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Confidentiality of information within this report

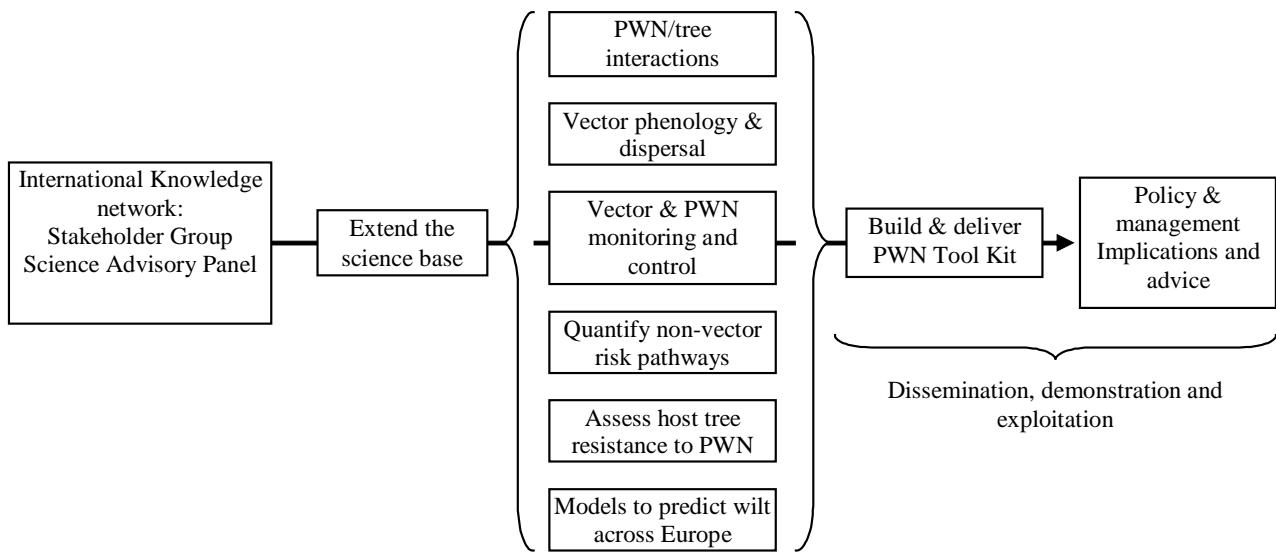
The results for B5 (JKI) in this report remain confidential to the authors until such time as thesis submission and acceptance and any other form of wider publication has taken place. They must not be reproduced without express permission of the authors.

The information will be made available as soon as practicable so that the results can be disseminated more widely.

Note from January 2016. The results have now been submitted for publication and, therefore, all information in this report is now available for dissemination.

Project Objectives for the Period

REPHRAME addresses the requirements of the Call KBBE.2010.1.4-09 (Analysis of the potential of the pine wood nematode (*Bursaphelenchus xylophilus*) to spread, survive and cause pine wilt in European coniferous forests in support of EU plant health policy). It does this through the structured approach below, which covers the principal topics highlighted in the call text:



As summarised in Annex I to the Grant Agreement, the objectives of REPHRAME are being addressed through 9 Work Packages whose progress is described in below (although WP1 is designated for Management & Coordination and is dealt with at the end of this report).

The first periodic report indicated the progress made by the project and also the major issues arising from the late issuing of the Grant Agreement and the subsequent problems in administration. The review carried out by Dr Ilaria Pertot, who attended the Consortium meeting in October 2012 and subsequently reported in January 2013, commended the work done at that stage, but also recommended a 12 month extension to the project. Consequently, and after discussion with EU Science Officer, a formal request was made for a 9 month extension. After great uncertainty which had a very negative impact on the Consortium members, especially at their meeting in Vienna in February 2014, the extension was finally granted just before the original end of project date. Despite these uncertainties, there has been considerable progress under REPHRAME as will be apparent in the rest of this document.

With regard to the useful comments provided by Dr Pertot in relation to the first periodic report, please see the separate Annex document (REPHRAME responses to recommendations by Dr Pertot).

Work Package 2: Behaviour and dynamics of PWN in infested trees

Objectives

The objectives of this WP are:

- to determine factors governing association of the nematode with the vector;
- to determine factors governing transfer of the nematode from the vector to the tree;
- to understand the behaviour of the nematode as it progresses inside the tree vascular system, and factors governing expression or latency of wilt expression, including clarification of the role of potentially pathogenic bacteria associated with PWN.
- to develop better sampling methods for the nematode in trees.
- to synthesise the above into optimised, statistically reliable survey and early detection regimes for inclusion in the PWN Tool Kit.

Deliverables

D2.1: Factors governing association of PWN with vector beetles: Collation of research describing links between and its vector beetles in the genus *Monochamus* especially specificity of links. Month 33

D2.2: Factors affecting departure of PWN from vector beetles: Determination of factors governing departure open from vector beetle established in relation to different host tree species. Month 33

D2.3: Pathogenicity of PWN in host tree species: Factors governing movement of PWN in different host tree species and relationship to expression of wilt established, as well as clarification of the role of bacterial associates in pathogenesis. Month 45

D2.4: Methods to detect PWN in trees: At least one new method for detecting PWN in European pine species validated. This will include work on the Chinese techniques for rapid PWN detection in standing trees. Month 45

Work carried out by B3, B4, B5, B6, B7, B10

B5 (JKI)

In the current reporting period B5 spent 4.76 PM (productive hours) in this work package. Therefore the proposed six person months have already been delivered, though the task "Assessment of different sampling techniques" within Task 2.3 needs to be carried out. These investigations are postponed to the vegetation period 2014.

2.1 Determination of factors governing association of the nematode with the vector

In spring, *B. xylophilus* third stage dispersal juveniles (JIII) begin to aggregate near the larval galleries and pupal chambers of the vector and then moult into dispersal JIV larvae (dauer larvae). Subsequently, these dispersal stages associate with the *M. galloprovincialis* callow adults inside the pupal chamber, just a few days before the emergence from the wood. The stimuli promoting the attraction, moulting and entry of the nematodes into this specific development stage of the *Monochamus* vector insect will be studied in tests under controlled conditions in the laboratory.

Pine bolts will be cut from healthy pine trees and inoculated with a suspension of PWN. Artificial pupal chambers will be made in the bolts and late-instar *M. galloprovincialis* pupae or callow adults will be placed individually in these. These procedures will be repeated with other Cerambycidae species also found in wilted pines, such as *Arhopalus syriacus*, *Acanthocinus griseus* and *Spondylis buprestoides*, but which are not recorded as vectors of PWN. Pupal chambers containing *M. galloprovincialis* + another cerambycid will also be created. At intervals treatments will be opened and the pupae/callow adult of the various species analysed for

nematode presence outside and inside the body. The wood adjacent to the chamber will also be sampled to quantify PWN numbers.

A sample of the bolts will also be kept at 25°C in the dark and checked daily for 60 days for emergence of adult beetles, which will be sampled for nematode infestation.

Cytological studies of PWN migration to the pupal chamber will be performed and chemical analysis of volatile sand of other secondary and primary metabolites will be carried out to determine how the nematodes find the vector and whether species of beetle other than *M. galloprovincialis* might become infested under these conditions. B6 to lead.

B6 (INIAV)

Deliverable 1 - Factors governing association of PWN with vector beetles

Natural infested pines with the four main cerambycid species (*Monochamus galloprovincialis*, *Arhopalus syriacus*, *Acanthocinus griseus* and *Spondylis buprestoides*) were collected from the field.

Final instar larvae are being extracted to assess anatomy differences of the pupae and callow adult's spiracle structure and the morphology of the respective pupal chambers that may prevent the nematode entry to non-vector species. Some of the larvae will be placed in individual containers and the air inside the chamber will be collected weekly using a syringe, gently piercing through the diaphragm. The air samples will be analysed on a Gas Chromatograph (GC), equipped with a TCD detector and a CTR column to separate main air compounds (O₂, N₂, CH₄, CO and CO₂).

2.2 Determination of factors governing departure of PWN from the vector

The fourth-stage dispersal larva of PWN (also known as JIV or dauer larvae) is the developmental stage carried by the insect vector into a new tree host, either by maturation feeding or oviposition activity. The stimuli that mediate and regulate the exiting of the nematodes from the vector's body into the new pine host are not known, and may partially explain why some pines are attacked by the nematode and may suffer from wilt disease while others do not.

The responses of the JIV larvae to stimuli from host plants volatiles will be studied under controlled conditions in the laboratory. The influence of plant materials from host trees on the mobility and attractiveness to PWN will be studied by exposing recently-cut branches with small artificial wounds in the bark simulating the vector's maturation feeding to Petri dishes with pure nematode suspensions. Some of the branches will be analysed immediately to detect nematodes on the bark surface or inside the tissues, while the remaining material will be kept at 25°C 70%RH for one week to incubate and increase the populations of colonising nematodes, which will later be extracted from the wood and counted. Resistant and non-resistant pines (relating to the species used in the WP4 experiments) will be tested, along with other non-hosts such as broadleaf trees.

Selected chemical substances from host pines will also be tested similarly.

B6 to lead

B6 (INIAV)

Last instar *Monochamus galloprovincialis* larvae are being extracted from natural infested wood to be used in greenhouse studies to compare maturation feeding preference between five year old, healthy, watered *P. pinaster* (PWN susceptible) and *P. pinea* (never found to be infested).

2.3 Investigation of movement of PWN inside the host

Movement of PWN within the host tree will be studied under greenhouse conditions with 2-3 year-old trees by B7 and in the field in Portugal by B6. In the greenhouse, selected healthy pines 2-3 year-old pines (*P. pinaster*, *P. pinea* and *P. sylvestris*) will be artificially inoculated, both at the base of the plant as well as at the top, at specified time intervals. Three pine seedlings inoculated with nematodes and one pine seedling inoculated with distilled water will be sampled from each pine species 1, 2, 3, 7, 15 and 21 days after inoculation, time-points previously determined by B7 as being the most representative of the histological changes

induced by PWN in *P. pinaster* stems. Stem samples collected 1,5cm above the inoculation point will be fixed and critical point-dried or embedded in Historesin or in epoxy resins for SEM, general histology or TEM respectively to observe the broad spectrum of changes induced in tissues and cells in the course of the infestation. The main classes of metabolites produced by pine tissues, in response to infestation, will be localized in situ by conventional histochemical tests. Histo- and cytochemical studies will be also performed to localise cellulase and pectinase activities in infested stems in an attempt to gain insight into the migration process of PWN through the tissues. In order to examine whether histological characteristics are related to resistance, the anatomical structure of the non-resistant, resistant and tolerant pines will be compared and the lumen area, number and pattern of distribution of the resin canals evaluated. Enough replicates will be used in order to provide material for nematode counts as well as for sectioning for histopathological observations, to understand the nematode trajectory and locate its presence at different time intervals. With this task we expect to elucidate the step-specific anatomical and cellular changes induced by PWN in the host tissues and contribute to the knowledge of the invasion and migration processes of nematodes through the trunks during infestation. We also hope to gain some evidence about the relationship between pine resistance, tolerance and susceptibility and their anatomical structure.

In the field, selected healthy pines of a range of species will be artificially inoculated with the PWN at different times of the year on the roots; on the trunk or on lower canopy branches. Periodically, the sanitary condition of the trees will be followed and some pines from each subset will be felled and thoroughly sampled at different heights for the presence of the PWN, regardless of symptoms. If the pine proves to be infested, wood samples will be collected to identify the exact location and distribution of the nematodes within the woody tissues, by sectioning and selective staining for microscopic examination. B7 to lead.

Based on the experience of B5 in PHRAME with population dynamics of PWN in seedlings as well as of PWN and *B. mucronatus* in freshly cut pine logs (EUPRESCO sub project PEKID), inoculation trials with *B. mucronatus* in *P. sylvestris* plants (15 to 20 years old) will be carried out by B5. 10 trees will be inoculated in the crown (simulating maturation feeding of *M. galloprovincialis*) and the population dynamics of this population of PWN will be assessed by detailed analysis of nematode distribution through the trunk and branches. Periodically one tree will be cut and wood discs will be cut along the tree and nematode occurrence will be assessed in the sap- and heartwood as well as in the bark based on gram dry weight. In addition different sampling techniques (chainsaw, different drill sizes, chips etc.) as well as processing techniques will be assessed concerning the influence of the methods on survival of the nematodes. B5 to coordinate.

Similar studies will be done by B7 and using field trees already being analysed for a national project. The aim of this subtask is to gather information on most suitable conventional sampling techniques.

Recent observations by Chinese colleagues have indicated a possible role for bacteria associated with PWN and *B. mucronatus* in the pathogenic process of PWN. The bacteria which are acquired during the nematode migration into the insect pupal chamber and carried inside the tree will be investigated together with the natural endophytic bacterial flora inside pine trees. A national project investigating this issue will provide partial support for this task. This work will address key issues such as: (1) Isolation and characterization of bacteria associated with the nematode in different stages of the *B. xylophilus* life-cycle, in vector pupae and within infested trees. (2) Taxonomic identification of bacterial isolates using routine molecular biology methods, such as 16S rRNA gene sequencing. (3) In vitro and In vivo pathogenicity tests of bacteria in *Pinus pinaster*. B7 to lead.

Duration: Y1, 2 & 3

B5 (JKI)

Objective of subtask: Study of the population dynamics of PWN in 7-8 year old *P. sylvestris*

(Subtask proposed by B5 is completed)

Issues:

How does population dynamics (nematode density per tree segment and sampling day) of *B. xylophilus* develop in 7-8 year old pines?

Can differences be observed between population dynamics of *B. xylophilus* in young pine saplings (for example 3-4 year old pines tested in PHRAME) and 7-8 year old pines?

Materials and methods:

Experimental set-up:

Ten 7-8 year old *P. sylvestris* trees with a mean height of 2.50 m (features described in Table 2.1) were tested in a greenhouse in tubs (height: 45 cm, diameter: 57 cm). The trees were pre-air-conditioned for 3.5 weeks.

Table 2.1 Overview of features of tested *P. sylvestris* trees - Geographic distribution, Dt. Herkunftsgebietsverordnung (Forstvermehrungsgut) – Silvicultural regions of provenance (Forest reproduction item) (HkG), age, height, stem diameter, diameter of breast height (DBH)

Geographic distribution (Provenance)	Age [Years]	Height [m]	Stem diameter (stem basis) [cm]	DBH [cm]
Upper Vogtland and North East Bavarian uplands (HkG 851 12)	7-8	2.5	5	3.5

Extracted PWN from wood located in Portugal in the year 2013 (provided by Dr. E. Sousa – B6) were reared on non-sporulating *Botrytis cinerea* on 1.5 % malt extract agar medium at approx. 20 °C. On 17th June 2013 10000 *B. xylophilus* of this isolate PT-7 (w) (number of the isolate in the reference culture collection of the Institute for National and International Plant Health, Julius Kühn-Institut Braunschweig, Germany) were inoculated into the main shoot using a 600 µl inoculum.

The inoculation of all tested trees was conducted according to the methodology used in the Julius Kühn-Institut Braunschweig. A modified technique, to mimic the natural spread of PWN with vector beetles of the genus *Monochamus* to host trees, was applied to guarantee nematode access to all tissues up to the cambial layer. Whereas the beetle can transmit *B. xylophilus* during maturation feeding or oviposition, the modified technique mimics maturation feeding. During this time fresh crown parts are preferred by the beetles, resulting in PWN being inoculated in the main shoot of the previous year.

The inoculation steps were done in the following way (as illustrated in Figure 2.1):

- The needles were removed from the selected shoot part of the previous year below the youngest whorl.

- A 1-2 cm (or in the case of 7-8 year old pines a 2-3 cm) long I-shaped slit was cut in the stem bark using a scalpel. The inner cortex was separated from the bark without damaging of the cambial layer.
- A cotton strip (for 300 µl suspension: 9 x 1 cm, for 600 µl suspension: 9 x 2 cm) was inserted in the slit using a scalpel and folded.
- A plastic strip surrounding the stem over the cotton was sealed at the lower end with an adhesive tape (Leukoplast®, Hamburg, Germany).
- The prepared nematode suspension, or sterile tap water in the case of control trees, was pipetted from the Eppendorf tube onto the cotton strip, which precisely absorbed the 300 µl (for small trees) or 600 µl (7-8 year old trees) suspension. This provided contact to the inner cortex of the stem for nematode migration.
- The top end of the plastic strip was sealed with an adhesive tape to prevent drying out of the inoculum.

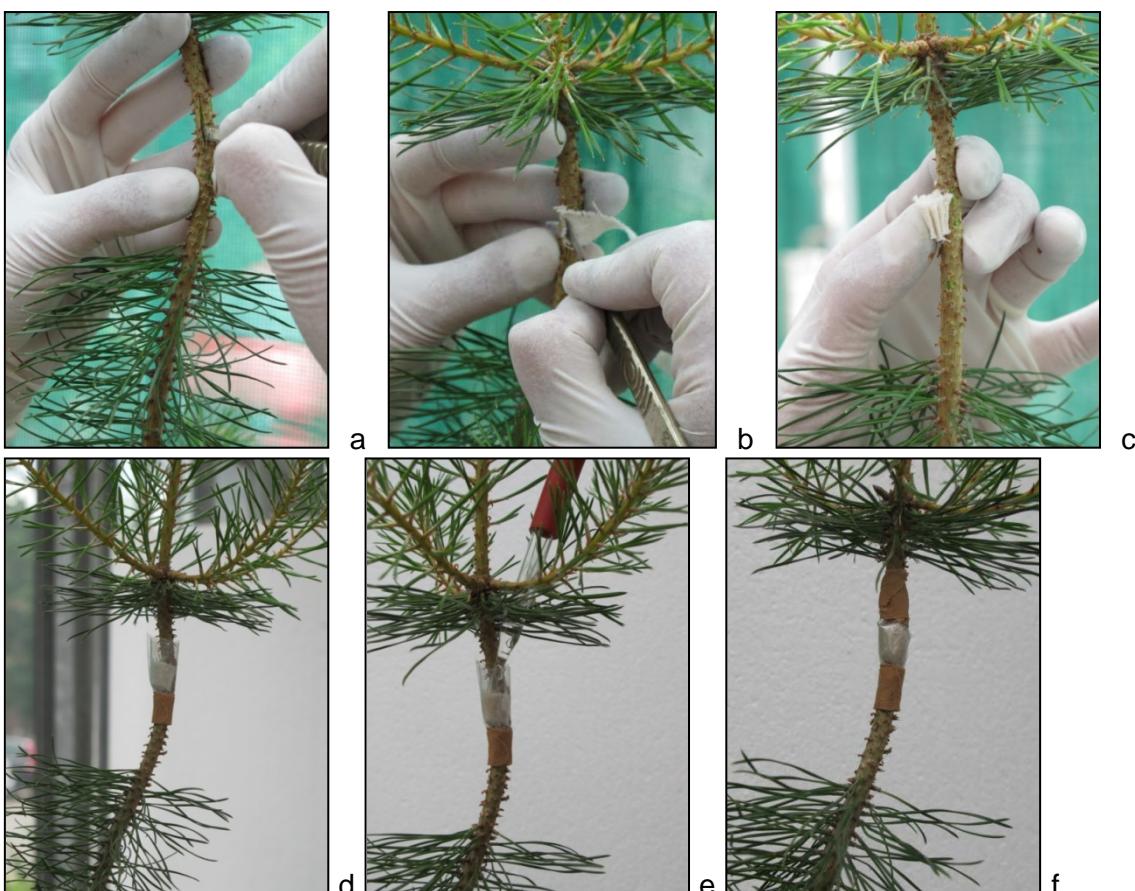


Figure 2.1 Tree inoculation technique for *Pinus sylvestris* trees (here shown at 3-4 years old); a: Cutting of an I-shaped slit in the stem bark of the shoot of the previous year using a scalpel, b: Insertion of a cotton strip in the bark slit, c: Folding of cotton strip, d: Sealing of the plastic strip surrounding the stem over the cotton at the lower end with adhesive tape, e: Transferring of prepared nematode suspension or sterile tap water in the case of control trees on the cotton strip, f: Sealing of the top end of the plastic strip with adhesive tape

The test was run at 25 C and on average 80% relative humidity (rH). The trees were watered as required, randomised and evaluated for pine wilt symptoms. The sampling dates (Table 2.2) and test end (13th August 2013) were determined during the test

period depending on time, wilt symptom development and population dynamics to give the best overview of population dynamics with the available tree number.

Table 2.2 Overview of samplings with time after inoculation

Sampling	Days after inoculation
1	2
2	4
3	9
4	11
5	16
6	22
7	35
8	42
9	51
10	67

Segmentation of *P. sylvestris* saplings:

On each sampling day, one tree was sampled and divided in the following 46 wood segments plus root collar and root (Figure 2.2) to give a detailed overview of how population dynamics develop in 7-8 year old *P. sylvestris*. Branch segments around the tree crown of the same age were aggregated to one sample. The root sample consisted of the main roots as well as sawdust and wood shavings obtained after drilling, several times, in the big root core with a 20 mm diameter drill bit (Drill: DP4003, Makita Corporation, Anjo, Aichi, Japan) to fill two 480 ml beakers. The biggest stem segments 18, 25 and 34 were cut in two cm wide discs using branch shears. The discs were alternately (= approx. 50 %) used only for fresh weight recording and disposed or in addition cut into smaller pieces for nematode extraction. Furthermore needles of year 8 and 7 were collected from all tree heights and mixed before collecting one 900 ml beaker as the sample for each year.

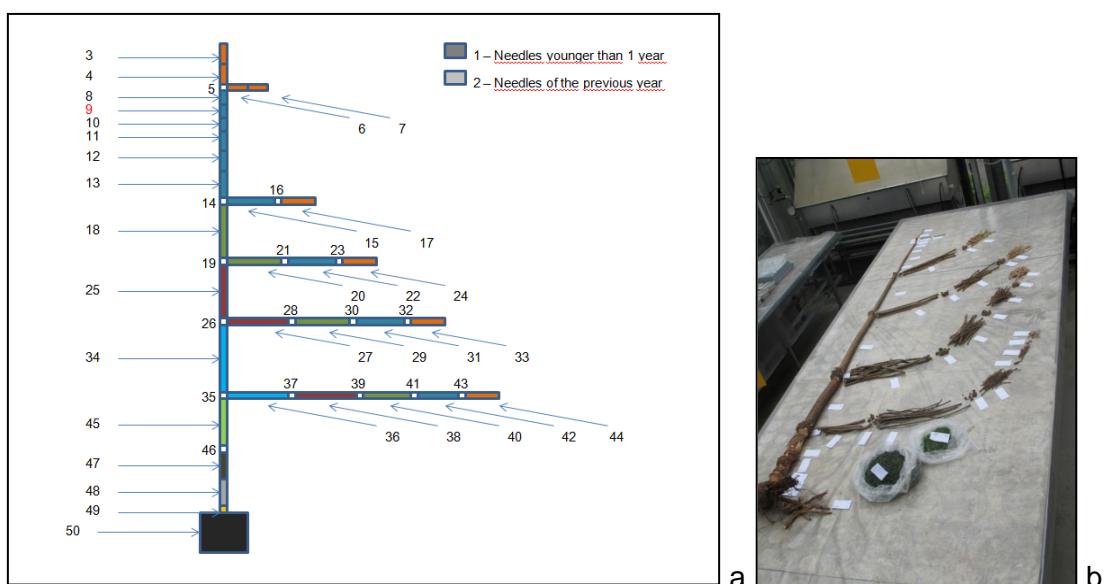


Figure 2.2 a: Tree segmentation into 50 parts to study population dynamics inside the host tree relative to MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site); Yellow segment: root collar; Black segment: root; White segments: whorls; Other segments of the same colour: stem/ shoot parts of

identical age (from downstairs year one (oldest part) to upstairs year 8 (youngest part)); Side shoot segments of the same age aggregated); b: Segmented *P. sylvestris* tree (sampling day 4)

Population development and assessment of pine wilt:

To determine the sampling dates, the trees were evaluated daily for wilt symptoms according to the rating scheme of wilt classes according to Daub (2008) (Table 2.3). The six wilt classes (0 to 5) represent the percentage of needle discolouration of the whole foliage, which is related to the physiological condition of the plant.

Table 2.3 Rating scheme of wilt classes for assessment of pine wilt (Daub 2008)

Wilt class	Tree coverage of needle discolouration [%]	Physiological condition
0	0	alive
1	1 – 25	
2	26 – 50	
3	51 – 75	
4	76 – 99	
5	100	dead

Six trees were sampled at wilt class 0. The final trees were cut for nematode extraction during the next wilt classes (one tree per wilt class). The tree of wilt class 4 was sampled only at 95 % tree coverage of needle discolouration to describe the population dynamics between wilt class 4 and 5 close to tree death. All 48 wood and root segments (segment no. 3 to 50) were cut in 5 mm to 10 mm (wide, length, height) pieces using a hand saw, branch shears with a gear unit (L98, PowerGear™, FISKARS, Finland) and ambos shears and were sampled for nematodes using the Baermann-funnel technique (Baermann 1917) modified for plant parasitic nematodes according to Decker (1969). Nematodes were preserved using 80°C hot fixative solution (890 ml Aqua dest., 100 ml formaldehyde solution (35 %), 10 ml glacial acetic acid referring to Dropkin 1989) for later counting. Wood and needle moisture content (segment no. 1 to 48) were calculated according to the Dt. Institut für Normung - German Institute for Standardisation DIN 52183 (1977). After nematode identification and counting, the nematode density (per gram of dry matter of plant tissue) was calculated.

Results:

For an appropriate overview of the population dynamics of *B. xylophilus* in the test trees, the sampling dates were determined during the experiment dependent on the time spans, results of the last extractions and the wilt class development. In Table 2.4 an overview of the wilt class development of all pines on each sampling day is given together with the information about the wilt class of the sampled tree per date. For all trees, which were not sampled already, it could be shown that wilt class 0 lasted for more than 2 weeks. During the next 7.5 weeks the remaining pines reached higher wilt classes till wilt class 4.

Table 2.4 Overview of wilt class development of tested PWN inoculated pines according to sampling dates, days after inoculation and sampling intervals; Wilt classes of the sampled trees are framed for each date; Last tree tested short before wilt class 5

Tree no.	Wilt class									
B. x. 1	0									
B. x. 2	0	0								
B. x. 3	0	0	0							
B. x. 4	0	0	0	0						
B. x. 5	0	0	0	0	0					
B. x. 6	0	0	0	0	0	0				
B. x. 7	0	0	0	0	0	1	1			
B. x. 8	0	0	0	0	0	1	2	2		
B. x. 9	0	0	0	0	0	0	1	3	3	
B. x. 10	0	0	0	0	0	0	2	4	4	4
Sampling	1	2	3	4	5	6	7	8	9	10
Days after inoculation	2	4	9	11	16	22	35	42	51	67

The population dynamics during the 67 days investigation time is illustrated in Figure 2.3 to Figure 2.12. Shoots of the same age were aggregated. Compared to the completely studied segments from the root segment the main roots as well as the wood shavings to fill two 480 ml beakers and from the biggest stem segments 18, 25 and 34 each second disc (approximately 50 %) were analysed for nematodes.

At the beginning the distribution of the nematode densities showed that nematodes could be found in the inoculation part with the highest class of 51-500 nematodes/g_{dry matter} at this time and in the adjacent segments with ≤ 50 nematodes/g_{dry matter}. Tree no. "B. x. 4" was (11 days after inoculation) the first tree with the next higher density class of 501-2000 nematodes/g_{dry matter} found in the inoculation segment. In "B. x. 5", 16 days after inoculation, the nematodes reached all stem segments for the first time, whereby the highest density was still at and around the inoculation height. The outer shoot parts of the lower tree end were still free from nematodes, but the root collar and root were PWN infested. Although tree no. "B. x. 6" had lower densities compared to B. x. 5 all trees were still in wilt class 0. "B. x. 7" with wilt class 1 showed similar population dynamics compared to tree no. "B. x. 5". At wilt class 2 and 3 PWN were found in the sampled pines in all or nearly all segments with similar population dynamics. The highest nematode densities are in the stem segments till the root collar and the shoot parts, which connect to the stem. At the end of the test, just before wilt class 5 was reached, "B. x. 10" showed in the top (including the inoculation site) lower nematode densities compared to earlier time points. However three-fourths of the whole stem length and the shoots in the middle tree height except the outer parts still showed high nematode densities. In the root segment the density was lower compared to the earlier time points. During all tests the highest values were found in the inoculation segment of pine no. "B. x. 5" and in several parts of tree no. "B. x. 8" (upper crown part) and "B. x. 10" (middle crown part) with the class > 10,000 nematodes/g_{dry matter}.

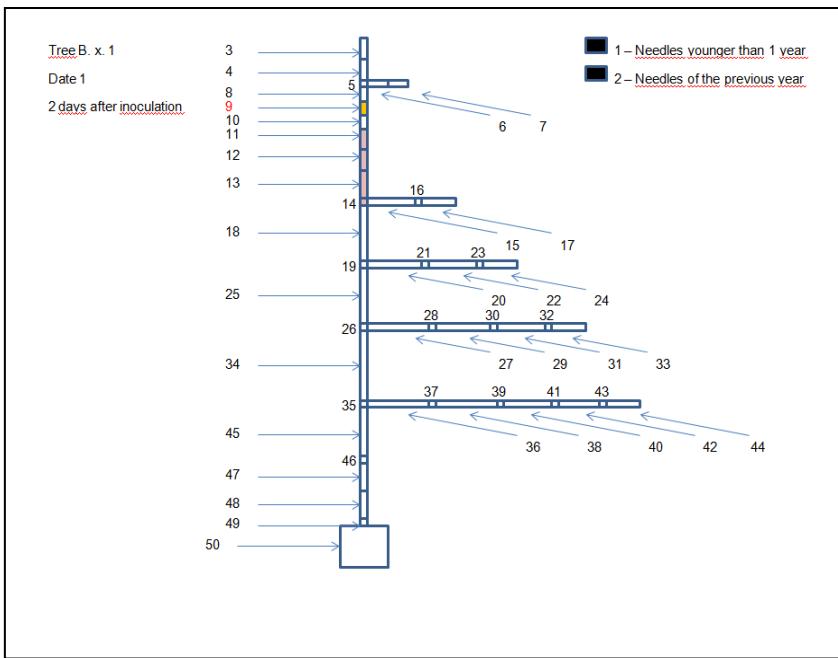


Figure 2.3 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 1 at sampling date 1; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

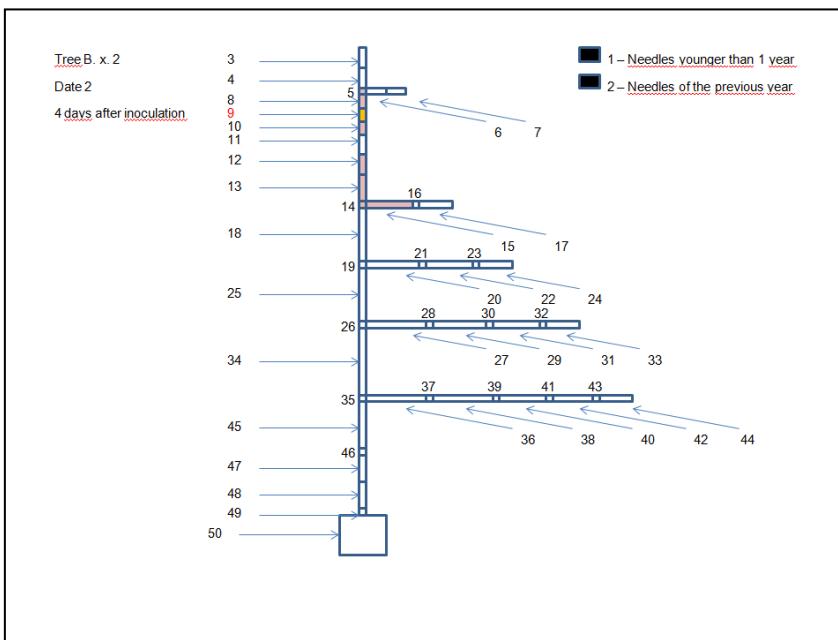


Figure 2.4 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 2 at sampling date 2; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

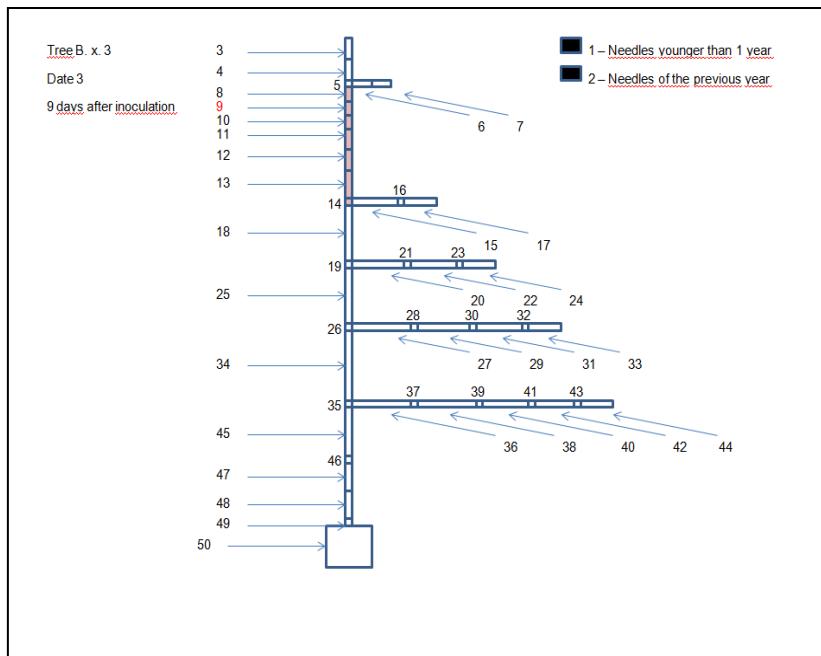


Figure 2.5 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 3 at sampling date 3; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

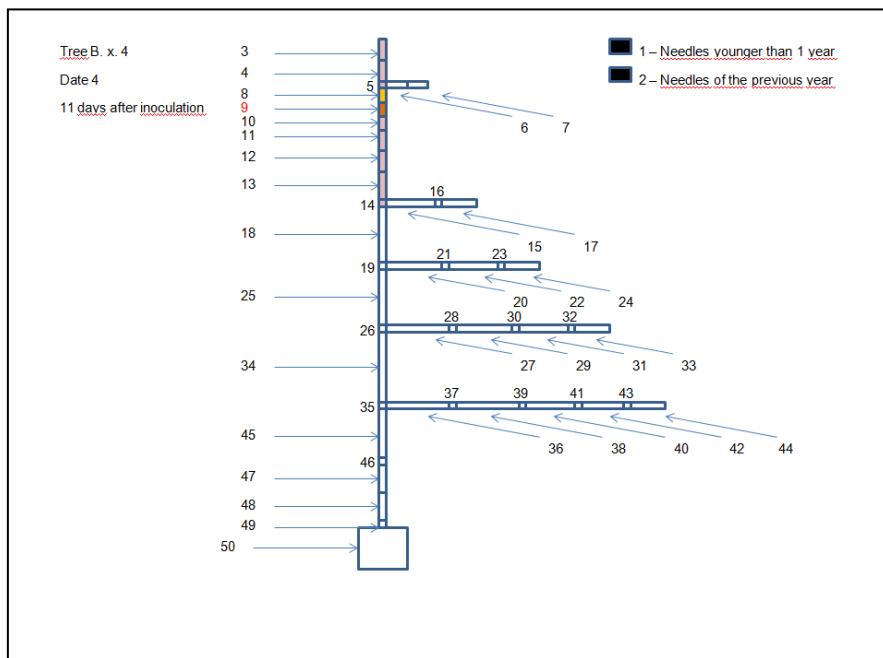


Figure 2.6 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 4 at sampling date 4; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

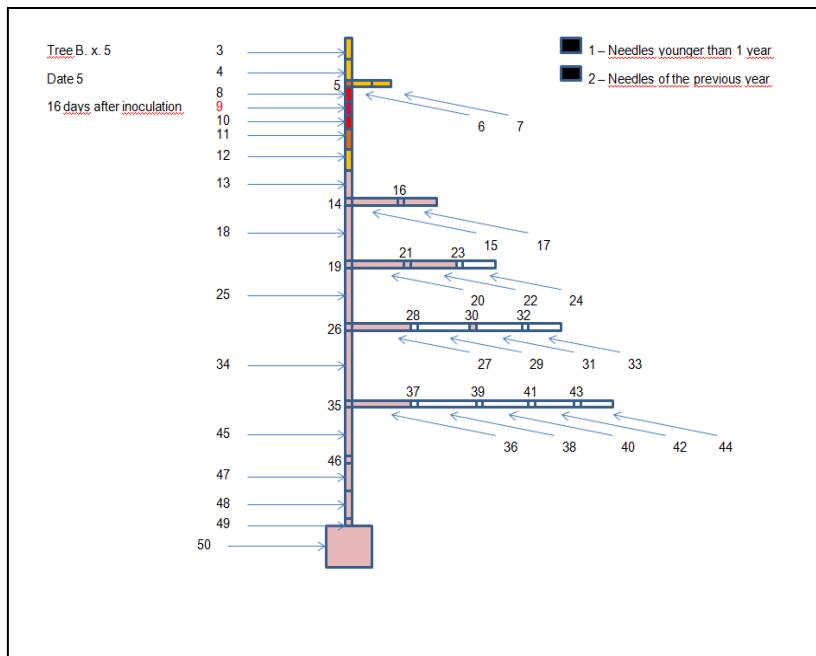


Figure 2.7 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 5 at sampling date 5; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

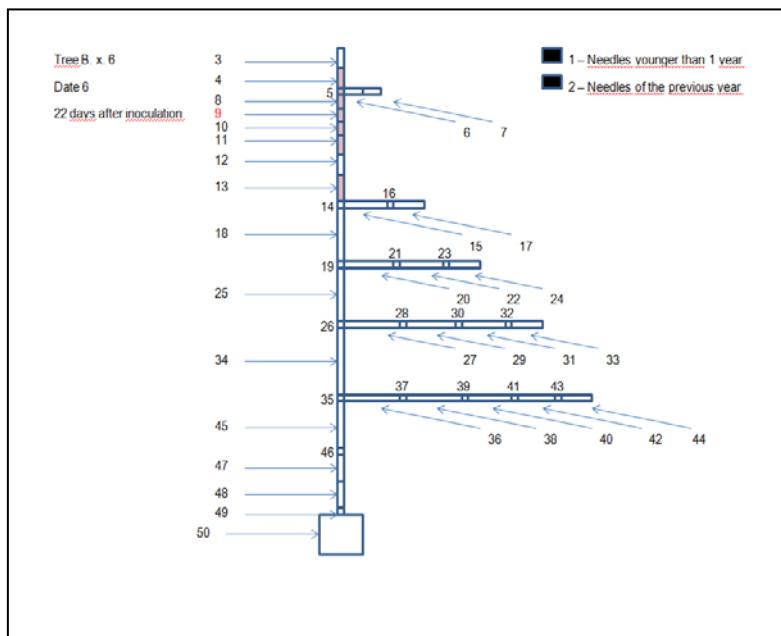


Figure 2.8 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 6 at sampling date 6; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

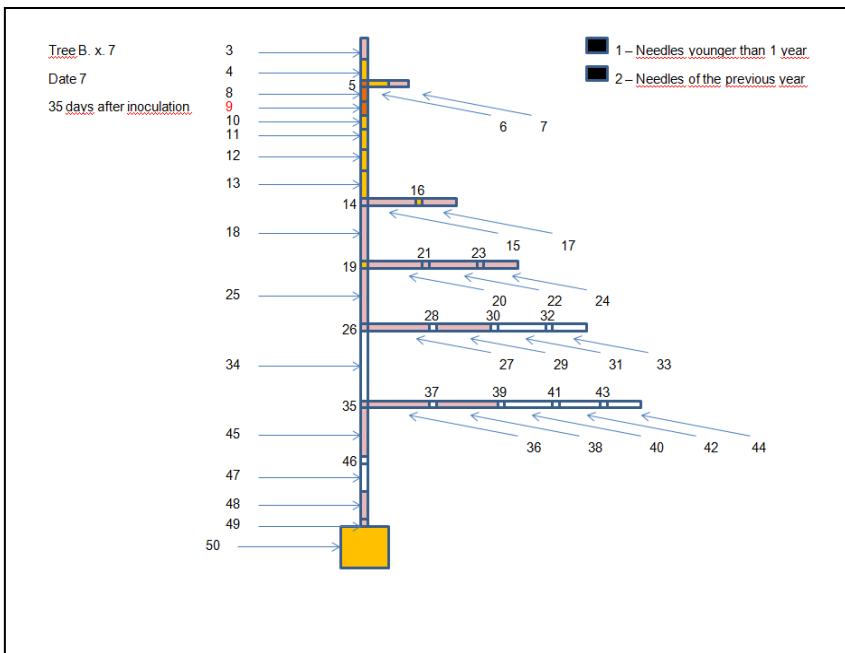


Figure 2.9 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 7 at sampling date 7; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

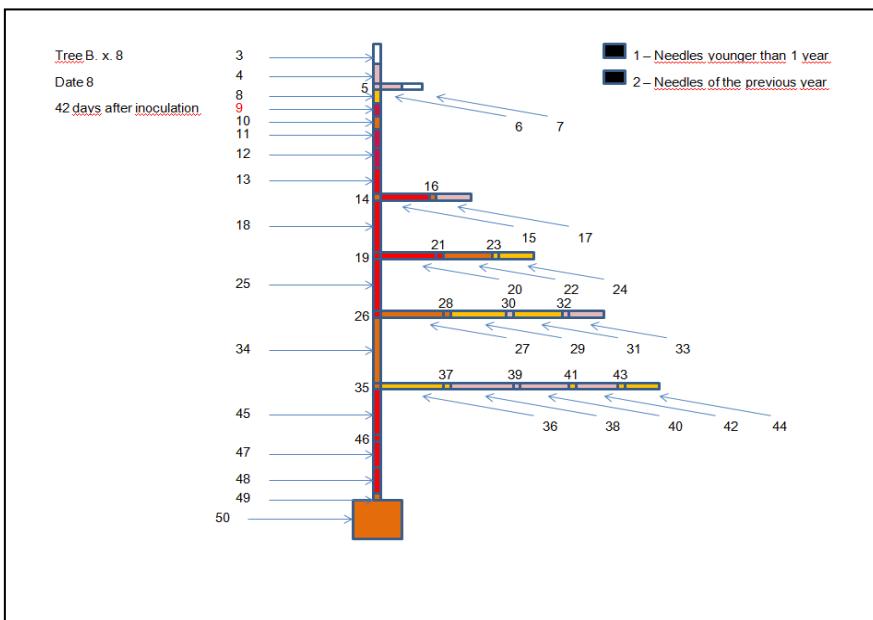


Figure 2.10 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 8 at sampling date 8; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

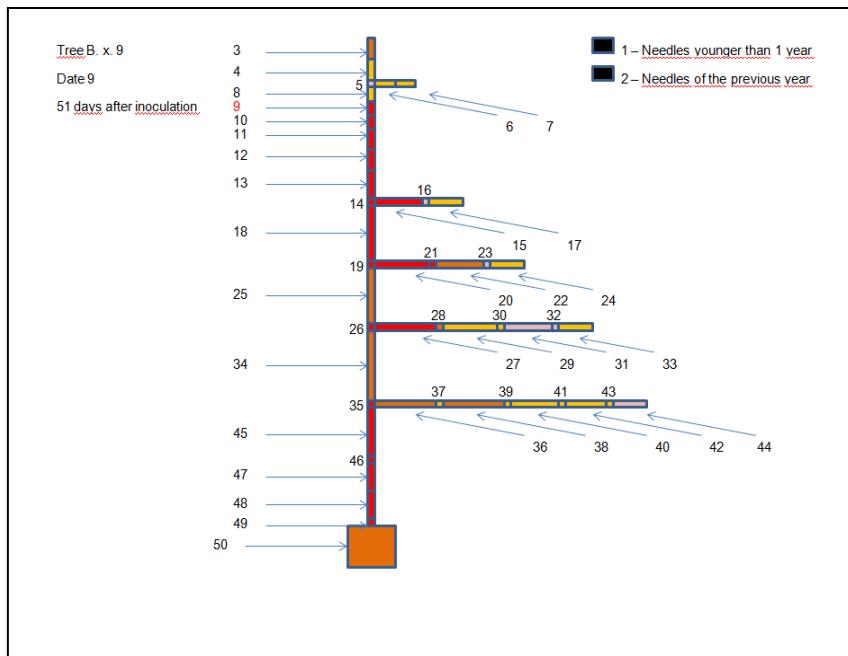


Figure 2.11 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 9 at sampling date 9; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

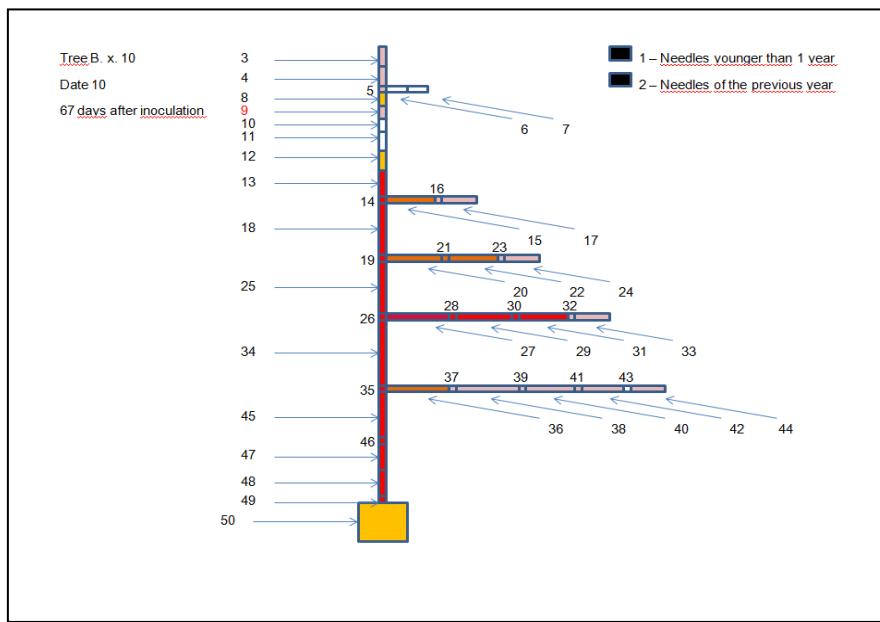


Figure 2.12 Nematode density classes (white: 0, rose: ≤ 50 , yellow-orange: 51-500, orange-brown: 501-2,000, red: 2,001-10,000, wine-red: $>10,000$ nematodes/g_{dry matter}) for tree segments of tree no. B. x. 10 at sampling date 10; 1 and 2: needle samples (without nematodes); 3-48: Stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated; 49: root collar; 50: root

The MC of wood and needles (per sampled tree illustrated for 48 tree segments in Figure 2.13 to Figure 2.22) decreased during higher PWN population dynamics starting from tree no. "B. x. 8" with wilt class 2 to "B. x. 10" with wilt class 4. Up to 5 weeks after inoculation until "B. x. 7" with wilt class 1 the MC classes between 101-400 % MC predominated, only few whorls showed the class 51-100 % MC. At wilt class 2, seven days later, the lowest MC classes 0-50 % and 51-100 % predominated and were the only recorded MC classes at wilt class 3 (again nine days later). At the end of wilt class 4 with 95 % wilt symptoms 9.5 weeks after inoculation, nearly all parts had not more than 50 % MC. Concerning possible differences between the segments per tree, whereby shoots of the same age were aggregated, it could be observed that the upper crown part had the highest MC in the most cases. At wilt class 2 some of the youngest shoots in the lower crown part and one stem segment in the middle tree height still had higher values than the other parts. The two lowest MC classes were reached in a similar way without characteristic differences between the segments, only at the end of the test was the lowest MC class reached for nearly all segments.

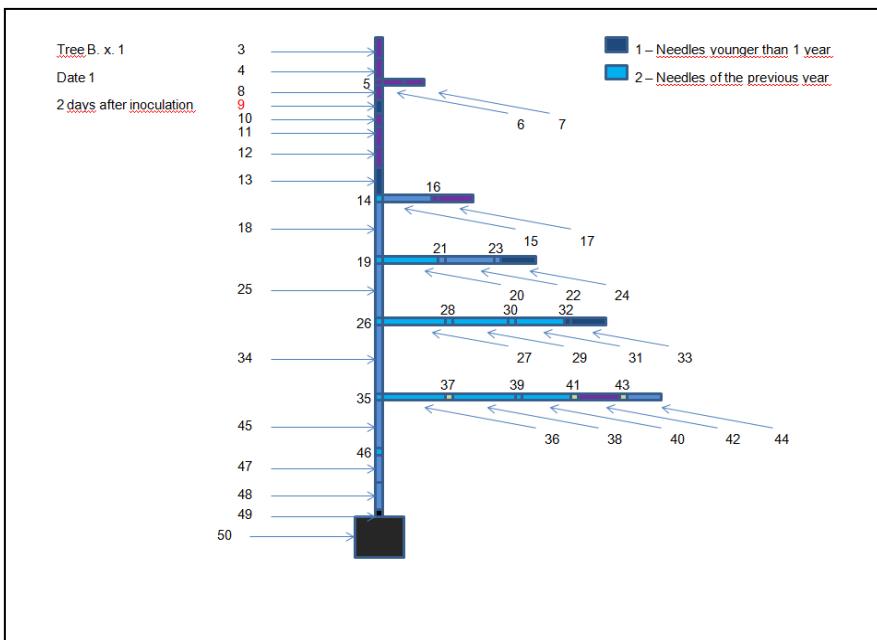


Figure 2.13 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 1 at sampling date 1; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

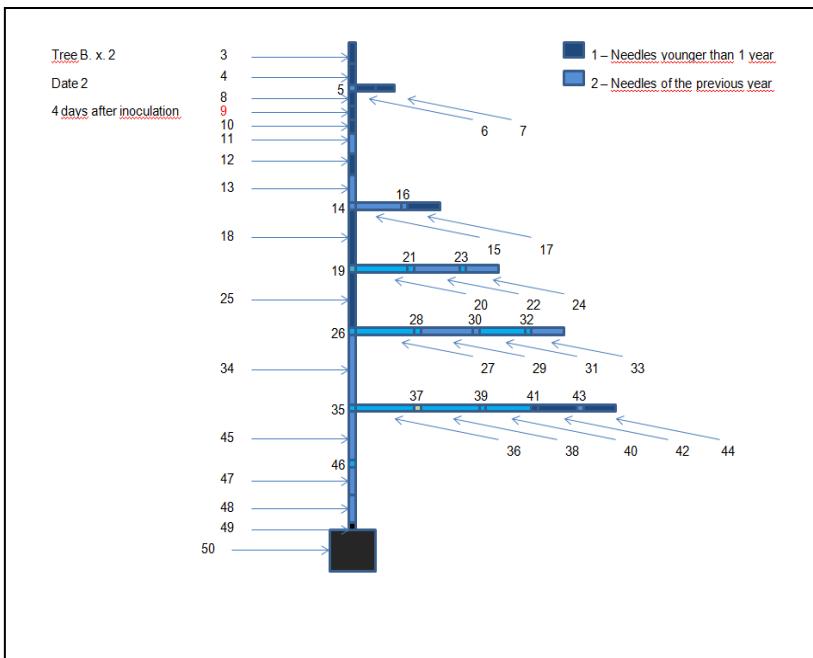


Figure 2.14 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 2 at sampling date 2; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

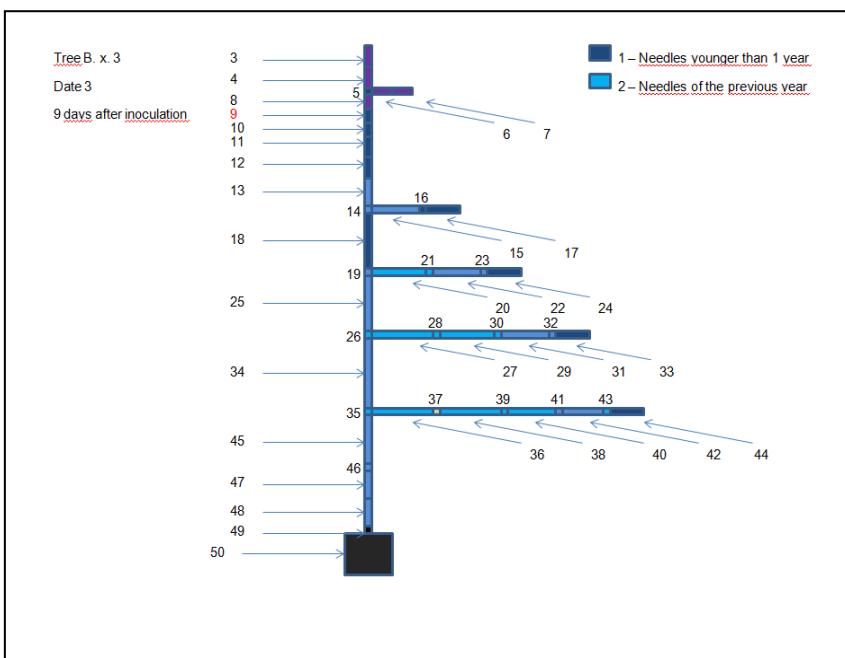


Figure 2.15 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 3 at sampling date 3; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

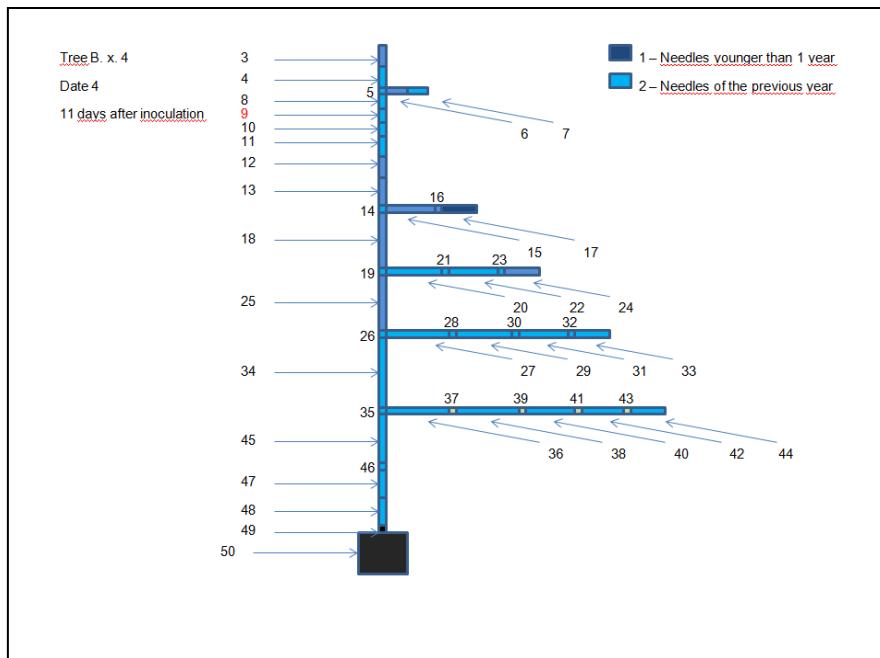


Figure 2.16 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 4 at sampling date 4; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

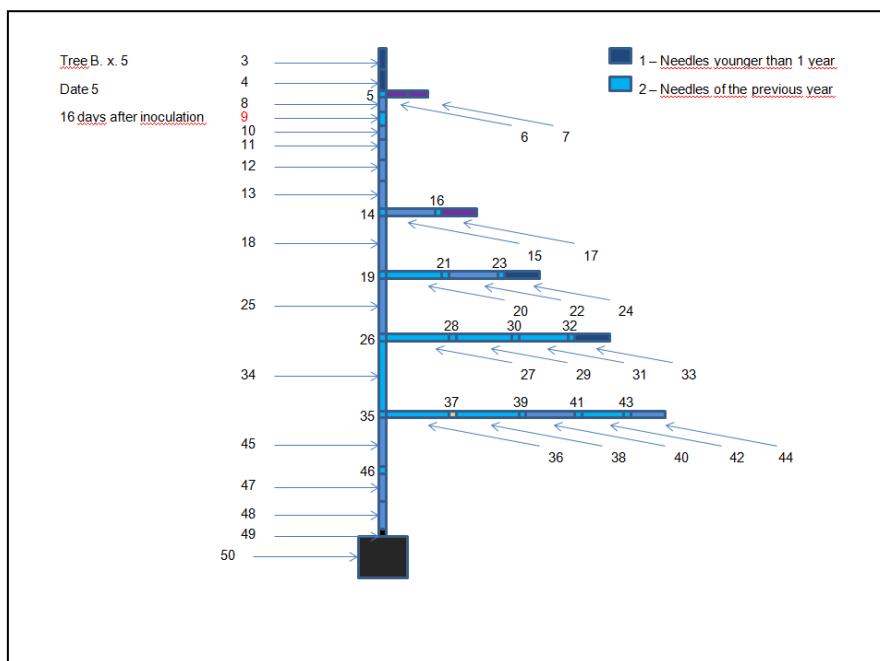


Figure 2.17 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 5 at sampling date 5; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

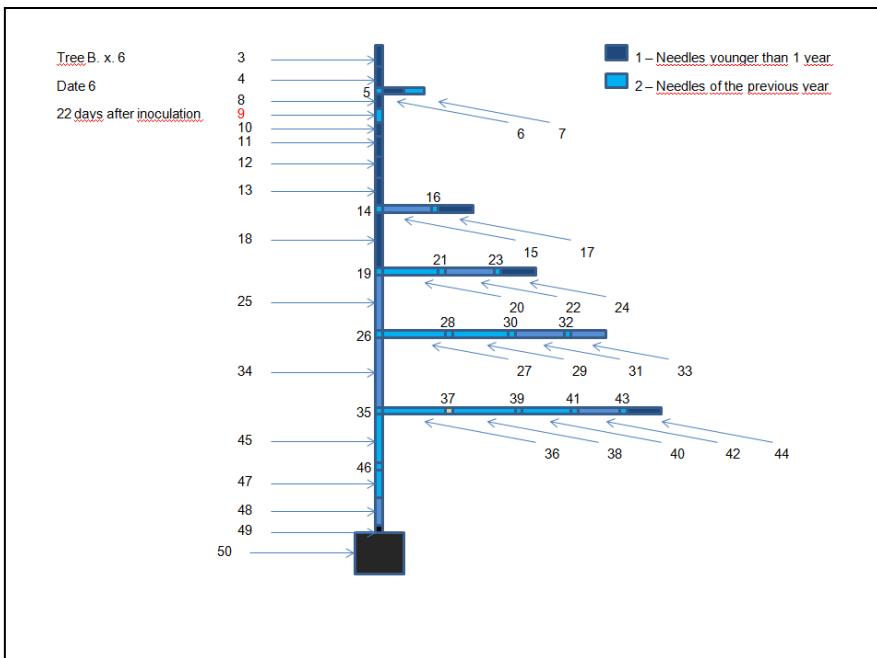


Figure 2.18 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 6 at sampling date 6; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

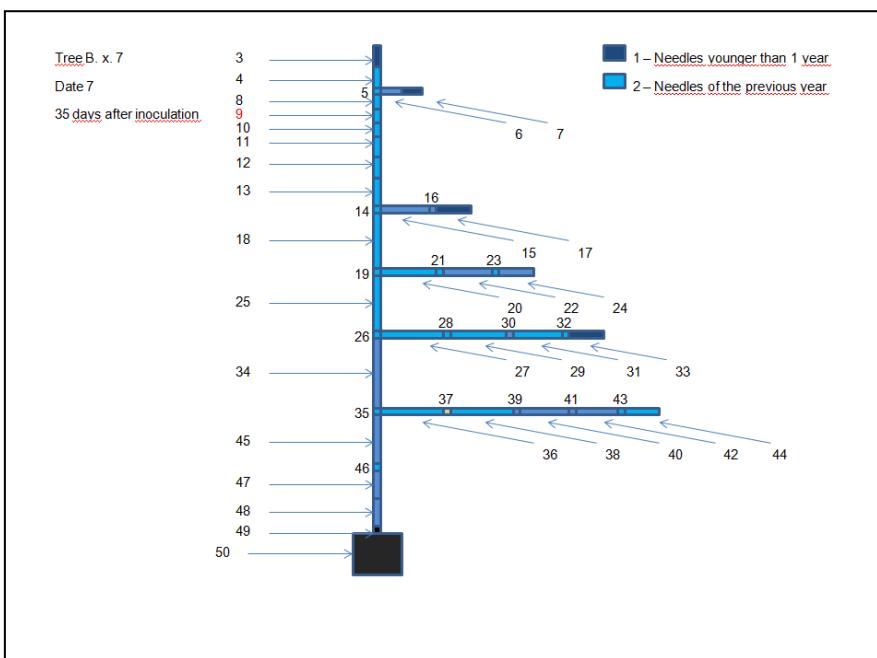


Figure 2.19 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 7 at sampling date 7; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

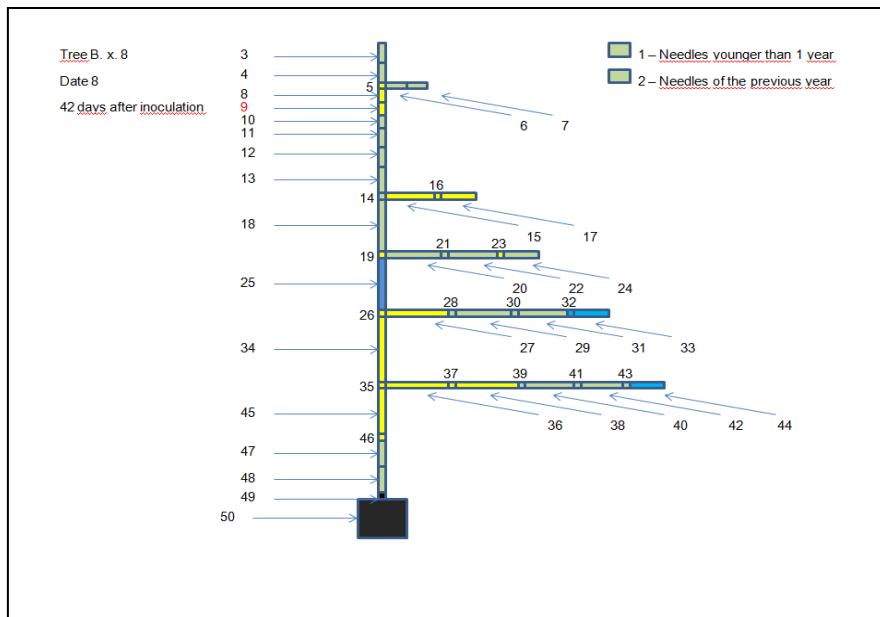


Figure 2.20 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 8 at sampling date 8; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

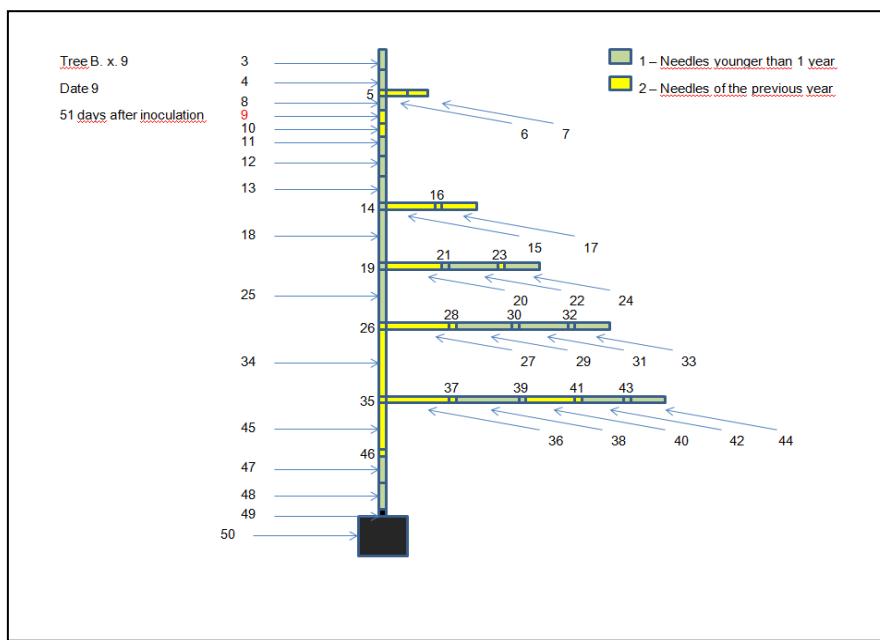


Figure 2.21 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 %, purple: 301-400 % MC) for tree segments of tree no. B. x. 9 at sampling date 9; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

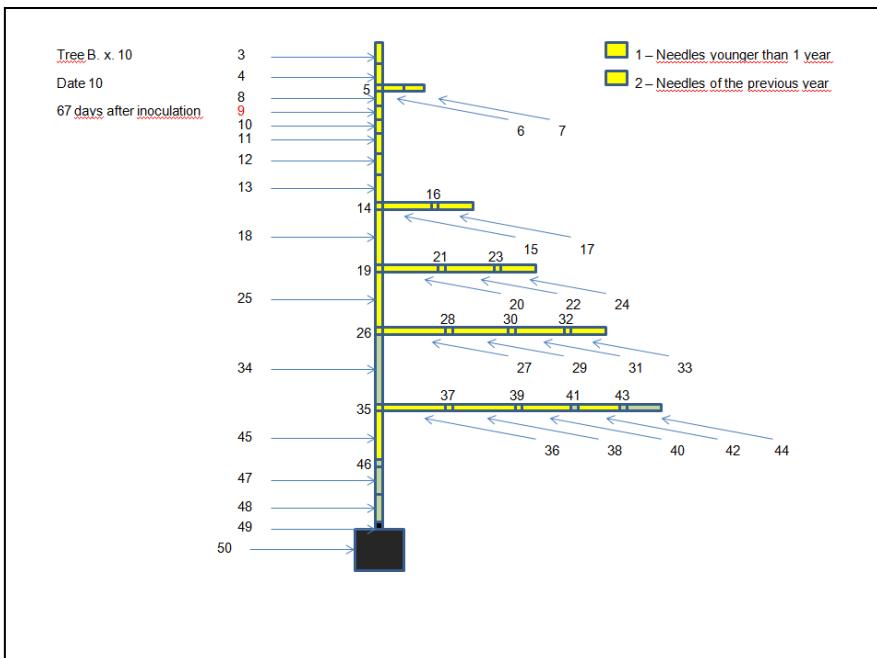


Figure 2.22 Moisture content classes (yellow: 0-50 %, green: 51-100 %, turquoise: 101-150 %, mid-blue: 151-200 %, dark blue: 201-300 % MC) for tree segments of tree no. B. x. 10 at sampling date 10; Black segments: root + root collar without information about MC; 1 and 2: needle samples; 3-48: stem and shoot segments (segment 9 with inoculation site, smallest segments: whorls); Side shoot segments in the same age aggregated

Objective of subtask: Determination of the pathogenicity of PWN towards 7-8 year old *P. sylvestris*
(Subtask proposed by B5 is completed)

Issues:

Has *B. xylophilus* a pathogenic effect on 7-8 year old pines?
 Can differences be observed between pathogenicity of *B. xylophilus* in young pine saplings (for example 3-4 year old pines tested in PHRAME) and 7-8 year old pines?

Materials and methods:

Experimental set-up:

This trial is connected with host selection of *M. galloprovincialis* dependent on host odours in relation to *B. xylophilus* (see WP4 Work done by B5), for which an extension of the test was necessary.

After cutting out the pines from the nursery field and replanting them back in the field over winter, in order for the roots to heal, in spring 2013 twenty 7-8 year old *P. sylvestris* trees with a mean height of 2.50 m (features described in Table 2.1) were tested in a greenhouse analogue to the population dynamics test described before. The trees were pre-air-conditioned for nearly five weeks. The following tree variants were tested:

- 6 PWN inoculated trees
- 6 control trees (inoculated with tap water)
- 6 drought stressed trees (inoculated with tap water) and
- 2 double control trees (without any inoculation and cutting of an inoculation slit).

On 26th June 2013 10000 *B. xylophilus* of the isolate PT-7 (w) per 600 µl inoculum were inoculated in one of the 2-3 year old main side shoots. This inoculation point

differs from the other tree experiments of B5 because of the collection of volatiles on the inoculated and neighbouring branch of a special age for WP4. The test conditions were 25 C and on average 78 % rH. The trees were randomised and watered specifically with regards to the evidence for existing/ not existing drought stress.

Watering of test trees:

The ordered pines were delivered with two types of soil, a clayey soil of the nursery field around the root ball (covered with a root net) and a container substrate consisting of turf, wood fibres and clay, which surrounded the root net. Replanting of the trees, to offer the identical amount and type of soil to calculate the right watering amount for a certain water capacity of the soil for all plants, was not possible in this tree dimension and without wounding of the roots. In May (one month before test start) the pines were watered until water saturation of the soil. The weight (portable electronic scale: DE 150K2DL, KERN, Germany) of the whole plant including the tub and soil was recorded (Figure 2.23). In the following weeks before test start, the soil condition was visually observed for water consumption of the trees under the mentioned greenhouse conditions and the water holding capacity of the soil. Together with tensiometer (Digital tensiometers with manometer, 33 cm, STEP SYSTEMS GMBH, Germany) and Scholander bomb (SKPM 1400, UP UMWELTANALYTISCHE PRODUKTE GMBH, Germany) measurements of a stepwise change of the percentage weight of the unit plant + soil + tub + water compared to the 100 % weight after water saturation was conducted to determine a percentage weight limit as watering target. Tensiometer values starting between 120-250 mbar (depending on the soil) and higher indicate dehydration of the soil. Drought stress studies of Rust (2000) in German pine stands showed that predawn water potential measured with a Scholander bomb of less than -1 MPa is an indication of drought stress. The percentage weight limit was fixed at 75 %. The watering of the drought stressed trees was stopped only one day after test start to create the same start conditions for all trees. During the whole test period (daily from Mondays to Fridays with the exception of Thursdays -because of Scholander bomb measurements) the pines were weighed and watered with a watering can and a three l beaker for measuring the watering amount according to the determined target weight in weight percentage, whereby one litter is equal to one kg of water. The watering amount was calculated with Equation 2.1. During the longer weekend period the water demand per day was roughly estimated with the necessary mean watering amount to reach the percentage weight limit on the respective last Wednesday (last day during controlled watering according to the scale after the weekend and before the Thursday break).



Figure 2.23 *P. sylvestris* trees watered till water saturation of the soil; Portable electronic scale and tensiometer to control the water supply

Equation 2.1 Calculation of the watering amount

$$H_2O = m_{75\%} - m_{actual} [\text{kg}] \text{ or } [l]$$

$H_2O =$ Watering amount [kg] or [l]

$m_{75\%} =$ 75 % weight (of plant + soil + tub + water) of the weight (of plant + soil + tub + water) after water saturation of the soil (= 100 %) [kg]

$m_{actual} =$ Actual weight (of plant + soil + tub + water) [kg]

The trees were evaluated weekly for pine wilt symptoms for 12 weeks. The sampling of the trees took place at wilt class 5, otherwise at the end of this study.

Susceptibility of pines, drought stress detection and nematode population development:

The pine wilt symptoms of the trees were evaluated according to the rating scheme of wilt classes according to Daub (2008) (Table 2.3). The mortality as part of the wilt assessment was calculated as the percentage of trees with wilt class 5 (dead trees). The drought stress (one of the test variants) could be shown with a Scholander bomb and tensiometers. The applied method was the pressure chamber method according to Scholander ET AL. (1965). The Scholander bomb measures the water potential of the needles in bar (= 0.1 MPa). The plant is the best indicator for drought stress, because not only the soil is taken into account. The tensiometer provides a proxy measure for the soil moisture tension in mbar (0,1 MPa = 1 bar = 1000 mbar) and can be used as additional measurement. With the Scholander bomb only one tree per variant was measured because of the wounding of the tree during pulling off the needles and connected possible changes of VOCs (connection to WP4). Referring to Rust (2000) these measurements were conducted once per week on each Friday morning at predawn and additionally each third Friday at the apex of the sun's motion. Watering of the plants directly one day before the Scholander bomb measurements would lead to artificially good results in the morning; therefore the last watering was conducted on Wednesday evenings. Per tree, three needle pairs of the main shoot and three needle pairs of different 1-2 year old main side shoots (in each case from the previous year segment under the last whorl) were chosen, whereby the common wooden end of the needle couple was carefully, manually stripped off. The needle couple was placed in a high pressure head (for over 20 bar) of the Scholander bomb as described in Figure 2.24. During the process the pressure was increased, which caused the water to be pressed out from the common needle end. If the first water drop could be observed by use of a loupe and a flash lamp, the shown value was recorded.

The tensiometers were checked on each weighting day before watering. Each tub was provided with a tensiometer, which was inserted 15-18 cm deep in the soil in the outer area of the root zone and 5-8 cm from the tub wall.

Four stem segments (described in Figure 2.25) were cut in 5 mm to 10 mm (wide, length, height) pieces using a hand saw, branch shears with a gear unit and ambos shears and sampled for nematodes using the Baermann-funnel technique (Baermann 1917) modified for plant parasitic nematodes according to Decker (1969). The MC of the wood was recorded according to DIN 52183 (1977). After nematode identification and counting, the nematode density (per gram of dry matter of plant tissue) was calculated.

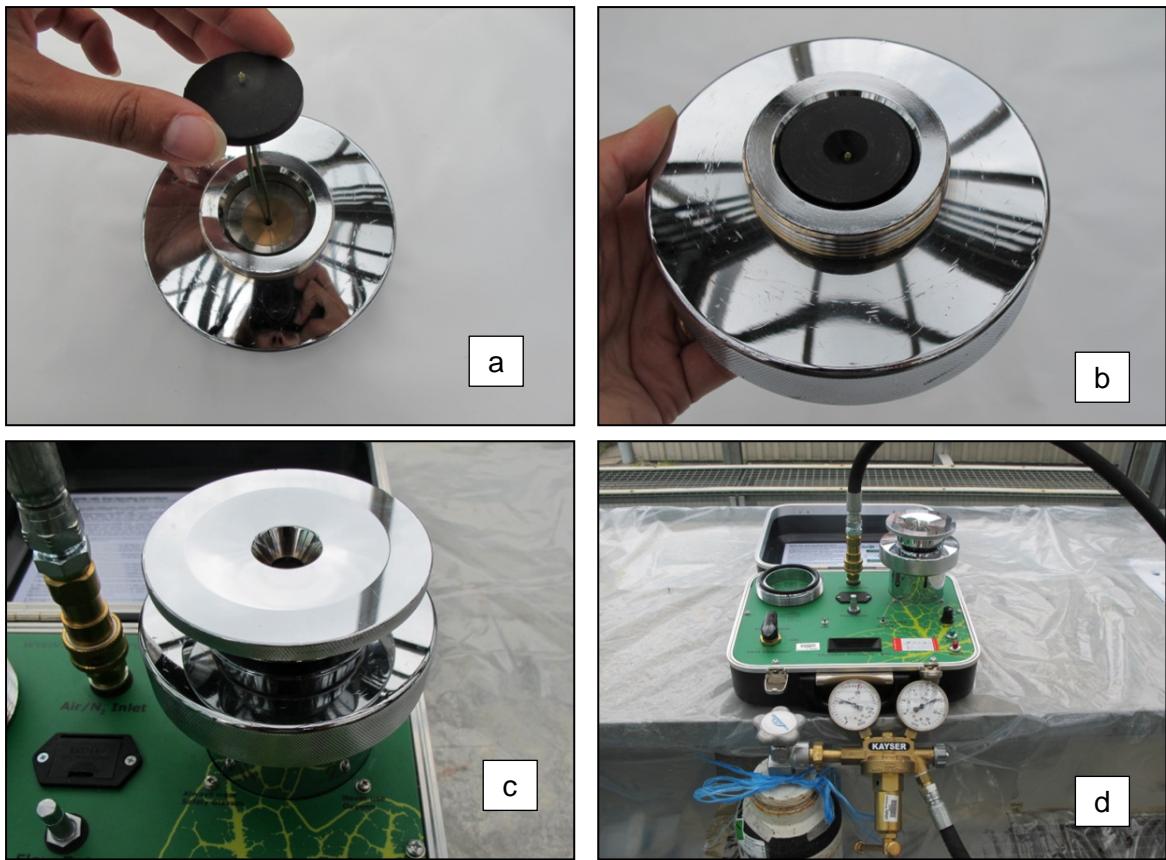


Figure 2.24 Scholander bomb (a-b: Pine needle pair insertion in the high pressure head of the Scholander bomb; c: High pressure head (with needle) installed to complete the Scholander bomb; d: Scholander bomb with compressed air bottle and 40 bar pressure regulator)

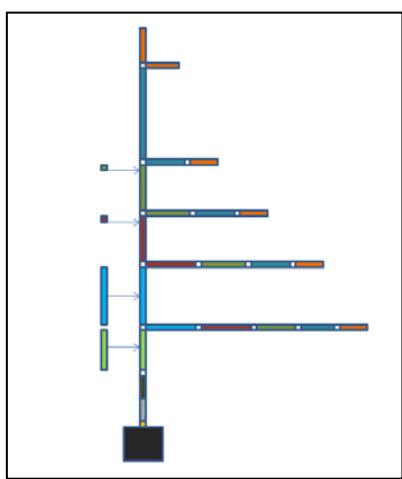


Figure 2.25 Tree structure (White segments: whorls; Black segment: root; Yellow segment: root collar; Other segments of the same colour: shoots of the same year (from bottom year one to top year 8)) with sampled segments on the left side (longitudinal length: two upper segments: 1.5-2 cm; lower parts: the whole stem part between both whorls)

Results:

All plants were water saturated and pre-tested by watering to find a good percentage weight limit. After the beginning of the test, three pine groups were regularly watered to 75 % (percentage weight of the unit plant + soil + tub + water) of their weight after water saturation. One day after test start on 27th June (day 178 in 2013) only the variant drought stressed pines were no longer watered, with the following changes to the percentage weight over time (Figure 2.26):

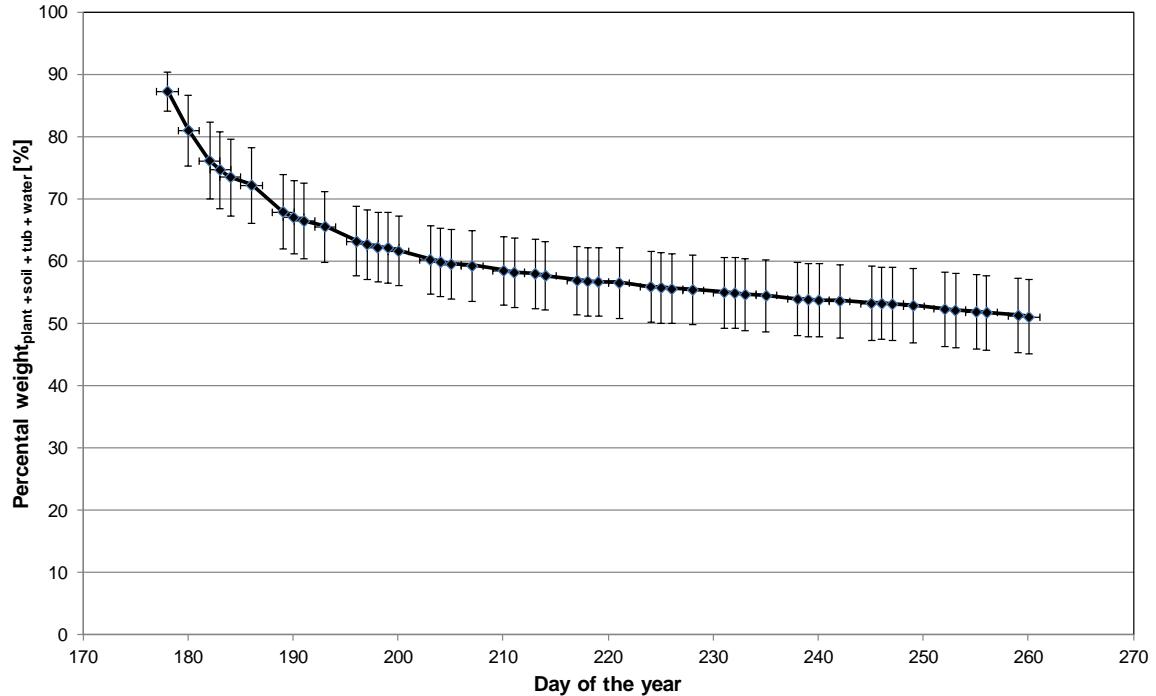


Figure 2.26 Percentage weight [%] (means and standard deviation (SD)) of the unit plant + soil + tub + water of the drought stressed pine group dependent on the time

The percentage weight loss was bigger at the beginning and slowed down towards the end, but did not reach a final state of dryness during the test period. With a mean percentage weight of 87% to 51% the percentage weight loss was 36% during the 12 weeks test period until 17th September 2013.

To monitor drought stress during the same period a Scholander bomb, for water potential measurements on needles of one tree per variant, and tensiometers for all tubs, for soil moisture tension measurements, were used. The tensiometer values, dependent on the tree variant and test time, ranged between 0 (very wet) and >750 mbar (very dry). The results of the water potential of the needles are illustrated in Figure 2.27 to Figure 2.30 for all pine variants.

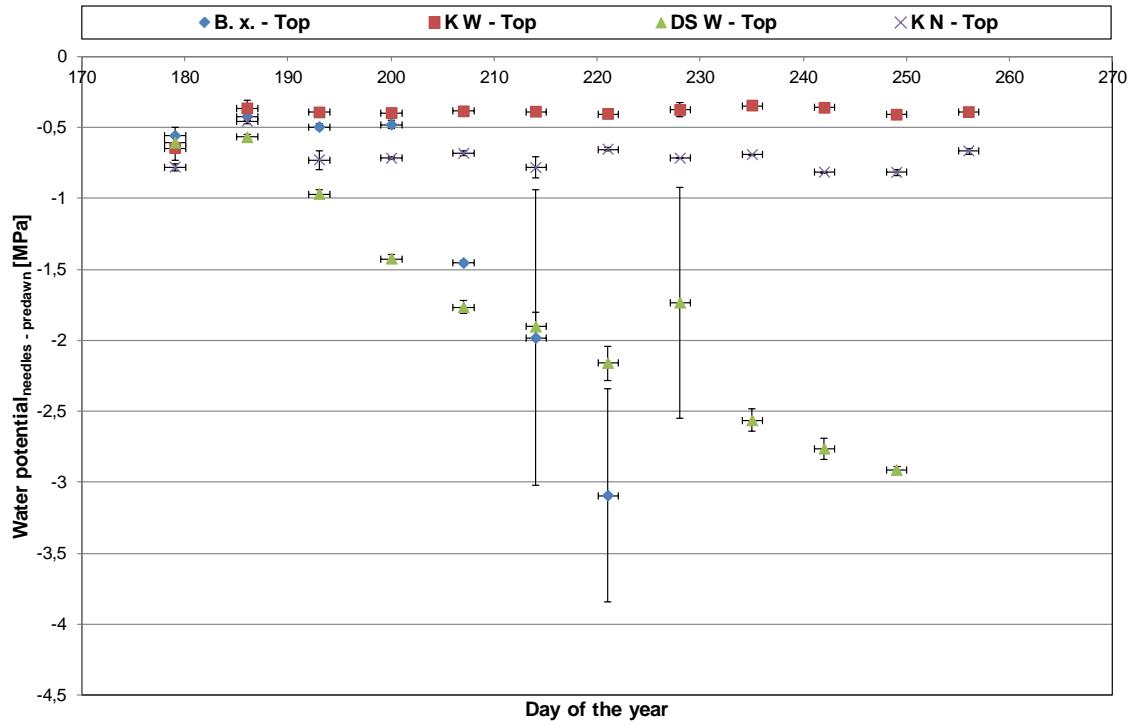


Figure 2.27 Water potential_{predawn} ψ_{PD} [MPa] (means and SD) of three needle pairs (n=3) of the main shoot (Top) of one tree of the *B. xylophilus* (B. x.), water inoculated control (K W), drought stressed (DS W) and non-inoculated control trees (K N), n=1

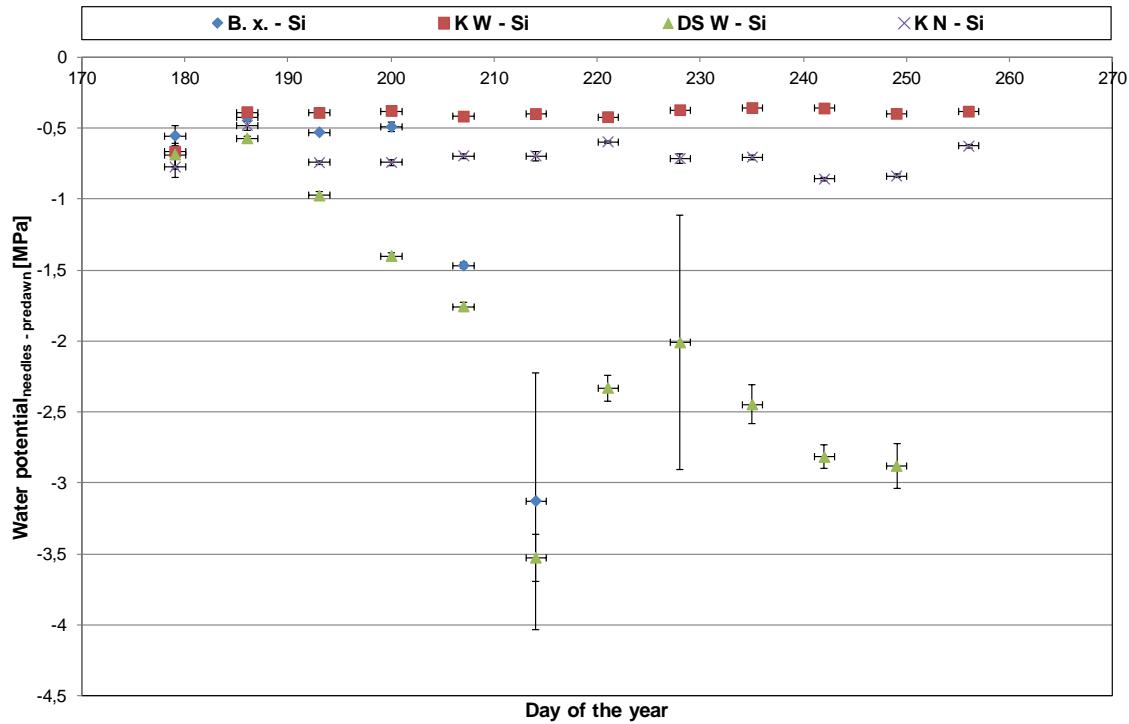


Figure 2.28 Water potential_{predawn} ψ_{PD} [MPa] (means and SD) of three needle pairs (n=3) of different 1-2 year old main side shoots (Si) of one tree of the *B. xylophilus* (B. x.), water inoculated control (K W), drought stressed (DS W) and non-inoculated control trees (K N), n=1

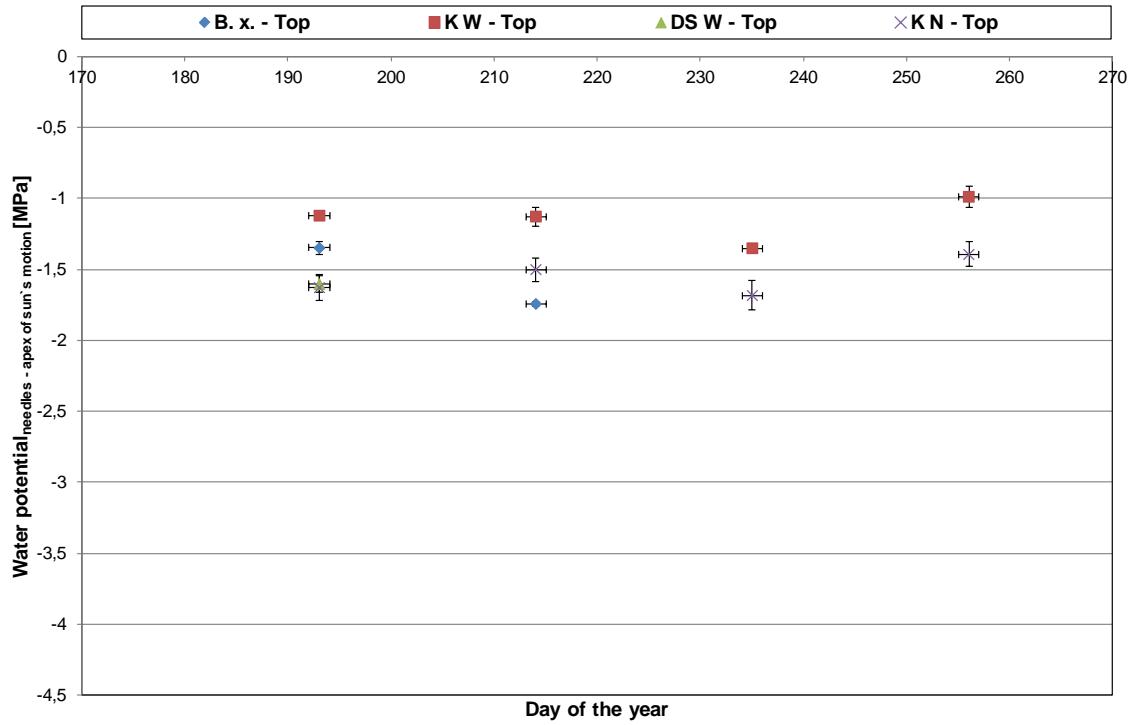


Figure 2.29 Water potential_{apex of the sun's motion} ψ_{PD} [MPa] (means and SD) of three needle pairs (n=3) of the main shoot (Top) of one tree of the *B. xylophilus* (B. x.), water inoculated control (K W), drought stressed (DS W) and non-inoculated control trees (K N), n=1

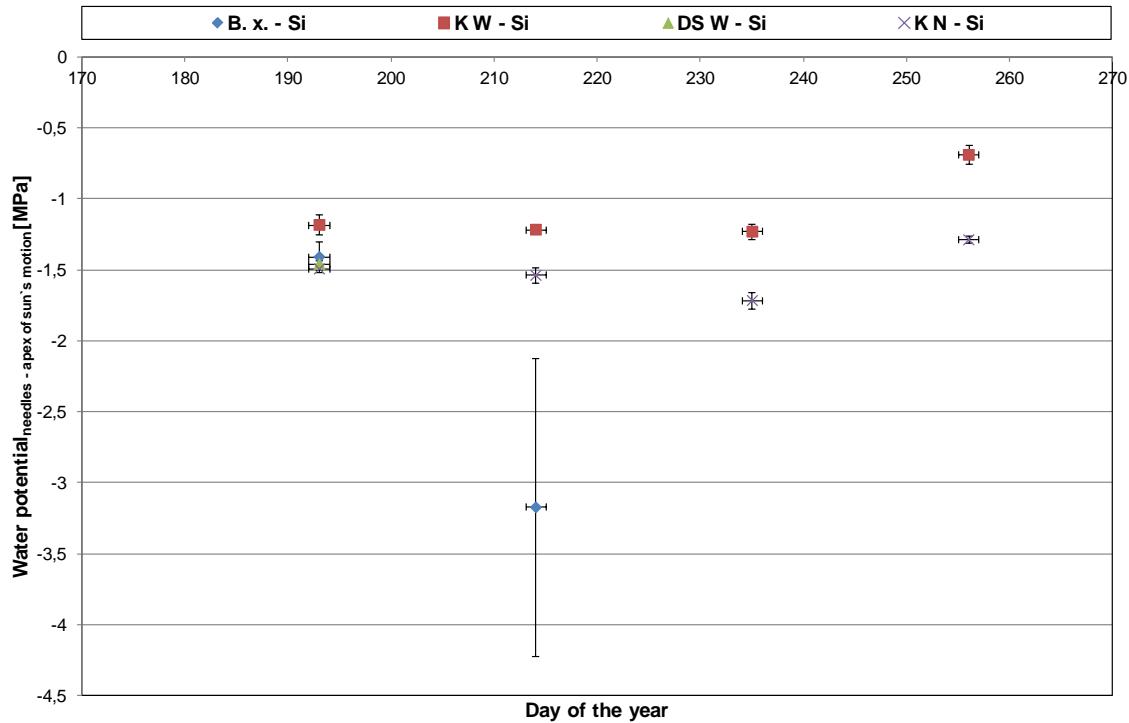


Figure 2.30 Water potential_{apex of the sun's motion} ψ_{PD} [MPa] (means and SD) of three needle pairs (n=3) of different 1-2 year old main side shoots (Si) of one tree of the *B. xylophilus* (B. x.), water inoculated control (K W), drought stressed (DS W) and non-inoculated control trees (K N), n=1

All figures show an approximately constant water potential of the needles of the control trees (with and without water inoculation), but a strong decrease of the values of the

PWN inoculated tree and drought stressed tree, which means an increase of drought stress. On each measuring day shown in the graphs, all pine variants were tested. Values over 40 bar, which means lower than -4 MPa, could not be recorded with this Scholander bomb (connected with a compressed air bottle and a 40 bar pressure regulator). Therefore already at predawn the complete decrease of the water potential of the nematode infested and drought stressed pines cannot be shown in detail. The predawn values of both control variants never reached -1 MPa. At the apex of the sun's motion, higher drought stress could be observed compared to predawn for the nematode-infested and drought-stressed tree. Also the control trees of both variants showed lower values at this time of day, but never reached -2 MPa and at predawn, had high values as before. Needles of the 1-2 year old main side shoots showed (compared to needles of the main shoots, all selected from the previous year part under the last whorl), similar tendencies. Concerning possible early detection of pine wilt disease, a correlation between the needle water potential of the PWN infested tree and its wilt class development could be assumed. Wilt class 1 started at week 4, as the water potential suddenly began to quickly decrease (Figure 2.27 and Figure 2.28), similar to the fast development of the other wilt classes, until wilt class 5 after ten weeks (Table 2.5).

Table 2.5 Wilt class development of tree B. x. 6 dependent on the week after inoculation (additional expressed as day of the year for comparison with the water potential graphs)

Weeks after inoculation	Day of the year	Wilt class
0	177	0
1	184	0
2	191	0
3	198	0
4	205	1
5	212	2
6	219	3
7	226	3
8	233	4
9	240	4
10	247	5
11	254	5
12	261	5

The drought stress development of the drought stressed pine was approximately linear with time, in contrast to the PWN infested tree, which for approximately four weeks held predawn values of round about -0.5 MPa and only then rapidly lost its water potential.

In Figure 2.31 to Figure 2.33 the number of trees per wilt class (with 0: 0 %; 1: 1-25 %; 2: 26-50 %; 3: 51-75 %, 4: 76-99 % and 5: 100 % tree coverage of needle discolouration) of all PWN inoculated pines and water inoculated control as well as drought stressed pines is illustrated in relation to time.

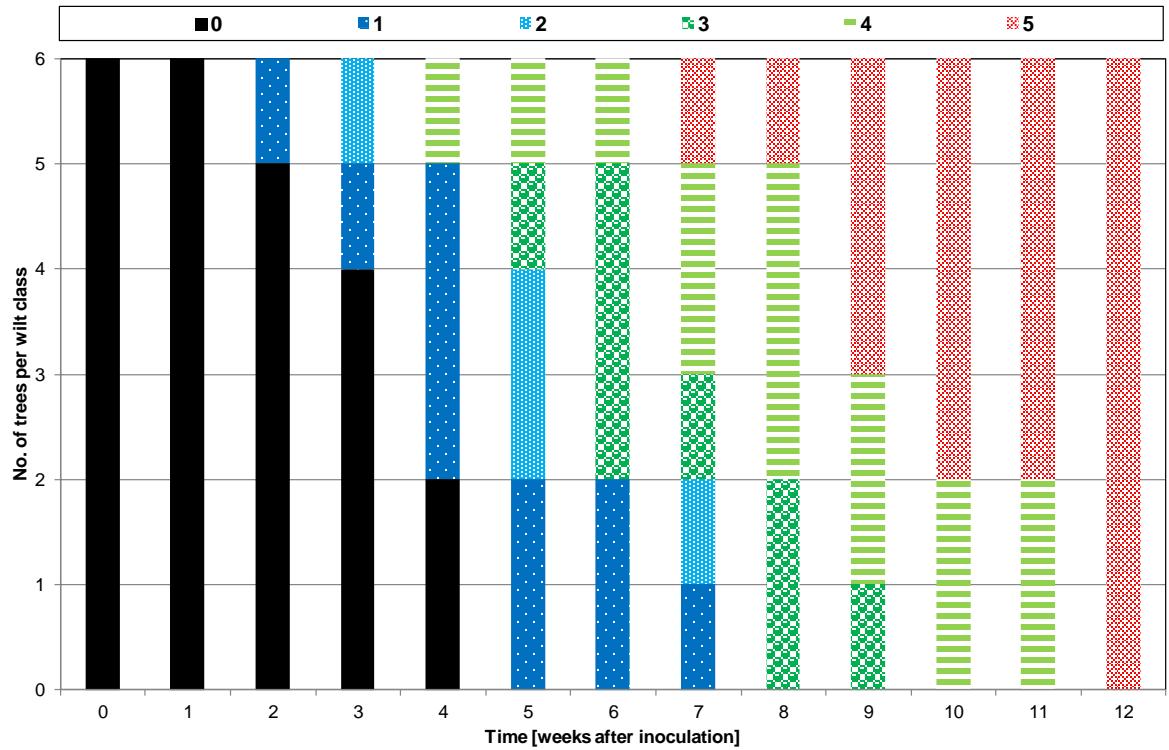


Figure 2.31 No. of trees per wilt class of the PWN inoculated trees over time [weeks after inoculation], n=6

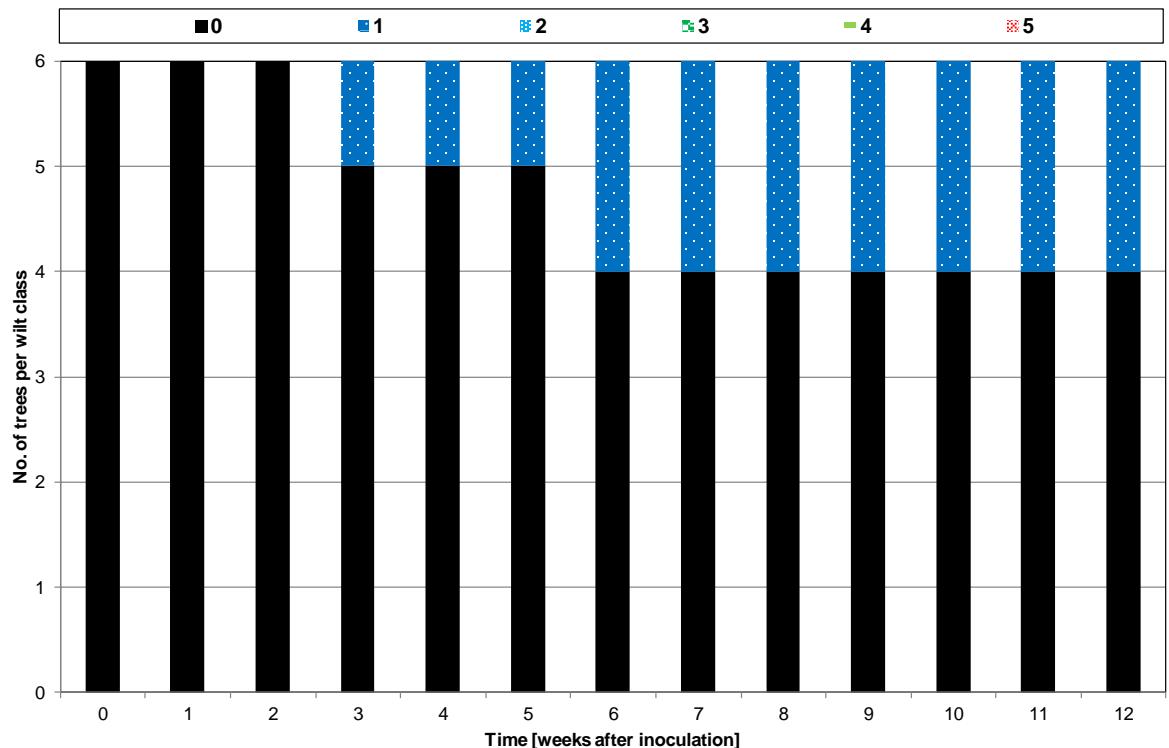


Figure 2.32 No. of trees per wilt class of the water inoculated control trees over time [weeks after inoculation], n=6

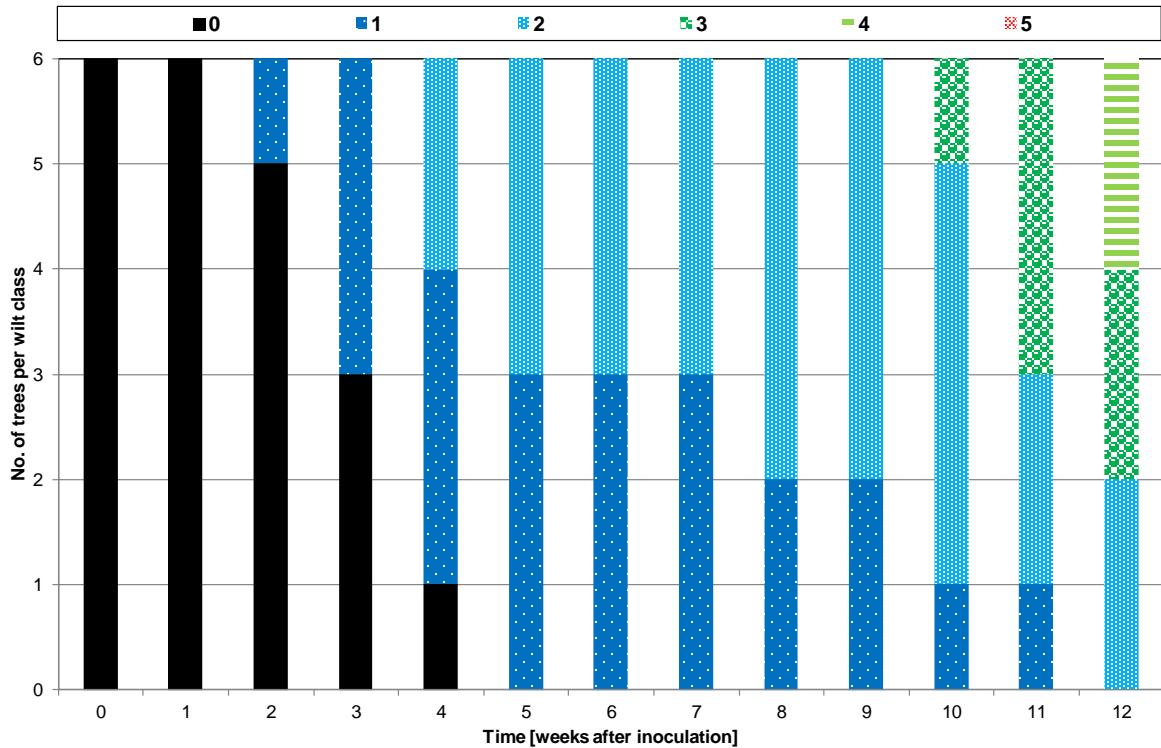


Figure 2.33 No. of trees per wilt class of the water inoculated drought stressed trees over time [weeks after inoculation], n=6

All trees showed wilt class 0 (no symptoms) until one week after inoculation. At test end after 12 weeks the control trees mainly held wilt class 0 or developed wilt class 1 in contrast to the PWN inoculated trees with a mortality of 100 % (all six trees with wilt class 5) and the drought stressed trees with an equal number of trees with wilt classes 2, 3 and 4. One of both double control trees without any inoculation (not illustrated) developed wilt class 1, the other held wilt class 0.

No PWN were extracted from the control, double control and drought stressed pines. All PWN inoculated trees were infested in all investigated segments, and showed the following nematode densities in the studied stem segments (Table 2.6):

Table 2.6 Nematode density [nematodes/g_{dry matter}] of the analysed stem parts of the PWN inoculated trees (1: 1.5-2 cm disc of the sixth year stem segment (under the next whorl); 2: 1.5-2 cm disc of the fifth year stem segment (under the next whorl); 3: complete fourth year stem part; 4: complete third year stem part), n=6

Stem part	Nematode density [nematodes/g _{dry matter}]		
	Median	Min	Max
1	13339.3	3185.2	30784.3
2	5044.8	1257.9	10865.4
3	889.4	249.7	5618.0
4	1470.2	403.8	2517.8

The biggest nematode density of the tested segments was found in the highest part (1), which was above the inoculation site.

The MC (Table 2.7) of the tested stem parts show high values of both control variants compared to the drought stressed pines and PWN inoculated trees with the lowest MC of 52% (median). The median of 68% of the drought stressed variant is an indicator for

successfully induced drought stress. In contrast to this variant, the low MC of the nematode inoculated trees developed in spite of normal watering.

Table 2.7 Summarized moisture content MC [%] of all four sampled stem segments of the *B. xylophilus* (B. x.), water inoculated control (K W), drought stressed (DS W) and non-inoculated control trees (K N), n=6 (except the variant K N with n=2)

Moisture content [%]			
Variant	Median	Min	Max
B. x.	51.5	45.7	54.5
K W	197.7	178.4	230.2
DS W	67.9	54.3	97.5
K N	191.1	189.4	192.8

Objective of subtask: Validation of different sampling techniques for *B. mucronatus* as substitute organism for *B. xylophilus* concerning the influence on extraction of living nematodes from artificially infested *P. sylvestris* logs

Issue:

Does the number of extracted living nematodes differ between various sampling techniques?

This investigation is postponed to the vegetation period 2014.

References:

- Baermann, G. (1917): Eine einfache Methode zur Auffindung von Ankylostomum- (Nematoden)- Larven in Erdproben. Welteureden. Batavia. Geneesk. Lab. Feestbundel, pp. 41-47.
- Daub. M. (2008): Investigations on pathogenicity, invasion biology and population dynamics of the Pine Wood Nematode *Bursaphelenchus xylophilus* (Steiner und Buhrer 1934) Nickle 1970 in European conifers. Bonn: Dissertation Rheinischen Friedrich- Wilhelms-Universität zu Bonn, 110 pp.
- Decker, H. (1969): Phytonematologie - Biologie und Bekämpfung pflanzenparasitärer Nematoden. VEB Deutscher Landwirtschaftsverlag Berlin, 526 pp.
- Dropkin, V. H. (1989): Introduction to Plant Nematology. JOHN WILEY & SONS, New York, Second edition, 304 pp.
- Rust, S. (2000): Untersuchungen zum Trockenstress in unterschiedlich stickstoffbelasteten Kiefernbeständen in Nordostdeutschland/ The effect of nitrogen input on drought stress in pine ecosystems in Northern Germany. Allgemeine Forst- und Jagdzeitung Band 171 Heft 4: 61-67.
- Scholander, P. F., Hammel, H. T., Bradstreet E. D., Hemmingsen, E. A. (1965): Sap Pressure in Vascular Plants. Negative hydrostatic pressure can be measured in plants. Science, Vol. 148: 339-346.

B7 (UE)

Studies on movement of the PWN inside the tree, which are coupled with histopathological observations, have progressed during this period, especially using *P. pinaster*. Following the agreement made with B6 (INIAV) in 2012/2013 our team has had access to the greenhouses in Oeiras. From the histopathological studies, some interesting results, obtained in collaboration with a top plant anatomist from Lisbon University (Prof. Lia Ascensão), have been produced. These results have been presented at meetings, and will now be submitted for publication in a major scientific journal (Mota, L., B. Moura, R. Galupa, P. Barbosa, M. Mota & L. Ascensão. 2014. Histological changes induced by *Bursaphelenchus xylophilus* in the stem tissues of *Pinus pinaster* seedlings. Paper in preparation).

In summary, debarked apical shoots of two-year old seedlings were inoculated with 2,000 PWNs from the Portuguese virulent strain HF. Seedlings not inoculated or inoculated with water only were used as controls. Samples collected at several time-points, from 1 day to 7 weeks after inoculation, were fixed with glutaraldehyde and prepared for SEM or embedding in Leica Historesin®, following standard methods. Stained resin sections were used for general histology.

The initial symptoms of disease began about 1-3 weeks after inoculation, but symptom development varied among specimens. Since the beginning of infestation, the number of tanniniferous idioblasts increased as well as the layers of cells surrounding the lumen of the resin ducts. At the “early stage” of infestation, when no external symptoms are visible, a certain degree of degradation in the parenchyma cell walls was observed and PWPs were detected within the lumen of cortical resin ducts. Severe tissue degeneration occurred during the “advanced stage” of infestation, when external symptoms were plainly visible. Cortical parenchyma cells were degraded or even collapsed as well as cambial and epithelial cells of the vertical and horizontal resin ducts. Cavities with irregular boundaries developed from degraded resin ducts and surrounding parenchyma cells. PWN were present in the xylem, vertical and horizontal ducts and their number per duct increased markedly. The cambial zone underwent degradation and the cavities expanded and fused as the disease progressed. Seven weeks after inoculation, the number of nematodes increased dramatically and all pine tissues were severely damaged.

The following images (Figure 2.34) illustrate aspects of the nematode pathology, both in light (LM) and scanning electron (SEM) microscopy.

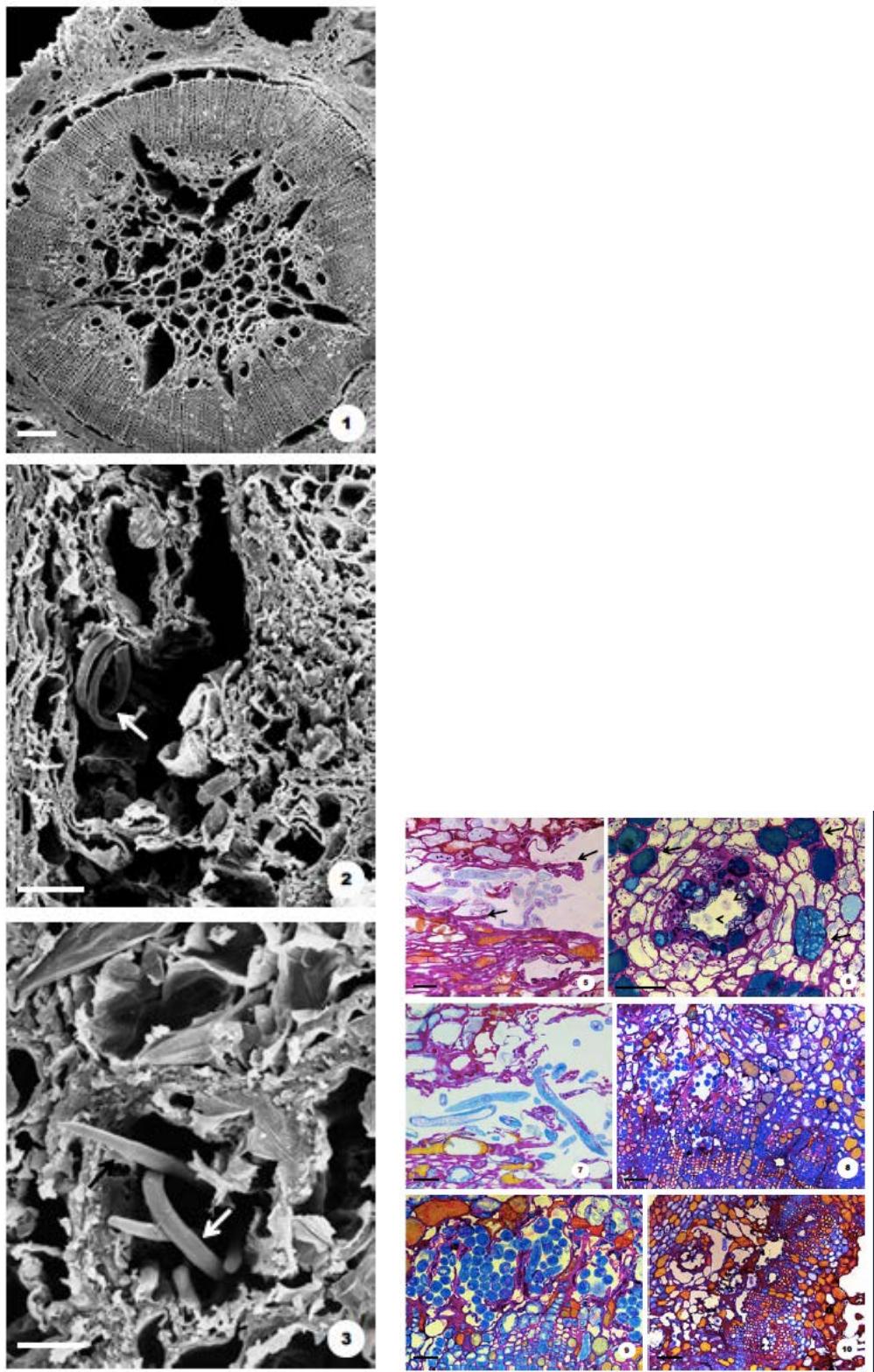


Figure 2.34 light (LM) and scanning electron (SEM) photomicrographs of nematode infested *Pinus pinaster* (Mota et al., 2014)

Further studies have been made, now using 2-yr old *P. halepensis* and *P. sylvestris* for comparative purposes. The HF, highly virulent, Portuguese isolate of *B. xylophilus* isolate was used. Response to infestation was significantly different in both species: *P. halepensis* behaved apparently as a resistant species (Figure 2.35), whereas

P. sylvestris proved to be even more susceptible (Figure 2.36) than *P. pinaster*.

This is of particular significance if we remember that *P. sylvestris* is the most widespread species of pine in Europe, and also the fact that the nematode and the disease have recently spread to Spain.

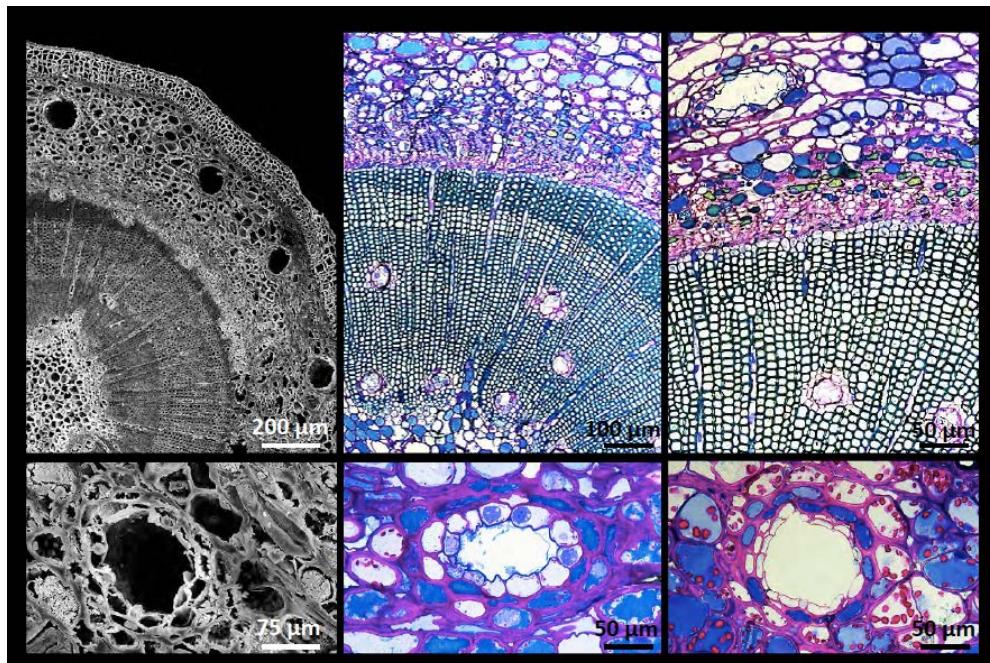


Figure 2.35 SEM and conventional photomicrographs of *P. halepensis* inoculated with *B. xylophilus* (Vieira da Silva et al.)

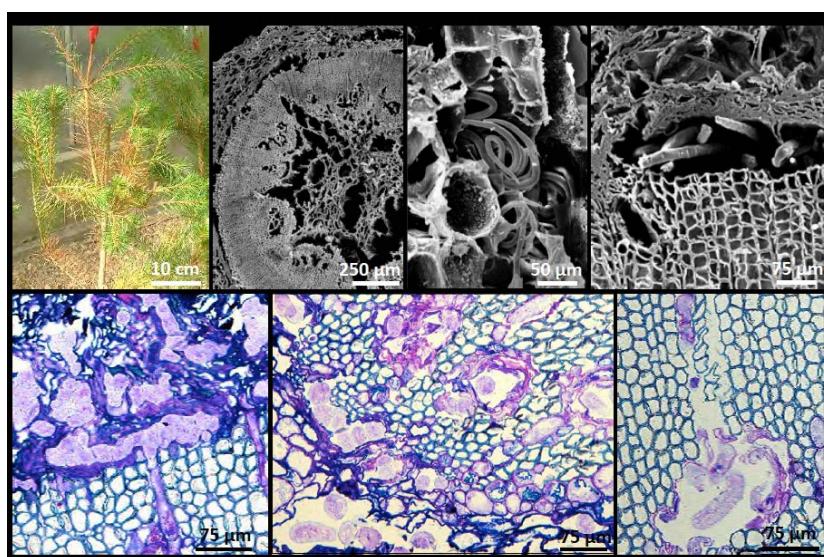


Figure 2.36 SEM and conventional photomicrographs of *P. sylvestris* inoculated with *B. xylophilus* (Vieira da Silva et al.)

Work on bacteria:

The research related to the role of bacterial communities associated with the pine wilt disease system has proceeded in 2013/ 2014, with some significant added information, namely the potential role of some bacterial species in protecting the nematode from oxidative stress (generated from the plant as reaction to the nematode entry and invasion), as well as identification of nematode communities associated with the insect vector. The results of these findings have been published in high impact factor journals, which are presented as annexes to this report:

- (1) Vicente, C. Ikuyo, Y., Mota, M. and K. Hasegawa. 2013. Pine wood nematode-associated bacteria contribute for oxidative stress resistance to *Bursaphelenchus xylophilus*. *BMC Microbiology* 2013, **13**:299 doi:10.1186/1471-2180-13-299.

Background: Pine wilt disease (PWD) caused by the pine wood nematode *Bursaphelenchus xylophilus* is one of the most serious forest pests and diseases in the world. Investigation of the role of *B. xylophilus*-associated bacteria in the PWD, and in the interaction with the nematode, has recently been the target of substantial research. Several studies report a potential contribution of bacteria for PWD development, either as a helper of the nematode or as a pathogenic agent expressing interesting traits related to lifestyle host-adaptation.

Results: We investigated the nematode-bacteria interaction under severe oxidative stress (OS) conditions (pro-oxidant hydrogen peroxide) and explored the adhesion ability of these bacteria to fix in the nematode's cuticle. Our results clearly showed a beneficial effect of *Serratia* spp. (isolates LCN-4, LCN-16 and PWN-146) towards *B. xylophilus* under OS conditions. In this scenario, *Serratia* spp. were extremely OS-resistant, and contributed to a considerably lower mortality percentage of *B. xylophilus* and down-regulation of *B. xylophilus* catalase genes (*Bx-ctl-1* and *Bx-ctl-2*). In addition, we used two isolates of *B. xylophilus*, virulent (Ka4) and avirulent (C14-5), and observed that Ka4 showed better fitness under OS conditions than C14-5. The bacterial effect was similar for both *B. xylophilus* isolates. We could not observe a strong and specific adhesion of these bacteria on *B. xylophilus* surface.

Conclusions: New insights into nematode-bacteria interaction are given in this study. We report, for the first time, that *B. xylophilus* associated bacteria may assist the nematode opportunistically in the disease, and that virulent *B. xylophilus* was able to better tolerate OS conditions than avirulent.

- (2) Vicente, C., F.X. Nascimento, M. Espada, P. Barbosa, K. Hasegawa, M. Mota, and S. Oliveira. Characterization of bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis*, the insect vector of the pinewood nematode *Bursaphelenchus xylophilus*. *FEMS Microbiology Letters* (in press; accepted, July 29, 2013) DOI: 10.1111/1574-6968.12232.

Abstract: Pine wilt disease (PWD) has a tremendous impact on worldwide forestlands, both from the environmental and economic viewpoints. *Monochamus* sp., a xylophagous insect from the Cerambycidae family, plays an important role in dissemination of the pinewood nematode, *Bursaphelenchus xylophilus*, the primary pathogenic agent of PWD. The present study investigates, for the first time, the bacterial communities of *Monochamus galloprovincialis* collected from Portuguese *Pinus pinaster* trees and *B. xylophilus*-free, using a metagenomics approach. Overall, our results show that natural bacterial communities of *M. galloprovincialis* are mainly composed by γ -proteobacteria, Firmicutes and Bacteroidetes, which may be a reflection of their feeding diet and habitat characteristics. We also report different bacterial communities' composition in the thorax and abdomen of *M. galloprovincialis*,

with high abundance of *Serratia* sp. in both. Our results encourage further studies in the possible relationship between bacteria from the insect vector and *B. xylophilus*.

A major review on this subject has now been submitted to another journal:

(3) Nascimento, F., Cláudia S.L. Vicente, Pedro Barbosa, Margarida Espada, Paulo Vieira, Koichi Hasegawa and Manuel Mota. 2014. Bacterial role in pine wilt disease development – review and future perspectives. *Environmental Microbiology, EMI Reports* (submitted).

Mutualistic and beneficial relationships between nematodes and bacteria are highly present in nature, mostly occurring because of nutritional dependence and pathogen protection, and intrinsically related with the environment, the ecological conditions and the nematode life stages. Thirty-three years have passed since the first hypothesis suggesting a bacterial role in pine wilt disease (PWD) that was initially thought to be caused only by the phytopathogenic nematode *Bursaphelenchus xylophilus*. In the late 70's, researchers reported that bacteria associated with the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, could produce toxins that lead to PWD in pine seedlings. It was also suggested a double vector system for PWD, where bacteria were vectored by the PWN, and PWN vectored by an insect from the *Monochamus* spp. The specific involvement of bacteria in such complex disease is still controversial, although recently the number of studies focused on the importance of bacteria has increased considerably. This review is up-to-date comprehensive perspective and brings new insights on the role of bacteria in PWD.

2.4 Development of early detection methods for PWN in trees

2.4.1. Trapping methods for PWN

Beneficiary 10 in China has developed a sampling method involving attraction of the different stages of PWN in infested trees, symptomatic or asymptomatic, by specific blends of host volatiles for either juvenile or dispersive nematodes. They allow rapid detection of PWN within two hours. However, these methods have been validated for only two species of pines in China, and their applicability to the European species of conifers susceptible to infestation by PWN will be assessed in Portugal by B6.

In two PWN-infested stands, the trees will be classified into categories based on the wilt symptoms they express. Ten trees from each category will be randomly felled for sampling. Tubes containing the standard Chinese attractive blend will be inserted into sampling holes at intervals between crown and root and their effectiveness at attracting PWN assessed. If these results are positive, experiments on boles artificially loaded with known population densities of PWN will be carried out to define relationships between PWN density and trapping results, and to optimize the attractive blend. If the results are negative, it will be necessary to identify the specific chemical blends with potential to attract the different stages of PWN from *P. pinaster* trees. The experimental approach used by B10 for identifying the attractive blends will be used, including the sampling of volatiles from both larvae/pupae of *Monochamus galloprovincialis* and pupal chambers, and behavioural bioassays with these volatiles. Data gained in Task 2.1.will be relevant here.

Similar experiments will be carried out to test the method the other species of conifers, especially *P. sylvestris*, *P. nigra*, *P. pinea* and *P. halepensis*, and other species of *Monochamus* to design attractive blends. When the experiments are carried out in non PWN-infested regions, *B. mucronatus* will be used as a substitute. B3 and B6to lead.

B3 (BFW)

2.4.1. Trapping methods for PWN. The planned testing of the PWN trapping method for detecting other *Bursaphelenchus* spp. in pines in Austria had to be postponed to 2014. Surveys for nematodes were continued in 2013 to identify localities where the nematode trapping method could be tested in 2014. Overall, the prevalence of *Bursaphelenchus* spp. in pine forests in Austria was very low; *Bursaphelenchus* sp. (non-*xylophilus*) was found only on one site in the federal province of Salzburg in 2013.

B4 (INRA)

As the preliminary tests done in 2012 using the Chinese blends designed for Asian pines were not as positive as expected, it appeared necessary to identify new chemical blends which could specifically attract PWN out of infested *Pinus pinaster* trees.

In early June 2013, volatiles were sampled in Portugal from PWN-infested and non-infested trees with the help of partner B6. Samplings included the 4 following categories of volatile emissions:

- 5th -instar mature larvae of *Monochamus galloprovincialis* (5 samples)
- pupae of *M. galloprovincialis* (3 samples)
- pupal chambers of *M. galloprovincialis* (4 samples)
- healthy *Pinus pinaster* (2 samples)

The volatile collection was performed simultaneously on the different categories using different types of SPME fibres (Figure 2.37). The fibres were then analysed by GC-MS at INRA. Most of monoterpenes and sesquiterpenes were identified and chromatograms were compared in order to find out possible “candidates” for a specific attractive blend.

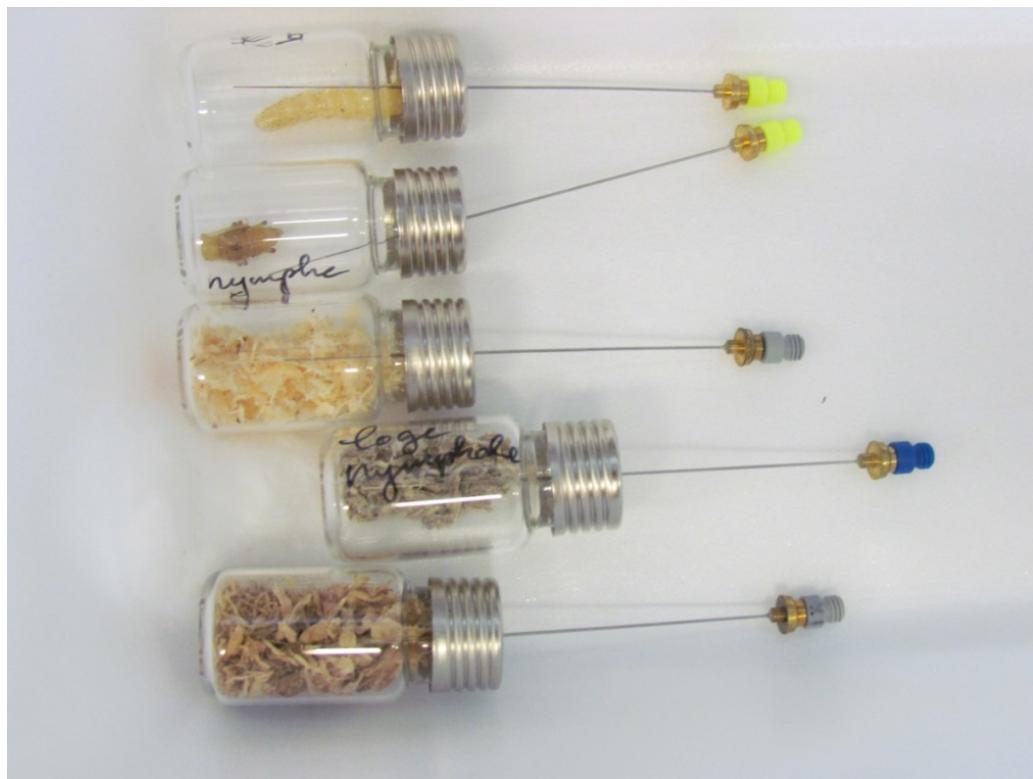


Figure 2.37 Sampling design for the four categories (two controls are shown); from top: larva; pupa; healthy pine; pupal chamber, healthy pine.

Figure 2.38 presents initial results. The numbers in brackets refer to the peaks identified on the chromatograms. In summary;

- Alpha-pinene (1) and beta-pinene (2) were present in every sample but with various relative amounts
- Ipsenol (3) amount is dominant in pupal chambers
- An unidentified peak (4) (rt 7.8mn) seems to be present only in pupal chambers
- Sesquiterpenes (longifolene (5), caryophyllene (6), ...) are present in wood samples (pupal chambers and control *Pinus pinaster* samples)

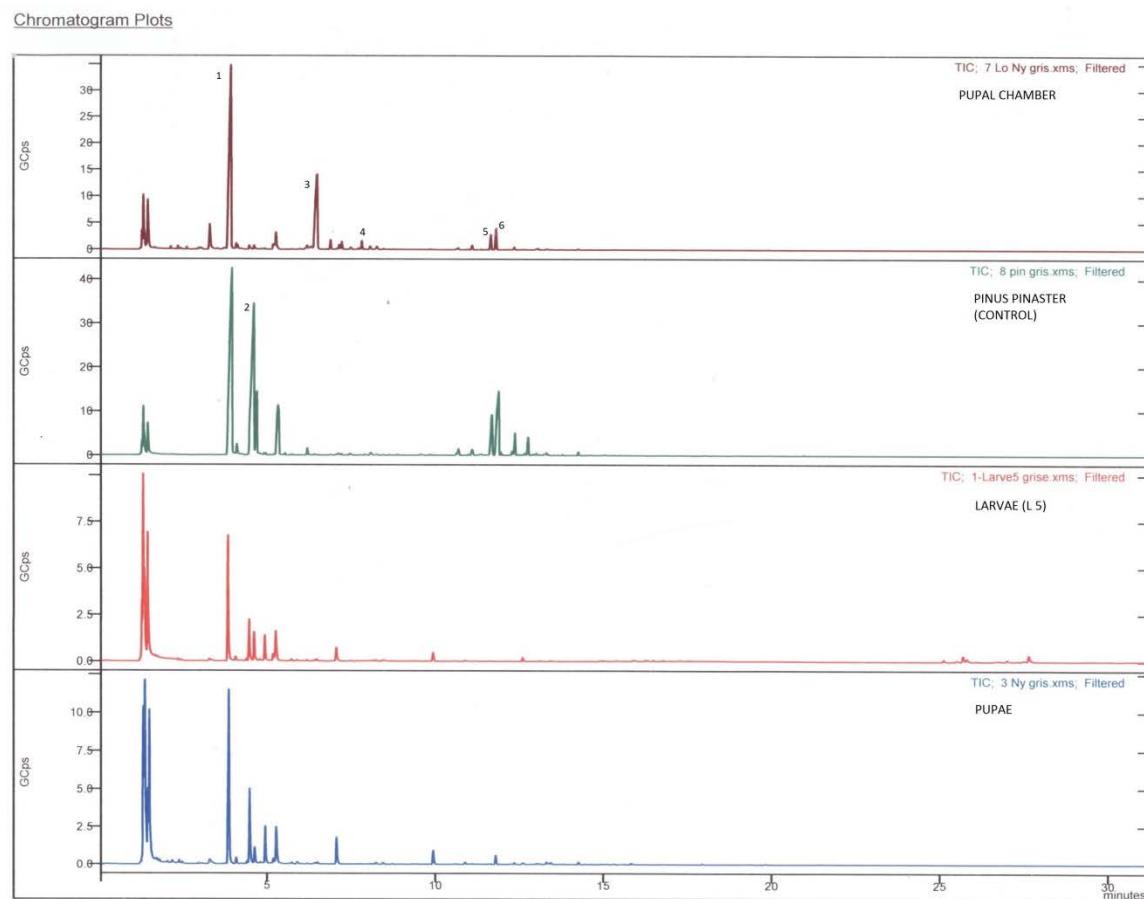


Figure 2.38 Examples of chromatograms obtained for a set of volatile emissions sampled at the same time. From top: pupal chamber, healthy pine larva; pupa.

Some peaks still need to be identified. Each peak has also to be precisely quantified and the relative amounts calculated so that the chromatograms obtained from each volatile sample can be compared.

After this stage, the experimental approach developed by the Chinese colleagues (B 10) for identifying the PWN- attractive blends will be used and bioassays tentatively performed using these volatiles.

2.4.2. Investigation of DNA detection

Several methods regarding detection of PWN DNA have already been developed, mainly by Japanese and Chinese colleagues. One method in particular, using the “nested-PCR” technique appears to be promising(Takeuchi et al., 2006) and is based on finely crushing a wood sample, utilizing a specific probe to detect the presence of the nematode DNA, amongst the wood material, with no need to isolate the nematodes from the wood. B7 will conduct testing of this method to samples obtained either from PROLUNP (annual survey) or using healthy wood artificially inoculated with PWN. Other similar DNA detecting methods will be tested in parallel byB4. Novel methods for detecting gene products, resulting from the pathogenic action of the nematode will also be investigated by B7.

A second method will also be tested in Portugal by B6. This new method was developed in Japan and is available as a kit. It is based on an enzymatic reaction that can extract nematode DNA from the wood tissue followed by Loop-mediated Isothermal Amplification (LAMP), targeting a specific DNA region of the PWN gene. It requires no expertise in microscopic/morphological techniques and is a time and labour-saving way to identify the nematode.

In each year adult pine trees with no wilting symptoms and with variable wilting symptoms will be selected, characterised and geo-referenced. In the spring and summer the oleoresin flow of the various selected trees will be assessed by making small punch holes in the bark and examining the subsequent resin flow, as the decrease of resin flow is a very early indication of wilt disease in maritime pines. All trees failing to produce oleoresin will be tested with the Japanese kit at breast height. To validate the results of the Kit, segments of wood will be collected from all the trees tested and analysed under laboratory conditions, in order to detect and quantify the presence of PWN. The sanitary condition of all the selected trees will be evaluated monthly, and periodically tested using the Kit. In winter the same procedures will be conducted in both non-symptomatic trees and in pines with isolated wilted branches which may result from late or reduced nematode infestations. Subsequently, these trees will be felled and the wood sampled quantitatively for the presence of PWN at different heights, in order to validate the results of the Japanese Kit.

At the B6 laboratories in Portugal, three-year old seedlings of *P. pinaster*) will be inoculated with one of two concentrations (“low” or “high”) of PWN diluted in sterile water. Following the inoculations, the sanitary conditions of the pines will be assessed periodically, and the presence of PWN verified using the Japanese detection kit. After a few months the trees will be subjected to a final sample to detect and quantify PWN using classical morphological methods, in order to validate the results obtained with the Kit. These procedures will also be conducted in other pine seedlings inoculated either with *B. xylophilus* or with similar related *Bursaphelenchus* species found in Portugal, such as *B. mucronatus*. This experiment will test and confirm the specificity of the Japanese Kit to *B. xylophilus*, in order to avoid false-positives when related nematodes of the same genus are found in the wood.

B6 (INIAV)

Two methods regarding rapid and accurate detection of PWN DNA, either in individual nematodes or in wood tissue from adult *Pinus pinaster* trees, were tested.

The first method was based on the internal transcribed spacer (ITS) region of the ribosomal DNA amplification with two sets of species-specific primers: the first set including the forward primer ITS1 (TACGTGCTGTTGAGTTGG) and reverse primer ITS2 (GCACGGACAAACAGTGCGTAG) (Takeuchi, et al., 2005) and a second set with forward primer XF (ACGATGATGCGATTGGTGAC) and reverse primer XR (TATTGGTCGCGGAACAAACC) (Matsunaga and Togashi, 2004).

For testing the sensitivity and specificity of this ITS-PCR method, total DNA from wood shavings and from one, five and ten nematodes was used. Pure cultures of the two closely related nematode species *B. xylophilus* and *B. mucronatus* and nematode-free wood tissue from young seedlings were used as positive and negative controls.

The detection of *B. xylophilus* in wood tissue of *P. pinaster* by ITS- PCR assay with the first set of primers was successfully established.

A second method, the Loop-mediated Isothermal Amplification (LAMP) method was also tested, using the kit developed by Nippon Gene Co., Ltd in Japan. *P. pinaster* wood chips with and without *Bursaphelenchus xylophilus* were evaluated. The *Bursaphelenchus xylophilus* presence is judged by the existence of emission of fluorescence (Figure 2.39).

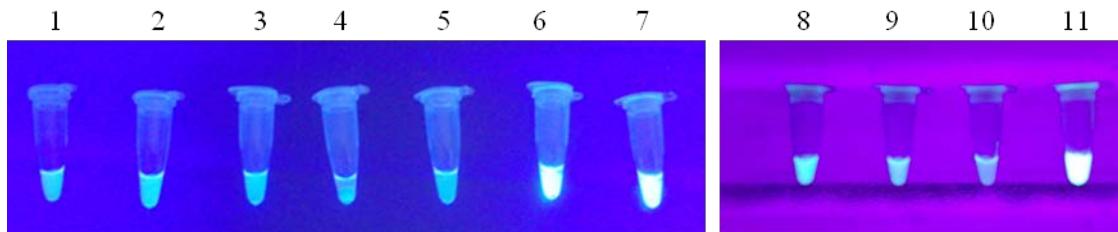


Figure 2.39 LAMP fluorescence images. 1 – Negative control (kit); 2 – *Bursaphelenchus mucronatus*; 3, 4, 5 - *P. pinaster* wood chips without *B. xylophilus*; 6 - *Bursaphelenchus xylophilus*; 7 – Bx positive control (kit); 8 - Negative control (kit); 9 - *P. pinaster* wood chips with *B. xylophilus* from the field (naturally infested); 10 - *P. pinaster* wood chips with *B. xylophilus* (infested in the laboratory); 11 – Bx positive control

The fluorescence obtained with the *P. pinaster* wood chips with *B. xylophilus* is very similar to the negative control and to the other wood chips without *B. xylophilus*.

B7 (UE):

The diagnostic methods to detect the pinewood nematode are (already) well developed but the mechanisms by which the nematode parasites its host are still not completely clear. To determine some of the factors that contribute to pine wilt expression we decided to analyse the transcriptome of the nematode in interaction with the plant host.

To identify some of the proteins that might be involved in the pathogenicity of the PWN, we inoculated 50 one-year-old *Pinus pinaster* trees with a mixed-life stage Portuguese isolate. The nematodes were collected from pines after 6 and 15 days, by the Baermann funnel method, then pelleted by sucrose flotation and cleaned in a buffer solution. The nematode pellet was frozen in liquid nitrogen and the total RNA was isolated. Three biological replicates were sequenced for each time point. The analysis of the RNAseq data revealed a list of highly expressed genes during host infestation, known as effectors, which are most likely to play a role in plant-nematode interactions (Table 2.8). These proteins might be involved in food digestion, plant cell wall degrading enzymes and protection and suppression of host defences. Also, for several highly expressed genes, the proteins have not been included on published databases, meaning that these proteins are specific for this nematode (and other) nematodes and haven't been identified so far, which make them an interesting case study.

Table 2.8 Summary of the categories and speculative function of the highly expressed genes identified in RNAseq analysis.

Speculative function	Effector
Food digestion – protein degradation	Peptidases, Cathepsin/cysteine, Putative Lysozymes, metalloproteinase, Aspartyl protease
Plant Cell wall degrading enzymes	Pectate lyase, GH45, Expansin
Protection and suppression of host defences	Glutathione-S-transferase, cytochrome P450, UDP, epoxide hydrolase,
Unknown Function	Unknown Protein domain function – novel genes; venom allergen proteins

From the highly expressed genes, it was hypothesized that they have a signal peptide for secretion and lack a transmembrane domain, possibly produced in the oesophageal glands and injected within the plant tissues via the stylet. To confirm the expression of the new candidate genes in the nematode oesophageal gland cells, *in situ* hybridisation was performed using DIG-labelled DNA probes designed to amplify a 250bp fragment of the gene (Figure 2.40).

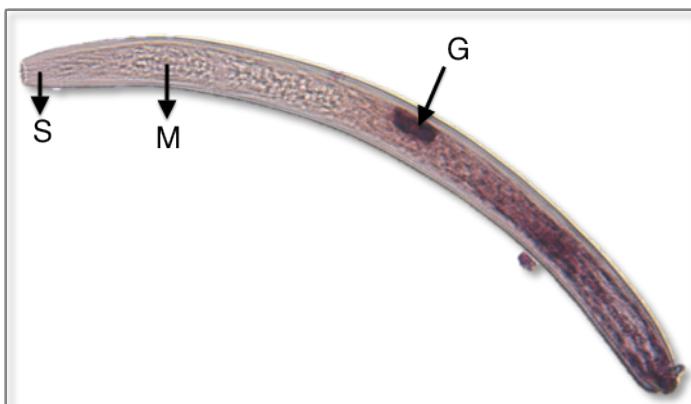


Figure 2.40 Localization of one of the effectors transcripts in the oesophageal gland cells of *B. xylophilus* by *in situ* hybridization. Nematode sections were hybridized with antisense digoxigenin-labelled cDNA probes. G, oesophageal glands; S, stylet; M, medium bulb.

So far, some of the candidate genes were expressed in the gland cells, such as glutathione-S-transferase, venom allergen proteins and several novel genes, which are potential targets. This is ongoing work which will continuously generate a huge amount of data to explore in order to find which proteins are responsible for the pathogenicity of the nematode. Our main goal continues to be the target of these proteins and try to find a way of controlling the parasite or for intervention in the plant host.

Work Package 3: Assessing phenology and dispersal capacities of PWN vectors

Objectives

The objectives of this WP are:

- To establish the influence of climatic conditions on vector emergence in order to define flight periods at different geographic locations across Europe.
- To establish vector physiological and behavioural parameters related to flight capacity
- To determine mean and maximum vector dispersal in the different phases of the adult life span
- To determine vector dispersal capacity in different forest environments, using behavioural and molecular techniques

Deliverables

D3.1: Vector flight capacity related to physiology: Determination of flight capacity of *Monochamus* vectors, particularly *M. galloprovincialis*, in relation to physiological status over the lifetime of the adult vector. Month 33

D3.2: Vector dispersal related to forest condition: Determination of vector field dispersion related to forest condition and structure, especially within closed canopies and open spaces. Month 33

D3.3: Vector dispersal related to population genetics: Determination of vector dispersal in relation to genetic diversity and population barriers. Month 45

D3.4: Climate influences on vector dispersal: Determination of climatic influences on phenology and dispersal of vectors. Month 33

Participants: B3, B4, B6 and B9.

3.1 Vector flight phenology under different climate conditions (Delivery date: 33 months).

The emergence period of the vector in central and northern Portugal will be studied by keeping pine wood containing *M. galloprovincialis* in meshed boxes at ambient temperatures in both completely shaded and sun-exposed situations, recording the air temperatures and the solar radiation of the wood. B6 to coordinate.

The study will use: a) wood with different development instars (eggs and larvae) sourced from local dead pines in the field; and b) branches cut from healthy trees and taken to the laboratory to be submitted to oviposition the female beetles (monthly from May to October), and then maintained under outdoors conditions until emergence.

The flight period of the vector in central and northern Portugal will also be studied by placing traps baited with attractants in selected pine forests from March to November, correlating local climatic parameters (temperature and precipitation) with the captures.

Similar studies, extending to different *Monochamus* species (e.g. *M. sartor*, *M. sutor*, *M. urosovii*) will be conducted in Austria (B3), France (B4), Spain (B9) and Northern Europe (B11), i.e., along a latitudinal gradient, allowing direct comparisons of emergence and flight periods under various climatic conditions.

The developmental thresholds and thermal requirements of *M. galloprovincialis* larvae have already been studied in Portugal, resulting in a simple linear method (the modified sine wave) driven by air temperatures used to predict the emergence in southern Portugal. The model can be a useful tool to predict insect emergence in other European regions after refinement. Thus, refinement and adjustment of the existing model with the emergence patterns from other locations of central and northern Portugal and Europe will be carried out. In addition, a validated map of annual *M. galloprovincialis* emergence patterns for the Iberian peninsula based on

degree-day accumulations in the different climatic regions will be developed. Similar studies will be made for other *Monochamus* species, as indicated above. B6 to coordinate.

Years 1 and 2: Evaluation of emergence at different climatic locations;

Years 2 and 3: Refinement and adjustment of the emergence model; creation of a validated map of annual emergence patterns for *M. galloprovincialis*.

Task 3.1 - Vector flight phenology under different regions

Activities carried out by B3

B3 studied the flight phenology of *Monochamus* spp. in Austria, with the monitoring of the flight phenology in the field (a) and the study of emergence from logs – breeding experiments with *M. sartor* (b).

a) Monitoring flight phenology in the field

Material & Methods: Multifunnel traps (Econex, Spain) baited with Galloprotect-2D and α-pinene (SEDQ, Spain) were set up in various regions in Austria, stretching from lowland pine forests in the East to mountainous spruce forests on the northern and southern side of the eastern Alps (Figure 3.1). Therefore, sampling sites covered typical areas for *M. galloprovincialis*, *M. sutor*, and *M. sartor*. Traps were installed in May 2013 and checked weekly until catches ceased in October; lures were replaced every 6 weeks. Capture was compared with temperature and precipitation data from nearby weather stations operated by ZAMG Austria. On two sites we additionally recorded air temperatures with ELB-USB-2 data loggers (Lascar Electronics, UK) set up near the traps.

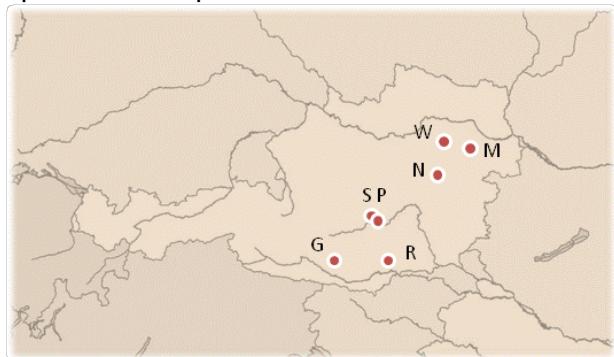


Figure 3.1 *Monochamus* trapping sites in Austria (with main tree species and elevation): M = Baden-Mitterberg (*Pinus nigra*, 330 m), W = Weyersdorf (*Pinus sylvestris*, *Picea abies*; 458 m), N = Naßwald (*P. abies*, *L. decidua*; 1240 m) P = Pöls (*P. abies*, *L. decidua*, some *P. sylvestris*; 930 m), S = St. Oswald (*P. abies*, *L. decidua*; 1490 m), R = Ragglbach (*P. abies*, *P. sylvestris*; 583 m), G = Gerlitzen (*P. abies*, *L. decidua*; 1490 m).

Results: Capture of *Monochamus* spp.: Traps in the Austrian pine forests Mitterberg caught exclusively *M. galloprovincialis*. This species and *M. saltuarius* were caught in the lowland mixed coniferous stand in Weyersdorf. On all other sites, *M. sutor* was the dominant species. Higher catches occurred in mountainous locations. In Naßwald, also *M. sartor* was caught in high numbers (Table 3.1).

M. galloprovincialis were caught first on June 5, *M. sutor* on June 10, and *M. sartor* on June 14. Once flight had begun it continued until weather conditions became unfavourable in the second half of August. No clear flight peaks became apparent during this period (Figure 3.2). The final captures occurred in late October during a dry and warm period. Generally, trap catches were correlated positively with air temperature (Table 3.2). No beetles were usually caught in weeks when daily mean temperatures dropped below 10-12°C and maxima below ca. 20°C (Figure 3.3).

Table 3.1 Total trap catches of *Monochamus* spp. from May to October 2013

	<i>M. gallo.</i>	<i>M. sutor</i>	<i>M. sartor</i>	<i>M. salt.</i>
Baden, Mitterberg I	143	0	0	0
Baden, Mitterberg II	91	0	0	0
Weyersdorf I	2	0	0	2
Weyersdorf II	2	0	0	2
Nasswald Waldeben	0	195	92	0
Nasswald Rainerboden	0	265	57	3
Pöls	4	114	0	0
St. Oswald	0	212	0	0
Gerlitzen	0	194	1	0
Ragglbach	2	19	0	0

Apart from *Monochamus* spp. other cerambycids, buprestids, scolytines and clerids were also determined. Hence, the vector flight monitoring also gave good insight into the occurrence of other phloeo-xylophagous beetles and important predators.

Table 3.2 Correlation between weekly mean air temperature and number of trapped beetles (n = 18)

Species	Location	Kendall's τ-b	P
<i>M. galloprovincialis</i>	Baden I	0.427	0.012
	Baden II	0.377	0.031
<i>M. sartor</i>	Naßwald-R.	0.434	0.017
	Naßwald-W.	0.557	0.002
<i>M. sutor</i>	Naßwald-R.	0.742	<0.001
	Naßwald-W.	0.624	<0.001
	Pöls	0.646	<0.001
	St. Oswald	0.657	<0.001
	Ragglbach	0.447	0.019
	Gerlitzen	0.729	<0.001

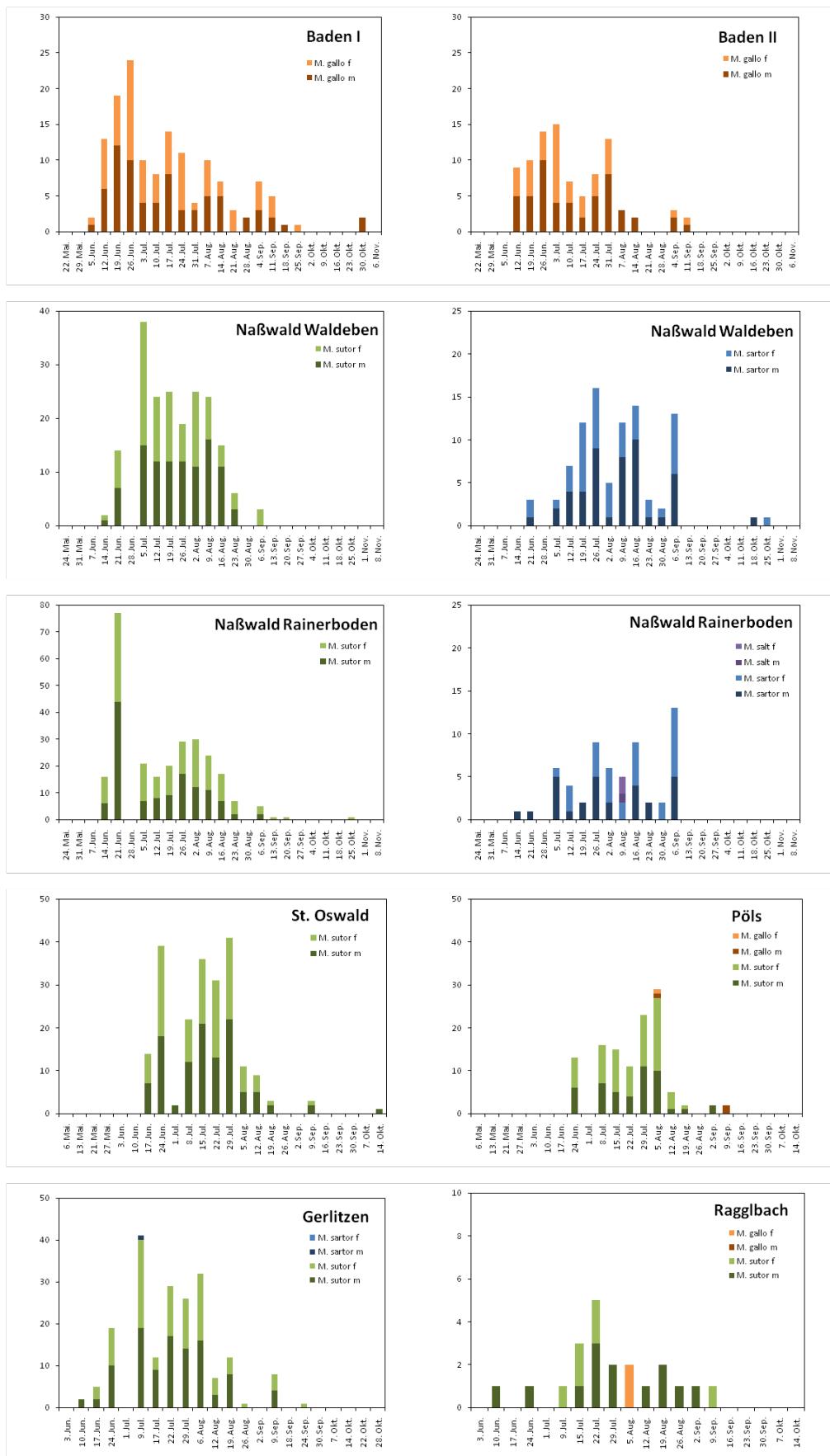


Figure 3.2 Weekly trap catches of *Monochamus* spp. (separated for male and female) in multifunnel traps baited with Galloprotect-2D plus α -pinene.

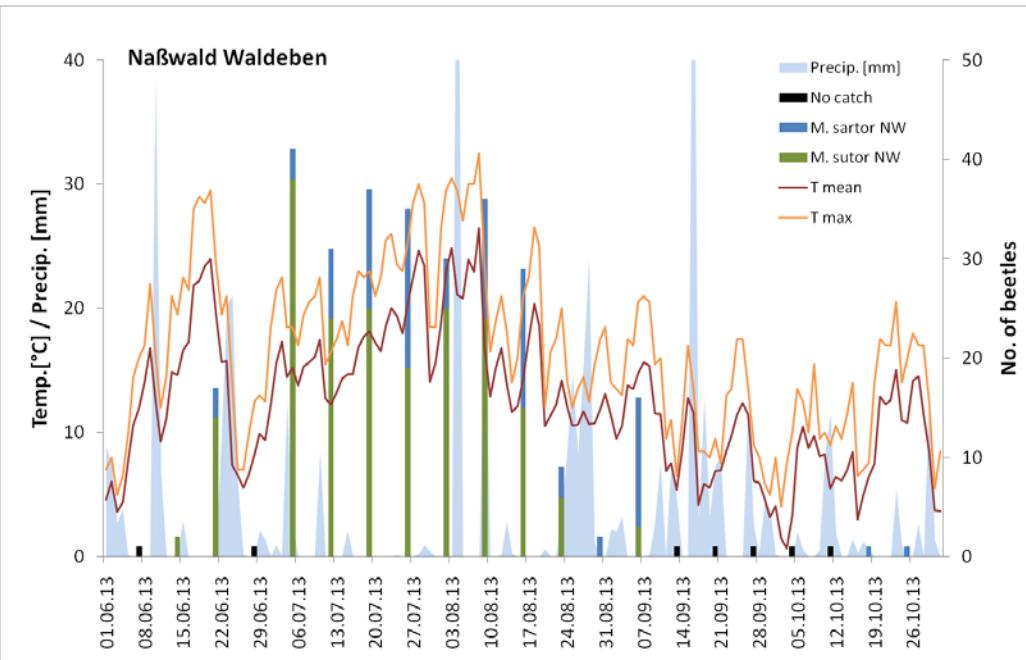


Figure 3.3 Weekly catches of *M. sartor* and *M. sutor* in Naßwald-Waldeben in comparison to daily mean and maximum air temperatures recorded on the site and precipitation on a nearby weather station.

Conclusions: Our trap network in various regions of Austria provided important information on flight phenology of the four *Monochamus* spp. that occur in our coniferous forests at different elevations. This is the first time such data were collected systematically in Austria. The study also demonstrated the feasibility of trap and lures developed for *M. galloprovincialis* for catching *M. sartor*, *M. sutor*, and *M. saltuarius* in lowland and mountain forests. Data show a continuous flight season from June to October with short episodes of no flight due to low temperatures (often together with high precipitation). Trap catches were positively correlated with air temperature.

b) Emergence from logs – breeding experiments with *M. sartor*

Material & Methods: Breeding experiments were initiated in the first full field season of the project (i.e. in the first reporting period) and have been going on continuously. *M. sartor* beetles emerging from field collected, infested logs and beetles caught in the field throughout the summer of 2012 were kept pairwise in cages in the laboratory, fed with *P. abies* twigs and provided with *P. abies* logs (50 cm long) for oviposition. The logs were checked regularly and replaced if necessary. On October 31, 2012 half of the logs were transferred to a shelter outdoors (i.e. not exposed to direct sunlight), the others remained in the laboratory at room temperature. Logs were moistened regularly and checked for larval activity and beetle emergence. Ambient temperature was recorded hourly with ELB-USB-2 data loggers. Breeding experiments were continued in the summer of 2013; emergence from logs will be monitored in 2014.

Results: First adult beetles emerged from logs continuously incubated under laboratory room temperature on February 25, 2013 after an incubation time of 177 days (Figure 3.4). Emergence from logs overwintering under outdoor conditions started on June 21, 2013 after approximately 330 days post oviposition and after daily mean temperatures reached more than 20°C for several days. All outdoor beetles emerged in the short period until July 5. No further emergence occurred during subsequent periods with similar or higher temperatures. However, larval feeding activity was evident in the summer months indicating that a part of the population will show a two-year development.

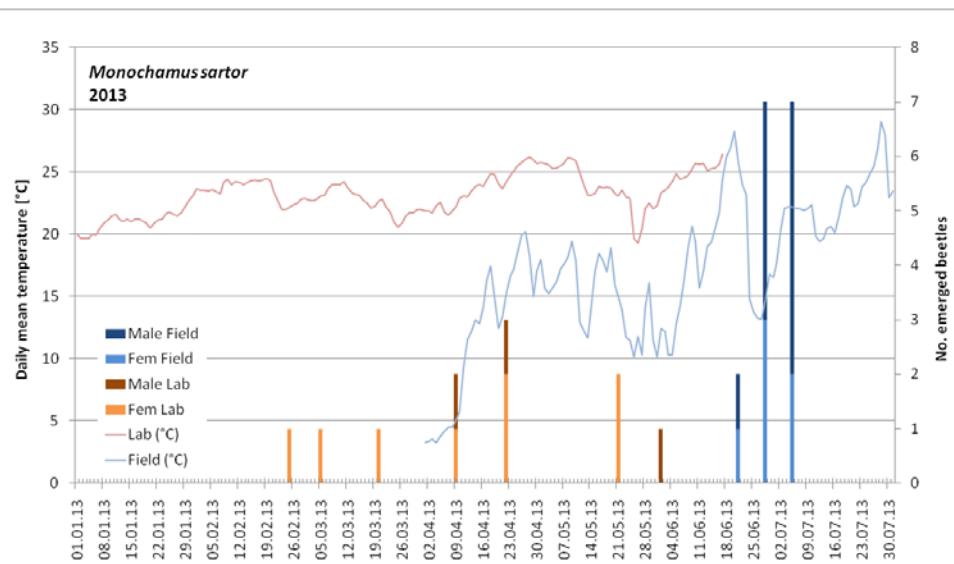


Figure 3.4 Emergence of *M. sartor* beetles from logs incubated under outdoor conditions (blue) or under laboratory room temperatures (orange-red) and respective daily mean temperatures.

Effective temperature sums were calculated by tentatively using the 12.2°C developmental threshold of *M. galloprovincialis*. Laboratory reared *M. sartor* emerged after accumulating 2407 ± 108 degree-days (dd); beetles reared under outdoor conditions emerged at 1536 ± 43 dd. While laboratory room temperature averaged 23.7°C and dropped slightly below 20°C only in a few exceptional cases, outdoor temperatures were continuously below the threshold between Nov. 1 and April 11. After this cold period, beetles accumulated 359 ± 8 dd prior to emergence from the logs.

Conclusions: When kept at room temperature, *M. sartor* was able to complete development from oviposition to adult emergence in approximately 6 months. Under outdoor temperatures, this period was significantly prolonged to approximately 11 months. Some logs showed continued larval activity in summer with no beetle emergence, indicating a two-year development. All *M. sartor* from the outdoor rearing emerged within two weeks from late June to early July while emergence in the lab extended over more than three months. The outdoor emergence period corresponded with results from 2012, when all beetles from logs collected in the field in May emerged in June after the first consecutive days with mean temperatures above 20°C.

Activities carried out by B6

a) Studies on artificial diets

B6 initiated studies on the comparison of eight artificial diets to rear the pine sawyer *Monochamus galloprovincialis* under laboratory conditions with reduced costs and high efficiency, in order to obtain a tool useful for studies on insect development under controlled conditions. Tested diets included various artificial media commercially available for other insects and media based on dried or fresh pine phloem. Diets #1 to #3 differed in the insect culture medium selected, which corresponded to around 1/4 of the composition. Diets #4 and #5 excluded the culture medium, but incorporated cellulose powder as a key ingredient. Diets #6 to #8 included an autoclaved pre-mixture (pre-mix) with ascorbic and propionic acids added to prevent contamination with fungus or with other life forms. In these diets the pre-mix and all the pre-autoclave ingredients were prepared in a large and sterile container, to which the phloem cambium was added later. All the ingredients were then autoclaved for 15 minutes at

121°C. After sterilization, all procedures were conducted in a biological safety chamber to avoid contaminations. The methyl paraben, ascorbic and propionic acids were added to the mixture inside a fume hood using anti-acid gloves and safety mask. A total of 240 glass Petri-dishes (90mm diameter) were sterilized and filled with 50cm³ of diet each.

Holes were created on the diet's surface to insert the recently-hatched larvae, numbering 30 per diet. Sex and weight of emergent adults were recorded. Diets were evaluated and compared based on the development time, number of adults obtained and weight.

Among the eight tested variations, diet #5 was the most efficient with 80% eclosions of adult beetles, while diet #6 allowed the fastest development, with the first adult emerging within 53 days. The diets enabled adult insects to be reared with a minimum mean of just 95 days, which implies a significant reduction of the normal larval development time, thus allowing the rearing of two or three sequential generations per year instead of the single annual generation observed under field conditions. This is a potentially useful tool for future studies on insect development under laboratory conditions. These studies produced one publication:

Petersen-Silva, Naves, Sousa & Pujade-Villar (2014). Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets. Journal of the Entomological Research Society, 16(1): 61-70.

b) Studies on emergence pattern of *Monochamus galloprovincialis*

M. galloprovincialis emergence pattern was studied in three locations in Portugal during 2013: Marinha Grande, Odeceixe & Monte Gordo. During the year only a few beetles emerged from these locations, with numbers insufficient to enable determination of the local emergence pattern. The low emergence was caused by extensive larval mortality due to abiotic and biotic factors, including several parasitoid and fungal species which were reared from the dead larvae and recorded for the three locations. Two publications were produced from these studies:

Petersen-Silva, Naves, Godinho, Sousa & Pujade-Villar (2014). Distribution, hosts and parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in mainland Portugal. *Silva Lusitana*, in press.

Petersen-Silva, Henriques, Inácio, Naves, Godinho, Sousa & Pujade-Villar (2014). Susceptibility of larvae and adults of *Monochamus galloprovincialis* to entomopathogenic fungi under controlled conditions. Submitted to publication journal.

In 2014, wood infested by the vector insect has been collected and is now being kept under both natural (sun-exposed) and protected (shaded) conditions at Oeiras while recording ambient air temperatures, to allow for the natural emergence of adult *M. galloprovincialis*. Observations of emergence will be compared with predictions made by the Degree-Day model, with input of daily maximum and minimum temperatures. Additionally, partner B6 is trying to obtain information on the captures of *M. galloprovincialis* within the national grid of traps to monitor this pest, along with data on daily temperature, to run the Degree-Day model and compare the prediction of emergences with captures of beetles for several locations in continental Portugal.

Activities carried out by B9

2012-13:

Experiment 1:

P sylvestris baited logs were left in the field in June-July 2012 for *M. galloprovincialis* field colonization (n= 15; 120cm long, 8-15 cm Ø). Logs were left under field conditions until mid-November, and then transferred to outdoor cages. Temperature was monitored (outdoor, within the cage, within the wood) from January 1st to end of adult emergence in August 2013.

2013-14:

Experiment 2:

Similarly to the above experiment, *P sylvestris* baited logs were cut and left in the field in July/August/September (2013) (n= 10 each time) for *M. galloprovincialis* colonization. After being in the field until mid-October, logs were transferred to outdoor cages. Temperature is being monitored (outdoors) from January to end of emergence in summer 2014.

Data from emergence are currently being compiled and will be analysed to check for a degree-day model.

2013

Experiment 3:

As in 2012, flight phenology was tracked in baited traps (Galloprotect 2D) in three localities of Sierra de Gata (Salamanca, Spain), within the demarcated 20 km radius area of the nematode focus in Valverde del fresno (Cáceres), from May to mid-October.

Results: data analysis is underway (Figure 3.5).



Figure 3.5 Trap locations for flight phenology of *M. galloprovincialis* (S. de Gata, Spain)

DELIVERABLES Task 3.1 - Vector flight phenology under different regions

Results from the ongoing experiment and those from previous years from the various beneficiaries will contribute to Deliverable 4 “Influence of climatic conditions on vector emergence and dispersal”.

3.2 Life time audit of vector flight behaviour and physiology (Delivery date: 33 months).

Flight capabilities and flight behaviour of *Monochamus galloprovincialis* will be tested using sophisticated flight mills adapted from those designed for insects of the same size and weight, and including electronic recording of both single and multiple, successive flights. The tested insects will be reared under controlled conditions.

Populations originating from different geographic areas and host trees will be compared in order to assess possible variability in flight capabilities within and between populations. Performances will be compared with regard to sex, size, age after emergence, and density of nematodes per beetle (using artificial infestation, *B. mucronatus* being used as a substitute to *B. xylophilus* if necessary), and under different temperature conditions.

A key target will be to assess whether the flight capabilities vary over the duration of the life cycle in relation to inoculation of PWN. Thus, the same individuals will be tested at emergence, before maturation feeding and after maturation feeding during searching for mating and oviposition.

B4 to coordinate, with input from B6 and B9. The study will be extended to *M. sartor* and *M. sutor* in a second step by B3.

Half of the pre-mature beetles used in the above experiments will be killed to compare physiological parameters (lipid content, carbohydrates) and development of flight muscles with flight performances. All the remaining beetles will be analysed in the same way after the maturation feeding.

Years 1 and 2: Evaluation of flight capacities of *M. galloprovincialis* using adapted flight mills;

Year 2: comparison of physiological parameters and flight muscles;

Years 2 and 3: Evaluation of flight capacities of other *Monochamus* species from Europe.

Activities carried out by B3

a) Flight mill experiments with *M. sutor*

Material & Methods: Two flight mills were provided by H. Jactel, INRA Bordeaux (B4). *M. sutor* that emerged from logs collected in the field in 2012 and adult beetles captured in the field in 2013 were used in preliminary tests on the flight mills. Beetles were reared individually in glass jars with ventilated lids and fed with spruce twigs. For flight tests, beetles were attached to the mills and attempts were made to trigger flight by gently blowing in their face. Once a beetle started to fly, rotations were recorded until the beetle stopped. Then a new attempt was made to start flight. The beetle was removed and returned to the rearing containers until used in the next session. Overall, we used 6 field collected *M. sutor* males and 9 females (4 reared out of logs and 5 field collected).

Results: Of the tested males, 5 performed extended flights (> 5 min) at least during one session; one male showed only flight attempts. All 5 field collected females flew > 5 min at least once; only one of the 4 females reared out of logs did so. The others showed flight attempts. Of the fliers in our preliminary experiment, females showed on average more extended flights than males travelling approximately 2 km in half an hour (Table 3.3). The longest recorded flight lasted 58 min extending over 5.6 km.

Table 3.3 Distance, time and speed of flight (mean ± SE) of those *M. sutor* males and females that exhibited extended flight > 5 min on the flight mill.

	Distance (m)	Time (s)	Speed (m/s)
Males	1272 ± 348	1413 ± 253	0,92 ± 0,44
Females	2008 ± 510	1907 ± 298	1,02 ± 0,51

Conclusions: The preliminary flight mill tests gave first insight in the flight capability of *M. sutor*. The experiments will be continued in 2014 with more insects and comparing different points in the adult lifespan. These values from the laboratory are in accordance with the recapture data from the field that showed that *M. sutor* easily dispersed through the entire study plot.

Activities carried out by B6

Collaboration with B4 in 2013, with studies conducted at the INIAV labs in Portugal with automatically recording flight mills to compare the effect of body size, sex, age and nematode load on the flight performance of adult *M. galloprovincialis* (Figure 3.6). Results of the collaboration are presented by B4.



Figure 3.6 *M. galloprovincialis* adult male on automatic flight mill.

Activities carried out by B9

Continuation of the experiments to study adult development from emergence through shoot feeding, initiated the previous year, was carried out in 2013

Experiment1: Fat content

Fat content of 140 *M. galloprovincialis* adults (80 ♀♀ and 60 ♂♂) was measured after 0, 4, 8, 14, 18 and >18 days of feeding from emergence.

Results: Data analysis is still preliminary, but freshly emerged adults were found to have a highest fat content , followed by some reduction to reach stability during shoot feeding. Fat content of emerged adults was higher and accounted for 15-18% of dry weight and was soon reduced to about 11%. Feeding did not increase the fat content of adults afterwards (Figure 3.7).

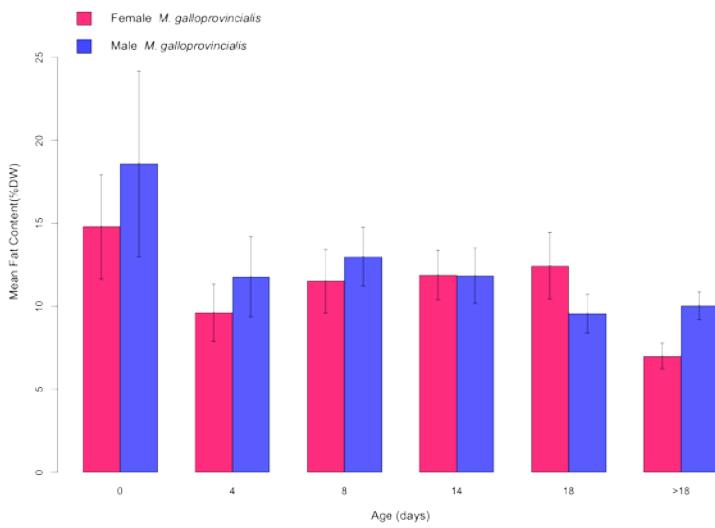


Figure 3.7 Fat content of *M. galloprovincialis* adults from emergence through shoot feeding.

Experiment 2: Flight muscle content

Eighty three *M. galloprovincialis* adults (43 ♀♀ and 40 ♂♂) were dissected and measured for flight muscle content after 0, 4, 8, 14, 18 and 40 days of feeding from emergence.

Results:

Though still preliminary, wing muscle of freshly emerged adults was about 16% of thorax dry weight and it steadily increased through shoot feeding

Experiment 3: Flight muscle content of unfed flight exercised adults

Ten *M. galloprovincialis* unfed adults (5 ♀♀ and 5 ♂♂) were exercised for various flying distances in a wind mill over 7 days (Figure 3.8). Flight muscle content was determined afterwards.

Results:

Preliminary results, but very few immature adults succeeded to undertake flight.



Figure 3.8 Flight exercising of unfed *M. galloprovincialis* adults in the flight mill.

Experiment 4: 3D scan imaging of flight muscle and reproductive organs

Six *M. galloprovincialis* adults (3♀♀ and 3♂♂), either freshly emerged, 8 or 14 days fed, are being scanned in a 3-D scanner, in cooperation with researchers of the U. of Granada (Spain), to record and measure development of flight muscle and reproductive organs.

Results: experiment is still underway, and will be completed in 2014.

DELIVERABLES Task 3.2 - Life time audit of vector flight behaviour and physiology

Results from these experiments, together with those from previous years will allow achieving Deliverable 1 "Vector flight capacity related to physiology".

3.3 Assessment of flight capacity in nature (Delivery date: 45 months).

There are several approaches to estimate dispersal of animals, including the mark and recapture of released individuals, which is frequently used for insect population studies. The efficiency of this method is dependent on the effectiveness of the recapture method, and systems based on effective traps and attractants are usually suitable. The kairomone-pheromone attractant recently developed for *M. galloprovincialis* and other potential attractants as developed in WP4, will represent convenient tools to assess flight distance for this species.
B3,
B4, B6 and B9 in collaboration.

3.3.1. Mark-release-recapture experiments within and between forests.

Dispersal of *Monochamus* adults will be tested under different forest conditions: contiguous forest at commercial stocking densities, open forest/woodland with low density, scattered host trees, mixed forest of host and non-host tree species, open field (with no host trees). Freshly emerged *Monochamus* spp. adults will be obtained from field collected colonized host material or from laboratory mass rearing. They will be kept without feeding and tagged individually, measured and weighed then field released in the centre of concentric circles of baited traps set at increasing distances. Released beetles will be captured by multiple funnel or cross-vane traps baited with kairomone-pheromone attractants, geo-referenced and weighed again. The adults will then be re-released from traps. Adults found dead in the traps and a proportion of the living captured adults will be analysed for determination of muscle development and physiological parameters. In areas where PWD disease is present, some of the live captured adults will be dissected to determine nematode load and others tested for their ability to transmit nematodes to host plants. Models will be applied to analyse natural dispersal (e.g., diffusion models). Ratios of marked/wild captured specimens will also provide data on population density (assessed using appropriate mark-capture models) and on rates at which beetle populations can be affected by the trapping system (connection to WP3.4)

In another experiment, marked insects will be released at the entrance of a small cross-section (about 50cm height and width) tunnel-like greenhouse structure (about one kilometre length). At regular distances from the entrance, fresh pine branches and logs will be placed to provide flight stimuli and assess their effects on adult flight interruption. Both nematode-free beetles and beetles with the PWN *B. xylophilus* will be used, and their flight distance evaluated and compared.

Additionally, to investigate the dispersal of the wilt disease in the new nematode focus in the centre of Portugal, 50m wide transects of about 7,000m length will be made to locate symptomatic pine trees. Wood samples will be collected to confirm the infestations, and traps will be placed to collect dispersing insects. B6 and B9 to carry out, with input on other *Monochamus* spp by B3.

Years 1 and 2: Mark-release-recapture experiments in the field;

Year 2: Study of insect movement in a tunnel-like greenhouse structure in the field;

Years 1 to 3: Implementation of transects to study the dispersal of the PWN in the centre of Portugal.

Activities carried out by B3

b) Assessment of flight capacity in nature

One study plot on a mountain slope for testing lures against *M. sartor* and *sutor* was used to assess flight of beetles. Living beetles captured in the traps were marked individually and released in the centre of the plot throughout the study periods in July-August 2012 and 2013. In 2012, four *M. sartor* and five *M. sutor* were recaptured (out of 317 and 92, respectively that had been released). In 2013, six *M. sartor* and seven *M. sutor* were recaptured. In both years, beetles dispersed in all directions, uphill as well as downhill and also crossed mature spruce stands; one *M. sutor* male (2012) and one female (2103) were recaptured in the most distant trap at 380 m. Median time between release and recapture was 7 days in both years (maximum 25 days).

Activities carried out by B6

After the mark-release-recapture assay within a maritime pine stand at Comporta conducted in 2013 and presented in the previous report, identical experiment is planned for 2014 but this time in a pine-free area, in a location with cork-oak (*Quercus suber*) and agricultural plantations at Samora Correia County, southern Portugal (Figure 3.9). Closest pine stand is over 4 km east from centre of presented image. Left of image is the river Tagus.



Figure 3.9 Approximate location of dispersal assay in a terrain without pines.

Natural infested wood with *M. galloprovincialis* has already been taken from Comporta to INIAV laboratory at Oeiras, and the removal of last instar larvae and obtaining of nematode-free insects to mark and release has begun and will be completed by the end of May.

Log-traps will be placed at different distances to recapture immature and mature beetles released on the centre of the plot, while in the neighbouring pine stands multifunnel traps with Galloprotect 2D Plus will be installed to recover long-distance flyers.

Activities carried out by B9

2013-2014

Analysis of dispersal experiments previous to REPHRAME, carried out in 2009 and 2010, based on mark-release and recapture of adults was performed. It has led to a Dispersal model and the estimation of the Effective Sampling Area, that was used for mass trapping studies (see work carried out within WP4). A paper on this is almost ready and is intended to be soon sent for publishing.

These findings will be used as a basis for the analysis of the experiments carried out in 2011 in Spain and Portugal on the dispersal of mature and immature adults within a forest stand.

Experiment 1:

After the successful experiment of the past year, a second experiment was carried out to determine flight dispersal capacity of emerged, unfed, adults in open areas deprived of any tree hosts, this time for checking longer flight distances.

The experiment was similar to the previous year. The experimental site was the same, an area of agricultural crops, with no pines, except for two small (ca 2 ha each) 50 year old *P. pinaster* stands, 150 m apart. A grid of 26 traps (Teflon-coated multifunnel, ECONEX, Spain) baited with Galloprotect Pack (SEDQ, Spain) was set within the stands to recapture the released beetles that were able to reach the stand.

Forty freshly emerged, unfed beetles were released downwind at each of 1000m, 1500m, 2000m, 2500m, and 3000m distance to the closest pine trees in the trapping stands. As before, we also released mature insects (14 days old), 20 for each distance, for checking and comparison. In total, 200 immature and 100 mature beetles were released from June 20th to October 17th. Sampling was performed twice weekly.

Results

Results were in agreement with those found in 2012 (distances at 1000 and 1500m were also tested in 2012). Pooled together, data from both experiments showed that freshly emerged adults were able to disperse at least over 2000 m without any feeding. No significant differences were found between mature and immature insects (Figure 3.10).

These results, together with those from physiological studies clearly point to the ability of recently emerged *M. galloprovincialis* to disperse over quite long non-forested distances to find a host for feeding.

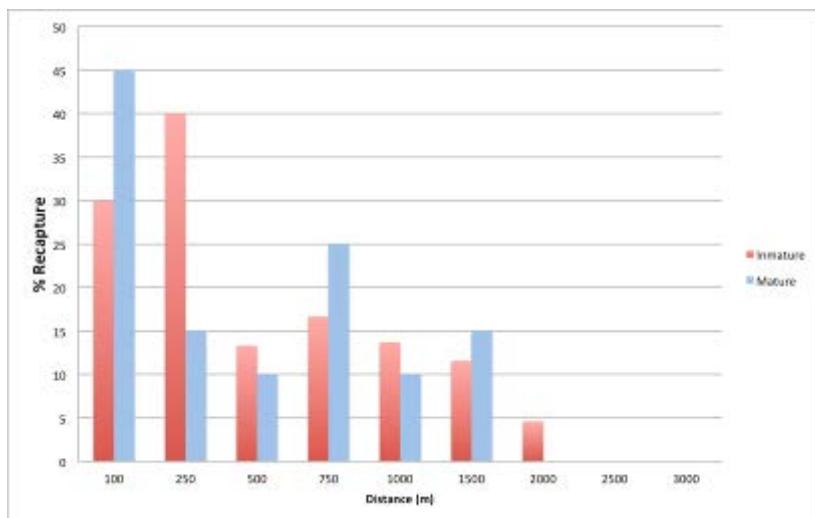


Figure 3.10 Distances of recapture on mature and immature beetles in areas deprived of hosts (2012 and 2013 experiments pooled)

Task 3.3.2. - Monitoring of flight behaviour with remote detection techniques

Activities carried out by B3

Nothing to report

Activities carried out by B6

Nothing to report

Activities carried out by B9

After unsuccessful tests to use radio telemetry, due to excessive weight of the lightest radio transmitter available during 2012, attention was focused on the possibilities of using harmonic radar techniques. Unsuccessful attempts were made to obtain (borrowing, renting) a RECCO harmonic radar location finder. It was considered that cost of acquiring such equipment was too high balanced against the likelihood of being successful in recording dispersal at long distances within the forest, upon reports already published on other insect species.

No further attempts to pursue this task were made.

DELIVERABLES Task 3.3 - Assessment of flight capacity in nature

These experiments, together with those from previous years and from other beneficiaries, represent a remarkable progress towards Deliverable 3 “Vector dispersal related to forest conditions”.

3.4 Development of molecular techniques to assess vector dispersal and dispersal routes. (Delivery date: 45 months).

Studies were done in parallel by B4, 6 and 9.

Adult dispersal is a critical life-history trait of any insect pest, affecting its survival, gene flow and its spread of associated pathogens. In combination with direct or indirect observational approaches described previously, population genetic studies offer an alternative and powerful approach for examining dispersal patterns over geographic and temporal scales.

Our objective was to examine population genetic structure of *Monochamus galloprovincialis* over fine and large geographic scales, to determine the level of gene flow that occurs at various spatial scales and in different environments, in order to define the barriers to vector dispersal. We used different approaches using two independent molecular markers, i.e. microsatellites (B4, in the frame of the PhD thesis of Julien Haran, University and INRA Orléans) and mitochondrial DNA (B6).

3.4.1. Analysis of vector dispersal in the field by estimation of ongoing gene flow.

Dispersal of the vector depends on both its population density and on host availability. While *Monochamus* spp. are generally rare in healthy forests, their population densities and ranges have greatly expanded in infested areas. Highly polymorphic genetic markers have proved to be effective in studying the genetic structure of populations and in detecting movement of individuals within expanding and dispersing populations.

In order to conduct a fine-scale genetic analysis of the vector dispersal, the study will be developed in areas of 30 km-radius in PWN-infested zones in the Iberian Peninsula, and non-infested zones in Portugal, Spain and southern France, using 2 areas per country. Adult beetles will be captured in baited traps and genetically compared with large systematic grid sampling of larvae carried out in the selected study areas. B4 to coordinate with input from the other Beneficiaries contributing to this WP.

Year 1: Development of microsatellite DNA markers (18 polymorphic loci) and first collections of adult/larvae (10 sampling areas, 10 sites per area, 5 individuals per site)

The objective of this task was to conduct a fine-scale genetic analysis of the vector dispersal using highly polymorphic molecular markers (microsatellites), which have proved to be effective in detecting movement of individuals within expanding and dispersing populations.

Activities carried out by B4

3.4.1.1 - Development of microsatellite DNA markers (B4)¹:

The development of microsatellite libraries was performed through a 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. A number of 386 loci were identified and 48 were selected for specificity and polymorphism assessment. Among the 48 loci, 22 were validated as specific at one locus. Polymorphism was assessed on 18 loci and 12 were found to be polymorphic for 14 individuals tested from various points of the vector distribution.

The genetic parameters are presented in Table 3.4. We observed a moderate allelic richness over all loci. The number of alleles per locus ranked from 2 to 8, with an average of 3,75. No significant linkage disequilibrium was detected in this set of loci after FDR correction. An excess of homozygotes was found at locus Mon17, Mon27, Mon30 and Mon35, suggesting the presence of null alleles for those markers in the population studied. Cross priming assessment gave positive results for the 4 species tested (Table 3.5). At least 5 loci were fully or partially amplified for each species. *M. sutor* gave the best results, with 9 of the 12 loci amplified. The loci Mon 31, 36, 8 and 17 were fully or partially amplified for all the species.

Table 3.4 Details of loci characteristics and basic genetic parameters for one population (Orléans, France, n=30), including the multiplexing of primers "Multiplex" and relative amount of each primer "Amount". (*) = potential presence of null alleles at this locus for this population.

Loc us	Primers sequence 5' -> 3'	Multiplex	Amount (pmol)	Mot if	Size range	N a	Ho	He	Fis
Mon 01	F: TTCACGCACATCATTCTT TG R:TCAAGCAGGAAACGAA AAGC	1	6	(aac)5	122-146	2	55	51	94
Mon 08	F: TGGTGTCTGTAGAACGCT TCA R:GCTTATTAGCTCTCATC AGTATGCTC	3	2,5	(tatc)5	192-196	2	74	71	38
Mon 17*	F: TAGTTTACTGGGCCAA TG R:GAACTCATGAACGGATA TAAATGAA	3	4	(gt)6	149-153	3	86	87	13
Mon 23	F: ATTATTCAAATTGCCAA TACTACA R:GTGTAAGGTGGAAGTG TCAAAGC	1	5	(ca)7	142-144	2	79	07	34
Mon 27*	F: ACAATCTTTCGATACC GTTGA R:TTTGCTACAAAGATGTTCTAAA	3	5	(tg)7	118-124	4	67	17	77

¹ J. Haran, F. Koutroumpa, E. Magnoux, A. Roques, G. Roux-morabito. Ghost mtDNA haplotypes generated by cryptic NUMTs can deeply disturb infraspecific genetic diversity and phylogeographic pattern. Submitted to Mol. Ecol. Res. (March 2014)

Loc us	Primers sequence 5' -> 3'	Multip lex	Amount (pmol)	Mot if	Size range	N a	H o	H e	Fis
	AGT								
	F:								
Mon 30*	TTCTAGTTGCCTGTATC	1	4	(ag) 8	236-248	4	0,1 33	0,3 40	0,6 08
	CCG								
	R:AAAGCGGGTGTGAAGT								
	ACCA								
	F:								
Mon 31	GTAAGAGAACCCAACCAC	2	1,6	(ag) 8	146-150	3	0,5 86	0,4 90	0,1 97
	CG								
	R:TTATCCTCACCGGACCG								
	TTA								
	F:								
Mon 35*	TTTCATCTGACTTAATTTC	2	18	(tc) 8	110-126	8	0,5 19	0,7 84	0,3 39
	CTTTCTCA								
	R:AGGGACGTGCAGATTAGGAA								
	F:								
Mon 36	ACGGTGAGAACTAAAGT	2	2	(tg) 8	196-200	3	0,4 48	0,4 37	0,0 26
	TAGCC								
	R:GACTCGGACGGAGCTT								
	CT								
	F:								
Mon 41	ACGGTAGCGCAATCTTGA	3	2	(ct) 9	116-120	3	0,2 00	0,2 09	0,0 45
	GT								
	R:ACGTGAGCAGTCCTGTT								
	GC								
	F:								
Mon 42	CCTTATTTAGGTCAAGAAT	1	4	(gt) 9	138-146	5	0,5 86	0,6 27	0,0 65
	TCGC								
	R:GCGTGTCAATTTCGAA								
	GGAC								
	F:								
Mon 44	AACCTGGACCTAGCTCGG	2	1,6	(act) 10	98-113	6	0,5 52	0,6 67	0,1 73
	AA								
	R:								
	TAGGAGAAGGTGGAGCA								
	GGA								

Table 3.5 Transferability of the 12 loci developed for *M. galloprovincialis*. Values indicate the rate of positive amplification ; “_”= no amplification.

Species	Individuals tested	Mon 1	Mon 23	Mon 30	Mon 42	Mon 31	Mon 35	Mon 36	Mon 44	Mon 8	Mon 17	Mon 27	Mon 41
<i>M. sutor</i>	26	_	100	11	_	100	23	100	_	42	88	19	11
<i>M. sartor</i>	4	_	_	75	_	100	_	100	100	100	100	_	_
<i>M. rosenmuelleri</i>	2	_	_	100	100	100	_	100	100	100	100	100	_
<i>M. alternatus</i>	2	_	_	100	_	100	_	50	_	100	100	100	_

3.4.1.2 - *Monochamus* sampling (B4, B6, B9)

Partners 4, 6 and 9 provided samples for this study.

Intensive *Monochamus* trapping was performed in 2011, 2012 and 2013 in different forests, not only where the nematode was detected but also in areas potentially at risk:

- Forest plots in PWN-infested and non-infested zones in Portugal (partners 6 & 4)
- Forest plots in PWN-infested and non-infested zones in Spain (partners 9 & 4)
- Forest plots in non-infested zone in southern France, with a focus on altitudinal/latitudinal zonation (the Pyrenean barrier, but also altitudinal transect in the Alps and Massif Central) (partner 4)

A total of 2.686 *Monochamus* adults were captured in multi-funnel traps baited with attractant in 43 sites in Portugal (1 to 4 individuals per site,) and 135 sites in Spain (1 to >10 individuals per site) gathered in infested and non-infested areas. Different altitudinal transects were performed in the Pyrenees, Massif Central and the Alps.

3.4.1.3 - Definition of environmental barriers to gene flow :

A- Role of the Pyrenean relief (B4)

Objective: evaluate the effect of altitudinal barriers to the dispersal of the vector.

In order to respond to the objectives of this sub-task, sampling was carried out during the summer 2013 by Julien Haran (PhD student at INRA Orléans – B4), with a particular focus on collecting *Monochamus* samples in infested and non-infested areas (especially for populations with less than 10 individuals in Portugal) as well as in areas potentially at risk (the Pyrenean barrier). Specimens were captured at 24 sampling sites using baited multi-funnel traps. A total of 3 traps was set up at each site from early June to the end of October and emptied every 3 weeks and specimens were stored in 99.5° ethanol at 4°C. Sampling sites were spread as follows: 11 on the French side of the Pyrenees and 13 on the Spanish side, forming 4 North-South transects (Table 3.6 and Figure 3.11). Transects were placed along the main road axes crossing the Pyrenees, corresponding to the main pathway, where altitude is more likely to allow migration of *M. galloprovincialis*. Additional sampling sites from the Spanish side of the Basque country were obtained from previous trapping performed in 2012 by B9.

Results: Despite intensive trapping, *M. galloprovincialis* was difficult to capture in elevated sampling sites. This was the case for the 3 localities above 1400 m (Font-Romeu, col la Pierre Saint Martin, Canfranc), without apparent effect of the pine species sampled. This species was not recorded in Vielha (Spain, *P. sylvestris*) where only *M. sutor* was captured. *M. sutor* was present in the traps in all localities above 800 m, although some specimens were trapped in lower localities on the Northern side of the chain (Prades, 410 m).

Table 3.6 Site characteristics for the transects across the Pyrenees.

	Population	Size	Long.	Lat.	Altitude	Dominant <i>Pinus</i> species
1	Tossa de Mar	15	2,93	41,72	21	
2	Castellbell	30	1,84	41,62	190	<i>P. halepensis</i>
3	Olvan	18	1,88	42,05	520	<i>P. pinaster/nigra</i>
4	Baga	18	1,86	42,24	794	<i>P. sylvestris</i>
5	La Pobla de Segur	18	0,94	42,25	646	<i>P. halepensis</i>
6	Castillonroy	10	0,52	41,88	402	

	Population	Size	Long.	Lat.	Altitude	Dominant <i>Pinus</i> species
7	Camarassa	6	0,84	41,85	271	<i>P. halepensis</i>
8	Torla	1	-0,11	42,62	1197	
		18				<i>P. halepensis/sylvestris</i>
9	Huesca Alep		-0,72	42,29	552	
10	HuescaPignon	7	-0,63	42,14	419	<i>P. pinea</i>
11	Jaca	18	-0,64	42,54	870	<i>P. sylvestris</i>
12	Canfranc	17	-0,52	42,71	1502	<i>P. nigra</i>
13	Col St Martin	10	-0,79	42,96	1590	<i>P. a crochets</i>
14	Falces	15	-1,79	42,38	360	
15	Beriain	10	-1,67	42,73	613	
16	Marcalain	10	-1,69	42,89	550	
17	Iruzun	1	-1,83	42,92	553	
18	Lekunberri	2	-1,90	43,00	507	
19	Ona	9	-3,44	42,77	1055	
20	espejo	3	-3,04	42,81	715	
21	Vitoria	7	-2,57	43,01	760	<i>P. nigra</i>
22	Aramayona	5	-2,56	43,05	627	
	Villanueva de Mena	5	-3,31	43,07	452	
23	Itziar	14	-2,32	43,28	200	<i>P. nigra</i>
24	Irun	18	-1,91	43,33	23	<i>P. pinaster</i>
25	Etchalar	12	-1,63	43,22	273	<i>P. radiata</i>
26	Sare	18	-1,60	43,26	303	<i>P. nigra</i>
27	St Vincent Tyrosse	18	-1,31	43,67	32	<i>P. pinaster</i>
28	Orthez	18	-0,56	43,43	180	<i>P. nigra</i>
29	Oloron	16	-0,61	43,15	302	<i>P. strobus</i>
30	Vieuzos	28	0,45	43,24	449	<i>P. nigra laricio</i>
31	Garros	18	0,50	43,09	560	<i>P. nigra laricio</i>
32	Font-Romeu	18	2,14	42,50	1432	<i>P. sylvestris</i>
33	Prades	17	2,40	42,61	410	<i>P. halepensis</i>
34	Font froide	18	2,89	43,09	235	<i>P. pinaster</i>

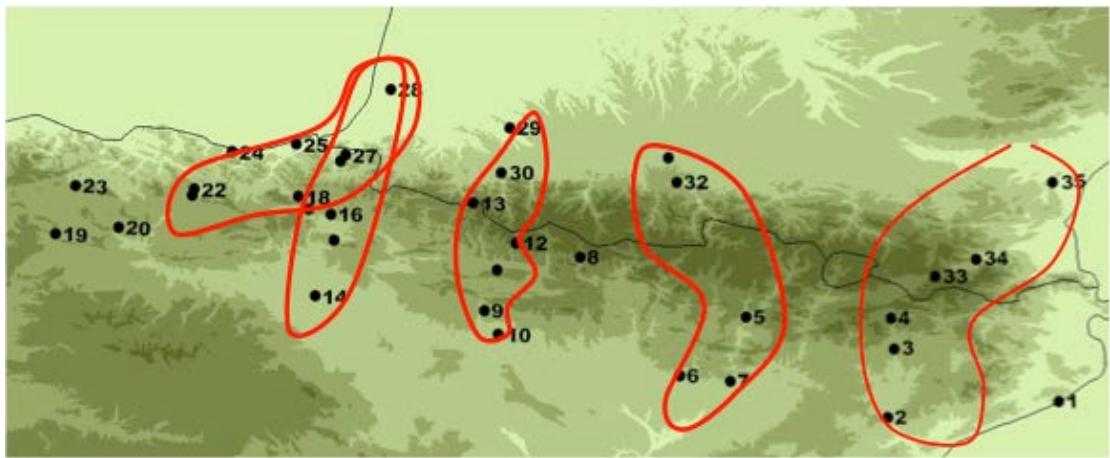


Figure 3.11 Locations and site numbers for transects across the Pyrenees

The genotyping of 26 populations (466 individuals of *M. galloprovincialis*) was performed for each of the 12 polymorphic loci defined. F statistics revealed that populations of *M. galloprovincialis* are genetically divergent, especially when considering populations trapped from each part of the mountain along the third and fourth transects, which display the highest Fst values (0.1437 and 0.1716). Conversely, populations collected on the same part of the relief, or from traps along the west part of the Pyrenees, display the lowest Fst values (Fst ranging from 0 to 0.09), suggesting gene flow between populations. The genetic clustering of populations was computed using the Bayesian approach provided by the software STRUCTURE V.2.3.4. The genotypes were analysed using 10 repeats of a 100000 burn-in period followed by 500000 replicates of Markov Chain Monte Carlo (MCMC), for K values from 1 to 10. Figure 3.12 shows the outcomes of the best assignment with Structure for K=2, genetic admixtures were present in populations 14, 15, 16, 17, 21, 22 (also shown on Figure 3.13). Spatial output of genetic clusters of *M. galloprovincialis* is shown on Figure 3.14 for K=2 under GENLAND. Black lines represent the clines of posterior probabilities to belong to the Spanish cluster. White to red shading indicates a high to low posterior probability of membership.

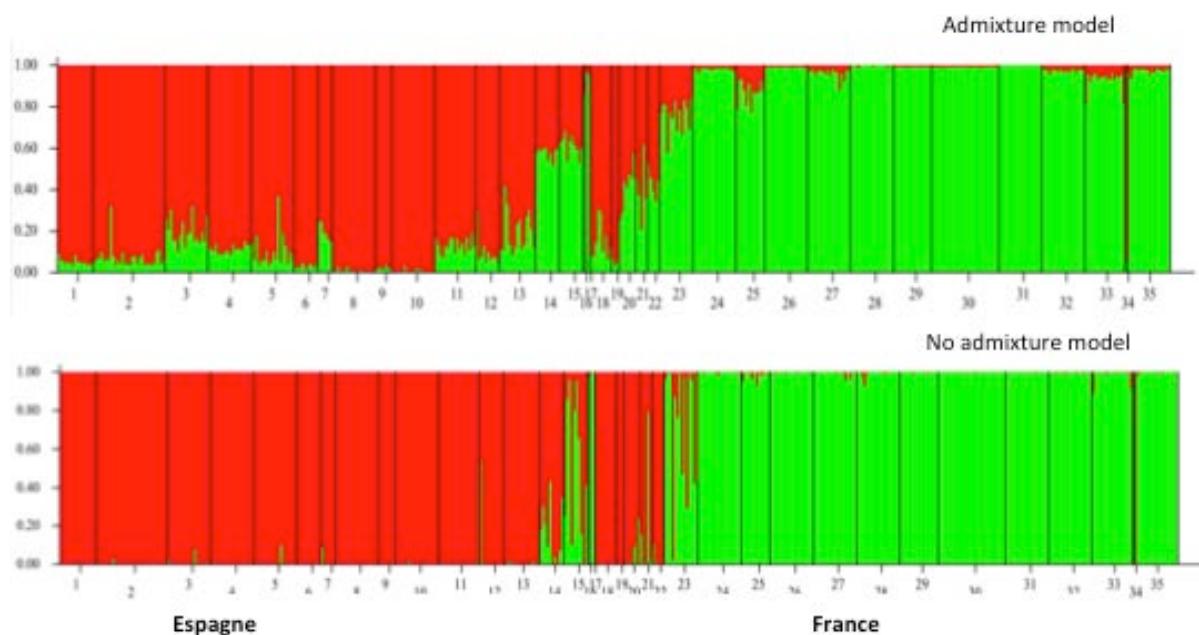


Figure 3.12 Genetic divergence in the 35 *M. galloprovincialis* populations as illustrated by STRUCTURE. Each vertical line represents an individual, and each colour represents a cluster in the histograms of STRUCTURE

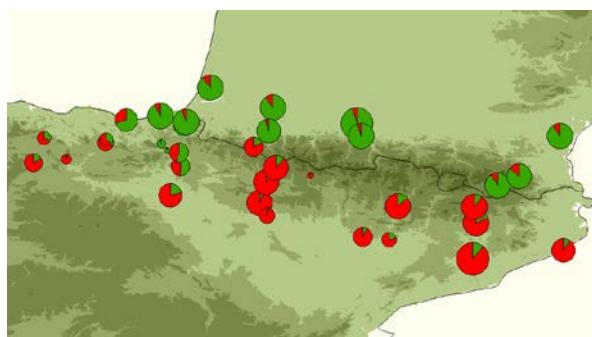


Figure 3.13 Distribution of the genetic divergence obtained by STRUCTURE, for K=2

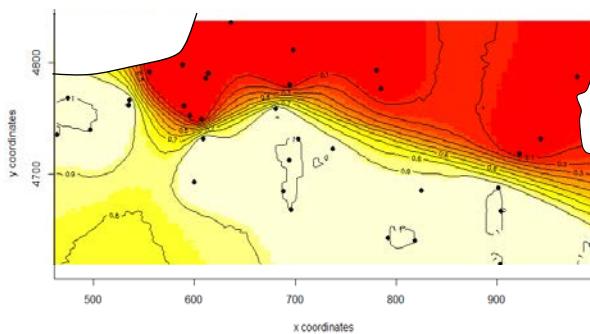


Figure 3.14 Map of posterior probability to belong to Spanish cluster, using GENLAND for K=2.

Modelling the dispersal of the vector *M. galloprovincialis* across the Pyrenees Mountains in relation to the genetic structure of its populations was started during months 19-36 (see analysis conducted for D7.5 [PART 2] by B4). The aim of this

approach is to compare the dispersal pattern simulated under various scenarios with the dispersal pattern shown by the genetic analysis.

Work to be done:

A sampling effort is planned in summer 2014, to complete our sampling along the east side of the Pyrenees, in order to evaluate natural pathways of the vector and to characterise the effect of elevation on the genetic structure of *M. galloprovincialis*.

B- Fine scale genetic structure in Iberian Peninsula (B4, B6, B9) (ongoing task)

Objectives: estimate the effect of landscape heterogeneity on dispersal patterns of the vector and evaluate the consequences of PWN infestation on *M. galloprovincialis* population genetic structure.

B4 carried out the genotyping for the 12 loci selected for a total of 1.022 individuals, corresponding to 130 populations sampled with the collaboration of B6 and B9.

Mean allelic richness varied between populations (Figure 3.15 and Figure 3.16), maximum allelic richness was observed in central Spain, whereas the lowest values were observed along the western coast of the Iberian Peninsula.

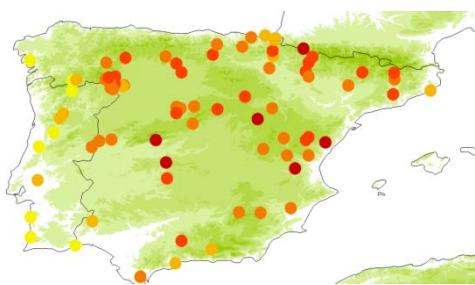
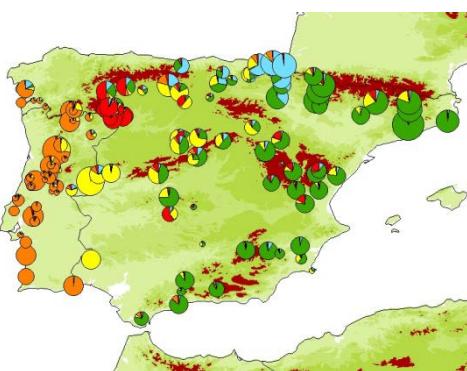


Figure 3.15 Mean allelic richness among *M. galloprovincialis* Iberian populations
low ● ● ● ●



Activities carried out by B6

B6 collaborated in the collection of *M. galloprovincialis* larvae and adults from different locations in continental Portugal and Madeira Island, for molecular analysis coordinated by B4 (see description in “Activities carried out by B4”). Grid sampling covered both PWN-infested locations and buffer and non-infested zones throughout the pine distribution area in Portugal.

In 2013, beetles were collected from 3 new locations from southern Portugal, contrasting in the degree of pine wilt disease expression:

- Sines (location with PWD)
- Odeceixe (boundary location of PWD)
- Monte Gordo (location without PWD)

Adult insects were captured in multi-funnel traps baited with attractants, and stored in alcohol. Larvae were hand-collected from dead maritime pine trees.

3.4.2. Analysis of dispersal routes by molecular determination of population structure and variation

Historical events and movements of *M. galloprovincialis* through a phylo-geographic study would provide effective information on the future expansion of the range of the nematode in Europe. Different populations and geographic sub-sets of *M. galloprovincialis* in Europe will be studied using mitochondrial genes and microsatellite loci. Both intensive (in Iberian peninsula) and extensive (rest of Europe) studies will be conducted, using traps baited with pheromone-kairomone attractants: Intensively, specimens will be collected from 70 sites selected in a 10x10 Km grid; Extensively, adults from 40 populations from a 100x100 Km grid of 10 European countries will be sampled. Sequencing of the target mitochondrial genes and genotyping of microsatellites will be performed by standard procedures. B4 to coordinate with input from other Beneficiaries in this WP.

Year 1: Collection of adults through the study area. mtDNA extraction and sequencing 10 individuals per population). Performance of phylo-geographic study and definition of the genetic variation of populations and their geographic variation.

Year 2: Collection of adults in miss-sampled populations for mtDNA and collection in specific geographic areas, defining population variation and gene flow using microsatellites (12 loci, 30 individuals per population). Definition of putative barriers to individual dispersal.

Year 3: Collection of adults and validation of results by collecting adults in random selected areas.

Activities carried out by B4

Objective: Uncovering historical dispersion patterns through phylogeographic study, in order to provide effective information on dispersal patterns and capacities of the insect vectors, which represent a key to define effective management strategies.

This phylogeographic study will enable reconstruction of the evolutionary history of this species, to define historical events and movements of *M. galloprovincialis*, its potential glacial refugia and recolonisation pathway. This study aims to characterize the occurrence of different lineages within the species. Furthermore, population genetics, especially microsatellites, have been shown to elucidate long distance dispersal or human-mediated transport.

Different populations and geographic sub-sets of *M. galloprovincialis* in Europe were studied using mitochondrial genes (B9) and microsatellite loci (B4). Sampling has been performed by B4, B6, B9 and from other beneficiaries in the project (Table 3.7).

Table 3.7 Geographic sources of populations of *M. galloprovincialis* in Europe.

Nb	Country	locality	N	X	Y	Dominant Pinus species (when available)	collector
1	Portugal	Madeira island	14	-16.9	32.75	<i>P. pinaster</i>	L. Bonifacio
2	Portugal	Santo Andre	18	-8.77	38.02	<i>P. pinaster</i>	E. Sousa
3	Portugal	Castro daire	24	-7.92	40.92	<i>P. pinaster</i>	J. Haran
4	Spain	Ourense	24	-7.54	42.01	<i>P. pinaster</i>	J. Haran
5	Portugal	Vale feitoso	24	-6.98	40.07	_	P. Naves
6	Spain	Zamora	30	-6.31	41.92	<i>P. pinaster</i>	J. Pajares
7	Spain	Segovia	30	-4.51	41.07	_	J. Pajares
8	Spain	Murcia	24	-1.16	38.28	<i>P. halepensis</i>	J. Pajares
9	Spain	Cuenca	28	-1.96	40.01	<i>P. nigra/pinaster</i>	J. Pajares
10	Spain	Malaga	27	-4.36	37.32	_	J. Pajares
11	Spain	Castellbell	30	1.84	41.62	<i>P. halepensis</i>	J. Pajares
12	Spain	Huesca	25	-0.72	42.29	<i>P. halepensis</i>	J. Pajares
13	Spain	Villalba de Guardo	25	-4.82	42.72	_	J. Pajares
14	Spain	Mallorca	20	3.23	39.70	_	J. Pajares
15	France	Conca	31	9.33	41.73	<i>pinea/halepensis</i>	A. Roques
17	France	Sare	30	-1.60	43.26	<i>P. nigra</i>	J. Haran
18	France	Vieuozos	28	0.45	43.24	<i>P. nigra</i>	A. Bernard
19	France	Marcheprime	30	-0.85	44.67	<i>P. pinaster</i>	H. Jactel
20	France	Vayrières	27	1.34	44.61	<i>P. sylvestris</i>	A. Roques
21	France	Orleans	30	1.91	47.83	<i>P. sylvestris</i>	G. Roux
22	France	Hagueunau	24	7.79	48.81	_	A. M. Chappe
23	France	St Beauzely	17	3.01	44.18	<i>P. nigra/sylvestris</i>	A. Roques
24	France	Gignac	30	3.54	43.64	<i>P. halepensis</i>	A. Roques
25	France	Meyrargues	30	5.52	43.63	<i>P. halepensis</i>	J. Hintzy
26	France	Villar-St-Pancrasse	22	6.62	44.87	_	A. Roques
27	Italy	Tregmago	24	11.16	45.52	_	Andrea Battisti
28	Italy	Monfalcone	30	13.53	45.80	_	Andrea Battisti
29	Austria	Baden	21	16.23	47.99	_	G. Hoch
30	Sweden	Gotska Sandön	29	19.19	58.39	_	M. Schröder
31	Sweden	Grasö	17	18.46	60.37	_	M. Schröder
32	Poland	_	20	19.15	51.91	_	J. Pajares
33	Croatia	Zadar	15	15.19	44.18	_	N. Lackovic
35	Bulgaria	Yakoruda	24	23.65	42	_	D. Doychev
36	Serbia	_	30	20.34	44.11	_	M. Glavendekic
37	Macedonia	Bitola	24	21.32	41.01	<i>P. peuce</i>	I. Papazova
38	Bosnia	_	18	18.34	43.88	_	M. Dautbasic
39	Italy	Palermo	29	13.36	38.11	_	A. Battisti
40	Tunisia	_	23	9.52	37.25	_	M.L. Ben Jamaa
41	France	Rennes	24	-1.71	48.12	_	A. M. Chappe
42	France	Dole	26	5.49	47.08	_	A. M. Chappe

A total of 38 populations (24-30 individuals per population), were genotyped for the 12 loci previously defined.

The study is still under analysis but preliminary results (Figure 3.17) showed that the highest mean allelic richness was observed for populations localized in central Spain, and to a lesser extent, in eastern European countries, suggesting the existence of potential glacial refugia for this species in these areas (but additional populations in Balkans are necessary to confirm this).

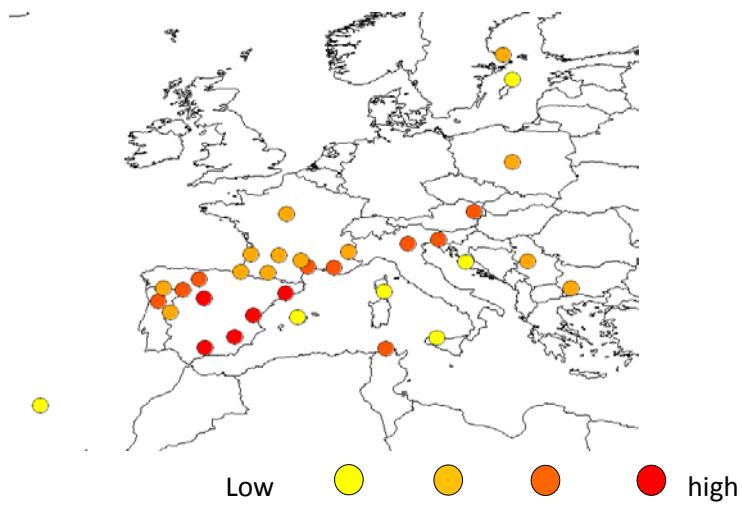


Figure 3.17 Mean allelic richness among *M. galloprovincialis* European populations

F statistics revealed that *M. galloprovincialis* populations are genetically divergent ($F_{ST} = 0.2$ to 0.5 for populations from different countries). A Mantel test revealed a significant correlation between genetic distances and geographic distances, indicating the effects of Isolation By Distance (IBD) on population divergence. Bayesian assignment with STRUCTURE indicated that $K=2$ is the best for describing the genetic structure of these populations (Figure 3.18). One cluster included all populations from Iberian Peninsula plus Corsica, and the remaining populations, mainly localized in more northern areas, grouped into the second cluster. Nevertheless, geographical structuration obtained for $K=6$ (data not shown) indicated that four main lineages occurred in Eastern and Western part of the Iberian Peninsula, in Central and Eastern Europe.

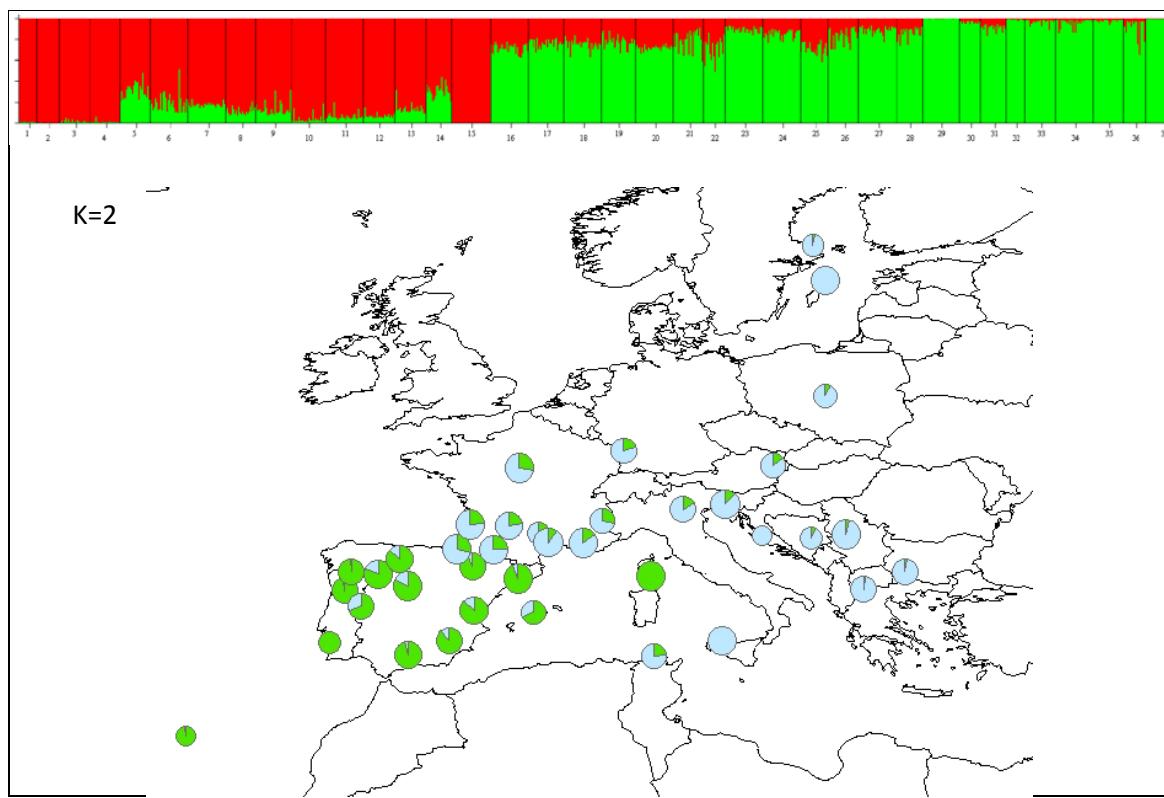


Figure 3.18 Genetic divergence in the 38 *M. galloprovincialis* populations as illustrated by STRUCTURE. Each vertical line represents an individual, and each colour represents a cluster in the histograms of STRUCTURE.

Work to be done: *Monochamus* sampling should be completed, especially in north-African and Mediterranean countries in order to respond to the deliverable previously defined (40 populations) and to recover historical dispersion patterns of this species.

Activities carried out by B9

Experiment 1:

In 2011 and 2012 over 700 live adults from 127 localities were sampled all over Spain (Iberian Peninsula and Balearic Islands). In 2013 the Sampling of 4 new localities was carried out. Besides, there was the exchange of all samples with beneficiary B4, so both teams will work with same specimens and produce coherent results.

The mtDNA analysis of 499 individuals was carried out following standard protocols, and an algorithm was implemented to analyse the haplotype network of the populations.

Results

Though still preliminary until the remaining samples are analysed, results show that there was a high number of haplotypes, many of them exclusive to the Iberian Peninsula (Figure 3.19). By contrast, French haplotypes were intermediate to Iberian and Eastern European haplotypes. Some haplotypes occurred in several countries. These data suggest that a west-east pattern may exist, with strong evidence of population mixing. This needs to be confirmed after all data have been analysed.

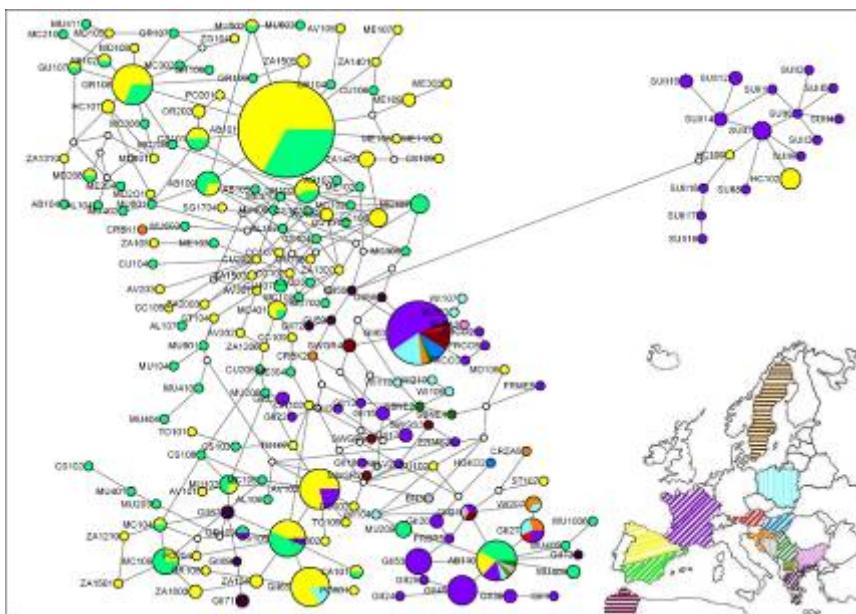


Figure 3.19 Current haplotype network obtained from the 499 samples analysed.

DELIVERABLES Task 3. 4. Development of molecular techniques to assess vector dispersal and dispersal routes

These experiments, together with those from previous years and from other beneficiaries, contribute directly to Deliverable 3 “Vector dispersal related to forest conditions, using molecular techniques”.

PUBLICATIONS

- David G, Jactel H, Piou D, Naves P & Sousa E (2013). Flight performances of *Monochamus galloprovincialis*, insect vector of the Pine Wood Nematode. In: Schroder, T. (ed.), Pine Wilt Disease Conference Book 2013, pp. 20, Braunschweig, ISSN: 1866-590X.
- Haran J. Approche multidisciplinaire de la dispersion chez un invasif : application au nématode du Pin (*Bursaphelenchus xylophilus*) et de son insecte vecteur (*Monochamus galloprovincialis*). Poster présentation. Séminaire des doctorants du département EFPA, INRA. Dourdan Novembre 2012
- Gruffudd H, Evans H, Haran J, Roux-Morabito G, Roques A, Robinet C (2013) How could climate change affect the potential spread of pine wilt disease in Europe? ClimTree 2013, Zurich (Switzerland), 2-4 Sept 2013 (oral, invited)
- Haran J., Garcia J., Bernard A., Roques A., Robinet C. & Roux- Morabito G. (2013). Etude des processus de dispersion de *Monochamus galloprovincialis*, vecteur du nématode du pin : rôle potentiel de la chaîne pyrénéenne. GEFF. Bedoin (France) 2oct. 2013.
- Haran J., Roques A., Roux- Morabito G. (2013). Assessing potential expansion of the Pine Wood nematode (*Bursaphelenchus xylophilus*) from the spatial genetic structure of the vector (*Monochamus galloprovincialis*). IUFRO 7.02.10 "Pine Wilt Disease Conference 2013" Braunschweig, Germany; 15–18 October 2013.
- Haran J., Roux- Morabito G., Roques A. (2013). Assessing potential expansion of the Pine Wood Nematode (*Bursaphelenchus xylophilus*) from the spatial genetic structure of the vector (*Monochamus galloprovincialis*): First results of a STSM stay in Portugal. PERMIT meeting, Treviso (Italy), 1-4 October 2013
- Haran J., Roques A., Roux- Morabito G. (2014). Evolutionary history and ongoing gene flow of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae), vector of

- the Pine Wood Nematode. IUFRO 7.03.14, 7.03.06, 7.03.01 Joint Meeting, 9-14 April 2014 Antalya, Turkey.
- Haran J., Roques A., Robinet C. & Roux- Morabito G. (2014). Assessing dispersal routes and ongoing gene flow of the vector of the Pine Wood Nematode, *Monochamus galloprovincialis*, at different spatial scales. XXIV IUFRO World Congress. 5-11 October 2014 Salt Lake City, United State.
- Petersen-Silva R., Naves P., Sousa E. & Pujade-Villar J. (2014). Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets. *Journal of the Entomological Research Society*, 16(1): 61-70.
- Petersen-Silva R., Naves P., Godinho P., Sousa E. & Pujade-Villar J. (2014). Distribution, hosts and parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in mainland Portugal. *Silva Lusitana, in press*.
- Petersen-Silva R., Henriques J., Inácio L., Naves P., Godinho P., Sousa E. & Pujade-Villar J. (2014). Susceptibility of larvae and adults of *Monochamus galloprovincialis* to entomopathogenic fungi under controlled conditions. Submitted to publication.

Conclusions on work done in WP3

- Good progress on Deliverable 3.1 vector flight capacity related to physiology (due m. 33). Completing of some test will be done.
- Steady progress towards vector dispersal related to forest condition (deliverable D 3.2 due m. 33). Dispersal models are well advanced. Checking models with non-used data (2011) will be carried out.
- Good progress is made towards vector dispersal related to population genetics (Deliverable 3.3 due m.45). Analysis to be concluded.
- Good data is available for establishing climate influences on vector dispersal (Deliverable 3.4 due m.33). Analysis to be concluded.

Statement on deviations from Annex I, and on failing to achieve critical objectives and/or not being on schedule:

There has not been any significant deviation from planned objectives by any beneficiary on WP3.

Statement on the use of resources

Resources have been used to the consecution of WP3 objectives by all beneficiaries.

Work Package 4: Development of new methods for monitoring and control of *Monochamus* spp and PWN based on vector trapping

Work carried out by B2, B3, B4, B5, B6 and B9

Objectives

- to optimise trapping systems for *M. galloprovincialis* to capture all life stages
- to develop traps for detecting the presence of beetles infested with PWN
- to determine whether trapping can be used to reduce populations of the vector
- to develop traps and lures for other European *Monochamus* species

Deliverables

- D 4.1: Lure for *M. galloprovincialis*. Month 33
D 4.4: Development of traps for monitoring & control. Month 12
D 4.3: Effectiveness of mass trapping for vector control. Month 45
D 4.4: Development of lures for other *Monochamus* spp. Month 45

4.1 Testing and improvement of synthetic lures for *M. galloprovincialis*

Partners involved: B2, B3, B4, B5, B6 and B9

Attractants recently discovered for *M. galloprovincialis* by Beneficiaries 9 and 2 are based on a blend of kairomone compounds from hosts and bark beetle competitors with a male-emitted aggregation pheromone that is strongly synergized by the kairomones. New variations on the kairomone-pheromone blends and dispensers will be tested against *M. galloprovincialis* in different forests in Austria, (B3), France (B4), Germany (B5), Portugal (B6), Spain (B9); and other countries as appropriate, deployed in multiple-funnel traps in a randomized block design. Captured adults will be analysed for physiological and physical parameters and for the presence of *Bursaphelenchus* spp. Overall coordination of this work will be by B9.

It is likely that the sensitivity of adult *Monochamus* spp to host or sex attractants will vary significantly over the lifetime of the adult beetles. Recently emerged *M. galloprovincialis* adults are sexually immature and they may not respond so well to the kairomone-pheromone blend that might be attractive to more mature adults. Thus development of age-specific attractants based on host pine volatiles will be investigated (B2, B3, B6 and B9), particularly in the post-emergence phase, when PWN inoculum is at its highest. Host species found to be highly preferred for maturation feeding and oviposition in previous experiments (WP3) will be selected. Host odours emitted at shoot level during the feeding phase of insect vectors will be captured by head space collection from intact plants (B5). Electrophysiological tests (GC-EAD) (B2) and olfactory bioassays in a wind-tunnel (B6) will be used to detect semiochemical cues of host selection for feeding (B2). Potential attractants will be identified and field tested by Beneficiaries as above, comparing different compounds mixtures and doses linked to the ages of the adult beetles.

Volatiles emitted from *P. sylvestris* trees with different physiological condition (healthy trees, dying from drought stress and dying because of PWN infestation) will be assessed and compared. Experiments will be carried out under quarantine conditions. Volatiles will be collected and processed by B5 and GC-MS-EAD analysis will be carried out by B2. Olfactometer bioassays to test the response of *M. galloprovincialis* will be carried out if differences between dying and PWN infested trees are shown.

Research on *M. galloprovincialis* by B9 has also shown that unidentified sex contact pheromones in the cuticles of females are present and they might further improve the power of the lure. Cuticular compounds of *M. galloprovincialis* will be analysed for sex contact pheromone determination. Cuticular compounds of males and females will be obtained by solvent extraction and by solid-phase microextraction (SPME), analysed by GC-MS and identified on the basis of mass spectra, GC retention times and comparison with synthetic standards by B2.

Candidate compounds will be tested in contact recognition and short range olfaction bioassays and then on their ability to improve catches in traps baited with the optimised kairomone/pheromone lure.

Progress since the mid-term report

Previous work within REPHRAME has shown:

- . The effectiveness of the standard lure (the aggregation pheromone plus two bark beetle kairomones; commercialized as GALLOPROTECT 2D; SEDQ, Spain).
- . The host volatile α -pinene may significantly increase attraction to the pheromone-kairomone lure (G 2D + α -pinene : G Pack; SEDQ, Spain)
- . Freshly emerged, immature, adults do not respond to these lures until they have fed for about 10 days.
- . Some smoke volatiles may also significantly increase attraction to the pheromone-kairomone lure.
- . Limonene reduced attraction to G 2D but not to G 2D + α -pinene

Based on these results, the objectives for the following period were:

- To check if smoke volatiles may replace α -pinene as synergist of G2D (advantages: similar increase of attraction, cheaper and easier to release, and more target specific).
- Test if immature adults can be attracted to host volatiles in areas deprived of hosts (absence of competitive sources; i.e. ports, warehouses)
- Test if limonene can reduce attraction to G2D when it is released together with α -pinene in proportions close to natural Stone pine ratios (i.e. 1:8)

Activities in 2013:

Lure experiments 1, 2 and 4 were carried out within a naturalized *P. halepensis* stand, located in Sierra Espuña (Murcia; SE Spain), in complete randomized designs of 7 blocks, deployed on Teflon-coated 12 funnels multifunnel traps, with extended collector for live trapping (Econex SA, Murcia, Spain) (see deliverable 4.2), and weekly sampling.

Experiment 1/13: (B2 and B9)

One experiment was carried out from June 24th to August 12th to continue evaluating the synergistic effect of smoke volatiles compared to α -pinene on the standard lure. Tested treatments were: G2D (standard lure); G2D + α (Galloprotect Pack); G2D + S1 (Smoke volatile 1: 2-methoxyphenol); G2D + S2 (Smoke volatile 2: 4-methyl-2-methoxyphenol); G2D + SB (Smoke volatile blend) and G2D + α + SB. Smoke dispensers were prepared by B2 and all the other dispensers were provided by SEDQ, (Barcelona, Spain).

Results:

Addition of smoke volatiles, or of α -pinene, did not significantly increase catches obtained by the standard lure G2D alone. However, mean catches on the lure incorporating Smoke compound S1 were 15% higher than catches in the standard lure (Figure 4.1).

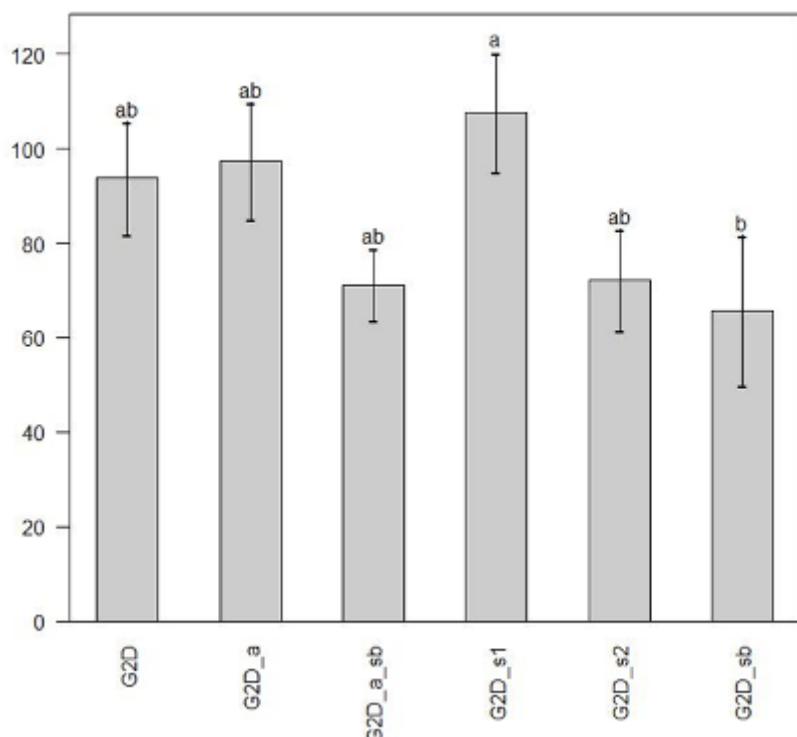


Figure 4.1 Results of experiment 1/13 on smoke volatiles in Spain (2013)

Experiment 2/13 (B2 and B9)

As results of previous experiments pointed to a synergistic effect of smoke compound 1, a second experiment was carried out from August 12th to September 22th to test two dosages of this compound (single and triple) and also to evaluate the synergistic effect of two non-previously tested compounds, a smoke volatile (Smoke volatile 3: methylphenol) and a pine terpene (terpinolene). Dispensers were provided as in experiment 1.

Results:

There were no significant differences among treatments (Figure 4.2). Thus, addition of any of the tested synergists significantly increased catches obtained by the standard lure (G2D). However, as in the previous experiment, mean catches obtained by releasing the smoke compound 1, in a single dosage, were highest, and 22 % higher than standard lure catches.

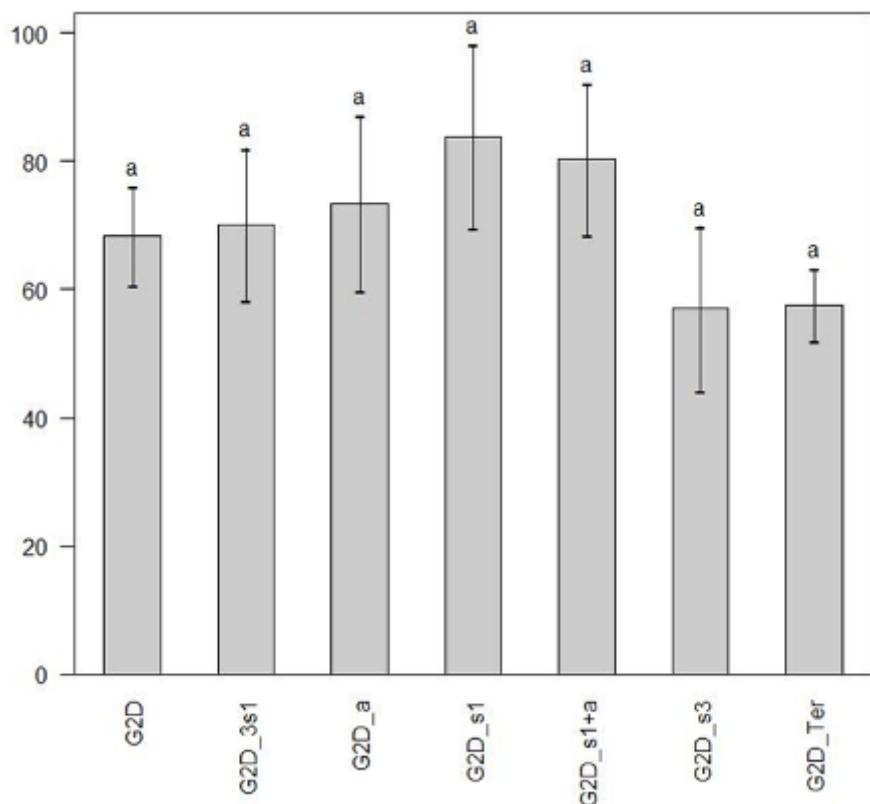


Figure 4.2 Results of the experiment 2/13 on Smoke volatiles in Spain (2013)

These two experiments, together with results gathered in 2011 and 2012, point to the fact that smoke volatile 1 could be a suitable candidate for replacing α -pinene as synergist of the standard pheromone-kairomone lure. Even though synergistic effect, compared to G2D alone, was not always significant, it was always somewhat higher than the effect obtained by α -pinene.

Experiment 3/13 (B2 and B9)

An experiment was set to test if immature, unfed, beetles can be attracted to host volatile releasing lures in areas deprived of hosts, that is, if competitive sources of attraction are absent. Thus, an assay was carried out in a non-forested area deprived of any pine trees (Cervatos de la Cueza, Palencia, Spain) from July 25th to August 9th.

One hundred and ninety (190) freshly emerged, unfed, immature beetles were released within 5 days in the centre of three concentric circles of traps, at 100, 250 and 500m from the release point. Each of these circles was composed of 4 pairs of 12 Teflon-coated multiple funnel traps (Econex, Spain) deployed in the four cardinal points (Figure 4.3). Traps within each pair were 30m apart, and one of them was baited with a mixture of host volatiles (α -pinene 500 mg/day + β -pinene 500 mg/day + 3-carene 500 mg/day, + terpinolene 500 mg/day), whereas the other was left unbaited. Sampling was performed every 3/4 days. The experiment compared the attraction of released immatures control vs. host lured traps.

Results:

No immature beetles were recaptured in any of the 24 traps

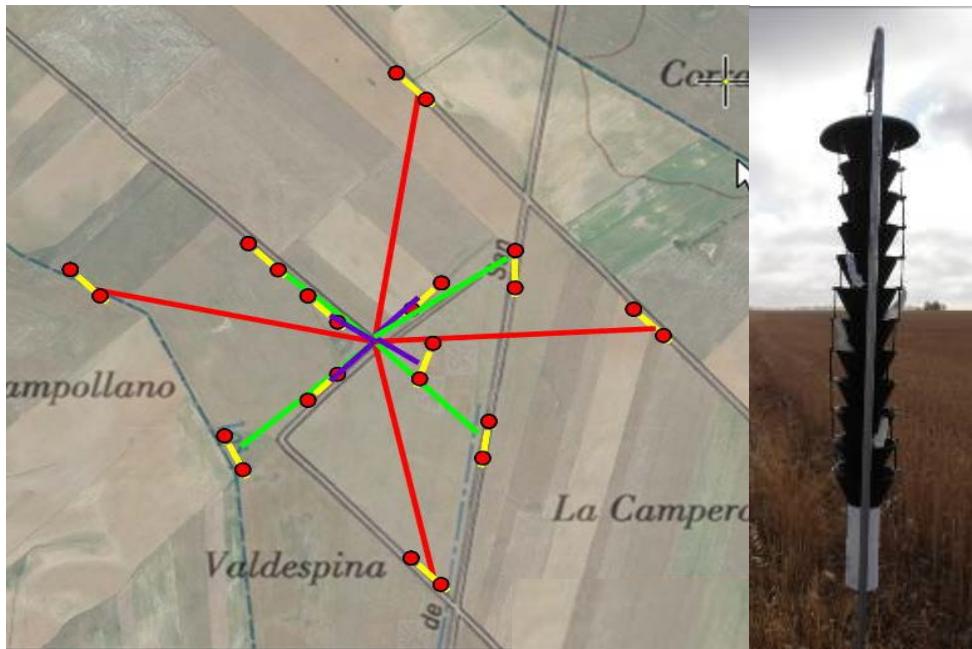


Figure 4.3 Set up in experiment 3/13 for attracting immature beetles in Spain (2013).

In previous experiments (2011), addition of several pine volatiles to the standard lure was unsuccessful in attracting immature *M. galloprovincialis* adults within a pine stand. Results now show that even a mixture of host volatiles (that were attractive as synergists to mature beetles), released at a high dosage of 2 gr/day in an area deprived of any other pine volatiles and deployed with strong visual targets, were not attractive to immature beetles.

Experiment 4/13 (B2 and B9)

A new experiment was conducted from July 24th to August 12th to test again the repellent effect of limonene. This time the aim was to determine whether limonene can reduce catches to G2D when it is released together with α -pinene, in proportions close to those naturally occurring in Stone pine (i.e. ratio pinene-limonene 1:8). Thus tested treatments were: G2D; G2D + α pinene (released at 250 mg/day), G2D+ limonene (released at 250 mg/day) and G2D + α pinene + 8 limonene.

Results:

There were no significant differences among treatments (Figure 4.4). Releasing 250 mg/day of limonene reduced, non-significantly, catches by the G2D lure by 17% (in 2011, release of 500 mg/day of limonene significantly reduced catches in the standard lure by 60%). However, when α -pinene was also emitted, even increasing the limonene dosage 8 times (2000 mg/day), this repellent effect was not increased (reduction in catches was only 10%).

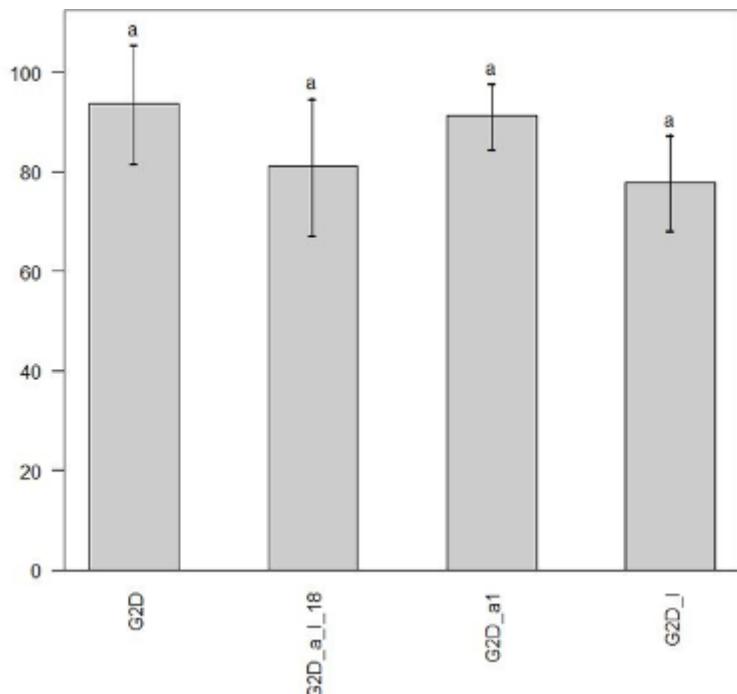


Figure 4.4 Results of experiment 4/13 on the limonene effect in Spain (2103)

These and earlier results in 2011 and 2012, suggest that even if limonene shows a repellent effect to beetles being attracted to pheromone-kaiomone baited traps, such effect is low if attractive terpenes, such as α -pinene, are also emitted, thus making practical use of this terpene for protective aims is unlikely.

Experiment 5/13(B5).

Several tests were run in 2012 and 2013 to check if *M. galloprovincialis* adults originating in Germany respond to the trap&lure combination in the same way as demonstrated in other countries. For this, Galloprotect Pack, was tested in six federal States within Germany in 2012 and in five in 2013. Traps used were standard multiple funnel traps (Comtech, Canada) in 2012 and Teflon-coated multiple funnel traps with extended collectors for live trapping (Econex, Spain) in 2013. All traps were placed either on trap hangers or on wooden poles or hanging on a rope between trees. Small branches were placed inside the collection cups to prevent beetles disturbing each other. Beetles were collected weekly at least.

Results:

Among the total of 6,441 insects that were captured in 2012 containing 40 taxa, only 29 *M. galloprovincialis* adults were recorded in the 49 traps in 2013, in only 3 out of the 6 Federal States studied (Table 4.1). In contrast, catches were much higher in 2013: a total of 12,078 insects was captured in 2013, containing 80 taxa, among which 442 specimens were identified as *M. galloprovincialis* in four out of the five States studied. The results of Lower Saxony (Forest Service) indicate that the traps are suitable to catch *M. galloprovincialis* in high numbers if populations are high. Only in one of the States, Northrhine-Westfalia, no *Monochamus* were obtained in both years. As a result of this investigation, several German plant protection services of the Federal States intend to use the tested trap system in their monitoring according to the EU-Commission implementing decision 2012/535/EU

Table 4.1 Number of specimens caught in multi-funnel traps attached with Galloprotect Pack lure in six German Federal States in 2012 and 2013.

Species							
<i>M. galloprovincialis</i>	Lower Saxony JKI (10)	Saxon y (10)	Northrhine -Westfalia (10)	Bavaria (9)	Baden Wurtenberg (10)	Brandenburg	Su m (49)
2012	0	3	0	2	0	24	49
	Lower Saxony JKI (5)	Lower Saxony Forest Service (10)	Saxony (10)	Northrhine -Westfalia (10)	Brandenburg Forst (4)	Brandenburg NPPO (10)	Su m (49)
2013	0	306	33		39	59	442

It became clear that the great differences found between both years were due to the beetles escaping from the non-Teflon traps in 2012. To check for this possibility, a small experiment was carried out in 2013. The results of the test; whether or not beetles stay in the collection cup or escape, showed clearly that *M. galloprovincialis* can very easily climb out of the uncoated collection cups as well as out of the funnels. The data show that 100% of the beetles placed in the uncoated cups escaped within 24 hours (in most cases within minutes) whereas 100% of the beetles placed in the Teflon-coated cups stayed there for the complete test period.

Experiment 6/13(B5).

An experiment was run to test the volatile organic compounds (VOCs) of different treated, nearly mature *P. sylvestris* (PWN infested pines, drought stressed pines, control pines and double control pines). It was aimed to see if chemical differences exist between the VOCs of the different treated pines and if *M. galloprovincialis* distinguish between the VOCs of the different treated hosts. Besides, it will be valuable to see if an early detection of PWN infestation is possible with the help of VOCs.

Studied trees were: controls (inoculated with tap water) (6 trees), PWN inoculated (6 trees), drought stressed (inoculated with tap water) (6 trees) and double controls (without any inoculation and cutting of an inoculation slit) (2 trees). VOCs were collected using the CLSA method (Figure 4.5) on two 2-3 year old main side shoots of 7-8 year old *P. sylvestris*, were subsequently eluted and GC-MS and EAD analysed. For the antennal analysis, we used beetles that had emerged from the pine logs and additionally captured in traps.



Figure 4.5 Collection of VOCs with CLSA method on 7-8 years old *P. sylvestris*

Results:

The EAG results of the beetle antennal reactions towards the VOC samples were compared with the GC records. First dose-response-curves of some compounds were produced, which have to be tested for significant differences. This work will be done/ continued in 2014.

The analysis of all compounds of the GC results and the determination of the concentrations of the VOC samples as well as the concentration borders for the main compounds to which *M. galloprovincialis* could react, will be done/ continued in 2014.

Highlights of results obtained in Task 4.1

- 1) Results in Spain, Portugal, France, Germany and Austria have confirmed the effectiveness of the pheromone-kairomone lure for *M. galloprovincialis* commercialized as GALLOPROTECT 2D (SEDQ, Barcelona Spain), in trapping both sexes of *M. galloprovincialis*. Thus, this combination is recommended as the standard lure for *M. galloprovincialis* monitoring.
- 2) Release of the host volatile α -pinene (500 mg/day) together with G 2D (GALLOPROTECT Pack, SEDQ) usually increases attraction by 20/30%, though sometimes not significantly. This terpene however is non target specific and attracts many xylophagous beetles and bark beetle predators. Besides, it has other disadvantages (bulky, flammable).
- 3) Some smoke volatiles can also act as synergists, increasing attraction to the standard pheromone-kairomone lure. Though results were not completely conclusive, they suggest that compounds including 2-methoxyphenol may satisfactorily replace α -pinene as synergist. Thus, it is worth continuing to test these compounds, commercially produced, during the extension period.
- 4) It should be stressed that these lures attract only adults that have fed for at least 10 days. Attempts to attract unfed, immature beetles using several pine terpenes, either within forests or in areas deprived of hosts, have been unsuccessful so far.
- 5) Limonene reduces catches, sometimes significantly, sometimes not, of *M. galloprovincialis* in baited traps. However, such repellence is overcome if attractive terpenes, especially α -pinene, are also emitted, which may be the case for pines. Effective use of this terpene in the protection of particular trees seems unlikely.

Thus Deliverable 4.1 Lure for *M. galloprovincialis* (due M. 24) is basically accomplished. A highly efficient lure, GALLOPROTECTD 2D, is available and can be recommended for monitoring and control. The efficiency of this lure can be increased to a certain degree by the addition of other kairomones (+ α -pinene = GALLOPROTECT Pack; smoke volatiles) but its convenience would depend on the aimed objectives (monitoring, direct control).

Such a lure has been shown to have great potential in controlling the vector population by mass trapping (see Task 4.3) and it is currently used for mass trapping in the eradication programmes in the three PWN foci in Spain. However, a lure for newly emerged adults is still lacking.

4.2 Development of traps for live trapping and for mass-trapping of *Monochamus*

Partners involved:, B4, B6 and B9

Different trap designs to maximise captures of *M. galloprovincialis* will be tested under different field conditions. These will include multiple-funnel traps, various cross-vane traps, the INRB prototype and other commercially-available traps, baited with the optimised kairomone/pheromone blend. Trapped beetles will be killed inside the traps by use of cardboard impregnated with contact insecticide or by drowning in a liquid preservative. Experiments will be carried out in various countries as in Task 4.1 using randomized block designs. Traps will be deployed on poles at least 2m high along forest roads, firebreaks or clearings to maximize target visibility to the insects. Trapped beetles will be identified, aged and sexed.

Monochamus adults are very agile and most escape easily from traps if they are not killed. However, keeping the captured beetles alive is vital for obtaining valuable information such as physiological parameters and detection and quantification of nematode loads in the beetles. Several trap modifications for avoiding escape of live *Monochamus* adults such as one-way funnels, coating inner surfaces with fluon, etc., will be tested.

B9 will work closely with the other Beneficiaries to carry out this work.

Outputs from Tasks 4.1.-4.3. will feed directly into work on detection of PWN in WP2 and studies of dispersal of the vector beetles in WP3.

Objectives:

- Trap designs to maximise captures of *M. galloprovincialis*: multiple-funnel, various cross-vane, the INRB prototype and other commercially-available traps.
- *Trap modifications for avoiding escape of live adults: one-way funnels, coating inner surfaces with fluon, etc.

Highlights of results obtained in Task 4.2

Activities for this task were carried out in 2011 and 2012. As the proposed objectives were accomplished, no further activities have been undertaken in 2013. As it was outlined in the previous Report:

- 1) The experiments in 2011 and 2012 showed that the two Teflon-coated traps tested performed significantly better in the trapping of the pine sawyer. Both of them appeared similar in effectiveness and in other desired features (durability, ease of handling and transportation). However, durability of Teflon treatment under field conditions has been only demonstrated during two consecutive seasons. Long term durability of the Teflon treatment is unknown.
- 2) Both trap designs allowed for the trapping of live beetles in their Teflon-coated, wire screened bottom containers (exp1/11 in Spain). Again, durability of the Teflon treatment is key for long term use of this favourable feature.

Deliverable 4.2: Development of traps for monitoring & control (due M. 12) is therefore accomplished. At least two commercially available Teflon-coated traps tested, multifunnel and crossvane, are efficient enough to be recommended for monitoring and for control of *M. galloprovincialis*. They are being operationally used in Spain in the eradication programs. For maximizing the trapping of live adults, the use of the extended collector cup in the multifunnel trap is greatly recommended.

These traps are currently available at Econex SL (Murcia, Spain) and are marketed as:

- CROSSTRAP® (with Crosstrap Collection Cup 2 Liters)
- ECONEX MULTIFUNNEL-12® (with Econex Multifunnel Extended Collection Cup)

However, the unknown long durability of the Teflon treatments must be taken in consideration. Tests are underway by the manufacturer to provide users with an easy way to re-coat the traps with Teflon (i.e. spraying).

In any case, it is likely that new designs would appear in the future that would require further testing.

4.3 Assessing of potential mass-trapping of vectors

Partners involved: B3, B4, B5, B6 and B9

The potential for management of vector populations by mass-trapping will be studied under different stand and landscape characteristics (host tree density, stand management, landscape fragmentation and heterogeneity) with different beetle population densities (from very low to outbreak). Population densities will be estimated by mark-release-recapture trials and by population assessment in infested host material. Trapping experiments will use lures and traps developed above. Trap setting and trap density required to maximize beetle captures and population reduction will be determined. Overall, the effects of mass trapping on beetle populations will be assessed by monitoring *Monochamus* spp. population dynamics before and after mass trapping. Field testing will be carried out in Austria, (B3), France (B4), Germany (B5), Portugal (B6); Spain (B9), and other countries as appropriate.

In terms of sustainability, this approach has the advantage that the traps are easily deployed and are rather specific for the target species, *M. galloprovincialis*. The cost will depend upon the density of traps required, but several European companies produce the traps that will be evaluated during this research and they will be kept informed of results and encouraged to produce the optimised design. Similarly a Spanish company already produces the kairomone lures on a commercial basis and is proposing to add the pheromone to the commercial product under guidance from B9 and B2.

This task is aimed to "determine whether trapping can be used to reduce populations of the vector". For that, mass-trapping has to be assessed under different stand and landscape characteristics, with different beetle population densities, using the lures and traps developed above.

Initially scheduled for the years 2012 and 2013, but to take advantage of the developed lures and traps, it was re-scheduled for 2013 and 2014 seasons.

Activities in 2013:

The above objective addressed the following two questions:

- 1) what is the optimal trap density for mass trapping of the vector (trap distance)? and, provided that such optimal density is applied,
- 2) what proportion of the beetle population could in this way be removed?

Experimental designs for addressing these questions were previously discussed. For answering question 1 it was decided to test two different approaches by the different partners.

Experiment 7/13 (B9)

For answering question 1, partner B9 investigated the “*Effective Area of Sampling*” concept by Turching & Odendaal, (Env. Entomol 25: 582-588, 1996), that can be regarded as the area around the trap from which all captured insects originate if the trap catches 100% of the insects within this area and no insects from outside. The EAS can be estimated by data obtained in mark-release-recapture experiments. We used data from previous experiments to set the ranges of trap densities (trap distances) to be tested.

Thus, an experiment to test optimal trap density for mass trapping was carried out in Cuellar (Segovia, Spain) within a *P. pinaster* low density forest stand where the local *M. galloprovincialis* population was estimated to be moderate (see 2011 dispersal experiment, WP3). Seven mass trapping 600x600 m plots, testing 4 trap densities (distances), were randomly distributed within the stand. Adjacent plots were separated by a 100m wide buffer.

Trap densities (distances) tested were: 0.02 trap/ha (600m) replicated 3 times, 0.11 trap/ha (300m) replicated 2 times, 0.25 trap/ha (200m), 0.44 trap/ha (150m). Traps were checked weekly

For answering question 2, how much of the population is being removed, it is necessary to determine the beetle population density. For this, we used the Model approach “Spatially Explicit Capture Recapture” by M.Efford (SECR; R library), which is based on the capture-mark- release and recapture of native beetles. For this, 20 traps were randomly deployed to estimate beetle density within a second experimental area of about 2 x 2 km, near to the mass trapping area. Native trapped adults were marked and released weekly near the place of capture. For extra information, 120 lab obtained immature adults were also released within this area.

In total 56 traps were deployed in both experiments from June 4th to October 20th.

Results

Accumulation during the whole season was quite high. Highest mean trap/catches were obtained in the 0.02 trap/ha (trap distance 600m) and 0.11 trap/ha (trap distance 300m) than in the 0.25 trap/ha (trap distance 200m) and 0.44 trap/ha (150m), but differences were only significant between the latter three treatments.

However, when total accumulated catches are considered per area (plot or ha), the highest trap density caught far more beetles than the others: 1570, that is 43,6 beetles/ha, compared to 3,2/ha, 14,4/ha and 25,2/ha in the 0,002, 0,11 and 0,25 trap/ha densities respectively.

Population density models, fitted with data from the other experimental subareas, showed positive results and allowed to estimate the local population density of *M. galloprovincialis* to be 60,4 beetles/ha. These results lead us to estimate that the population that was removed in the mass trapping area ranged from 5,3% in the lowest trap density to a remarkable 70,2% obtained when traps were placed every 150m (Figure 4.6).

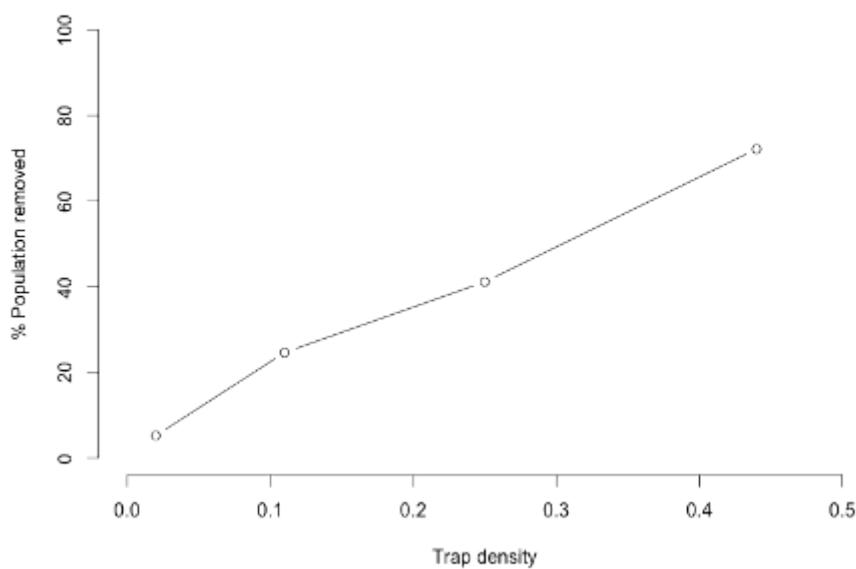


Figure 4.6 Results of experiment 7/13 on mass trapping in Spain (2103)

These results show that mass trapping with the lures and traps used has great potential for population control or even eradication in the infestation foci. The optimal trap density among those tested was 0.44 traps/ha, however higher trap densities were not tested so it is unknown if even better results can be achieved. Beetle densities on the other hand are surely another factor that would affect mass trapping efficiency. Application of our model to data obtained previously in other mark and release experiments suggests that high population removal by mass trapping can be achieved with more spaced traps at lower *Monochamus* densities

Experiment 8/13 (B4)

Partners B4 and B6 devised a different approach for answering question 1, based in the concept of Attraction Radius (AR), which represents the radius of a passive "sticky" sphere that would intercept the same number of flying insects as the attractant. This concept is of particular interest for optimizing the density of trapping network. In theory, trap capture efficiency is expected to reach an optimum for distance D_{Opt} between traps equal to twice AR.

In 2013 an experimental method was developed to be applied in France and in Portugal to estimate AR in the field, using pairs of pheromone traps at increasing distances from each other. Five distances between traps were tested: 25, 50, 100, 200, 300m with 3 replicates i.e. 3 clusters of 5 pairs (total $3 \times 10 = 30$ traps). Crosstrap® (Econex, Spain) baited with Galloprotect pack (SEDQ, Spain) were used.

Results

A logistic function to model the relative capture per trap and day in relation to distance between traps was applied. An asymptotic curve was fitted ($R^2 = 0.98$, $P = 0.001$) that levelled out at ca. $D_{Opt} = 200$ m thus giving an $AR = 100m \pm 30m$.

Experiment 9/13 (B6)

A similar experiment was carried out by B6 in Portugal, based on the same approach. The 36 multifunnel traps baited with Galloprotect-2D were deployed in pairs at 6 tested distances (25m to 400m) forming three blocks, inside a maritime pine stand from 15 August until 11 September, with weekly removal of insects.

Results

A total of 1.476 *M. galloprovincialis* was caught with higher numbers when distance between traps was 300 meters (Table 4.2).

Table 4.2 Number of *Monochamus galloprovincialis* caught in the traps in Exp 9/13

Traps/ distance	25m	50m	100m	200m	300m	400m	T
Block 1 (a01-a12)	65	48	69	63	82	65	392
Block 2 (a13-a24)	52	50	66	49	154	113	484
Block 3 (a25-a36)	61	96	73	181	91	98	600
	178	194	208	293	327	276	1476

Based on the main result of the assay, 300 meters appears to be the optimal distance in the placement of adjacent traps, which leads to a trap density recommendation of one trap every 1.25 hectares.

Highlights of results obtained in Task 4.3

Results so far have shown the potential of the trapping system developed (lures and trap) for mass trapping of PWN vectors in particular areas of interest.

- 1) Experiments in Spain have indicated that a network of regularly spaced traps at 0.44 traps/ha (i.e. traps spaced every 150m) is able to remove 70% of the beetles in a moderate-high density population of *M. galloprovincialis* (60 beetles/ha) during a whole trapping season.
- 2) Based on a different approach, experiments in France indicated that the optimal distance between traps for maximizing mass trapping may be in about 200m (France) or 300m (Portugal).
- 3) Disparities among these results, other than experimental approaches, may be related to differences in stand features, particularly beetle densities.

Thus considerable progress has been made towards achieving Deliverable D 4.3: Effectiveness of mass trapping for vector control, due month 45. Further experiments on mass trapping during 2014, as planned, will help to determine optimal trap density (trap distance) and the relevance of factors affecting it, such as beetle density.

4.4 Development of attractants for other European Monochamus

Responses to potential kairomone compounds by *M. sutor* will be laboratory tested in olfaction and electrophysiological (GC-EAD) bioassays (B9 and B2). Bioactive candidates will be field tested in traps as above. Existence of a sex or aggregation pheromone will be determined by collection and GC-MS analysis of male and female volatiles. Bioactivity of candidates will be determined by GC-EAD and bioassays. Positive candidates will be field tested as above. Synergism between kairomones and pheromones will be also studied. Similar work will be

carried out for *M. sutor* and any other potential vector species identified during the project, with input from B3, B4 and B5

This task aims to develop traps and lures for other European *Monochamus* species considered likely to be vectors of PWN, particularly *M. sutor* and *M. sartor*.

It has been found, during the first part of the project, that *M. sutor* aggregation pheromone produced by males is 2-undecyloxy-1-ethanol, which is identical to that produced by *M. galloprovincialis*. Field testing showed that males and females are attracted to a blend of pheromone and kairomones and that Galloprotect 2D resulted in the best attractive blend tested for *M. sutor*.

These results, together with those obtained in Sweden and in China by other colleagues, have led to the joint publication: Pajares et al, 2013. 2-(Undecyloxy)-ethanol is a major component of the male-produced aggregation pheromone of *Monochamus sutor*. *Entomologia Experimentalis et Applicata* 1-10

Activities in 2013:

Experiment 10/13: (B2 and B9)

Previous results in 2012 showed no differences between bark beetle kairomonal blends tested as pheromone synergists. Besides, there were other untested potential kairomonal compounds, such as chalcogran. Thus one experiment was carried out in Spain to determine what individual kairomone is the better synergist of the pheromone and to determine if there is a 2-compound kairomone blend that is better than an individual kairomone as synergist of the pheromone.

Preliminary antennal studies showed that *M. sutor* males gave EAG responses to chalcogran (Figure 4.7).

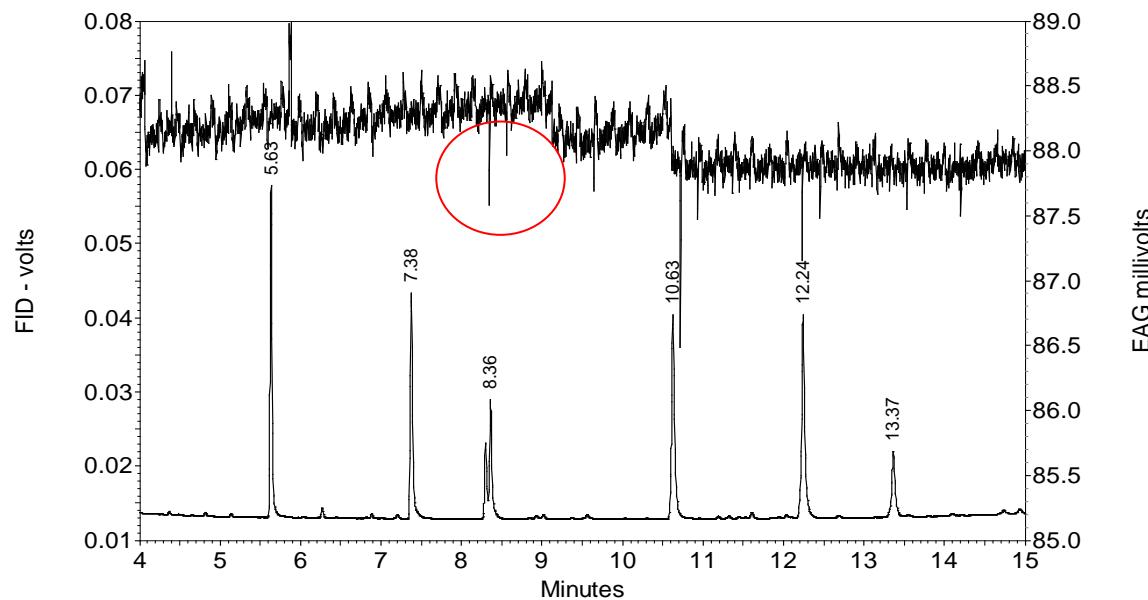


Figure 4.7 GC-EAG of chalcogran with *Monochamus sutor* males.

A field experiment to test the single kairomones and some blends as synergists of *M. galloprovincialis* pheromone was carried out from July 4th to Sept 4th 2011 within a

P. uncinata stand, in the Pyrenees (Huesca; Spain), using a complete randomized block design of 5 blocks. Lures were deployed in multifunnel traps (extended collector for live trapping) that were sampled every 7-10 days. The seven treatments were: Blank, P (*M. galloprovincialis* pheromone), P+ipsenol, P+ Methyl-butenol, P+ Chalcogran, P+ Ipsenol + Methyl-butenol, and P+ Ipsenol + Chalcogran.

Results:

Results showed clearly that chalcogran was not bioactive in the field. Pheromone was greatly synergized by ipsenol, but not by either M-butenol or chalcogran. Addition of M-butenol to the (Pheromone+Ipsenol) blend enhanced response, though not significantly (Figure 4.8).

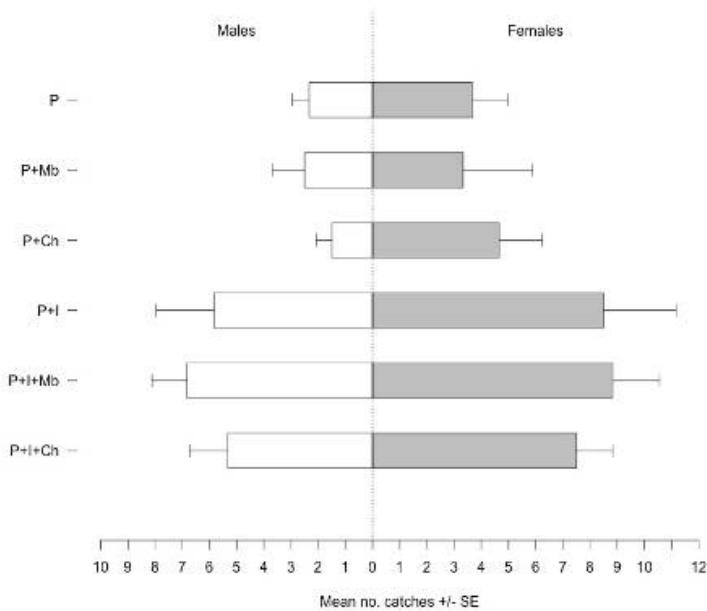


Figure 4.8 Mean catches of *M. sutor* in experiment 10/13 in Spain.

A similar experiment, carried out in cooperation with researchers of the University of Uppsala in Sweden produced identical results.

Experiment 11/13: (B3)

One experiment was set to further confirm the synergist effect of the bark beetle pheromones on *M. sutor* catches in Oiswald Natural Forest Reserve (Austria) during 6 weeks from July to August 2013. The kairomonal blend Ipsenol+methyl-butenol was assayed either alone or as synergist of the *M. sutor* pheromone in multiple funnel traps deployed in 5 randomized blocks.

Results

Results confirmed the much higher attractive effect of the standard blend (Pheromone+Ipsenol+Methyl butenol= IMP) compared to the kairomone alone (Ipsenol+Methyl butenol= IM) (Figure 4.9).

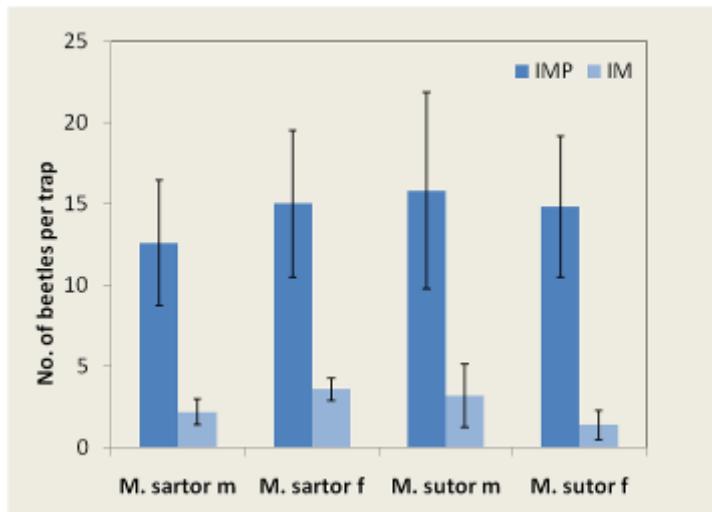


Figure 4.9 Mean catches of *M. sutor* and *M. sartor*. in experiment 11/13 in Austria.

Experiment 12/13: (B2 and B3)

Another experiment was carried out in Austria (Dürrenstein Wilderness Area) to check if attraction of the standard blend can be enhanced if methyl-butanol was replaced by chalcogran. Three treatments (P=Pheromone, PIM=P+ipsenol + methyl butenol and PIC=P + ipsenol + chalcogran) were deployed in multiple funnel traps during 6 weeks in July and August.

Results

Results were similar to those found in Spain and Sweden, as replacement of M-butanol by chalcogran did not produce any increase in attraction (Figure 4.10).

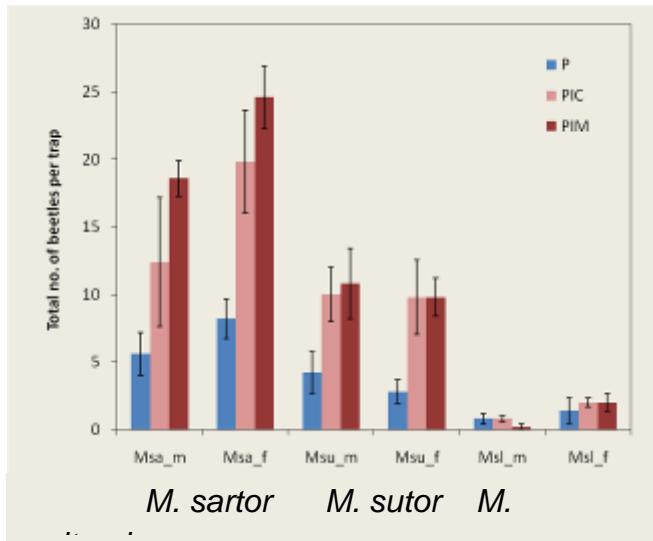


Figure 4.10 Mean catches of *M. sutor*, *M. sartor* and *M. saltuarius*, in exp12/13 in Austria.

Experiment 13/13: (B2 and B3)

Earlier work showed that *M. sartor* responded to *M. galloprovincialis* and *M. sutor* pheromone. A study was undertaken to isolate and identify the pheromone produced by *M. sartor* males. Beetles were collected in Austria by B4 and sent to B2 for pheromone study.

Results

FID-GC analysis showed that beetles produced a male specific peak (Figure 4.11) that was identified to be, as hypothesized, 2-undecyloxy-1-ethanol, the same pheromone produced by *M. galloprovincialis*, *M. sutor* and other conifer *Monochamus* species.

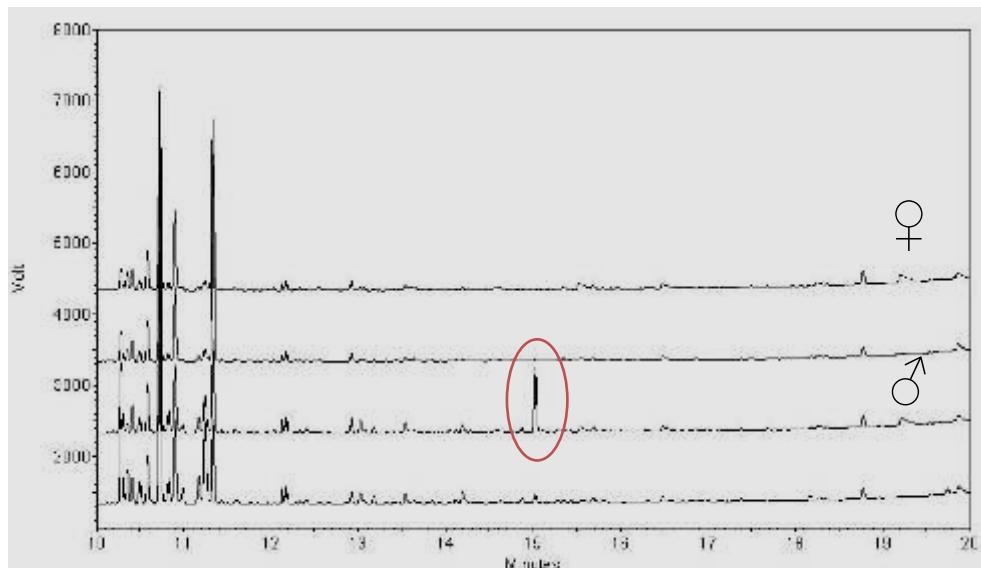


Figure 4.11 GC of volatiles released by *Monochamus sartor* males and females

Experiment 14/13: (B2 and B4)

Experiments 2 and 3 above, carried out for *M. sutor* served also to test the response by *M. sartor* to the pheromone and the kairomones, as both species were present in the experimental areas.

Results

It can be seen in Figure 4.9 and Figure 4.10 that responses by *M. sartor* to the pheromone and to the kairomones, either alone or in blend with the pheromone, were completely similar to those found for *M. sutor*. The standard blend for *M. galloprovincialis* also resulted in an efficient lure for this species. Few catches of the rare *M. saltuarius* were also recorded in the traps.

Experiment 15/12 (B2 and B9)

Previous work pointed to the role of contact sex pheromones in *M. sutor* mate discrimination, as in *M. galloprovincialis*. Besides quantitative differences between males and females, the occurrence of two male specific peaks was noteworthy, also present in *M. galloprovincialis* males. An experiment was conducted to further explore the role of these male specific compounds in mate discrimination of *M. galloprovincialis* and *M. sutor*.

Separation from the rest of the extract of the two *M. sutor* male peaks was achieved by fractionation of cuticular extracts from *M. sutor* males. Fractionated extracts applied to freeze killed females or female decoys (glass rod) were bioassayed against *M. sutor* males.

Results

Few beetles could eventually be tested so results are quite preliminary. Males accepted decoys treated with fractionated male extracts that had the male peaks removed, but reject them if the male peak extracts were re-applied, suggesting that male specific peaks are key in *M. sutor* male mate discrimination.

Highlights of results obtained in Task 4.4

- 1) Results obtained in Spain, Austria and Sweden show that a simple and effective lure for *M. sutor* trapping can be composed of just the pheromone plus ipsenol. However, the standard lure for *M. galloprovincialis* (including also methylbutenol) obtained the highest catches.
- 2) *M. sartor* males produce 2-undecyloxy-1-ethanol, the same pheromone so far found in several species of conifer dwelling *Monochamus* in Europe, Asia and North America. Similarly to *M. sutor*, the standard pheromone plus bark beetles kairomones blend described for *M. galloprovincialis* can be efficiently used for *M. sartor* monitoring and control.
- 3) Male cuticle specific peaks maybe involved in *M. sutor* mate discrimination, as in *M. galloprovincialis*.

Thus Deliverable D 4.4 Development of lures for other *Monochamus* spp. (due M 35) is complete. *M. sutor* and *M. sartor* pheromones have been identified. There is an effective lure already available for monitoring, and even control, of these species (Galloprotect 2D).

Progress in our understanding on mate discrimination chemical ecology, particularly the role of specific peaks of both *M. galloprovincialis* and *M. sutor* has also been made.

Statement on deviations from Annex I, and on failing to achieve critical objectives and/or not being on schedule:

No further deviations from Annex I and from critical objectives, than those reported in the first midterm report, have occurred during this second period. As it was already indicated, initial delay on funding led to propose the one year delay (to month 45) of the studies aimed to "Deliverable D4.3 Effectiveness of mass trapping for vector control". Now, these studies have begun during 2013 and will be finished in 2014.

Statement on the use of resources

None of the beneficiaries have declared any deviation on the use of resources for this WP.

Work Package 5: Determine risk of non-vector spread of PWN through various pathways to healthy forests

Objectives

The principal objective of this WP is to investigate the hypothesis that wood containing PWN maybe a pathway for the dispersal of PWN even when there is no association with *Monochamus* spp. This will be investigated through the following pathway-associated sub-objectives:

- to determine the distribution, survival and population dynamics of PWN in wood, relative to wood moisture content, and in wood chips;
- to assess the possibilities for transmission of PWN to healthy trees from infested wood chips, infested wood and infested bark in the absence of the vector beetle;
- to assess the possibility of transmission of PWN from tree to tree in the absence of the vector beetle;
- to assess the possibility of transmission of PWN from infested wood to non-infested wood in the context of international use of wood packaging;
- to develop a set of microsatellite genetic markers for use in PWN genetic characterisation;
- to assess, through genetic characterisation of PWN, the invasion routes into Europe, with potential linkage to specific pathways.

Deliverables

D5.1: Distribution of PWN in wood and wood chips: Data on distribution of *B. xylophilus* in wood (*Pinus sylvestris*) and wood chips, survival and population dynamics depending on wood moisture content and temperature on a laboratory scale basis. Month 33

D5.2: Transmission of PWN to trees with wood chips/bark: Assessment of non-vector transmission of *B. xylophilus* to healthy trees with infested wood, wood chips or bark. Month 45

D5.3: Direct tree to tree transmission of PWN: Assessment of transmission from tree to neighbouring tree through the soil or by root contact. Month 33

D5.4: Wood to wood transmission of PWN in wood packaging: Assessment of transmission of *B. xylophilus* from infested wood to non-infested wood in storage and transit. Month 45

D5.5: Microsatellite markers for PWN identification: Validation of a set of PWN-specific microsatellite markers usable at the individual level. Month 12

D5.6: PWN genetic diversity as indicators of invasion history: Assessment of genetic diversity of European PWN populations in relation to invasions. Month 45

Overall comment for WP 5: As already stated in the first report, B6 and B1 carried out a research project covering similar questions to those raised in WP 5 of the REPHRAME proposal. This project was carried out together with the industry (CHEP) using boards and blocks of sizes used for pallet production (referred to as "CHEP-project" in the following text). This project took place when the proposal for the REPHRAME project was under development. Some of the proposed work in REPHRAME was already addressed by the CHEP-project as mentioned above. Due to a confidentiality agreement with the industry, the involved partners (B1 and B6) were not able to inform other partners like B5 of the detail of the research and therefore some work was additionally proposed to be carried out in REPHRAME. Because the CHEP investigation has been completed and the results published by B6 in the EPPO Bulletin (Sousa E.; Naves, P.; Bonifacio, L.; Henriques, J.; Inacio, ML; Evans, H.

(2011): Assessing risks of pine wood nematode *Bursaphelenchus xylophilus* transfer between wood packaging by simulating assembled pallets in service. EPPO Bulletin 41: 123-431.), some of the proposed work in WP5 does not need to be carried out again. The relevant parts were highlighted in the first report and the results were cited. The scheduled person months for B5 and B6 in the REPHRAME proposal have, therefore, been shifted within WP 5 to allow other tasks to be investigated in more detail or with a higher sample size than originally planned.

5.1 B. xylophilus distribution in wood and wood chips: survival and population dynamics depending on wood moisture content

(5.1.1) Pine wood (*Pinus sylvestris*) logs of different dimensions will be inoculated with PWN at different times of the year but within a time frame corresponding to the period where *Monochamus galloprovincialis* carries out its maturation feeding. Inoculation will be carried out using fresh wood to mimic PWN transmission during maturation feeding and should lead to distribution of the nematodes throughout the logs. Following different time intervals the wood will be sampled to determine nematode population development, including assessment of developmental stages (males, females, LIII larval stage and other larval stages).

(5.1.2) In parallel, wood will be assessed after drying to particular moisture contents to assess the influence of wood moisture content on nematode development.

(5.1.3) Studies concerning the long term survival of PWN in wood will be established immediately after project start with different variants using fresh wood: inoculated logs, shrink-wrapped in plastic bags; inoculated logs allowed to air dry; boards sawn from infested logs with dimensions used e.g. in pallet production, bark free and air dried. B6 to lead. Similar investigations will be done with wood chips by B5.

(5.1.4) The influence on the survival of *Bursaphelenchus* species of naturally occurring temperatures in piles of wood chips will be assessed using native *B. mucronatus* as substitute test organism. Infested wood chips will be placed in nets in different zones of chip piles and will be assessed for nematode survival after a storage period under operational conditions. Temperature and air moisture contents throughout the chip piles will be documented using data loggers, so these data may be used to test *B. xylophilus* infested chips under quarantine conditions. B5 to coordinate

Work done by B5 (JKI):

In the current reporting period B5 spent 9,97 PM (productive hours) in this work package. Together with the amount of delivered PM in the first reporting period B5 spent already 6,37 PM over the proposed 16 PM to be delivered in this WP.

Objective 5.1.1 (Work of B5): Determination of the population development and assessment of developmental stages of PWN after inoculation in non-infested *P. sylvestris* logs at different times in the year during the maturation feeding of *Monochamus galloprovincialis*

Materials and methods:

Experimental set-up:

In 2012 from May until September each month approximately 8 year old *P. sylvestris* trees were felled in a pine stand (Essehof near Braunschweig/ Lower Saxony/ Germany), cut into segments, sealed with hot paraffin at the log ends and stored at 5 C in a climate room for eight days. Ten logs per month were tested. After storage, the logs were cut to a length of 20 cm, weighed and sealed again with hot paraffin at the ends. In addition five 2 cm wide discs per stem with an equal distribution in the stems were cut for control of nematodes and the determination of the moisture content.

Extracted PWN from wood located in Portugal in the year 2012 (provided by Dr. E. Sousa – INIAV) were reared on non-sporulating *Botrytis cinerea* on 1.5 % malt extract agar medium at approx. 20 C. A nematode suspension was prepared. One inoculum consisted of one ml tap water with 4000 PWN of the isolate PT-6 (w) (number of the isolate in the reference culture collection of the Institute for National and International Plant Health, Julius Kühn-Institut Braunschweig, Germany).

For inoculation of the nematode suspension, holes were drilled (driller: DP4003, Makita Corporation, Anjo, Aichi, Japan) with 10 mm diameter to the centre of the log. Per log two holes (one per cut log end with 5 cm distance to the end) were prepared. The suspension was inoculated using a pipette. The holes were closed with wooden pegs. Each artificial inoculated log was sealed in a plastic bag (Figure 5.1) for incubation at 25 C in a climate chamber (RUMED® Rubarth Apparate GmbH) for 35 days referring to Schröder et al. (2009).



Figure 5.1 *P. sylvestris* logs after inoculation with *B. xylophilus* - Sealed in plastic bag; Ready for incubation

Population development and assessment of developmental stages of nematodes:

After the 35 day incubation period, each 20 cm log was unsealed from paraffin and cut in 2 cm wide discs with a laboratory band saw (EBS-250A, Rexon industrial corp., LTD; Taichung, Taiwan). With ambos shears the wood discs were cut into approx. 20 x 5 x 5 mm pieces maximum size according to Schröder et al. (2009). PWN were extracted by placing the wood chip samples on funnels (Baermann-funnel technique according to Baermann (1917) modified for plant parasitic nematodes according to Decker (1969)) after weighing. The moisture content of the wood chips was determined according to the Dt. Institut für Normung - German Institute for Standardisation DIN 52183 (1977). Nematodes were preserved using 80 °C hot fixative solution (890 ml Aqua dest., 100 ml formaldehyde solution (35 %), 10 ml glacial acetic acid referring to Dropkin 1989) for later counting. After nematode identification and counting, the nematode density referred to gram dry matter plant tissue with differentiation of males, females and juveniles was calculated.

Results:

In the tested pine stem discs from Essehof no nematodes were found before the inoculation of PWN took place. The stems were felled between May and September 2012. The MC medians (Table 5.1) of the tested stem discs ranged between 171 % (July) and 261 % (June) at inoculation start (after stem end sealing and eight days storage at 5 °C). In the spring the MC was higher than in the summer months. The logs had a mean diameter of 8 ± 1 cm (n=50).

Table 5.1 Moisture content MC (%) of stem discs at the inoculation date of the logs for each test month in 2012, n=5

Inoculation date	Moisture content _{stem discs} [%]		
	Median	Min	Max
22 nd May	212.4	195.0	215.5
25 th June	260.8	240.5	272.6
24 th July	171.0	165.9	174.8
23 rd August	190.7	157.7	226.7
26 th September	172.5	156.9	210.7

On the nematode extraction dates after 35 days incubation time at 25 °C the MC medians of the cut 2 cm wide wood discs of the sealed logs were similar to the MC medians of the wood discs at test start with a range between 161 % and 285 %.

The extraction of nematodes showed the following results:

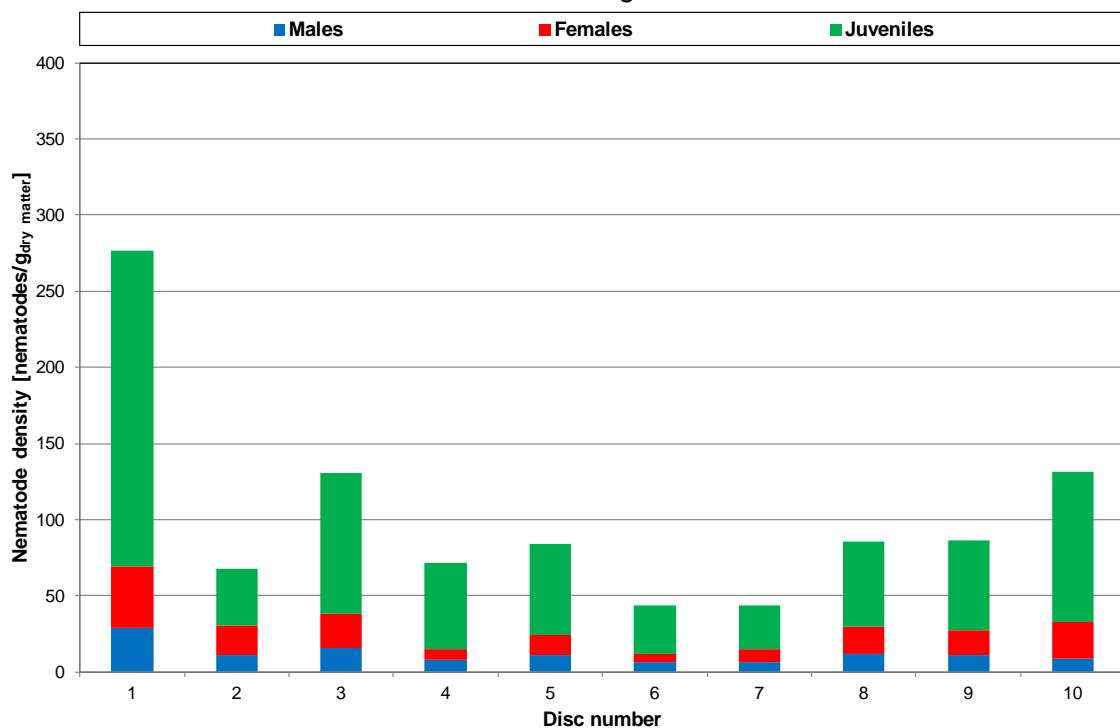


Figure 5.2 Population development: Nematode density [nematodes/g_{dry matter}] (medians) differentiated for males, females and juveniles in all 2 cm wide wood discs of the pine logs for incubation month 1 (22nd May - 26th June), n=10

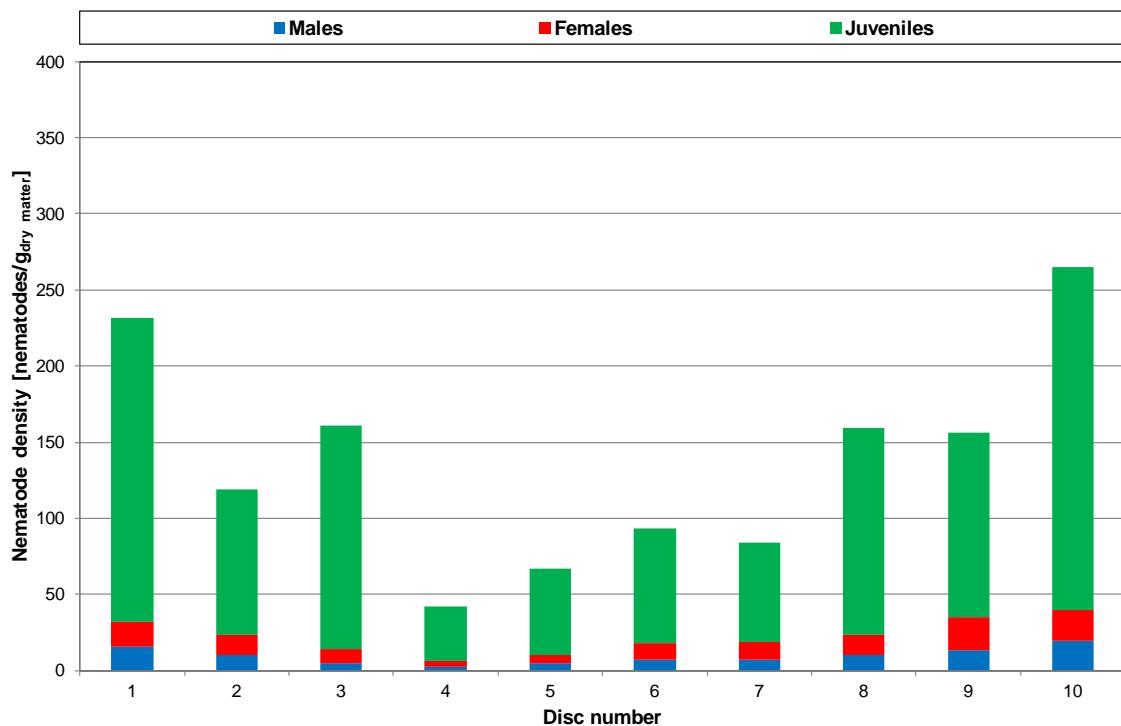


Figure 5.3 Population development: Nematode density [nematodes/g_{dry matter}] (medians) differentiated for males, females and juveniles in all 2 cm wide wood discs of the pine logs for incubation month 3 (24th July - 28th August), n=10

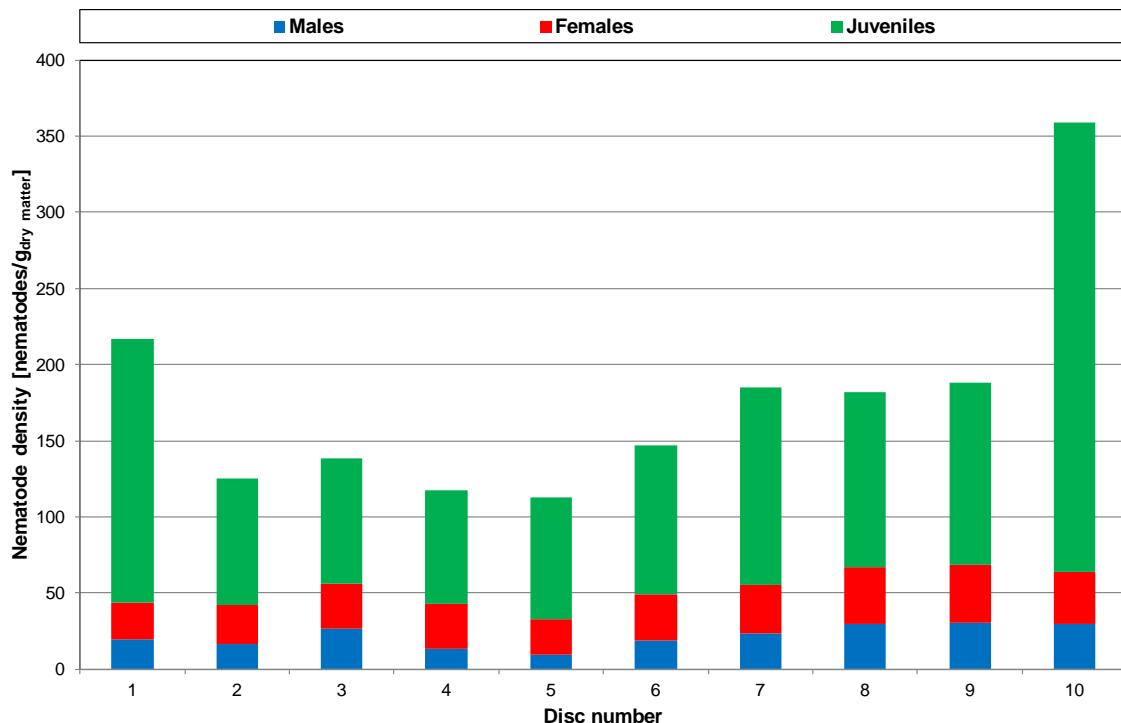


Figure 5.4 Population development: Nematode density [nematodes/g_{dry matter}] (medians) differentiated for males, females and juveniles in all 2 cm wide wood discs of the pine logs for incubation month 4 (23rd August - 27th September), n=10

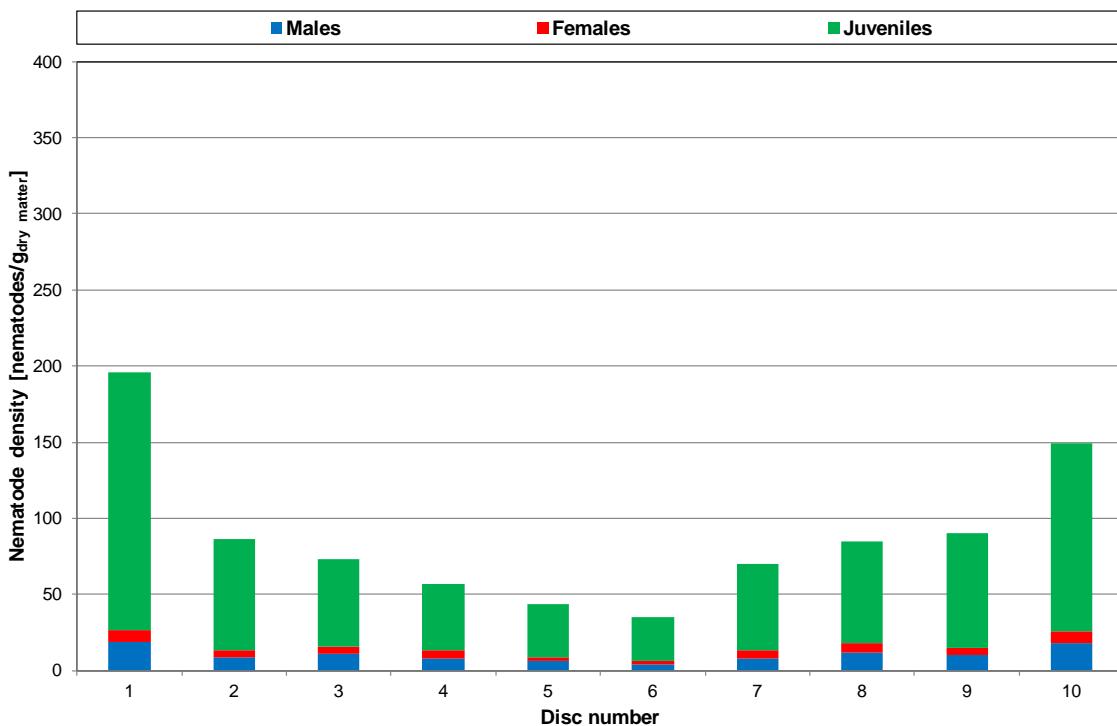


Figure 5.5 Population development: Nematode density [nematodes/g_{dry matter}] (medians) differentiated for males, females and juveniles in all 2 cm wide wood discs of the pine logs for incubation month 5 (26th September - 31st October), n=10

The results of incubation month 2 are excluded from the result part because of mistakes during the test execution. The nematode density in total and differentiated for males, females and juveniles after an incubation time of 35 days at 25 °C was not equal distributed between the pine log segments. In each test month in nearly all cases the highest nematode densities were found in both stem discs of the log ends, followed by the nematode inoculation sites disc no. 3 and 8. The lowest nematode densities occurred on different places between the inoculation sites. The maximum nematode density appeared in disc no. 10 in month 4 (23rd August - 27th September) with 2168 nematodes/g_{dry matter}, whereby this month showed on average the highest densities (medians). In disc no. 7 in month 3 (24th July - 28th August) the minimum nematode density was found with 1 nematode/g_{dry matter}. On average the lowest densities (medians) appeared in month 5 (26th September - 31st October). In all segments in each test month all studied nematode stages - males, females and juveniles – could be recorded. The number of juveniles was higher compared to the adult stages. Males and females were found in a similar proportion.

Objective 5.1.3 (Work of B5): Test of the long-term survival of PWN in infested *P. sylvestris* wood chips under different environmental conditions for one year

Materials and methods:

Experimental set-up:

In total 4000 g PWN (PT-6 (w)) infested *P. sylvestris* wood chips were tested. Information about inoculation, incubation and wood chip production is given in chapter 5.2.1. The size of the tested wood chips was limited according to the 2 cm sieve size of the laboratory mill (RETSCH, Germany) with 10 x 20 x 4 mm. After the wood chip production on 27th August 2012, the wood chips were mixed and split in plastic bags one day later. The remained wood chip bags in chapter 5.2.1 (work of B5) were stored for five weeks at 25 °C in the greenhouse until start of the long-term survival

experiment. On 2nd October 2012 the wood chips were mixed and split in samples. Five 22 g samples were tested for the infestation with PWN and moisture content at test start. According to the amount of wood chips, the minimum sample size, variants, replicates and investigation dates approx. 22 g-samples were tested for each variant. Sample size seems to be very small, but it was assumed if nematodes survive under these conditions, they probably would survive in bigger lots as well. So the current test could be seen as worst case scenario. The tested variants were PWN infested wood chips allowed to air-dry and sealed in plastic bags (Figure 5.6) at 15 °C (on average 76 % rH) and 25 °C (on average 58 % rH) in greenhouse compartments and climate chambers. The sealed samples were covered to avoid heating and therefore perspiration by sunlight. After 41 days 5 samples per variant were regularly examined for living PWN on nine extraction dates for one year (together 180 samples).



Figure 5.6 *P. sylvestris* wood chips infested with *B. xylophilus* (left: allowed to air-dry, right: sealed in plastic bag)

Nematode survival in wood chips:

PWN were extracted by placing the wood chip samples on funnels (Baermann-funnel technique) after weighting. The moisture content of the wood chips was determined. PWN were identified, fixated and counted as nematodes per gram dry matter.

Results:

Different samples were used per extraction date. Lower nematode densities were found over time of wood chip storage. During the investigation the nematode density of the 22 g wood chip samples decreased from 374 nematodes/g_{dry matter} (median of five samples) initially to 0 nematodes 369 days for the wood chips allowed to air-dry at 15 C and 25 C (Figure 5.7 and

Table 5.2). During the whole test time on nine extraction dates with 41 day time spans, these two variants showed the lowest values, especially the 25 C variant. Nematodes could be extracted from the 25 C air-dried wood chips up to 123 days (over 17.5 weeks) after the test start. Sealing of the logs had a positive influence on the nematode survival. At the end of the test, after more than one year, nematodes were still present in both sealed variants. The higher temperature combined with a lower relative humidity of the air resulted in a reduced PWN density by the air-dried variants, but did not show this tendency for all samples sealed in plastic bags. For most dates the 15 C variant had higher nematode densities, but at test start and test end the 25 C variant showed bigger values. Therefore at trial end the highest nematode density was found for the variant wood chips in plastic bags at 25 C with 57 nematodes/g_{dry matter} (median).

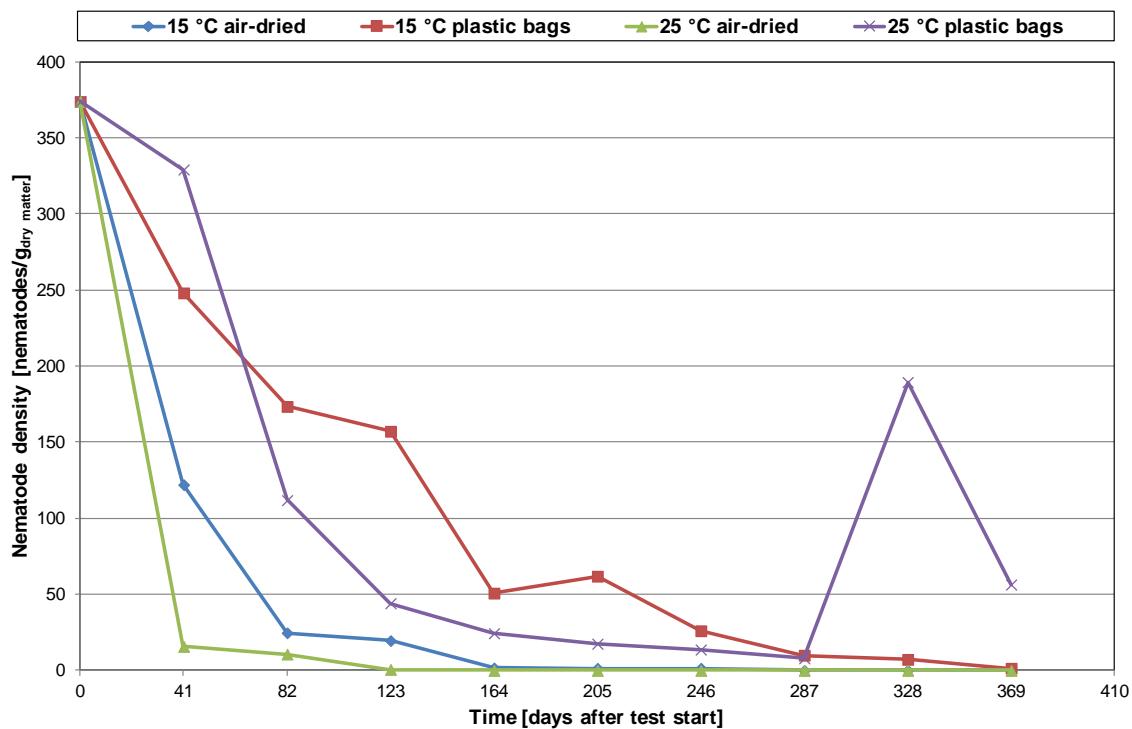


Figure 5.7 Nematode density [nematodes/g_{dry matter}] (medians) dependent on storage temperature, condition (open/ sealed) and time, n=5

Table 5.2 Nematode density [nematodes/g_{dry matter}] (median, minimum, maximum) dependent on storage temperature, condition (open/ sealed) and time, n=5

Variant	Time [days]	Nematode density [nematodes/g_{dry matter}]		
		Median	Min	Max
Start	0	373.9	189.7	487.4
15 °C air-dried	41	122.1	30.0	172.6
15 °C plastic bags		248.1	197.8	312.3
25 °C air-dried		15.8	10.1	106.4
25 °C plastic bags		329.3	267.2	369.7
15 °C air-dried	82	24.7	15.9	51.4
15 °C plastic bags		173.5	39.4	194.0
25 °C air-dried		10.7	6.8	24.0
25 °C plastic bags		111.9	82.3	188.8
15 °C air-dried	123	19.6	5.1	27.7
15 °C plastic bags		157.2	119.4	187.7
25 °C air-dried		0.7	0.4	2.0
25 °C plastic bags		44.2	35.5	51.4
15 °C air-dried	164	1.6	1.3	7.9
15 °C plastic bags		50.8	22.2	69.2
25 °C air-dried		0.0	0.0	0.0
25 °C plastic bags		24.5	11.4	30.7
15 °C air-dried	205	1.4	0.0	4.2
15 °C plastic bags		61.9	1.0	117.2
25 °C air-dried		0.0	0.0	0.0
25 °C plastic bags		17.5	6.8	23.5
15 °C air-dried	246	1.4	0.0	4.0
15 °C plastic bags		26.1	0.4	39.2
25 °C air-dried		0.0	0.0	0.0
25 °C plastic bags		13.7	10.5	69.6
15 °C air-dried	287	0.0	0.0	1.4
15 °C plastic bags		10.0	1.8	20.3
25 °C air-dried		0.0	0.0	0.0
25 °C plastic bags		8.0	4.4	152.9
15 °C air-dried	328	0.1	0.0	0.3
15 °C plastic bags		7.3	3.8	11.3
25 °C air-dried		0.0	0.0	0.0
25 °C plastic bags		189.5	70.5	252.6
15 °C air-dried	369	0.0	0.0	0.0
15 °C plastic bags		1.4	0.3	2.2
25 °C air-dried		0.0	0.0	0.0
25 °C plastic bags		56.5	0.0	150.5

Figure 5.8 shows the MC of all wood chip variants (different samples per extraction date) during the storage time. After the test start the MC decreased for the air-dried variants from 210 % (median) to 22 % (at 15 °C) and 18 % (at 25 °C) over a period of 41 days to come closer to the equilibrium moisture content under the storage conditions, which was with 192 % the biggest drop. Afterwards the MC did not change very much, which would be expected for wood that had reached equilibrium MC. The sealed variants maintained the MC from the test start. The temperature in combination

with the relative humidity of the air influenced the air-dried variants only. At 25 C and lower rH the wood chips were drier compared to 15 C.

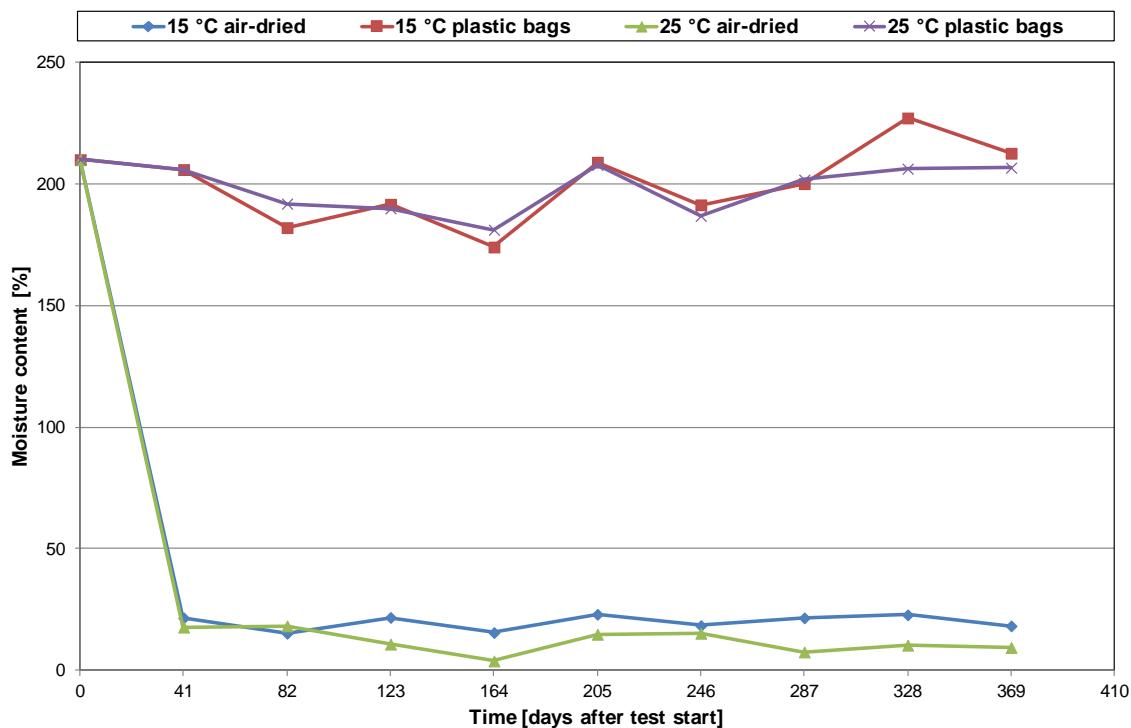


Figure 5.8 Moisture content MC [%] (medians) of wood chips dependent on storage temperature, condition (open/ sealed) and time, n=5

Objective 5.1.4 (Work of B5): Determination of the effect of temperatures occurring in wood chip piles mentioned in literature and the period at the same temperature level on the survival of PWN using *Botrytis cinerea* infested agar Petri dishes as substitute material

This test will replace the original plan to test *B. mucronatus* as a native substitute organism for *B. xylophilus* in a wood chip pile on the field of the Julius Kühn-Institut Braunschweig. A literature review already gave good results which temperature occurs in smaller chip piles. The review already showed that large chip piles are necessary to reach the known lethal temperatures for PWN. Negotiations with chip producers to carry out the planned tests under operational conditions failed, because the scientific needs (insertion of measurement equipment, test organisms) including to keep the chip pile on hold, could not be guaranteed. Placing a big chip pile on the institute field would result in costs of up to €10,000, which is not covered by the budget of B5. Smaller chip piles would be cheaper and within the project budget, but suffer from too low temperatures. Therefore the tests will be changed and the temperatures to be tested will be based on literature data as well as the results of investigations of heat development in bark chip piles carried out by B6. Using those temperatures, laboratory scaled investigations on survival of *B. xylophilus* over the time will be carried out using *B. cinerea* infested agar Petri dishes and climate chambers. This test will be carried out in 2014.

5.2 Transmission of *B. xylophilus* with wood, wood chips or bark to healthy trees.

5.2.1: Laboratory scale investigations will be carried out concerning potential transfer of *B. xylophilus* in wood chips to living pine trees. Different sizes of wood chips infested with *B. xylophilus* will be placed at a range of distances from the trees, superficially or incorporated in the soil, to healthy, wounded and cut seedlings of *Pinus sylvestris*. Trees will be assessed for symptom development and nematode occurrence under temperature conditions optimal (20°C) and sub-optimal (<20°C) for nematode development. B5 to coordinate.

5.2.2: Corresponding outdoor investigations are planned to be carried out in the native range of PWN (i.e. Canada and USA) in areas where pine wilt expression has not been recorded and which, therefore, would provide a proxy for conditions in northern Europe where it is not currently expected that pine wilt would be expressed. B5 to coordinate with inputs by other Beneficiaries in this WP and by collaboration with international contacts.

5.2.3: In Portugal, laboratory experiments will take place during Spring and Summer using small *P. pinaster* (3-4years old) placed in individual containers with sandy soil, in greenhouse at the laboratory of B6. Randomly selected trees will be distributed through one of eight treatments. These will be based on sandy soil mixed with chips of PWN-infested wood, regularly watered to keep it continuously wet, sandy soil mixed with chips of PWN-infested wood, with minimum watering to mimic soil moisture deficit, but while keeping the pines alive and the corresponding two sets of control conditions using chips of uninfested wood. Each set of conditions will be tested with pines with intact roots and pines with artificially damaged roots. Humidity content and temperature of the soil will be recorded continuously. The sanitary condition of the pines will be evaluated weekly; samples of the wood chips and soil will be periodically recovered to study the abundance and persistence of the PWN. As soon as any wilting symptoms appear, the pines will be fully sampled for the presence of the PWN, divided into crown, stem and root zones. At the end of the experiment, all pines will be sampled for PWN infestation regardless of the symptoms. The quantities of nematodes in the woodchips and in the soil (near and distant from the roots) will also be analysed, along with samplings of the fungal community in the wood substrates and any mycorrhizal fungi present. B6 to lead.

5.2.4 Transmission of *B. xylophilus* or closely related species of the genus from sawn wood boards to healthy trees. Laboratory experiments will be conducted using 4 to 5 year old seedlings of the species *P. sylvestris*. Small pieces of wood infested with PWN will be attached to the stems either with wounding the tree (e.g. attaching the wood with needles or thumbnails) or not wounding the tree by fitting the wood pieces by lashing. Outdoor experiments using trees of different ages and therefore different thickness of bark will be carried out with boards infested with the non-quarantine species *B. mucronatus*. Seedlings and trees will be assessed at various time intervals to see whether and after which time nematodes can be isolated from the tree. B5 to coordinate.

5.2.5 PWN infested bark will be produced by artificial inoculation of *P. sylvestris* under quarantine conditions. Similar to the above investigations will be carried out using bark pieces instead of wood chips. The work will be done using *P. sylvestris* seedlings 4-5 years old. B5 to coordinate.

Objective 5.2.1 (Work of B5): Test of the non-vector spread of PWN from artificially infested *P. sylvestris* wood chips to non-infested *P. sylvestris* saplings under laboratory conditions

Materials and methods:

Experimental set-up:

Production of wood chips:

At the end of July 2012 young *P. sylvestris* trees from a pine stand close to Essehof near Braunschweig/ Lower Saxony/ Germany were felled, cut into segments and checked randomly for the absence of nematodes. The log ends were sealed with hot

paraffin wax and stored at 5 C in a climate room for eight days. At the beginning of August about 80 kg of the stored logs (20 to 80 cm long) with a diameter of 8 cm were chosen and inoculated with the PWN isolate PT-6 (w). Holes with 10 mm diameter were drilled (driller: DP4003, Makita Corporation, Anjo, Aichi, Japan) to the middle of the logs with a distance of about 10 cm from each other. One ml suspension of tap water with 1050 nematodes was inoculated in each hole using a pipette. All together 200 ml suspension was used for 80 kg wood. After the holes were closed with wooden pegs the logs were sealed in plastic bags for incubation at 25 C in a climate room for 24 days. The wood chips were produced using a laboratory wood mill (RETSCH, Germany) after stripping of the bark with a peeler iron. According to the 2 cm sieve size of the mill the applied maximum wood chip size was 10 x 20 x 4 mm. Those relatively small chips were not separated into different size fractions, so chips of various sizes (as occurring under operational conditions) were used. The wood chips were mixed and ten 100 g samples which were equally distributed through the pile were tested for nematode density and moisture content at test start. The whole amount of wood chips was divided in 100 g samples and filled in plastic bags.

Test trees:

After at least two weeks pre-air-conditioning of the 3-4 years old *P. sylvestris* saplings (features described in Table 5.3).

Table 5.3 Overview of features of tested *P. sylvestris* trees - Geographic distribution, Dt. Herkunftsgebietsverordnung (Forstvermehrungsgut) – Silvicultural regions of provenance (Forest reproduction item) (HkG), age, height, stem diameter

Geographic distribution (Provenance)	Age [Years]	Height [m]	Stem diameter (stem basis) [cm]
Middle and East German lowland (HkG 851 04)	3-4	0.7	1.4

100 g PWN infested wood chips were placed in each three litter pine pot of the infested variants on 29th and 30th August. The test was run in two greenhouses at 15 C and on average 92 % rH and at 25 C and on average 66 % rH. Figure 5.9 shows all tested variants. Uninjured, stem- or root-injured and cut pines were combined with direct contact of PWN infested wood chips to the stem, wood chips mixed in the soil or wood chips on the soil separated from the stem. The control pines were tested without wood chips. With the stem injury damages during timber harvesting in the forest was mimicked. A scalpel was used to remove one strip of bark at the beginning of the stem part. To produce a root injury ambos shears were used to cut inside the root ball surface. The cut-off trees were felled short above the soil. In both cases for the direct contact to the stem as well as for wood chips on the soil with distance to the stem a sawn off ring of a drainpipe (radius: 3.5 cm, height: 5 cm) was used in the first case to fix as many as possible wood chips directly on the stem and in the second case to guaranty the distance to the stem. For the variation wood chips mixed in the soil wood chip layers and soil layers were one by one put inside the pot because of quarantine reasons (Figure 5.10). For all treatments at least two people were necessary, one who only worked with the trees and soil, another for handling only the wood chips by use of plastic bags and a scoop. Per variant 20 trees were examined (totalling 640 plants).

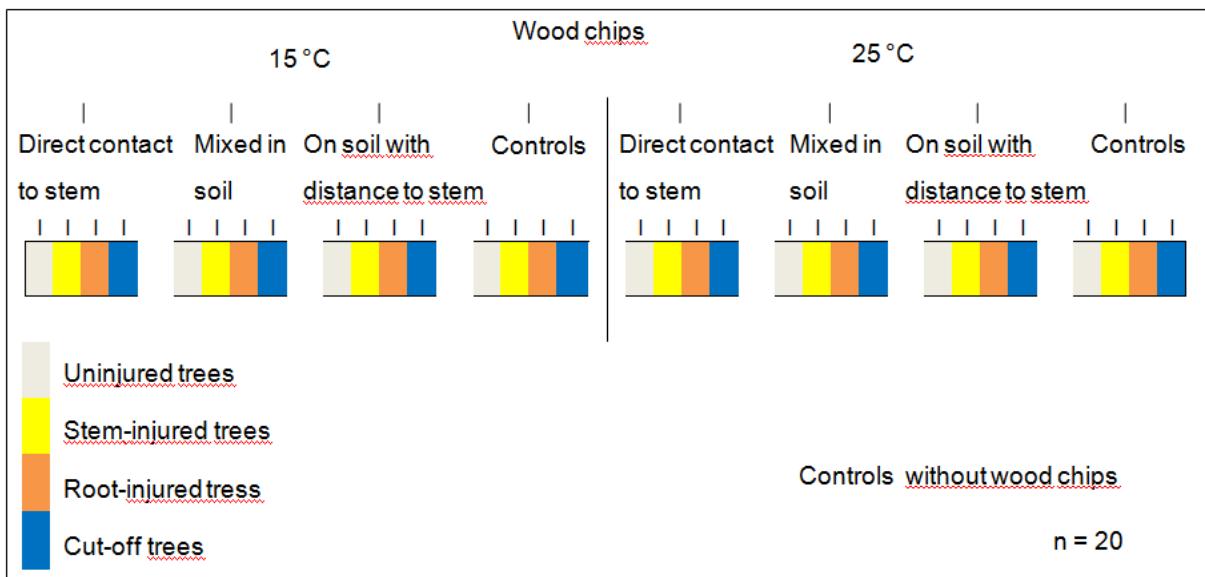


Figure 5.9 Test variants of combined *P. sylvestris* saplings, PWN infested wood chips and temperatures

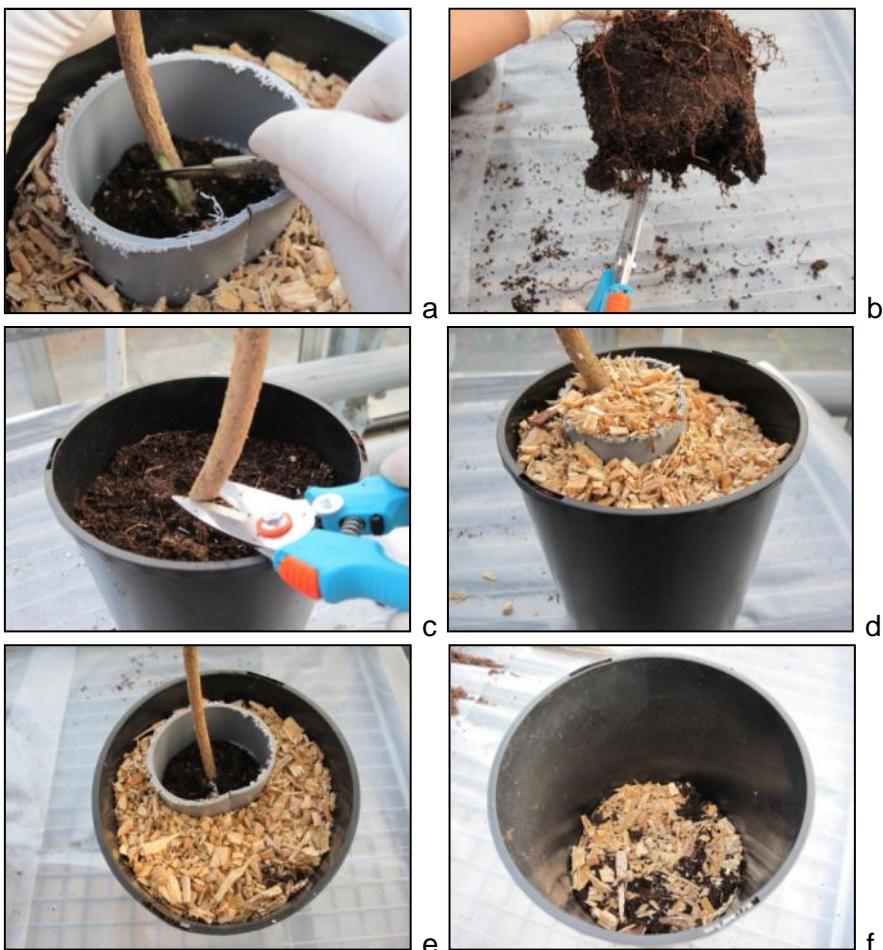


Figure 5.10 Examples for tested wood chip and tree variants; a: Stem-injured pines, b: Root-injured pines, c: Cut-off pines, d: Direct contact of wood chips to the stem, e: Wood chips on the soil with distance to the stem, f: Wood chips mixed in the soil

The trees were watered as required, randomised and evaluated for pine wilt symptoms weekly for 12 weeks. The sampling of trees took place at wilt class 4, which represents 76 – 99% needle discolouration and certainty that the tree will die. Once wilt class 4 is reached no recovery of the tree is possible. Wilt class 5 relates to mortality of the tree but most of the above ground plant parts are dry. According to the results of Daub (2008) nematodes densities tend to decrease in the above ground plant parts with increasing wilt classes. This leads to the situation that at wilt class 5 the risk is high that no nematodes can be re-isolated even though the plant has been killed by pine wilt disease. Although this is also the case for the late stages of wilt class 4 it was decided to sample the tree at wilt class 4 to increase the chance to extract living nematodes. If wilt class 4 was not reached the sampling took place at the end of the study after three months

Assessment of pine wilt and occurrence of PWN in trees:

To evaluate the wilt symptoms of the pine saplings a rating scheme of wilt classes according to Daub (2008) (Table 5.4) was used. The six wilt classes (0 to 5) represent the percentage of needle discolouration of the whole foliage, which is related to the physiological condition of the plant.

Table 5.4 Rating scheme of wilt classes for assessment of pine wilt (Daub 2008)

Wilt class	Tree coverage of needle discolouration [%]	Physiological condition
0	0	alive
1	1 – 25	
2	26 – 50	
3	51 – 75	
4	76 – 99	
5	100	dead

The saplings were sampled by cutting off the stem using ambos shears above the contact point with the infested wood chips at the lower end of the stem. The small lower stem part under the cut-off point was excluded to reduce possible contamination by external PWN on the wound and bark. Exclusively for the cut-off pine variants the root parts including the root collar were tested for nematodes. For all other variants exclusively the plant part above the cutting point was analysed. In addition, the moisture content of the upper plant material (without needles) was recorded. PWN were identified, fixed and counted as nematodes per gram dry matter.

Results:

The 100 g samples of wood chips, which were placed inside the pot of each test pine, were infested with on average 552 ± 252 PWN/g_{dry matter} (mean wood MC: $194 \pm 6\%$, n=10).

The distribution of wilt classes of all tested pine variants, except the cut-off tree variants, dependent on the time are illustrated in the following figures:

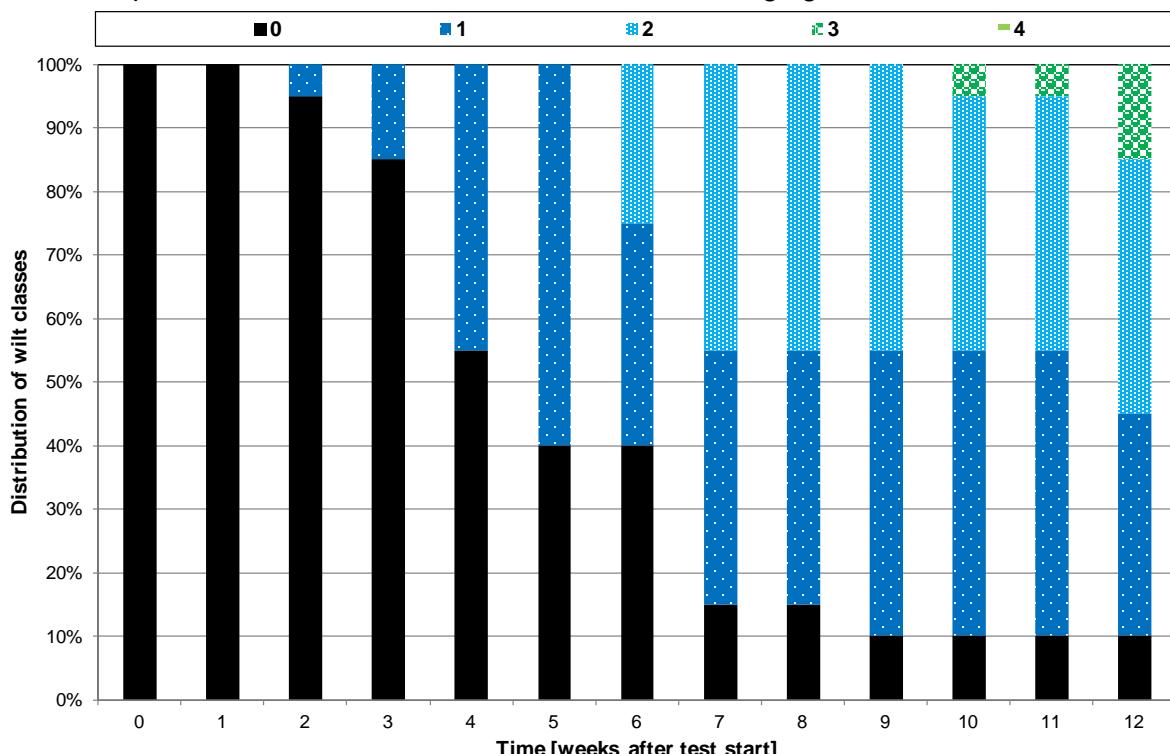


Figure 5.11 Distribution of the wilt classes 0-4 [%] of the variant W 15 D (uninjured pines at 15 °C with direct contact of wood chips to the stem) over time [weeks after test start], n=20

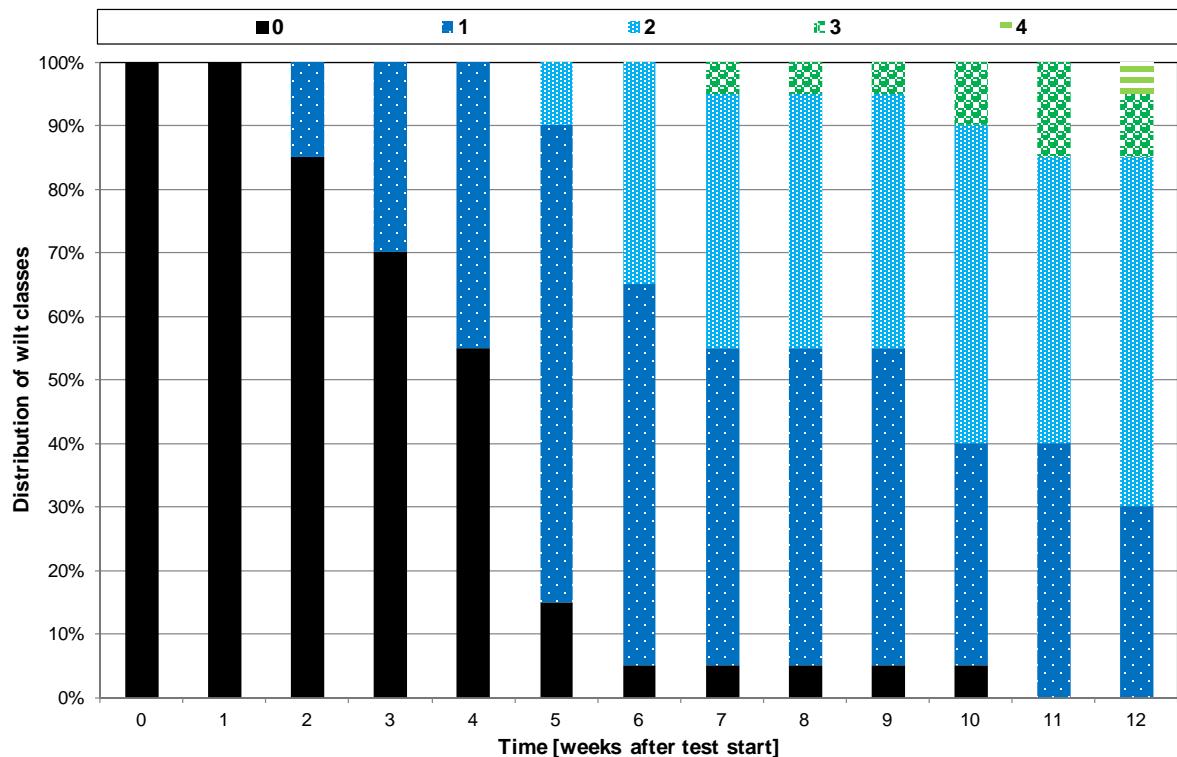


Figure 5.12 Distribution of the wilt classes 0-4 [%] of the variant Y 15 D (stem-injured pines at 15 °C with direct contact of wood chips to the stem) over time [weeks after test start], n=20

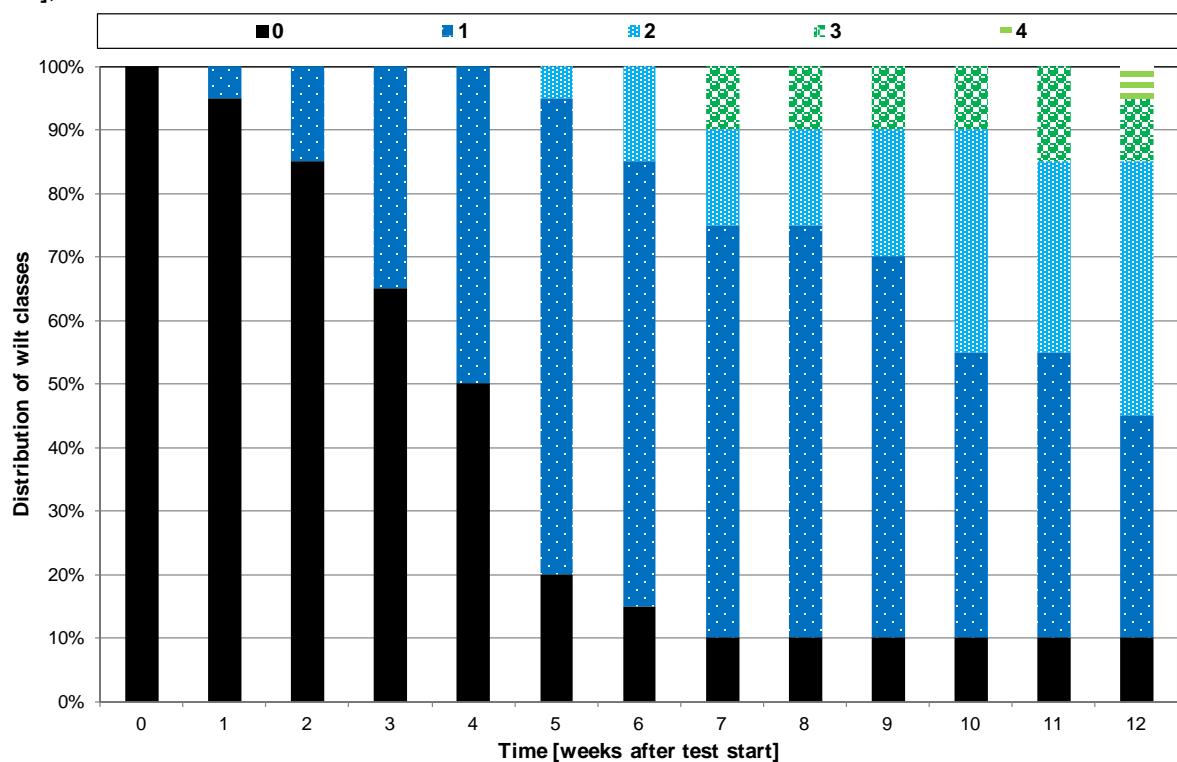


Figure 5.13 Distribution of the wilt classes 0-4 [%] of the variant O 15 D (root-injured pines at 15 °C with direct contact of wood chips to the stem) over time [weeks after test start], n=20

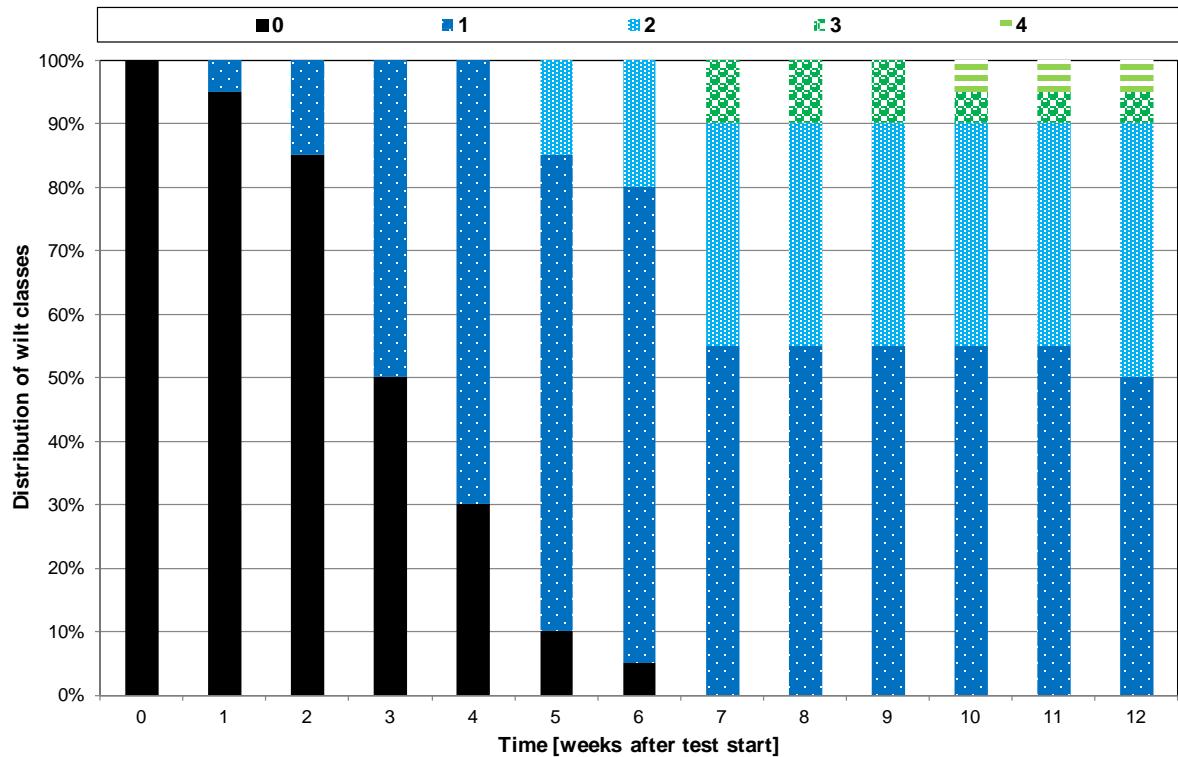


Figure 5.14 Distribution of the wilt classes 0-4 [%] of the variant W 15 I (uninjured pines at 15 °C with wood chips mixed in the soil) over time [weeks after test start], n=20

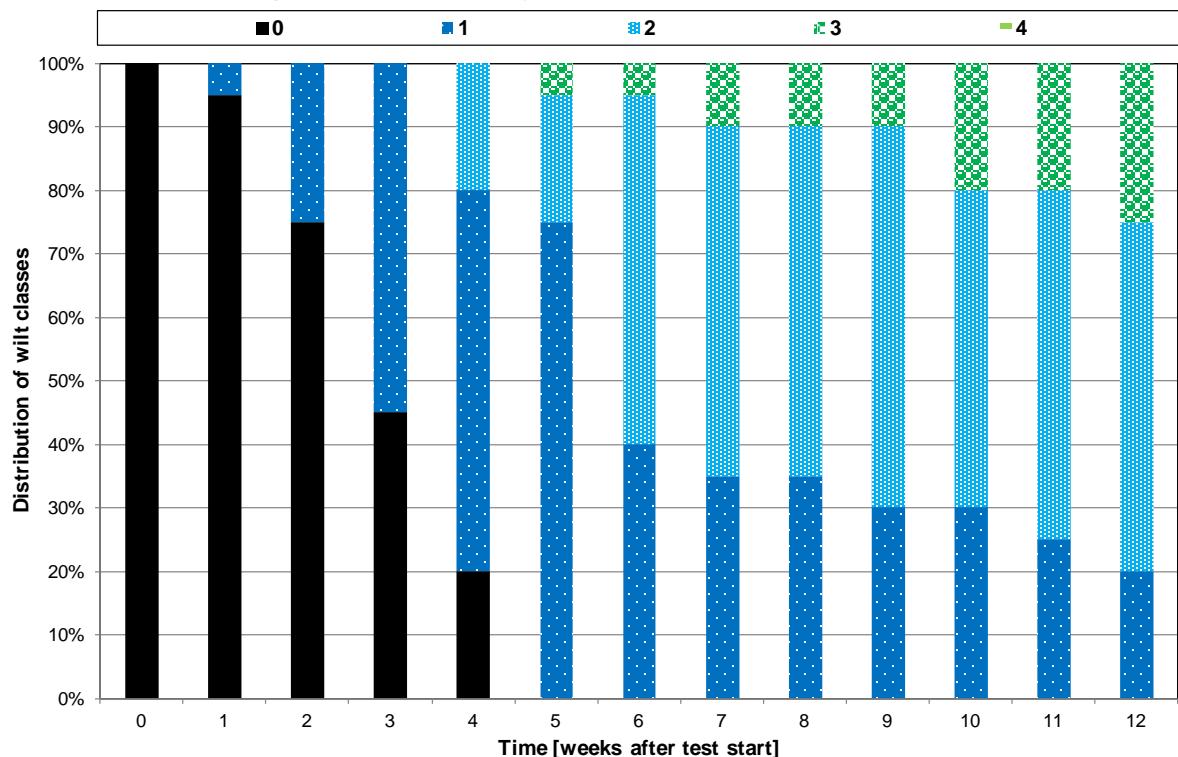


Figure 5.15 Distribution of the wilt classes 0-4 [%] of the variant Y 15 I (stem-injured pines at 15 °C with wood chips mixed in the soil) over time [weeks after test start], n=20

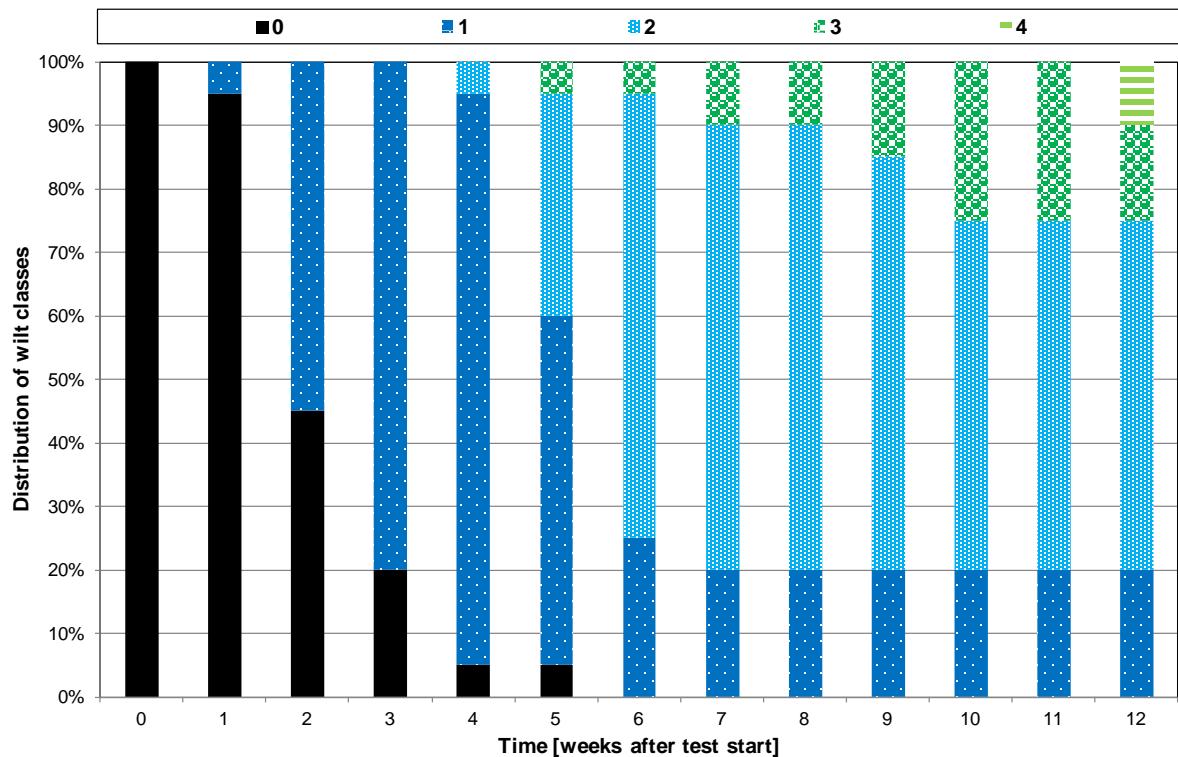


Figure 5.16 Distribution of the wilt classes 0-4 [%] of the variant O 15 I (root-injured pines at 15 °C with wood chips mixed in the soil) over time [weeks after test start], n=20

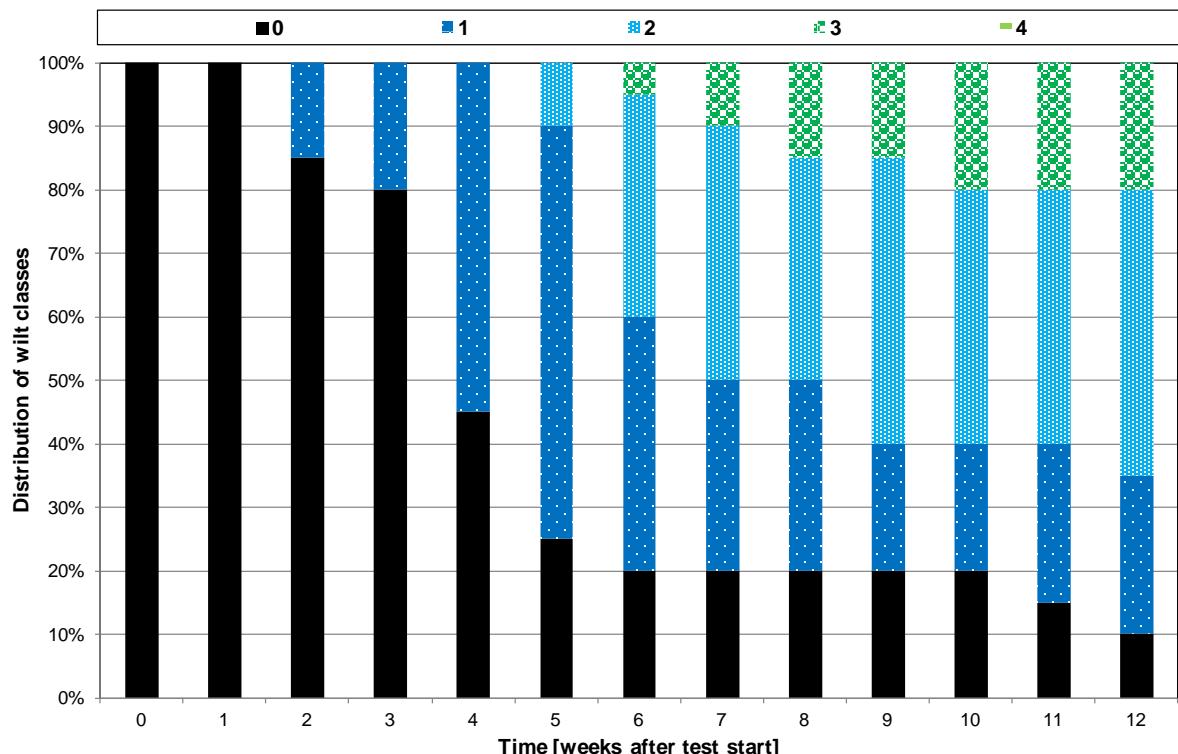


Figure 5.17 Distribution of the wilt classes 0-4 [%] of the variant W 15 A (uninjured pines at 15 °C with wood chips on soil with distance to the stem) over time [weeks after test start], n=20

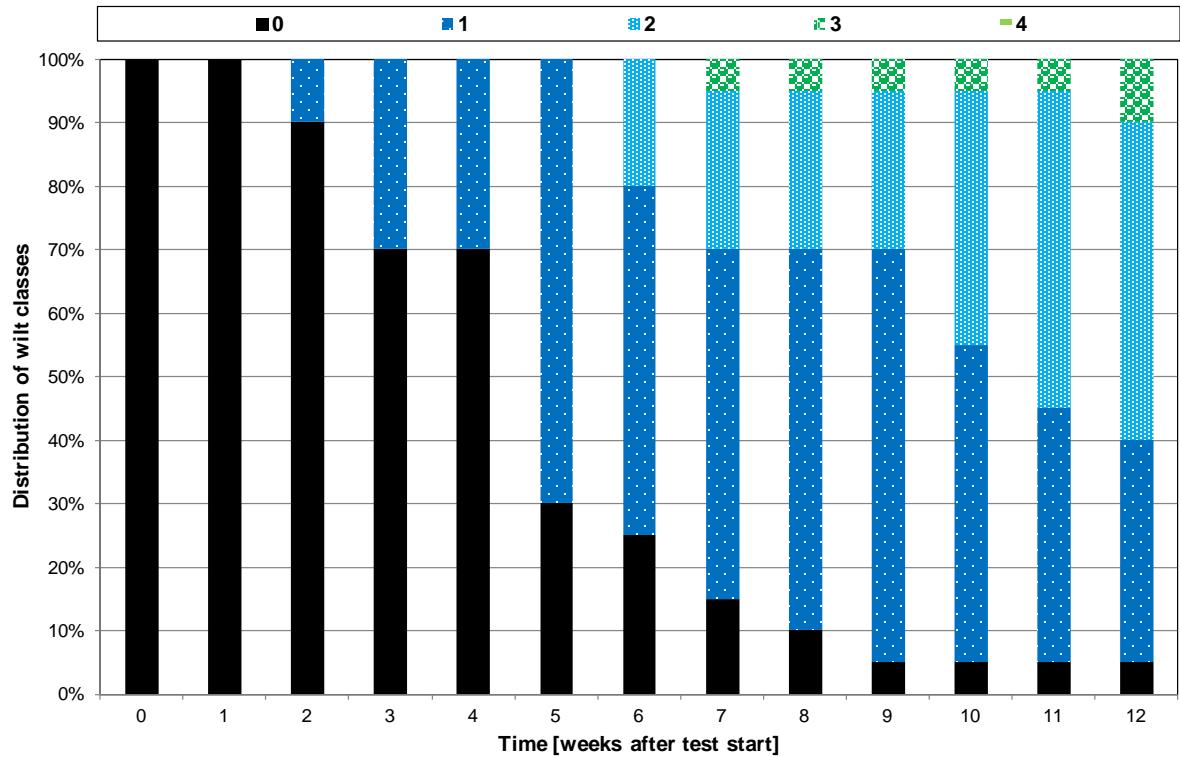


Figure 5.18 Distribution of the wilt classes 0-4 [%] of the variant Y 15 A (stem-injured pines at 15 °C with wood chips on soil with distance to the stem) over time [weeks after test start], n=20

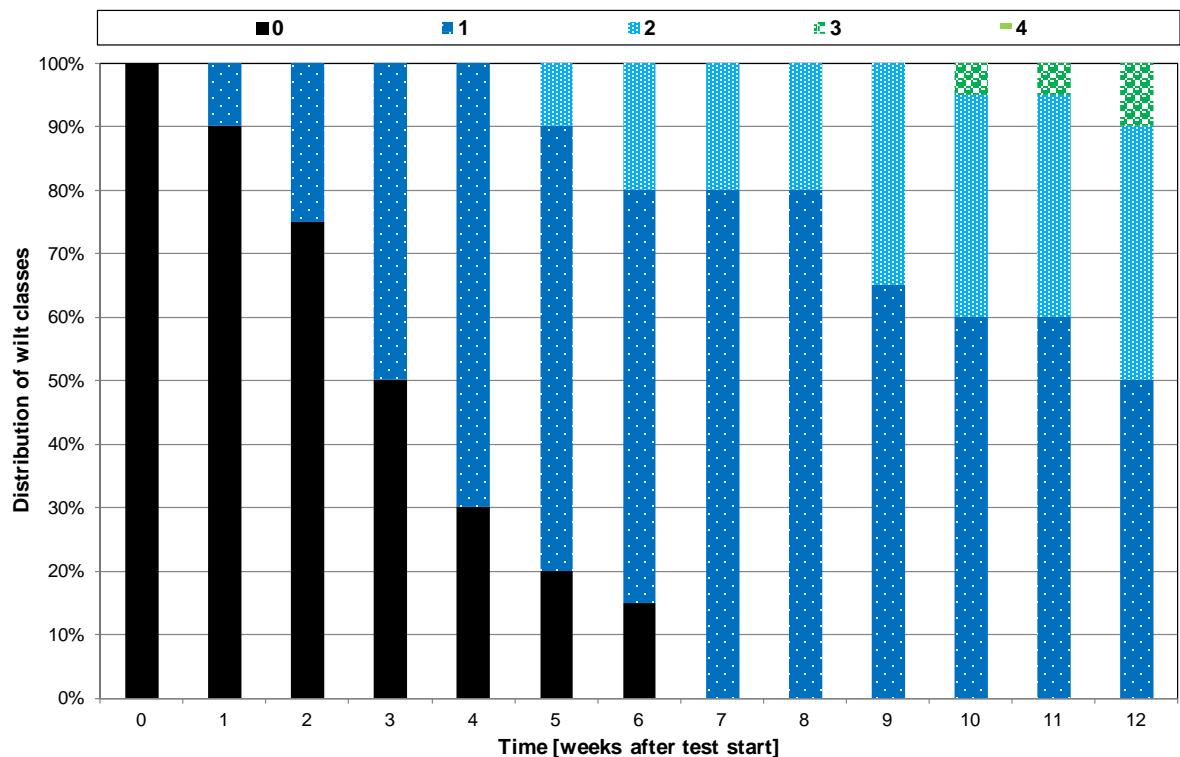


Figure 5.19 Distribution of the wilt classes 0-4 [%] of the variant O 15 A (root-injured pines at 15 °C with wood chips on soil with distance to the stem) over time [weeks after test start], n=20

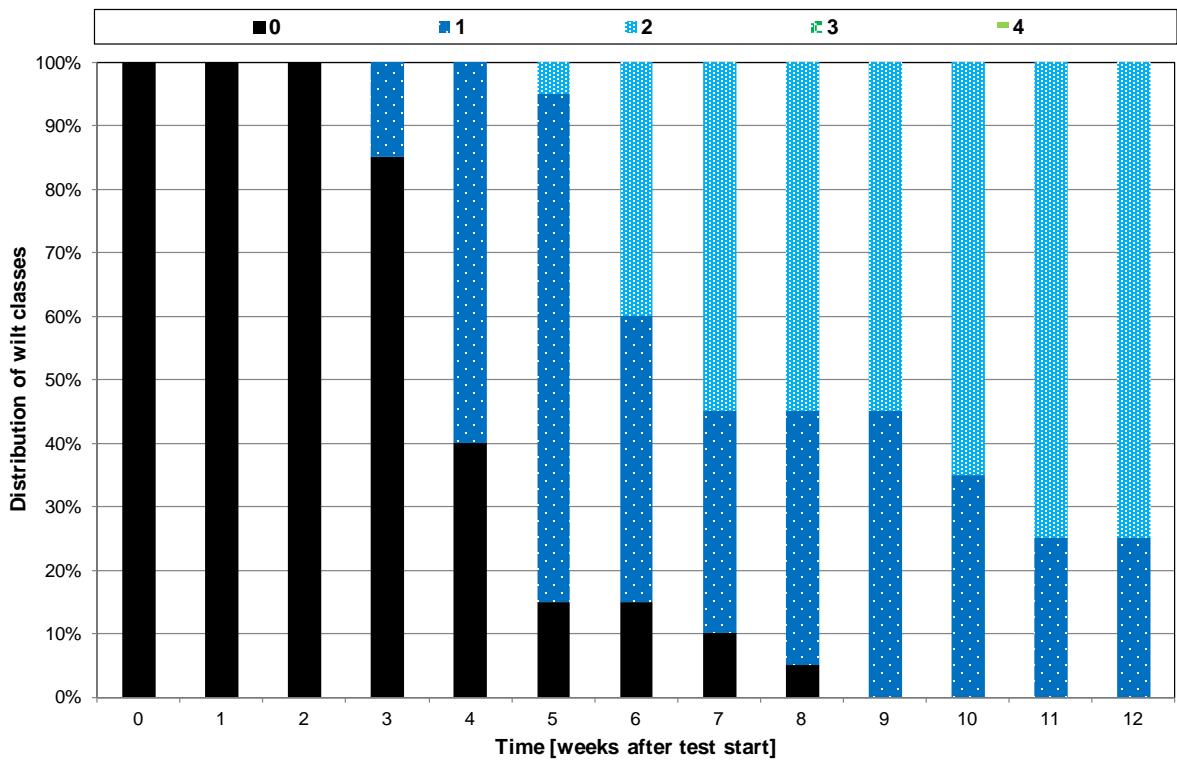


Figure 5.20 Distribution of the wilt classes 0-4 [%] of the control variant W 15 K (uninjured pines at 15 °C without wood chips) over time [weeks after test start], n=20

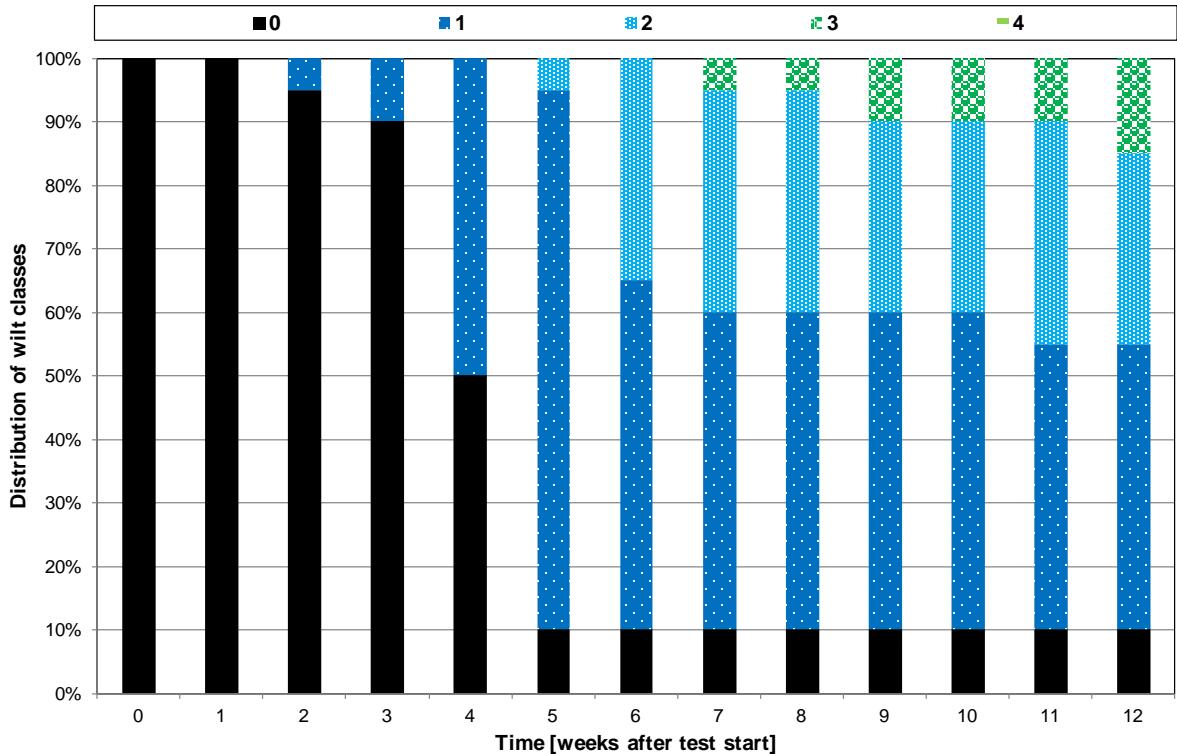


Figure 5.21 Distribution of the wilt classes 0-4 [%] of the control variant Y 15 K (stem-injured pines at 15 °C without wood chips) over time [weeks after test start], n=20

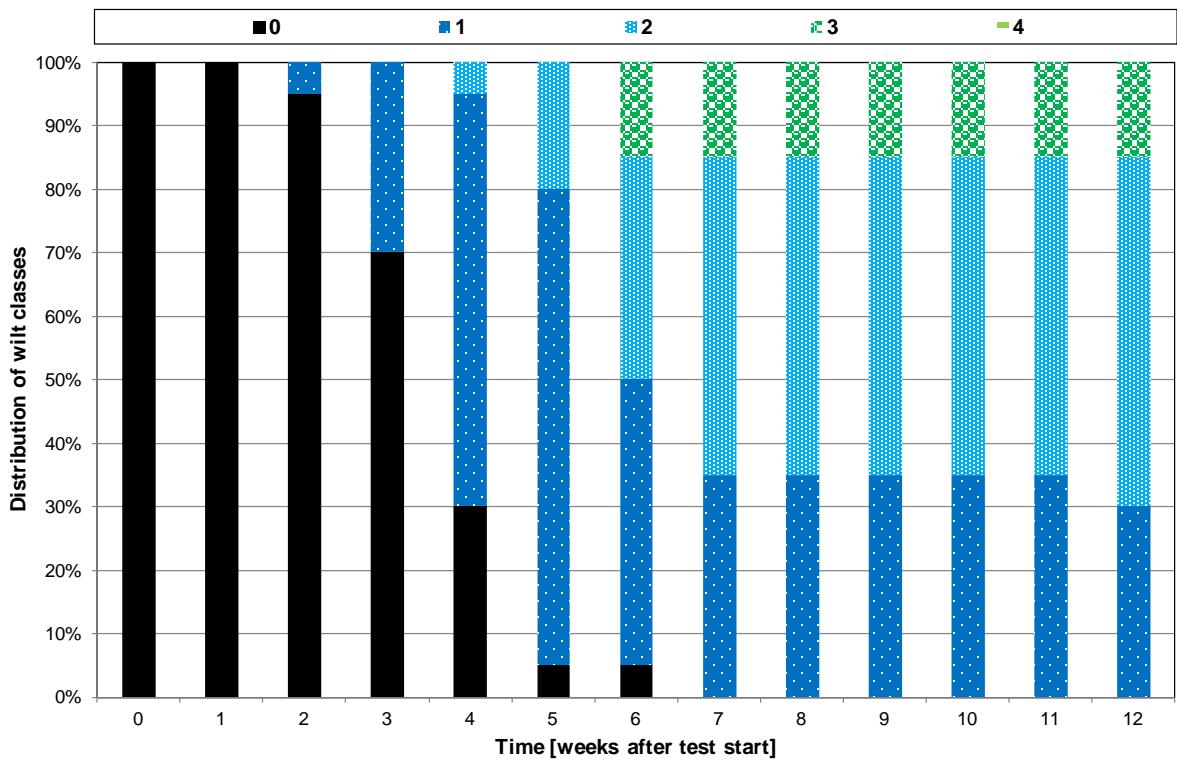


Figure 5.22 Distribution of the wilt classes 0-4 [%] of the control variant O 15 K (root-injured pines at 15 °C without wood chips) over time [weeks after test start], n=20

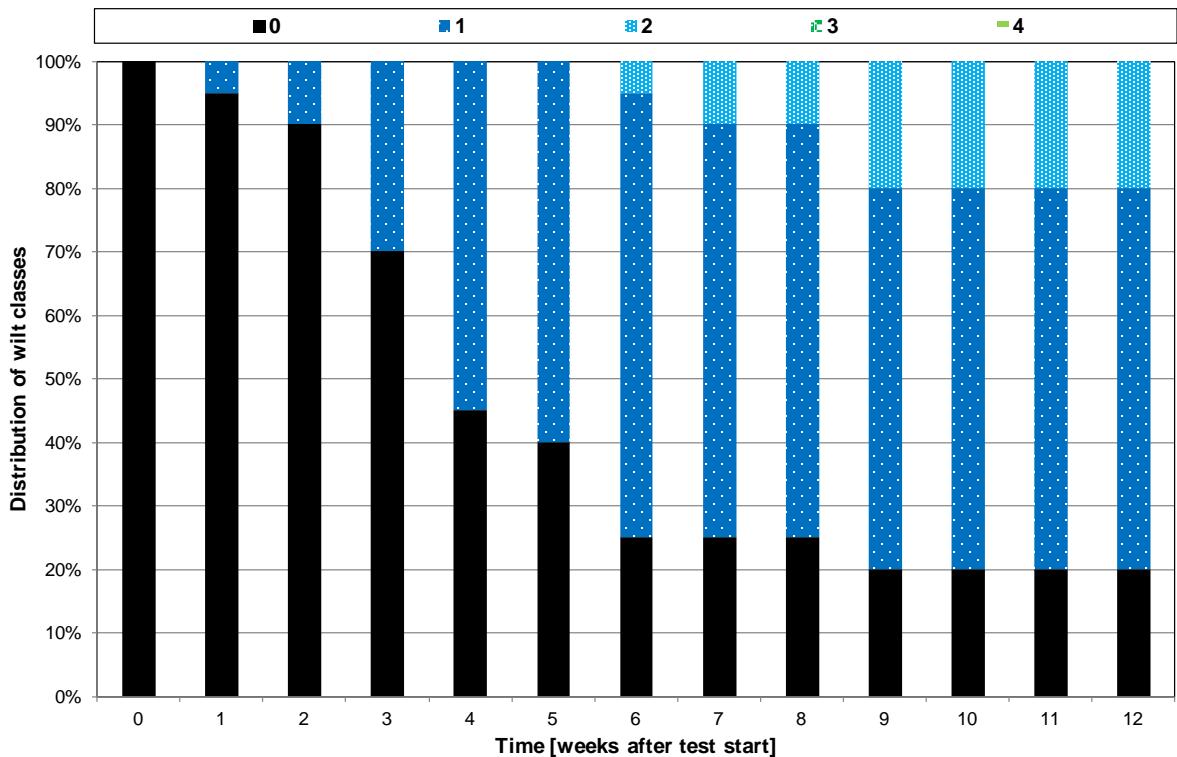


Figure 5.23 Distribution of the wilt classes 0-4 [%] of the variant W 25 D (uninjured pines at 25 °C with direct contact of wood chips to the stem) over time [weeks after test start], n=20

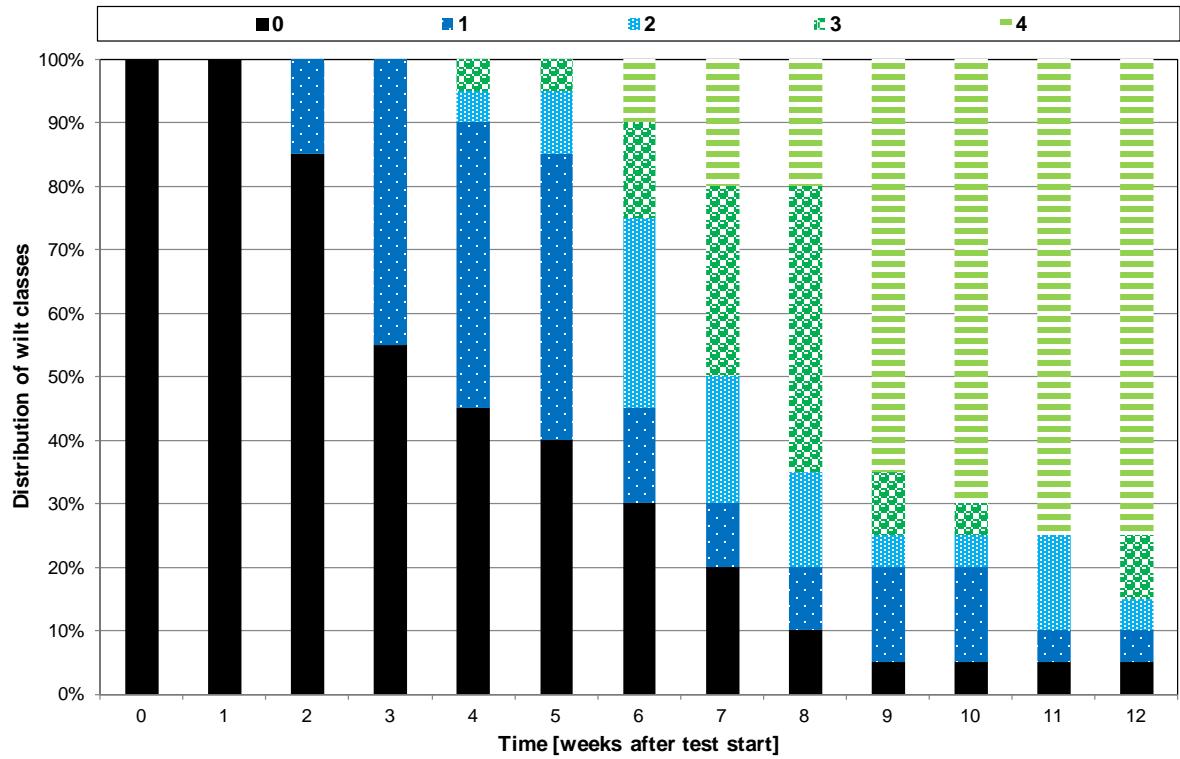


Figure 5.24 Distribution of the wilt classes 0-4 [%] of the variant Y 25 D (stem-injured pines at 25 °C with direct contact of wood chips to the stem) over time [weeks after test start], n=20

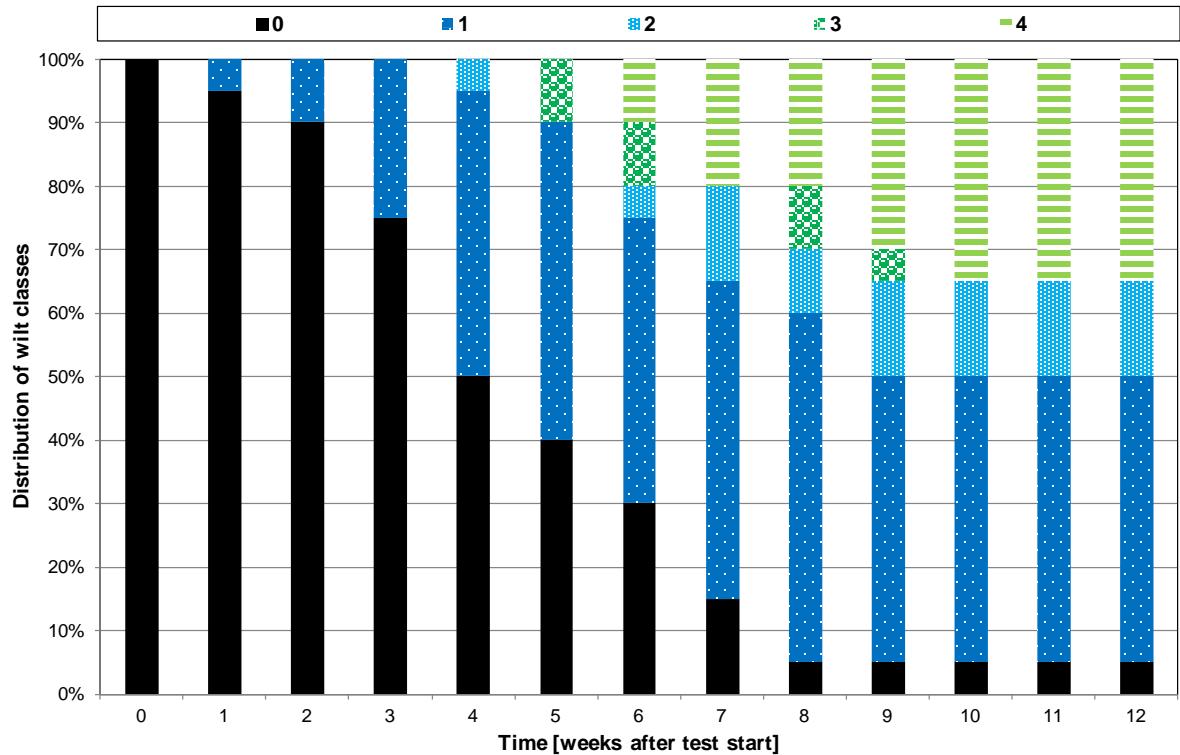


Figure 5.25 Distribution of the wilt classes 0-4 [%] of the variant O 25 D (root-injured pines at 25 °C with direct contact of wood chips to the stem) over time [weeks after test start], n=20

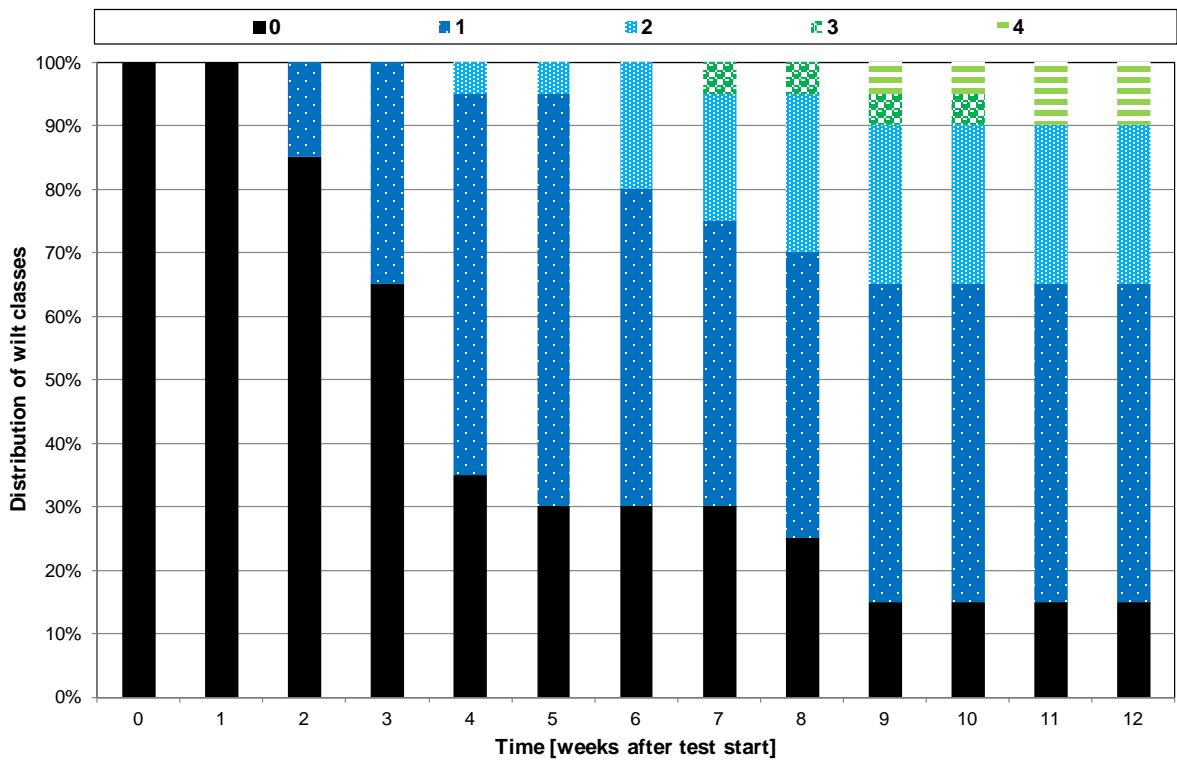


Figure 5.26 Distribution of the wilt classes 0-4 [%] of the variant W 25 I (uninjured pines at 25 °C with wood chips mixed in the soil) over time [weeks after test start], n=20

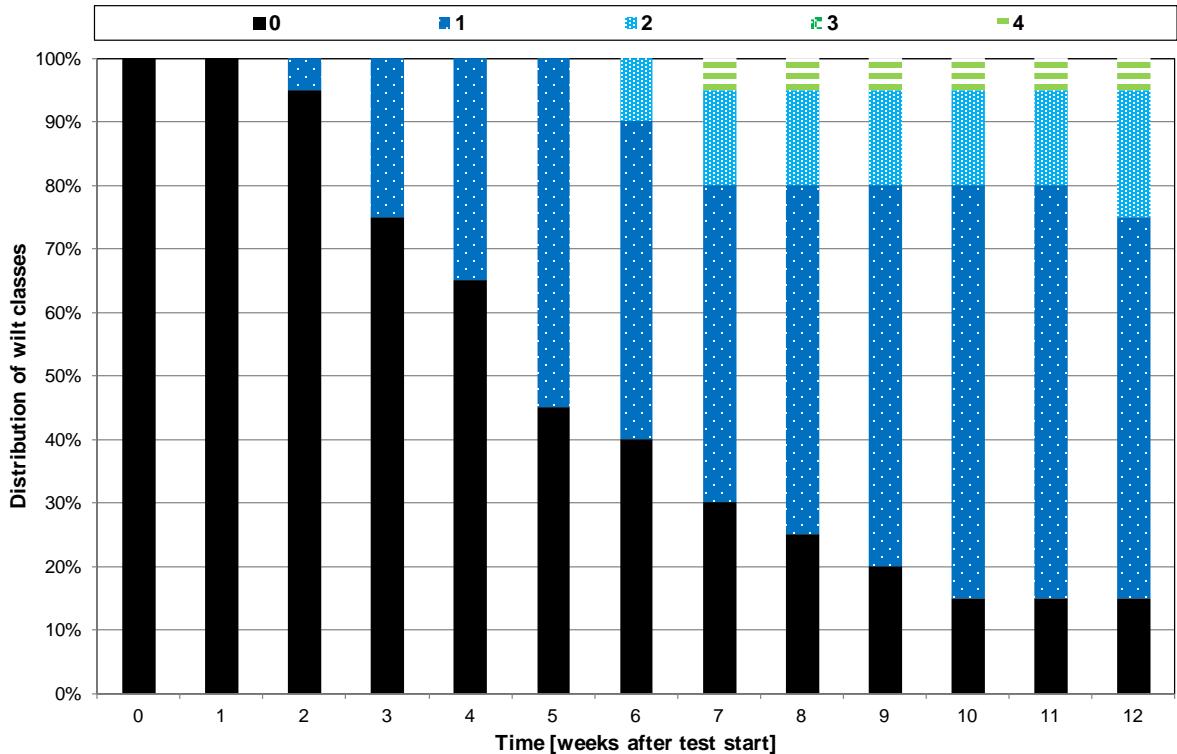


Figure 5.27 Distribution of the wilt classes 0-4 [%] of the variant Y 25 I (stem-injured pines at 25 °C with wood chips mixed in the soil) over time [weeks after test start], n=20

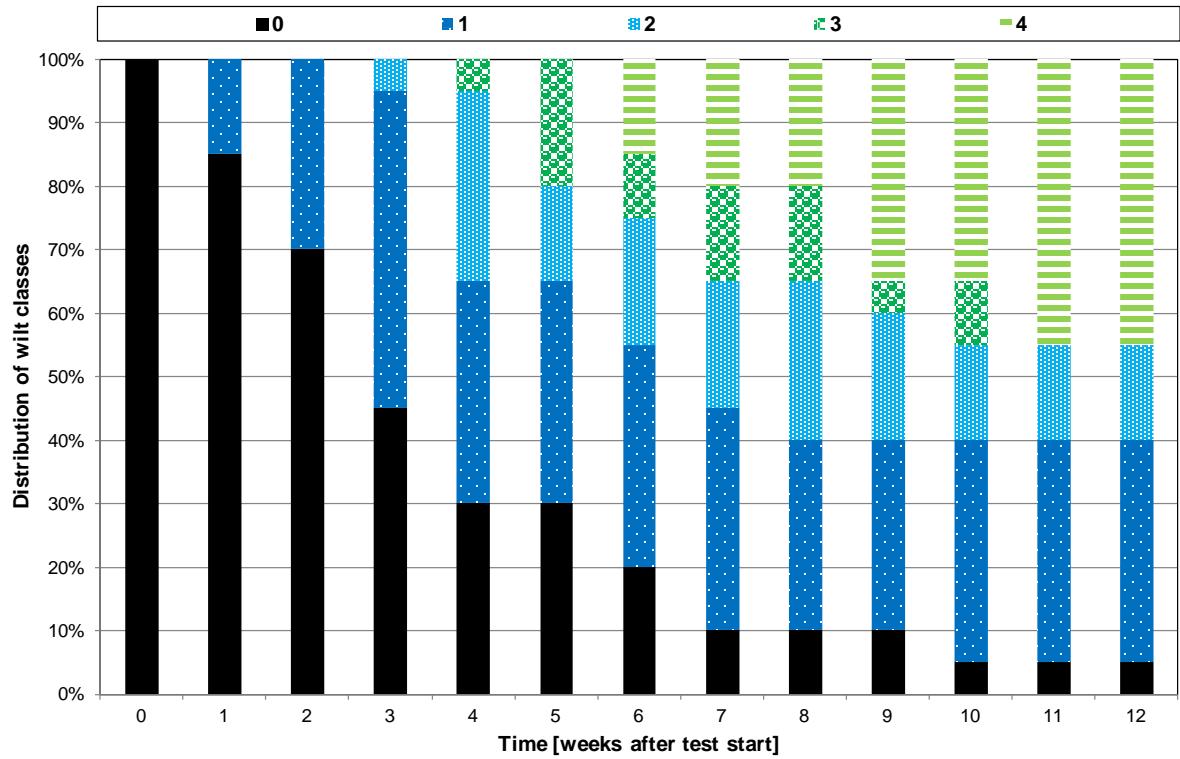


Figure 5.28 Distribution of the wilt classes 0-4 [%] of the variant O 25 I (root-injured pines at 25 °C with wood chips mixed in the soil) over time [weeks after test start], n=20

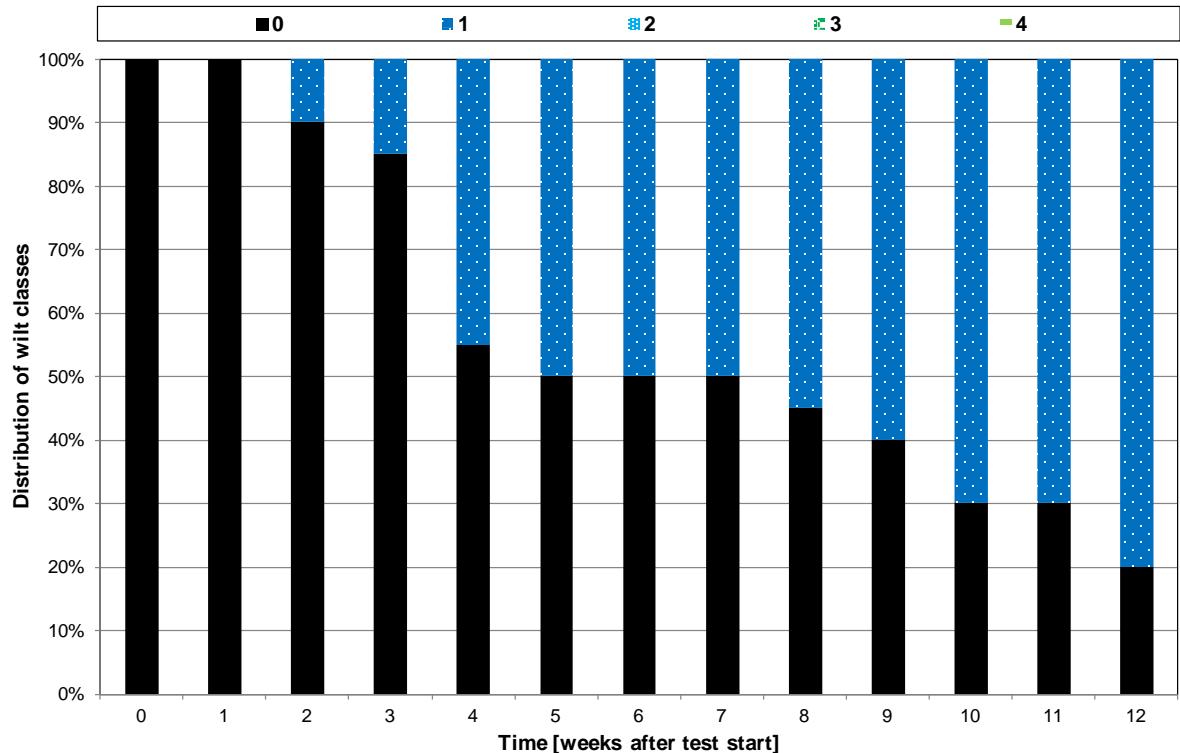


Figure 5.29 Distribution of the wilt classes 0-4 [%] of the variant W 25 A (uninjured pines at 25 °C with wood chips on soil with distance to the stem) over time [weeks after test start], n=20

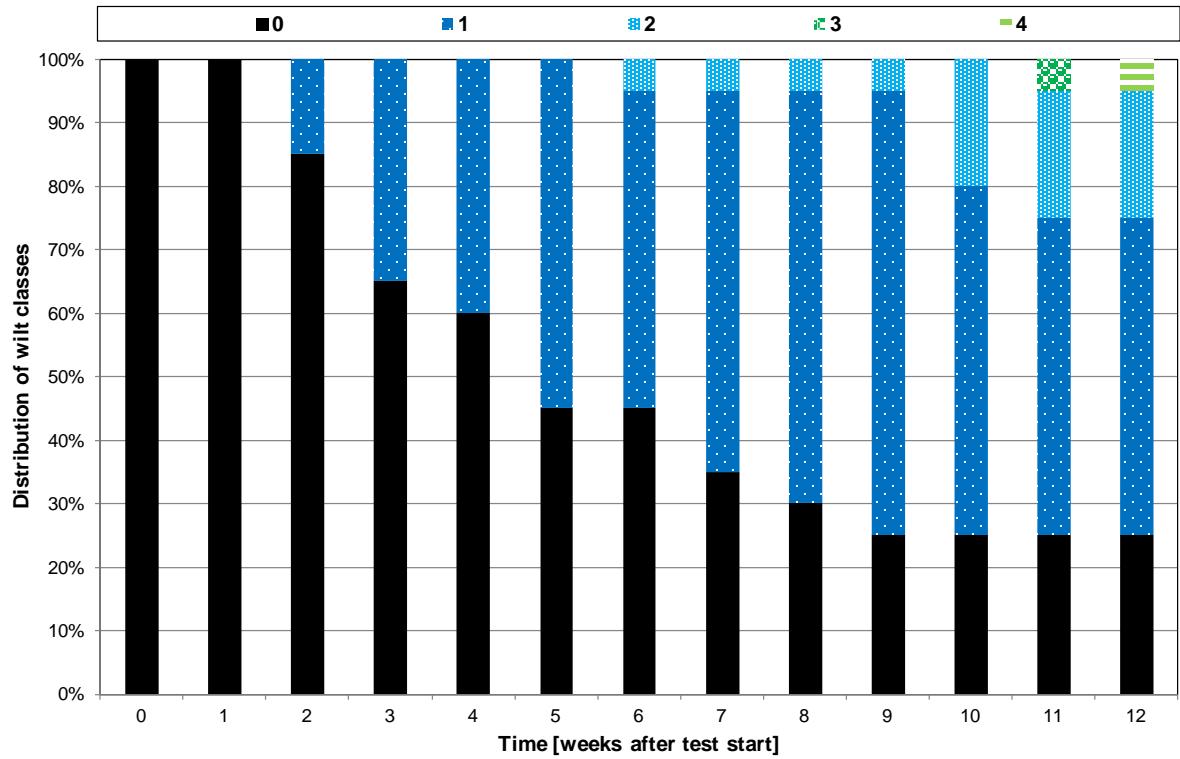


Figure 5.30 Distribution of the wilt classes 0-4 [%] of the variant Y 25 A (stem-injured pines at 25 °C with wood chips on soil with distance to the stem) over time [weeks after test start], n=20

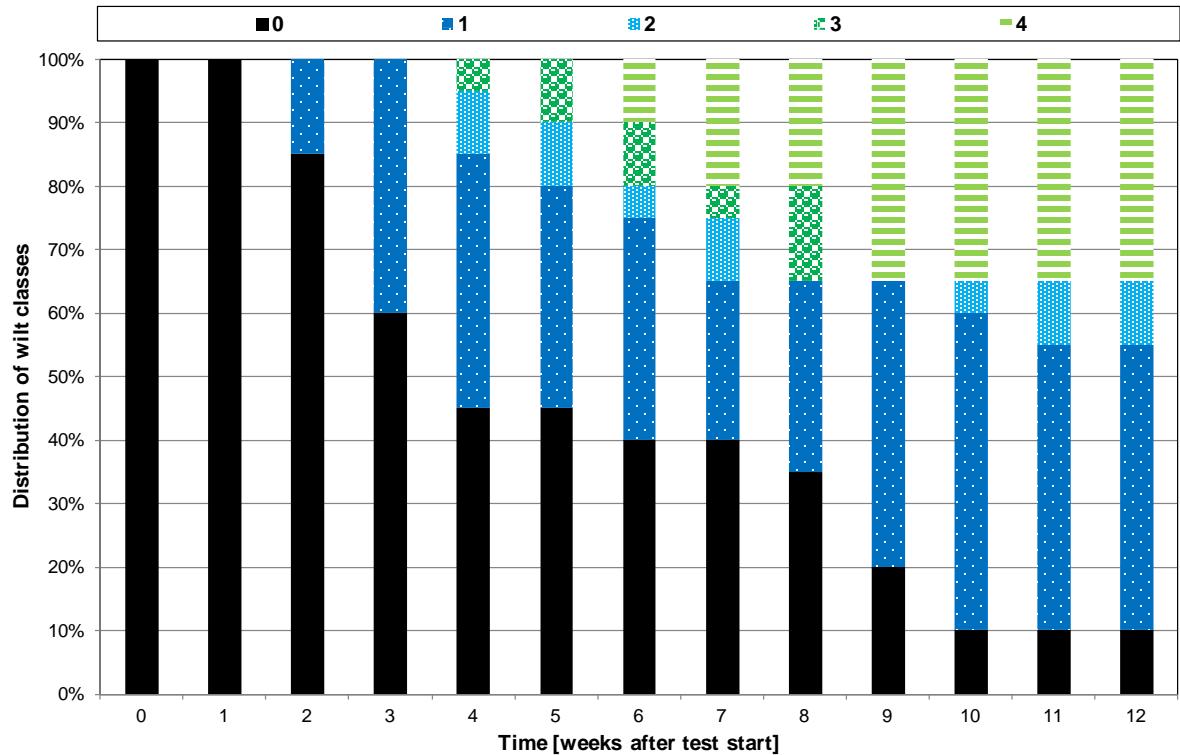


Figure 5.31 Distribution of the wilt classes 0-4 [%] of the variant O 25 A (root-injured pines at 25 °C with wood chips on soil with distance to the stem) over time [weeks after test start], n=20

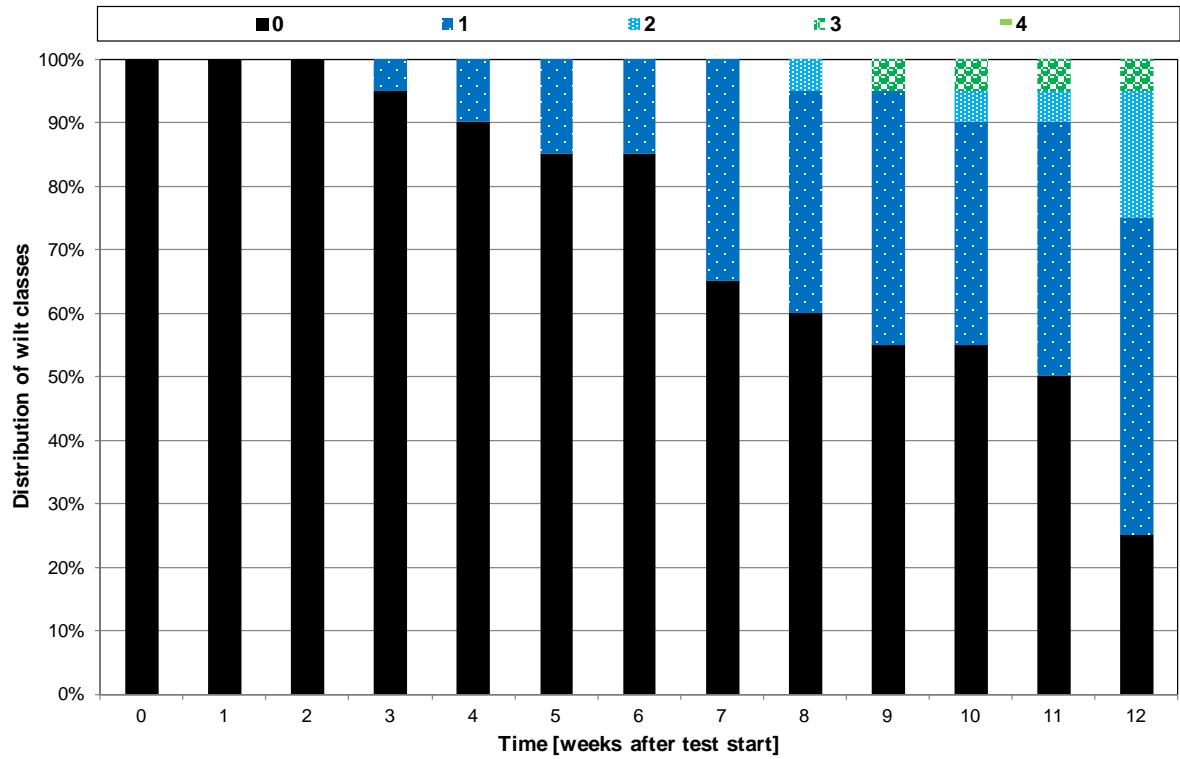


Figure 5.32 Distribution of the wilt classes 0-4 [%] of the control variant W 25 K (uninjured pines at 25 °C without wood chips) over time [weeks after test start], n=20

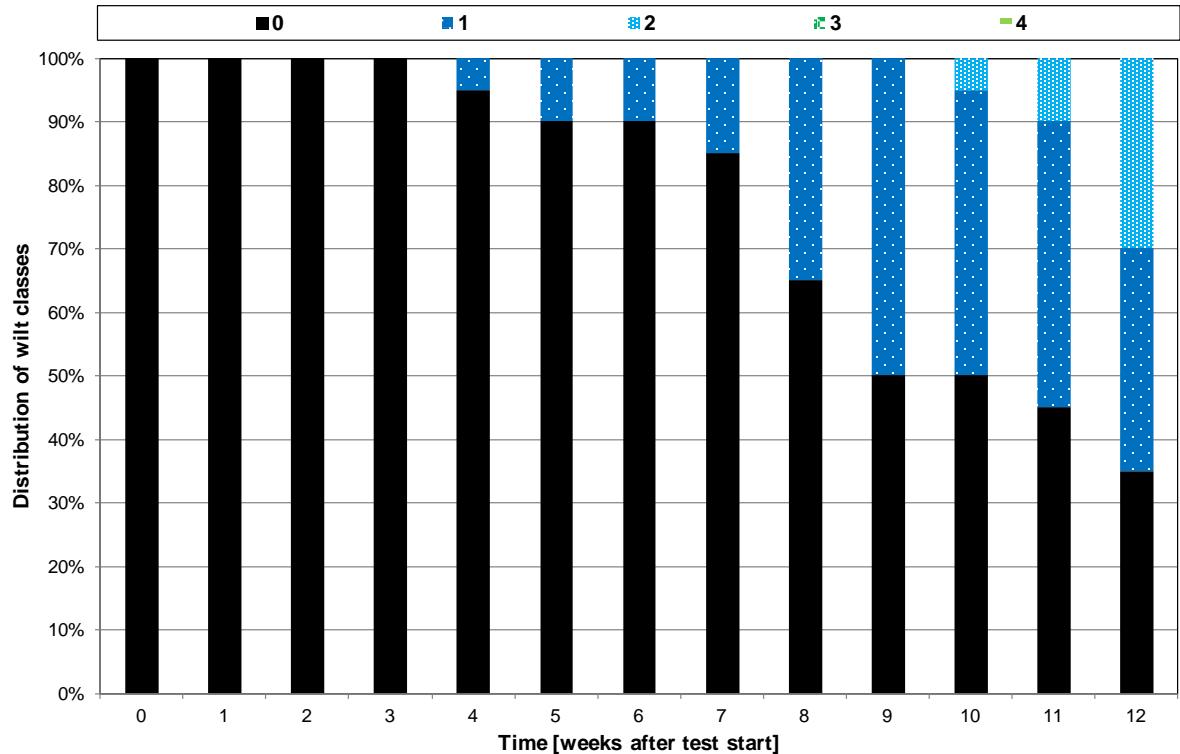


Figure 5.33 Distribution of the wilt classes 0-4 [%] of the control variant Y 25 K (stem-injured pines at 25 °C without wood chips) over time [weeks after test start], n=20

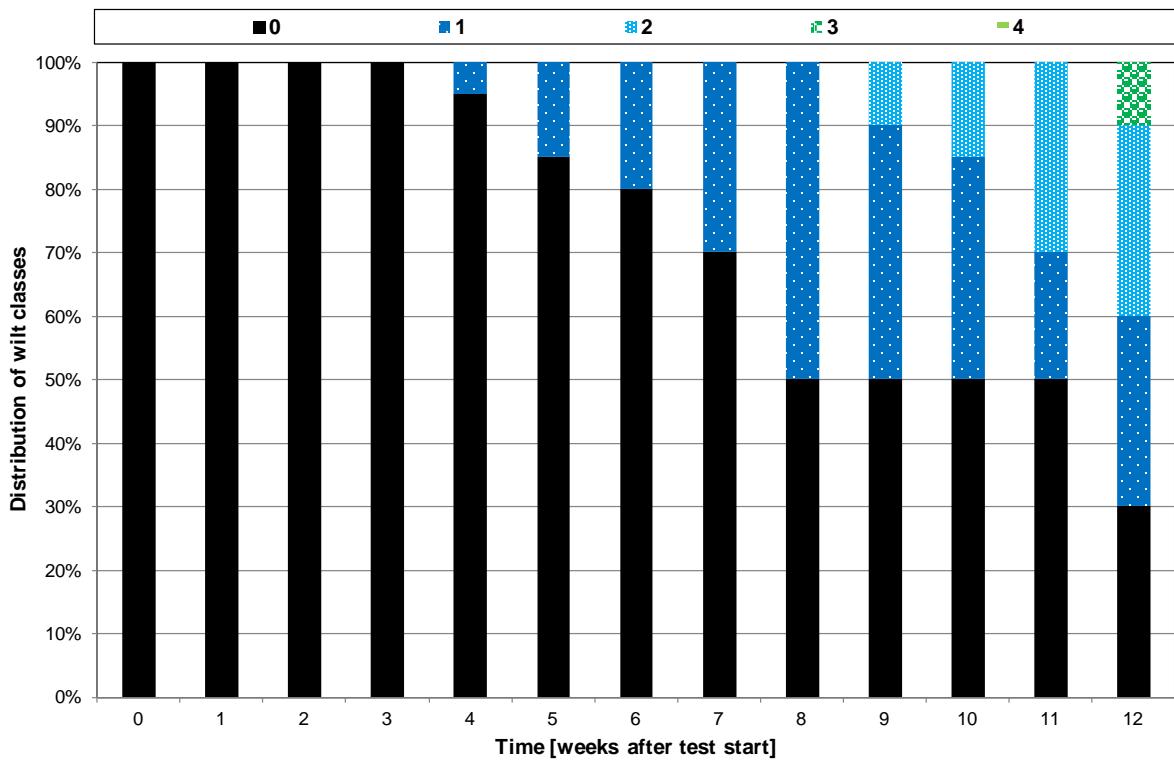


Figure 5.34 Distribution of the wilt classes 0-4 [%] of the control variant O 25 K (root-injured pines at 25 °C without wood chips) over time [weeks after test start], n=20

In general at test start all trees (without the cut-off variants) showed no symptoms (= wilt class 0). By week 12 wilt class 4 could be found for both test temperatures, but at 25 C more saplings developed higher wilt classes and over a shorter time span. The control trees without wood chips until test end reached mainly wilt classes 0, 1 and 2, but not wilt class 4. Wilt class 4 was developed by trees for all three wood chip positions at 25 C. At 15 C only pines with wood chips in the soil or in direct contact with wood chips, but not with wood chips separated from the stem reached wilt class 4. For all three tree conditions except the cut-off variant, pines with wilt class 4 were observed at both temperatures. Wilt class 1 and 2 were the most frequent classes until the end of week 12 for nearly all variants (uninfested trees). Exceptions were the variants Y 25 D (stem-injured pines at 25 C with direct contact of wood chips to the stem) with 75 % and O 25 I (root-injured pines at 25 °C with wood chips mixed in the soil) with 45 % wilt class 4 as the most frequent class at test end. At 15 C wilt class 4 could not be observed for the most variants. Some exceptions showed wilt class 4 with 5 % and only the variant O 15 I (root-injured pines with wood chips mixed in the soil) had 10 % pines with wilt class 4. At 25 C some variants did not show wilt class 4, apart from the two mentioned variants with the most percentage of pines within wilt class 4 and groups with 5 or 10 % trees of this wilt class, O 25 D showed wilt class 4 with 35 % and also O 25 A with 35 % (root-injured pines with direct contact of wood chips to the stem or wood chips on the soil with distance to the stem).

47 pines of all 480 non-control trees (9.8 %) were PWN infested. 44 of 47 infested trees developed wilt class 4 during the 12 weeks' test period. The other three pines were in classes 2 and 3. No PWN could be extracted from the control pines. The number of PWN infested trees (without control variants) sorted according to the independent variables is illustrated in Table 5.5:

Table 5.5 Number of PWN infested and uninfested saplings per independent variable (a: Temperature; b: Wood chip position; c: Tree condition) without control trees

Temperature [°C]	Tree No. PWN infested	Tree No. Not PWN infested
15	3	237
25	44	196

a

Wood chip position	Tree No. PWN infested	Tree No. Not PWN infested
Direct contact	27	133
In soil	12	148
On soil with distance	8	152

b

Tree condition	Tree No. PWN infested	Tree No. Not PWN infested
Uninjured	2	118
Stem-injured	22	98
Root-injured	23	97
Cut-off	0	120

c

The chi-square test produced a highly significant result, which indicates an association between test temperature and infestation of pines with PWN spreading from wood chips. At 25 C many more pines were affected compared to 15 C. The same test was also done for the variables wood chip position and tree condition. Also, in these two cases, an association between the independent variables and the variable PWN infestation was found. Only the variant cut-off trees resulted in no PWN spread. Significant differences between direct contact of wood chips to the stem and wood chips mixed in the soil as well as between direct contact and wood chips on the soil separated from the stem could be found, but not between the other two variants. The highest number of infested trees was found for direct contact of wood chips with 27 pines. Also, for the independent variable tree condition, significant differences between nearly all possible double combinations of uninjured, stem- and root-injured and cut-off pines, but with two exceptions, could be found. The result for uninjured trees was not significantly different from these, which were cut-off with two and no infested trees and the result of stem-injured trees was not significantly different from these with root injuries with 22 and 23 infested trees.

The nematode densities extracted from the upper plant part above the contact point with wood chips and the corresponding nematode infested tree number per affected variant can be found in Figure 5.35.

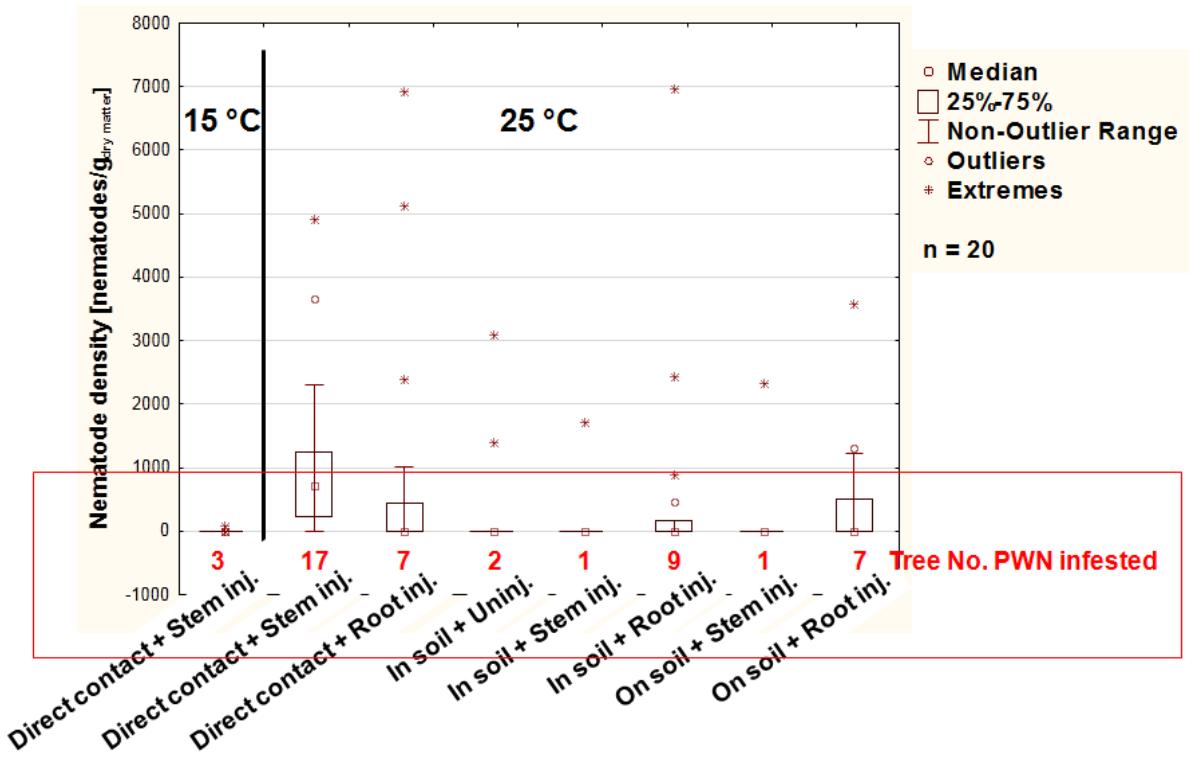


Figure 5.35 Nematode density [nematodes/g_{dry matter}] in the upper plant part above the wood chip contact of PWN infested trees with median, 25 % and 75 % quantiles, non-outlier range, outliers and extremes and number of infested plants per variant

At 15 C, all three independent variables combined show successful PWN spread only for the variant direct contact of wood chips to the stem of stem-injured trees. At 25 C all combinations of the independent variables direct wood chip contact to the stem, wood chips mixed in the soil and wood chips on the soil separated from the stem resulted in PWN spread both for stem- and for root-injured trees. Uninjured trees were only affected in the case of wood chips mixed in the soil. The most PWN infested pines, with 17, belonged to the variant 25 C direct wood chip contact to the stem of stem-injured trees. Also the median for the nematode density was, with 709 nematodes/g_{dry matter}, higher compared to all other variants (with 0 nematodes/g_{dry matter}), but high extreme values (maximum 6,969 PWN/g_{dry matter}) of single trees could be found for nearly all infested variants. The maximum density was not higher than 71 PWN/g_{dry matter} only at 15 C. In accordance with the four variants with the highest number of PWN infested pines, the highest percentages of trees with wilt class 4 up to week 12 were found with 35% for root-injured trees at 25 C with direct contact of wood chips to the stem or with wood chips on the soil with distance to the stem, with 45% for root wounded trees at 25 C with wood chips mixed in the soil and with 75% stem wounded trees at 25 C with direct wood chip contact to the stem. The earliest detection of wilt class 4 at 25 C was in week 6. At 15 C only one of the infested trees developed wilt class 4 in week 12. A successful PWN spread often led to reduced moisture contents of the wood. At test end the median of the uninfested pines (without the cut-off variants) was 180 % (n=433) and of the infested trees 38 % (n=47).

Objective 5.2.1 (Work of B6 & B11 = subtask 5.2.3): Test of the non-vector spread of PWN from artificially infested *P. pinaster* wood chips to non-infested young trees of *P. pinaster* under laboratory conditions

Materials & Methods

Transmission studies from infested wood chips to 5-year-old *P. pinaster* with intact and artificially wounded roots started in September 2013. Chips were produced from PWN inoculated logs. Trees were planted in sand mixed with two litres of chips containing 300 nematodes per gram (fw.) wood (Figure 5.36). In controls sand was mixed with fresh chips. In all treatments n =12.

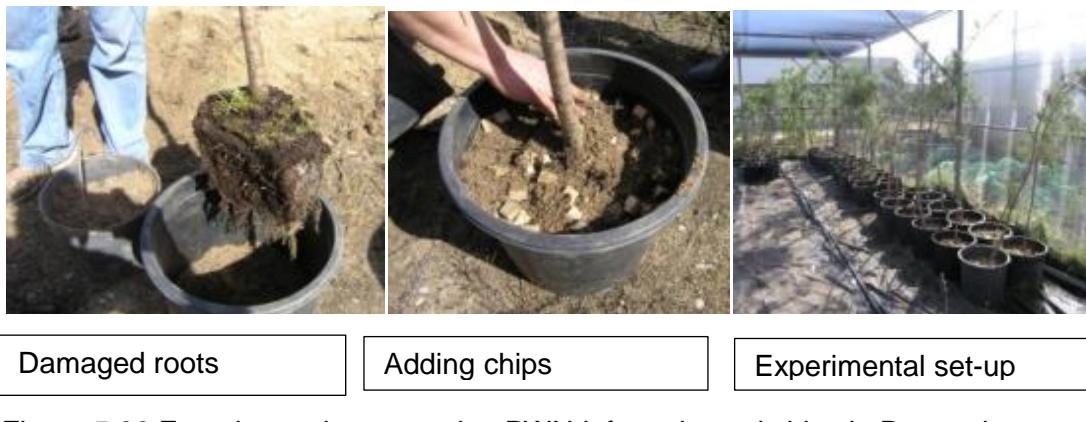


Figure 5.36 Experimental set up using PWN-infested wood chips in Portugal.

Results

At 90 days from the start of the experiment 25% of the trees that received PWN-infested chips were dead, with nematode densities ranging from 0 to 16200 per 100 gram stem wood (fw) (Table 5.6). Also one of the control trees with intact roots was dead at 90 days.

Table 5.6 Numbers of PWN per 100 g of stem wood in plants exposed to PWN-infested wood chips

TREATMENT *300 PWN per gram wood chips (fw)	PLANT NO.	PWN per 100 g stem wood (fw)
Control	8	0
Infested chips* damaged root	40	1 650
Infested chips* damaged root	41	0
Infested chips* damaged root	44	420
Infested chips* damaged root	48	16 200

The nematode content in infested chips decreased drastically during the first ten days of the experiment and continued to decrease until day 90 (Table 5.7).

Table 5.7 PWN densities over time in PWN-infested wood chips.

EXPERIMENT PERIOD	PWN per 100g of chips (fw)
Day 0	29 700
Day 5	1 079
Day 10	114
Day 30	0
Day 60	65
Day 90	20

Objective 5.2.2 (Work of B5): Test of non-vector spread of *B. xylophilus* from artificially infested *P. sylvestris* wood chips to non-infested *P. sylvestris* trees in the native range of PWN (e.g. Canada, USA), where PWD has not been recorded and similar climate conditions to North European countries occur

Investigations regarding non-vector spread of PWN with wood chips to saplings outside the native range of PWN and under laboratory conditions were done by B5 in 2012 (see objective 5.2.1). For the outdoor investigations as foreseen in the project proposal in summer 2013 contacts between B5 and the Canadian Forest Service in Victoria took place. The feedback was not promising as it seems that there are some internal restrictions to carry out those outdoor trials (see also comment in the first report). The negotiations continued without any positive feedback until now, which mainly is due to the problem of financing the investigations in Canada. Because the preparation of this study should already have started, it seems that is too late to conduct such a time intensive experiment in the last few months of the project. This situation reflects the problems sound scientific ideas are faced with. Though scientists agree on the need of those experiments, political issues and financial problems cannot be foreseen during proposal development and, as in this case, it may become necessary to abandon the planned work.

Objective 5.2.4: Transmission of *B. xylophilus* from boards to trees.

Concerning the overall comment see report 1. Investigations will be carried out by B6 according to the report 1. The boards are prepared and the trial will start in the vegetation period 2014, so it is expected to get results at month 45.

Objective 5.2.4: Transmission of *B. xylophilus* with bark.

This sub-task has been deleted as agreed after report 1. The connecting results with wood chips have been delivered by B5 and will be completed by B6 & B11 at month 45.

5.3 Assessment of transmission from tree to neighbouring tree through the soil or by root contact

Studies will be carried out in field and laboratory to clarify the possibility of nematode infestation from infested trees to the soil, its persistence within this element and subsequent entrance to nearby healthy hosts. In the field 10-15 old *P. pinaster* trees will be trunk-inoculated with 30,000 *B. xylophilus* in Year 1. Nearby young trees will be covered with nets to prevent maturation feeding by *M. galloprovincialis*. The trees will be inspected regularly over the 3 years of the project for wilt symptom development. For laboratory experiments, 60 3-year-old plants of *P. pinaster* (year 2) will be planted in pairs in forest soil in 30 5-litre pots. In April of Year 2 one tree in each pot will be stem inoculated either with 400 *B. xylophilus* (n=15) or filtered nematode suspension water (n=15). The experiment will be continued for 12 months. As symptoms appear in the non-inoculated tree, or at the end of the experiment shoots and roots of each tree will be harvested and analysed for nematodes. The soil will also be extracted and analysed for nematodes. The experiment will be carried out at the premises of B6, working with B11.

A similar experiment but using *Pinus sylvestris* will be carried out by B5 under quarantine conditions in a climate chamber.

Objective 5.3 (Work of B5): Determination of the non-vector spread of PWN through the soil or by root contact from one infested tree to another neighbouring uninfested one

Materials and methods:

Experimental set-up:

3-4 year old *P. sylvestris* trees (features described in Table 5.8) were divided in two groups for natural root contact (80 pines) and artificial root grafting (80 pines).

Table 5.8 Overview of features of tested *P. sylvestris* trees - Geographic distribution, Dt. Herkunftsgebietsverordnung (Forstvermehrungsgut) – Silvicultural regions of provenance (Forest reproduction item) (HkG), age, height, stem diameter

Geographic distribution (Provenance)	Age [Years]	Height [m]	Stem diameter (stem basis) [cm]
Middle and East German lowland (HkG 851 04)	3-4	0.7	1.4

Root contact by root grafting:

At first the pines were tested at the Büsgen-Institut, Department of Forest Botany and Tree Physiology, Labor für Radioisotope, Georg-August-Universität Göttingen. On 16th, 20th and 23rd April 2012 one/ two strongest roots of at least 3 mm diameter per tree were root grafted between two pines. Both pines were planted in separate 1 l pots to exclusively test the root contact as the only possible pathway for PWN. The two roots which were used for the grafting were about 2-2.5 cm longitudinal cut from the lower end upwards to remove 0.5-1 mm of the diameter using a scalpel referring to Fischer et al. (1960). The wounded sides of both roots were carefully pressed to each other. Small brackets held this construction during the grafting process and insulating tape was wrapped around the root contact point. Finally wound balsam for trees was used to cover the contact point and the rest of both roots outside the soil as a second protection layer to reduce dehydration (Figure 5.37). On 25th July after 12.5 weeks for root growth in a greenhouse at room temperature (approx. 20 C) the success of the root grafting was tested by use of the phosphorus isotope ³³P to test the efficiency of PWN inoculations. In the case of successful root contact the behaviour of PWN concerning non-vector spread by roots could be clearly shown. ³³P is a radioactive isotope with 25.4 days half-life period and β⁻ decay (GisChem 2012). According to experiences of the radioisotope laboratory in Göttingen on one of two pines per couple 9 ml 1,4 MBq ³³P were added during the regular watering. After seven and 14 days phosphor imager pictures (phosphor imager type FLA 5100, Fuji) of needles of both neighbouring trees were made to detect radiation to determine the success of the root grafting. In August in the case of successful root grafting the inoculation of PWN (PT-6 (w)) in one pine per tree couple was planned.



Figure 5.37 Steps of root grafting between 3-4 year old *Pinus sylvestris*; a: Cutting of roots using a scalpel, b: Placing of wounded root sides on each other held by small brackets; c-d: Wrapping of self welding insulating tape around the root contact point, e: Covering of the root grafting with wound balsam for trees

Natural root contact:

On 24th April 2012 two *P. sylvestris* trees of the second pine group were planted together per 5.5 l pot to allow natural root contact (Figure 5.38). After 2.5 months on 05th July the inoculation of PWN in one of two pines per tree couple was conducted after 1.5 months pre-air-conditioning.



Figure 5.38 *P. sylvestris* pair for development of natural root contact

PWN inoculation:

4000 nematodes per 300 µl inoculum were inoculated in the main shoot of one tree per pair (inoculation steps see WP2, Work done by B5).

Per pine group 20 PWN inoculated and 20 control (inoculated with tap water) pine pairs were planned. The trees were tested in a greenhouse at 25°C and on average 61% rH, watered as required, randomised and evaluated weekly for pine wilt symptoms for more than one year (59 weeks). The sampling of the trees took place at wilt class 4 or 5, otherwise at the end of this study.

Assessment of pine wilt and occurrence of PWN in trees:

The wilt symptoms of the pine trees were evaluated according to a rating scheme as explained in chapter 5.2.1 (work of B5). After 6.5 weeks, irrespective of wilt class, two branches from the lowest stem part per PWN-inoculated tree were sampled for nematodes. In the case of unsuccessful PWN detection in the branches, all inoculated trees were severed at the root collar at wilt class 5 (to prolong the time for a possible tree contact) or at test end using ambos shears. The upper plant part was sampled. The neighbouring trees were sampled by cutting off the tree over the root collar without pretesting of branches and already at wilt class 4 (to increase the chance to extract living PWN). In the case that no PWN could be found in the upper plant part, the roots and root collar of the inoculated and the neighbouring tree were analysed after the extraction of the last tree per pair. For the upper plant part (without needles) the moisture content was recorded. PWN were identified, fixed and counted as nematodes per gram dry matter.

Results:

The artificial root contact was not successful. The phosphor imager pictures of the needles of the neighbouring trees did not show radioactivity, only in the ^{33}P watered pines radiation could be observed from blue (less radioactivity) to red (high radioactivity). For exact figures of radioactivity a scintillator would be necessary. Additionally the same results could be shown for the root contact points by use of the phosphor imager. Therefore the planned inoculation of PWN was not conducted for this pine group.

The planting of two pines per pot to allow natural root contact was done 2.5 months before the inoculation and with 1.5 months pre-air-conditioning at 25 C. After a maximum common growing time span in the pot of 16 months at test end, the pine roots of both trees could be separated with the fingers. If a connection between the roots of different trees had occurred no contacts between big roots were observed. A test with radioactivity or other markers to validate a possible root contact was not conducted to increase the chance to extract living PWN.

The results illustrated as distribution of wilt classes of the subsequent inoculation of water or PWN in one of both trees after 2.5 months common growing time can be found for all variants (with 0: 0 %; 1: 1-25 %; 2: 26-50 %; 3: 51-75 %, 4: 76-99 % and 5: 100 % tree coverage of needle discolouration) dependent on the time in the following figures.

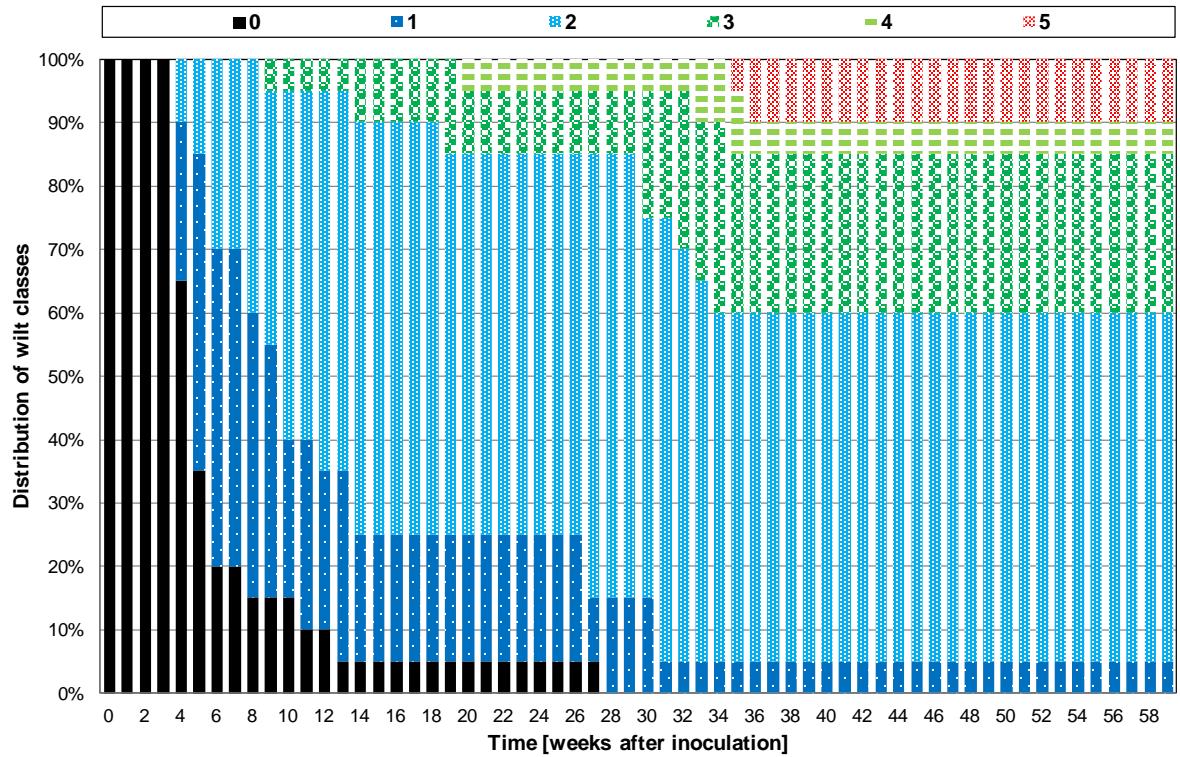


Figure 5.39 Distribution of wilt classes [%] of the water inoculated control trees over time [weeks after inoculation], n=20

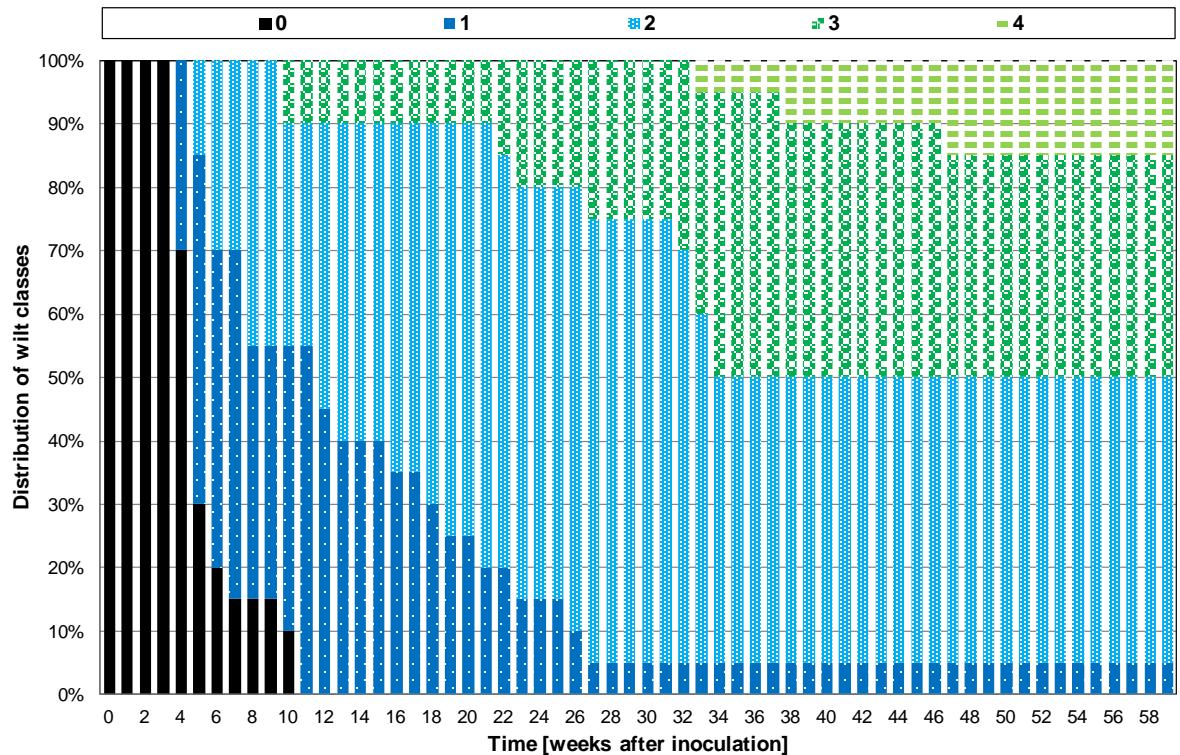


Figure 5.40 Distribution of wilt classes [%] of the neighbouring trees of the inoculated control trees over time [weeks after inoculation], n=20

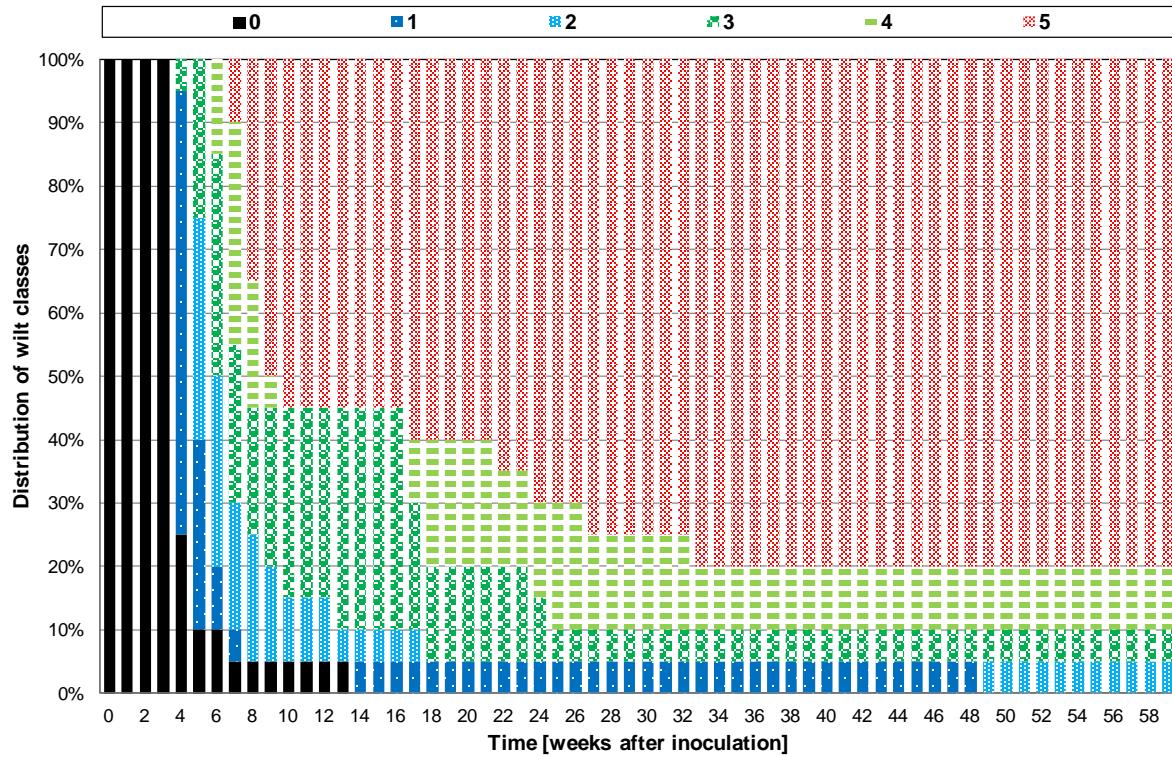


Figure 5.41 Distribution of wilt classes [%] of the PWN inoculated trees over time [weeks after inoculation], n=20

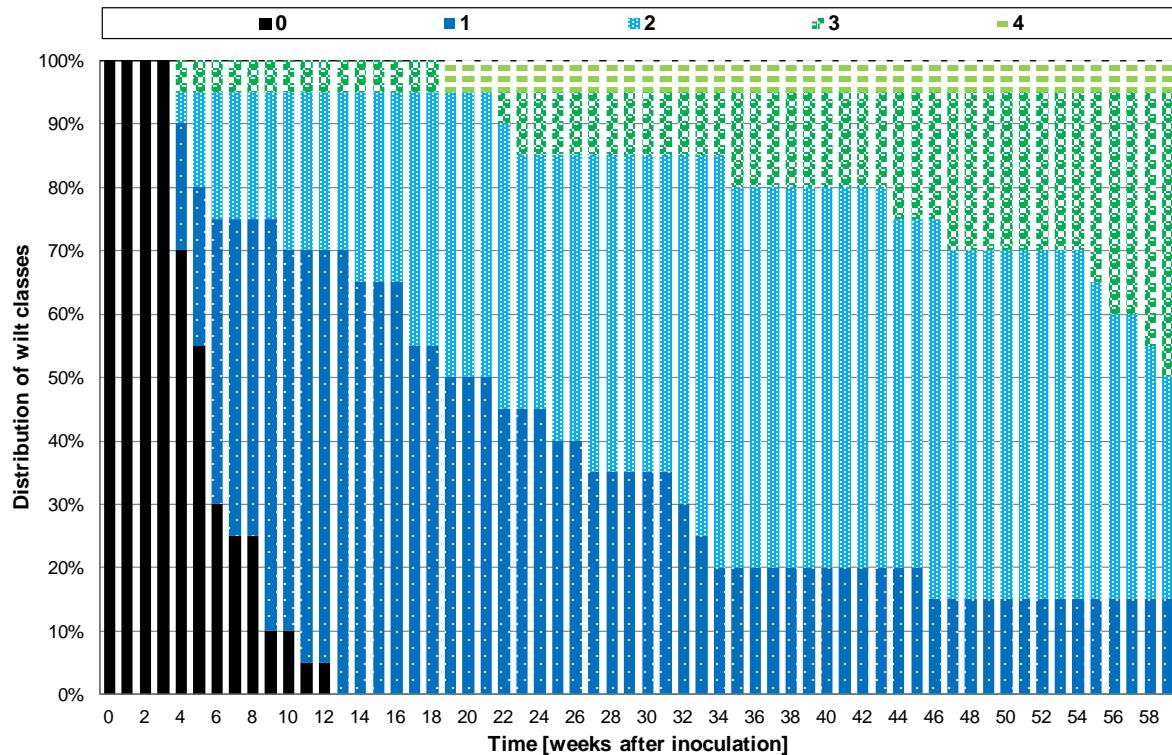


Figure 5.42 Distribution of wilt classes [%] of the neighbouring trees of the PWN inoculated trees of over time [weeks after inoculation], n=20

In general in the first four weeks all trees showed wilt class 0 (no symptoms). The control trees and the neighbouring trees of the control and PWN variants had similar distributions of wilt classes; only the PWN-inoculated trees developed very high wilt

classes quickly. For this group wilt class 5 could be recorded as soon as seven weeks after nematode inoculation and from week eight it was the most frequent class in this group. Starting at week 33 80% of these pines were dead. At test end, 59 weeks after inoculation, 10 % of the trees belonged to wilt class 4 and one tree per class 3 and 2 were found. Since week ten for the water inoculated control trees wilt class 2 was the most frequent class. Until the last extraction date, one tree showed wilt class 4, another class 1 and two trees wilt class 5. 55 % developed wilt class 2, followed by 25 % of wilt class 3.

Both neighbouring pine groups irrespective of whether they were grown next to water- or PWN-inoculated trees, developed mainly wilt class 1 and 2 during the first test half. In the second half of the test, classes 2 and 3 dominated with a few trees with classes 1 and 4. The neighbouring pine groups were cut at wilt class 4, therefore wilt class 5 was only recorded for the water or nematode inoculated trees.

From eleven of 20 PWN-inoculated trees, PWN could be extracted from two lower branches after 6.5 weeks irrespective of wilt class. The median of the nematode density was 1 nematode/g_{dry matter} (n=20), whereas the maximum value was 225 nematodes/g_{dry matter}. The minimum wood MC of the branches was 22 % (tree with wilt class 5) and the maximum MC 240 % (tree with wilt class 3).

Because of the incomplete detection of PWN in all sampled branches, all trees of the nematode inoculated variant were cut to extract all living nematodes of the plant part above the root collar at wilt class 5 or at the end of the test. Together with the results of the nematode extraction of the other pine variants at wilt class 4 or 5 or at test end involving the upper plant part as well as the root including the root collar indicated that only the nematode inoculated pine group was infested with *B. xylophilus* in the upper plant part. The median of the nematode density for this pine group (upper plant part) was 11 nematodes/g_{dry matter}, the maximum 3611 nematodes/g_{dry matter} and the minimum 0 nematodes/g_{dry matter}, because from four pines, which did not develop wilt class 5 during the test time, no PWN were extracted.

In Table 5.9 the wood MC of the upper plant part per pine variant can be found. The variant PWN inoculated trees showed the lowest median with 71 % MC, which, compared to its neighbouring trees is 97 % reduced.

Table 5.9 Wood moisture content MC [%] per pine variant with *B. x. – B. xylophilus* inoculated, K – Controls, N – Neighbouring trees, n=20

Variant	Wood moisture content [%]		
	Median	Min	Max
K	164.6	16.2	187.7
N K	162.9	122.5	183.7
B. x.	70.9	20.2	153.4
N B. x.	168.0	159.1	191.7

Objective 5.3 (Work of B6 & B11): Determination of the non-vector spread of PWN through the soil or by root contact from one infested tree to neighbouring uninfested tree/trees.

Materials and methods

Green House

Root transmission of PWN is studied on 5-years-old *Pinus pinaster*. The trees are potted in pairs in 30 plastic 80 L boxes. In the nematode treatment (n=15) one tree in

each pair was inoculated with 6 000 PWN at 6 points along the stem. For controls (n=15) one tree in each pair was inoculated with water..

Field

The experiments on root transmission in the field are carried out in the Lisbon area and are located at Herdade da Comporta, Companhia das Lezírias and Mata da Machada (Figure 5.43).



Experiment installation at Companhia das Lezírias



Experiment installations at Herdade da Comporta

Figure 5.43 Experimental installations for root transmission in the field in Portugal

Results

Green house

The experiment with paired trees of *P. pinaster* has been running for 1 year; water inoculated control pairs (n=16) and PWN inoculated (n=16). As indicated in Table 5.10, for *P. pinaster*, one tree dies in the untreated control (6%) compared with 11 dead in the PWN inoculated plants (69%). None of the neighbours of inoculated trees are showing symptoms.

Table 5.10 Preliminary results of greenhouse inoculation experiments with *Pinus pinaster*.

CONTROL		INOCULATED 6 000 PWN / donor	
Donor (n=16)	Recipient (n=16)	Donor (n=16)	Recipient (n=16)
1 dead	0 dead	11 dead	0 dead

Field

So far no results are available. The experimental installations in the forests have just been made and the donor trees are waiting to be inoculated with PWN.

5.4 Transmission of *B. xylophilus* from infested wood to non-infested wood in storage and in transit

Task 5.4.1: Laboratory scale experiments will be carried out with artificially infested pine wood. Horizontal and vertical distribution potential of PWN to neighbouring wood samples will be investigated. Outdoor experiments using wood cut to the typical dimensions of pallets will also be done but using native *B. mucronatus* as a non-quarantine species. In this framework, different moisture contents and climatic conditions such as dry periods or rain will be simulated. B5 to lead with links to other Beneficiaries in this WP.

Task 5.4.2: Importance of infestation bridges from forest soil to sawn wood. Nematode free blocks of *Pinus sylvestris* intended for pallet construction will be laid out on the forest floor in a randomized block design. The experiment will include 5 experimental blocks, each with 55 wood blocks. Sampling will be done destructively every second month during Year 1 and 2. Fungi colonizing the wood will be identified, isolated and tested for their host status for *B. xylophilus*. Any nematodes naturally colonizing the wood will also be quantified and identified. After sampling for fungi, *B. xylophilus* will be inoculated into the sampled wood and the nematode multiplication will be followed for 2 months under quarantine conditions in the laboratory. The experiment will be carried out in a pine forest (*P. sylvestris*) in eastern Norway and conducted by B11 in collaboration with forest mycologists of the Forest and Landscape institute in Ås.

Task 5.4.1 was covered in Periodic Report 1.

Task 5.4.2 had to be omitted by B11.

5.5 Validation of a set of PWN-specific microsatellite markers usable at the individual level.

So far, the molecular markers that have been developed for the genetic characterization of PWN (e.g., RAPD,AFLP) are not sensitive enough to enable analysis at the individual level (Vieira et al., 2007; Cheng et al.,2008). In addition, their use could not efficiently differentiate European populations (Vieira et al., 2007), thus precluding any precise analysis of their relationships or further comparative analysis with populations of foreign origins. Therefore, the hypothesis that the PWN population(s) that invaded Portugal is (are) of Asian origin is still to be unambiguously demonstrated. Preliminary experiments on PWN populations from China indicated that microsatellite markers may be better suited to reveal the genetic structure of populations (Zhou et al., 2007).Therefore, it is proposed to use a set of microsatellite markers identified from the nematode genome to locally characterise PWN populations and their genetic relationships, resulting from direct field sampling (including the newly infested area in Madeira) but also in retrospective analysis of the variability in the existing distribution of PWN in Portugal. These studies will help to resolve basic questions such as the tree-to-tree transmission of the nematode, its capacity for long-term persistence and dispersal (independently of the transmission mode), etc. Recently developed computer simulation methods (Ciosi et al., 2008; Guillemaud et al., 2010) will be used to quantitatively compare the different introduction scenarios for the European populations. The results will possibly be used in tree resistance studies (see WP6) to assess whether different variants of the nematode have variable pathogenicity and also whether nematodes encountering tree resistance factors are altered genetically. Moreover, it is expected that such studies may be linked to the analysis of the genetic variability of the vectors(see WP3), in order to evaluate the possibility that some co-evolution has occurred between the nematode and insect populations. If this would be the case, as can be reasonably hypothesized, it could be of obvious practical interest to identify the

genotypes that are the most fit together, and those that probably could not be found associated. B4 will coordinate this work, which will also link closely to the other WPs, as indicated.

5.6 Assessment of genetic diversity of European PWN populations in relation to invasions.

Proteomics are extremely useful when looking for differential “functional” elements (parasitism, adaptability, ecological value,) within a species’ common genetic pool, and is becoming increasingly valuable when studying the natural variations within a population of any species (Biron et al., 2006; Karr, 2008) Adaptive processes are the key in parasite evolution. The interspecific and intraspecific population variations can be easily identified by using bidimensional electrophoresis with nematode samples (Navas et al, 2002). The use of protein sequences has clear advantages. The number of loci that are detectable in a single gel are so large that it provides an extremely efficient method to analyse genetic changes and changes in peptide sequences (Calvo et al, 2005). Therefore it is proposed to describe the proteome of *B. xylophilus* by creating a database of the positions of the proteins in bidimensional gels isolated from *B. xylophilus* populations of European and Asian origin and identify the genetic changes which have occurred during the evolution and distribution/movement of *B. xylophilus*. Current data bases that have been created using bidimensional gels have proven to be extremely useful and complementary to the genomic based approaches, therefore the possibility of using proteomic methodology in the project opens up a wide range of future opportunities. This task compliments 5.8. B8 will coordinate and provide linkage to other WPs, as appropriate.

Work of B4 during the reporting period (23 man-months delivered)

B4: INRA UMR 1355 / University of Nice Sophia Antipolis / CNRS UMR7254, Institut Sophia Agrobiotech (Scientist: Dr. Philippe Castagnone-Sereno, Scientist: Dr. Thomas Guillemaud, Scientist: Dr. Pierre Abad, PhD student: Sophie Mallez, Research Assistant: Chantal Castagnone.; Collaboration with other partner(s) of the project: B7: University of Evora, Portugal (Dr. Manuel Mota, Dr. Paulo Vieira, Margarida Espada).

Objective: to assess, through genetic characterisation of PWN, the invasion routes into Europe, with potential linkage to specific pathways (deliverable D5.6 partly achieved).

- Genetic diversity of the PWN in its native area

As stated in the previous report, working with fresh samples was considered a prerequisite to decipher the worldwide invasion routes of the PWN. Therefore, with the help of P7, useful contacts have been established with scientists from the USA (John Eisenback, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Blacksburg and Mark Harrell, Nebraska Forest Service, University of Nebraska, Lincoln), that allowed the research team to obtain nematode individuals directly sampled from infested trees (and not artificially multiplied in the laboratory on fungi).

The field samples were extracted from wood pieces that were collected directly from four field locations in Nebraska (NE) and Missouri (MO) states. Each field sample corresponded to a single tree and consisted of between 15 and 31 individuals of mixed life stages. The trees from NE were close to each other (less than 5 meters) and about 500 km away from the MO trees, which were about 50 km from each other. A total of 85 individual nematodes were genotyped using the microsatellite markers characterized in the first year of the project (see previous report). The total number of alleles per locus over all samples varied from 2 to 11, with a mean of 4.7. In MO samples, more than four microsatellite markers displayed five alleles or more. The expected heterozygosity per locus over all samples ranged from 0.142 to 0.825. The mean number of alleles per sample ranged from 1.1 to 3.5. The mean allelic richness and the mean expected heterozygosity ranged between 1.49 and 3.13 and between 0.144 and 0.385, respectively.

Nematodes from the four field origins were significantly differentiated either within or between States (Fisher's exact tests, $p < 10^{-5}$). Moreover, the analysis of molecular variance revealed that the majority of the genetic variance was explained by the variation between individuals within trees (75.27%) and that the proportion of variance was much more important between States (16.64%) than between trees within States (8.09%). The genetic structure of the samples was analysed using both the Bayesian assignment approach implemented in Structure version 2.3.4 and the multivariate method using Discriminant Analysis of Principal Components (DAPC). Results are visualized and summarized in Figure 5.44.

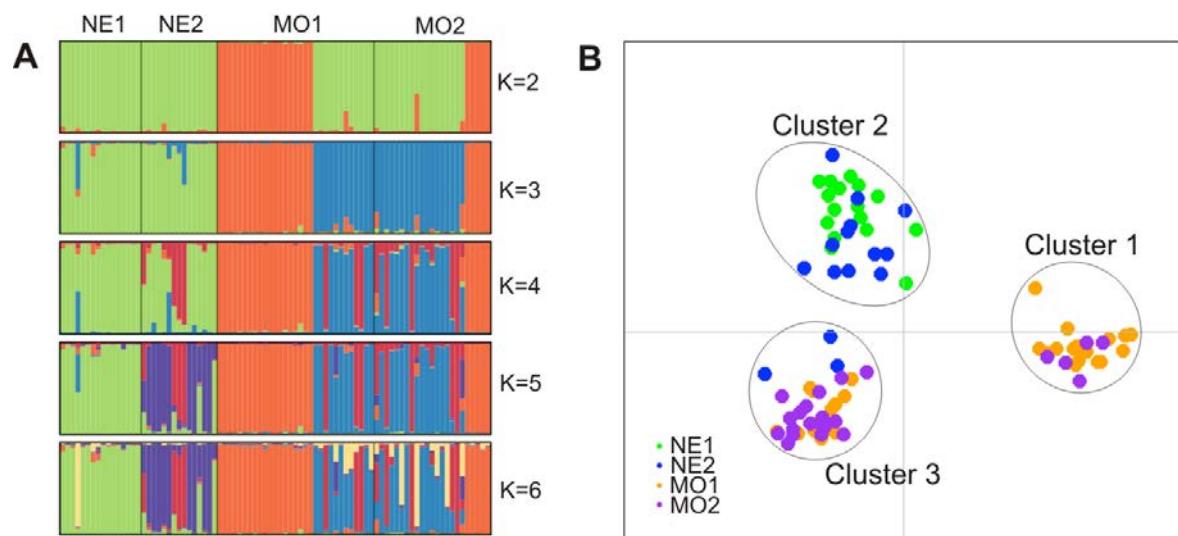


Figure 5.44 Genetic structure of the PWN field samples from the USA. (A) Barplots of Structure of the coefficient of co-ancestry for $K = 2, 3, 4, 5$ and 6 clusters. Each bar corresponds to one individual nematode and each cluster is represented by a colour. The number of clusters inferred was $K = 3$. (B) DAPC scatterplot showing the first two principal components of the DAPC for $K=3$, the number of cluster being inferred from the Bayesian Information Criterion (BIC).

Both methods suggest the existence of three clusters. These three clusters were supported by a mean Structure co-ancestry coefficient larger than 93%. In DAPC, all individuals were assigned to the three clusters so that no 'ghost' population was inferred. The three clusters inferred by the two different methods were very similar, with one identical cluster and with only four individuals (NE1-4; NE2-6; NE2-8 and NE2-9) assigned differently to the two remaining clusters. One of the clusters consisted of individuals from Nebraska and the two others were shared between the two samples from MO, mixing individuals from different trees. The four individuals from NE mentioned above were assigned either to the NE's cluster or to one of the two clusters found in MO, depending on the method used.

Finally, the various samples displayed significant genetic differences, highlighting the existence of a spatial genetic structure. Spatial differentiation exists at very short scale, with neighbouring trees of NE significantly differentiated. This suggests that PWN

dispersal, whether active or passive, can be spatially limited even at a short scale and that genetic drift may play an important role. Furthermore, both methods used here inferred three clusters among the field samples analysed. Each cluster consisted of individuals from different trees, reinforcing the existence of a genetic structure within and between trees. Different clusters were identified within trees scale (MO trees) suggesting that different beetles carrying genetically differentiated nematode populations infested a single tree. The individuals from the NE trees, close to each other, were grouped in a single cluster. In addition, both MO trees exhibited the same two genetic clusters. These local genetic similarities probably result from efficient short distance dispersal mediated by the insect vector. Some nematodes from NE were also assigned to a cluster mainly formed by Missouri individuals (results of DAPC method) or presented hybrid genotypes between NE and MO clusters (results of Bayesian method) despite the large geographical distance between them (more than 500 km). This result is an agreement with the potentially important role of the human-induced dispersal of the PWN. However, too few samples were used in this study to provide clear evidence of long-distance dispersal. With that respect, a hierarchical sampling scheme with nematodes sampled from various trees located in different groups of trees situated in different forests has been implemented, and the resulting data are expected soon.

- Genetic diversity of the PWN in Portugal

a) Using satellite DNA (satDNA) as a marker:

This part was done in close collaboration with Partner 7. The experimental work was mainly done by Paulo Vieira in the INRA laboratory in Sophia Antipolis.

As indicated in the previous report, 23 *B. xylophilus* isolates collected at the likely point of entry of the nematode in Portugal (Setúbal Península; Figure 5.45) were characterized by cloning and sequencing monomers of the *MspI* satDNA previously identified in this species.

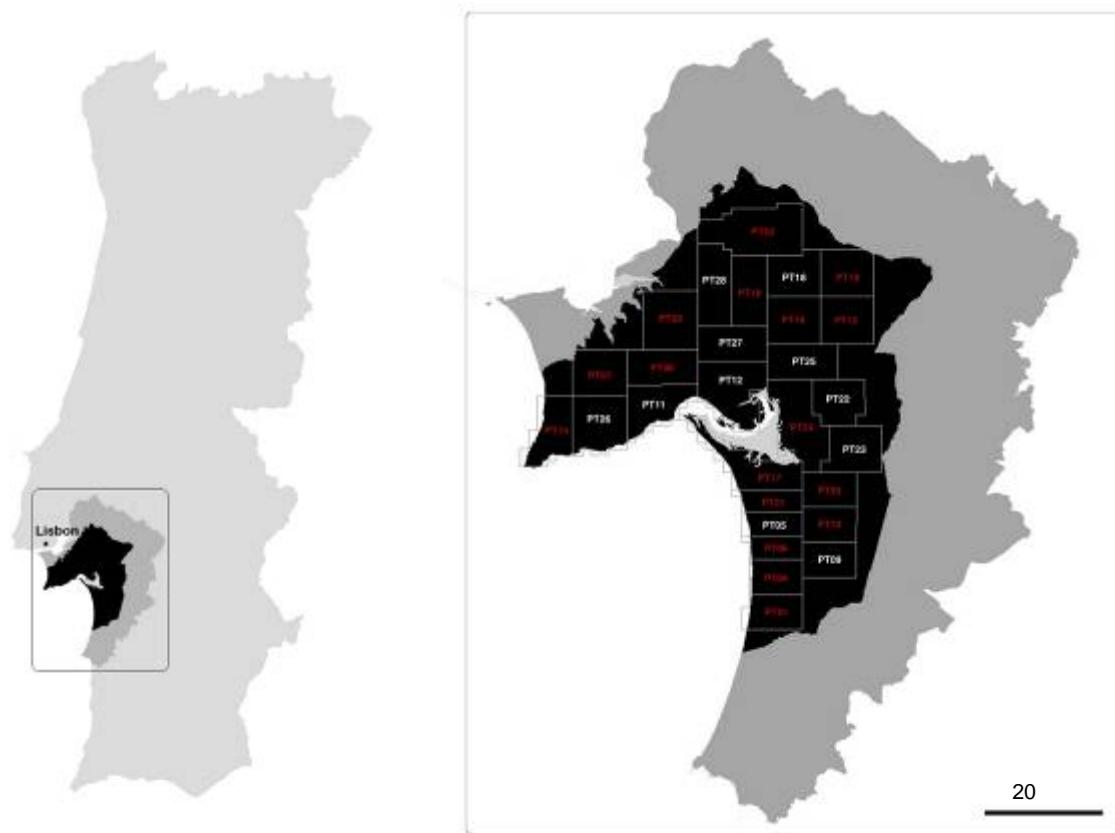


Figure 5.45 Left image: Portugal continental and location of initial point of entry and dispersion of the pine wilt disease. Right image: Location of *Bursaphelenchus xylophilus* isolates obtained (text in red) from different blocks within this affected area.

Alignment of the sequences of the 207 monomers cloned exhibited internal sequence variability, and pairwise comparison of the monomers revealed 88.1% sequence similarity between them. The distribution of nucleotide diversity was measured by sliding window analysis among the sequences. This analysis revealed an alternation of highly to moderately variable and conserved domains within the individual monomeric sequences of the Peninsula of Setubal isolates. The two most highly variable domains within this satDNA accumulated in the sequence inner-central regions between the base positions 22–40 and 78–97 bp. To analyse the extent of sequence variation among the different geographic isolates collected, we first calculated the genetic distances within and between the repeats of each geographic isolate. Genetic distances within monomers of the same isolate ranged from 0.165 to 0.047 (*p*-mean distances), showing a similar magnitude of sequence diversity obtained against different geographic isolates collected within the Peninsula of Setubal (0.172–0.051) (Table 5.11).

Table 5.11 Pairwise genetic distances (*p*-distances) among different *Bursaphelenchus xylophilus* isolates collected from the Peninsula of Setubal.

	PT1	PT1a	PT1b	PT2	PT3	PT4	PT4a	PT6	PT7	PT8	PT10	PT13	PT14	PT15	PT16	PT17	PT19	PT20	PT21	PT21a	PT24
PT1	0.111																				
PT1a	0.103	0.091																			
PT1b	0.089	0.076	0.064																		
PT2	0.116	0.112	0.100	0.143																	
PT3	0.131	0.125	0.117	0.141	0.148																
PT4	0.089	0.069	0.058	0.101	0.118	0.047															
PT4a	0.116	0.107	0.100	0.122	0.134	0.096	0.127														
PT6	0.104	0.088	0.075	0.114	0.122	0.071	0.110	0.094													
PT7	0.104	0.083	0.075	0.113	0.127	0.061	0.106	0.086	0.081												
PT8	0.140	0.144	0.137	0.147	0.152	0.142	0.146	0.145	0.149	0.165											
PT10	0.123	0.121	0.113	0.126	0.146	0.110	0.126	0.124	0.120	0.148	0.148										
PT13	0.095	0.084	0.070	0.108	0.121	0.068	0.106	0.085	0.085	0.140	0.117	0.081									
PT14	0.086	0.073	0.057	0.101	0.113	0.051	0.098	0.072	0.069	0.135	0.112	0.069	0.053								
PT15	0.126	0.126	0.117	0.129	0.148	0.119	0.129	0.129	0.128	0.148	0.131	0.122	0.118	0.146							
PT16	0.093	0.080	0.065	0.107	0.122	0.059	0.103	0.079	0.076	0.144	0.117	0.075	0.060	0.124	0.070						
PT17	0.118	0.115	0.104	0.127	0.140	0.103	0.126	0.116	0.116	0.148	0.132	0.112	0.100	0.136	0.109	0.137					
PT19	0.125	0.127	0.117	0.135	0.142	0.119	0.133	0.127	0.129	0.149	0.137	0.118	0.117	0.139	0.123	0.137	0.156				
PT20	0.122	0.125	0.113	0.127	0.145	0.117	0.130	0.125	0.128	0.143	0.129	0.119	0.113	0.129	0.121	0.131	0.135	0.135			
PT21	0.137	0.131	0.128	0.139	0.144	0.126	0.134	0.134	0.132	0.172	0.139	0.131	0.126	0.140	0.133	0.143	0.145	0.140	0.124	0.153	
PT21a	0.104	0.095	0.083	0.114	0.125	0.081	0.111	0.096	0.094	0.138	0.119	0.092	0.080	0.124	0.088	0.114	0.124	0.119	0.130	0.101	
PT24	0.098	0.083	0.071	0.108	0.122	0.066	0.104	0.084	0.082	0.141	0.117	0.080	0.068	0.121	0.074	0.110	0.124	0.119	0.130	0.090	0.082

The average within-isolate genetic distance is shown in bold and highlighted in grey cells.

Our analyses showed that no specific nucleotide substitutions or indels were identified in *Mspl* satDNA monomers, which could discriminate single populations or groups of geographically close populations at the scale of the Peninsula of Setubal. Such a lack of intraspecific molecular structure in satDNA sequences has been suggested to arise due to the combination of both past and current gene flow events among populations and independent homogenization mechanisms favouring monomers shared among the populations. Considering the very recent invasion of Portugal by the PWN, and the limited dispersion capacity of the nematode, it is very unlikely that a significant amount of gene flow has occurred within the investigated area. However, the natural dispersion of the PWN from tree to tree is totally dependent on its insect vector, and linked to flight of the adult beetles. As we have been working with isolates from a relative limited area, this suggests that intermingling of isolates could result from insect dispersion within this area, resulting in uninterrupted crossing among the established genetic background within the Peninsula of Setubal. Nevertheless, the exchange of infested wooden goods within these areas by human activities cannot be excluded, as they represent one of the most effective ways of nematode dispersal into new areas, particularly over longer distances.

Finally, the lack of correlation observed herein between satDNA nucleotide diversity and geographic distribution of the nematode precludes the use of this kind of repetitive sequence as a relevant marker for population genetics studies in *B. xylophilus*, which are of crucial importance to decipher the invasion routes of this pest and anticipate further expansion of the PWD in Europe. As an alternative approach, investigation of the geographic diversity of *B. xylophilus*, including a higher number of isolates distributed worldwide among the native and invaded regions, has been set up using more powerful molecular markers, i.e., microsatellites (see above). Definitely, population genetics analyses should provide a better resolution on the genetic variability within this species.

b) Using microsatellites as markers:

Nematode samples were obtained from nine locations from mainland Portugal and Madeira (169 individuals). All individuals were extracted from wood samples collected directly from field locations. Each location sample originated from a single tree and consisted of seven to 30 individuals, at various developmental stages. All individual nematodes were genotyped using 16 microsatellite loci in three multiplex PCRs: MA28

(5 microsatellite loci), MB28 (5 microsatellite loci) and MC33 (6 microsatellite loci), as described previously. Eight of the nine samples were genetically identical and 162 individuals (of the 169 sampled) had identical homozygous multi-locus genotypes. Clustering analysis inferred a single cluster, grouping together the samples from mainland Portugal and Madeira.

A remarkable finding in this study was the very low level or even complete absence of genetic diversity in the invaded area. Indeed, the large number of monomorphic markers and the similarity between them across individuals (fixed alleles) are surprising at first glance for a sexual species and for microsatellite markers. However, these findings may be accounted for by the low level of intra-sample genetic diversity observed in the native area, together with the genetic bottlenecks and founder events often occurring during the introduction of species into new areas. Moreover, biological invasions tend to occur over short timescales, so mutational processes have very little effect on the genetic structure of invasive populations in the short term. Consequently, genetic structure is shaped mostly by demographic processes, such intense demographic bottlenecks resulting in intense genetic bottlenecks, as in the present cases.

- First insight into the invasion routes of the PWN

In order to determine the source of the European invasive populations and the number of introduction events in Europe, we investigated the relationships between populations in native and invaded areas. For that purpose, we first genotyped additional field samples from Japan (seven locations, 210 individual nematodes) that were kindly provided by Drs. Takuya Aikawa (Tohoku Research Center, Forestry and Forest Products Research Institute, Morioka, Iwate), Mitsuteru Akiba (Forest Pathology Laboratory, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki) and Hajime Kosaka (Kyushu Research Center, Forestry and Forest Products Research Institute, Kumamoto). The three samples from Iwate in the North of Japan were genetically identical, presenting one fixed allele for all the markers. All the remaining samples appeared to display significant differentiation (Fisher's exact tests, $p < 10^{-5}$), with extremely high corrected FST values, ranging from 0.63 to 0.99. Strong genetic structure was also detected at the individual tree scale.

We analysed the FST values corrected for null alleles between each Portuguese sample and each American or Japanese sample: the most probable source of a target invasive population sample i is considered to be the population with the lowest corrected FST values with i . We also plotted a neighbour joining (NJ) tree, based on Cavalli-Sforza & Edwards genetic distances. The robustness of the tree topology was evaluated by carrying out 2000 bootstrap replicates over loci. The most probable source of a target invasive population sample i is considered to be the population from outside Europe whose sample is clustering closest to i in the tree. The results of the various analyses performed to clarify the relationships between the populations in different geographic areas are visualized and summarized in Figure 5.46 and Figure 5.47. The lowest FST values obtained with Portuguese samples always corresponded to American samples (Figure 5.46). Thus, the populations of Portugal/Madeira seem to be closer to the American populations than to the Japanese populations, on the basis of FST . On the NJ tree, the Portuguese samples were closer to the Japanese samples than to the American samples, for trees generated with both uncorrected and corrected Cavalli-Sforza & Edwards distances (Figure 5.47).

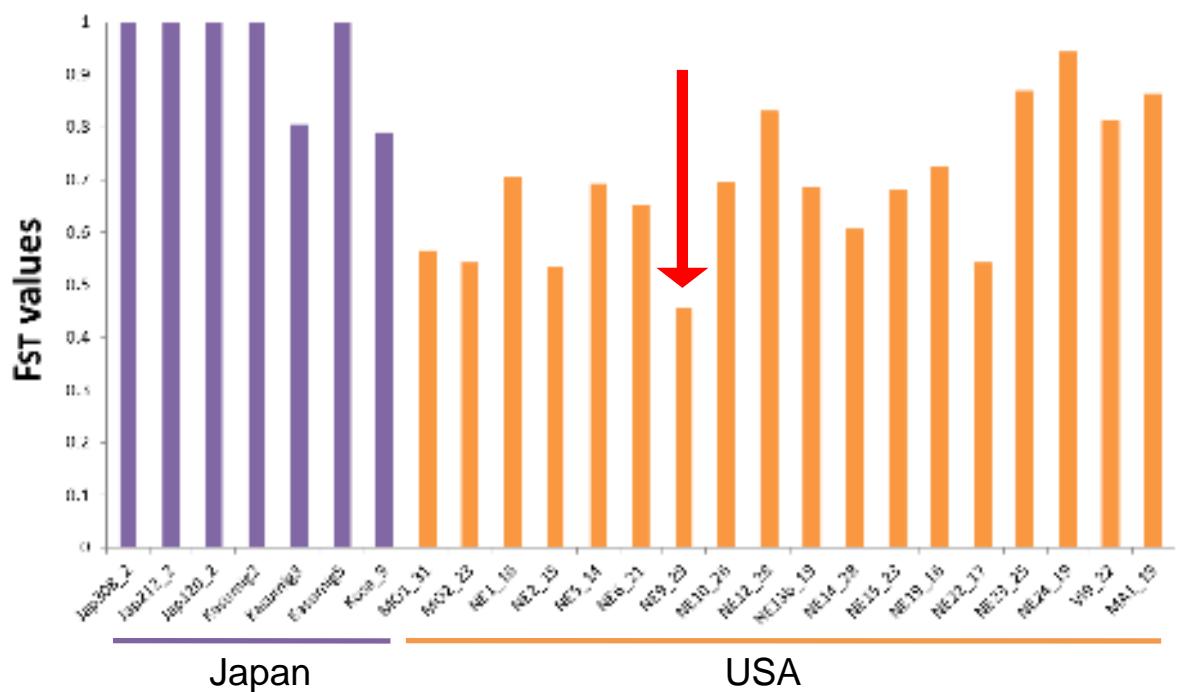


Figure 5.46 Estimates of F_{ST} corrected for null alleles between TR1, a Portuguese population, and each Japanese (in purple) or American (in orange) population. The lowest F_{ST} value is indicated with a red arrow.

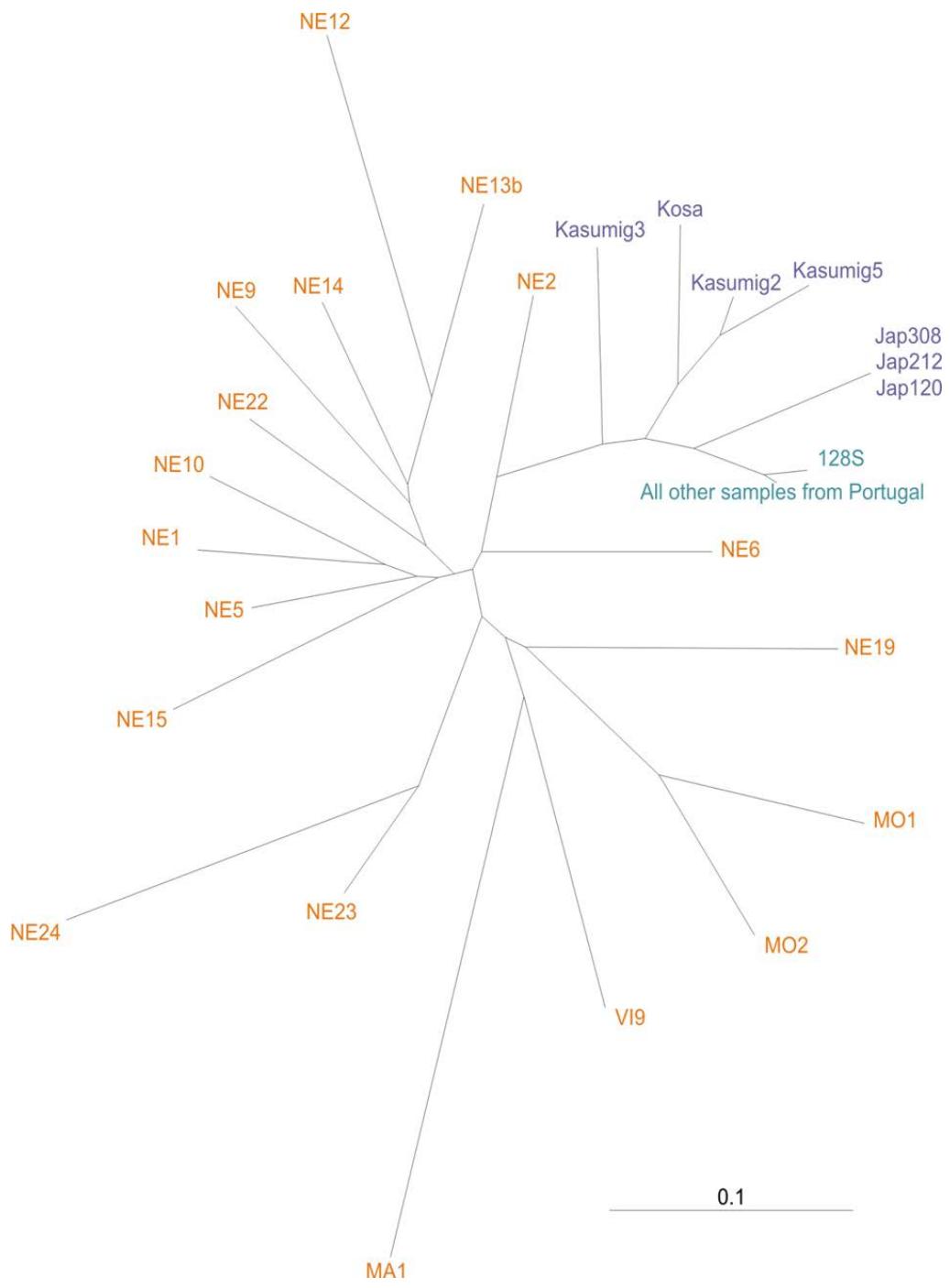


Figure 5.47 NJ tree based on Cavalli-Sforza & Edwards distances. Portuguese, Japanese and American populations are indicated in green, purple and orange, respectively.

Depending on the analysis and the method used, we alternatively inferred two possible origins, North America and Asia, for the Portuguese outbreaks. The *FST* and mean *FST* values across samples suggested an American origin for all the Portuguese samples, whereas the NJ tree and the Bayesian clustering analysis suggested a Japanese origin for these samples. These inconclusive results highlight a major problem with traditional methods: a lack of statistical confidence evaluation for inferences of the source population of invasion. No statistical tests are carried out and no probabilities or type I or type II errors are calculated for classical and Bayesian

clustering or distance methods. This makes it difficult to determine which result is the most likely when several alternatives are proposed. Given the limitations of classical and Bayesian methods, the use of recent model-based methods, such as the approximate Bayesian computation may prove useful, and such analyses will be conducted in the coming months.

Work of Beneficiary 8 (MNCN-CSIC)

satDNA mathematical limits function: Sequencing of the species specific satDNA (represented as a 154 bp-repetitive unit organised in tandem arrays) was carried out in order to compare DNA sequences of Iberian isolates (intercepted from Portugal and reported in Spain) with 13 known published sequences (Castagnone-Sereno et al., 2008), being a total of 45 isolates. In spite of genetic continuity shown by the populations (or isolates) when they are classified according to this marker (Vieira et al., 2013), they might be grouped in different clusters if the sequences reach a level of "mathematical" saturation. Accordingly, we are testing the extraordinary variability of satDNA under mathematical analysis in order to define the numbers of sequences which are needed to use this marker as an efficient phylogenetic tool due its importance in *Bursaphelenchus* taxonomy. We are using a Montecarlo approach considering numbers of changes (a minimum of 0 to 5 changes) in sequences among different clones obtained (minimum 6 clones) from the same isolate. At the moment we have obtained 450 sequences expressed in 2250 changes combination. Asymptotic values for any population/isolate are defined according the calculated limits of Miller and Wiegert (1989) function: $F(x) = a(1-e^{-bx})$.

Comparison of *Bursaphelenchus xylophilus* proteomes: We are using Proteomics iTRAQ after 2DE approach. Significant differences of total proteomes in gel spots were distinct among five studied *B. xylophilus* selected populations, as detailed in 2-DE comparison of total proteins by imaging software PDQuest 8.1. Variations in spot intensity in identically located spots due to small modifications were considered as normal phenotypic variations. The fact that most of the spots were detected in all the studied *B. xylophilus* gels served to avoid differences due to allelic variations manifested in protein changes. There were at least 540 spots clearly expressed, most of which are matched in the same position on the gels representing the studied populations. The five compared *B. xylophilus* proteomes were phenotypically different based on optical density of the spots and according to the ANOVA results for overall signification ($P < 0.001$). At the moment we have identified 60 proteins combining the studied populations. These Proteins were generally identified based on at least two qualified MS/MS spectra. Most of the spots are matched to nematode proteins of the database (94.66% identification rate). Results have been obtained for a 1% rate of false positives (FDR) at peptide level. From the total of assayed proteins, thirty two can be considered which express real and significant differences (at least 2 peptides per protein) as taxonomic biomarkers comparing the five entities. The experiment is currently in progress and will be completed in next 6 months.

References:

- Baermann, G. (1917): Eine einfache Methode zur Auffindung von Ankylostomum- (Nematoden)- Larven in Erdproben. Welteureden. Batavia. Geneesk. Lab. Feestbundel, pp. 41-47.
- Daub, M. (2008): Investigations on pathogenicity, invasion biology and population dynamics of the Pine Wood Nematode *Bursaphelenchus xylophilus* (Steiner und Buhrer 1934) Nickle 1970 in European conifers. Bonn: Dissertation Rheinischen Friedrich-Wilhelms-Universität zu Bonn, 110 pp.
- Decker, H. (1969): Phytonematologie - Biologie und Bekämpfung pflanzenparasitärer Nematoden. VEB Deutscher Landwirtschaftsverlag Berlin, 526 pp.

- Dropkin, V. H. (1989): Introduction to Plant Nematology. JOHN WILEY & SONS, New York Second edition, 304 pp.
- Fischer, F., Bazzigher, G., Kobert, H. (1960): Künstlich hergestellte Wurzelverwachsungen. Mitteilungen Schweizer. Anstalt f. d. forstl. Versuchswesen 36: 15-32.
- GisChem (2012): Phosphor-33 (Nuklidlabor). Arbeitsmediziner/ Betriebsrat, www.gischem.de, 7pp.<https://www.google.de/search?q=25%2C3+Tage+Halbwertszeit+von+P33&ie=utf-8&oe=utf-8&aq=t&rls=org.mozilla:de:official&client=firefox-a> [26/01/2013]
- Schröder, T., Krehan, H., Jurc, D., Roversi, P. F. (2009): EUPHRESCO Final Report. Euphresco Phytosanitary ERA-NET, PEKID, 117 pp.<https://www.google.de/search?q=EUPHRESCO+Final+Report&ie=utf-8&oe=utf-8&aq=t&rls=org.mozilla:de:official&client=firefox-a> [12/03/2012]
- DIN 52183 (1977) Testing of wood; determination of moisture content

Publications based on results of WP 5:

- Hopf, A.; Schröder, T. (2013): Non vector spread of *Bursaphelenchus xylophilus* via wood chips. In: Schröder, T. (ed.) Pine Wilt Disease Conference 2013, Abstracts, Berichte aus dem Julius Kühn-Institut 169: 46-47.
- Mallez S., Castagnone C., Espada M., Vieira P., Eisenback J.D., Mota M., Guillemaud T. and Castagnone-Sereno P. (2013). First insights into the genetic diversity of the pinewood nematode in its native area using new polymorphic microsatellite loci. *PLoS ONE* 8 : e59165.
- Schröder, T.; Welling J.; Aukamp-Timmreck C. (2013): Efficacy of kiln drying as phytosanitary treatment against wood borne nematodes. In: Schröder, T. (ed.) Pine Wilt Disease Conference 2013, Abstracts, Berichte aus dem Julius Kühn-Institut 169: 51-52.
- Vieira P., Castagnone C., Mallez S., Espada M., Navas A., Mota M. and Castagnone-Sereno P. (2014). Sequence variability of the *MspI* satellite DNA family of the pinewood nematode *Bursaphelenchus xylophilus* at different geographic scales. *Molecular Phylogenetics and Evolution* 70 : 120-129.

Scientific communications in international meetings:

- Mallez S., Castagnone C., Espada M., Mota M., Guillemaud T. and Castagnone-Sereno P. (2012). Development of *Bursaphelenchus xylophilus*-specific microsatellite markers to assess the genetic diversity of populations from European forests. Proceedings of the 31st International Symposium of the European Society of Nematologists, 23-27 September 2012, Adana, Turkey : 88.
- Mallez S., Castagnone C., Espada M., Vieira P., J. Eisenback, Mota M., Aikawa T., Akiba M., Kosaka H., Castagnone-Sereno P. and T. Guillemaud T. (2013). First insights into the genetic diversity of the pinewood nematode in its native area and around the world. (Poster). Abstracts of the XIV Congress of the European Society for Evolutionary Biology, 19-24 August 2013, Lisbon, Portugal : 536.
- Mallez S., Castagnone C., Espada M., Vieira P., Eisenback J., Mota M., Aikawa T., Akiba M., Kosaka H., Castagnone-Sereno P. and Guillemaud T. (2013). Genetic diversity of the pinewood nematode around the world – First insights in its invasion routes. Abstracts of the 2nd International Congress of Biological Invasions, 23-27 October, Qingdao, China : 96.
- Vieira P., Castagnone C., Mallez S., Espada M., Navas A., Mota M. and Castagnone-Sereno P. (2013). Sequence variability of the *MspI* satellite DNA family of the pinewood nematode at different geographic scales. (Poster). Abstracts of the 52nd Annual Meeting of the Society of Nematologists, 14-17 July 2013, Knoxville, USA.

Vieira P., Castagnone C., Mallez S., Espada M., Navas A., Mota M. and Castagnone-Sereno P. (2013). Comparative analysis of *Mspl* satellite repeats of the pinewood nematode, *Bursaphelenchus xylophilus*, at different geographic scales. (Poster). Abstracts of the IUFRO / REPHRAME International Conference on Pine Wilt Disease, 15-18 October 2013, Braunschweig, Germany : 123.

Work Package 6: Host tree resistance to PWN and its vectors for future planting

Objectives

The objectives of this WP are:

- to investigate further resistance of *Pinus* to PWN and establish the genetic basis for this;
- to investigate preferences of the vector for different species during maturation feeding or oviposition;
- to obtain hybrid progenies segregating differently in relation to PWN as an approach to obtain resistant plant material and to study quantitative trait loci involved in disease resistance;
- to investigate the potential of pattern mosaics, rather than monocultures, of tree species as a measure to reduce PWN impacts by field observation of pine wilt expression in existing mixed forests in Portugal.

Deliverables

D6.1: Susceptibility of *Pinus sylvestris* provenances to PWN: Determination of the susceptibility of different *Pinus sylvestris* geographical provenances to PWN. Month 45

D6.2: Construction of cDNA libraries from sensitive and resistant genotypes of *Pinus*: Construction of cDNA libraries from sensitive and resistant genotypes of *Pinus*. Month 12

D6.3: Identification of PWN resistance genes in pines: Identification of genes of pines involved in disease resistance to PWN . Month 45

D6.4: Resistance of pines to feeding by *Monochamus*: Identification of resistance in pine species, provenances and families for the feeding of *Monochamus* beetles. Month 33

D6.5: Host preferences for *Monochamus* oviposition: Identification of host preference and resistance in pine species to the oviposition of *Monochamus* beetles. Month 45

D6.6: Hybrid progenies with different tolerance/resistance to the PWN: Hybrid progenies with different tolerance/resistance to the PWN. Month 18

D6.7: Tree species mosaics to reduce PWN impact: Determination of pattern mosaics of tree species to reduce PWN impact in Portugal. Month 45

6.1 Resistance of *Pinus* to PWN

B5 will coordinate this task and use a standardised artificial inoculation procedure developed and used successfully in PHRAME. Single trees will be inoculated with 4000 PWN each. Different geographical provenances of *P. sylvestris* will be used as test trees, all of which will be grown under controlled conditions in climate chambers. If available, *Pinus* clones will be used to reduce the genetic diversity between individual plants from one origin. Comparisons, using the same procedures, will also be made with European *Pinus* species previously regarded as resistant or tolerant to PWN; in these cases it may not be possible to obtain different provenances.

In Portugal the same experiments will be done by B6 in the laboratory using pine shoots collected from different origins and will be tested using a hierarchical approach, i.e. pine species > provenances > families. B6 will use plant material from different *Pinus pinaster* and *P. pinea* provenance trials. Families will also be studied in *P. pinaster* half-sib progeny trials. Symptom development will be carried out according to the rating scheme already used in PHRAME by several partners. Needle discolouration and wilting will be assessed and divided into 6 classes from healthy to dead. Time until symptom development as well as time until trees may die will be assessed. If significant variation in resistance/tolerance is recorded, the plants will be analysed biochemically. Confirmation of nematode breeding will be made in all trees tested,

irrespective of symptom development. B6 and B7 will link to the other Beneficiaries in this WP to coordinate the biological and molecular elements of the work.

To be done in Year 2 and Year 3

Task 6.1.1 - Symptom development

Activities carried out by B5

Objective: Investigation of eight German *P. sylvestris* provenances regarding to the pathogenicity of *Bursaphelenchus xylophilus*

Issues:

Has *B. xylophilus* a pathogenic effect on all tested *P. sylvestris* provenances? Do differences between the tested *P. sylvestris* provenances exist concerning wilt expression and development and therefore the pathogenicity of PWN?

Materials and methods:

Experimental set-up:

Two to three year old *P. sylvestris* saplings (Figure 6.1) of eight different German *P. sylvestris* provenances (Figure 6.2), which are described in the table of Fig. 6.2 were planted in 1 l pots and at least two weeks pre-air-conditioned in the quarantine green house. Deviating from the project proposal only eight instead of 10 provenances could be tested, because of limited available plant material in the nurseries. For each provenance, 20 controls (inoculated with tap water) and 20 PWN inoculated saplings were tested.



Figure 6.1 Pathogenicity test of *P. sylvestris* saplings towards *B. xylophilus* in the greenhouse

Geographic distribution (Provenance)	Height [m]	Stem diameter (stem basis) [cm]
Mecklenburg (HKG 851 02)	0.37	0.7
Heide and Altmark (HKG 851 03)	0.43	1.2
Vogtland, Thuringian Forest and Franconian Forest, montane level (HKG 851 08)	0.36	1
Upper Rhine rift (HKG 851 13)	0.29	1
Neckarland and Franconian plate (HKG 851 14)	0.29	0.8
Middle Franconian hill land (HKG 851 15)	0.26	0.8
Black Forest, montane level (HKG 851 20)	0.38	1
Alps, submontane level (HKG 851 22)	0.29	1

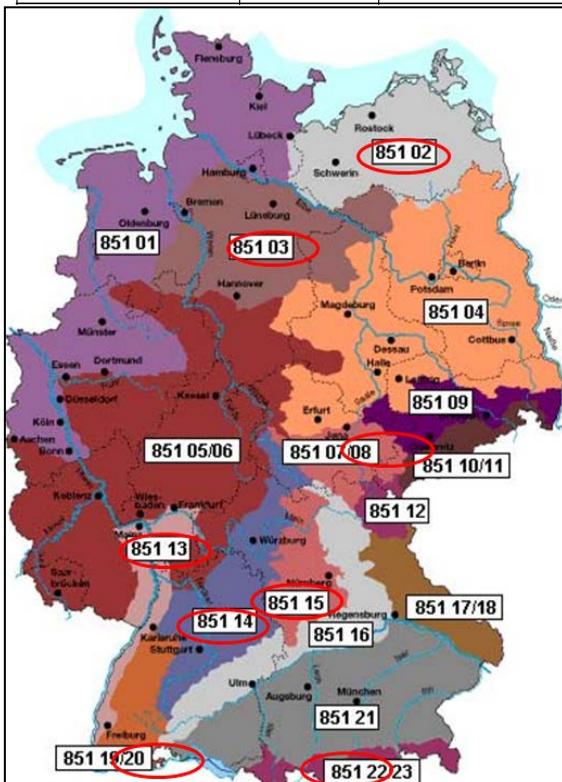


Figure 6.2 table: Overview of features of tested *P. sylvestris* saplings - Geographic distribution, Dt. Herkunftsgebietsverordnung (Forstvermehrungsgut) - Silvicultural regions of provenance (Forest reproduction item) (HkG), height and stem diameter; map: geographical overview of tested German *P. sylvestris* provenances

Extracted PWN from wood located in Portugal in the year 2013 (provided by Dr. E. Sousa – B6) were reared on non-sporulating *Botrytis cinerea* on 1.5 % malt extract agar medium at approx. 20 °C. On 11th and 12th June 2013 4000 PWN of this isolate

PT-7 (w) (number of the isolate in the reference culture collection of the Institute for National and International Plant Health, Julius Kühn-Institut Braunschweig, Germany) were inoculated in the main shoot of the previous year below the youngest whorl (inoculation steps see WP2 Work done by B5) using a 300 µl inoculum. The saplings were placed in a greenhouse at 25 °C and on average 80 % rH, watered as required, randomised and weekly evaluated for pine wilt symptoms for 12 weeks. The sampling of the trees took place at wilt class 5, otherwise at the end of this study after 12 weeks. Susceptibility of provenances and nematode population development:

The wilt symptoms of the pine saplings were assessed using a rating scheme of wilt classes according to Daub (2008) (Table 6.1). The six wilt classes (0 to 5) represent the percentage of needle discolouration of the whole foliage, which is related to the physiological condition of the plant.

Table 6.1 Rating scheme of wilt classes for assessment of pine wilt (Daub 2008)

Wilt class	Tree coverage of needle discolouration [%]	Physiological condition
0	0	alive
1	1 – 25	
2	26 – 50	
3	51 – 75	
4	76 – 99	
5	100	dead

The mortality as part of the wilt assessment was calculated as the percentage of saplings with wilt class 5 (dead trees). With ambos shears, the upper plant part above the root collar was cut to 5 mm to 10 mm (wide, length, height) pieces and sampled for nematodes using the Baermann-funnel technique (Baermann 1917) modified for plant parasitic nematodes according to Decker (1969). Nematodes were preserved using 80 °C hot fixative solution (890 ml Aqua dest., 100 ml formaldehyde solution (35 %), 10 ml glacial acetic acid referring to Dropkin 1989) for later counting. After nematode identification and counting, the nematode density was related to gram dry matter plant tissue and the wood moisture content according to the Dt. Institut für Normung - German Institute for Standardisation DIN 52183 (1977) were recorded.

Results:

The distribution of wilt classes (with 0: 0 %; 1: 1-25 %; 2: 26-50 %; 3: 51-75 %, 4: 76-99 % and 5: 100 % tree coverage of needle discolouration) of all PWN and water inoculated pines per provenance dependent on the time are illustrated in Figure 6.3 to Figure 6.18.

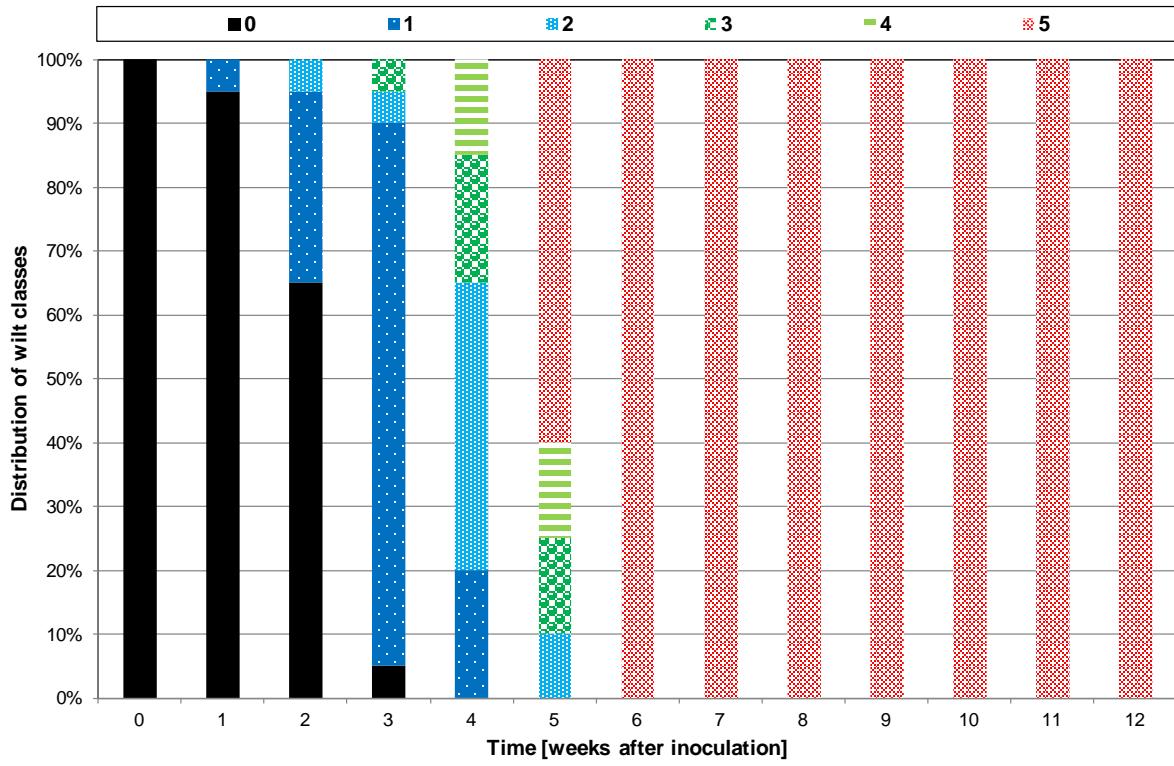


Figure 6.3 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 02 over time [weeks after inoculation], n=20

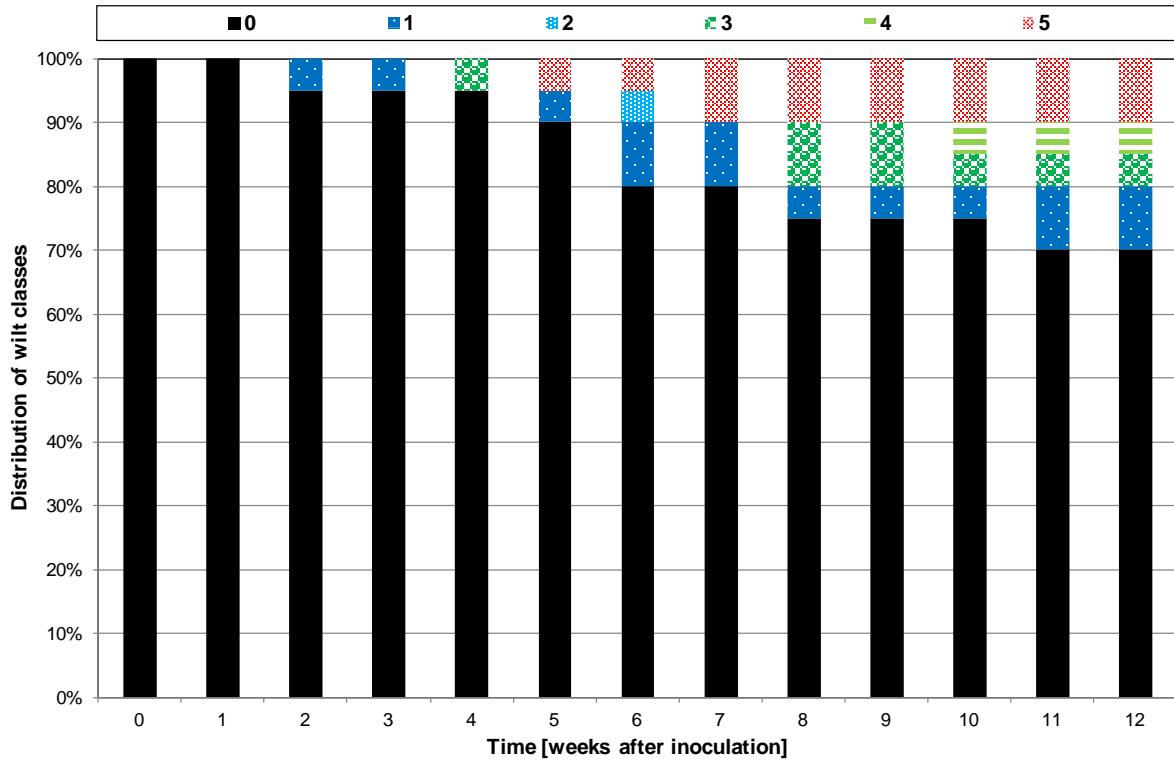


Figure 6.4 Distribution of wilt classes [%] of the control trees of the provenance 851 02 over time [weeks after inoculation], n=20

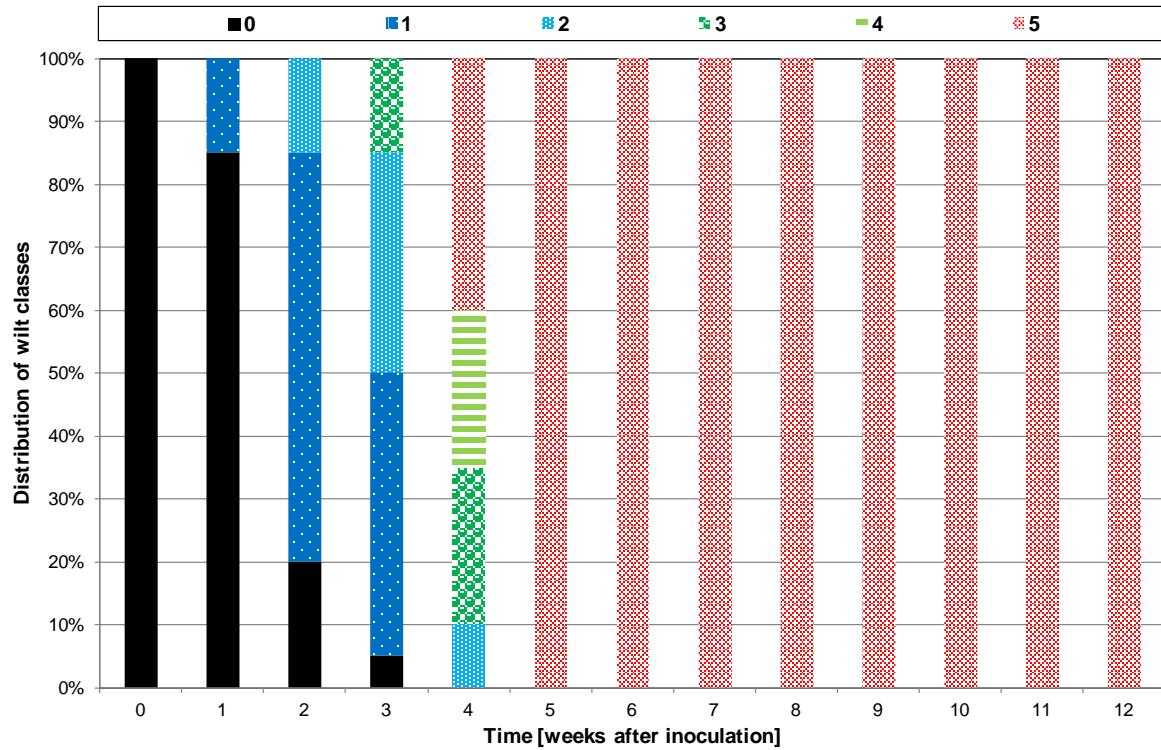


Figure 6.5 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 03 over time [weeks after inoculation], n=20

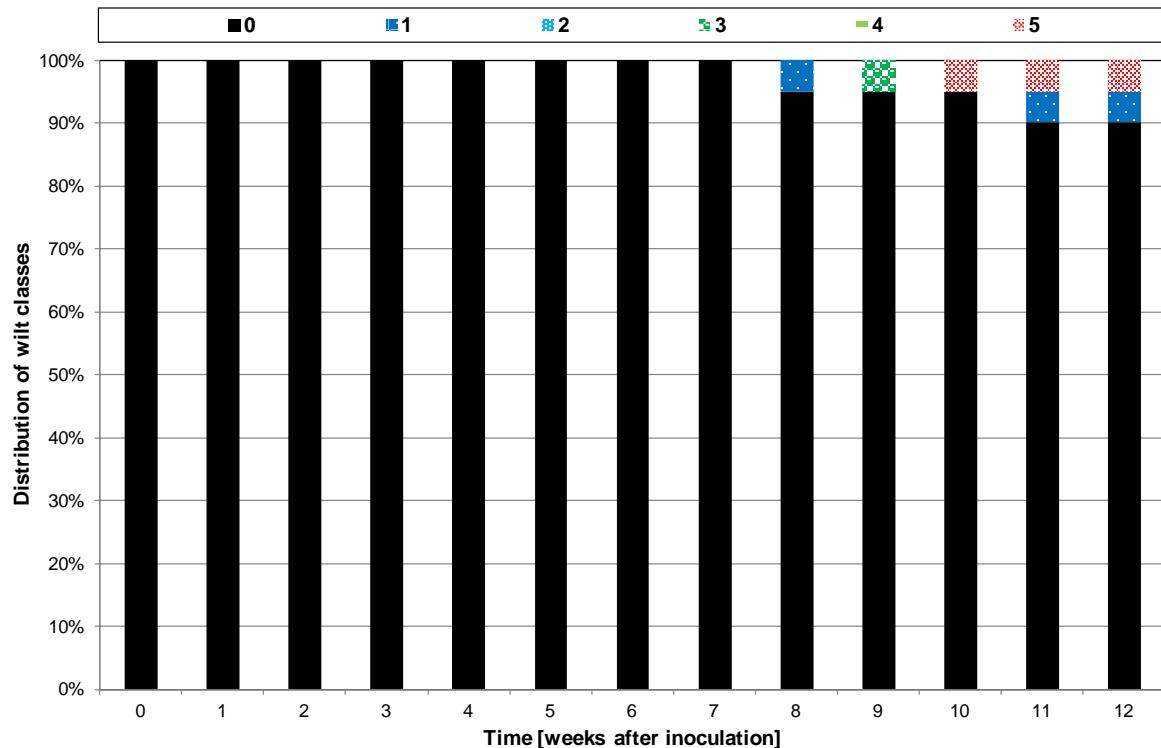


Figure 6.6 Distribution of wilt classes [%] of the control trees of the provenance 851 03 over time [weeks after inoculation], n=20

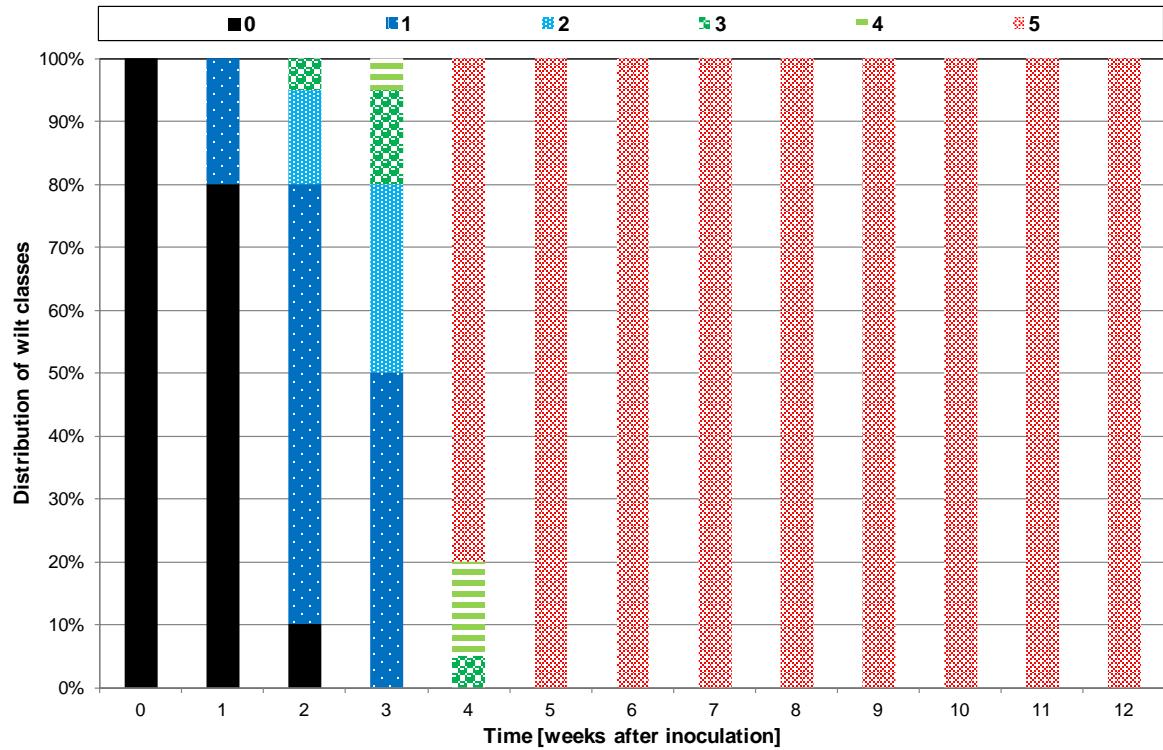


Figure 6.7 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 08 over time [weeks after inoculation], n=20

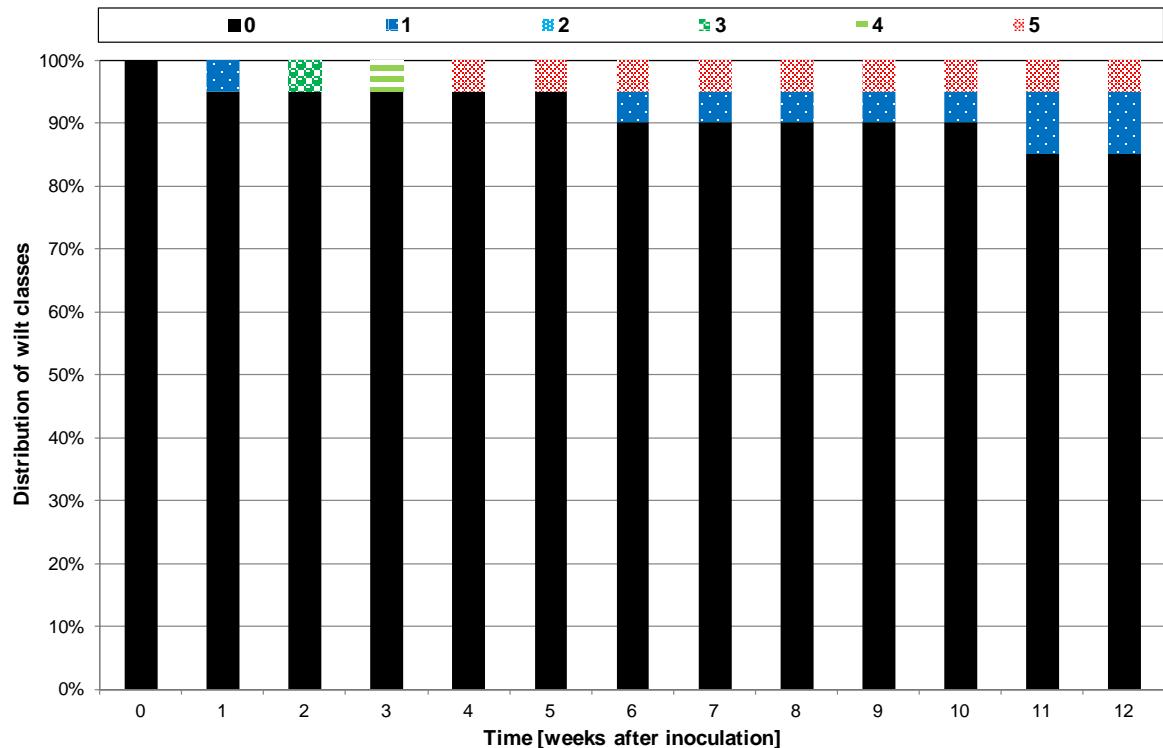


Figure 6.8 Distribution of wilt classes [%] of the control trees of the provenance 851 08 over time [weeks after inoculation], n=20

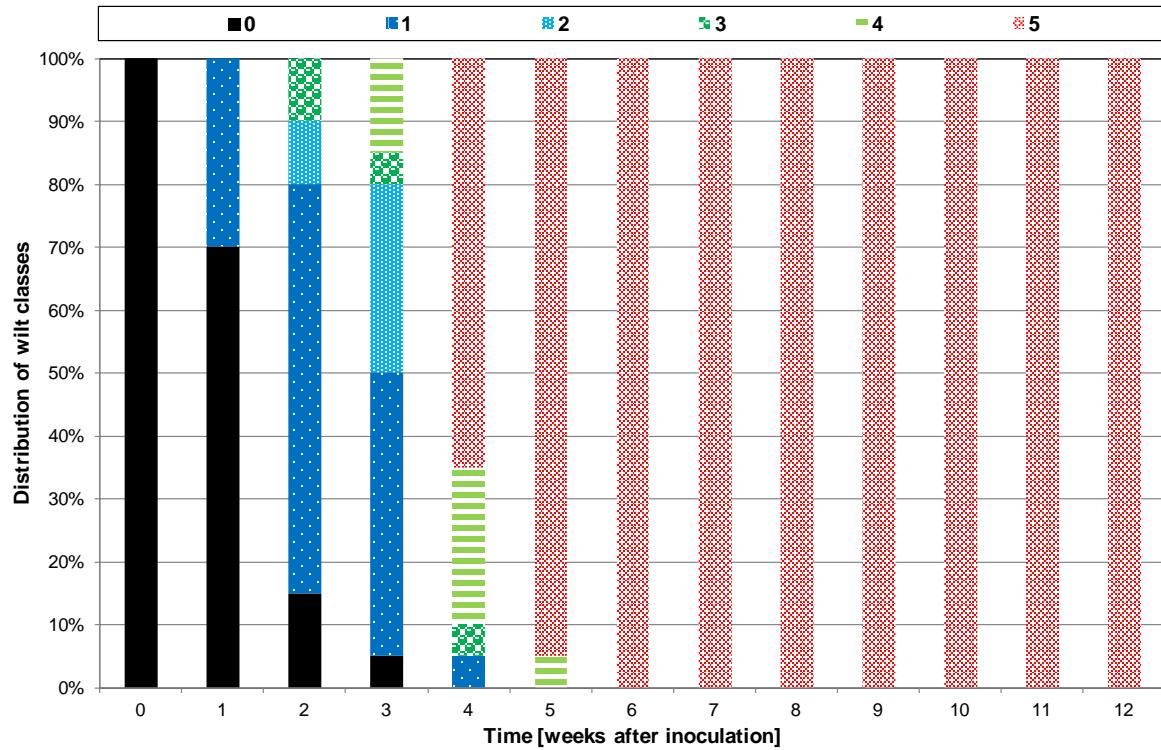


Figure 6.9 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 13 over time [weeks after inoculation], n=20

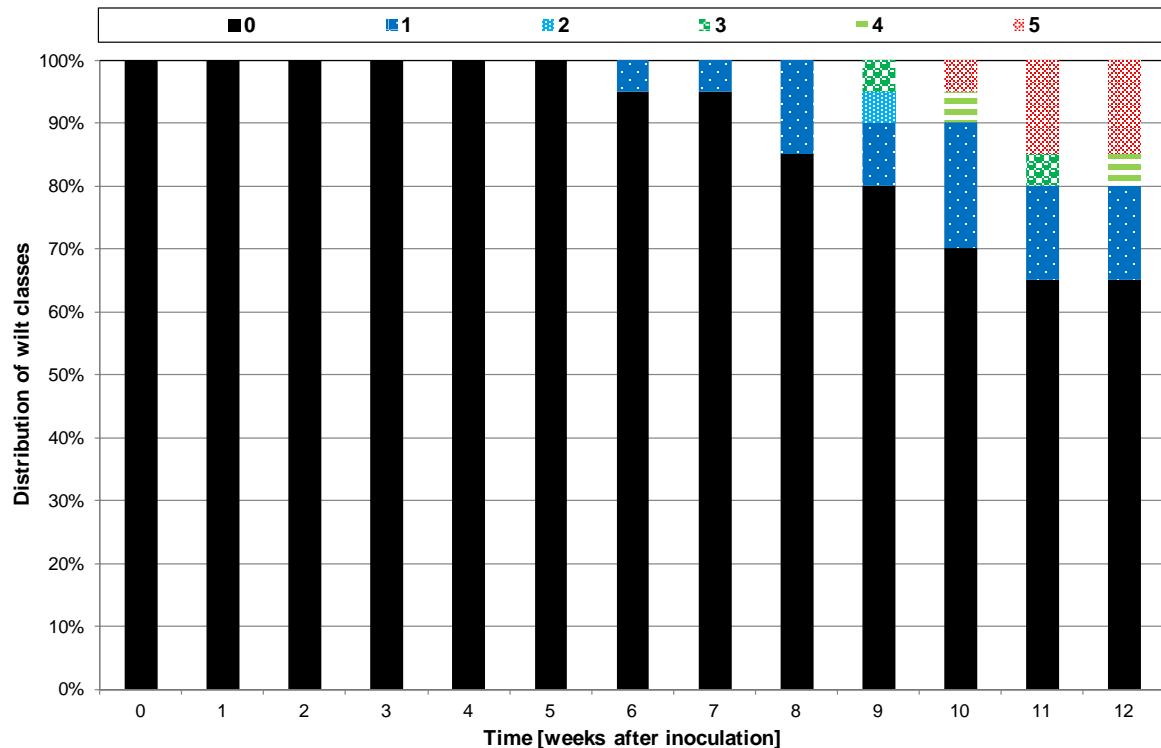


Figure 6.10 Distribution of wilt classes [%] of the control trees of the provenance 851 13 over time [weeks after inoculation], n=20

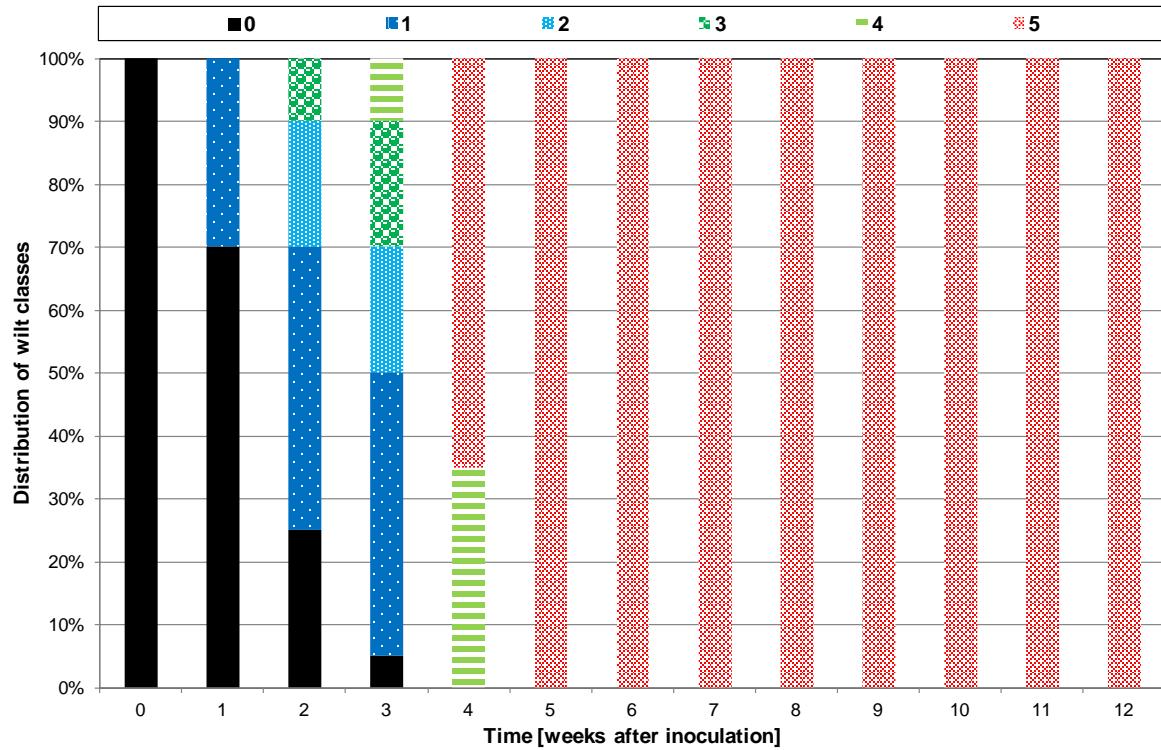


Figure 6.11 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 14 over time [weeks after inoculation], n=20

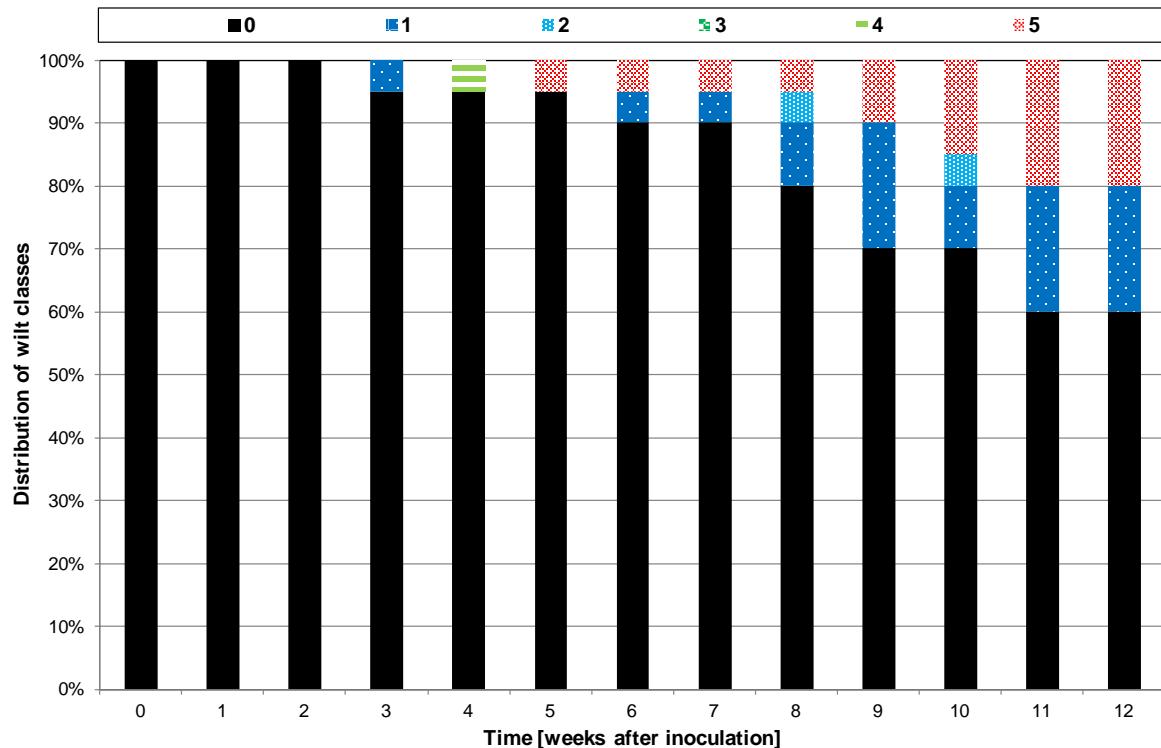


Figure 6.12 Distribution of wilt classes [%] of the control trees of the provenance 851 14 over time [weeks after inoculation], n=20

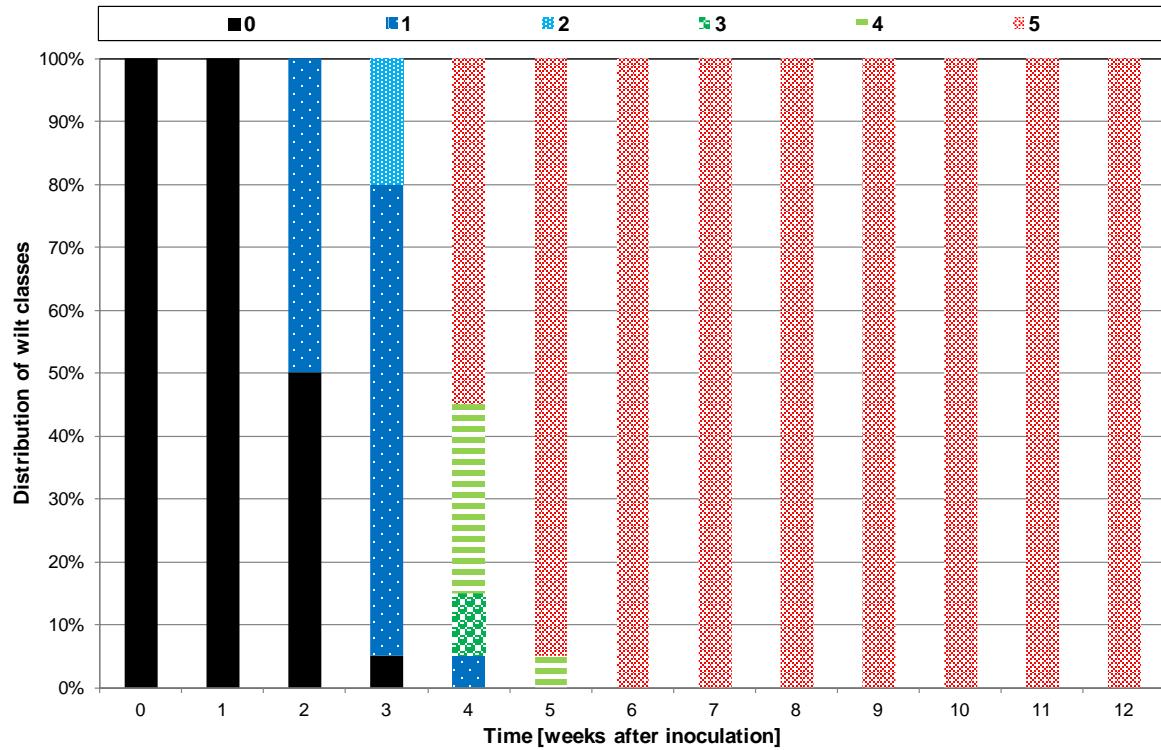


Figure 6.13 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 15 over time [weeks after inoculation], n=20

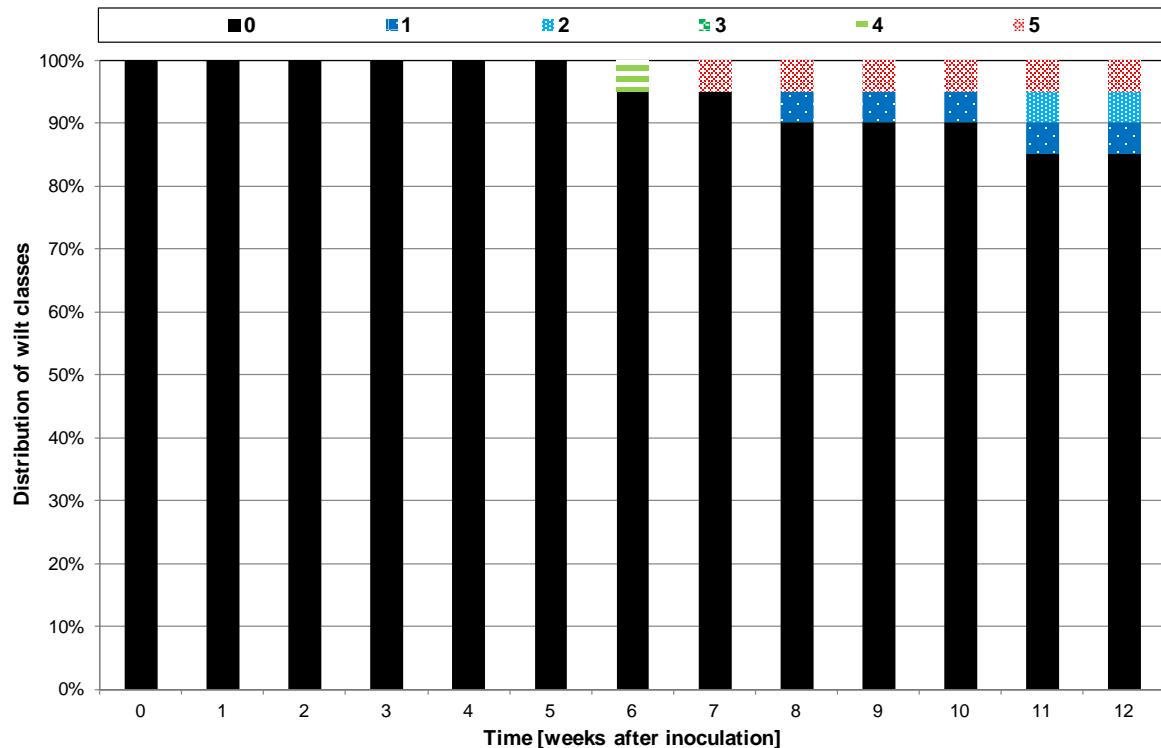


Figure 6.14 Distribution of wilt classes [%] of the control trees of the provenance 851 15 over time [weeks after inoculation], n=20

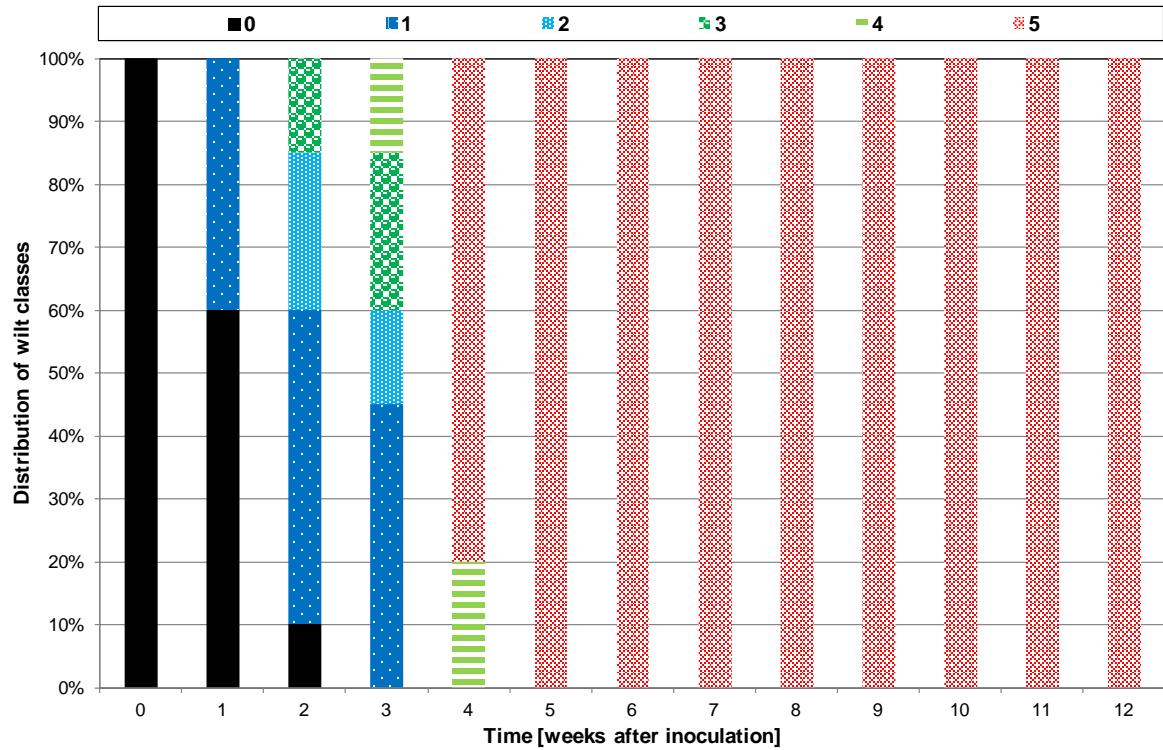


Figure 6.15 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 20 over time [weeks after inoculation], n=20

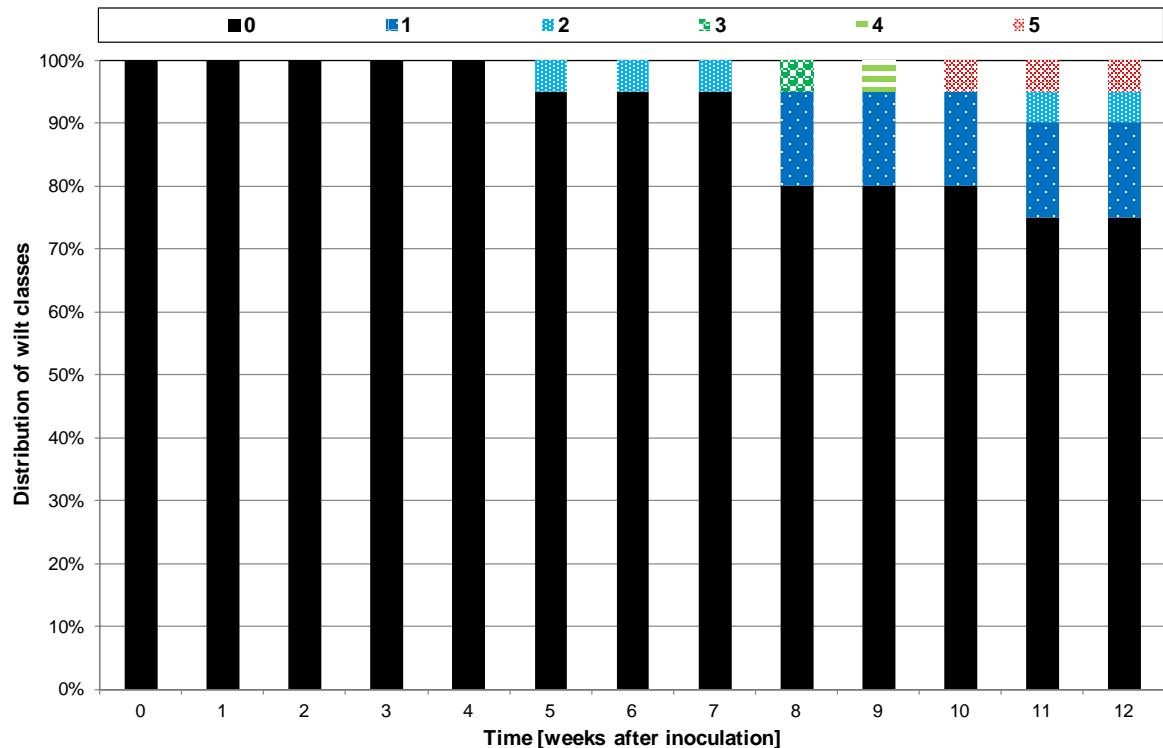


Figure 6.16 Distribution of wilt classes [%] of the control trees of the provenance 851 20 over time [weeks after inoculation], n=20

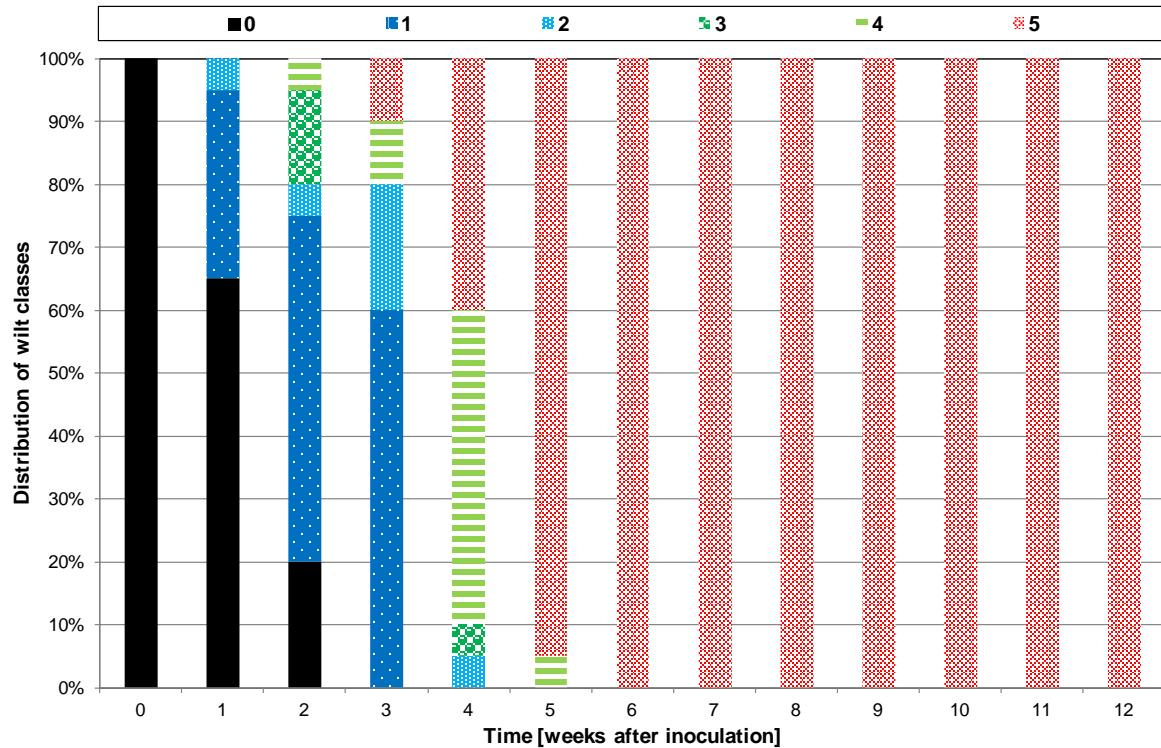


Figure 6.17 Distribution of wilt classes [%] of the PWN inoculated trees of the provenance 851 22 over time [weeks after inoculation], n=20

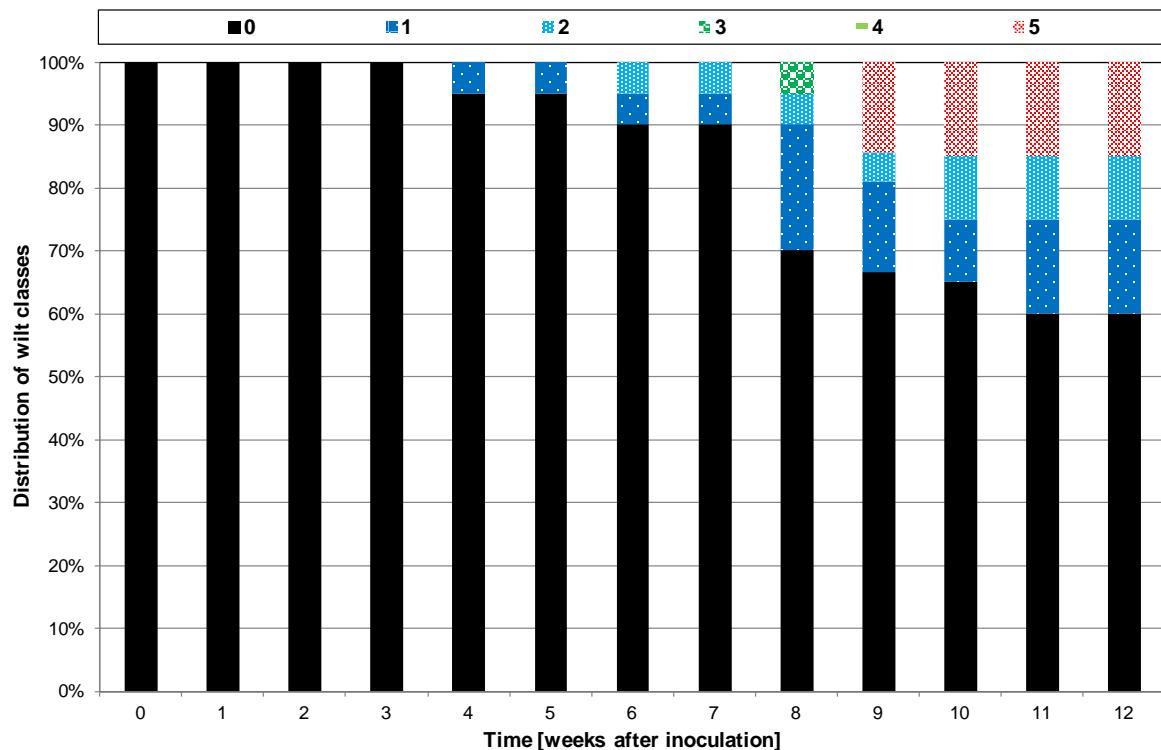


Figure 6.18 Distribution of wilt classes [%] of the control trees of the provenance 851 22 over time [weeks after inoculation], n=20

At test start all trees showed no symptoms (= wilt class 0). In the course of the testing weeks the distribution of wilt classes of the different provenances developed similarly.

Until test end the control trees could hold wilt class 0 for 60-90 % of the saplings per provenance. Wilt class 1 could be found with 5-20 % per provenance and wilt class 2 and 3 with 5-10 % (but not for all provenances). Only for the provenance 02 (from now on short versions of the provenance numbers used) wilt class 4 could be found for one tree. Wilt class 5 was developed with 5-20 % per provenance (control saplings) until 12 weeks after test start.

After five or six weeks, for all PWN inoculated saplings of all eight provenances, wilt class 5 was observed. After one week only the provenance 15 still showed wilt class 0 with 100 %. Two and three weeks after test start in nearly all cases wilt class 1 was the most frequent class and the wilt classes 2 and 3 occurred. A fast development from wilt class 1 to 4 and 5 could be observed. In week four the wilt class 4 or 5 was dominant, except by the provenance 02. Here the last wilt class was only one week later dominant. The earliest detection of wilt class 5 was three weeks after inoculation for the provenance 22. With a mortality of 100 % *B. xylophilus* was, for all saplings in a similar time span, pathogenic independent from the provenance.

No PWN were extracted from the control pines. In all nematode inoculated pine groups PWN could be extracted. The nematode densities extracted from the upper plant part above the point of intersection and the corresponding number of pines with successful PWN extraction per provenance can be found in Figure 6.19 and Table 6.2.

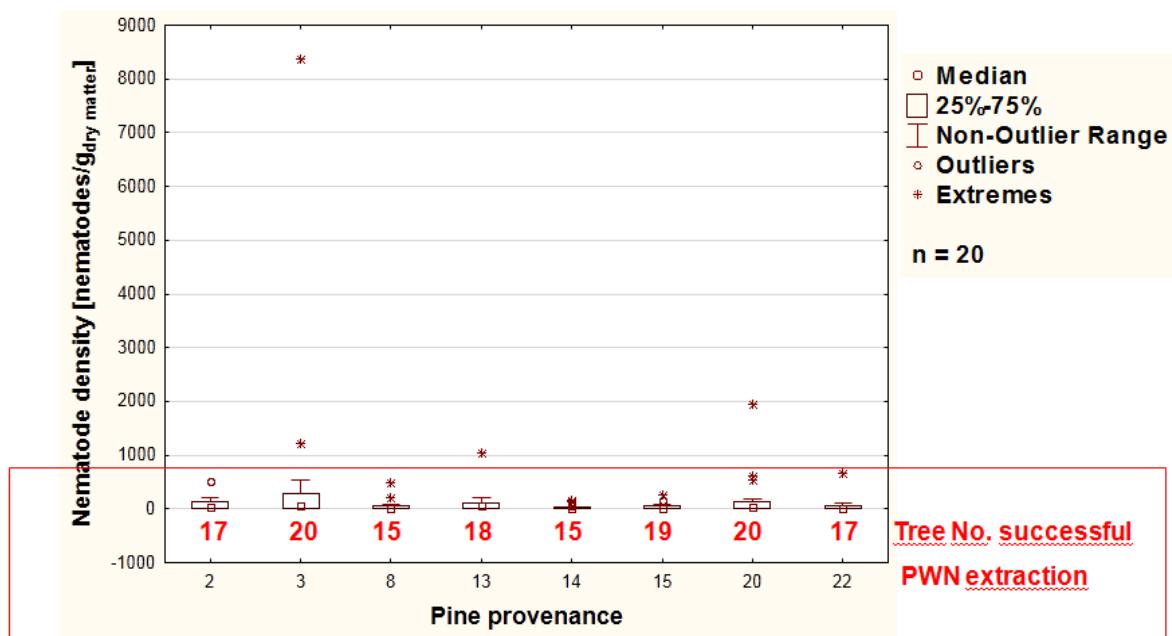


Figure 6.19 Nematode density [nematodes/g_{dry matter}] in the upper plant part above the intersection point with median, 25 % and 75 % quantiles, non-outlier range, outliers and extremes and number of plants with successful PWN extraction

Table 6.2 Nematode density [nematodes/g_{dry matter}] of the PWN inoculated pine variant per provenance, n=20

Variant	Nematode density [nematodes/g_{dry matter}]		
	Median	Min	Max
02 B. x.	24.5	0.0	513.3
03 B. x.	68.2	2.0	8372.4
08 B. x.	13.9	0.0	488.7
13 B. x.	52.8	0.0	1031.9
14 B. x.	6.4	0.0	154.6
15 B. x.	14.0	0.0	265.4
20 B. x.	22.8	1.1	1935.4
22 B. x.	9.9	0.0	662.1

The maximum nematode density was found with 8,372 PWN/g_{dry matter} for one sapling of the provenance 03. The medians for each provenance of the nematode inoculated variants range between 6 (provenance 14) and 68 nematodes/g_{dry matter} (provenance 03).

In Table 6.3 the wood MC at test end are listed for each test variant. PWN infestation led to 132-186 % reduced wood MC (based on medians) compared to the control saplings for each provenance.

Table 6.3 Wood moisture content MC [%] at test end per pine variant (provenance and inoculation type) with B. x. – *B. xylophilus* inoculated, K – Controls (with water inoculation) and last numbers of silvicultural regions of provenance, n=20

Variant	Wood moisture content [%]		
	Median	Min	Max
02 B. x.	70.8	16.7	150.0
02 K	202.5	63.9	328.6
03 B. x.	70.6	39.4	167.1
03 K	218.0	142.1	258.3
08 B. x.	42.4	4.2	90.0
08 K	211.8	94.7	284.6
13 B. x.	42.8	6.3	118.8
13 K	204.6	25.0	269.2
14 B. x.	59.6	35.4	150.0
14 K	207.2	17.9	229.4
15 B. x.	44.6	16.1	112.4
15 K	220.2	52.9	294.7
20 B. x.	33.3	23.3	93.8
20 K	219.7	163.2	247.6
22 B. x.	36.7	22.2	100.0
22 K	217.7	28.4	269.2

Activities carried out by B6

The evaluation of the susceptibility to PWN was compared in July 2013, using plants from maritime pine and from Mediterranean stone pine. Fifty three year' old seedlings from two different stone pine and one maritime pine provenances were used to evaluate the existence of different levels of resistance/tolerance to the pinewood nematode (PWN). The treatments were arranged in a split-plot under a randomized

complete block design, using 50 plants/provenance, distributed in four replications in the greenhouse. All the plants were measured for the total height and diameter at the base. Weekly, the plants were evaluated for the symptoms and 40 plants were destroyed for the evaluation of the number of PWN, using the modified Baermann method.

DELIVERABLES Task 6.1.1 - Symptom development

Results from the ongoing experiment will contribute to Deliverable 1 “Susceptibility of *Pinus sylvestris* provenances to PWN”.

Task 6.1.2 - Comparison of the transcriptomes of *Pinus pinaster* and resistant genotypes of *Pinus* in response to *Bursaphelenchus xylophilus* infestation for detection of ESTs and candidate genes involved in disease resistance to PWN.

Activities carried out by B6

Team

Cândida Trindade and Rita Costa – RNA isolation and sequencing;
Cândida Trindade, Margarida Fontes, Teresa Valdivieso, Amélia Palma, Lurdes Inácio and Rita Costa – Inoculation assays with *Bursaphelenchus xylophilus*.

Sample preparation and sequencing

Eight cDNA libraries of *Pinus pinaster* and *Pinus yunnanensis* inoculated with *Bursaphelenchus xylophilus* were sequenced using IonProton. The RNA was isolated separately from each plant for 6 different time points, 34 plants in total. Following the previous analysis of expression made by qRT-PCR for 6 candidate genes of resistance to *Bursaphelenchus xylophilus*, 4 time points were selected as the most relevant for further sequencing (6, 12, 24, 48 hours after inoculation - hai). For reduction of costs, eight samples were prepared as a pool, according to the following scheme:

Sample 1 - 2 plants of *Pinus pinaster* wounded and not inoculated - Control;

Sample 2 - 3 plants of *Pinus pinaster* wounded and inoculated with *Bursaphelenchus xylophilus* and collected in 2 time points after inoculation respectively 6hai (hours after inoculation) and 12hai;

Sample 3 - 3 plants of *Pinus pinaster* wounded and inoculated with *Bursaphelenchus xylophilus* and collected 24hai;

Sample 4 - 3 plants of *Pinus pinaster* wounded and inoculated with *Bursaphelenchus xylophilus* and collected 48hai;

Sample 5 - 2 plants of *Pinus yunnanensis* wounded and not inoculated - Control;

Sample 6 - 3 plants of *Pinus yunnanensis* wounded and inoculated with *Bursaphelenchus xylophilus* and collected in 2 time points respectively 6hai and 12hai

Sample 7 - 3 plants of *Pinus yunnanensis* wounded and inoculated with *Bursaphelenchus xylophilus* and collected 24hai

Sample 8 - 3 plants of *Pinus yunnanensis* wounded and inoculated with *Bursaphelenchus xylophilus* and collected 48hai.

Sequences of poor quality were removed and the first output is shown in Table 6.4:

Table 6.4 Number of reads and average length.

Sample	Number of Reads	Average length of reads
Amostra 001	47.903.109	122
Amostra 002	38.483.969	119
Amostra 003	44.943.925	122
Amostra 004	44.951.165	121
Amostra 005	43.708.074	113
Amostra 006	40.281.442	114
Amostra 007	39.601.162	109
Amostra 008	45.734.269	108

Analysis of Differential Expression

The transcriptome SustainPine from *Pinus pinaster* was used as the reference and the reads of each sample were mapped against it (Table 6.5).

Table 6.5 Percentage of reads aligned in each sample.

Sample	Percentage of alignment
001	82.24%
002	83.16%
003	83.34%
004	81.30%
005	78.60%
006	79.39%
007	76.30%
008	78.81%

The differential expression of each transcript was determined in each sample using the software RSEM. The normalization of reads was done using the method Trimmed Mean of M-values (TMM). The determination of differentially expressed transcripts between samples was determined with package R EdgeR, using the following parameters: significance of 0.05 and 4x differentially expressed between samples (fold). The number of transcripts differentially expressed between samples is presented in Table 6.6.

Annotation

The functional annotation of transcripts was done with the pipeline Trinotate. This pipeline identifies the transcripts with homology using BlastX e BlastP against the database SwissProt. It also identifies the protein domains through HMMER/PFAM. The annotations of each transcript with the expression values were added to a database for better analysis and visualization

Table 6.6 Number of transcripts differentially expressed between samples.

Samples	002	003	004	005	006	007	008
001	12.909	7.269	4.420	16.649	17.007	20.453	16.995
002		5.453	9.930	18.737	16.627	16.819	20.275
003			4.676	14.171	13.371	14.574	15.149
004				13.541	13.963	19.731	13.591
005					954	8.198	2.227
006						3.373	2.369
007							6.596

Analysis of Results

The highest number of transcripts differentially expressed is observed between sample 2 (*Pinus pinaster* 6h+12hai) and sample 8 (*Pinus yunnanensis* - 48hai). The highest number of transcripts, differentially expressed for the same species, was obtained for *Pinus pinaster* in the first time point (6h+12hai). The number of transcripts differentially expressed is much higher in *Pinus pinaster*, between the different time points, than in *Pinus yunnanensis*. The analysis will proceed for the identification of candidate genes of resistance to *Bursaphelenchus xylophilus* and validation by quantitative PCR.

DELIVERABLES Task 6.1.2 - - Comparison of the transcriptomes of *Pinus pinaster* and resistant genotypes of *Pinus* in response to *Bursaphelenchus xylophilus* infestation for detection of ESTs and candidate genes involved in disease resistance to PWN

Results from the ongoing experiment will contribute to Deliverable 2 “Construction of cDNA libraries from sensitive and resistant genotypes of *Pinus*” (m. 45), and Deliverable 3 “Identification of PWN resistance genes in pines” (m. 45).

6.2 Comparison of the transcriptomes of *Pinus pinaster* and resistant genotypes of *Pinus* in response to *Bursaphelenchus xylophilus* infestation for detection of ESTs and candidate genes involved in disease resistance to PWN.

To identify candidate genes involved in disease resistance to PWN, the transcriptomes of infested and healthy tissues, collected from sensitive and resistant genotypes of *Pinus* sp will be sequenced, using ultra high throughput pyrosequencing to identify, characterize and quantify the transcripts and putative candidate genes involved in the molecular mechanism of response to biotic stress related with PWN infestation. Different species of *Pinus*, namely *P. pinea*, *P. halepensis* and *P. brutia* will be assessed regarding its susceptibility to PWN. The genotypes revealing the highest levels of resistance will be used for the sequencing of transcriptomes.

Differential gene expression between resistant and sensitive species of *Pinus*, namely *Pinus pinaster* will be used to generate Expression Sequence Tags (ESTs) and select candidate genes involved in disease resistance to PWN. The ESTs will make possible discovery of single nucleotide polymorphisms (SNPs) and these genetic resources will be made available for association genetics approaches to management of PWN.

Conifers are difficult genetic organisms in many respects because of long generation times, large genomes, lack of well-defined mutants and inefficiencies in transformation. However, their relatively undomesticated condition and large unstructured natural populations provide an ideal situation for association genetics. Linkage disequilibrium is expected to be limited in such populations, making a candidate-gene-based approach optimal and certainly more feasible and cost-effective than a genome-scan approach. On average the SNP frequency in conifer genome coding regions is in the order of 1 in 50 nucleotides. This is ample polymorphism to conduct candidate-gene-based association studies. Initial studies in trees have found ample nucleotide diversity in candidate genes to perform association studies and single nucleotide polymorphisms have been associated with economic and adaptive traits. B6 to coordinate.

Tasks 6.2.1 and 6.2.2

These Tasks have been eliminated.

Task 6.2.3 Evaluation of resistance level and identification of resistance markers during the reproductive phase

Activities carried out by B6

B6 has begun a study to determine the preference of *M. galloprovincialis* to lay eggs on wood with and without the pine wood nematode (PWN). Studies will be made under laboratory conditions during 2014, to evaluate preference for:

- ***P. pinaster* bolts without PWN vs *P. pinaster* bolts with PWN;**
- ***P. pinea* bolts without PWN vs *P. pinea* bolts with PWN;**
- ***P. pinaster* bolts without PWN vs *P. pinea* bolts without PWN;**

- ***P. pinaster* bolts with PWN vs *P. pinea* bolts with PWN;**

Trials have already begun, with the rearing of beetles and inoculation of wood with the PWN. Results are expected for early summer 2014.

DELIVERABLES Task 6.2.3 Evaluation of resistance level and identification of resistance markers during the reproductive phase

Results from the ongoing experiment will contribute to Deliverable 5 “Host preferences for *Monochamus* oviposition” (m. 45).

6.3 Testing of resistance of *Pinus* spp to PWN insect vectors

6.3.1. Evaluation of resistance level and identification of resistance markers during the maturation feeding phase by *Monochamus* spp. on cut shoots

Preliminary bioassays will be carried out on cut pine shoots to provide initial screening for possible resistance to young *Monochamus* adults during their maturation feeding phase. The beetles will be obtained from intensive laboratory rearing. Pine shoots from different origins will be tested using a hierarchical approach, i.e. pine species > provenances > families. B6 will use plant material from different *Pinus pinaster* and *P. pinea* provenance trials. Families will also be studied in *P. pinaster* half-sib progeny trials. A sub-sample of trees will be submitted to different water supplies to test for genetic-environment effects on resistance to *Monochamus* during the feeding phase. The relative amounts of bark fed upon will be used to compare host preferences. Volatile organic compounds and bark extracts will be collected from the same pine shoots and analysed at the laboratory (GC/HPLC – MS) to search for biochemical markers of pine resistance. B6 to coordinate.

To be done in Year 1 and Year 2

Activities carried out by B6

Half-sib progenies obtained from 120 plus trees selected at Comporta were sowed at Obidos nursery (Quinta do Furadouro) in November 2012. In April 2014, all the 8700 plants were transferred to Oeiras. The resistance /tolerance tests for the estimation of genetic parameters (genetic variability, heritability) for pinewood nematode tolerance/resistance will start in June/July 2014.

6.3.2. Evaluation of resistance level and identification of resistance markers during the maturation feeding phase by *Monochamus* spp. on seedling trees

Depending on results from the preliminary screening, further tests will be carried out to determine whether potential resistance is maintained when seedling trees under a range of environmental conditions are subjected to *Monochamus* spp. feeding pressure. The use of seedling trees with intact root systems will remove the possible influence of reduced water supply and plant stress that may have influenced the results from Task 6.1.1. The same methods will be employed to identify putative biochemical markers of pine resistance. B6 to coordinate.

To be done in Year 1 and Year 2

Activities carried out by B6

The objective of this study is to perform artificial pollination between two pines to obtain hybrid seedlings for further testing the resistance to *Bursaphelengus xylophilus*.

Artificial hybridization between *P. pinaster* x *P. halepensis* and *P. halepensis* X *P. pinaster* were performed. In both species seed maturation takes approximately 20 months.

Seeds from the 2012 artificial hybridization between *P. pinaster* x *P. halepensis* were collected by November 2013. *P. halepensis* X *P. pinaster* were performed by February 2014 and seeds will be mature by November 2015.

2012 *P. pinaster* x *P. halepensis* crosses

Flowers from *P. pinaster* x *P. halepensis* controlled crosses performed on 2012 at Quinta do Marquês located at Oeiras, Portugal produced 19 mature cones. The trees that were selected as female and male parents were young and medium age, parasite free, vigorous and with abundant flowers. Pollination was carried out from 3rd to 7th of March, with fresh pollen.

Male flower of *P. halepensis* and female flowers of *P. pinaster* occurred simultaneous and during female receptivity while pollen of *P. pinaster* was not yet dehiscent. Therefore, in order to perform crosses between *P. pinaster* X *P. halepensis* next year (2013) *P. pinaster* pollen has been collected, processed and stored (Figure 6.20).



Figure 6.20 Pollen handling at laboratory, pollen collection, filtrating, drying and -80°C storing

As a precaution against cross-contamination, female flowering cones were bagged after third pollination (Figure 6.21). The needles were removed to accommodate the marked bags. The pollination bags were removed after three weeks.



Figure 6.21 Female flowers with pollination bags

P. halepensis male strobilis were removed from trees for pollen collection. At laboratory the pollen was filtrated and dried at 27°C for 20% moisture. Pollen was stored into labelled vials at -80°C to be used in future pollinations.

Flowers from *P. halepensis* X *P. pinaster* didn't develop

2013

Pollination bags from 2013 were destroyed by wind.

2014

Flowers from *P. halepensis* X *P. pinaster* are evolving and if the process reaches the final stage seeds will be available by November 2015

Results from 2012 crosses *P. pinaster* x *P. halepensis*

- ❖ 1332 seeds from 19 fully mature seed cones from *P. pinaster* x *P. halepensis* crosses, performed in 2012 at Quinta do Marquês
- ❖ Only 16 cones had seeds
- ❖ Seeds were manually extracted, examined, and buoyancy test was used to discard empty seeds- 497empty seeds
- ❖ A total of 835 seeds were sowed in containers from January 30- February 4, 2014 (fig 6.22)
- ❖ Germination data are summarised in Table 6.7
- ❖ by April 2014 the seedlings size average varies from 3-5 cm. All seedlings have definitive needles

Table 6.7 germination data from the seeds from controlled crosses *P. pinaster* x *P. halepensis*

Cones code	Sowing date	n. of viable seeds	N of empty seeds	Germination onset	Germination %
CT-1.14	30-01-2014	0	10		0
CT-2.14	30-01-2014	5	21	01-03-2014	0
CT-3.14	30-01-2014	4	29	01-03-2014	50
CT-4.14	30-01-2014	28	12	01-03-2014	21
CT-5.14	30-01-2014	34	33	01-03-2014	9
CT-6.14	30-01-2014	57	6	01-03-2014	33
CT-7.14	30-01-2014	41	23	01-03-2014	27
CT-8.14	30-01-2014	74	17	01-03-2014	20
CT-9.14	04-02-2014	91	24	01-03-2014	46
CT-10.14	04-02-2014	39	80	01-03-2014	51
CT-11.14	04-02-2014	63	120	01-03-2014	100
CT-12.14	04-02-2014	47	42	01-03-2014	51
CT-13.14	04-02-2014	108	26	01-03-2014	44
CT-14.14	04-02-2014	142	20	01-03-2014	44
CT-15.14	04-02-2014	65	46	01-03-2014	69
CT-16.14	04-02-2014	27	6	01-03-2014	62



Figure 6.22 aspect of seedlings by March 2014 from controlled crosses *P. pinaster* x *P. halepensis* performed on March 2012

The putative hybrids require confirmation by molecular genetic markers.

DELIVERABLES Task 6.3 — Breeding for resistance to PWN disease

Results from the ongoing experiment will contribute to **Deliverable 6 “Hybrid progenies with different tolerance/resistance to the PWN” (m. 33).**

6.3.3. Evaluation of resistance level and identification of resistance markers during the reproductive phase.

Comparative studies of host selection will be conducted using mature adults after mating. Pines to be tested will include species such as *P. pinaster*, *P. pinea*, *P. halepensis*, *P. eldarica*, *P. brutia* and *P. sylvestris*, among others. Oviposition preferences will be determined in replicated no-choice tests, and subsequent hatching of the eggs and development of the larvae will be followed. Morphological characteristics of the pines, such as bark thickness and wood density, as well as resin duct density and movement of the nematode, will be correlated with the results on host selection and suitability. If appropriate, chemical studies of volatiles and bark will be undertaken as above to search for any biochemical markers associated with the preferences observed. The preference of the vector to lay eggs in maritime pine logs with and without the presence of PWN will be determined similarly. B6 to coordinate.

6.4 Breeding for Resistance to PWN Disease

6.4.1. Estimation of genetic parameters of pine resistance to PWN insect vectors

Both direct (damage) and indirect (biochemical markers) resistance parameters will be estimated to evaluate the relevance of tree breeding as preventive control method of PWN disease. Variability, heritability and genetic correlations (e.g. between resistance and growth parameters) will be estimated in genetic field trials (e.g. progeny and diallel tests) to estimate genetic gains. B6 to coordinate.

To be done in Year 2 and Year 3

6.4.2. Hybridizations for resistance to PWN

Artificial F1 hybrid progenies within Mediterranean *Pinus* species will be assessed for expression of PWN resistance. *Pinus halepensis* and *P. brutia* and *P. eldarica* referred as PWN resistant, and the Portuguese native *Pinus pinaster* as sensitive. Crosses will be done between *Pinus pinaster* and *P. halepensis*, *P. brutia*, *P. eldarica* and *P. nigra*, already established in Portugal. Foreign pollen of resistant *Pinus* species, not available in Portugal, will be introduced for artificial crossing using *P. pinaster* as female genitor.

Inoculation tests with PWN will be done for assessment of susceptibility of the progenitors and F1 progenies in the future. B6 to coordinate.

6.5 Potential of mosaics of tree species as a measure to reduce PWN impacts in Portugal.

The mortality and severity of infestation by the PWN will be investigated by field observations of wilt expression in both monoculture stands and in mixed pine-other species forests in Portugal. Wilted trees will need to be sampled for the presence of the PWN. Plots will be created within the pure and mixed stands, georeferencing all trees and following their sanitary condition through the time, in order to detect differential patterns of PWN infestation and tree mortality. B6 to coordinate.

To be done in Year 1, 2 and 3

Activities carried out by B6

From the two plots delimited in maritime pine stands on Madeira island, one (located at Gaula) was completely burned by the huge forest fires that occurred during summer 2012 (Figure 6.23).



Figure 6.23 Location of plots in Madeira Island and incidence of forest fires during summer 2012.

The plot at the East of the island (Prazeres) had 391 pine trees with 28 cm of average diameter at breast-height, and 36 were dead (mortality rate of 9.2%) (Figure 6.24).

Seven of the dead trees were felled and colonizing bark and wood-boring beetles surveyed. Four pines were heavily attacked by the pine shoot beetles (*Tomicus* sp.) but all showed the trunk colonized by the PWN vector, and it started at a remarkably low height.

In fact, all Madeira's pines had the trunk colonized by *M. galloprovincialis* below canopy level and in three the first pupal chamber was less than 2 meters from the ground, very rarely observed in continental Portugal.

However, the bark thickness at the pupal chambers was between 2.34mm and 10.26mm, within the limits detected for *M. galloprovincialis* (less than 15mm). The pines surveyed at Madeira had thinner bark than in continental Portugal allowing *M. galloprovincialis* to lay eggs in lower trunk.

During summer 2014 new evaluation of mortality will be done to assess the sanitary evolution of the plot.

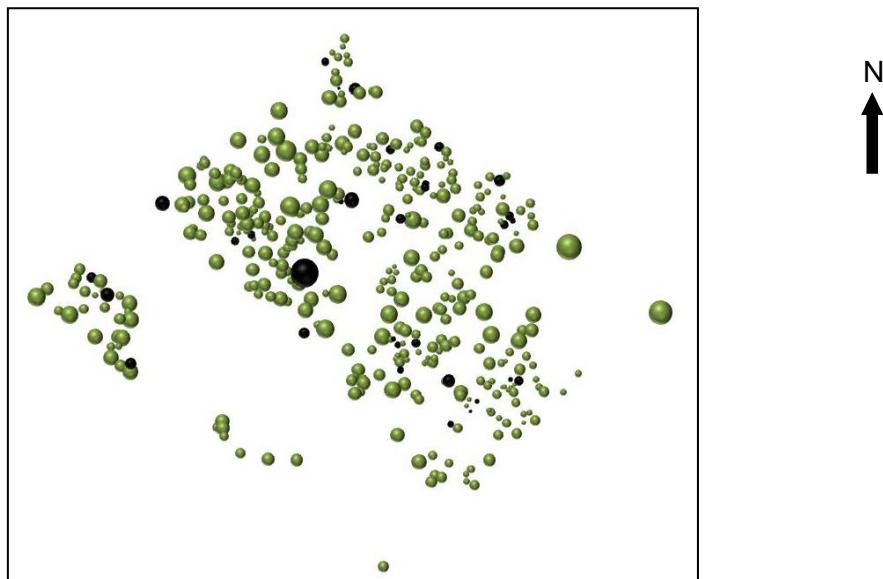


Figure 6.24 Plot delimited in a maritime pine stand at Prazeres, Madeira Island in 2012. Size of bubbles reflect diameter at breast-height of pines. Black bubbles represent dead pines when plot was delimited.

DELIVERABLES Task 6.4 – Potential of mosaics of tree species as a measure to reduce PWN impacts in Portugal

Results from the ongoing experiment will contribute to Deliverable 7 “Tree species mosaics to reduce PWN impact”.

References:

- Baermann, G. (1917): Eine einfache Methode zur Auffindung von Ankylostomum- (Nematoden)- Larven in Erdproben. Welteureden. Batavia. Geneesk. Lab. Feestbundel, pp. 41-47.
- Daub. M. (2008): Investigations on pathogenicity, invasion biology and population dynamics of the Pine Wood Nematode *Bursaphelenchus xylophilus* (Steiner und Buhrer 1934) Nickle 1970 in European conifers. Bonn: Dissertation Rheinischen Friedrich- Wilhelms-Universität zu Bonn, 110 pp.
- Decker, H. (1969): Phytonematologie - Biologie und Bekämpfung pflanzenparasitärer Nematoden. VEB Deutscher Landwirtschaftsverlag Berlin, 526 pp.
- Dropkin, V. H. (1989): Introduction to Plant Nematology. JOHN WILEY & SONS, New York, Second edition, 304 pp.

Directory of norms:

- DIN 52183 (1977) Testing of wood; determination of moisture content.

Work Package 7: Prediction of pine wilt expression across eco-climatic zones, taking account of latency

Objectives

- The process model developed in PHRAME will be extended, refined and a simplified sub-model developed to provide assessment of the risk of pine wilt expression at a range of scales and climate scenarios, current or future. This will include an economic dimension to quantify the full impact of wilt expression.
- The conditions that result in latent infestation of trees and delayed expression of wilt will be investigated and quantified through the model approach, leading to improved sampling methods accounting for latency.
- A PWN range-expansion model taking into account both vector and human influences for possible long-range introductions will be developed, building on work in China.

Deliverables

D7.1: Refinement of core model: Refinement of the core model, including testing by end users in the course of development. Month 44 – Progress reported

D7.2) Field verification of process model: Field experimentation to verify and refine the process models with initial work in Portugal, followed by parallel experiments in Canada and, if feasible in relation to inputs by REPHRAME researchers working in the country, Japan. The latter will depend on practicality and potential for collaboration with research groups in Japan under their ongoing research programmes. Month 45 – Field experimentation was not possible but verification was done in Japan using results from literature.

D7.3: Latency sub-model: Development of a sub-model to account for latency in wilt expression. Month 44 – Progress reported.

D7.4: Analysis of PWN history in Portugal: Analysis of the history of infestation and wilt expression in Portugal. Month 42 – Progress reported.

D7.5: PWN spread model: Development of a PWN spread model taking into account human influences. Month 44 – Progress reported.

Work carried out by B1, B4, B7 with some inputs of B5, B6, B10

Progress

All the tasks of WP7 should be delivered at the end of the project since results from other WPs are needed to refine the modelling. A description of progress to date is given hereafter for each deliverable. Regarding the new schedule (resulting from the project extension), the deliverables should be provided on time. The only deviation concerns D7.2 but an alternative approach was developed by using published data on tree infestation and mortality and running the model to assess its accuracy against those data.

7.1 Refinement of the core model (M44)

The process model developed in PHRAME is based on ForestETp, an existing, fully coupled, point scale and daily time step soil-vegetation-atmosphere transfer (SVAT) model, which predicts vertical and lateral water movement from soil, through the plant to the atmosphere, as well as gross primary productivity (GPP). Based on the Penman-Monteith equation, the model simulates various 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. 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 potential

to produce photosynthates and to utilise water, determined in particular by nutrient availability (light, water and nitrogen), temperature and CO₂ for both current and, particularly, future climatic conditions.

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 a new approach to understanding and predicting the impacts of PWN on living trees, ForestGROWTH has been extended to allow simulation of host tree-PWN pathogen interactions principally by simulating the extent of xylem cavitation. 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 can distribute throughout the tree.

Assumption 3. Following infestation, the host tree will change its biomass allocation strategies; consuming reserves of non-structural carbon to activate defence responses to the PWN. In the longer term, carbon remobilisation from storage also contributes to lowering host resistance, reducing the reserves available for flushing and re-growth in the year following infestation (a factor in latency of expression of pine wilt).

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 and is measured as negative xylem water potential.

Assumption 5. By directly and negatively affecting tree ecophysiology in advance of visual symptoms being observed, PWN leads to host death where, either in combination, or whichever occurs sooner:

- 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.

7.1.1. Refinement of the core model

B1 will carry out refinement of the model, both to improve and verify its predictions and also to produce a simplified version (PHRAME light) to enable non-specialist users to input parameters to test ‘what-if’ scenarios of regional impacts of PWN under current and future climates. Forward prediction to account for future climates will be a core activity for both the full and ‘light’ models, and will count on the collaboration of B7, following up on the predictive model (scenario) elaborated under PHRAME, in 2005/ 2006. Most of the initial effort will be in testing the sensitivity of the model to the quality of data inputs and assessing which can be reduced or approximated without compromising the quality of the outputs. Working with economists in Forest Research, the model will be extended to assess the economic, as well as environmental, impacts of PWN.

Work carried out by B1

General summary of progress:

Since the last reporting period significant progress has been made in refining the core model. In the previous report we considered both the ETP (Evapo-transpiration) model and the FG (Forest Growth) model. We noted that although the ETP model was the easier one to refine and simplify, it did however lack some of the outputs that the FG model could offer, i.e. carbon storage. We believed that the amount of “stored energy” the tree had available to it could play an important role in defending itself against PWN.

However the fundamental step in the FG model is to allocate energy to growing new roots, shoots and leaves, while we want to restrict this process in order to model a tree suffering from PWD. We conclude that the FG model is unsuitable to use and that the modified ETP model from the PHRAME project does not currently have an element to account for available energy. As a result we have made new modifications to the original ETP model, which we will call ETPN (Evapo-transpiration + Nematode) model.

Details of modifications and refinement of core model

Modifications have been made to the model to incorporate the presence of pine wood nematodes (PWN), specifically *Bursaphelenchus xylophilus*, inside a tree by restricting the flow of water through the system. We have made 3 significant changes to the original model: introduced an energy source that we can add to and take from, included an element to model the population growth of nematodes inside the tree and restricted the photosynthesis that can occur by relating it to the nematode population. We consider each element in turn.

Available Energy Element

It is widely believed that PWN kill pine trees by restricting the flow of water through the xylem. Furthermore, observations in the field suggest that a tree can survive a summer following inoculation without displaying any symptoms until the following growing season (Zhao *et al.*, 2008). The cause of this is unknown, however it has been suggested that, providing a tree has enough stores of energy it can continue growing new xylem cells to allow it to transfer water to the crown.

In order to model this behaviour we have created a store of energy that we can add to and take from. We use net photosynthesis to calculate the energy a tree has (after respiration) and we relate the amount of energy a tree uses in defence (against PWN) to the number of nematodes inside the tree. The following equation is used to model the available energy $E_{A,i}$ on day i :

$$E_{A,i} = E_{A,i-1} + E_{Ph,i} - E_{R,i} - E_{G,i} - E_{D,i} \quad (7.1)$$

where $E_{Ph,i}$ is the energy from photosynthesis on day i , $E_{R,i}$ is the energy used in respiration on day i , $E_{G,i}$ is the energy used for growth on day i and $E_{D,i}$ is the energy used in defence day i . Furthermore, $E_{A,0} = C$, where C is initial storage energy.

We note that we can combine the energies from photosynthesis and respiration as the energy from net photosynthesis $E_{Phn,i}$:

$$E_{Phn,i} = E_{Ph,i} - E_{R,i} \quad (7.2)$$

Since the model is static, we cannot allocate some of this available energy to grow new roots, shoot and leaves. Realistically, a tree would allocate a lot of this energy to growth. Instead, we include it in available energy, since a tree that is being severely attacked by nematodes would shift energy use from growth to defending itself. So we remove the $E_{G,i}$ term from equation (7.1) and include it in $E_{A,i}$.

Equation (7.1) becomes:

$$E_{A,i} = E_{A,i-1} + E_{Phn,i} - E_{D,i} \quad (7.3)$$

The net photosynthesis is calculated in the original model, so we don't need to calculate this, only relate it to energy produced. In the original ETP model, the units for net photosynthesis are $\mu\text{mol m}^{-2}\text{s}^{-1}$ given at mid-day. To convert this to energy we need

to express it as Joules. $2000 \mu\text{molm}^{-2}\text{s}^{-1}$ is equivalent to 900Wm^{-2} (Vince and Zoltán, 2011), where 1 Watt is 1 Joule per second. Hence, one $\mu\text{molm}^{-2}\text{s}^{-1}$ is $0.45 \text{Jm}^{-2}\text{s}^{-1}$. We can convert the Joules per area in to total energy by multiplying 0.45 times the photosynthesis rate by the total leaf area (specified in the model parameter file). This will give us an energy rate of Joules per second (value at mid-day). We can estimate the photosynthesis over a particular day by using a sinusoidal function (Burgess, 2009). Let Ph_{md} be the mid-day (max) value of photosynthesis, then we can calculate the total amount of photosynthesis Ph_{tot} over a particular day by calculating the area under the sinusoidal curve. We obtain:

$$Ph_{tot} = Ph_{md} \frac{2h_d}{\pi} \quad (7.4)$$

where h_d are the hours of daylight (calculated in the ETP model).

We are now able to express equation (7.3) as:

$$E_{A,i} = E_{A,i-1} + \frac{0.9h_d}{\pi} Ph_{md} A_{tc} - E_{D,i} \quad (7.5)$$

A_{tc} is the total canopy area.

Finally, we relate the energy used in defence to the number of nematodes inside the tree. We note that the amount of energy a tree can use to defend itself on day i depends on how much available energy the tree has on day $i-1$:

$$E_{D,i} \leq E_{A,i-1} \quad (7.6)$$

We want a proportion of the energy available to the tree to be used in defence. The proportion will depend on the number of nematodes in the tree and also how resistant the tree is. There exists a positive correlation between the number of inoculated nematodes and the rapidity and severity of pathological responses and disease development (Mamiya, 1983 and Myers, 1986). We make the amount of energy used in defence proportional to the number of nematodes inside the tree with the added constraint that the energy used is bounded by zero and the available energy. Hence, a logistic curve relationship (an S-shaped curve, bounded below by zero and bounded above by one) will model our situation well. We note that a similar idea was used in the PHRAME project (Ikegami, 2009) with percentage loss in conductivity curves.

We express equation (7.5) as:

$$\begin{aligned} E_{A,i} &= E_{A,i-1} + \frac{0.9h_d}{\pi} Ph_{md} A_{tc} - \psi(n) E_{A,i-1} \\ &= [1 - \psi(n)] E_{A,i-1} + \frac{0.9h_d}{\pi} Ph_{md} A_{tc} \end{aligned} \quad (7.7)$$

Where $\psi(n)$ is a logistic curve defined by:

$$\psi(n) = \frac{1}{1 + \exp\{a[n - b]\}} \quad (7.8)$$

For a tree with no nematodes we would expect that no energy will be used for defence. That is, for $n=0$, $\psi(n)=0$. For large nematode numbers we want $\psi(n)$ to tend to 1. We require that the coefficient a is negative and of small magnitude (less than one) so that we have a very gentle slope in the logistic curve. The coefficient b needs to be very large since we would not expect to use much energy in defence until the nematode numbers reach a significant amount.

We also want to add an element that depends on tree resistance, such that if a tree has high resistance then it will use less energy in defending itself. It is difficult to quantify what we mean by tree resistance so we have defined it as a proportion (0.0-1.0), where 1 corresponds to very high resistance (will survive attack by PWN) and 0 corresponds to no resistance at all (tree will die from PWD in a very short time). We define tree resistance as σ then $0 \leq \sigma \leq 1$ and we can express equation (7.7) as:

$$E_{A,i} = [1 - (1 - \sigma)\psi(n)]E_{A,i-1} + \frac{0.9h_d}{\pi} Ph_{md} A_{tc} \quad (7.9)$$

When $\sigma = 1$, a tree will not use any of its energy for defence, regardless of how many nematodes are in the tree. When $\sigma = 0$ a tree has no resistance and so the amount of energy used in defence is controlled by the number of nematodes in a tree. Realistically, the value should never be at either extreme so that both tree resistance and nematode number have an effect on the amount of energy a tree will use to defend itself.

It follows that we have an expression to calculate the available energy on a given day, given the available energy on the previous day, the amount of photosynthesis, daylight hours, the canopy area and the number of nematodes.

Nematode Population Element

In order to model the growth of the nematode population within a tree we use a discrete, single species population model (Murray, 1989). The equation we use calculates the number of nematodes n_i present on day i from the number of nematodes n_{i-1} that were present on day $i-1$, for a given daily growth rate r_i :

$$n_{i+1} = r_{i+1} n_i^{1-s} \quad (7.10)$$

for some constant s , where n_i^{1-s} is the population that survives to breed. We will later make s a variable, since we want to vary the proportion of nematodes that survive to breed depending on how much energy the tree has. We know that a tree infested with pinewood nematodes will use some of its energy defending itself; hence, not all nematodes will survive long enough to reproduce. The growth rate of the nematodes r_i will be made temperature dependent and we will also add an element of resistance to equation (7.10).

The PWN has two different modes in its life cycle: a propagation mode and a dispersal mode. Within these modes are different stages. Depending on the temperature and food availability juvenile pinewood nematodes can moult into a resistant third stage until conditions become favourable again. When referring to nematode number in equation (7.10) we make no distinction between the different stages of development. This would be far too complicated and even unnecessary. However, we do make r a function of temperature so that when temperature is low the nematode numbers remain at a low number.

We make n_i , the number of nematodes on day i , a function of temperature t_i on day i : $n(t)$ (we drop the subscripts i for convenience). We estimate this function using results from the literature.

We obtain information about temperature thresholds for development, life expectancies and the number of days for PWN to reproduce at different temperatures (OEPP/EPPO, 1986, Donald *et al.*, 2012, Dropkin *et al.* 1981, Oh *et al.* 2009, Stokes 1979 and Wang *et al.* 2012). Results of the disease development (Kuroda *et al.*, 1988) are also used to obtain accurate parameters for the model and we use details of the disease development, as documented in chapter 21 of Pine Wilt Disease (Zhao *et al.*, 2008), to validate modifications to the ETPN model.

Areas experiencing hot and/or dry summers are at much greater risk of developing the disease. In Japan, severe pine wilt occurs in areas with an annual mean temperature above 14°C (Mamiya, 1984). Furthermore, the average summer temperature of the northernmost location in Japan where PWD has been found is 23.2°C (Magnusson, 1986).

We combine all of the data from the literature to estimate an expression for r_i , the growth rate of nematodes on day i .

The following function estimates r_i :

$$r_i(t_i) = 1.7(40 - t_i)^{2.32} \exp\left\{\frac{-(40 - t_i)}{3.3}\right\} \quad (7.11)$$

Substituting equation (7.11) back into equation (7.10), we obtain:

$$n_{i+1} = 1.7(40 - t_{i+1})^{2.32} \exp\left\{\frac{-(40 - t_{i+1})}{3.3}\right\} n_i^{1-s} \quad (7.12)$$

We note that the initial nematode number n_0 is an input that the user is required to enter into the parameter file. The temperature on day i is included in the climate data file as part of the model input. The variable s depends on tree resistance and the energy available to the tree for defence. When a tree has high resistance, we would expect the number of nematodes to be restricted. Furthermore, if a tree has plenty of energy stores then it will be able to defend itself and keep nematode numbers low.

If we consider equation (7.10), we can see that in order for it to be realistic, we must impose some restrictions on s . For example, we require that $n_{i-1}^{1-s} \leq n_{i-1}$, i.e. the number of nematodes that survive to breed must be less than the total number of nematodes. We fix s on day i such that it is between 0 and 1 and introduce another logistic curve that will restrict the growth of the nematodes depending on the available energy that the tree has.

$$s = \phi(E_{A,i}) = \frac{1}{1 + \exp\{\lambda[E_{A,i} - \theta]\}} \quad (7.13)$$

We require that the logistic curve approaches 1 as the value for available energy increases. However, determining accurate values for the coefficients λ and θ is more

difficult. We use results from the literature as mentioned above to determine what these values should be. We can express equation (7.12) as:

$$n_i = 1.7(40 - t_i)^{2.32} \exp\left\{\frac{-(40 - t_i)}{3.3}\right\} n_{i-1}^{1-\phi(E_{A,i})} \quad (7.14)$$

We also add an element of resistance, as defined in the previous section; σ such that $0 \leq \sigma \leq 1$. We incorporate this into equation (7.14) to obtain:

$$n_i = 1.7(40 - t_i)^{2.32} \exp\left\{\frac{-(40 - t_i)}{3.3}\right\} n_{i-1}^{1-\sigma\phi(E_{A,i})} \quad (7.15)$$

It follows that we have a function to calculate nematode number on a particular day, given the temperature on that day, the number of nematodes on the previous day, the tree resistance (constant) and the available energy on that particular day.

Photosynthesis Restricting Element

The ETP model calculates the net photosynthesis from both the sunlit and shaded parts of the tree. The equations involved in calculating these are complex and trying to modify these directly would be impossible. Two different methods are used and then the model checks that these do not differ by more than a certain margin of error. Instead we modify the final values for net photosynthesis by using another logistic curve relationship. We define the restricted net photosynthesis (RPh) as a proportion of the original net photosynthesis from the sunlit and shaded parts (NPh_{sun} and NPh_{shade} respectively):

$$RPh = \varphi(n)[NPh_{sun} + NPh_{shade}] \quad (7.16)$$

Where $\varphi(n)$ is the logistic curve defined by:

$$\varphi(n) = \frac{1}{1 + \exp\{\alpha[n - \beta]\}} \quad (7.17)$$

We note that n , the number of nematodes is temperature dependent. Furthermore, we have omitted the subscript i in the equations in this section for convenience. The coefficients α and β determine the slope and shift of the curve respectively. When the nematode number is zero we require that the restricted photosynthesis is equal to the original values of photosynthesis, i.e. $\varphi(0) = 1$

The coefficient β gives the value of n at which the logistic curve is 0.5. We would expect to have a large number of nematodes in the tree before seeing a 50% drop in the photosynthesis.

Combining the above, we have developed a system for modelling the presence of PWN inside a tree and for determining how the presence of these nematodes will affect the processes of a tree. These modifications have been coded and incorporated into the original ETP model and we are now in a position to run the model to determine accurate parameter values and to determine what parameters and input are driving the model.

Figure 7.1 represents the movement of water and energy through the soil-plant-atmosphere system that is modelled by the ETPN model.

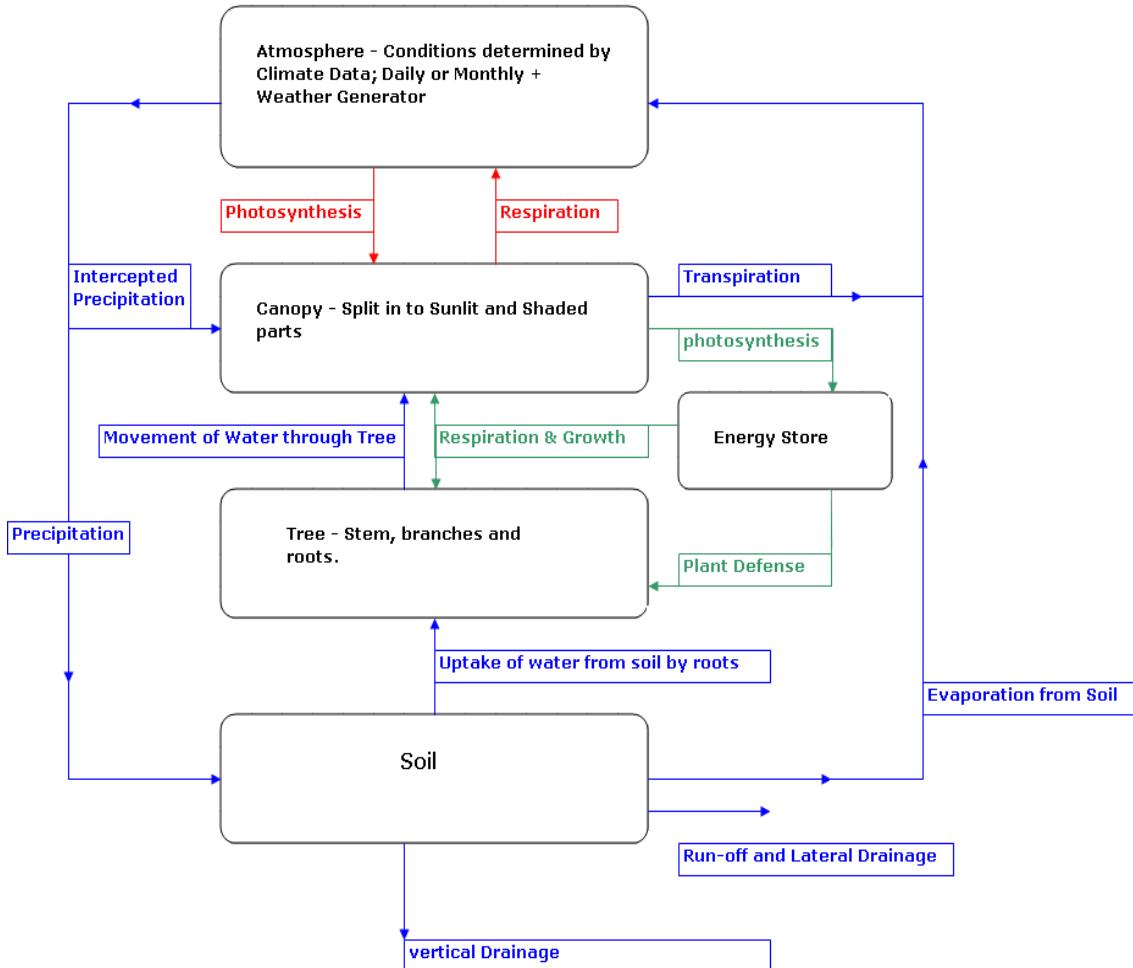


Figure 7.1 Diagram representing the key processes in the ETPN model.

Limitations of ETPN model

PWD depends on the existence of a longhorn beetle of type *Monochamus*. The beetle acts as a vector for the nematode, without which it would not be able to reach new hosts. The ETPN model predicts the likelihood of a tree dying from PWD provided it has been infested with pine wood nematodes. The ETPN model does not consider the role played by the beetle. We assume that the beetles are present and that nematodes have entered the tree. Furthermore, we note that certain pine trees are resistant to pine wood nematodes and that certain nematodes also appear to be avirulent. The ETPN model cannot differentiate between these things. The model assumes that we are using susceptible pine trees and virulent nematodes.

Model Validation

Data from Japan have been used to validate the ETPN model. There are limited data available for Portugal, since this is a relatively new disease there and because of strict control measures used to contain the disease. PWD has been killing pine trees in Japan since 1905 (first incidence reported in Nagasaki on Kyushu Island (Zhao et al., 2008)). Since then it has spread all the way to the north of Honshu Island. The disease has not reached Hokkaido Island, and this is believed to be due to the colder climate there.

Figure 7.2, taken from Mamiya (1988), shows the limit of the disease in Japan. It has affected all of Japan except the Hokkaido and Aomori Prefectures. The areas shaded with diagonal lines, vertical lines and dots represent areas affected during the 1930's, 1940-1960's and after 1971 respectively.

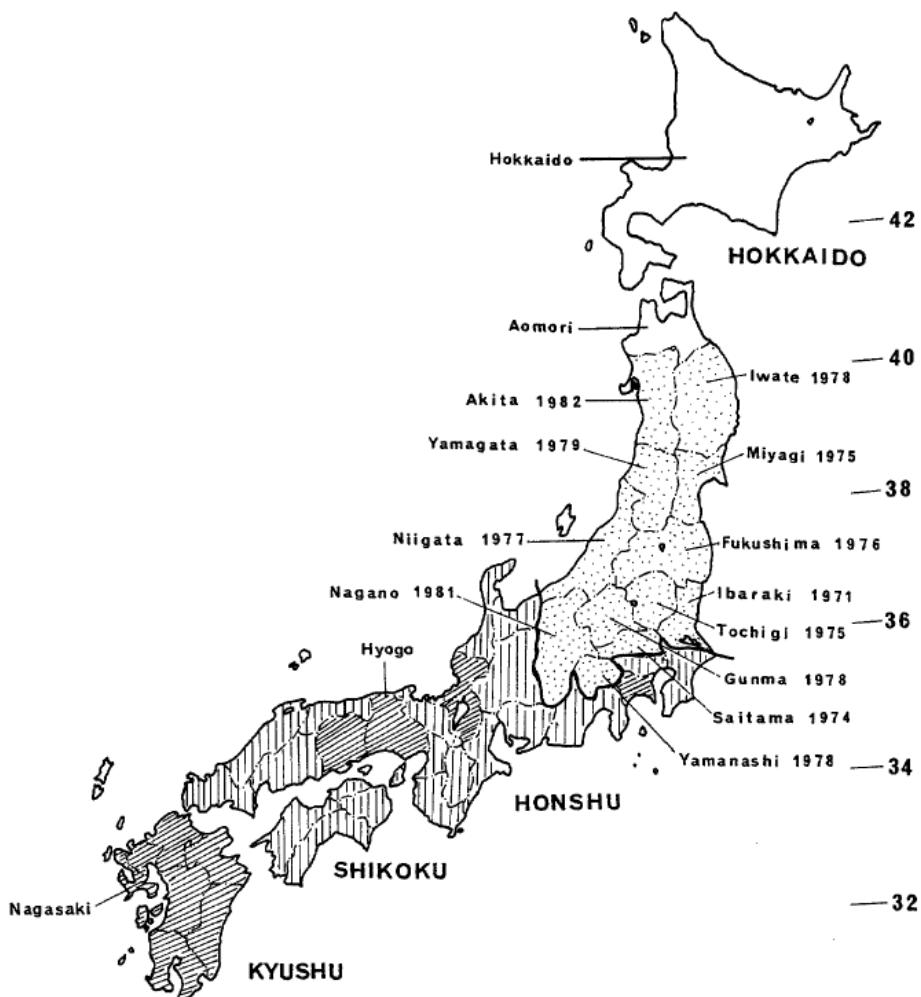


Figure 7.2 Map of Japan showing the current extent of PWD

Recent observations have found PWD in the Aomori Prefecture (Hoshi, 2011), located at the northern tip of Honshu.

We have run the ETPN model for the following locations in Japan: Abashiri, Akita, Aomori, Hiroshima, Kyoto, Morioka, Nagasaki, Tokyo, Tomakomai and Yamagata, varying the resistance between 0 and 1. If there were an equal number of trees in a stand with low resistance, medium resistance and high resistance then one could interpret these values as a percentage risk of death in these regions. However, we note that this is not usually the case; we would expect that a stand of trees would have a consistent level of resistance if all trees are of the same species, planted at the same time and that the land on which they are planted does not vary too much in geology. In a well-managed stand of trees, we would expect that weaker trees or trees not growing as well as others would be removed during thinning and so removing the trees of lower resistance.

The results, shown in Figure 7.3, indicate the limit of resistance that the model predicts, below which, we would not expect to see PWD. For example, at Nagasaki the ETPN model predicts PWD for all trees with resistance below 0.89. At Aomori, the ETPN model predicts PWD for all trees with resistance below 0.55. While at Abashiri, the ETPN model predicts PWD for trees with resistance below 0, i.e. no trees.

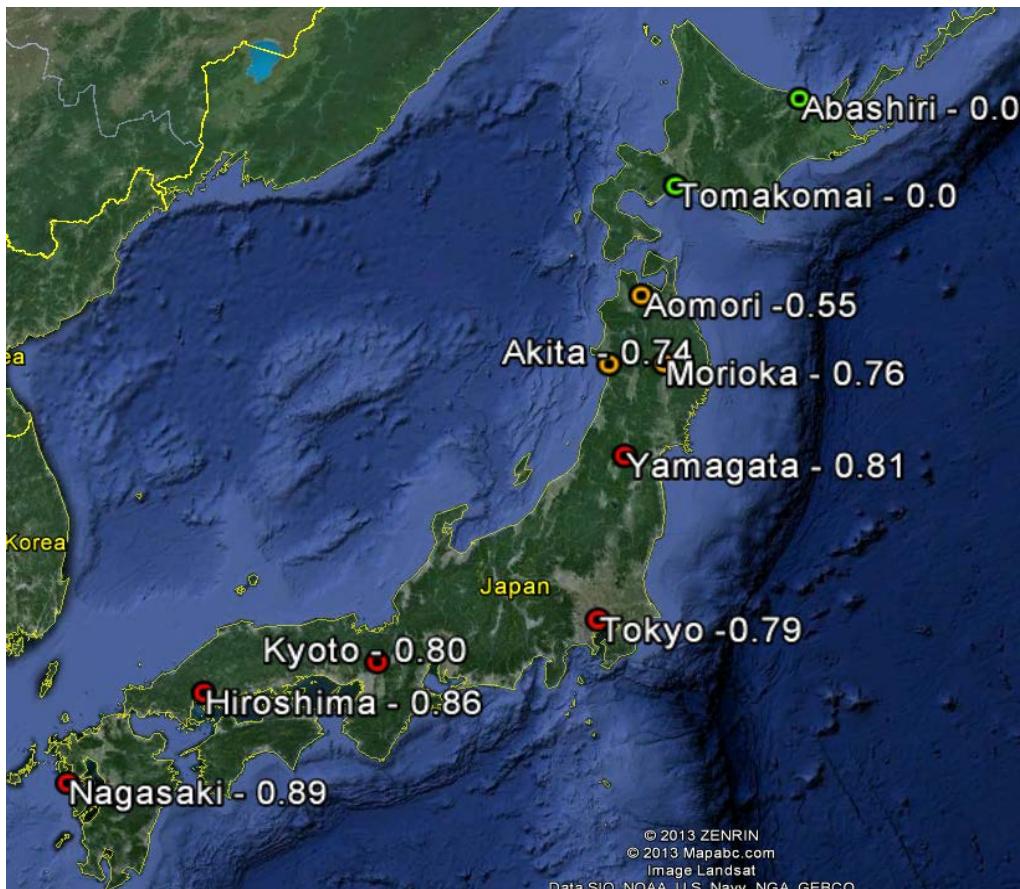


Figure 7.3 Map of Japan with results of simulations from ETPN model.

For trees in locations at the south of Japan; Hiroshima, Kyoto, and Nagasaki, for mid-value resistances, it takes an average of 5-6 weeks from inoculation until death. For those locations coloured amber: Akita, Aomori and Morioka, we see tree death in the growing season a year after inoculation. Tomakomai and Abashiri showed no signs of wilt for any resistances.

Model Input

The model requires climate data as input. Currently we use daily data (The National Climatic Data Center – National Oceanic and Atmospheric Administration, 2013) for the following variables: maximum temperature, minimum temperature, mean temperature, precipitation, relative humidity, wind speed and incoming solar radiation. However, the model has a coupled weather generator, which means that monthly averages can be used instead. For monthly data, the model requires maximum daily temperature (plus standard deviation), minimum daily temperature (plus standard deviation), precipitation of month, mean number of wet days in month and mean daily wind speed (plus standard deviation).

In addition to the climate data, the ETPN model also requires parameter values as input. These are numerous and relate to soil properties, properties of the tree, properties specific to a particular location and those specific to the refined ETPN

model. We will not modify any of the parameters of the original model, only those new parameters introduced into the ETPN model: number of nematodes entering the tree, the day of year that the tree is infested, the resistance of the tree (which we express as a proportion between 0.0-1.0), initial storage energy, diameter at breast height (DBH) and age of the tree.

Model Output

The ETPN model produces daily output for each day of the simulation. Some examples of the output are: transpiration, photosynthesis, soil evaporation, stomatal conductance, soil drainage and specific to the refined ETPN model we have nematode number, growth rate and available energy.

We use the following output to determine whether or not a tree at a particular location is likely to succumb to PWD: nematode number, available energy and photosynthesis. We deduce that a tree has died when the photosynthesis becomes zero and stays zero thereafter, which coincides with a rapid drop in available energy. Finally, we see a large increase in nematode number. There is evidence in the literature to suggest that nematode numbers remain relatively low until the final stage of disease, when the tree is almost dead and then we see a large increase in nematode numbers.

We demonstrate these three outputs for different regions in Europe. We use daily data over three years (2009-2011). The parameters are shown in Table 7.1:

Table 7.1 Parameter values for simulations in Europe.

Parameter	Value
Tree Resistance	0.7
Initial Nematode No.	100
Day of infestation	160
Initial Energy	10000
Age	25
DBH	14

We note that the units for the following output are: photosynthesis given in $\mu\text{mol m}^{-2}\text{s}^{-1}$ and available energy in Joules. We run the model for Evora in Portugal, Santander in Spain and Llanwddyn in Wales.

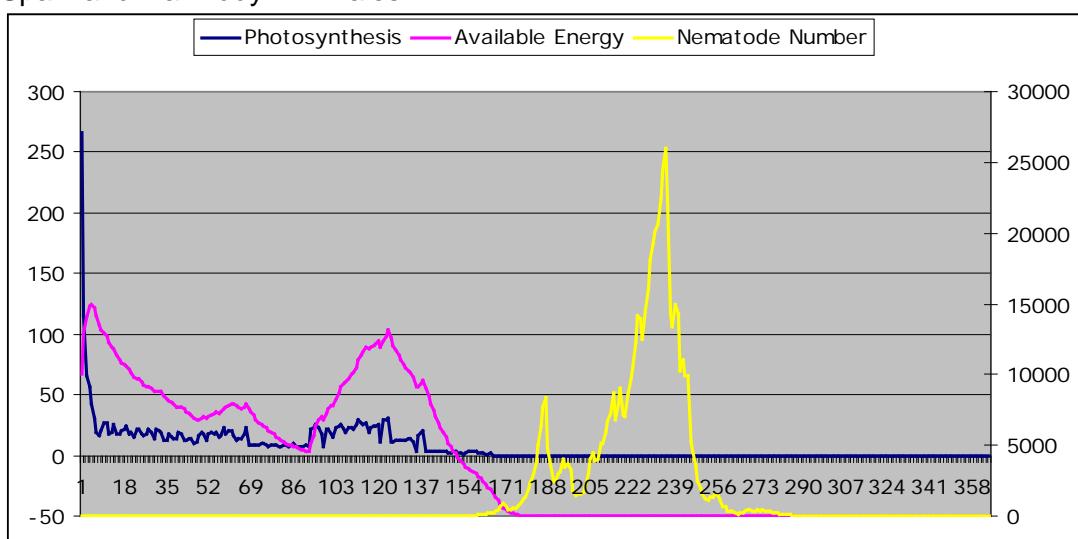


Figure 7.4 Outputs for Evora, Portugal. Death by PWN on Julian day 228.

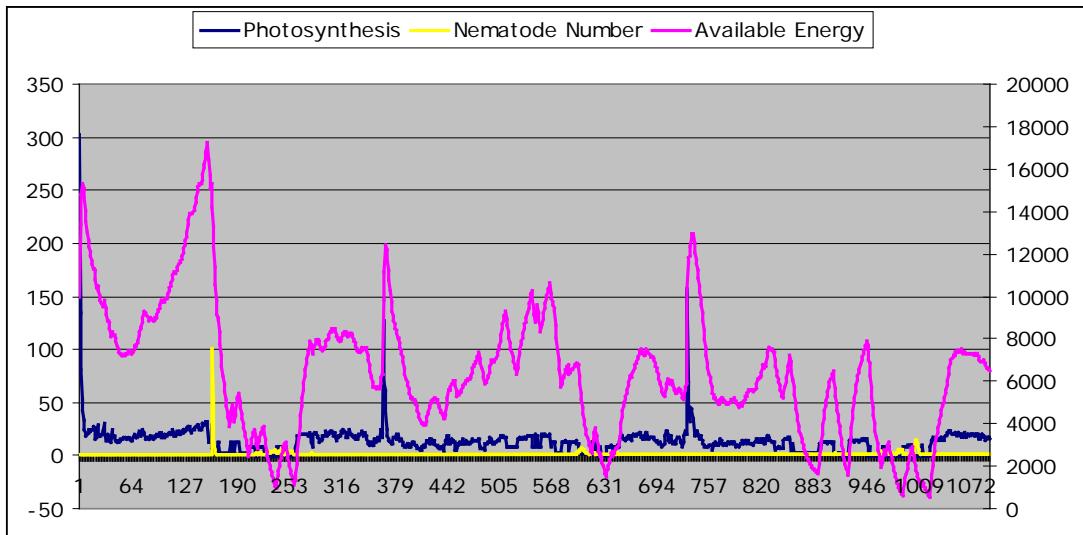


Figure 7.5 Outputs for Santander, Spain.

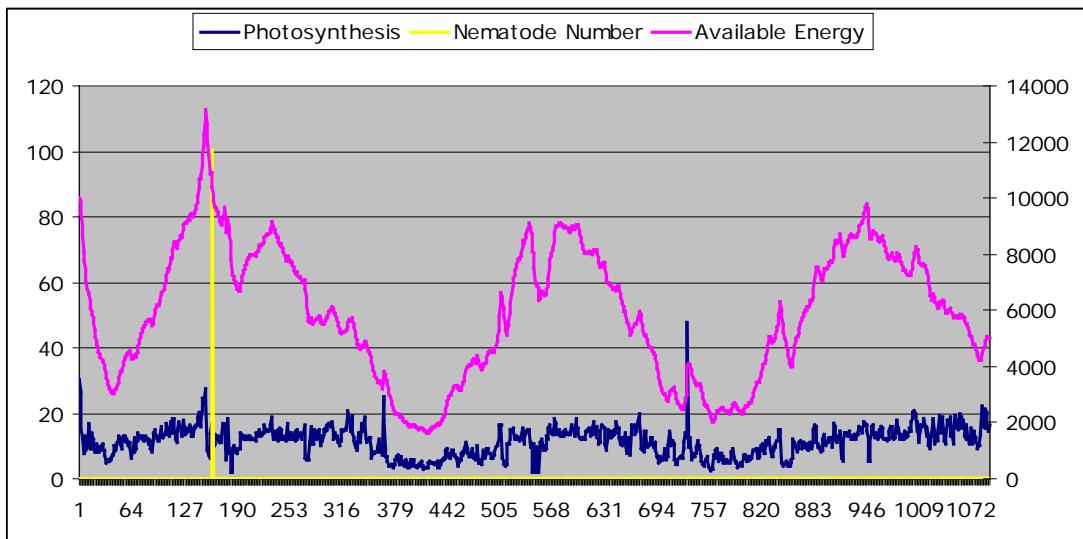


Figure 7.6 Outputs for Llanwddyn, Wales.

Output from the ETPN model predicts PWD at Evora in Portugal but not at Santander or Llanwddyn. Initial drops in the available energy and photosynthesis at Evora prior to infestation (day 160) are due to a particularly dry summer. Following infestation, the photosynthesis and available energy reduces followed by a peak in nematode numbers. Photosynthesis eventually becomes zero on day 228, almost 7 weeks after infestation.

At Santander, the nematode numbers remain low throughout the three years with very small peaks in the following two summers. The nematode numbers are not large enough to affect the photosynthesis or available energy suggesting that a tree at Santander will not succumb to PWD.

Finally, we consider the output for Llanwddyn in Wales. We have an initial peak in nematode number on the day of entry, however, due to the cooler climate at Llanwddyn, the nematode numbers are not able to multiply and we see the nematodes disappear shortly after inoculation.

We note that the initial energy that a tree has: $E_{A,0} = C$ is difficult to determine. However, the energy from photosynthesis is the main factor in determining the available energy a tree has on a particular day. We demonstrate this below with climate data from Llanwddyn in Wales, where we run the ETPN model for different initial values for $E_{A,0}$. (Figure 7.7).

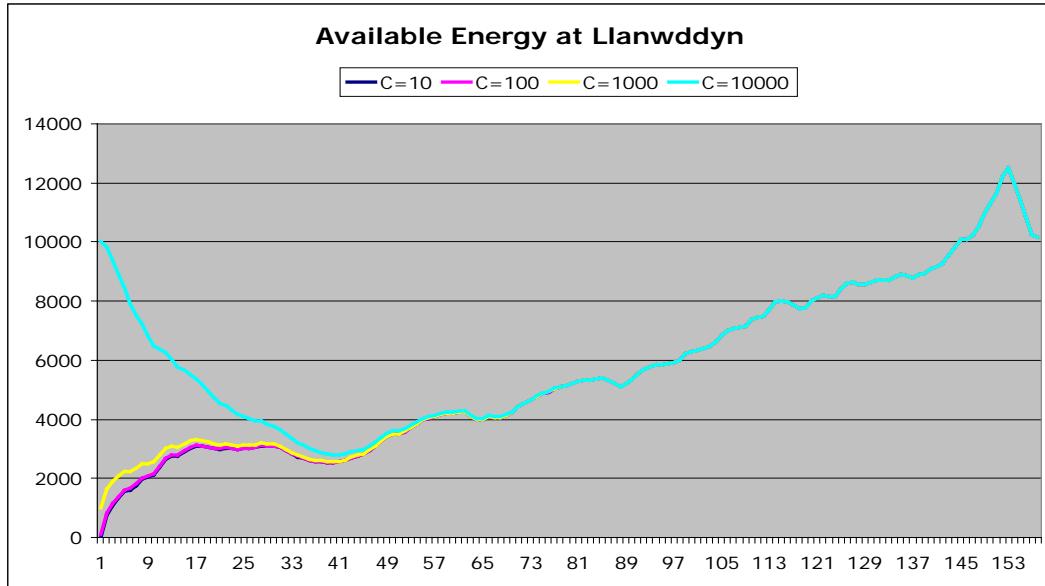


Figure 7.7 Variations in available energy, at Llanwddyn, given different initial values.

Figure 7.7 shows that if we vary the initial available energy from 10 to 10000, the available energy on day i converges at around day 60. This suggests that the trees ability to make energy from photosynthesis is more important than the value of energy it has on day zero. Also, the energy value converges weeks before we would expect infestation of nematodes. In the next section we see from results of the sensitivity analysis (Table 7.2) that the initial energy does not have a large effect on the model output. We conclude that the value that we specify for C does not matter too much; somewhere in the interval 10-1000.

Sensitivity Analysis

We have performed a sensitivity analysis for both the climate data and the new parameters introduced into the model. We use the software GEM-SA to perform the sensitivity analysis, which requires two text files, an input file and an output file. With regards to the parameters, we vary each parameter (through a range of values) one at a time and these values are placed in an input file. We run the model for each set of parameters and the output from the ETPN model that we require (nematode number, available energy and photosynthesis) are put into an output file. For the climate data, we fix the parameters and run the model for a range of different locations with a range of different climates. The output goes into a text file for the GEM-SA software and a summary of the climate data becomes the input file.

Table 7.2 lists the percentage of variance in output caused by each parameter. We have also included a column to give the range of values used for each parameter.

Table 7.2 Results of Sensitivity Analysis using model parameters.

Parameters	Range	Maximum Nematode Number	Minimum Available Energy	Minimum Photosynthesis
Tree Resistance	0.1–0.8	74.31%	8.45%	33.30%
Initial Nematode No.	1–5000	2.47%	1.07%	1.42%
Day of infestation	1–365	4.95%	44.77%	2.18%
Initial Energy (Joules)	0–50000	1.17%	2.22%	4.47%
Age	17–97	1.34%	17.22%	13.60%
DBH	11.5–63.4	0.87%	4.20%	3.42%
Joint Effects		Negligible	Negligible	Negligible

It is clear that different parameters are affecting different outputs. However, note that there are links in the model between photosynthesis, nematode number and available energy. Tree resistance causes an effect in all three outputs, especially the maximum nematode number and the minimum photosynthesis. Other parameters that are important are: day of infestation, tree age, initial energy and DBH.

What is driving the ETPN model even more than the parameters above is the climate data that we use, especially the temperature and precipitation.

Table 7.3 summarises the results of the sensitivity analysis for the climatic data, with the same output as in the previous sensitivity analysis: maximum nematode number, minimum available energy and minimum photosynthesis. Included is a column giving the range of values considered.

Table 7.3 Results of Sensitivity Analysis: percentage variance in output caused by climate data.

Input	Range	Max Nematode No.	Minimum Available Energy	Minimum Photo-synthesis
Max Temp (°C)	22.87–42.33	1.97%	9.63%	11.83%
Min Temp (°C)	-37.17–3.7	1.51%	2.01%	2.38%
Mean Winter Temp (°C)	-13.14–13.27	14.66%	16.76%	15.30%
Mean Annual Temp (°C)	0.46–18.81	22.93%	32.03%	28.27%
Mean Jul/Aug Temp (°C)	12.79–28.73	39.50%	7.42%	6.45%
Total Annual Precip (mm)	256.12–2581.4	1.11%	3.15%	3.05%
Mean Summer Precip (mm)	2.29–1060.45	0.89%	2.45%	3.52%
Mean Winter Precip (mm)	49.95–2094.99	1.93%	3.51%	2.81%
Mean Wind Speed (m/s)	1.33–10.71	0.28%	0.56%	0.84%
Mean Summer Humidity (%)	43.34–89.58	4.34%	1.11%	2.78%
Mean Winter Humidity (%)	53.83–94.94	0.33%	0.66%	0.96%
Maximum Humidity (%)	90.42–100	1.65%	1.68%	0.51%
Minimum Humidity (%)	23.15–65.79	1.76%	1.91%	1.01%

It is clear from the results of the sensitivity analysis that temperature is the main parameter that is affecting the nematode number, with mean July/August temperature

accounting for 39.5% of the variance. Mean winter temperature and mean annual temperature are also important parameters, accounting for 14.66% and 22.93% respectively. Temperature parameters account for more than 80% of the variance in maximum nematode number. Precipitation parameters combined only account for approximately 4% of the variance.

For the minimum available energy, the temperature parameters are having the biggest affect. Mean annual temperature accounts for 32.03% of the variance while winter temperature is more significant than the July/August temperature. Combined, the temperature parameters account for nearly 70% of the variance in the minimum available energy. The precipitation parameters only account for just over 9% of the variance.

Finally, with regards to minimum photosynthesis, the results of the sensitivity analysis show that the temperature parameters are the most important. Mean annual temperature accounts for the largest variance; 28.27%, while mean winter temperature and mean July/August temperature account for 15.30% and 6.45% respectively. Combined, the temperature parameters account for a total of nearly 65% of the variance. The precipitation parameters account for less than 10% of the variance.

We conclude from the sensitivity analysis that temperature is the key climate input affecting the output of the ETPN model and tree resistance is the main parameter. It is interesting to note that winter temperature has a significant effect on the output. Nematode development is affected at not only high temperatures but also low temperatures. In regions where winter temperatures are particularly low, nematode development is restricted over winter and if cold enough juvenile nematodes moult into a dormant dauer larvae stage. Depending on the winter temperature of a region, nematodes could continue to breed and multiply over winter or they may remain at a low and constant number.

Summary

To summarise, we have made modifications to the ETP model, now referred to as ETPN model. The three significant changes to the model have been to introduce an element to model the number of nematodes inside the tree and relate their growth to temperature, to restrict the photosynthesis, depending on the number of nematodes present in the tree and to introduce an energy element, where we calculate the energy available to use as defence against PWN, dependent on photosynthesis, the number of nematodes present and the resistance of the tree.

We have used an extensive amount of data from the literature to make these modifications and also to test that the model output is accurate.

A sensitivity analysis has allowed us to deduce what parameters and model input are driving the model which will assist us in developing a light user-friendly version of the model as well as a latency sub-model.

Work has begun on determining threshold values of temperature, below which we would not expect to see PWD.

References

- Burgess P (2009), Variation in Light Intensity at Different Latitudes and Seasons, Effects of Cloud Cover, and the Amounts of Direct and Diffused Light, School of Applied Sciences, Cranfield University, Presentation to Continuous Cover Forestry Group Scientific Meeting, Sept.
- Donald P A, Stamps W T and Linit M J (2012), Pine wilt disease. The Plant Health Instructor, The American Phytopathological Society.
- Dropkin V H, Foudin A, Kondo E, Linit M, Smith M, and Robbins K (1981), Pinewood Nematode: A Threat, Plant Disease, Vol. 65, No. 12.

- Hoshi H (2011), Learning from pine breeding for resistance to pine wood nematode in Japan,
http://www.efiatlantic.efi.int/files/attachments/efiatlantic/nematode_day/la_experiencia_en_jap_n.pdf
- Ikegami M (2009), PHRAME Project – Plant Health Risk and Monitoring Evaluation, Final Report, <http://www.forestry.gov.uk/fr/INFD-7XRFX9>
- Kuroda K, Yamada T, Kazuhiko M and Tamura H (1988), Effects of Cavitation on the Development of Pine Wilt Disease Caused by *Bursaphelenchus xylophilus*, Ann. Phytopath. Soc. Japan 54: 606-615.
- Magnusson C (1986), Potential for Establishment of *Bursaphelenchus xylophilus* and the pine wilt disease under Nordic conditions, EPPO Bulletin, 16, 465-471.
- Mamiya Y (1983), Pathology of the Pine Wilt Disease caused by *Bursaphelenchus xylophilus*, Annual Review of Phytopathology, 21: 201-220.
- Mamiya Y (1984), The Pine Wood Nematode, Plant and Insect Nematodes (ed. Nickle), 589-626, Marcel Dekker, New York.
- Mamiya Y (1988), History of Pine Wilt Disease in Japan, Journal of Nematology 20 (2): 219-226.
- Murray J D (1989), Mathematical Biology, Biomathematics Texts, Springer-Verlag.
- Myers R F (1986), Cambium Destruction in Conifers Caused by Pinewood Nematodes, Journal of Nematology 18 (3): 398-402.
- National Climatic Data Center - National Oceanic and Atmospheric Administration, (2013) <http://www.ncdc.noaa.gov/>
- OEPP/EPPO (1986), Data sheets on quarantine organisms No. 158, *Bursaphelenchus xylophilus*, Bulletin OEPP/EPPO Bulletin 16, 55-60.
- Oh W S, Jeong P Y, Joo H J, Lee J E, Moon Y S, Cheon H M, Kim J H, Lee Y U, Shim Y H and Paik Y K (2009), Identification and Characterization of a Dual-Acting Anti-nematodal Agent against the Pinewood Nematode, *Bursaphelenchus xylophilus*, PLoS ONE 4(11): e7593. doi:10.1371/journal.pone.0007593.
- Stokes D E (1979), Pinewood Nematode, *Bursaphelenchus lignicolus*, A Nematode Pest of some Pine Species, Nematology Circular No. 53.
- Vince Ö and Zoltán M, Plant Physiology, (2011) http://www.tankonyvtar.hu/en/tartalom/tamop425/0010_1A_Book_angol_01_no_venyelettan/ch03s03
- Wang F, Wang Z, Li D and Chen Q (2012), Identification and Characterization of a *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae) Thermotolerance-Related Gene: Bx-HSP90, Int. J. Mol. Sci. 13, 8819-8833.
- Zhao B G, Futai K, Sutherland J R and Takeuchi Y (2008) Pine Wilt Disease, Springer Publishing.

7.1.2. Field experimentation to verify and refine the process models (M45)

B1 will work with B6 and with researchers in North America and, using visits by researchers from REPHRAME augmented, if possible by existing research work being carried out by local Japanese research groups (to be investigated as part of international collaboration), Japan and to carry out field inoculations to verify the predictions of the model under conditions where wilt is expected (Japan and Portugal) and where no wilt is expected (North America). Inoculated trees will be instrumented to measure key physiological parameters of host trees and to link directly to onset of easily assessed field parameters, such as resin flow and detection of PWN using sensitive molecular techniques (see WP2).

Work carried out by B1

Field verification of the process model could not begin until a working, accurate version of the model was running. Although we are now at this stage, unfortunately, due to the uncertainty with regards to obtaining the extension it has not been possible to plan field experimentation. Also, negotiation had commenced with the Canadian Forest Service,

but it was not possible to obtain permission to carry out inoculation work in Canada and, therefore, the proposed field work in the native range of the nematode has had to be removed from the plans. However results of the spread of PWD in Japan have been used to verify the model along with results from the literature that describe the symptoms of the disease expression.

7.1.3. Development of a sub-model to account for latency in wilt expression (M44)

Both the core model and field experimentation in 'non-wilt' and marginal wilt areas will explore the conditions under which either delayed onset of wilt (latency) or non-wilt (eco-climatic resistance) in intrinsically susceptible trees is likely to occur. This will include both tree physiological parameters and climate-site interactions that are the core drivers of the process model. Outputs from the model will be particularly linked to WP1 and WP5. B1 will work closely with all Beneficiaries to develop the sub-model.

Work carried out by B1

With regards to this deliverable, a little progress has been made towards developing a latency sub-model. Completion is expected by the end of the project. By using the results of the sensitivity analysis that we have already performed for both the climate data and model parameters, we are able to deduce what input and parameters are causing a lag in the disease expression.

We extract data from the sensitivity analysis for only those locations that did show disease expression during model simulation and discard all the other data, where the model showed no expression. The results in Table 7.4 assume that a tree will die from PWD and looks at what parameters effect when we see disease expression. The ranges of values used are identical to those used in the sensitivity analyses above.

In the first sensitivity analysis, the climate data is fixed and we vary the parameters. Since the day of infestation is varied, we consider two model outputs: the Julian day on which the model predicts tree death and the number of days it takes for the tree to succumb to PWD.

Nematode Parameters

Table 7.4 Results of Sensitivity Analysis for model parameters.

	Day of Tree Death	Days taken for tree to Die
Tree Resistance	0.68%	3.45%
Initial Nematode No.	1.95%	5.64%
Day of infestation	55.8%	21.33%
Initial Energy	2.78%	0.05%
Age	7.99%	4.03%
DBH	7.31%	1.56%
Joint Effects	Negligible	Joint effects observed between day of infestation, age and number of nematodes entering the tree; percentage variance being around 1.15% for all 3 pairs.

For both outputs in Table 7.4, the day that the nematodes enter the tree is the main parameter that is affecting the day of disease expression; accounting for 55.8% of the variance in the day we observe tree death and 21.33% of the variance in the number of days it takes for a tree to die from PWD. The age and diameter at breast height are also important parameters, suggesting that the size of the tree affects the time it takes for the nematodes to kill the tree. The initial nematode number accounts for 5.64% of the variance in the time taken for a tree to die, which is also quite significant.

With regards to the sensitivity analysis for climate data (Table 7.5), the parameters are fixed, i.e. we are not varying the day that we inoculate the nematodes (fixed at Julian day 160), and hence we need only consider one output; day of tree death.

Climate Parameters

Table 7.5 Results of Sensitivity Analysis using climate data.

Input	Variance (%)
Maximum Temperature	3.5
Minimum Temperature	2.35
Mean Winter Temperature	7.09
Mean Annual Temperature	26.28
Mean July/August Temperature	27.98
Total Annual Precipitation	8.30
Mean Summer Precipitation	3.47
Mean Winter Precipitation	4.53
Mean Wind Speed	0.35
Mean Summer Humidity	1.41
Mean Winter Humidity	0.32
Maximum Humidity	2.20
Minimum Humidity	0.21

Temperature parameters also affect the day of year that the model output shows pine wilt expression, particularly the summer temperature and mean annual temperature. Total annual precipitation is the third highest accounting for 8.3% of the variance.

Both sensitivity analyses will need to be combined to develop a latency sub-model, but it is clear already that certain parameters and input are affecting how long it takes for a tree to die after infestation, particularly: temperature of the location, total annual precipitation, day of year in which (and amount of) nematodes enter the tree and the size (Age and DBH) of the tree.

7.1.4. Analysis of the PWN history in Portugal (M42)

To contribute to the refinement of the process model for prediction of wilt, B6 will compile all available information on the spatio-temporal evolution of pine wilt in the affected region south of Lisbon since its appearance in 1999. Information such as yearly accumulation of felled wilted and dead trees and the number of trees estimated to be affected by the nematode will be correlated with forest characteristics (species diversity, density, age, area) and geographic location (soil, climate). This information will be used to improve the wilt prediction model and compare its predictions with actual observations over time in Portugal.

The model will incorporate information from other existing models such as the detection-eradication model from Okland et al, which is adapted to northern areas without a suitable climate for PWD, although it can be adjusted to regions where the potential of PWN is present, being a tool in the work to evaluate surveys and eradication strategies for current infestations in

southern Europe (Okland et al., A simulation model approach to evaluate the chance of eradicating an introduction of the pine wood nematode – in prep.). Cross-linkage to other models, such as CLIMEX® will also be made, particularly by collaboration with researchers in Australia, where valuable interactions through the EU Marie-Curie project TRANZFOR will facilitate direct contact and exchange. Each of these will link to the economic consequences of the model outcomes.

All tasks under 7.1 will commence in Year 1 and continue through the full duration of the project, with deliverables at country level initially for all EU Member States and, through the delivery and testing of the evolving versions of the model, at more local scales and levels of detail by interaction with NPPOs in each country expressing interest in obtaining customised outputs.

Work carried out by B7

Progress report

From 2008 to 2011, there were 11425 samples made in symptomatic trees in Portugal, with 10597 negative results and 828 positive results (Figure 7.8). We summarize this information at UTM cell size, to allow us to quantify the severity of the disease in each UTM cell of Portugal. For this same grid we summarize also the climatic drivers and with this data we made a first modelling approach with actual data (Figure 7.9).

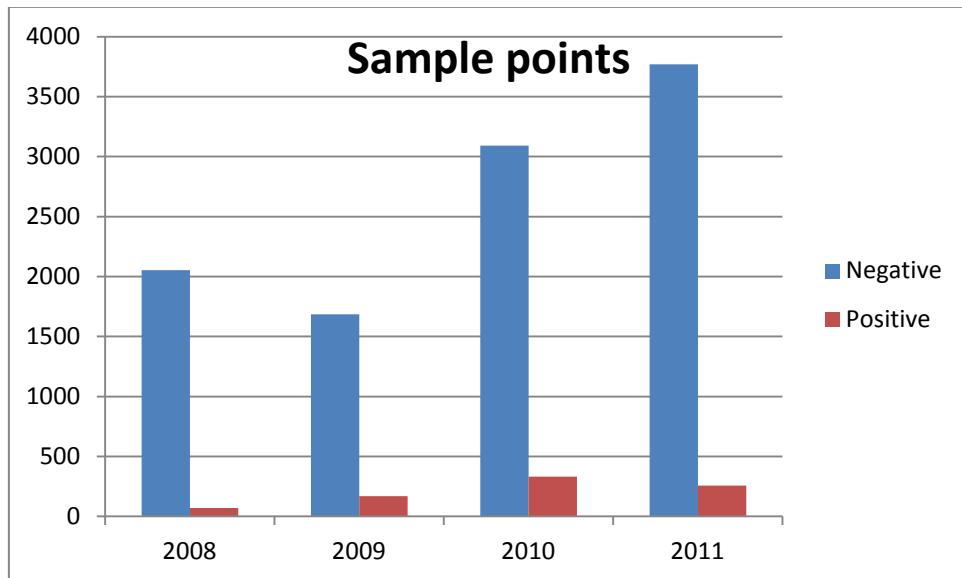


Figure 7.8 – PWD prospection made in Portugal from 2008 to 2011, with evolution of negative and positive samples

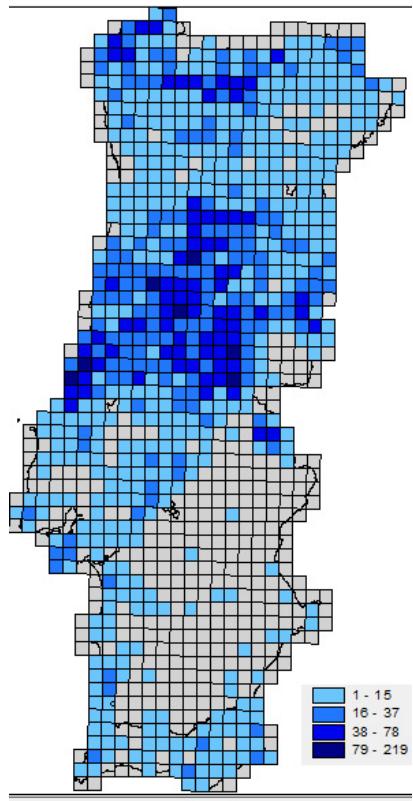


Figure 7.9 Sample effort in Portugal from one (light blue) to 219 (dark blue) of samples made in each UTM cell

The previous figure reflects the sample effort in Portugal. After the outbreak in 2008 in centre Portugal, it was concentrated in this region.

From the Forestry institute, we can see the actual distribution of *Pinus pinaster* in Portugal (Figure 7.10). Comparing Figure 7.9 and Figure 7.10, we see that the sampling effort was proportional to *Pinus* distribution in Portugal.

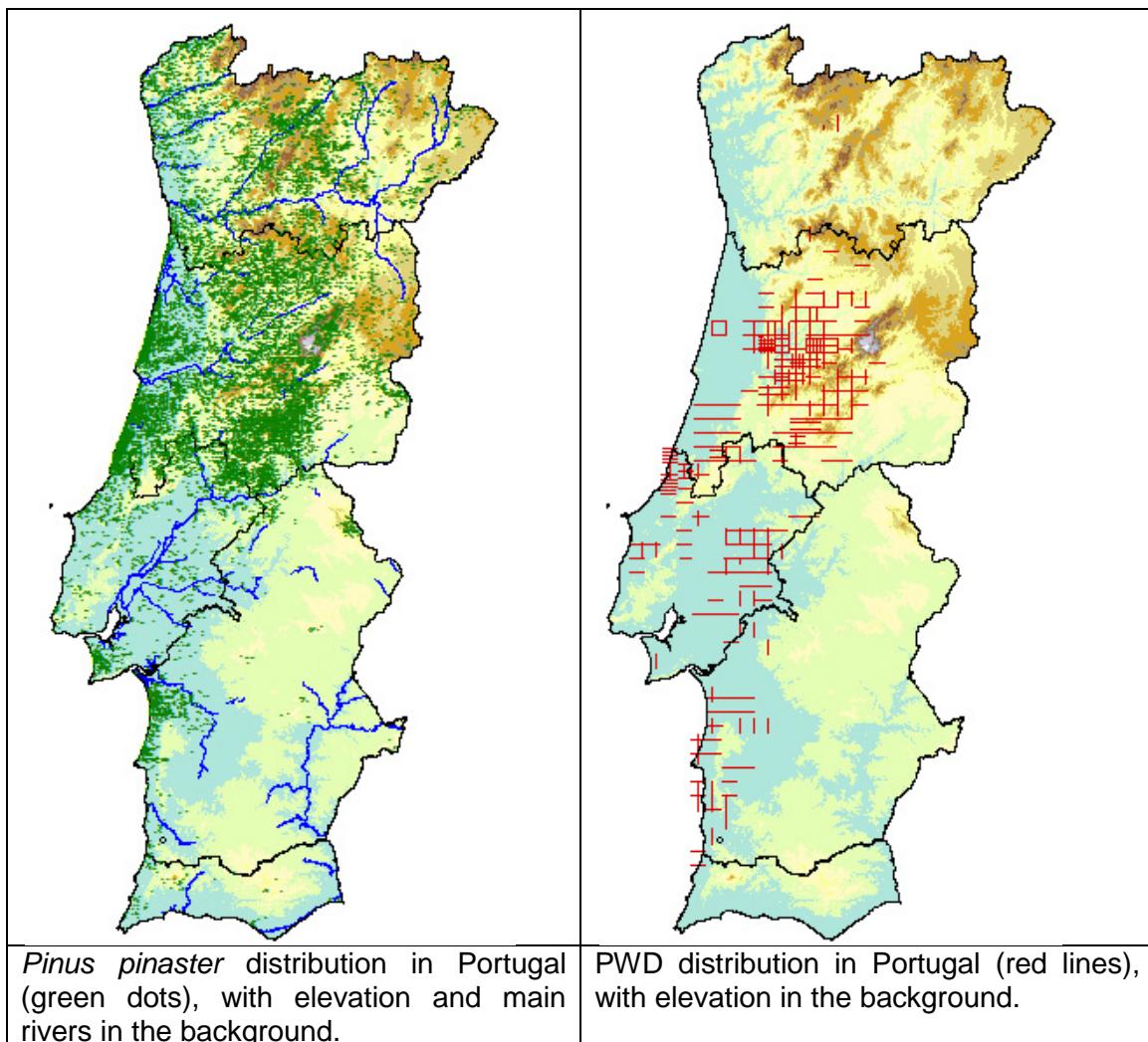


Figure 7.10 Distribution of *Pinus pinaster* (left) and PWD positive samples (right) in Portugal

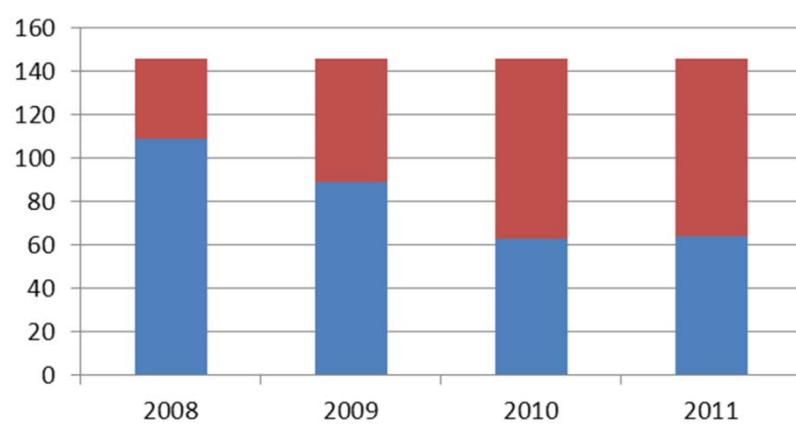


Figure 7.11 Evolution of positive samples for PWD in Portugal. Bars represent the 146 cells infested by PWD at least once between 2008 and 2011. Red represents the number of cells infested for the given year and blue represents the number of cells not infested this given year (but infested another year).

In the infested area, the percentage of infested cells increased from 2008 to 2011, but in 2011 it seems to have stabilized (Figure 7.11)

The number of positive samples in Portugal increased from 2008 to 2011 (Figure 7.12).

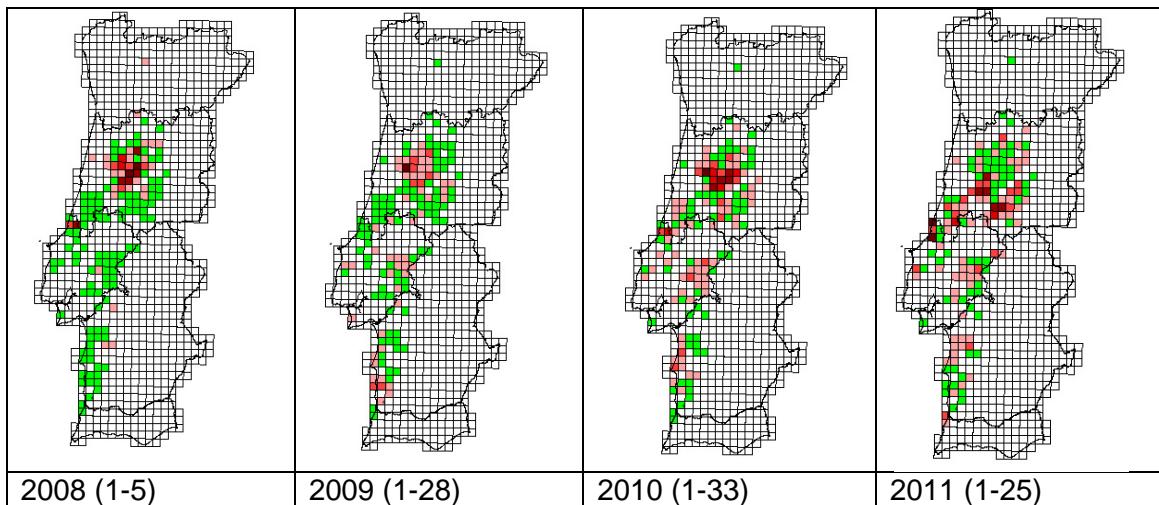


Figure 7.12 Evolution of number of positive samples to PWD by UTM grid cell. Between parenthesis, the range of positive samples (in 2008 there were a maximum of 5 positive samples, whereas in 2011 there were a maximum of 25). Green is a presence cell for PWD but with no positive results for that year in particular, and pink to red are positive samples for PWD in that year.

From this data we get the impression that the PWD is more important in the centre of Portugal. This was in contradiction with our field data given in the previous report, where coastal Nazaré and Sines were strongly attacked by PWD and this was visible in Satellite Imagery (Figure 7.13 and Figure 7.14).





Figure 7.13 Evolution of PWD in Lagoa de Santo André between 2004 and 2011.

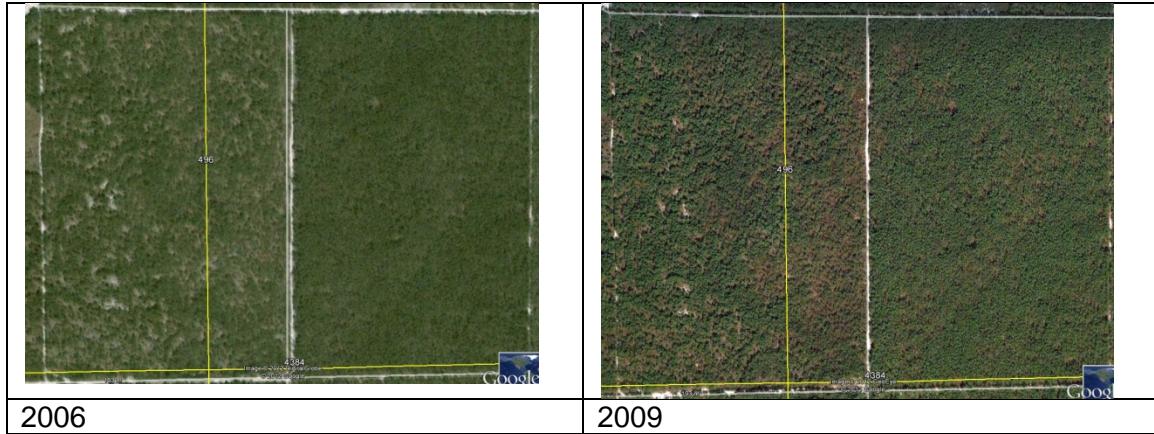


Figure 7.14 PWD in single stand *P. pinaster* forest, near Nazaré in 2006 and 2009.

From this data only, it is not possible to infer the severity of PWD in the territory. If we calculate the percentage of infested samples (number of positive samples by all inventory sampled in each UTM cell) it gives us a much better picture of PWD distribution in Portugal (Figure 7.15).

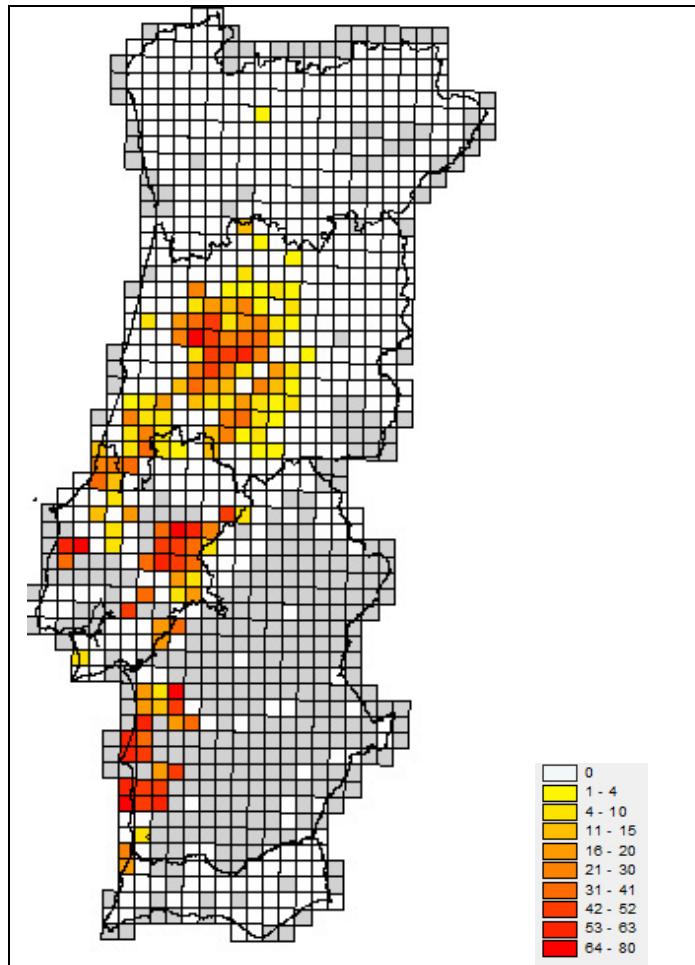


Figure 7.15 Percentage of PWD infested samples by UTM

This approach gives us a much better interpretation of the real situation of PWD in Portugal.

Regional analysis

In this map, yellow from orange reflects the PWD severity presence in Portugal, from 1% (yellow) to 80% (red) infested samples per UTM unit cell. As we see in this regional detail of Figure 7.16, centre region, LVT (Lisboa e Vale do Tejo) region and coastal Alentejo have high values of PWD percentage infested samples.

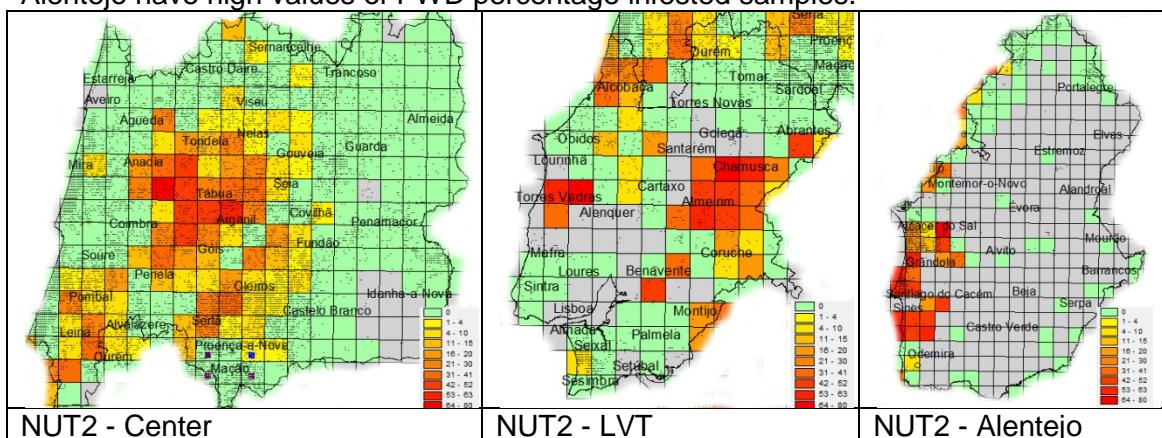


Figure 7.16 Regional distribution of PWD in Portugal

Modelling

The regression equation is:

$$PWD = 30.8 - 0.000003 [\text{distance to the sea}] - 1.13 [\text{August minimum temperature}] - 0.00534 [\text{precipitation}]$$

Predictor	Coef	SE Coef	T	P
Constant	30.806	9.449	3.26	0.001
MAR_CUSTO	-0.000000032	0.000000005	-6.72	0.000
TMIN_AGO	-1.1271	0.5643	-2.00	0.046
P_TOTIL	-0.005342	0.001369	-3.90	0.000

$$S = 12.6540 \quad R-\text{Sq} = 8.7\% \quad R-\text{Sq}(\text{adj}) = 8.3\%$$

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	3	10140.1	3380.0	21.11	0.000
Residual Error	665	106482.2	160.1		
Total	668	116622.3			

In R. Regression tree

Regression tree model in R-package with only three variables (Table 7.6), where:

AMT — August minimum temperature

SP — Summer precipitation

SE — South exposure (1 to the south, -1 to the north)

Table 7.6 Results of the first model. The last column represents the predicted percentage of infested trees.

AMT<14							0
AMT>14	AMT>16						0
	AMT<16	SP<26	SE>0.4				18.3
			SE<0.4				32.6
		SP>26	SP>71	SP>89			5.6
				SP<89			19.2
			SP<71	SP>40			5.1
				SP<40	MAT>15	MAT<15.5	0
						MAT>15.5	10
					MAT<15		18

From this model we can infer that AMT below 14°C and above 16°C are free of PWD.

In	a	second	model	(Table	7.7)
CD		is		Coast	
P		is		annual	
AMT	is		August	minimum	
ALT is altitude				distance	
				precipitation	
				temperature	

Table 7.7 Results of the second model. The last column represents the predicted percentage of infested trees.

CD>347	P<1000						0
	P>1000	P>1418					0
		P<1418	CD>1237				2
			CD<1237	AMT>28			7.6
				AMT<28	CD<397		5.7
					CD>397	ALT>234	19
						ALT<234	36
CD<347	P>800	CD<307					4
		CD>307					20
	P<800	AMT>30					0
		AMT<30	P<610				0
			P>610				19

This model indicates that the climatic envelope of PWD is more complex than previously reported, and efforts should be done to better understand climatic driven variables as determinant to the future spreading of PWD.

Future work

- Modelling will be extended to Iberia
- Field data (2010-2014) and 2003 data (PROLUNP) will be integrated to the modelling
- Projections will be done for several climate change scenarios.

7.2 PWN spread model (M44)

A spread model will be developed at the European scale based upon the previous results taking into account local dispersal (results of WP3) and inadvertent transportation by humans at long-distance (results of WP4). This risk will be calculated using the human population density, and eventually some connection means (such as road network and ports) potentially involved in the transportation of infested materials. Because the transportation of infested material from an infested region to a pest free region is a random process, a stochastic model will be chosen and the probability of occurrence of PWN and PWD will be calculated at different years in the future using several climate scenarios. For this purpose, environmental factors involved in the expression of PWD, the survival of PWN, and host tree susceptibility will be identified from the full-detail model (Task 7.1) and summarizing indicators that could be used at a large scale will be determined (B4 in collaboration with B1 and B6). These thresholds will be combined in the spread model to determine the possibility for the PWN presence and PWD expression. This spread model will be validated at small scale using the history of infestation in Portugal (Task 6.1.4)

This task will start in the 2nd year (require inputs from Task 7.1 and from other WPs)

Work carried out by B4 (other partners involved in WP7 provided some data or information needed to develop the model and/or estimate some parameters)

Summary of progress

This deliverable is divided in two independent parts:

- D7.5 [PART 1]: Modelling the potential spread of pine wood nematode and pine wilt disease across Europe. This part is the main one: a new model will be developed to describe more precisely the potential spread of the pine wood

nematode and wilt disease in Europe taking into account the latest findings from other WPs.

- D7.5 [PART 2]: Modelling the vector dispersal across the Pyrenees Mountains in relation with the genetic structuration of the vector populations. In this part, we consider the spread model previously developed (Robinet et al. 2009, 2012) to test the effects of various factors on the potential spread across the Pyrenees Mountains and we compare the results of the model to the genetics results (WP3, D3.3).

PART 1

During months 1-18, the theoretical framework of the spread model was developed. This model is divided into several sub-models describing various mechanisms involved in the spread of the pine wood nematode. During months 19-36, this framework was adapted to the data actually available and to the new findings from the other WPs. The flow chart in Figure 7.17 describes the sub-models.

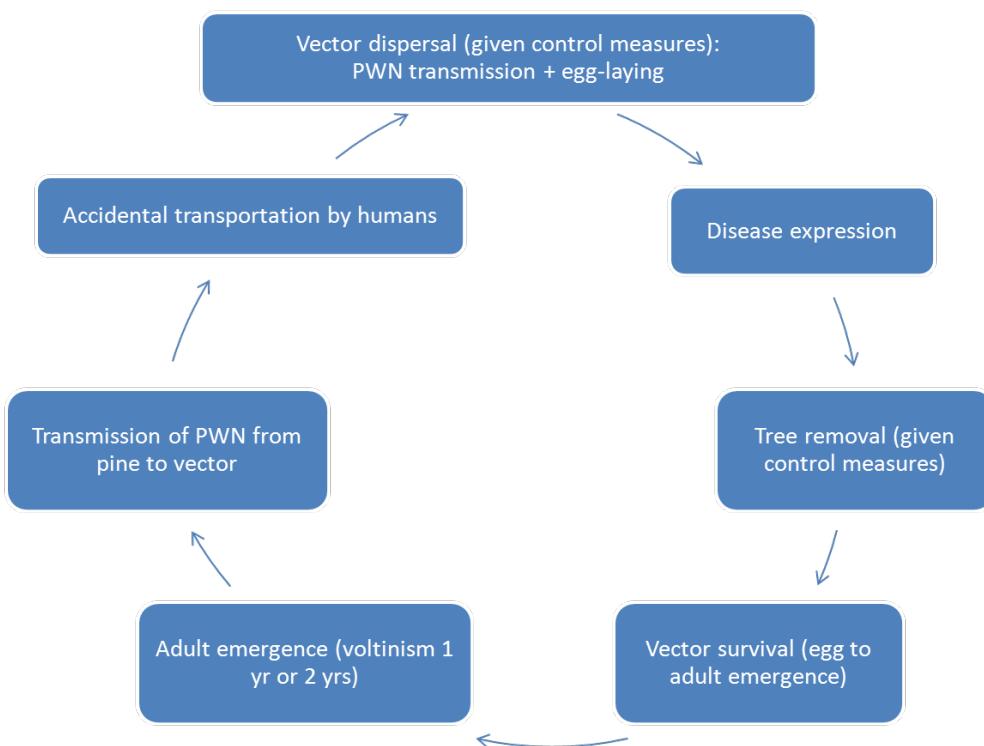


Figure 7.17 General flow chart of the spread model

The first step was to develop an individual-based model to describe the vector dispersal, nematode transmission from the vector to the trees, and vector oviposition under various scenarios of nematode control: no control, clear cut belts of 500 m, 1000 m and 3000 m around the infested tree that released the infested beetles.

With this individual-based model, it is possible to keep track of each individual over a continuous space between adult emergence and adult death. We simulate dispersal based on a dispersal kernel fitted to the distance the beetles were able to fly in the flight mill experiment (see D3.1; David et al. 2013). However we assume that they do not fly straight and that they make stops and turns. The distance flown between the emergence point and the final point is therefore lower than the total accumulated distance recorded on the flight mill (Table 7.8). As mature beetles were tested on the flight mill once per week, it is not clear whether the distance recorded is the distance they can potentially fly every day or over one week. Therefore, we consider three

scenarios. In scenario (D, “daily”), we assume that the distance recorded once per week is representative of the daily flight performance. In scenario (W, “weekly”), we assume that the distance recorded once per week is representative of the flight performance over one week. Thus, to have a daily estimate, we divide this distance by 7 in scenario W. In reality, the beetle flight performance is probably between these two scenarios. Therefore, we consider an intermediate scenario (M, “medium”).

Table 7.8 Estimate of the dispersal distance according to scenarios W, M and D. Numbers in brackets indicate the standard deviation based on the dispersal simulation of 100 beetles.

Distance type	Scenario W	Scenario M	Scenario D
Mean daily dispersal distance of flying beetles (m)	324	1296	2268
Mean accumulated distance over an adult life (km)	18.7 (1.4)	73.6 (6.4)	132.8 (10.9)
Mean distance between release point and final location (km)	3.3 (1.8)	12.3 (6.0)	19.5 (9.9)

At each stop, we consider that beetles unload a given proportion of nematodes (depending on their age; Naves et al. 2007) and that females can lay eggs (depending on their age, the total number of eggs they can lay over their life span and the tree species from which they emerged). In the model, we distinguish three groups of pines: *Pinus pinaster*, *P. sylvestris* and other *Pinus* species as they can affect the female fecundity and larval survival. The latter group is represented by data found on *P. nigra*. We assume that females do not choose the pine species on which they lay eggs. We distribute the eggs on the tree groups depending on their proportion. Data about transmission rate of the nematode from vectors to trees, female fecundity and larval survival were found in literature (for the European vector).

After the beetle dispersal, nematode transmission (during maturation feeding and egg-laying), and egg-laying, we simulate clear cut belts. In the model, we assume that infested trees are removed over a given radius from the tree releasing the infested beetles and then we calculate the remaining infested trees beyond this radius (Figure 7.18).

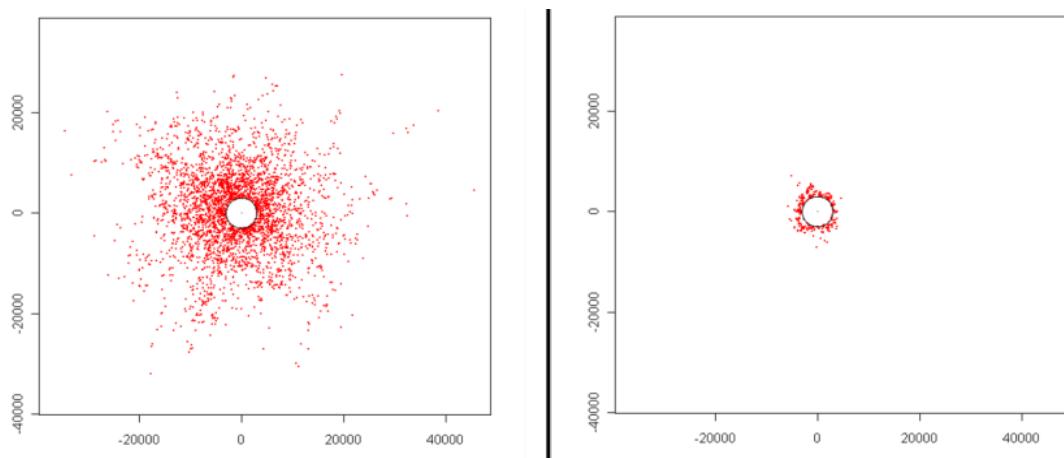


Figure 7.18 Simulations of the nematode transmission rate in scenarios D (on the left) and W (on the right) in case of a clear cut zone 3 km radius (represented by the black circle), resulting from the individual based model and assuming that 100 infested beetles are released at (0,0).

This dispersal model at the individual-based level should be validated with release-recapture experiment data (see D3.2).

To obtain a model at the population level (not individual based) and over a large area (not on a continuous space), we calculate the mean transmission rate and the mean number of eggs in grid cells covering the total infested area. As we should develop a model at fine scale (over the Iberian Peninsula, for validation) and at large scale (over Europe, for projections), we make these calculations over two grid resolutions: 1 km and 10 km. To summarize the work done for this first sub-model, we consider:

- Dispersal kernel of mature beetles and immature beetles derived from data collected on flight mill
- Transmission of the pine wood nematode from the vector to the tree via feeding activity
- Oviposition and transmission of the pine wood nematode from the vector to the tree via oviposition
- Simulations of clear cut zones
- Results of the individual-based model
- Results of the model over fine and large scale grids (1 km and 10 km resolution)

Then, we can combine this sub-model with the other ones (Figure 7.17):

- Development of the pine wilt disease: From D7.1 (refinement of the core model), a simplified model for the disease expression should be derived to be applied at large scales and be inserted in the spread model. In the spread model, we will consider three cases: the disease can develop during the year of infestation, it can develop only the year after (in case of latency), or it cannot develop.
- Tree removal (depending on control measures of the pine wood nematode)
- Survival of the vector from eggs to adult emergence (depending on the tree species and number of eggs laid). Data were found in literature to estimate the related parameters for the European vector.
- Emergence of adult vectors: From the degree-day model previously developed (Naves & Sousa 2009), it is possible to estimate the proportion of the vector population which makes its development over one year versus two years. We derived a simplified model to estimate these proportions at large scales. However, the degree day model was developed on Portuguese populations so it is necessary to check whether the adult emergence time is correctly predicted for other populations (see Task 3.1).
- Transmission of the pine wood nematode from pine to vector. Very little information is available. We will make various scenarios: 33, 66 and 100% of beetles emerging from an infested tree carry the nematode.
- Long distance dispersal of the pine wood nematode. Very little information is available. For now, it is not possible to identify long distance dispersal based on the genetic analysis of the nematode populations or vector populations. From observation data, it seems that the infestation in Central Portugal (near Coimbra) probably resulted from an accidental introduction (Paulo Pereira, Pers. Com.). For the model validation, we make several scenarios to determine which one is the more likely:
 - only natural spread from Setubal since 1999
 - natural spread from Setubal since 1999 and an introduction near Coimbra at different years (e.g., 2002, 2003, 2004, 2005, 2006).
 - For the model projection, we can test an introduction at various locations where an introduction is the most likely such as areas where there are numerous wood factories or other strategic areas (e.g., near the forest of

Les Landes, France). Risk maps based on the combination of human population density, road network density and wood factories density have been developed (Figure 7.19). These maps can be used to identify areas where the pine wood nematode could be introduced.

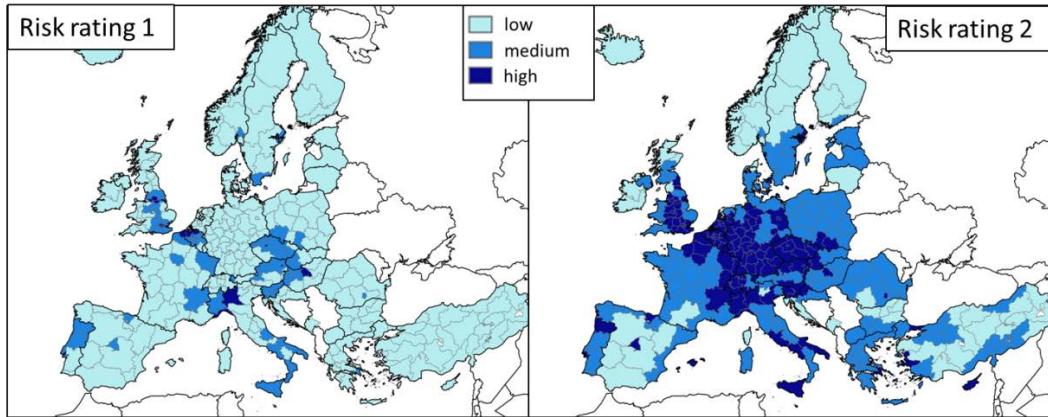


Figure 7.19 Risk maps representing the risk of introduction of the pine wood nematode at long distance based on human population density, road density and wood factory density.

Validation of the model will be done at fine scale (South West Europe, 1999-2011, 1 km resolution) and projection of the spread in the future will be done at large scale (Europe, 2011-2030, 10 km resolution) as indicated in Figure 7.20.

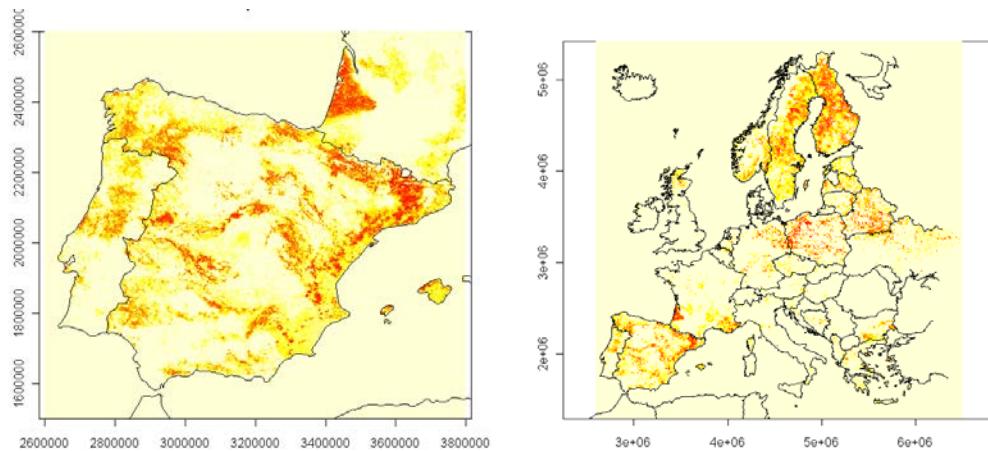


Figure 7.20 Pine density over the fine scale and over the large scale area (light yellow: very low proportion of pines to red: pines cover 100% of the area). Source: EFI (<http://www.efi.int/>).

All the model parameters have been estimated based on literature search or new findings in the project. The simplified model for disease expression is not yet available so it will be included later in the model. Some preliminary simulations were conducted at fine scale. A sensitivity analysis will be done at fine scale to determine the parameters which have the strongest effects of the results.

PART 2:

During months 19-36, we started to model the potential dispersal of the vector population across The Pyrenees, a mountainous area that could be a barrier for the natural and human-assisted spread of the nematode from the Iberian Peninsula to other parts of Europe. This study is complementary to the previous one as it focusses on this critical area that the nematode should cross to reach other European countries, and it is closely related to the genetic analysis conducted for D3.3. This part is done in the frame of the PhD thesis of Julien Haran (INRA Orléans; partner B4). The aim of the model is to test the role of various factors in the spread of the vector across The Pyrenees and to compare the dispersal pattern simulated under various scenarios with the dispersal pattern shown by the genetic analysis. We use a reaction-diffusion model similar to the one developed for the nematode spread in China (Robinet et al. 2009, 2012). Given the uncertainty about the dispersal distance, we tested various values, notably the estimate found in China, 7.5 km/yr as it was consistent with the distance derived from flight mill experiment (between 3.3 and 19.5 km / adult life span, see Table 7.8) but we also tested lower values as the beetles may not disperse so far when the resource is available locally. In the model, we consider elevation by taking into account the difference in elevation between the grid cells in the distance that the beetles have actually to fly to disperse around. In addition, we have tested:

- the effect of a maximal elevation limit of 1580 m (because there is a very low number of captures above this elevation limit),
- the effect of minimal temperatures via the beetle survival (binary variable depending on the temperature threshold considered), and
- the effects of pine density on the carrying capacity (maximal population density)

From preliminary results, it seems that the temperature constraint could be the best explanatory factor for the dispersal barrier in The Pyrenees but additional tests and simulations will be conducted.

Oral communications related to this task

- Robinet C (2013) Expansion potentielle du nématode du pin : vers une modélisation calibrée sur l'Europe. GEFF, Brens (France), 22-24 Oct 2013.
- Haran J, Garcia J, Bernard A, Roques A, Robinet C, Roux-Morabito G (2013) Etude des processus de dispersion de *Monochamus galloprovincialis*, vecteur du nématode du pin : rôle potentiel de la chaîne pyrénéenne. GEFF, Brens (France), 22-24 Oct 2013.
- Robinet C, David G, Piou D, Roques A, Jactel H (2013) Simulating the dispersal of *Monochamus galloprovincialis* based on its flight mill performance and testing several pest management scenarios. Pine Wilt Disease Conference 2013, Braunschweig (Germany), 15-18 Oct 2013.
- Gruffudd H, Evans H, Haran J, Roux-Morabito G, Roques A, Robinet C (2013) How could climate change affect the potential spread of pine wilt disease in Europe? ClimTree 2013, Zurich (Switzerland), 2-4 Sept 2013 (invited presentation)
- Haran J., Roques A., Robinet C. & Roux- Morabito G. (2014). Assessing dispersal routes and ongoing gene flow of the vector of the Pine Wood Nematode, *Monochamus galloprovincialis*, at different spatial scales. XXIV IUFRO World Congress. 5-11 October 2014 Salt Lake City, United State.

References

- David G, Giffard B, Piou D, Jactel H (2013) Dispersal capacity of *Monochamus galloprovincialis*, the European vector of the pine wood nematode, on flight mills. Journal of Applied entomology, DOI: 10.1111/jen.12110
- Naves P, de Sousa E (2009) Threshold temperatures and degree-day estimates for development of post-dormancy larvae of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae). J Pest Sci, 82:1-6.
- Naves PM, Camacho S, de Sousa EM, Quartau JA (2007) Transmission of the pine wood nematode *Bursaphelenchus xylophilus* through feeding activity of *Monochamus galloprovincialis* (Col.: Cerambycidae). J Appl Entomol, 131: 21-25.
- Robinet C, Roques A, Pan H, Fang G, Ye J, Zhang Y & Sun J (2009) Role of human-mediated dispersal in the spread of the pinewood nematode in China. *PLoS ONE* 4(2): e4646. doi:10.1371/journal.pone.0004646.
- Robinet C, Van Opstal N, Baker R, Roques A (2011) Applying a spread model to identify the entry points from which the pine wood nematode, the vector of pine wilt disease, would spread most rapidly across Europe. *Biological Invasions* 13:2981-2995.

Work Package 8: EU and international cooperation and collaboration

Objectives

Since *Bursaphelenchus xylophilus* was confirmed as the causal agent of catastrophic pine mortality in Japan in the late 1960s, there has been an enormous research effort to improve knowledge on this extremely damaging pest. This Work Package builds on this accumulated knowledge base by gathering together data and fostering effective international cooperation and collaboration, with particular reference to the requirements of call KBBE.2010.1.4-09. The WP will achieve its objective of developing EU and international cooperation and collaboration by assessing or interacting with:

- Previous relevant EU projects.
- Current relevant EU projects
- Phytosanitary organisations globally
- EUMAINE MSc program in Nematology
- International PWN research groups

Overall comment for B5

In the current reporting period B5 spent 1.28 PM (productive hours) in this work package. Therefore the proposed two person months nearly have already been delivered (0.09 PM missing).

Deliverables

D8.1) Knowledge from previous EU projects: Synthesis of knowledge from previous EU projects. Month 12

D8.2) Interaction with EU/International projects: Interaction with current EU and international projects and final synthesis. Month 45

8.1 Synthesis of knowledge from previous EU projects

The purpose of this task is to reflect on the results, conclusions and lessons learned from earlier EU programs, directly or indirectly related to PWD, particularly:

- PHRAME (<http://www.forestryresearch.gov.uk/fr/infd-63kgf>);
- PortCheck (<http://www.portcheck.eu.com/>).

This Task will be enhanced by fact that several members of the present consortium have participated in one or both projects (B1, B3, B4, B5, B6, B7, and B8).

B5 analysed both the EU-research project RISKBURS and PHRAME as previous partner. The main issue covered the questions of pathogenicity tests and population dynamics. As in RISKBURS as well as in REPHRAME, small seedlings served as host trees for investigations on pathogenicity in combination with population dynamics, WP 2 of REPHRAME included mature trees as well. Techniques, assessment schemes, isolation techniques and so on were transferred from experiences with small seedlings to mature trees. In addition, in previous projects pathogenicity tests focused on any available tree provenance. Therefore in WP 6 different provenances will be tested. To guarantee comparable results the main parameters of similar investigations in RISKBURS and PHRAME were transferred to REPHRAME.

8.2 Synthesis and interaction with current EU projects

Beneficiaries of this project will liaise with current EU projects of relevance including:

- COST872, a COST project on plant-nematode interactions (coordinated by SCRI, Scotland, UK) <http://cost872.scri.ac.uk/>;
- PRATIQUE (Enhancements of Pest Risk Analysis Techniques, coordinated by FERA,) <https://secure.fera.defra.gov.uk/pratique/index.cfm>), B4 participating;

- QBOL (KBBE-2008-1-4-01: Development of new diagnostic methods in support of plant health policy, coordinated by The Netherlands, Switzerland and INRA) <http://www.qbol.wur.nl/UK>, B4 participating;

- EUPHRESCO (ERA Net on Plant Protection, <http://www.euphresco.org/>);
- ISEFOR (Increasing Sustainability of European Forests: Modelling for Security Against Invasive Pests and Pathogens under Climate Change, coordinated by Aberdeen University, B1 and B4 participating).

- Other bilateral ongoing collaborations have been established between Beneficiaries and several EU or neighbour countries, such as the Czech Republic, Slovenia, Russia, Turkey.

B7 will coordinate linkages and, where they exist already, those Beneficiaries involved in EU topics will ensure a high level of bilateral exchange.

Relevant activities of International Phytosanitary Organisations include:

- the European and Mediterranean Plant Protection Organisation (EPPO) is working on the EPPO standard PM 9 "Procedures for official control with the aim of containing and eradicating *Bursaphelenchus xylophilus*".

- The EU-Expert Working Group on PWN advises the Standing Committee on Plant Health (SCPH) concerning contents of the EU-emergency measures for PWN (Decision 2006/133/EC).

- The International Forestry Quarantine Research Group (IFQRG) is working on a protocol for the assessment of new phytosanitary treatments against PWN in wood. This is closely related to the work of the IPPC/CPM Technical Panel on Forestry Quarantine of the FAO (IPPC TPFQ).

B1, B5 and B6 are partners of the EPPO EWG, and the EU EWG on PWN; B1 and B5 are members of the IFQRG and B5 is a member of the IPPC TPFQ. B7 has extensive international cooperation with colleagues from Asia, EU and North America. These Beneficiaries will work closely together to coordinate interaction between REPHRAME and international phytosanitary organisations.

The Erasmus Mundus are considered to be the "flagship" of EU post-graduate level higher education. The EUMAINE (<http://www.eumaine.ugent.be/index.asp>) MSc Programme in Nematology is coordinated by Ghent University, a major nematological centre in the EU, together with three more EU universities (Bielefeld, Germany, Jaen, Spain, and Evora, Portugal), and having four more nematological centres (Univ. Kiel, Germany; SCRI, UK; Wageningen, The Netherlands; Catholic Univ., Leuven, Belgium) as satellite partners. Although all types of nematological research are being undertaken, PWD is the focus given in one Beneficiary institution (B7).

This task will aim at providing information on PWD to the EUMAINE programme in order to sensitise EU colleagues and students to the seriousness of PWD, providing a major route to disseminate this information among scientists, students and the public at large. B7 will coordinate.

International links directed at obtaining all possible experience from:

- countries that have PWN but whose forests are not directly affected by it (Canada, USA) and are sources of indigenous populations;

- countries that have PWN and are seriously affected by its presence (Institute of Zoology of the Chinese Academy of Sciences, Beijing, China; University of Tokyo Japan; Korea);

- other countries, such as Australia and Russia (Sukachev Institute of Forest and Wood, Siberian Branch of the Academy of Sciences, Krasnoyarsk), where scientists are already working on the potential impact of PWN.

The inclusion of non-EU teams as collaborators through this WP will also allow for the necessary contacts to obtain material (nematode isolates from centres of origin, in particular), access to field conditions to experiment (inoculation of trees in non-wilt areas in Canada or USA), and transfer of technology already available for testing of rapid and early detection methods (China, Japan, Korea). All Beneficiaries with formal input to this WP will contribute.

8.3 Interaction with international phytosanitary organizations, relevant to PWD

8.4 Interaction with the EUMAINE MSc programme in Nematology, based in Ghent

8.5 International and collaboration with international PWN research groups

Progress against all the above tasks is summarised below.

In February 2014 the ISEFOR consortium held a workshop in Finland on “predicting invasive pests and diseases of forests” where B 5 attended. One of the presented organisms was *B. xylophilus*. The participants were able to use the model developed by the ISEFOR consortium to create a risk map of potential declining trees under given and changeable climate parameters. The model of ISEFOR will be cross-linked with WP 7 in REPHRAME as B1 and B4 are also partners of ISEFOR. During the discussion on the ISEFOR model the following issues were raised which need to be taken into account either in improving the ISEFOR model or the one to be developed in WP 7:

- Include asymptomatic trees
- Difference in spread of pine wood nematode and pine wilt disease
- Flight distance of vector beetles
- Stochastic number of entry points
- First and last year of entry
- Directional distribution of winds

For a spread model the effect of clearcuts (as required according to the current EU-quarantine legislation: Implementing decision 2012/535/EU) and border control should be included.

Objective: Transfer of questions arising from discussions in EU, EPPO and IPPC Working groups and addressing of research needs in relevant WPs as possible.

B5 is a member in several expert groups regarding forest health on EU, EPPO and IPPC level. Currently PWN is of high importance. On IPPC-level (B5 and B 11 involved) a new International Standard of Phytosanitary Measures (ISPM) on the international movement of wood was finally drafted during the reporting period. In this framework the phytosanitary risk of infested wood chips and bark in the framework of non-vector transmission is still not quantified. Questions concerning this issue influenced the work in WP 5 and vice versa. Another issue which was discussed over the years in the International Forestry Quarantine Research Group (B1, B5 involved) as well as in the IPPC-Technical Panel On Forestry Quarantine (B5 and B 11 involved) was a guidance paper in the form of an Annex for ISPM 15. This guidance paper should help companies to plan their investigations on the efficacy of a new phytosanitary treatment. One of the key- organisms to be tested is *B. xylophilus*. Questions on how to create artificially infested wood influenced the work in WP 5.

The question of non-vector spread also influenced the discussion in developing a standard on procedures for official control of *B. xylophilus* and its vectors within the standard series PM 9 (National regulatory control systems) of EPPO. This standard was published during the reporting period under the contribution of several partners of the REPHRAME consortium (B1, B3, B5, B6).

The exchange with scientists all over the world and especially in the framework of the IUFRO unit Pine Wilt Disease was used to develop the venue for an International Conference on Pine Wilt Disease which was carried out in cooperation with REPHRAME. Details are reported in WP 10. There was also a specific session on *B. xylophilus* at the 2nd International Congress on Biological Invasions held in Qingdao, China in late October 2013. This included papers from several REPHRAME participants and is also reported in WP 10.

Technology transfer between REPHRAME project partners 7 and 5

A PhD student of B5 employed in REPHRAME visited several places in Portugal from 29th April until 4th May 2013. The exchange program in Portugal was supervised by B 7. Aim of the exchange was to transfer knowledge in the framework of Pine Wood Nematode management and technologies either used for sampling trees or processing samples in the laboratory. In this exchange program also a scientist from the Chiba University in Japan was involved (former PhD student of B7), who reported on the current investigations in PWN in Japan. Aim of the exchange program was to gather knowledge either from previous research projects on PWN where B7 was involved, current investigations on PWN and PWD including management in Portugal as PWN infested country as well as information exchange with other scientists belonging to the wider range of the Portuguese plant protection service. The experience of this visit influenced work of B5 in the WPs 2, 4, 5 and 6.

Work Package 9: Synthesis and development of PWN Tool Kit for monitoring and management of PWN

Objectives

The key objective of this Work Package is:

- Development of a PWN Tool Kit (PTK). This will be a simple, web-based interface that will provide access to the analysed data from the project and, particularly, to practical advice and new or enhanced methodologies that REPHRAME will make available to end-users. The PTK interface will provide a structured decision-tree interface to enable non-specialists to post questions and interrogate data to access general and specific advice on key management options for PWN and Monochamus spp.

Deliverables

D9.1: PTK interface: Design of the PWN Tool Kit (PTK) interface. Month 12

D9.2: Beta testing of PTK modules: Synthesis of results, construction & Beta testing of PTK modules. Month 39

D9.3: Launch of PTK: Full public launch of the PTK. Month 44

9.1 PTK interface: Design of the PWN Tool Kit (PTK) interface

While the precise content of the PTK will evolve throughout the duration of the project, early attention will be paid to its format and user interface. The core will be a decision-tree expert system to enable users to interrogate the data and obtain general and specific advice and outputs on all aspects of the PWN research and development acquired during REPHRAME. Early versions of PTK will be populated with existing datasets (e.g. derived from the EU PHRAME project and other sources synthesised in WP8 and developed de novo within the project). B1 will work closely with B4 to develop the interface.

Developments on the PTK structure and interface

The original outline of the PTK was presented in the first interim report and discussed at the mid-term meeting and review held in Portugal in 2012. Whilst some progress had been made in the design of the content and initial interface, the EU Science Officer was not content to accept this as sufficient progress. Since that time there has been further development of the structure of the toolkit which, as indicated in the DOW, has to be based on progress in the other Work Packages within REPHRAME. Some of these elements were held up by the slow start to the project which has been recognised by the granting of the 9 month extension.

The outline structure of the PTK has, in the meantime, been refined and benefitted particularly from interaction with contributors to the Stakeholder Observer Group (SOG) (see WP10) through both email and face to face contact at several international meetings from 2012 to 2013. This is an ongoing process that has moved on to 9.2 (below).

9.2 Synthesis of results and construction of PTK modules.

Although it is not possible to be precise about the final modules likely to be included in the PTK, it is likely that, following analysis and synthesis of results, the following will be developed:

- (i) Statistically reliable survey methods for PWN in symptomatic and asymptomatic trees (WP2)
- (ii) Rapid and accurate diagnostic procedures for PWN (WP2)
- (iii) Statistically reliable improved survey methods for Monochamus spp. (WP4)
- (iv) Options for the management of vector populations (WP4)
- (v) Quantification of the potential for non-vector transmission of PWN to host trees (WP5)
- (vi) Recommendations for pathway-specific risk reduction (WPs2-5)

- (vii) Recommendations for choice of conifer species choice in relation to susceptibility to PWN (WP6)
- (viii) Prediction of pine wilt expression by eco-climatic zone (WP7)
- (ix) Factors to be accounted for in dealing with new PWN infestations, including optimised and statistically reliable survey techniques (WPs 2-5, 7)
B1 to coordinate, with input from all Beneficiaries in this WP.

The current version of the PTK content, which will form the basis for the on-line system, is shown in Figure 9.1. This was developed by interaction with the beneficiaries within REPHRAME and the ongoing outputs from the other Work Packages. Additional input has been provided by the members of the SOG, including direct discussions at the IUFRO Alien Invasive Species and International Trade meeting in Tokyo, Japan in June 2012, the joint REPHRAME/IUFRO PWN Group International Conference Braunschweig, Germany in October 2013 (see WP10), the 2nd International Congress on Biological Invasions in Qingdao, China in October 2013 and the International Forest Quarantine Research Group in Qingdao, China in November 2013.

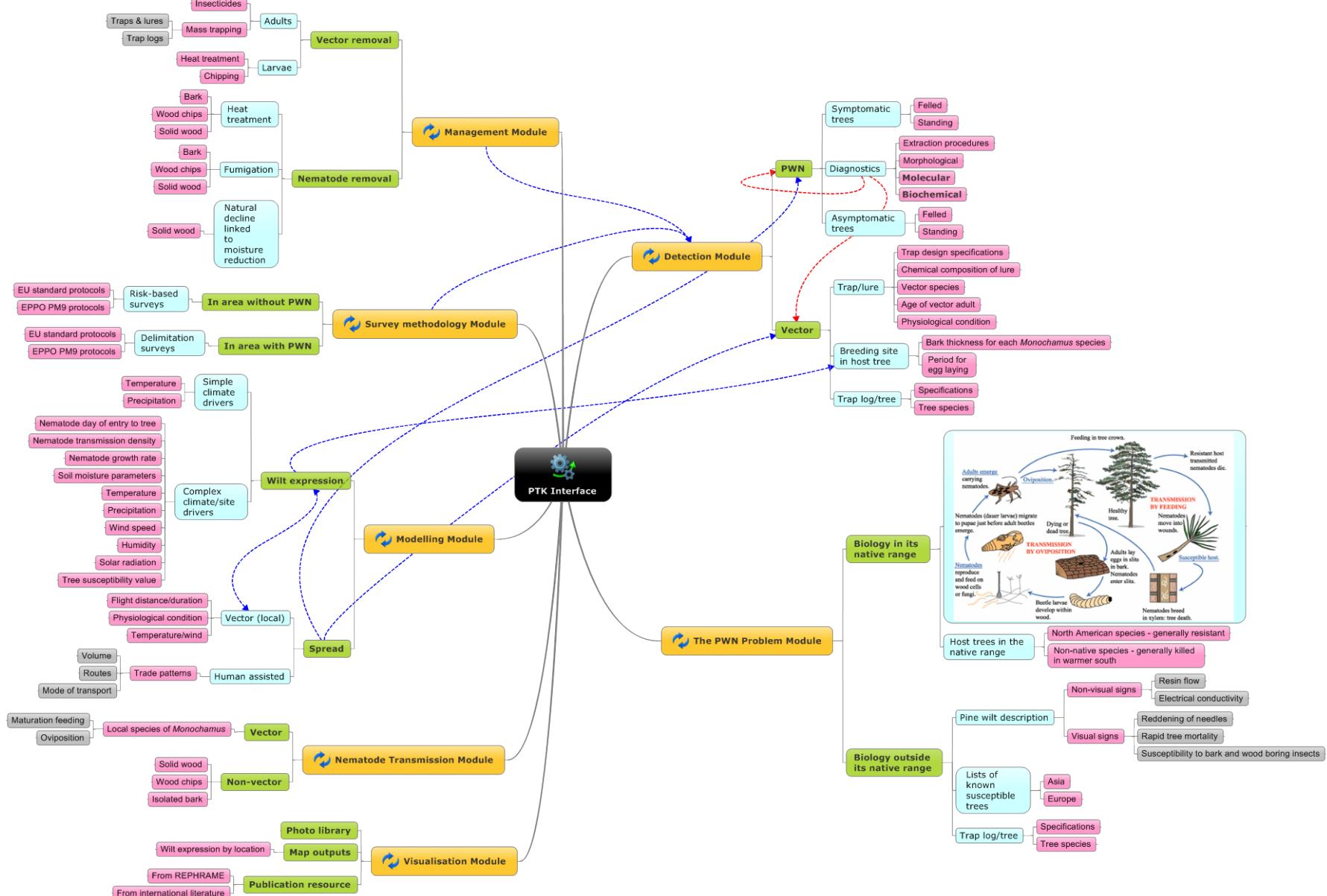


Figure 9.1: Structure of the PTK following analysis of progress in Work Packages and SOG engagement.

As indicated in Figure 9.1, we have now included most of the elements that have been studied within REPHRAME in the expanded structure of the PTK. We are now working to populate these elements with the text and graphics that will be included in the web version of the toolkit. This is proving to be a complex task since the interface will include the ability to customise the search terms so that end users can benefit from both general browsing of facts and more specific provision of advice and methodologies using user-selected search terms.

Some examples of the specific elements to be included within the online PTK are shown below (with reference to the WP and beneficiary responsible):

- Updated life cycle drawing (still under development) to provide a concise summary of the key elements in the saprophytic and pathogenic modes of *B. xylophilus* and its close relationship vectors in the genus *Monochamus* Figure 9.2. Work of B7 in WP8 (taken from a poster by Manuel Mota, Paulo Vieira and Fernando Correia used at a number of national and international meetings).

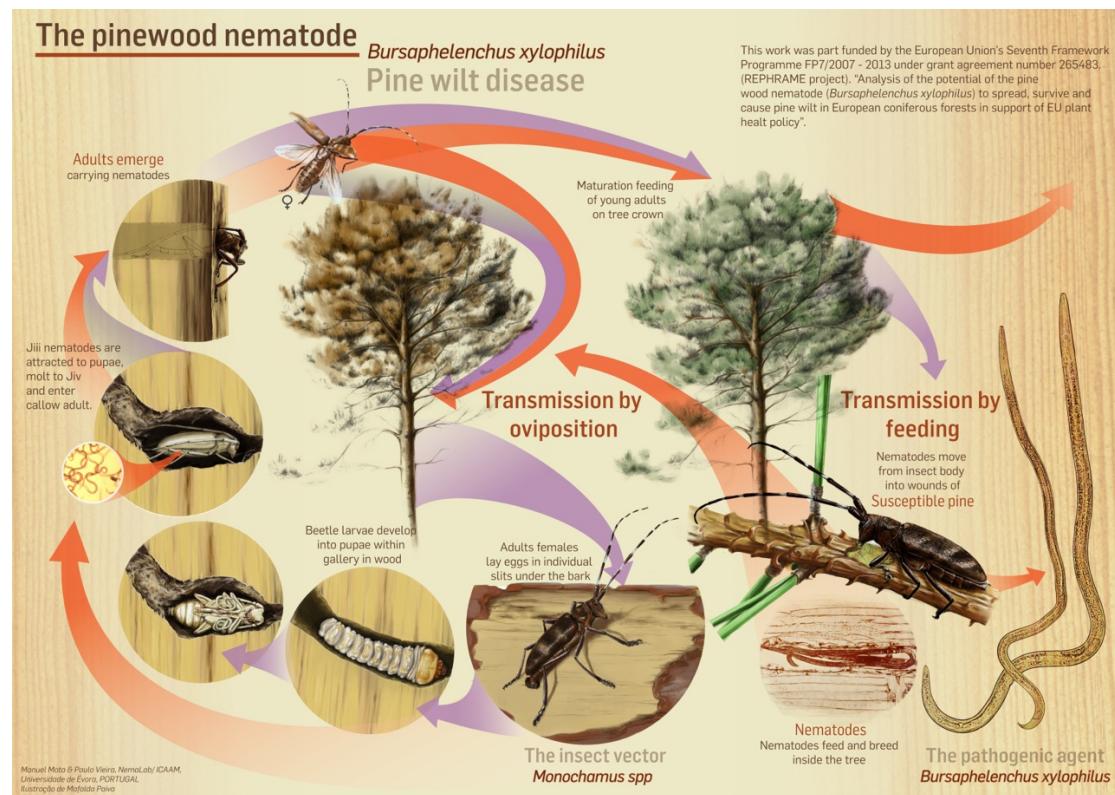


Figure 9.2: Updated life cycle illustration to summarise the PWN/tree/vector relationship (draft version prepared by Manuel Mota and Mafalda Paiva).

- Simulated time of the vector emergence (Figure 9.3) and simulated duration of the life cycle (Figure 9.4) for different zones of Europe, including the current known infested area and other countries in Europe. Work of B4 in WP7.

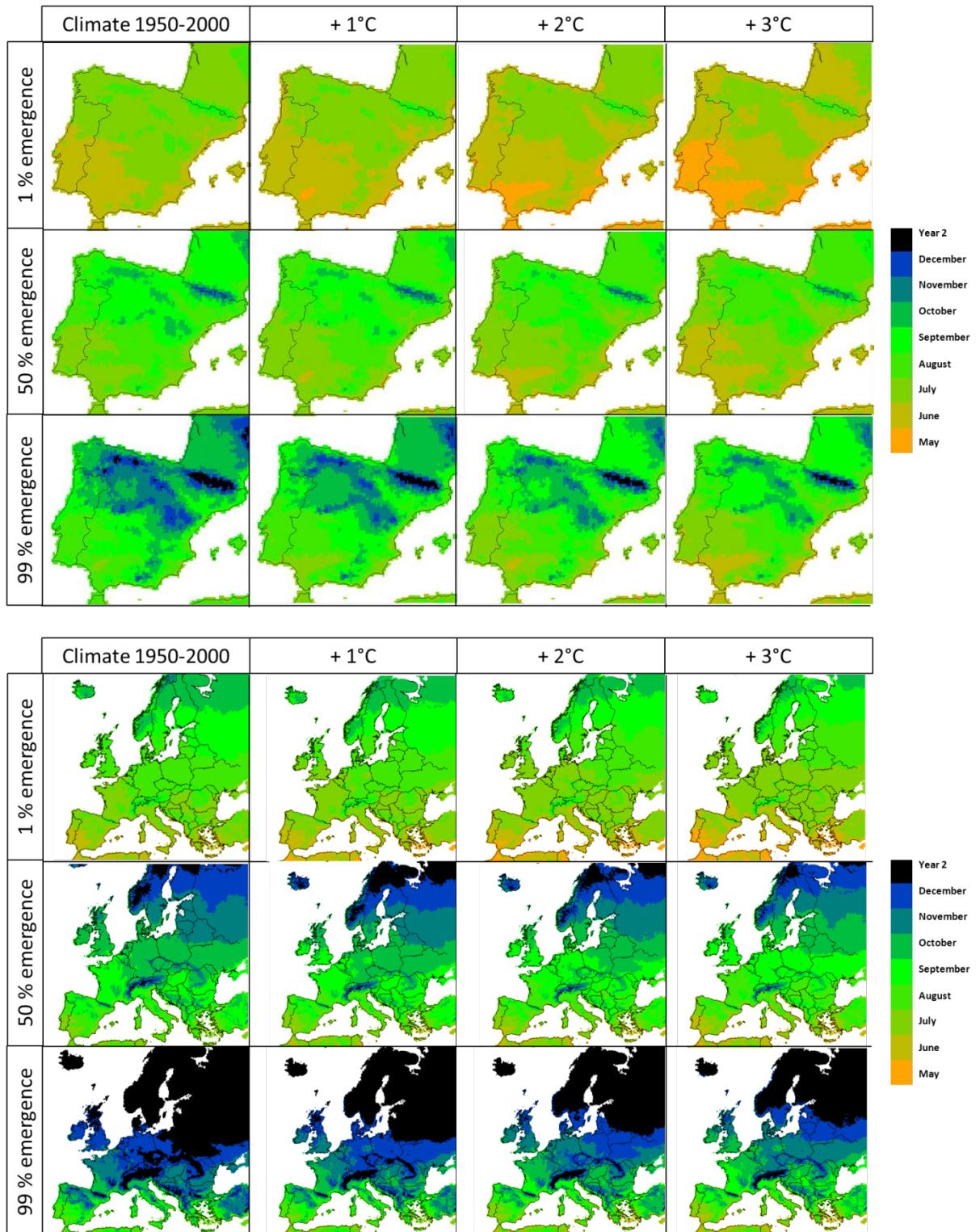


Figure 9.3 Emergence time of *Monochamus* spp. Simulated for Europe

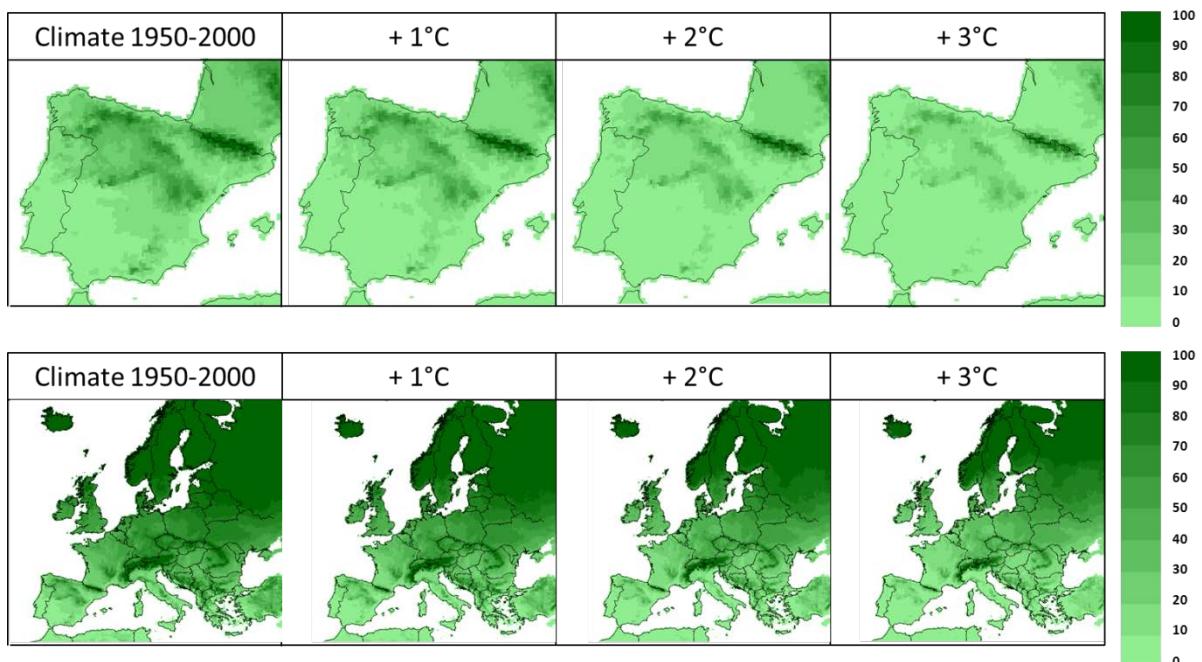


Figure 9.4 Simulation of proportion of the vector population having a 2-year cycle

9.3 Full public launch of the PTK

The full public launch of the PTK will take place during the final month of the project, although partial versions will be made available once particular modules have been completed. However, the full value of the PTK will be enhanced when all the data gathered during REPHRAME has been analysed and interpreted into suitable outputs. The launch is likely to coincide with the International Conference on PWN to be held during the final three months of the project (see WP10).

B1 to coordinate and ensure linkage to WP10.

Some progress on development of the PTK was reported at the “International Conference on Pine Wilt Disease 2013” organized by B5. The conference was a joint activity of the Consortium of REPHRAME, the IUFRO unit 7.02.10 “Pine Wilt Disease”, which was chaired by B5, the Julius Kühn-Institute and the German Scientific Society for Plant Protection and Plant Health (DPG). Useful discussion with the participants and with members of the SOG were held at the meeting and this has aided the further development of the PTK structure. Now that the project has entered the extension period, the PTK is receiving very active development and will be featured at the planned Workshops in Portugal and Spain during 2014 and the feedback will be used to refine the web version to be launched at the end of the project. It is likely that this will be a central component of a final workshop that has been requested to be held in Brussels (to be confirmed).

Work Package 10: Stakeholder Engagement & Dissemination

Objectives

The objective of this Work Package is to ensure successful communication between Beneficiaries, the European Commission and key stakeholders as part of an effective and flexible dissemination package.

The key objectives are:

- To ensure that all outputs are in appropriate formats and are disseminated as soon as possible after analysis and interpretation of data has been carried out.
- To establish the Stakeholder Observer Group and ensure that it is involved by electronic and direct contact with the consortium.
- To deliver themed Workshops along with the current implementation of the PWN Tool Kit.
- To hold an International Conference involving both REPHRAME Beneficiaries and international experts during the final year of the project.

Deliverables

D10.1: REPHRAME website launch & maintenance: Website constructed and initial version in place (Month 2). Regular updates and revisions for the remainder of the project duration. Month 2-36.

D10.2: Project leaflet: Project leaflet prepared and printed with e-copy on REPHRAME website Month 4.

D10.3: SOG minutes: Minutes of meetings of SOG. Month 12.

D10.4: Themed workshop 1: Themed workshops to be held in Portugal in years two (month 22) and three (month 34) Month 38.

D10.5: Themed workshop 2: Themed workshop to be held in Portugal in year three. Month 38.

D10.6: International Conference on PWN: International Conference on PWN and its vectors followed by publication of proceedings. Month 32.

D10.7: Plan for use & dissemination of foreground: Final plan for the use and dissemination of foreground. Month 45.

D10.8: Awareness & wider societal implications: Report on awareness and wider societal implications. Month 45

Work done by B5 (6 PM)

B 5 spent 5,28 PM on this WP (only productive hours). This was mainly due to the organization of the International Conference on Pine Wilt Disease. In addition time representing more than another person month was spent by persons who supported the conference: technicians running the technology during the conference, staff facilitating the coffee breaks, representatives from the management of the National park Harz and the North-West-German Forest Research Center who conducted the field trip, staff who launched the conference website. Those were not staff of JKI, so they do not fall under the equity ratio of JKI, nor were they paid by the project or conference fee.

10.1 Production of appropriate dissemination media for project outputs

- Website. This will be a key resource for informing stakeholders, the wider world and also for keeping Beneficiaries informed of progress, sharing information, and ensuring good communication.

- Project Leaflets. These can be a very effective and inexpensive way of informing people of the existence of the project, especially during face-to-face communication.
 - Reports. These are a key output of the project and form the main outputs of the work. These reports enable detailed descriptions of the work and findings to be communicated within the project, to the EU and to a wider audience.
 - Scientific Publications. Publication in the peer reviewed literature is the Quality Assurance on the scientific level of the work conducted during the project.
- B1 to lead and work closely with the other Beneficiaries.

Objective: Production of a website/ project leaflets/ reports/ scientific publications

Additional dissemination of REPHRAME work was done for WP5 “Non-vector spread of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhler) Nickle 1970, with wood chips to non-infested trees” during a poster session to the Young Scientist Forum of the Julius Kühn-Institut in Quedlinburg (Germany) on 4th December 2012.

An information leaflet concerning PWN and PWD to inform interested stakeholders and the public in Germany was produced:

http://www.etracker.de/lncnt.php?et=dQsHU3&url=http://www.jki.bund.de/fileadmin/dam_uploads/_veroeff/faltblaetter/kiefernholznematode_.pdf&lncname=http://www.jki.bund.de/fileadmin/dam_uploads/_veroeff/faltblaetter/kiefernholznematode_.pdf.

Scientific Publications

A total list of publications is being prepared and the following are some of the papers that have been produced from REPHRAME.

- David G, Jactel H, Piou D, Naves P & Sousa E (2013). Flight performances of *Monochamus galloprovincialis*, insect vector of the Pine Wood Nematode. In: Schroder, T. (ed.), Pine Wilt Disease Conference Book 2013, pp. 20, Braunschweig, ISSN: 1866-590X.
- Evans, H.F. (2013) The current situation on pine wood nematode, *Bursaphelenchus xylophilus* in Europe; from research to management. Abstracts of the 2nd International Congress of Biological Invasions, 23-27 October, Qingdao, China
- Gruffudd H, Evans H, Haran J, Roux-Morabito G, Roques A, Robinet C (2013) How could climate change affect the potential spread of pine wilt disease in Europe? ClimTree 2013, Zurich (Switzerland), 2-4 Sept 2013 (oral, invited)
- Gruffudd, H., Evans, H.F. and Jenkins, T. (2013) Using an evapo-transpiration model to predict the current and future range and severity of pine wilt disease caused by pine wood nematode, *Bursaphelenchus xylophilus* in Europe. Abstracts of the 2nd International Congress of Biological Invasions, 23-27 October, Qingdao, China
- Gruffudd, H., Evans, H.F. and Jenkins, T. (2013) Using an evapo-transpiration model to predict the current and future range and severity of pine wilt disease caused by pine wood nematode, *Bursaphelenchus xylophilus* in Europe. Abstracts of the IUFRO / REPHRAME International Conference on Pine Wilt Disease, 15-18 October 2013, Braunschweig, Germany
- Halbig, P., Hoch, G, Menschhorn, P., Hall, D.R., Krehan, H. (2013): Flight activity of longhorn beetles *Monochamus sartor* and *M. sutor*. Attractiveness of insect and tree produced volatiles. Meeting of the Deutsche Gesellschaft für allgemeine und angewandte Entomologie, March 18-21, 2013, Göttingen, Germany

- Halbig, P., Menschhorn, P., Krehan, H., Hall, D.R., Hoch, G.: Flugaktivität der Bockkäfer *Monochamus sartor* und *Monochamus sutor*: Attraktivität insekten- und baumbürtiger volatiler Substanzen. Silva Fera, in press
- Haran J. Approche multidisciplinaire de la dispersion chez un invasif : application au nématode du Pin (*Bursaphelenchus xylophilus*) et de son insecte vecteur (*Monochamus galloprovincialis*). Poster présentation. Séminaire des doctorants du département EFPA, INRA. Dourdan Novembre 2012
- Haran J., Garcia J., Bernard A., Roques A., Robinet C. & Roux- Morabito G. (2013). Etude des processus de dispersion de *Monochamus galloprovincialis*, vecteur du nématode du pin : rôle potentiel de la chaîne pyrénéenne. GEFF. Bedoin (France) 2oct. 2013.
- Haran J., Roques A., Robinet C. & Roux- Morabito G. (2014). Assessing dispersal routes and ongoing gene flow of the vector of the Pine Wood Nematode, *Monochamus galloprovincialis*, at different spatial scales. XXIV IUFRO World Congress. 5-11 October 2014 Salt Lake City, United State.
- Haran J., Roques A., Roux- Morabito G. (2013). Assessing potential expansion of the Pine Wood nematode (*Bursaphelenchus xylophilus*) from the spatial genetic structure of the vector (*Monochamus galloprovincialis*). IUFRO 7.02.10 "Pine Wilt Disease Conference 2013" Braunschweig, Germany; 15–18 October 2013.
- Haran J., Roques A., Roux- Morabito G. (2014). Evolutionary history and ongoing gene flow of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae), vector of the Pine Wood Nematode. IUFRO 7.03.14, 7.03.06, 7.03.01 Joint Meeting, 9-14 April 2014 Antalya, Turkey.
- Haran J., Roux- Morabito G., Roques A. (2013). Assessing potential expansion of the Pine Wood Nematode (*Bursaphelenchus xylophilus*) from the spatial genetic structure of the vector (*Monochamus galloprovincialis*): First results of a STSM stay in Portugal. PERMIT meeting, Treviso (Italy), 1-4 October 2013
- Hoch, G., Halbig, P., Menschhorn, P., Hall, D.R., Krehan, H. (2013): Flight activity of sawyer beetles *Monochamus sartor* and *Monochamus sutor*: Attractiveness of insect and host tree volatiles. 24th USDA Interagency Research Forum on Invasive Species, Jan 8-11, 2013, Annapolis, MD, USA
- Hoch, G., Halbig, P., Menschhorn, P., Hall, D.R., Krehan, H., 2013: Testing attractants for trapping *Monochamus sartor* and *Monochamus sutor*. In: T. Schröder (Ed.): Proceedings of the Pine Wilt Disease Conference 2013, 15 to 18 Oct 2013, Braunschweig. Berichte aus dem Julius Kühn-Institut, Braunschweig, (169): 103-104
- Hopf, A.; Schröder, T. (2013): Non vector spread of *Bursaphelenchus xylophilus* via wood chips. In: Schröder, T. (ed.) Pine Wilt Disease Conference 2013, Abstracts, Berichte aus dem Julius Kühn-Institut 169: 46-47.
- Lilin Zhao, Min Lu, Hongtao Niu, Guofei Fang, Shuai Zhang, And Jianghua Sun. 2013. A native fungal symbiont facilitates the prevalence and development of an invasive pathogen–native vector symbiosis. Ecology, 94(12), 2013, pp. 2817–2826
- Lilin Zhao, Shuai Zhang, Wei Wei, Haijun Hao, Bin Zhang, Rebecca A. Butcher, and Jianghua Sun. 2013. Chemical Signals Synchronize the Life Cycles of a Plant-Parasitic Nematode and Its Vector Beetle. Current Biology 23, 2038–2043
- Mallez S., Castagnone C., Espada M., Mota M., Guillemaud T. and Castagnone-Sereno P. (2012). Development of *Bursaphelenchus xylophilus*-specific microsatellite markers to assess the genetic diversity of populations from European forests. Proceedings of the 31st International Symposium of the European Society of Nematologists, 23-27 September 2012, Adana, Turkey : 88.

- Mallez S., Castagnone C., Espada M., Vieira P., Eisenback J., Mota M., Aikawa T., Akiba M., Kosaka H., Castagnone-Sereno P. and Guillemaud T. (2013). Genetic diversity of the pinewood nematode around the world – First insights in its invasion routes. Abstracts of the 2nd International Congress of Biological Invasions, 23-27 October, Qingdao, China : 96.
- Mallez S., Castagnone C., Espada M., Vieira P., Eisenback J.D., Mota M., Guillemaud T. and Castagnone-Sereno P. (2013). First insights into the genetic diversity of the pinewood nematode in its native area using new polymorphic microsatellite loci. *PLoS ONE* 8 : e59165.
- Mallez S., Castagnone C., Espada M., Vieira P., J. Eisenback, Mota M., Aikawa T., Akiba M., Kosaka H., Castagnone-Sereno P. and T. Guillemaud T. (2013). First insights into the genetic diversity of the pinewood nematode in its native area and around the world. (Poster). Abstracts of the XIV Congress of the European Society for Evolutionary Biology, 19-24 August 2013, Lisbon, Portugal : 536.
- Mallez, S., C. Castagnone, M. Espada, P. Vieira, JD. Eisenback, M. Harrell, M. Mota, T. Aikawa, M. Akiba, H. Kosaka, P. Castagnone – Sereno, T. Guillemaud. 2014. Worldwide routes of invasion of the pinewood nematode: what have we learned so far? (submitted to *Biological Invasions*, March 27, 2014).
- Moreira, C., von Asch, B., Pereira-Castro, I., Silva, R., Azevedo, L., Mota, M., Abrantes, I., Amorim, A. and F. Pereira. 2013. The mitochondrial genome of the pinewood nematode (*Bursaphelenchus xylophilus*) lineage introduced in Europe. *Mitochondrial DNA* (Posted online on July 10, 2013. (doi:10.3109/19401736.2013.809438)
- Mota, L., B. Moura, R. Galupa, P. Barbosa, M. Mota & L. Ascensão. 2014. Histological changes induced by *Bursaphelenchus xylophilus* in the stem tissues of *Pinus pinaster* seedlings (Paper in preparation).
- Nascimento, F., Cláudia S.L. Vicente, Pedro Barbosa, Margarida Espada, Paulo Vieira, Koichi Hasegawa and Manuel Mota. 2014. Bacterial role in pine wilt disease development – review and future perspectives. *Environmental Microbiology, EMI Reports* (submitted).
- Pajares et al, 2013. 2-(Undecyloxy)-ethanol is a major component of the male-produced aggregation pheromone of *Monochamus sutor*. *Entomologia Experimentalis et Applicata* 1-10
- Pereira F, Moreira C, Fonseca L, van Asch B, Mota M, et al. 2013. New Insights into the Phylogeny and Worldwide Dispersion of Two Closely Related Nematode Species, *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*. *PLoS ONE* 8(2): e56288. doi:10.1371/journal.pone.0056288
- Petersen-Silva R., Henriques J., Inácio L., Naves P., Godinho P., Sousa E. & Pujade-Villar J. (2014). Susceptibility of larvae and adults of *Monochamus galloprovincialis* to entomopathogenic fungi under controlled conditions. Submitted to publication.
- Petersen-Silva R., Naves P., Godinho P., Sousa E. & Pujade-Villar J. (2014). Distribution, hosts and parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in mainland Portugal. *Silva Lusitana*, in press.
- Petersen-Silva R., Naves P., Sousa E. & Pujade-Villar J. (2014). Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets. *Journal of the Entomological Research Society*, 16(1): 61-70.
- Petersen-Silva, Naves, Godinho, Sousa & Pujade-Villar (2014). Distribution, hosts and parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in mainland Portugal. *Silva Lusitana*, in press.
- Robinet C (2013) Expansion potentielle du nématode du pin : vers une modélisation calibrée sur l'Europe. GEFF, Brens (France), 22-24 Oct 2013.

- Robinet C, David G, Piou D, Roques A, Jactel H (2013) Simulating the dispersal of *Monochamus galloprovincialis* based on its flight mill performance and testing several pest management scenarios. Pine Wilt Disease Conference 2013, Braunschweig (Germany), 15-18 Oct 2013.
- Schröder, T.; Welling J.; Aukamp-Timmreck C. (2013): Efficacy of kiln drying as phytosanitary treatment against wood borne nematodes. In: Schröder, T. (ed.) Pine Wilt Disease Conference 2013, Abstracts, Berichte aus dem Julius Kühn-Institut 169: 51-52.
- Vicente, C. Ikuyo, Y., Mota, M. and K. Hasegawa. 2013. Pine wood nematode-associated bacteria contribute for oxidative stress resistance to *Bursaphelenchus xylophilus*. *BMC Microbiology* 2013, 13:299 doi:10.1186/1471-2180-13-299.
- Vicente, C., F.X. Nascimento, M. Espada, P. Barbosa, K. Hasegawa, M. Mota, and S. Oliveira. 2013. Characterization of bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis*, the insect vector of the pinewood nematode *Bursaphelenchus xylophilus*. *FEMS Microbiology Letters* (in press; accepted, July 29, 2013) DOI: 10.1111/1574-6968.12232.
- Vieira P., Castagnone C., Mallez S., Espada M., Navas A., Mota M. and Castagnone-Sereno P. (2014). Sequence variability of the *MspI* satellite DNA family of the pinewood nematode *Bursaphelenchus xylophilus* at different geographic scales. *Molecular Phylogenetics and Evolution* 70 : 120-129.
- Vieira P., Castagnone C., Mallez S., Espada M., Navas A., Mota M. and Castagnone-Sereno P. (2013). Sequence variability of the *MspI* satellite DNA family of the pinewood nematode at different geographic scales. (Poster). Abstracts of the 52nd Annual Meeting of the Society of Nematologists, 14-17 July 2013, Knoxville, USA.
- Vieira P., Castagnone C., Mallez S., Espada M., Navas A., Mota M. and Castagnone-Sereno P. (2013). Comparative analysis of *MspI* satellite repeats of the pinewood nematode, *Bursaphelenchus xylophilus*, at different geographic scales. (Poster). Abstracts of the IUFRO / REPHRAME International Conference on Pine Wilt Disease, 15-18 October 2013, Braunschweig, Germany : 123.
- Vieira P. C. Castagnone, S. Mallez, M. Espada, A. Navas, M. Mota and P. Castagnone-Sereno. 2014. Sequence variability of the *MspI* satellite family of the pinewood nematode, *Bursaphelenchus xylophilus* at different geographic scales. *Mol. Phylogenetics and Evolution* 70: 120-129.
- Zhao, L. M. Mota, P. Vieira, RA. Butcher, and J. Sun. 2014. Interspecific communication between pinewood nematode, its insect vector, and associated microbes (accepted, *Trends in Parasitology*, Apr. 13)

B3 presentations, outreach

Presentations for forestry practice

Introducing the problem of PWD, demonstrating vector monitoring methods, PWN emergency measures, national PWN contingency plan

- at Forest Protection workshop at the BFW Forest Training Centre Ossiach
- at regular meetings of forest protection officers of Austrian federal provinces

Internet

Article on the BFW website (in German) introducing the problem of PWD and research in the project REPHRAME (incl. link to project website).

10.2 Stakeholder Observer Group

- Set up the Stakeholder Observer Group (SOG) and elect Chairperson for the group. Beneficiaries already indicating willingness to contribute are EPPO, various EU NPPOs, IFQRG, IUFRO. B1 to lead.
- Establish specific Web portal for the SOG. This will also be a discussion forum to share information and ideas in parallel with, but independently managed from, the main REPHRAME consortium pages. B1, in liaison with Chair of the SOG.
- Meetings of the full SOG will be held at least once per annum, but the Chair will be invited to all consortium meetings. B1 in liaison with Chair of the SOG.

Although plans were made to establish a formal structure for the Stakeholder Observer Group (SOG), it was felt that a more informal mechanism would prove to be as effective and would enable contact to be maintained through both emails and *ad hoc* at international meetings. Thus, the membership of the SOG has evolved during the course of the project, bearing in mind that there was a very slow start to the project itself.

Meetings at which the aims of the project and the direction of work were discussed therefore include:

April 2011: Interaction with the EPPO Forest Quarantine Panel to coincide with the kick-off meeting of REPHRAME held in Funchal, Madeira. Particular focus was on the developing PWD impact on Madeira and the contribution of REPHRAME to the EPPO protocol on PWN.

June 2012: IUFRO Alien Invasive Species and International Trade meeting in Tokyo, Japan in June 2012. Since the situation on PWN in Japan has a very long history, there was strong focus on the nematode and its vectors and discussion with key Japanese researchers included a commitment to contributing to the SOG. A field visit contributed to the interaction with many researchers from Japan and elsewhere in the world who were attending the meeting.

October 2012: Meeting with the Nematode Group chaired by Dr Christoph Orazio. This was a very valuable opportunity to discuss PWN in a European context as indicated in the agenda in Table 10.1.

Table 10.1: Agenda for joint REPHRAME and Nematode Group meeting to develop the Stakeholder Observer Group and other interaction.

Joint meeting of REPHRAME project and the Nematode Group
22 October, 2012
Sana Hotel, Estoril, Portugal
Agenda
14.00 Meeting commences with introduction of participants from REPHRAME and Nematode Group
14.20 Outline of the work and purpose of REPHRAME, including the development and working of its Stakeholder Observer Group – Hugh Evans
14.50 Outline of the work and purpose of the Nematode Group – Christophe Orazio
15.20 Description of the new project FORRISK and consideration of collaboration and synergy between participants of all groups – Christophe Orazio and others
16.00 Discussion on research needs that can be addressed through joint working of the different projects – All to contribute
17.00 Future meetings

There has been regular contact with members of the nematode group and this will continue in the final phase of REPHRAME.

October 2013: Joint REPHRAME/IUFRO PWN Group International Conference Braunschweig, Germany in October 2013 (see 10.4). A short formal session to encourage additional membership of the SOG and to extend collaboration was held in addition to the normal scientific interchange at the meeting.

October 2013: 2nd International Congress on Biological Invasions in Qingdao, China. Several participants in REPHRAME presented papers at the Congress and Prof Hugh Evans gave an overview of the European situation on PWN at a special session on the nematode. During this presentation, he developed discussion with existing members of the SOG and encouraged further membership, which has proved fruitful in developing the PTK (WP9)

November 2013: International Forest Quarantine Research Group in Qingdao, China. A specific presentation and discussion session on PWN was included in the agenda and further interest shown in REPHRAME and the SOG, including additional membership.

Current membership of the SOG includes 22 scientists, plant health regulators, practitioners and timber trade representatives from Canada, China, France, Japan, South Korea, Netherlands, Portugal, South Africa, Spain, UK, USA, Vietnam.

The recent approach has been to use circular email interaction, with particular focus on the desired outputs on the PTK. Professor Keiko Kuroda from Japan has been especially helpful in providing feedback and additional ideas to add to the outline PTK and we look forward to further interaction with her and other members of the SOG.

10.3 Themed Workshops

- Two Workshops in the second and third years of the project will be organised. These will be themed to describe and enable users to use the PWN Tool Kit and to provide training and hands-on experience in laboratory techniques (Workshop 1) and field sampling and vector trapping techniques (Workshop 2). The Workshops will be held in Portugal and Spain. B1, B6, B7 and B9 to coordinate.
- Appropriate training and dissemination packages (hard copy and electronic) will be produced to accompany each Workshop. All Beneficiaries to contribute.

These workshops will be organised during 2014 during the extension period. Following a request from the EU Science Officer, we will also organise a specific Workshop in Brussels at the end of the project.

10.4 International Conference on PWN and its vectors.

- An International Conference on all aspects of PWN research and development will be held during the final three months of the project. This Conference will take place in the framework of the International Union of Forest Research Organisations (IUFRO) unit 7.02.10 “Pine Wilt Disease” which is currently chaired by B5.

The Conference will follow up on the 2006 (Lisbon, organised by B7) and 2009 (Nanjing) IUFRO international meetings on PWD. It was proposed by the last IUFRO-Unit meeting in Nanjing to carry out the next meeting in a non PWN-infested country to discuss on site the management options after an initial finding of PWN. As B5 chairs the IUFRO group it is proposed to hold this Conference in Germany which represents a country where PWN at this stage is not believed to cause symptoms on infested trees if introduced. The outputs from REPHRAME will form a substantial part of the Conference, but wide international involvement

will be actively pursued so that the outcomes of the Conference will provide a state of the art synthesis of knowledge. It is expected that more than 100 scientists from all countries where PWN occurs and others will take part. B5 to coordinate.

- The proceedings of the Conference will be published as a printed book (B5 to coordinate). In addition, e-copies of the presentations and a summary of the main findings and outcomes of the Conference will be made available on the REPHRAME website. (B1 assisted by all).

Objective: Organization of the International Conference on PWN and publishing of the conference proceedings

Because the original end date of REPHRAME was February 2014, the consortium agreed to have the proposed scientific conference at the end of the vegetation period 2013. Therefore from 15th until 18th October 2013 the “International Conference on Pine Wilt Disease 2013” was organized by B5. The conference was a joint activity of the Consortium of REPHRAME, the IUFRO unit 7.02.10 “Pine Wilt Disease”, which was chaired by B5, the Julius Kühn-Institute and the German Scientific Society for Plant Protection and Plant Health (DPG).

With the member background of these groups and the access to their mailing lists, it was possible to announce the conference to more than 10,000 scientists worldwide. This resulted in an unexpectedly high number of 87 participants representing 23 countries including Europe as well as Russia, Asia, North America and Australia.

In total 41 talks were given and 22 Posters were presented. The presentations covered the actual research on the following topics:

- The vector of PWN
- Systematic and Diagnosis
- PWN in international trade, pathways and phytosanitary treatments
- PWN interactions with bacteria
- PWD management and contingency planning
- PWN Biology, Population dynamics, epidemiology, modeling.

The conference proceedings were published in the series “Berichte aus dem Julius Kühn-Institut” which is an open access journal and can be downloaded under: <http://pub.jki.bund.de/index.php/BerichteJKI/issue/view/858>.

The conference received a high level of interest by public media. During the conference six radio interviews were given by B5 and two television reports were produced, one of which was 30 minutes long, broadcast by the local television station “TV38” on 25th Oct. 2013. Numerous reports in daily newspapers as well as print and online magazines were published including the German Press Agency (dpa). Using a google search with the search terms “Kiefernholznematode Braunschweig” results in more than 1,500 findings reporting in most cases on the conference and related issues.

The conference was announced and press releases were published on several websites, such as JKI: http://www.jki.bund.de/index.php?id=940&no_cache=1&press_id=187, DPG: <http://dpq.phytomedizin.org/de/pwdc2013/>, German Federal Ministry on Agriculture: http://www.bmeli-forschung.de/no_cache/de/startseite/presse/pressedetailseite/Pressemitteilung/fadenwurm-aus-uebersee-bedroht-kiefernwaelder.html. Even the Russian Plant Protection Service, Rosselkhoznadzor, reported on the conference <http://www.fsvps.ru/fsvps/news/7927.html?language=en>.



Participants of Pine Wilt Disease Conference 2013

Publications:

- Schröder, T. ed. (2013): Pine Wilt Disease Conference 2013. Berichte aus dem Julius Kühn-Institut 169, DOI 10.5073/berjki.2013.169.000: 141p.
Schröder T. (2014): Kiefernholznematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle. Informationsblatt des JKI, 2. aktual. Aufl. DOI 10.5073/jki2014.007: 2p.

Project management during the period
This was designated as WP1 in the original proposal.

Consortium management tasks, achievements and problems/solutions

The REPHRAME project started on 1 March 2011 on the advice of the EU Science Officer and with the purpose of being able to carry out fieldwork during the 2011 activity period of *Monochamus galloprovincialis*, the vector beetle of pinewood nematode. At this stage, the project was still in the final stages of negotiation and, although the science elements had been completed, the Grant Agreement had not been finalised or signed.

Although the contract negotiation was not complete, the Consortium kick-off meeting was held in Madeira on 1 April 2011 and successfully launched the project. This included immediate interaction with the EPPO Forest Quarantine Panel which was also meeting in Madeira (relevant to WP8 and WP10) and field visits to see the heavy damage arising from PWN infestation on the island. The work within REPHRAME was planned according to Annex I and on the assumption that contract negotiation and distribution of funds would follow quickly.

Unfortunately, the Grant Agreement was not finalised and signed until 27 July 2011, which posed problems for some beneficiaries in relation to being able to commit resources to the project. In addition, and more significant in relation to the work plan of the project, there were problems in setting up an interest-bearing bank account for the coordinator institute, Forest Research (FR). Although a Declaration of Honour concerning the normal rule of the UK Government that its own institutes did not hold interest-bearing bank accounts was submitted, this was refused by the EU Finance Officer and, therefore, special permission had to be sought for, exceptionally, FR to set up an appropriate account. Although beyond the control of FR, this took several months to obtain approval and to set up the account with the consequence that funding was not received for distribution to beneficiaries until mid-October 2011. Whilst some beneficiary institutes had been able to support resource allocation pending distribution of funds (i.e. on a financial accrual basis), not all were able to do so. This had particular consequences for B5 and, to a certain extent, B6 who were not able to commence work, including recruitment of staff, until funds were received.

Consequently, particularly as seen in the first periodic report, there were delays in commencement of some elements of the Work Packages which, because it meant losing a full activity period for the vector insect (May to late September), had the effect of delaying work on some topics by a whole year. As indicated above, this was acknowledged by the external reviewer Dr Ilaria Pertot and, subsequently, an application was made for a 9 month extension. After much uncertainty, this was granted eventually during February 2014, the final month of the original contract. Some work has, therefore, been lost irretrievably, but nevertheless the extension will enable the virtually all the original planned programme to be delivered by the new end date of 30 November 2014.

As is apparent from this comprehensive scientific report, there has been excellent and significant progress in most of the tasks planned for REPHRAME and these will form the basis of a comprehensive dissemination effort during 2014.

Consortium Agreement

A signed and agreed Consortium Agreement has been concluded by the beneficiaries. This remains an active document and, in fact, there have been a

number of substantial changes to some of the items included in the CA, which necessitated distribution of a revised version and a new round of signatures to ensure full agreement. The document provides for a wide range of activities, including IP protection and exploitation.

Project Meetings

1. Kick-Off Meeting, Funchal, Madeira, Portugal. 1-2 April 2011
2. Update and planning meeting, INRA Orleans, France. 12-14 March 2012
3. Mid-term meeting, Estoril, Portugal. 22-25 October 2012. This meeting included a Stakeholder Observer Group meeting with the Nematode Group organised by Dr Christophe Orazio (EFI).
4. Meeting within the joint REPHRAME/IUFRO PWN Group International Conference, Braunschweig, Germany. 15-18 October 2013.
5. Update and planning meeting, BFW Vienna, Austria. 10-11 February 2014.

Project planning

The main project planning events have been at the Consortium meetings, but there has been regular email correspondence between beneficiaries concerning work within and between Work Packages. This has also included full exchange visits (some of these have been outlined in the WP descriptions, especially WP8) to learn and share techniques and to plan laboratory and field work.

The issues over delays in starting the project have been indicated in the first report and above. These impacts have been generally overcome and will be managed through the project extension period.

There will be workshops for Portuguese and Spanish end users during the summer of 2014 (in planning stage) and, at the request of the EU Science Officer a final workshop in Brussels probably in November 2014.

Legal status changes

Beneficiary 6 (previously INRB) went through a major re-structuring during 2012 which had some impacts on the ability of the scientists to deliver the full planned work. Nevertheless, they have achieved much of the planned work despite considerable uncertainty concerning resources and infrastructure. The new name, address and contact information of the organisation is:

Instituto Nacional de Investigação Agrária e Veterinária, I.P.

Unidade de Silvicultura e Produtos Florestais
Av. da República, Quinta do Marquês, 2780-159 Oeiras
PORTUGAL
Tel: (+351) 21 4463700 Fax: (+351) 21 4463701
Principal Investigator: Dr Edmundo Sousa
Email: edmundo.sousa@iniav.pt

Project website

As indicated in WP10 the project website was initially:

www.forestry.gov.uk/fr/rephrame

but is now www.rephrame.eu.

Due to the delay in obtaining permission for the extended period, we are having to carry out a further procurement exercise to keep the external website going and this should be completed soon.

Dissemination

This has been summarised in WP8 and WP10. Consortium participants have been active in publication of results and in presentation of findings from the project at national and international workshops and conferences. This has publicised REPHRAME and fostered interaction and collaboration globally. This has also enabled face to face contact with members of the Stakeholder Observer Group which would not have been possible at a single meeting.