106. Table 50 depicts the explanatory variables for each level of split: these results confirmed the amount of precipitation, a surrogate of water availability, as the main determinant of low and high site suitability for pine trees.

Table 50: *P. pinaster* regression tree: explanatory variables for each suitability class (P: precipitation; TMA: August maximum temperature; Litol: Lithology; Pver: Summer precipitation; TmJ: January minimum temperature; Minc: Slope; Malt: altitude; PInv: Winter precipitation; Tcon: Difference between August maximum temperature and January minimum temperature)

Explanatory variables selected by the regression tree							Site suitability
P<887	TMA>31						Marginal
	TMA<31	Litol=acghjk	Pver<35				Marginal
			Pver>35	TmJ<1.1			Bad
				TmJ>1.1			Regular
		L dif	Minc>15.5				Marginal
			Minc<15.5	TmJ<5	TmJ>3.9	Pinv>179	Marginal
						Pinv<179	Regular
					TmJ<3.9		Good
				TmJ>5			Good
P>887	Malt>584						Regular
	M<584	L=efkl					Regular
		L dif	Tcon>25				Regular
			Tcon<25	Minc>8.9			Good
				Minc<8.9			Excellent

9.3.3 P. pinaster site suitability: heuristic modelling

Combining information on the distribution of the *P. pinaster* suitability classes (Figure 107A) with the actual *P. pinaster* distribution (Figure 107B), it was possible to highlight areas of abundant *P. pinaster* cover where the species presents a low productivity potential (Figure 107C). Such is the case of the largest pine area of Portugal, in Oleiros, Vila de Rei and Sertã municipalities, but also of another area on the west (Leiria and Pombal), and a third one on the northwest (Bragança and Chaves). These are especially sensitive areas to an attack by the PWN.

By applying a mask of pine cover density to the previous map (Figure 108), it was possible to conclude that, apart from the Setubal Peninsula, the area of most concern is the central region of Portugal (Oleiros, Vila de Rei and Sertã) where *P. pinaster* growths in high densities outside its optimal grow region (under hydrological stress) (Figure 108C).

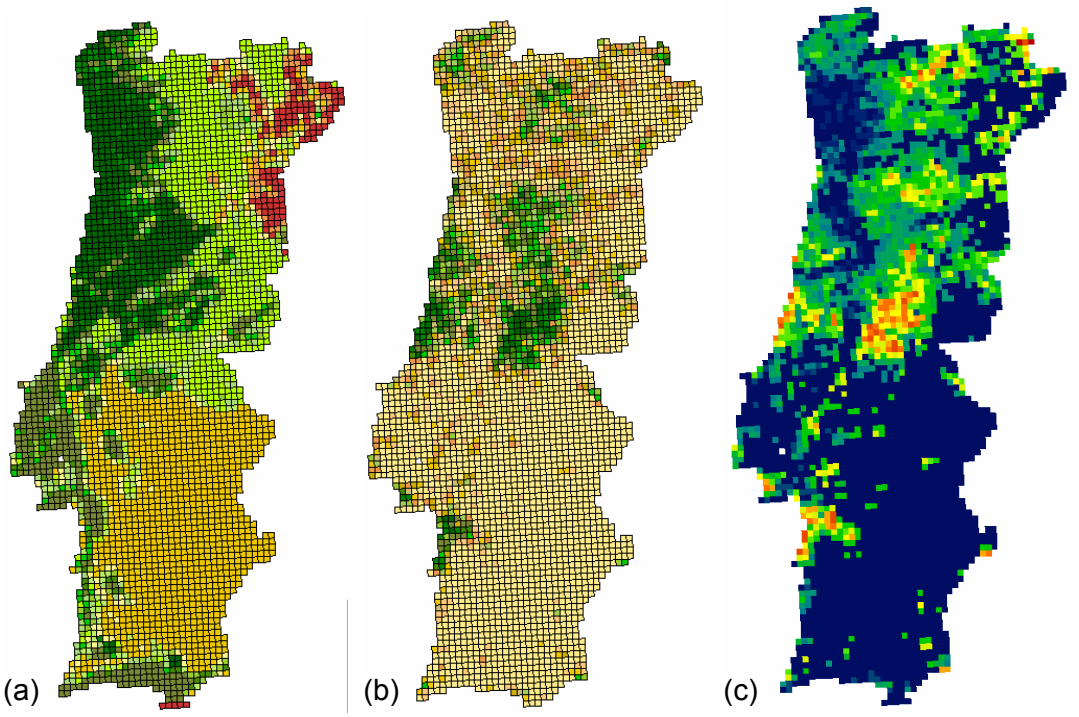

Figure 107: *Pinus pinaster* productivity model: (a) pine potential productivity (from red – marginal to dark green – optimal); (b) pine actual distribution (green – presence; brown – absence); (c) heuristic model based on the previous data layers (blue and green are safety zones; orange and brown are sensitive zones)

9.3.4 P. pinaster site suitability; empirical modelling

A regression tree analysis of the previous results further identified the factors governing the pattern revealed by the heuristic model. All together, the model selected 11 variables: altitude, annual precipitation, dry year precipitation, evapotranspiration, maximum year temperature, plus five bioclimatic variables: BIO5 = Maximum Temperature of Warmest Month, BIO7 = Temperature Annual Range, BIO9 = Mean Temperature of Driest Quarter, BIO10 = Mean Temperature of Warmest Quarter and BIO 17 = Precipitation of Driest Quarter.

And finally the spatial differences in site suitability for pine were, again, overlaid with information on the actual pine distribution (Figure 109).

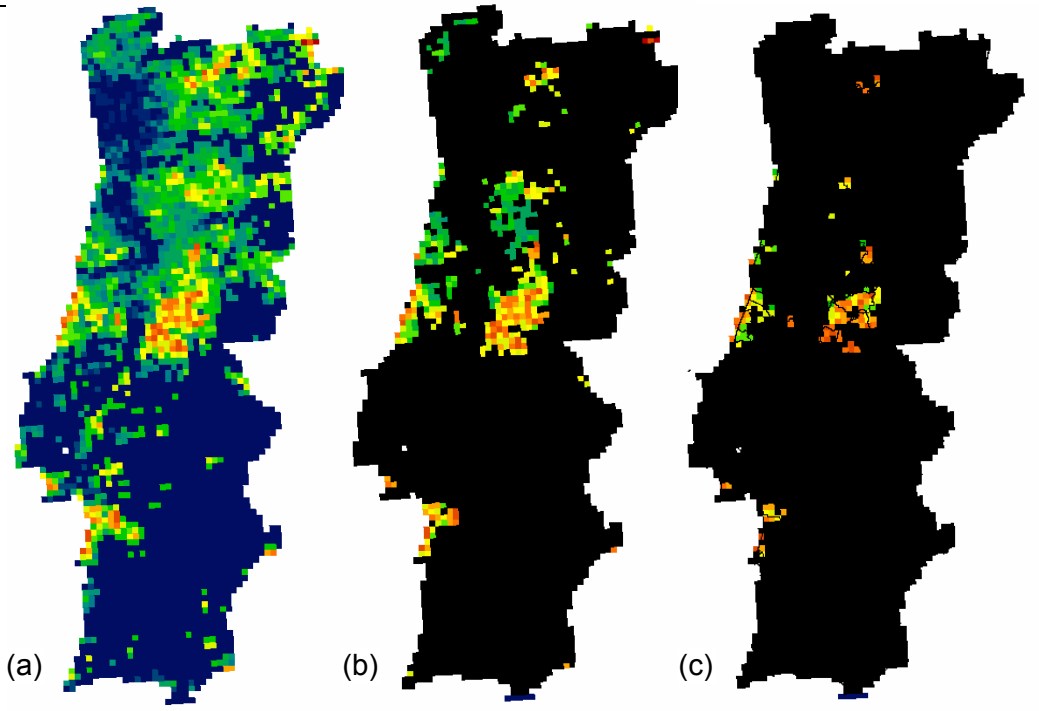

Figure 108: Site suitability heuristic models for *P. pinaster:* (a) the heuristic model; (b) the previous model with a black mask of UTM squares of pine cover < 28% overlaid; (c) idem, for pine cover < 54%; blue and green – safety zones; orange and brown – sensitive zones)

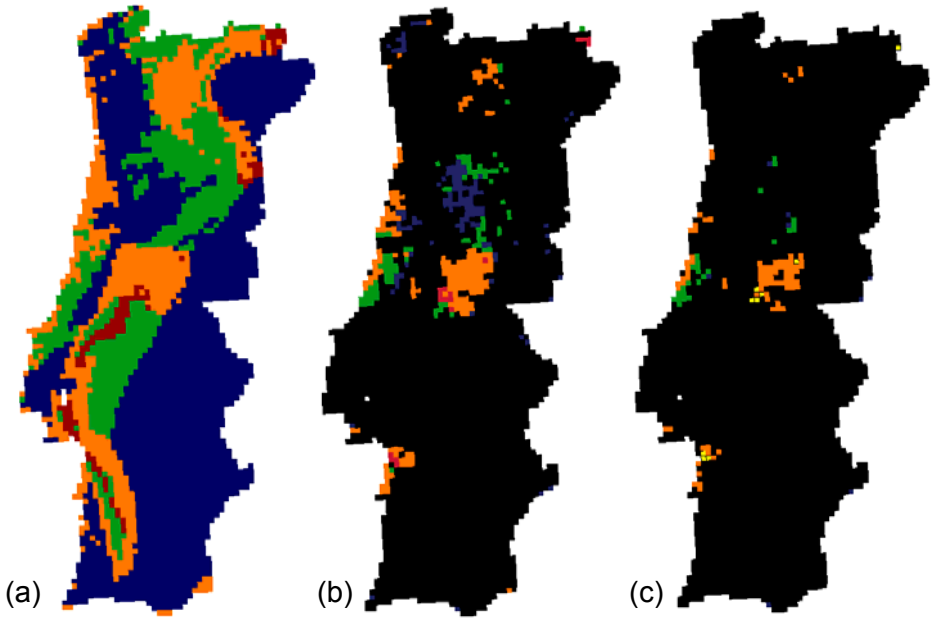

Figure 109: Site suitability empirical models for *P. pinaster* (a) the empirical model results for Portugal; (b) the previous model with a black mask of UTM squares of pine cover < 28%; (c) idem, for pine cover < 54%; blue and green are safety zones; orange and brown are sensitive zones

As pine stands' risk to PWD increases with pine density, this technique allowed us to discriminate among sites of low ecological suitability for pine. For instance, although the NE of Portugal, Bragança, is not particularly suited for pine, its low distribution in the region decreases the risk for PWD Figure 110).

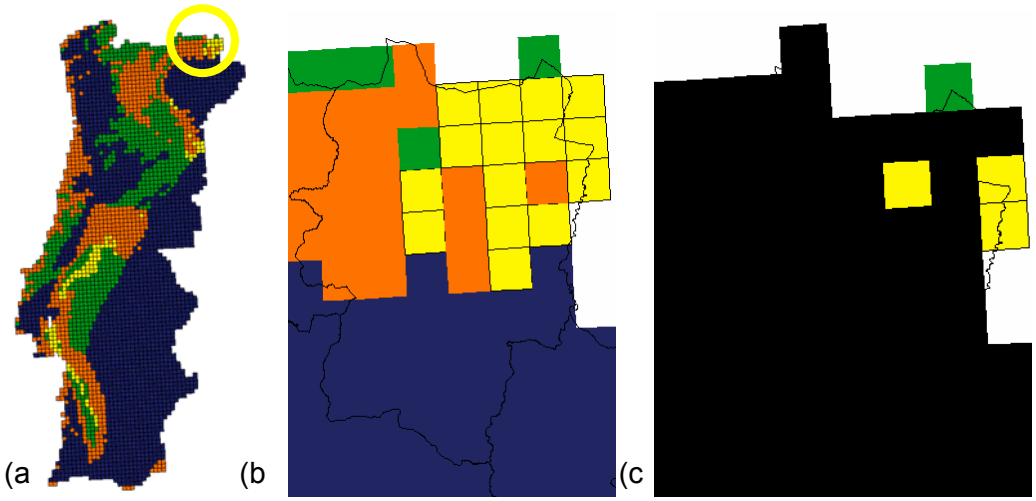

Figure 110: High risk areas for the PWD in Portugal (yellow color). (a) Portugal; (b) Bragança; (c) Bragança with a cover mask of pine cover < 40%

On the contrary, our results showed that high pine density on the West Coast indeed could make this a critical region for the PWD (Figure 111).

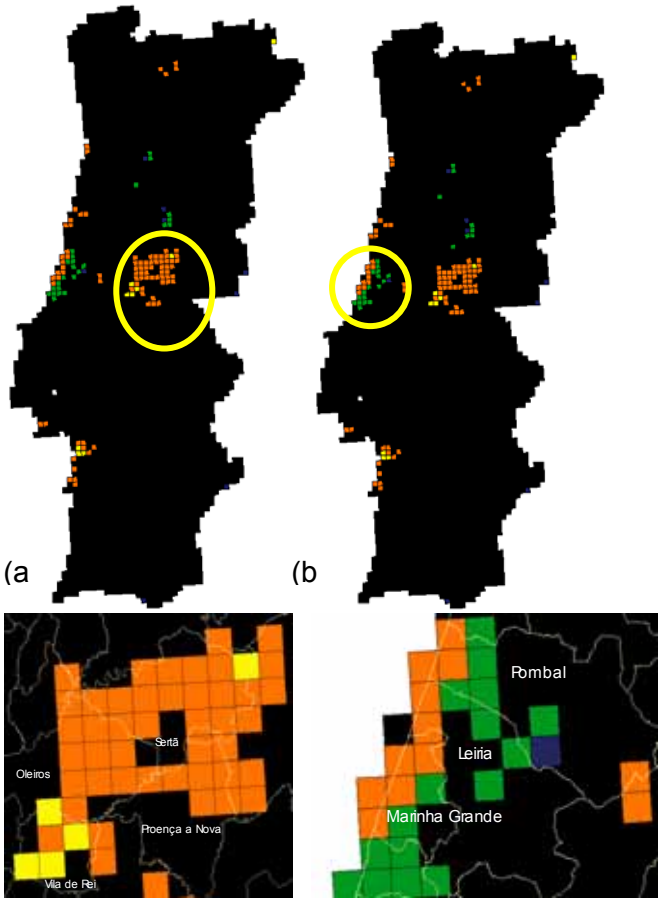

Figure 111: High risk areas for the PWD in Portugal (yellow colour). (a) Central Portugal, pine cover mask < 40%; (b) West coast, pine cover mask < 40%

9.3.5 Dispersal routes

The threshold of 2% pine cover showed a possible corridor of dispersion from the already affected zone to the centre of Portugal, a region that the previous models showed up as especially critical for the PWD (Figure 112).

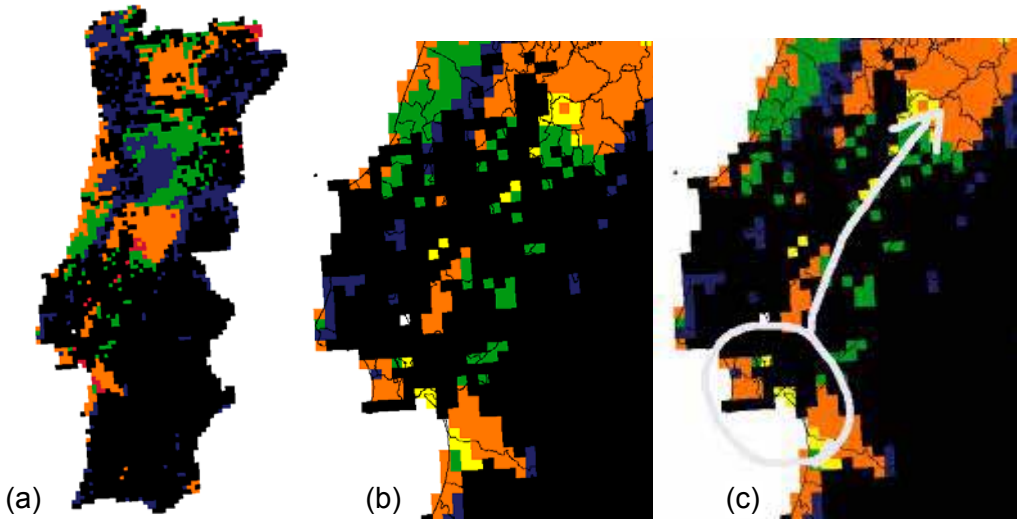

Figure 112: Model overlaid with a 2% pine cover mask, displaying possible corridors for the spread of the PWN: (a) Portugal; (b) Setubal Peninsula and Oleiros region; (c) possible path for the PWN dispersion

9.4 *Discussion*

When coupled with geographical information systems (GIS) technology, statistical modelling usually enables biological distributions to be extrapolated across large regions, thereby providing geographically complete information for many different environmental applications (Franklin, 1995; Austin, 1998; Guisan and Zimmermann, 2000). Population based models, in particular, are effective and especially used in modelling small areas (Yoshimura *et al*, 1999), though also they can fit country scale modelling if there is enough field information. For instance, Takusu (2000) successfully predicted the PWN dispersion in Japan modelling the whole country, for he could count on accurate information on PWD that occurs extensively throughout the country.

9.4.1 The Pest approach

In Portugal, the introduction of the PWN is recent, which implies that its local patterns of distribution and dispersion are scarcely known. Also the affected zone is of small size, which reduces the amount of environmental conditions that the nematode has faced so far in Europe. Another problem rose from the fact that the PWN surveys do not follow a stratified sampling design, a key element for a successful modelling effort (Austin, 1998; Legendre and Legendre, 1998). In addition, since PROLUNP is an eradication programme, infested trees are suppressed from the stand, which makes this a much forest destructive initiative. As a result (and this is in fact the intended result of PROLUNP), PWN dispersion within the Setubal Peninsula can hardly be expected to follow a natural dynamic, and instead is constrained by man. All this contributed to the failure of our approach to estimate rates of dispersion throughout the country counting on data from the Setubal Peninsula. Yet it must be stressed that this methodology can be successfully pursued in the future, given that the affected zone enlarges to encompass enough environmental variability.

9.4.2 The Host approach

In Portugal, the only tree species killed by the PWD is *P. pinaster*. Unlike the PWN, pines are widely distributed throughout the country and much is known about their ecological requirements. As empirical modelling still is one of the best options for modelling over large territories (Legendre and Legendre, 1998; Guisan and Zimmerman, 2002), we went on to predict the risk of PWN dispersion throughout Portugal focusing on the tree and site conditions. The literature shows that, in terms of the PWD development, the water status of pines plays a very important role in the pine-nematode relationship (Suzuki, 2002). Our results confirmed that water availability was indeed a key factor determining a site 'suitability for pines' growth: while identifying regions where pines grow in high numbers and bad conditions, we intended to pin point areas of high risk for the PWD. And one of the interesting results of the heuristic environmental modelling was that one of the three areas classified as most sensitive is the Setubal Peninsula (Figure 109C), the very region where the PWN has been so far identified. The fact that the data used in both models (*P. pinaster* productivity and *P. pinaster* spatial distribution) was independent of the PWN data certainly accounts for the model's good predictive value. This approach differs from the initial trial of extrapolation in that it uses information that covers the whole country, thus greatly increasing the statistical power of the model. This explains why the model succeeded predicting Setubal to be an area of high risk, even if the model did not take into account the already confirmed occurrence of the PWN in this area.

9.4.3 Ecological surrogates

The use of ecological surrogates allowed us to estimate patterns of risk to the PWD in Portugal. Interestingly enough, one of the important variables revealed by our productivity model, which lead to a "marginal" quality class, was August temperature over 31º (Table 50). If the tree is not able to grow properly, also is more susceptible to diseases: our results recall the literature that relates hot summer temperatures to nematode spreading (Rutherford *et al*., 1990; CABI and EPPO. 1996; Rebetez and Dobbertin, 2004). There are no reports of pines dying of pine wilt in those regions of Europe, North America, or Japan where mean summer air temperatures are less than 20 °C (Rutherford and Webster, 1987), a threshold that coincides with the temperature limit over which also tree growth is hampered (Rutherford *et al*., 1990).

At present, national surveys of the PWN are based on pine cover only. The differences in potential risk of infestation just described could be used to improve these surveys: a stratified sampling designed could be implemented, where areas with high risk would be more intensive sampled.

According to our findings, pine forests may be classified as:
Critical: when located at sites with high potential evapotranspiration (dry summers with high temperatures)
Safe: when located at sites with low potential evapotranspiration (mild summers with low temperatures).
Tree density also plays a role: it was possible to identify critical regions in Portugal where pine density is low (e.g. Bragança in Figure 110). It must be stressed that the areas of most concern are the ones environmentally critical and with a high pine cover (e.g. Oleiros and Sertã, Figure 111).

9.4.4 Possible corridors of dispersion

Although most problems are expected to arise in areas of abundant and poor growing pines, a low density tree cover may act as a dispersal corridor for the insect vector. Our models showed that there is in fact a possibility for the PWD to disperse from the Setubal Peninsula into one of the most critical regions (Figure 112). These models are, thus, a useful tool to explore potential corridors between affected and critical areas.

9.4.5 Main findings and recommendations for further research

1. Predictions of PWD dispersal over the whole of Portugal using the PWN occurrence data from the affected zone were not successful; yet if more data becomes available this type of modelling is worth exploring;
2. A tree based approach produced useful prediction models of the PWD risk for Portugal;
3. Central Portugal showed up as the area of most concern: high pine density growing in poor site conditions;
4. High temperatures and low precipitation showed up as the main drivers of tree stress, which indicates climate change as a future central area of research in dealing with the risk of PWN spreading throughout Europe;
5. Our models can be used to improve the PWN national surveys, focusing field effort at high risk areas.

9.5 *Process-based modelling and prediction of pine wilt expression*

9.6 *Introduction*

Pinewood nematode [PWN] (*Bursaphelenchus xylophilus*) is a saprophytic organism, in the Parasitaphelenchidae family, usually exploiting dead or dying coniferous tree species. It is native to North America, where it is widespread, but it has been distributed internationally through trade and its present geographical distribution ranges from Japan, Korea, Taiwan to China and also Portugal[3]. A key element of its life history, which is the initial determinant of its ultimate impact on living potential host trees, is maturation feeding by adults of its principal distribution vector, longhorn beetles of the genus *Monochamus*, (Coleoptera: Cerambycidae). During the feeding phase in the crowns of living trees, the vectors can introduce *B. xylophilus* into healthy trees, which in its native range has no apparent effect on plant activity (Futai and Furuno 1979; Rutherford and Webster 1987). Unusually, and in a number of geographical regions, PWN has been identified as the causal agent for the death of mature, healthy host plants within a short time after introduction to the living tree following maturation feeding by adult vectors (Mamiya and Enda 1972; Linit 1989). The symptoms of rapid wilting of trees have led to the description of the syndrome as Pine Wilt Disease [PWD] (Kiyohara and Tokushige 1971; Mamiya and Kiyohara 1972; Kishi 1995). The nematode is now known to be responsible for large-scale tree death in Japan, China, Korea and, since 1999, Portugal, and is identified as a major driver to successional processes in semi-natural stands (Fujihara et al. 2002), and a corresponding source of considerable financial loss to the timber industry as well as imposing restrictions on movement of conifer wood in international trade (EPPO 1986; EPPO 1989).

Although dispersal of the nematode is intimately linked to the dynamics of its only known effective vectors in the genus *Monochamus*, with considerable literature on the dispersive phase of its cycle (Mamiya and Enda 1972; Linit 1988), there is still much uncertainty concerning the factors that determine whether a tree that is infested through maturation feeding in the canopy is likely to succumb to PWD. The literature indicates that expression of PWD results from complex interactions within the host tree. This involves PWN movement within the tree, feeding behaviour, population dynamics, and effects on its host by disrupting physiological processes leading to destruction of cell structure and content. However, there is no mechanistic description of this process, which is essentially a 'black box'. At a generic level, the severity and time course of infestation includes visible wilt expression, accompanied by early reduction of resin flow (Mamiya 1972), chlorosis and stem/branch cell necrosis, followed by rapid death of the tree (Mamiya 1980). In turn, symptom severity varies according to the susceptibility of the host species (Futai and Furuno 1979; Guiran and Bouolbria 1985; Linit and Tamura 1987; Yang 1987; Baojun and Qouli 1989). It is further apparent that certain environmental conditions leading to stress of a healthy host, primarily elevated temperatures and restricted water availability, are required for visible symptoms to occur or for death to follow. However, the internal processes of the nematode-tree interaction and their linkage to external environmental factors are poorly understood, so that prediction of wilt expression arising from PWN presence can currently only be achieved using broad environmental parameters that, inevitably, provide only general predictors of wilt expression. The ecological and host tree suitability indices used by the University of Evora in the PHRAME topic (see section 9.2.3), suggest that regional variation in climatic and site factors can provide risk predictions at regional scales. This approach fits with the predicted range of potential wilt expression in both Japan and Europe which is based on July or August isotherms exceeding 25.2^{o}C (Yokobori 1986). While this broad-brush approach from the literature provides useful predictors in Pest Risk Analysis for the expression of PWD (Evans et al. 1996), it does not allow more precise assessments at local and regional scales. The approach by the University of Evora, takes this a step further in integrating various datasets in correlation analysis. The final stage of interaction at the tree level is the ultimate measure in determining likelihood of tree mortality and particularly, provision of breeding resources for *Monochamus* spp. and hence acceleration of

[3] http://www.eppo.org/QUARANTINE/nematodes/Bursaphelenchus_xylophilus/BURSXY_map.htm

spread of the nematode and this is the subject of the current modelling work by Forest Research and, for the field verification experiments in collaboration with Estacao Florestal Nacional and Nemalab, University of Evora, Portugal.

This paper reviews current understanding of the interactions of *B. xylophilus* and living trees and proposes a theoretical modelling framework describing the dynamics of physiological processes that can ultimately result in death of the host plant. The predictive ability of the modelling framework has been tested through a field experiment, with initial results presented here. It is further proposed that the model is suitable for developing a generic framework to predict the vulnerability of different tree hosts to PWN across a range of geographical regions.

9.7 *An overview of mechanisms*

Although PWN is introduced to conifer hosts through maturation feeding by adult beetles, this does not always result in further development and breeding by the nematode or in death of the host tree. Normal transmission, survival and further dispersal of the nematode is achieved through entry to the tree during oviposition by female *Monochamus spp* (Figure 113). This only takes place in dying or recently dead trees, which are attractive to the vectors and can support oviposition and subsequent larval development (Edwards and Linit 1992). Both PWN and *Monochamus* larvae develop within the outer wood of the tree, the former feeding primarily on fungi. Towards the end of the *Monochamus* cycle, PWN larvae aggregate preferentially around the pupal chambers and, through responding to chemical cues (Maehara and Futai 1997) moult to a specific stage called a 4th stage dauer larva. This stage is adapted to migrate to the callow adult stage of the vector and to enter the tracheae where it remains during subsequent flight by the newly emerged adults. The presence of other pathogens, such as blue-stain fungi, appears to increase the number of nematodes aggregating around such chambers, by facilitating their movement through the disaggregating wood structure (Maehara and Futai 2002). The relationship between PWN dauer larvae and *Monochamus spp* adults appears to be co-evolved because 4th stage dauer larvae do not appear to be formed when nematodes encounter other xylophagous insects within their tree hosts. The behaviour of the 4th stage dauer larvae within the adult vectors increases their likelihood of being able to transfer to new hosts either during maturation feeding or during oviposition by the beetles. Thus, on emergence from the tree, PWN 4th stage dauer larvae are found in the tracheal system of adult beetle vectors, facing inwards to the distal end and outwards to spiracles in tracheae. After the nematodes change orientation, the proportion of tracheae with nematodes facing outward increases with beetle age (Aikawa and Togashi 2000).

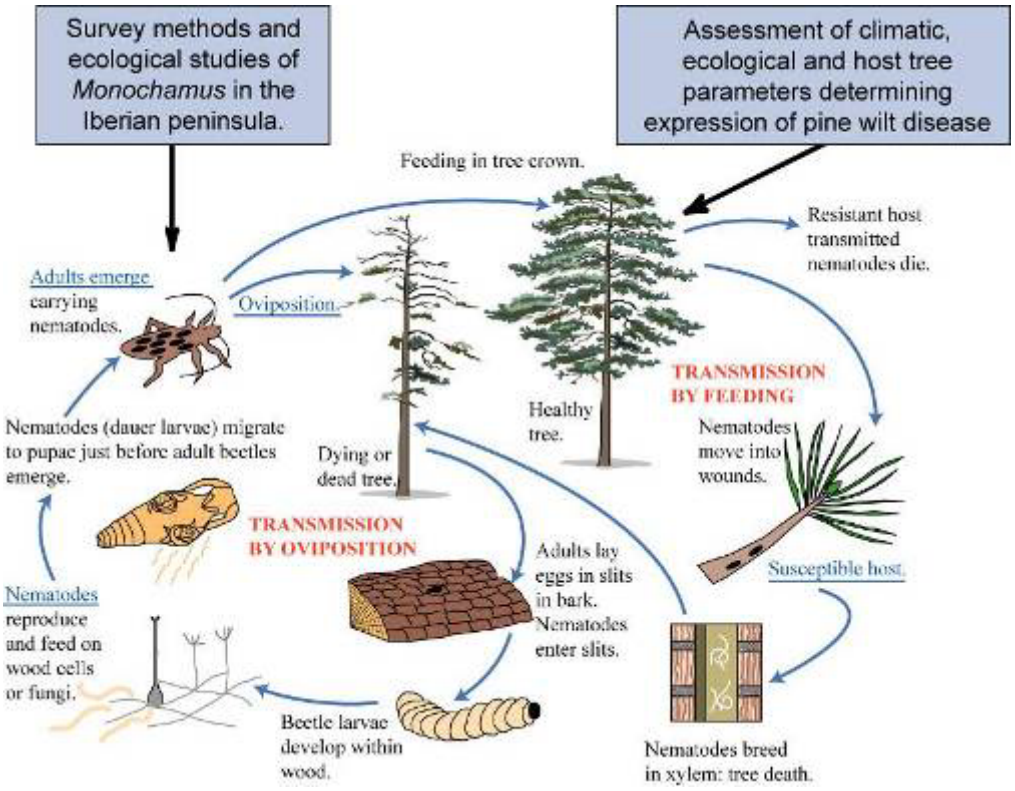

Figure 113: The interaction of *Bursaphelenchus xylophilus* and its *Monochamus* spp vectors in saprophytic and pathogenic phases of the cycle. After Wingfield (1987)

Monochamus spp. adult emergence tends to take place from late spring through the summer months, depending on temperature. Maturation feeding and oviposition take place throughout the adult activity period (see Chapter 6 and Figure 59). Crucial to understanding the process of nematode entry and subsequent activity within a living tree, maturation feeding in the branches of the canopy exposes the sapwood, and PWN larvae are introduced through the feeding wound into the wood tissue (Togashi 1985; Linit 1989). Once introduced into the tree, the pioneer PWN population appears to decline during the first few days by up to 90% (Suzuki 1984; Tamura 1984; Mamiya 1985) possibly reflecting a high degree of capture in the exposed resin at the feeding wound, with only a small proportion becoming established in the area immediately adjacent to the wound.

Studies on the distribution of inoculated nematodes in seedling trees, carried out within the PHRAME project by Schroeder (Section 5.4) and Sousa (Section 5.7) indicate that they move rapidly through the tree and gradually increase through reproduction, depending on temperature. The fate of pioneer populations of the nematodes depends on a range of host and environmental variables that are, currently, not well understood. If conditions are suitable for further development and spread within the tree, it is apparent that there are sizeable differences in PWN reproductive rates irrespective of initial *inoculum* loading, probably influenced markedly by external environmental pressures such as temperature (Mamiya 1975) and tree condition, making predictions of host dose responses difficult. From the literature and from studies within the PHRAME project it appears that PWN can disperse through the whole tree soon after introduction by moving along the living tissue pathway predominantly represented by the cambial zone. By feeding on this tissue the nematodes begin to break down cell content and structure, triggering local host defensive responses leading to increasing disruption of physiological processes. Evidence from the seedling studies described in Chapter 5 suggests a sequence of events that

confirms rapid nematode migration prior to compromising the conductive system of a susceptible tree, leading to wilt expression:

(1) Formation of the initial population inside the host in a confined area around the side of entrance,
(2) Rapid migration and colonisation of all tree parts by a highly active part of the initial population of nematodes entering the tree,
(3) Establishment of the nematode inside the host through exponential growth,
(4) Retreat of the nematode into the basal parts of the tree.

These observations confirm literature data that indicate PWN migration of up to 150 cm per day in wood tissues (Kuroda and Ito 1992) and the extent of tissue destruction appears proportional to the spread of the nematode. This suggests that PWN continues to migrate in the still living tree as increasing amounts of tissue structures collapse and, with increasing numbers, nutrient sources become scarce.

Hypotheses on the mechanisms of PWN-induced mortality in pine trees can be summarised into three broad categories of effects:

- The progressive physical destruction of living cells in wood arising from feeding by PWN. Histopathological evidence suggests that PWN tends to be found in undifferentiated, soft tissues such as cambium and living tissue (axial and vertical parenchyma) as pathways of movement through the host (Myers 1986; Myers 1988). The presence of nematodes in resin canals is also reported frequently. Meristematic tissue and other living tissue (parenchyma and epithelium cells) in the sapwood are, therefore, exploited both as a food source and as a conduit for movement of nematodes through the tree. Compounded by its own defence responses (hypersensitive reaction), the ability of the host tree to replace damaged cambial tissues and its ability to translocate photosynthate and synthesise defence compounds is severely compromised.
- Destruction of cambial and storage tissue during feeding may also lead to progressive physical blockage of vascular tissue in the xylem, as a result of resin secretion following wounding (Fukuda et al. 1992), or embolism induced by occlusion of tracheid border pits by feeding-induced debris (Nobuchi et al. 1984a; Nobuchi et al. 1984b; Kuroda et al. 1988; Kuroda 1989).
- Toxins (i.e. cellulase) produced either by PWN (Odani et al. 1985; Yamamoto et al. 1986) or accompanying bacteria (or both) (Zhao et al. 2003), may lead to a phytotoxic reaction.

The above effects may act independently or, more likely, in concert leading to host death. Their relative contributions may also change according to local circumstance and the timing of PWN penetration in relation to the host's own phenological cycle as well as external environmental drivers. Consequently, the effects of nematodes on susceptible living host trees can be regarded as a combination of direct and indirect effects, promulgated through defence-induced biochemical reactions following release/leakage of host-synthesised volatile monoterpenes and other carbohydrates in the xylem, inducing progressive hydrophobicity and occlusion of conductive tissue, leading to cavitation (Kuroda 1989) and, ultimately, expression of wilt and possible mortality in affected trees.

9.8 *Modelling the likelihood of wilt expression at the tree level*

The complex interactions between host, environment and pathogen would suggest recourse to a modelling solution to improve understanding of the process and to develop predictive solutions to improve risk assessment for this organism. Modelling is increasingly used to complement and integrate the hypothetico-deductive approach to experimentation, as a means of encapsulating the current, mechanistic knowledge base of process dynamics. In forests, tree growth is determined by the interactions between species and ecosystems driven by the terrestrial water, carbon and nitrogen cycles. A number of numerical models explicitly describing the interactions between trees

and their environment have been developed over some years. In particular, a number of such process (or mechanistic) models are now available to predict interactions between trees and forecasted impacts of climate change (Woodward et al. 1995). Such models lend themselves well to describing the interactions between host, environment and biotic damaging organisms. The model used here, and briefly outlined below, is fully described in Evans et al. (2005).

9.8.1 The ForestETp model

ForestETP is an existing, fully coupled, point scale and daily time step soil-vegetation-atmosphere transfer (SVAT) model, which predicts vertical and lateral water movement through the soil-plant-atmosphere continuum as well as gross primary productivity (GPP). The model simulates relevant terrestrial hydrology processes (rainfall interception, vertical and lateral soil water movement, runoff, soil and canopy evaporation, and photosynthesis-coupled transpiration) for a forest stand of known structure, growing in locally determined soil and climate: the model structure is illustrated in Figure 114.

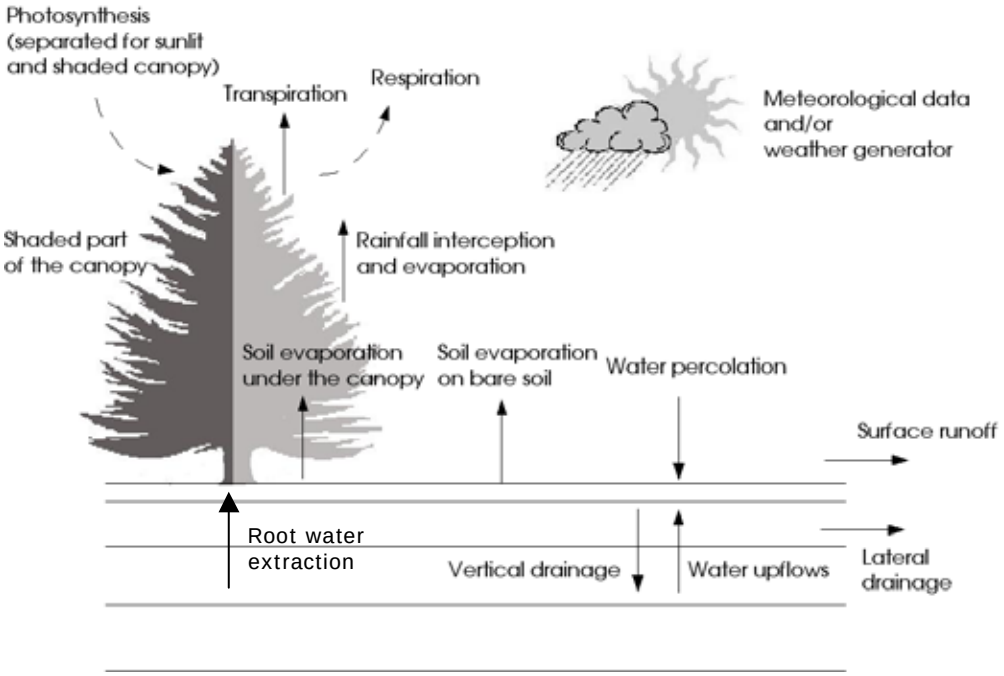

Figure 114: Schematic representation of water (solid lines) and carbon cycle (broken lines) processes simulated by ForestETP

At its core is the Penman-Monteith equation, extensively used in the literature to calculate free surface water evaporation and potential evapotranspiration. The equation simulates the theoretical rate (or reference evapotranspiration) at which water would be removed from the soil and plant surface by a reference crop, and does so by combining approximations of an energy balance and an aerodynamic formula, and without accounting for a structured canopy. In this model, the reference value is dynamically adjusted by the actual soil water available for plant growth, and further regulated by a number of physiological parameters such as (*inter alia*) species-specific stomatal conductance, air temperature, tree height and canopy size and structure. Of specific relevance in the context of potential for PWN to induce wilt, is the model's overall capacity to describe the impacts of dynamic environmental drivers on the tree's photosynthate production potential and water utilisation, determined in particular by nutrient availability (light, water and nitrogen), temperature and CO_2 for both current and, particularly, future climatic conditions.

The ForestETP model has been validated across a range of forest sites in Europe where, along with meteorological and soil water content, data on the water vapour and carbon dioxide content

moving through the forest system are measured on a continuous basis. These data provide high-resolution values of hourly and daily changes in fluxes through the soil-plant-atmosphere system, useful for model validation. Figure 115 illustrates the comparison between observed and simulated carbon dioxide (here expressed as gross primary productivity) and water fluxes (here expressed as evapo-transpiration or ETp) at three *Pinus sylvestris* stands in Europe. Spanning a number of growing seasons, the model appears well able to describe the fluxes of both carbon dioxide and water vapour observed in these forest systems.

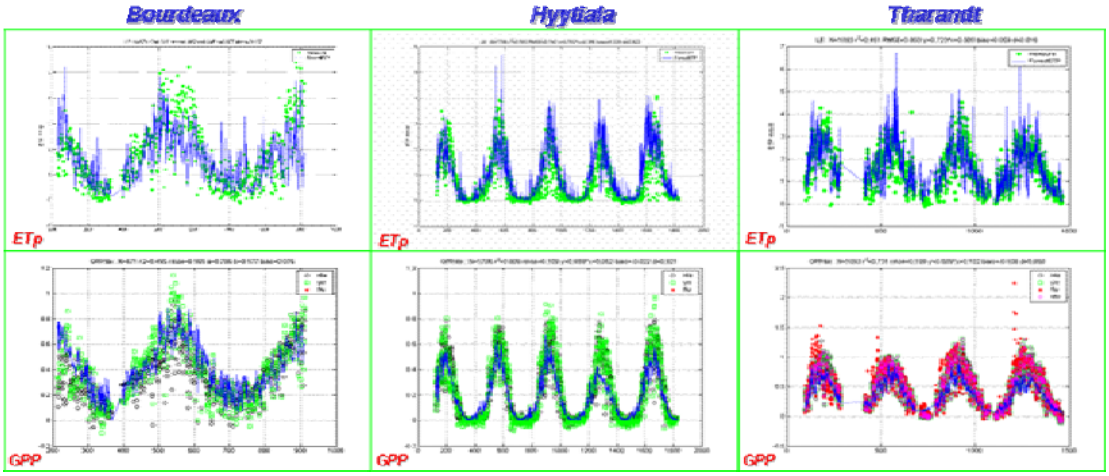

Figure 115: Comparison between simulated and observed values of water and carbon fluxes at stand scale, here expressed as evapotranspiration (ETp) and gross primary productivity (GPP)

9.8.2 The ForestGROWTH model.

As part of a larger mechanistic model (ForestGROWTH), units of net carbon/units of water outputted by ForestETp at the tree level, are dynamically allocated to a number of tree compartments (stems, branches, foliage, fine and coarse roots). In wood, C units are further sub-divided between different tissue types (meristematic, conductive, support and storage), in accordance with a user-defined phenological scheme and a modification of pipe theory (Deckmyn et al. 2006).

In a new approach to understanding and predicting the impacts of PWN on living trees, ForestGROWTH has been extended to allow simulation of host-PWN pathogen interactions under a number of assumptions, described below. These integrate existing knowledge, principally after Ikeda (1996), who found that cavitation occurred at normal pressure in infested trees and Kuroda (1989, 1995) who indicated that the site of cavitation did not always correspond with the location of initial embolism, and determined that gaseous blockages were caused, at least in part, by volatile terpenoids; overall he found that following PWN infestation, the xylem became more vulnerable to cavitation (Kuroda 1989; Kuroda 1995; Ikeda 1996). The model includes a number of assumptions concerning the dynamics of PWN after introduction to a susceptible tree (i.e. intrinsic susceptibility combined with environmental susceptibility).

Assumption 1. The PWN population will increase at known rates as a function of environmental drivers (e.g. internal tree temperature driven by air temperature).
Assumption 2. Once PWN is introduced into the living host tree, it immediately distributes throughout the tree.
Assumption 3. That, following infestation, the plant host will change its biomass allocation strategies; consuming reserves of non-structural carbon to activate defence responses to the PWN. The tree will divert photosynthate from growth, by increasing translocation of new or remobilised substances, to trigger local defence responses or to compensate for PWN-induced damage. In the longer term, carbon remobilisation from storage also contributes to lowering host

resistance further, and to reducing the reserves available for flushing and re-growth in the year following infestation.

Assumption 4. The host tree becomes vulnerable to irreversible cavitation due to the direct and indirect effects of PWN, arising from PWN consumption/destruction of living cells, release and movement of cytoplasm containing molecules possibly inducing embolism (i.e. tannins) or cavitation (i.e. monoterpenoids) acting on tracheids in the xylem. When cavitation occurs, water conductance is compromised in some or all of the xylem portion where it occurs (Zimmermann 1983); xylem water is then diverted elsewhere to avoid the affected part. Cavitation (the formation of air bubbles in conductive tissues) frequency can be measured as negative xylem water potential, approximated as the difference between the theoretical demand for soil water (potential transpiration) and the supply of available water to the canopy (calculated as actual transpiration). In dry regions, such as the Iberian Peninsula, cavitation may occur naturally in most trees during the summer and, depending on severity, can be reversible. Under extreme drought conditions, cavitation can be very extensive, as indicated by high negative xylem water potential, and may be irreversible resulting in wilt expression and tree death. When this occurs, all conductive tissue above the cavitation point, and canopy portions directly supported by affected conductive pipes, will exhibit reduced leaf water potential, lower transpiration and reduced photosynthesis. Correspondingly this lowers photosynthate availability for biomass production.

Assumption 5. By directly and negatively affecting tree ecophysiology in advance of visual symptoms being observed, PWN leads to host death (either in combination, or whichever occurs sooner) where:

- Net photosynthesis is less than respiration and growth maintenance costs over a threshold number of days.
- Irreversible cavitation accumulates over a threshold value above which the host tree cannot maintain the minimum necessary transpiration of water to the crown.

No assumptions are made about the number of maturation feeding events by the vector: their timing and number of nematodes are model input parameters.

9.9 *Model definition and parameterisation*

In the model, cavitation occurs in a conductive pipe, with each pipe in turn supporting a known portion of canopy. Cavitation may be local and not always permanent but, while it occurs, all conductivity is lost in the affected pipes. Thus whole tree conductivity reduces according to the frequency of separate cavitation events. With increasing negative xylem pressure, cavitation may be reversed, allowing affected tracheids to refill with water. The impact of increasing negative xylem potential values is expressed as a Percentage Loss of Conductance (%) [PLC]. Using equation 1 from Martinez-Vilalt and Pinol (2002), PLC is expressed by a vulnerability curve proportional to xylem pressure (Figure 116):

$$PLC = \frac{100}{(1 + e^{A(P-B)})}$$

where:

*A (*2.0) determines the slope of the curve, and correlates with the variance of maximum size of border pits and diameter of tracheid (after Zimmerman 1983);
B is the pressure causing a 50% loss of hydraulic conductivity (PLC50) [–3.0 to -3.9 MPa in pine species] (Martinez-Vilalta and Pinol 2002);
P is xylem pressure (MPa).

There are two conditions under which xylem cavitation can occur (Figure 116):

Condition 1: in *normal conditions*, expressed as the difference between the reference (potential transpiration Pt) and actual transpiration (At), as a function of climate, soils and tree characteristics. Where xylematic pressure recovers, the model assumes that tracheids affected by cavitation can be refilled overnight.

Condition 2: In the *presence of* PWN the range of negative xylem potential values is increased by assuming a vulnerability curve shift (set to –2.2 MPa, after Ikeda 1996) in order to simulate the loss of conductive tissue through feeding, embolism and loss of tissue cohesion: the shift is also proportional to population size and pathogenicity of nematodes. Living cells, such as parenchymic tissue cells, are believed to have an important role in refilling xylematic tracheids (Tyree and Sperry 1989; Canny 1995; Tyree et al. 1999). In an infested tree, these cells are consumed by nematodes: the model therefore assumes that refilling cannot occur in the infested tree. With cavitation, and by reducing canopy water content and photosynthate production, this affects biomass productivity, the re-deployment of carbon reserves to defence and death by drought. When needle water potential drops below –5.0 MPa, irreversible damage is assumed and the needle ceases to function.

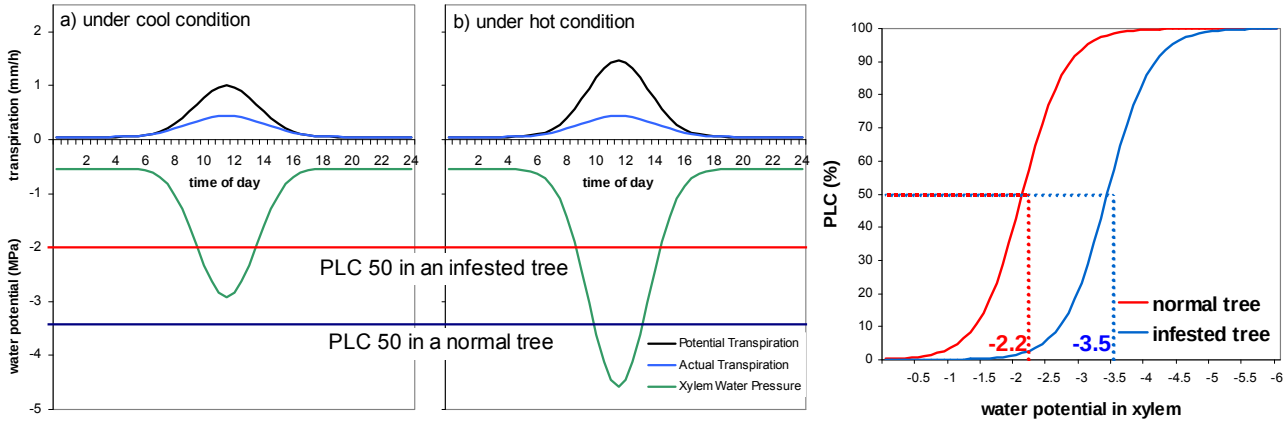

Figure 116: Simulated model interactions in xylem water potential between a healthy and a PWN-infested host, with interactions between climatic conditions and values of PCL50.

9.10 *Results*

9.11 *Modelling experiments*

The PWN process models have been run at the single tree level, simulating the impact on key physiological processes and on whole tree physiology, following PWN inoculation. Simulations 1-2 describe conditions typical of those observed in the area of Portugal currently affected by pine wilt disease; simulation 3 is broader in scope, and extends to two sites in the Iberian Peninsula and a third UK site. In simulation 4, the model has been run for the same tree inoculated with nematodes under a range of pathogenicities; here the results were used to express the likelihood of mortality following infestation.

Species-specific model input parameters determining condition 1 are from the literature; in the absence of measurements for trees affected by PWN, input values to simulate condition 2 are approximated, and determined by the speed with which loss of leaf water potential has been observed (Sobardo et al. 1992; Salleo 1996).

9.12 *Modelling simulations*

Modelling simulations have been undertaken of the trajectories of key physiological processes of a single tree following the inoculation of PWN and with increasing nematode population numbers. Simulations 1-2 are for conditions typical of those observed in the area of Portugal currently affected by pine wilt disease; simulation 3 refers to 2 sites in the Iberian Peninsula and to a third UK site.

SIMULATION 1. The model is run for 1 year (ending 31 Dec = day 365), with PWN inoculation taking place on julian day 180. Figure 117 provides trajectories for PWN population growth and key physiological parameters.

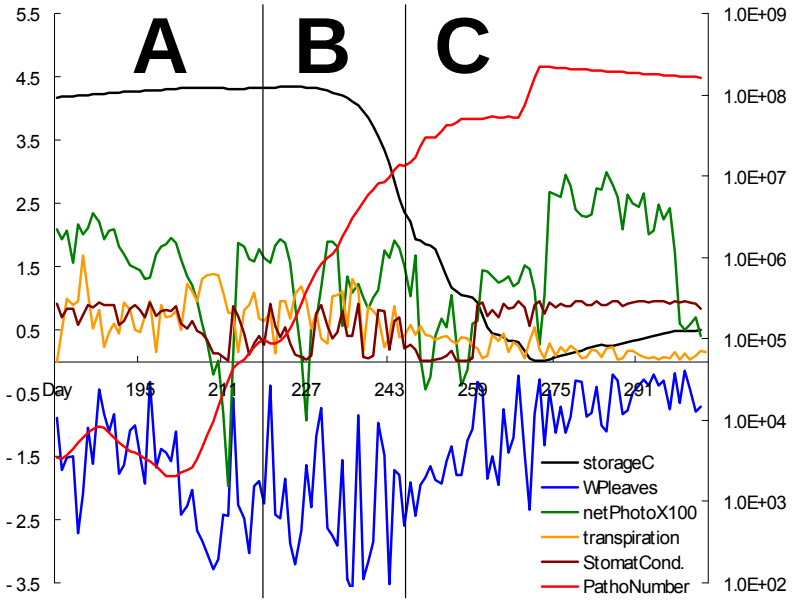

Figure 117: Simulation 1: simulated trajectories of physiological parameters following PWN infestation, over the period between day 180 and day 320.

Three phases can be observed:
Phase A. The PWN population is present in low numbers. Tree physiological parameters respond primarily to local environmental conditions.

Phase B. Favoured by high air and, therefore, tree temperatures, the PWN population increases. In conjunction with summer drought, and as a result both of PWN movement through the host and of feeding, cavitation becomes extensive. This is expressed by significant reductions in leaf water potential, net photosynthesis and stomatal conductance. Further, the host responds by decreasing the amount of non-structural C to reserves and towards defence mechanisms.
Phase C. With the onset of autumn, and a significant increase in soil water content and with temperatures limiting further PWN reproduction, the host has an opportunity for some recovery in physiological processes and photosynthesis.

SIMULATION 2. Using the same conditions as Simulation 1, the model is run over a two year period. Figure 118 shows key model outputs over the period 1 Jan (Day 1) to the date of tree death, occurring on day 541.

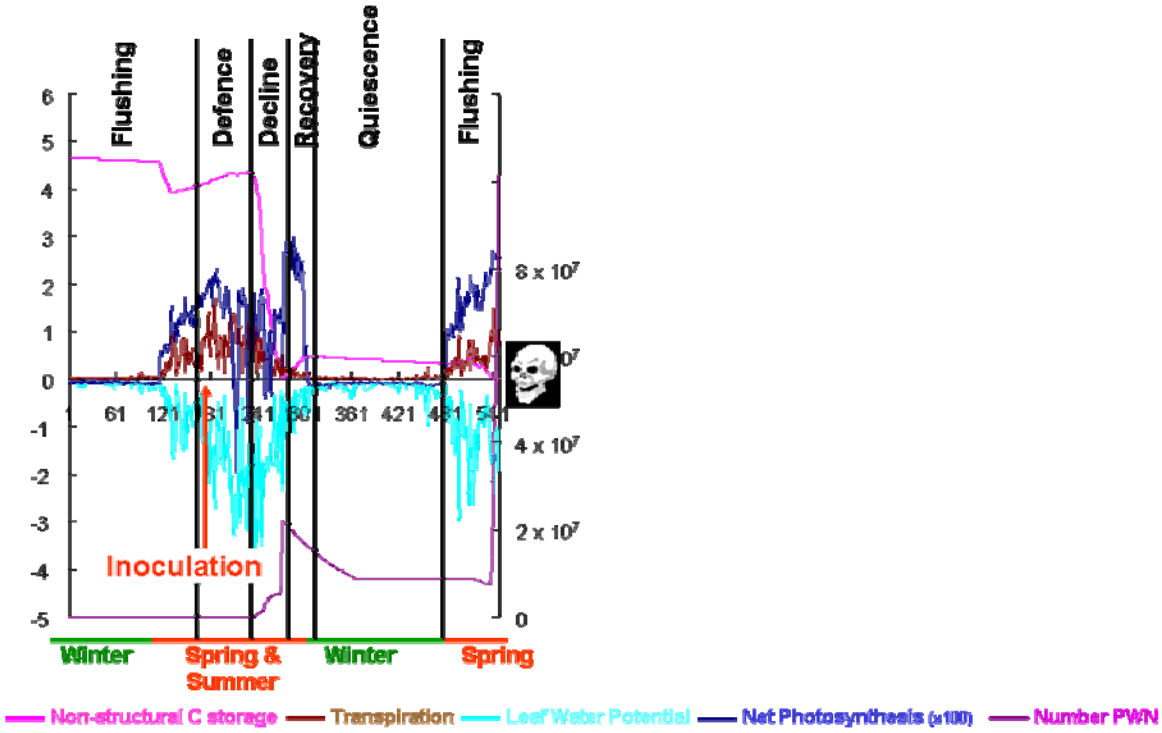

Figure 118: Simulation 2: simulated trajectories of physiological parameters following PWN infestation, over the period between day 1 and day 541.

Six phases can be observed:

Flushing year 1. Prior to inoculation with PWN on day 165, normal tree physiological activity is simulated prior to and after the onset of flushing. Non-structural carbon reserves decrease slightly in order to support the production of new biomass and in advance of both older and new needles becoming sources of carbon.
Defence. Again, the PWN population is present in low numbers and does not affect tree processes adversely. Tree physiological parameters respond primarily to local environmental conditions.
Decline. Cavitation occurs both as a result of environmental pressure and as a result of growth in the PWN population, resulting in significant reductions in photosynthate production alongside diversions of carbon from growth and storage to defence.
Recovery. With increasing soil wetness and lower air temperatures in the autumn, the host tree increases photosynthesis concurrently with fixed or decreasing PWN population. This improves tree tissue conductivity and allocates modest amounts of photosynthate to parenchyma storage sites of non-structural carbon.
Quiescence. Little or no activity is simulated during winter months in both PWN and the host.

Flushing year 2. Following bud-burst and to develop new foliage, the host draws on remaining reserves of non-structural carbon: these are however insufficient to allow concurrent usage for new biomass production, regeneration of damaged or destroyed conductive tissue and defence. With increasing temperatures and reducing capacity for defence by the host tree, the PWN population increases dramatically, entirely compromising the host's conductive tissue, and resulting in tree death.

SIMULATION 3. In this simulation, the model has been run over two years, at three sites with different climates, for the same species of tree: PWN is introduced on day 180 of year 1. For illustrative purposes, only simulated trajectories for leaf water potential and xylem water potential are shown in Figure 119.

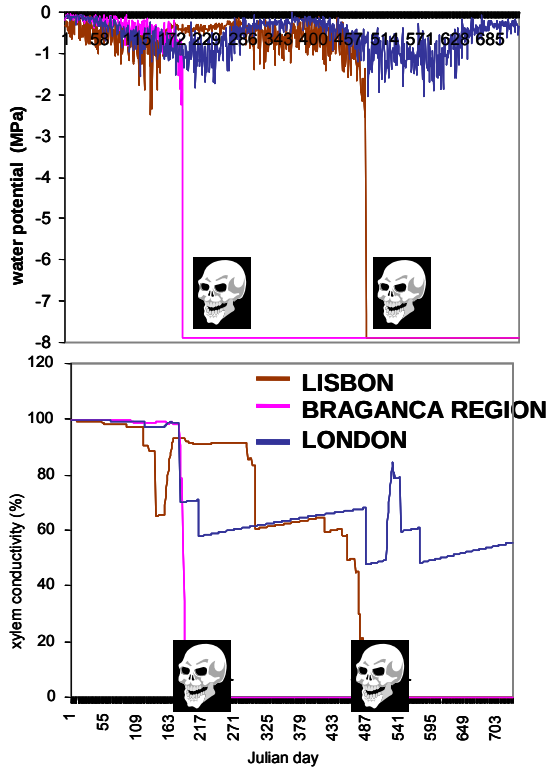

Figure 119: Simulation 3: simulated trajectories of leaf water potential (MPa) and xylem conductivity (as a %) at 3 sites over 2 years.

The results of simulations suggest that the timing of PWN infestation, the beginning of the drought season and the timing of flushing in the second year after infestation could be crucial for symptom development. In the Lisbon region, the host starts transpiration intensively before cambium growth and PWN infestation while Bragança and London show high transpiration after cambium growth. PWN infestation occurs in the dry season in the Lisbon area where, due to soil drought, the host will tend to close stomata, thus transpiration is highly regulated. As a result, although soil water potential has high negative values, the host can, through stomatal regulation, maintain adequate xylem water potential in the needles, which acts as a protection mechanism to avoid irreversible cavitation. In contrast, PWN infestation occurs during a relatively wet season in Bragança, thus the host still transpirates intensively which results in high negative xylem water potential that can accumulate cavitation quickly leading to host mortality in the year of nematode infestation. Tree hosts in the Lisbon area can, therefore, survive until the second year but will face a high transpiration period before renewing damage tissues, which can result in wilting and death in the second year.

Where environmental pressures are less marked and conditions unfavourable to PWN population growth, as at the UK site, while a reduction is xylem conductivity after inoculation is predicted, low

PWN numbers and higher soil water content result in only limited damage to the host, which appears able to localise the PWN population and to survive beyond the second year after infestation. In all simulations, the host is known to be intrinsically susceptible to PWD in areas with suitable climatic and environmental conditions.

SIMULATION 4. Given the sensitivity of the model to both host-environment and host-PWN interactions predicted by the prior simulations, this simulation was undertaken to predict the probability of tree death (as a percentage), following PWN infestation with various assumed nematode pathogenicities at selected sites in Portugal, where each has different environmental conditions. Figure 120 provides simulated values of the likelihood of host mortality occurring following inoculation.

Figure 120: Simulated values of the likelihood of host mortality occurring following inoculation of susecptible pine trees in Portugal.

Predicting a range of mortality values across the region, the model suggests that some 90% of infested tress will die in the Lisbon area, and are more likely to do so in the year following infestation. In the Bragança region the simulation suggests that around 40% of infested trees will die, and are more likely to do so in the year of infestation, dependent on the timing of high transpiration and the flushing period of trees.

9.13 *Model Validation*

9.14 *Introduction*

To date there has been a lack of field or laboratory data to allow verification of simulated physiological processes and PWN population dynamics. Validation of carbon and water flux outputs from the ForestETp model has elsewhere shown good agreement between (inter- and intra-annual) simulated and observed values (Evans *et al*, 2005). Indirect comparison with mortality rates at a site in the Setubal Peninsula, Portugal where PWD is prevalent and significant environmental stresses occur, indicates that simulated mortality values are similar to those reported across the PWN affected area.

9.15 *Materials and methods*

A manipulative, replicated experiment involving inoculation of trees at the crown level with 32,000 PWN/tree is taking place in Portugal. The nematodes used were obtained from an infested site in Troia peninsula, Portugal and reared in laboratory conditions at Nemalab, Evora University. Inoculation experiments were done using healthy cultivated 8-year old *Pinus pinaster* trees (8 m in height) at a site at Alcochet, 40km south-east from Lisbon, Portugal. Continuous instrumental monitoring of key environmental drivers regulating plant growth and a range of ecophysiological parameters is being undertaken to evaluate host responses to the pathogen. Monitoring of wilt symptom development has also been undertaken, along with semi-destructive sampling to assess the distribution and frequency of PWN in woody tissue of infested trees. The experiment is ongoing and will continue into the second growing season following inoculation: preliminary data are presented here to illustrate the range of symptoms observed and as initial comparison with model predictions. Full results will be described elsewhere.

9.16 *Results*

Monitoring of soil water at depth (Figure 121) indicates severe seasonal drying throughout the profile, and a source of considerable water stress to plants.

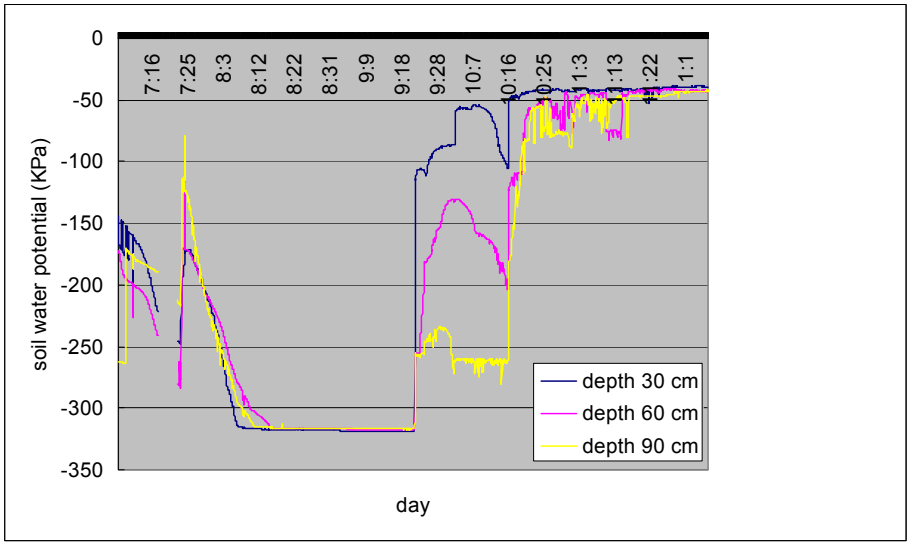

Figure 121: Soil water potential (KPa) at 3 depths measured over the period 9th July-6th November 2006.

Over the corresponding period, sap velocity (Figure 122) and needle water potential (pre-dawn and at midday) (Figure 123) were monitored in control (no PWN inoculation), asymptomatic and symptomatic trees both inoculated with PWN; at this stage, asymptomatic trees did not exhibit visible symptoms of pine wilt, expressed as progressive discoloration, browning and death of needles

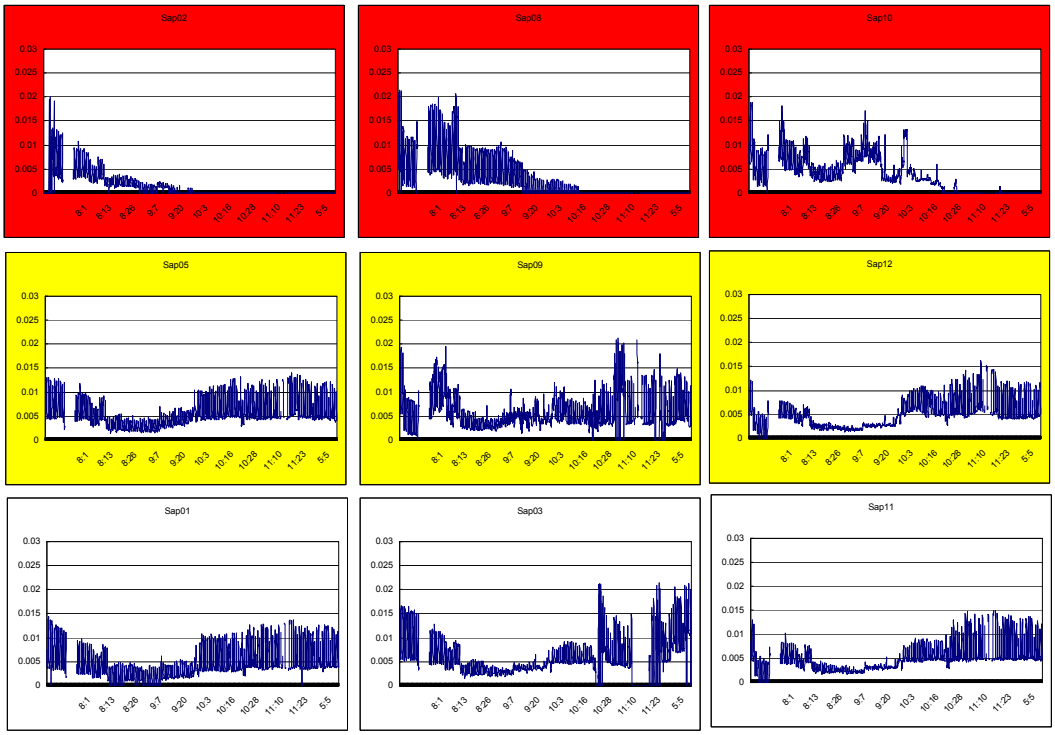

Figure 122: Sap flow velocity (cm/s) in symptomatic (red), asymptomatic trees (yellow) and control trees (white) over the period 9th July-6th November.

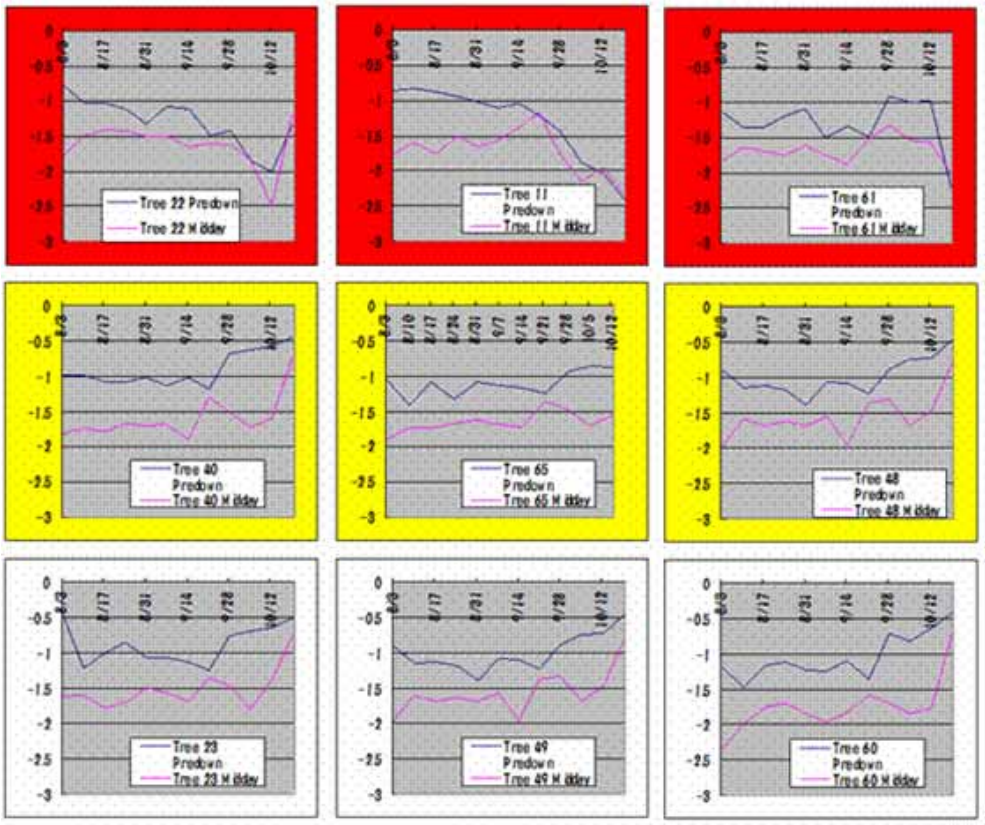

Figure 123: Needle water potential (MPa) in symptomatic (red), asymptomatic (yellow) and control trees (white) over the period 3rd Aug. - 19. Oct.2006.

Data from symptomatic trees indicate significant reductions in both sapflow and needle water potential, indicative of a progressive breakdown in the vascular conductive system. This is also accompanied by visible symptoms of wilting and progressive discolouration of the crown. Initial data also suggest progressive movement of PWN throughout the woody tissue, confirming repeated literature observations (Kiyohara and Suzuki 1978; Mamiya 1985; Kuroda et al. 1988) and laboratory/field assessments in the PHRAME project. Data for asymptomatic trees suggest that individual trees have contained the PWN inoculation at the site of wounding.

Initial model simulations, undertaken prior to the experiment and in the absence of field data, suggest a 90% likelihood of tree death over the two year period following infestation with PWN: at the time of writing (November 2006) observed tree mortality stands at 46 % of the sample. However the true extent of mortality among infested trees will not be known until flushing occurs in 2007.

An initial and only qualitative comparison between trends in the field assessed and simulated data suggests broad correspondence, supporting the view that the model appears able to describe, with good approximation, the complex interactions and sequence of activity following PWN infestation. It remains to be seen whether, following more accurate parameterisation, the model will successfully simulate the overall mortality rate observed in the field experiment.

9.17 *Discussion*

Using an existing mechanistic model of tree and stand scale carbon and water cycles that simulates tree growth dynamics under a range of conditions, here extended to describe PWN growth dynamics following host inoculation and nematode-induced cavitation, a series of model simulations have been undertaken to simulate and predict the impact of PWN infestation on host physiology in the Iberian Peninsula. The model makes a number of assumptions concerning plant host-pathogen interactions, particularly in relation to the environmental conditions of host growth.

Model results suggest that, under normal conditions, seasonal drought and/or high temperature significantly reduce host physiological activity, resulting in seasonal water stress and partial, reversible cavitation. Initially affecting the cambial zone, and with rapid extension to the conductive and reserve tissues, PWN infestation represents a significant, additional stress. This is expressed by the increasing presence of cavitation in conductive tissue that reduces canopy water supply and photosynthates to sites of meristematic activity and non-structural C storage. Ultimately, PWN activity, possibly in combination with the host's own defence mechanisms, results in degradation and destruction of cambial, conductive and storage tissues.

The model has been used to simulate tree mortality across a range of sites characterised by varying degrees of environmental pressure on tree physiology which, when coupled with the dynamics of PWN population growth, can result in tree death. Depending on local environmental conditions, the model suggests that infested trees may die either in the year of infestation or in the following year and following flushing. This result is, however, heavily dependent on the phenology and physiology of the host plant. In all simulations, the period of flushing was kept constant, but if it takes place prior to the high transpiration period, then a tree may be able to, at least partially, renew damaged tissues and could survive.

Initial model results are currently being compared with the results of an ongoing, observational experiment being undertaken in Portugal, using PWN extracted from a naturally infested site where significant pine wilting and mortality has been observed. Initial results suggest good agreement between observed values of host physiological parameters and those simulated by the model, as well as predicted rates of mortality. On the basis of these results it is proposed that, with further refinement and validation, the model may be suitable for developing a generic framework to predict the vulnerability of different hosts to the organism across a range of geographical regions. This represents a highly significant breakthrough in understanding the processes that result in pine wilt in some regions of the world. Conversely, it also explains why, in its natural range, despite having the same tree species and known exposure to the nematode through maturation feeding in the crowns of susceptible trees, little or no tree mortality occurs.

Chapter 10 Discussion

Each chapter has provided a discrete description of the progress made in a number of key aspects of the identity, biology, dispersal, actual and predicted impacts, and potential management of pinewood nematode, *Bursaphelenchus xylophilus*. Detailed discussion on each of the topics is provided at the end of each Chapter.

Irrespective of the fact that there has been a strong literature and, indeed, ongoing research on the effects of pinewood nematode globally since the early 1970s, one of the principal findings of the current programme is that a great deal of research and study has to be carried out to provide precise data on the interaction of *B. xylophilus*, its vectors and host trees in a new environment. This indicates strongly that extrapolation of data from other ecoclimatic zones has to be done with care and that the guidance provided from the literature must be tailored to local requirements. The research in this programme has done just that; it has looked at the fundamental biological aspects of the PWN/vector/tree/environment interaction and has provided a strong base of relevant and inter-related information that can be used to guide future policy on *B. xylophilus* in Europe. Since the approach is based on an enhanced understanding of processes, the data are also applicable to other regions of the world, where local parameters can be used to generate predictions.

Some elements of the research have confirmed the widely held understanding that the only effective and proven vectors of *B. xylophilus* are longhorn beetles in the genus *Monochamus*. Confirmation that *M. galloprovincialis*, a previously uncommon beetle in the Portuguese forests, has not only taken on the role of vector but has increased considerably in its incidence since PWN arrived in the country was provided by Sousa *et al* (2001). The inter-relationships between PWN and *M. galloprovincialis* have been studied in detail and this has provided valuable information on the biology of the vector under Portuguese conditions and elsewhere in Europe (Chapter 4, Chapter 6). Seasonal dynamics of the vector indicate that it has a single generation with a flight, and oviposition, peak in mid-summer. This period coincides with the introduction of nematodes to host trees either through maturation feeding – which could result in wilt expression – or through oviposition, which mirrors the normal biology of the nematode in its native range in North America.

A particularly significant finding is that when there was application of thorough eradication procedures, which took place at the experimental plots in Tróia and Companhia das Lezirias, no infested beetles emerged in the managed forest blocks. This indicated the potential of tree removal procedures for managing the nematode threat and also suggested that new infestations were caused by immigrant vector adults that carried out maturation feeding in the crowns of some trees. Further, the data also indicated that adult flight from the edges of forests into neighbouring forests and woodlands was also possible, suggesting that migratory flights might spread the nematode to adjacent, non-infested areas.

Studies of the range of *Bursaphelenchus* species and some close relatives, indicated that many species are present in the European Union (Chapter 3). Some of these are closely related to *B. xylophilus* and are difficult to differentiate using conventional morphological techniques. Nevertheless, the data gathered, particularly in Portugal, France and Spain, indicated that PWN was absent from the latter two countries, despite their proximity to Portugal. There has been extensive use of molecular techniques both to confirm the identities of the range of *Bursaphelenchus* species found in this project but also to study the range of variation within the species and to help understand its country of origin and pathway to Europe. Although not definitive, the combination of several techniques, including ITS-RFLP, ITS1/2, RAPD-PCR, ISSR, confirmed that the likely origin of the Portuguese strain(s) of *B. xylophilus* was SE Asia (Chapter 8). Satellite DNA techniques and ITS-RFLP also indicated that there are two distinct strains of PWN in Portugal, suggesting either separate incursions or a single incursion of a mixture of strains.

Field and laboratory data indicate that maritime pine, *Pinus pinaster*, which is grown widely in Portugal, is highly susceptible to expression of wilt symptoms when PWN is introduced to the crowns of healthy trees during maturation feeding by *M. galloprovincialis* (section 6.5). There was

also evidence that the vector did not attack and feed on umbrella pine, *P. pinea*. There was little prior information on the interaction between PWN and *P. pinaster* and, therefore, this provided focus for much of the research on the biology and pathogenesis of *B. xylophilus* under European conditions (Chapter 5). Of particular interest, especially in relation to development of risk models and for improvement to Pest Risk Analysis procedures, was the rate and scale of infestation of susceptible tree species. Most studies on this aspect were concerned with experiments involving inoculation of seedling trees with known quantities of nematodes. Results are described in detail in Chapter 5 but the common thread from studies carried out in Germany and Portugal (with linkage to work in Japan carried out by Mota *et al*), was that nematodes enter live trees and move immediately away from the inoculation site to colonise most of the circulatory system, including the roots. This method of colonisation of a tree only occurs in living trees and is dependent on interaction of the nematodes with living cells, initially in the cambial zone and, thereafter, the xylem. By contrast, nematodes introduced to trees during oviposition tend to remain near the oviposition pits made by the female beetles (section 6.6.4). Detailed information is now available on the temporal and spatial dynamics of PWN in living trees and this has aided the development of process models of the interactions taking place within living susceptible trees.

Although it is well known that *B. xylophilus* can survive in a wide range of coniferous trees and over a very wide geographical area (e.g. the whole boreal zone in Canada), there is remarkably little knowledge about the precise conditions under which maturation feeding can lead to wilt expression after introduction of nematodes into the crowns of susceptible trees. Correlative studies indicate that temperature is a key determinant of likelihood of wilt (de Guiran & Boulbria, 1986; Rutherford & Webster, 1987). However, such studies have taken only limited account of the interactions between the other principal variables such as tree species, soil type, soil moisture content and local meteorology in a given region. The approach adopted in the present project has been to integrate these variables into several modelling approaches to develop a risk analysis that can be tailored to local requirements. Using a range of heuristic and empirical models, maps of the areas of Portugal that are at high risk of expression of wilt have been generated. These match the characteristics of the sites for tree growth, particularly for *P. pinaster*, the main commercial species. This approach provides a regional matching exercise that is of value in assessing current as well as future risk of PWD developing.

A further approach to predictive modelling of wilt expression has also been successfully developed in the research programme. This is based on process modelling using current understanding of plant-host interactions as the basis for assumptions incorporated into a mechanistic model describing relevant process dynamics. The model describes plant host physiological behaviour following PWN infestation that ultimately may result in death of the host plant. Several simulations have been run for sites in the Iberian Peninsula, including those where fatal infestation of pines by PWN occur. These indicate a high likelihood of host death, both immediately and in the year following infestation. Globally in regions where the nematode occurs and where environmental conditions do not result in significant tree stress, PWN does not result in wilting and host death. An ongoing observational experiment using PWN-infested trees, suggests a good correlation between simulated and observed results. It is proposed that, with further refinement and validation, the model may be suitable for developing a generic framework to predict the vulnerability of different hosts to PWN and other organisms across a range of geographical regions.

Chapter 11 Conclusions

There is no doubt that *Bursaphelenchus xylophilus* is a significant threat to the pine forests of Europe and this is confirmed by the considerable tree mortality that has already been observed in the affected zone in Portugal. For example, surveys carried out in the affected zone during the winter of 2006 revealed around 200,000 symptomatic trees and, of the trees sampled for presence of PWN, approximately 22% were positive for *B. xylophilus* (Report of FVO Mission to Portugal, May 2007). Such data indicate that the scale of the problem is large and, therefore, access to definitive information on the interactions between PWN, its vectors and its host trees in a European context is essential. The results presented in this report provide a solid basis from which to determine optimised methods for management of this important new pest to Europe.

In relation to identification of the source of the *B. xylophilus* populations in Portugal, a combination of sophisticated molecular techniques have indicated that at least one race of the nematode originating in Asia has arrived in the country. There are indications that two sources of origin are included in the Portuguese population of PWN which suggests either multiple introductions or a single introduction of a mixed population of the nematode. While of academic interest, this information is valuable in assessing potential future pathways of arrival of further incursions of the pest to Europe. Extensive surveys of trees in the countries represented within the project have revealed no further findings of *B. xylophilus* outside the affected zone in Portugal. However, the surveys have revealed many other species of *Bursaphelenchus*, including the closely related *B. mucronatus* and *B. fraudulentus*, which are difficult to differentiate morphologically. The data provide encouragement that *B. xylophilus* itself is still restricted to the affected zone in Portugal. New molecular techniques from this research programme will aid rapid and accurate identification of the members of the genus *Bursaphelenchus* in future.

A key factor in the survival and spread of PWN in the field is association with a vector insect species. Extensive studies were made of the potential of members of the xylophagous insect fauna associated with PWN-affected trees to act as vectors of the nematode for further spread of the organism. A clear conclusion from the studies is that only *M. galloprovincialis* has taken on the role of vector in Portugal. Although there are nematodes associated with a number of bark and wood-boring beetles in Austria, Portugal and Spain, none of these has been shown to successfully transmit *B. xylophilus* to host trees. A further significant finding from the project is that despite clear demonstration of the presence of PWN in the roots of affected trees, there was no evidence of transmission of the nematode to adjacent trees either through root grafting or by direct migration through the soil. This information confirms the widely demonstrated results from international literature on PWN that it is only adult beetles in the genus *Monochamus* that act as effective vectors of the nematode.

Information on the biology and pathogenesis of PWN in relation to tree species potentially likely to exhibit wilt expression following introduction of the nematode to the crowns of trees during maturation feeding by *Monochamus* spp is critical and essential to understanding and predicting likelihood of wilt expression. Correlative studies in regions of the world where pine wilt disease has been demonstrated all indicate a close relationship with average summer temperatures, including hypotheses that monthly isotherms must exceed 24^{o}C to give rise to extensive tree mortality (Rutherford & Webster, 1987). While of general practical value, such correlations cannot account for local variation in eco-climatic conditions and it was recognised that more detail on the nature of pathogenesis of PWN in living susceptible trees was needed. Results from studies carried out in Germany, Portugal and Japan within this project have thrown considerable light on the nature of the process of wilt expression when *B. xylophilus* is introduced to living trees by maturation feeding. Of particular significance is the knowledge that movement of nematodes from the site of introduction is immediate and rapid, with some nematodes being found in the root area within days of inoculation. Such patterns of invasion can be explained by the ability of the nematodes to exploit living tissues both for nutrition and reproduction and also to move into less well defended areas of the affected trees. This matches with previous histopathological examination of nematode invasions that linked their rapid movement downwards to exploitation of the cambial zone, leading

to breakdown of tissue integrity and the opening of cavities that facilitated free movement of nematodes. Lodging of nematode populations in particular parts of the tree was also shown to provoke local defence reactions, which contributed to embolism and cavitation of the xylem. Presence of large numbers of nematodes in xylem cells also confirmed the ability of *B. xylophilus* to breed successfully in the living trees. Such data are essential building blocks in developing process-based predictive models for wilt expression.

With the demonstration that *M. galloprovincialis* is the only known vector in Portugal, data on the biology and dispersal of the vector have been gathered, starting from a low knowledge base on this insect prior to the appearance of PWN in Portugal. Results from the project studies have shown that the vector has a single generation per year, with clearly defined emergence, flight and oviposition peaks around mid-summer in Portugal. The proportion of adults carrying viable *B. xylophilus* dauer larvae emerging from trees killed by PWN can be high (ca. 75%), indicating a close relationship between tree mortality from PWN and subsequent exploitation of those trees for breeding by the vector. Such data confirm the cause and effect relationship between PWN, its host trees and the breeding success of the vector, the combination forming a feedback loop leading to increasing tree mortality and availability of breeding resources. The Portuguese strategy of early identification and removal of PWN affected trees before emergence of vectors insects that may have used them for breeding is, therefore, sound. Methods to increase the efficacy of sampling for vector insects are part of such an approach and considerable progress has been made in identifying and improving trap and chemical lure designs for use in both monitoring and, potentially, reduction of vector populations. Within this approach, attempts have been made to develop methods for early detection of infestation and potential wilt expression in PWN-affected trees. A simple method to measure oleoresin flow from a punch hole system, proved to be quite reliable for larger trees (> 20cm DBH) and gave early warning of subsequent wilt and tree mortality, but this was dependent also on the time of year and month of infestation by the nematode. Electrical conductivity methods did not provide sufficient warning of likely tree mortality.

Bringing together the detailed and more general information on the interactions between PWN and its host trees in an eco-climatic context, significant progress has been made in predictive modelling of wilt expression. Two main approaches have been adopted, namely use of correlative heuristic and empirical models to link to tree suitability parameters and a process-based modelling strategy that links directly to tree physiology in relation to local eco-climatic conditions. This complementary approach has started to yield valuable predictive tools for improved risk assessment in the future. The main conclusions from the correlative modelling approach were:
1. Predictions of PWD dispersal over the whole of Portugal using the PWN occurrence data from the affected zone were not successful; however if more data become available, this type of modelling is worth exploring;
2. A tree based approach produced useful predictive models of the PWD risk for Portugal - Central Portugal showed up as the area of most concern where there was high pine density growing in poor site conditions;
4. High temperatures and low precipitation showed up as the main drivers of tree stress, which indicates climate change as a future central area of research in dealing with the risk of PWN spreading throughout Europe;
5. The models can be used to improve the PWN national surveys, focusing field effort at high risk areas.

The process-based models employed and adapted existing tree growth models that have already been validated for pine species in Europe. Addition of PWN as a factor compromising the evapotranspiration models has allowed direct prediction of the effects of the nematode using moisture and temperature as key driving variables. Simulations carried out under a range of conditions in Portugal and in the UK as a contrast, indicated that high mortality can be expected in the Setubal region of Portugal, which confirms the situation observed in *P. pinaster* trees in this area. The model also predicted when mortality can be expected in relation to month of infestation in the field and, here, there were some surprising predictions in that some trees in the main affected area are predicted to die in the second year after infestation. This has profound implications for survey strategies based on symptomatic trees; some trees may be infested by the

nematode but not be declared symptomatic during a winter survey and could be missed. Current work includes field trials with inoculated standing trees to verify the predictions of the models. This work is ongoing but early results confirm the mortality and timescales arising from PWN infestation of susceptible trees.

In final conclusion, the combined data from this study have advanced knowledge on the interaction of PWN, its vectors, host trees and eco-climatic factors in both a regional and global context. Much has been learned about the PWN system and there are real prospects of using the advanced knowledge to provide customised Pest Risk Analysis predictions of PWN impact in any location in Europe. This will commence with use of the process-model to provide country and region specific predictions of likelihood of wilt expression.

Chapter 12 Exploitation and dissemination of results

During the course of the project, a number of publications, conference presentations and seminars have been written and presented by members of the consortium carrying out the research. These are listed at the end of this Chapter.

In addition and underpinning the information flow from the project, the University of Evora coordinated the gathering of key literature and other relevant information into appropriate databases. This was done by Prof Manuel M. Mota and a MSc student, Paulo R. Vieira.

12.1 *Electronic databases for pine wilt disease (PWD) and the pinewood nematode (PWN) Bursaphelenchus xylophilus.*

12.1.1 Introduction

Scientific information generated from a research project is usually presented in the form of scientific papers or at international meetings (oral or poster). This may include species descriptions, images, experiments, etc… (Figure 124).

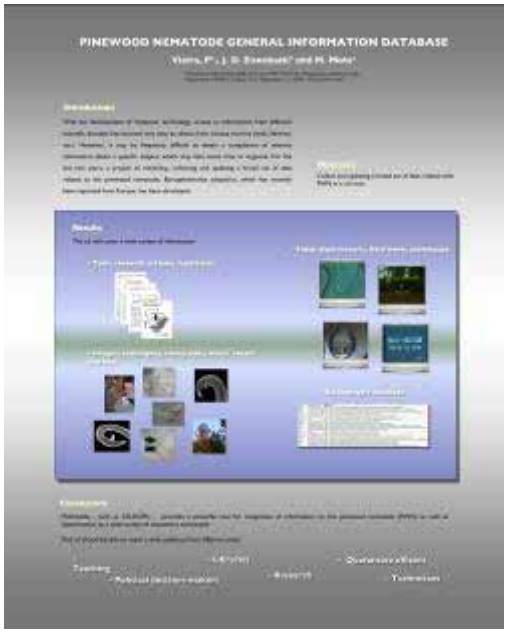

Figure 124: An example of a poster presentation arising from the PHRAME project

Complementary to this type of information, technical and legislative information may be generated, if the scientific subject is intimately connected to economic or political issues, in a particular country or continent. That is the case of the “Pine Wilt Disease” (PWD) issue. This disease, affecting conifer forests in several regions of the world, is elicited by the pinewood nematode, *Bursaphelenchus xylophilus*, its causative agent (Vieira & Mota, 2006). This nematode, and the consequences of the damage it has caused in East Asia, North America and now recently in Portugal, Europe (Mota et al., 1999) has been the subject of nearly 2000 scientific papers, mostly originated from Japan. Numerous technical bulletins and specific legislation have been generated over more than 30 years, more recently (1999-2006) from the European Union (EU). The genus *Bursaphelenchus* comprises more than 75 species (Ryss et al., 2005). It is essential to understand the diversity of the group and, in particular, the members belonging to the “xylophilus” group, closely associated with *B. xylophilus*.

Thus, the accumulated information on this issue has grown to a highly significant number. The need to compile these data, as well as other types of data such as e-keys, images, video, etc… in a coherent and logical manner seems obvious. The electronic format of presenting and making available scientific information has become generalised during the last decade (Eisenback et al., 2006). There are numerous advantages, among which is the ease of quick access, namely for scientists and decision makers, and the educational value, both for students and technicians.

The information presented here was organised in order to provide scientists, technicians and decision-makers dealing with PWD and the PWN with useful tools to help quick access to reflect on the progress of the science for each particular subject, e.g., a first report of the nematode in a certain country (Mota *et al*, 1999), to provide a clear and overall view of the existing species of *Bursaphelenchus*, to allow the use of an electronic key of the genus and also to access a specific technical issue. Undoubtedly, the most important resources for nematode taxonomy are the original species descriptions. Unfortunately, nematode descriptions have been published since the middle of the 18th century in a variety of forms including many relatively obscure journals and proceedings and sometimes in lengthy special publications. As a result, the task of collecting all of the descriptions of a single genus is daunting and has been repeated by numerous nematode taxonomists around the world. Furthermore, many of the papers were printed on acid containing paper and are rapidly deteriorating by yellowing and becoming brittle.

This information may also be useful for educational purposes, in particular for graduate students in nematology, forestry, entomology, etc…

12.2 *Materials & Methods*

Source material, such as original descriptions of species, from various journals, was used. All copyrightable material was verified for public dissemination, i.e., all journals were contacted and the appropriate authorisation obtained. The same applied to all images used. A large portion of this material is already in “PDF” (portable document format), thus making it easy to immediately include in a specific collection. Most printed material (scientific papers, etc..) was scanned with an Epson (http://www.epson.com/) professional scanner at 300 dpi. The bibliographic references program EndNote (http://scientific.thomson.com/products/endnote/) was used for organising the references on PWD and PWN. This program allowed export to other programs or to a simple text file. Direct access to CAB Abstracts (http://www.cabi-publishing.org/) was obtained via the University of Évora site licence.

Whenever travel opportunities were available (e.g. to Canada, USA, Russia, Czech Republic, China, Japan and Korea) local contacts were established and authorisation was obtained in order to copy relevant and often rare publications from these countries. Scanned material was either provided online and by e-mail to participants, per request, or were compiled into CD-ROM, with an appropriate label and are now either available or soon to be available commercially (http://www.mactode.com/).

12.3 *Results*

Three CD-ROMs have been produced so far, one on PWN taxonomy (database; 2 CDs, in fact, 1st and 2nd editions) (for example, Figure 125), another on the broader issue of PWD, also as an information database , and a third on the taxonomy of the closely related genus *Laimaphelenchus* (Ryss et al., 2004b). This genus may be confused by non-specialists with the genus *Bursaphelenchus*. The PWN taxonomy database contains all of the original descriptions of all of the species in the genus *Bursaphelenchus.* The documents can be examined with any software application that can open PDF files, most commonly Adobe Acrobat Reader. Additional information can be found in the README file.

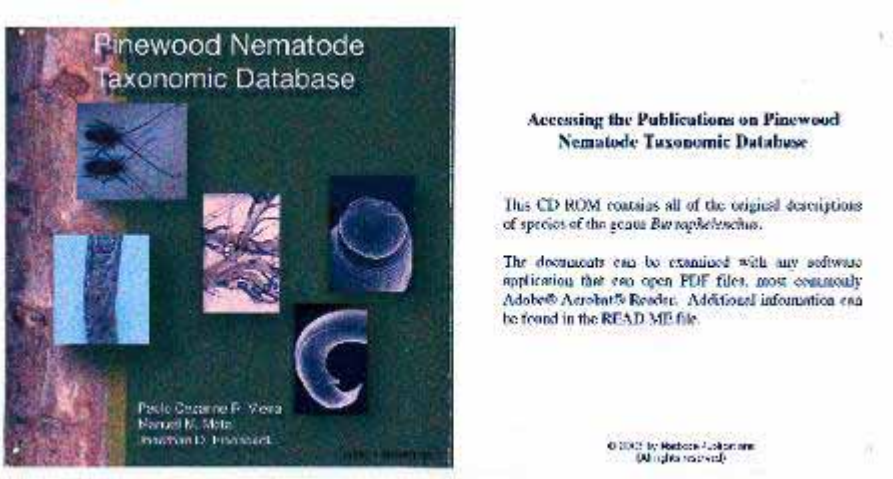

Figure 125: CD ROM containing the pinewood nematode taxonomic database

The second CD-ROM (in publication) contains an EndNote file with all listed publications on pine wilt disease (ca. 2000) with all the bibliographical details, in particular the abstract. This file may be opened with EndNote (Mac or Windows) and is easily abstracted, sorted and manipulated (Figure 126). It is expected to be available by the end of the year 2006.

Figure 126: Example of the Endnote bibliographic database on pinewood nematode

12.4 *Discussion*

Electronic databases are unquestionably a powerful and useful resource to help scientists sort through the thousands of references, taxonomic descriptions and overall information. The electronic databases now available will provide end-users with a tool to help them in their daily research activities, material to reflect upon and evaluate, or lecturers in their classes. The taxonomic information, combined with matrices of morphological (and other) characters) may be instrumental in the production of electronic, polythomous keys (Ryss et al., 2004a).

It is expected that technology should provide more ways to access, compile and analyse information in a meaningful way. Relational databases, for example, may also be used in this type of project. Although not a research topic in itself, it has gained popularity together with the use of the internet, and will certainly continue to be extremely useful in the future, despite the continuation of use of standard textbooks. New species descriptions of *Bursaphelenchus*, electronic keys and phylogenetic analyses, molecular biology data, images and video clips of critical experiments, as

well as field data (GIS) can be used in a way to provide scientists dealing with this forest problem with an integrated approach to studying and controlling this disease.

12.5 *Workshops arising from or linked to the PHRAME project*

Two workshops were organised during the final year of the project.

The first, held in Lisbon at the Gulbenkian Foundation from 10-14 July 2006 was an International Workshop on 'Pine wilt disease: A worldwide threat to forest ecosystems'. This was attended by 103 participants from 24 countries and three continents. Apart from the excellent range of papers on many aspects of the threat posed by *B. xylophilus*, there was also a session where the data and final conclusions of the EU PHRAME project were presented. The proceedings of the Workshop, as mentioned earlier, will be published by Kluwer-Springer.

The second was a specialised Technical Workshop with invited participants from the phytosanitary services of the EU Member States. This Workshop, organised by Dr Edmundo Sousa, was held in Lisbon and in the PWN Affected Zone on 17 and 18 October and was attended by around 40 participants. Following presentations on the key findings of the PHRAME programme on 17 October, there was a field meeting on 18 October at which the work of PROLUNP as well as the contributions of the PHRAME consortium were explained in a field context.

In addition to the formal Workshops, the coordinator, Dr Hugh Evans, gave a presentation on the work of the Consortium to the European Commission Standing Committee on Plant Health on February 24, 2006. This provided an excellent opportunity to summarise the main findings of the research and to answer the many queries from the Member States. Additional meetings were also held with the Portuguese authorities to explain the work of PHRAME and to consider field experimentation to verify the process-based models. These meetings were held in February 2006.

12.6 *Web publicity for the work of the Consortium*

The Consortium website describing the work of the project and providing links to the websites of the individual partners is accessible through the Coordinator's Institute website: (http://www.forestresearch.gov.uk/fr/INFD-63KGEF). This has been updated regularly and provides links to other relevant sites. A secure website to allow data transfer and confidential information flow between partners has also been set up. This is accessed by username and password and has proved useful in transfer of large documents.

12.7 *Presentations (published and conference/seminar) by PHRAME participants*

As indicated above, there has been strong written and verbal dissemination of results from the research programme members and much interaction with the scientific and policy communities as new data have been produced and analysed. The full list of dissemination outputs is shown below.

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Chapter 13 Policy related benefits

The main aim of the research carried out within the PHRAME project was to develop improved Pest Risk Analysis (PRA) techniques with particular reference to the serious and ongoing problems arising from infestation of pine trees in Portugal by pinewood nematode, *Bursaphelenchus xylophilus*. Consequently, the main policy drivers for the research are the various phytosanitary requirements of the EU and, indeed, the international community.

Within the EU the main requirements are to improve PRA methodology to underpin the Plant Health Directive Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community (OJ L 169, 10.7.2000, p. 1). The Directive identifies named organisms and the pathways by which they could move to the Community and consequently both *B. xylophilus* and its vectors are identified. Thus *B. xylophilus* is listed in Annex II, Part A - Harmful organisms whose introduction into, and spread within, all Member States shall be banned if they are present on certain plants or plant products; Section I - Harmful organisms not known to occur in the community and relevant for the entire community. Risks of direct importation of the nematode are, therefore, recognised and covered by certain prohibitions on plants for planting and in control of wood and wood products, including packaging wood. The latter is now controlled globally through International Standards on Phytosanitary Measures No. 15 (ISPM15) of the International Plant Protection Convention (IPPC) of the FAO.

Recognition of the role of vectors in international transfer of PWN is included in the fundamental Annex I; Part A - Harmful organisms whose introduction into, and spread within, all Member States shall be banned; Section I – Harmful organisms not known to occur in any part of the Community and relevant for the entire Community. In this category *Monochamus* spp. (non-European) is included because of the known association with PWN as principal vectors.

Results from the current study have provided tools for the improved evaluation of risk from PWN within the Community and elsewhere. Principal findings are:

- In keeping with the listings in Directive 2000/29/EC, the link between *B. xylophilus* and *Monochamus* spp. as vectors has been confirmed for the situation in Portugal where the local *M. galloprovincialis* has been proven as vector.
- The biology and chronology of vector activity have been defined and provide the known window of risk for flight and dispersal of both vector and, particularly, *B. xylophilus*.
- Detailed surveys and molecular analysis have confirmed that *B. xylophilus* is present only in the affected zone in Portugal. DNA analysis has revealed that the likely origin of the nematode is SE Asia and that there is a high likelihood of two identifiable races of the nematode, implying the possibility of separate introductions or a single introduction of a mixture of nematode races.
- Experimental studies of seedling and larger trees have revealed the speed of PWN infestation and effects on water relations in affected trees. This information has been developed for several tree species and temperature regimes and provides a strong basis from which to develop the process-based models for prediction of wilt at the tree level.
- Correlative models, particularly those linking to site suitability of different pine species, as well as to local climatic variables, have provided prediction of regional variation in risk of wilt at a Portuguese scale.
- Process-based models have been developed that provide a basis for site by site prediction of likelihood of wilt expression. These have provided accurate predictions of the incidence of pine wilt disease in the affected zone in Portugal and have made further predictions for a range of locations in Portugal and in the UK. This approach is now being extended to other Member States and will provide a basis for accurate risk analysis for PWN at a range of scales in Europe and globally.
- The combined predictive modelling approach is applicable to PWN and to other organisms that interact with host trees. It is hypothesised that by linking to tree physiological processes,

knowledge of the strategies employed by a wide range of pests and pathogens, can be incorporated into the models to provide a generic risk analysis tool for the future. Although further work is needed to provide this level of sophistication, the verification trials on PWN being carried out in Portugal will aid the development process.

Although the approach adopted in this project has been focussed on Europe, the principles that have been developed provide a basis for improved risk analysis in a global context. There is, therefore, applicability to the European and Mediterranean Plant Protection Organization (EPPO) and to other Regional Plant Protection Organizations as they work to improve risk analysis procedures. The modelling approaches are highly relevant to the work of IPPC in developing improved standards for Pest Risk Analysis and interaction with the IPPC Secretariat has already taken place. A likely development for the future is to produce generic risk profiling for organisms with particular biological attributes so that the principles of process and other models can be applied to commodity risk assessment, especially for difficult pathways such as Plants for Planting.

On a local scale within Europe, there has been regular interaction with the Portuguese Authorities, particularly the management of PROLUNP, in providing up-to-date information on the latest scientific developments within PHRAME and also in discussing strategies for monitoring and management of the PWN threat. It is likely that the risk models developed will have influence on the future policy on management of PWN both within Portugal and also through the EU Standing Committee on Plant Health.

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