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rephrame

Development of improved methods for detection, control and eradication of pine wood nematode

EU Project 265483 REPHRAME

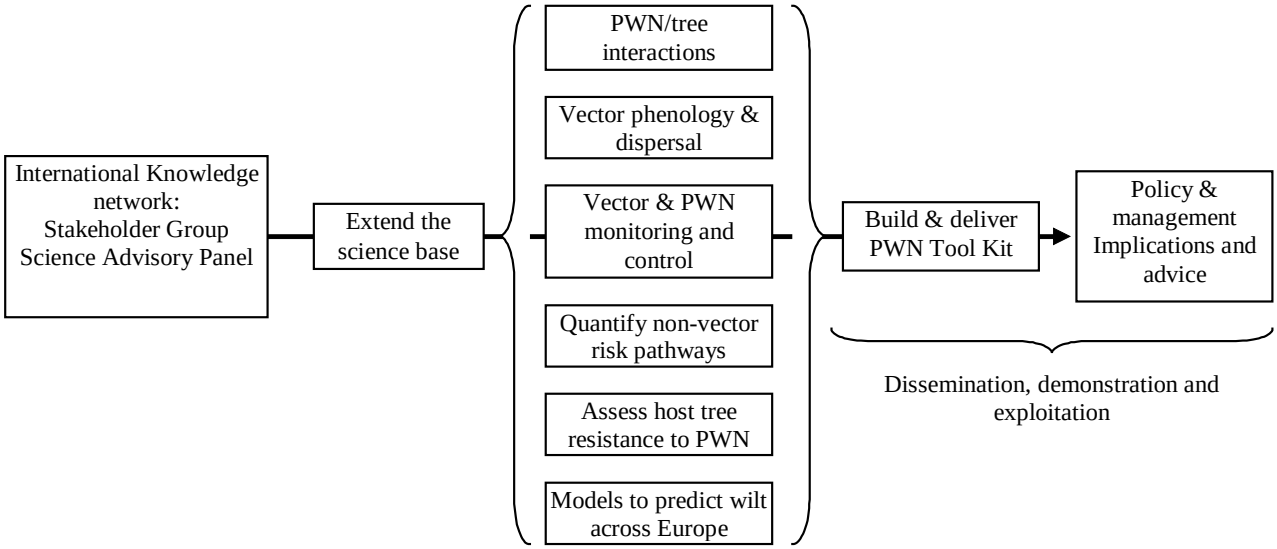
Third Periodic Report

1 March 2014 – 30 November 2014

Contents

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 for the current Period is described in detail below (although WP1 is designated for Management & Coordination and is dealt with at the end of this report).

The first and second periodic reports 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 substantial further progress in the final 9 months of REPHRAME as will be apparent in the rest of this document. We have disseminated these results in both planned Workshops and also an additional seminar in Brussels (requested by the Science Officer) and a Webinar, jointly with another FP7 project, ISEFOR.

WP2 Behaviour and dynamics of PWN in infested trees

Work done from March 01, 2014- November 30, 2014

Beneficiaries reporting: **B3, B4, B5, B6, B7, B10**

Objectives for the period
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 synthesize the above into optimized, statistically reliable survey and early detection regimes for inclusion in the PWN Tool Kit.

Deliverables
D 2.1: Factors governing association of PWN with vector beetles (30-11-2013)
D 2.2: Factors affecting departure of PWN from vector beetles (30-11-2013)
D 2.3: Pathogenicity of PWN in host tree species (30-11-2014)
D 2.4: Methods to detect PWN in trees (30-11-2014)

There is nothing to report for WP2 from **B3 (BFW**). Based on the overall very low prevalence of *Bursaphelenchus* spp. (non-*xylophilus*) in Austria found in 2013 and inconclusive results with the nematode trapping method by B4 (INRA), no tests were performed in Austria.

Task 2.1: Determination of factors governing association of the nematode with the vector (**B6**, leader)

Work carried out by partner B6 (INIAV)
D 2.1: Factors governing association of PWN with vector beetles
Experimental tests took place from 6 June to 22 September 2014 and started with 238 longhorn beetle larvae (177 *Monochamus galloprovincialis* and 61 *Arhopalus syriacus*) extracted from naturally infested pines cut during winter. The larvae were placed in individual glass tubes closed with silicone septa, to allow air sampling. The tubes were kept at the INIAV laboratory in Oeiras at room temperature 21,0 ± 0,7 ^{o}C (Hobo Pro V2 data-logger, Onset Computer Corp., Cape Cod, Massachusetts, USA). Every three/four days, 30 tubes were selected and the inside air composition was determined using a GC 8000 Top (CE Instruments), with a TCD detector and CTR I column (Alltech) (O_2 and CO_2) and GC TRACE Ultra (Thermofisher), with a FID detector and TG-Bond Alumina column (Thermo Scientific) for hydrocarbon detection. These assessments were carried out on 13 occasions and after each analysis, all tubes were opened to allow the renewal of the air and to weigh the insects (Figure 2.1).

Figure 2.1 Larvae and pupae ready for weighing, near the tubes used for air composition determination

Insect development was monitored and the metamorphosis phases were registered (pupae and callow adult formation). The *M. galloprovincialis* pupae needed, on average, 13,1±3,6 days to form the callow adult while the *A. syriacus* pupae needed only 9,5±3,3 days.

Abnormal and very high mortality was registered during this experiment. The mortality occurred mainly in the larval stage but was also very high in the pupal stage, such that, in the end, only 33 adult insects were obtained, 29 *M. galloprovincialis* (Table 2.1, Figure 2.2) and four *A. syriacus* (Table 2.1, Figure 2.3).

Table 2.1 Number of larvae of *Monochamus galloprovincialis* and *Arhopalus* syriacus used in the assay for gas exchange assessment and mortality registered in each developmental stage.

	M. galloprovincialis		***A. syriacus***	
	No.	%	No.	%
Initial larvae	**202**		**61**	
Dead Larvae	150	74,3	55	90,2
Pupae	**52**	**25,7**	**6**	**9,8**
Dead Pupae	23	44,2	2	33,3
Adults	**29**	**14,4**	**4**	**6,6**

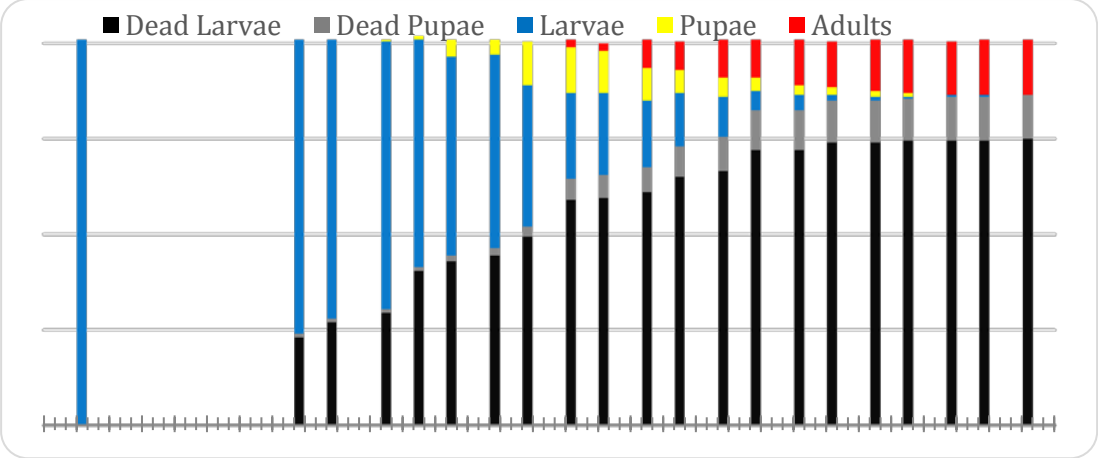


Figure 2.2 Development of the *Monochamus galloprovincialis* larvae during the experiment.

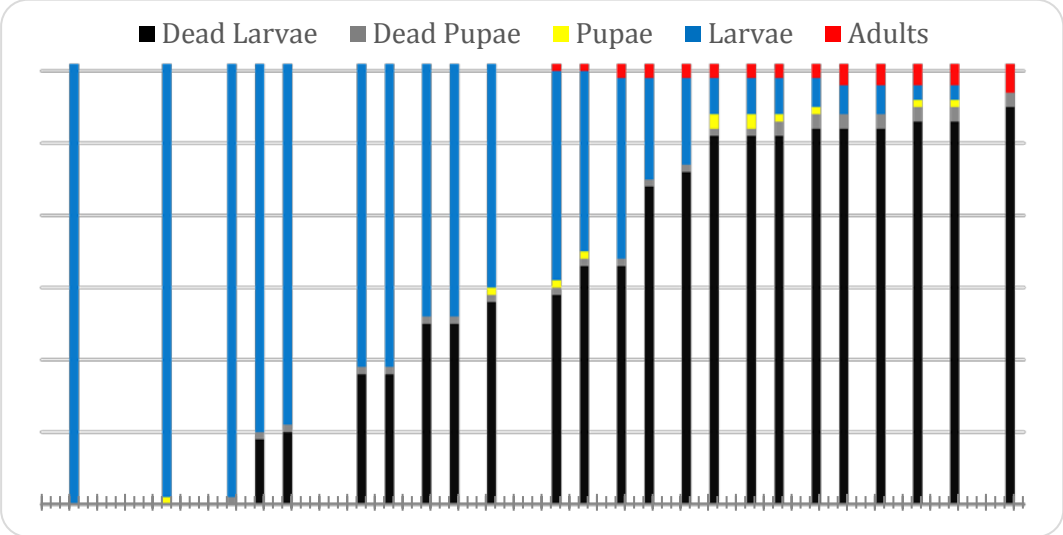


Figure 2.3 Development of the *Ahropalus syriacus* larvae during the experiment.

The *M. galloprovincialis* specimens, in all developmental stages, were heavier than *A. syriacus* (around 3,5 times), however, the number of *A. syriacus* pupae and adults obtained was very small therefore results obtained must be considered carefully (Table 2.2).

Table 2.2 Weight of different developmental stages of *Monochamus galloprovincialis* and *Arhopalus* syriacus (mean ± standard deviation). Larvae and pupae measurements were taken just before changing stage.

	M. galloprovincialis	***A. syriacus***
Larvae	0,3292±0,1470	0,0971±0,0638
Pupae	0,2703±0,1337	0,0795±0,0481
Adults	0,2517±0,1240	0,0725±0,0419

Both species experienced a loss of weight, mainly in the formation of the pupae, by 18,0% of initial weight and less in callow adult formation (6,9% in *Monochamus* and 8,9% in *Arhopalus*).

Since there was a significant correlation between the CO_2 release and the weight of the specimens (r= 0,68) the absolute emission rate readings were transformed into mass-specific CO_2 emission rates (in ppm CO_2 per gram of insect weight and per day). Data were also log-transformed to equalize variances.
The metabolic activity of the insects at all developmental stages was influenced by the air temperature (r= 0,16), so future work should be done in controlled temperatures, and considering that *M. galloprovincialis* pupal formation starts in April and adults begin to emerge on late May, the experiment only covered part of the cycle Figure 2.4).

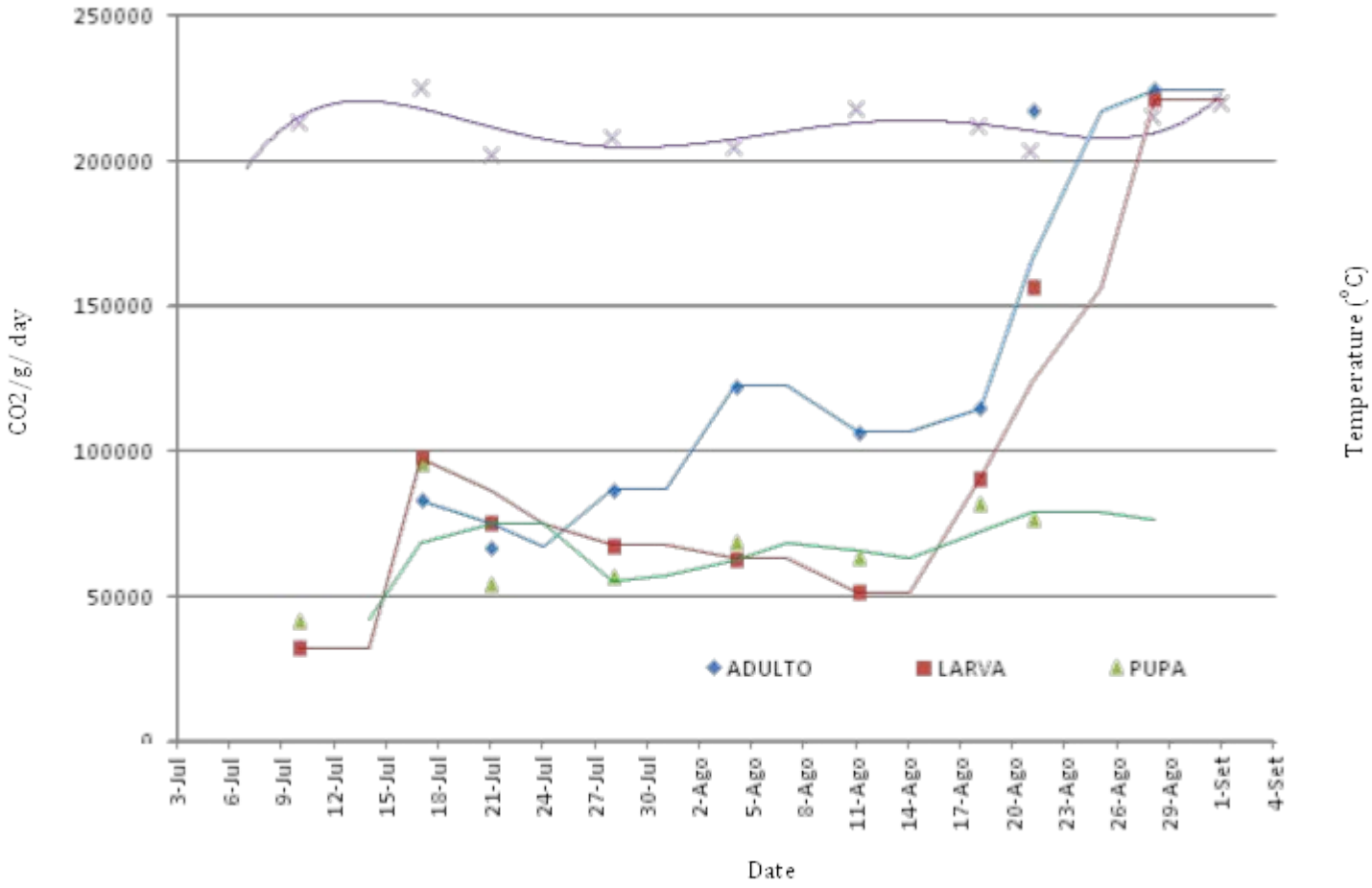


Figure 2.4 – Mass-specific CO_2 release rates of different development stages of *Monochamus galloprovincialis* (ppmCO_2/g/day)*10^{-4} and air temperature (^oC).

The gas monitoring results showed that *M. galloprovincialis* larvae and pupae released significantly less CO_2 than adults, while in *A. syriacus* adults also released more than immature stages but without statistical significance (probably as a result of the variability in the few data obtained for pupae and adults) (Table 2.3).

Table 2.3 – Results obtained of statistic ANOVA applied to each immature stage mass specific CO_2 release rate (ppmCO_2/g/day) log transformed for *Monochamus galloprovincialis* and *Arhopalus syriacus* (mean ± standard deviation).

	A. syriacus	***M. galloprovincialis***
Larvae	87.764,2±58.551,0 (a)	66.638,6±45.583,6 (a)
Pupae	116.493,7±63.323,3 (a)	68.199,4±26.337,6
Adult	161.233,3±80.107,1 (a)	110.689,3±39.952,3
Statistical results	$F_{(2,55)}$= 2,1380; p= 0,1276 NS	$F_{(2,135)}$= 5,0402; p= 0,0077*

The Mass-specific CO_2 release rates increased significantly from larval to adult formation, both in *A. syriacus* and *M. galloprovincialis*, in the last latter with higher emphasis on the formation of the adults. Large standard errors observed in *A. syriacus* are due to the small numbers of pupae and adult insects obtained, as previously stated.

This study must be considered as exploratory due the unexpected mortality that conditioned the number of adult insects obtained. Moreover, the new experiment to consolidate these results should start earlier to overlap the insects' development and PWN transmission cycle in nature (April to August)

Considering that the pinewood nematodes migrate from the wood of the dead host tree to the insect vector only when the callow *M. galloprovincialis* adult is formed, the significant increase of the CO_2 release rate found in this experiment (over 1,6 times from both pupae and larvae) may be an important signal for the nematode, as referred by Miyazaki *et al.* (1978) for *M. alternatus.* In fact statistical analysis indicated that only in the larval stage was there no significant differences between species ($F_{(1,90)}$= 0,7374; p= 0,3928), while for pupae ($F_{(1,72)}$= 6,69; p= 0,0117) and adults ($F_{(1,28)}$= 3,26; p= 0,0818) the differences between species were significant (Figure 2.5), however this result must be confirmed with more data for pupae and adults of *A. syriacus*.

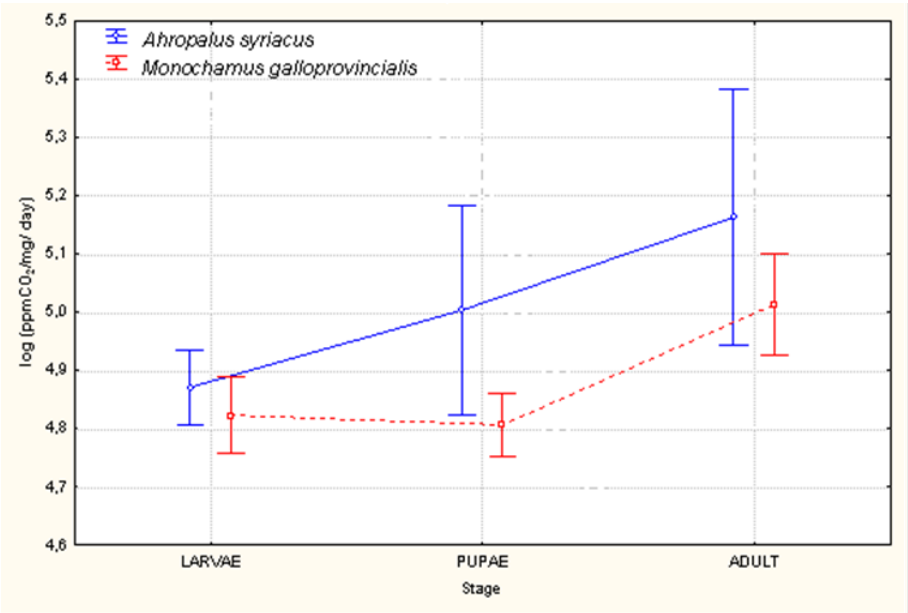


Figure 2.5 Mass-specific CO_2 release rates of different development stages of *Arhopalus syriacus* (log transformed - ppmCO_2/g/day). Vertical Bars denote 0,95 confidence intervals.

References

Miyazaki, M.; Yamaguchi, A.; Oda, K. (1978) - Behaviour of Bursaphelenchus lignicolus in response to carbon dioxide released by respiration of Monochamus alternatus pupa. Journal of the Japanese Forestry Society. 60(7): 249-254

Task 2.2: Determination of factors governing departure of PWN from the vector (B6, leader)

D 2.2: Factors affecting departure of PWN from vector beetles (Luis Bonifácio)

The problems that caused the massive delay in task 2.1 made impossible to even start task 2.2.

Task 2.3 Investigation of movement of PWN inside the host tree (B5, B6, B7)

Following our previous findings in 2013, during 2014 two major contributions were given on the subject of *Bursaphelenchus xylophilus*-associated bacteria.

Bacterial role in pine wilt disease development – review and future perspectives Francisco X. Nascimento, Koichi Hasegawa, Manuel Mota, and Cláudia S.L. Vicente, Environmental Microbiology Reports (2014) doi:10.1111/1758-2229.12202

Mutualistic and beneficial relationships between nematodes and bacteria are frequently present in nature, mostly occurring because of nutritional dependence and pathogen protection, and intrinsically related with the environment, ecological conditions and life stages of the nematode. Thirty-four years have passed since the first hypothesis suggesting a bacterial role in pine wilt disease (PWD), associated with the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. In 1980, researchers reported that bacteria associated with the PWN could produce toxins that lead to PWD development in pine seedlings. It was also suggested that there was a double vector system for PWD, where bacteria were vectored by the PWN and the PWN vectored by an insect from the *Monochamus* genus. Presently, the specific involvement of bacteria in such complex disease is still controversial, even though the increased number of studies focused on the potential role of bacteria has increased considerably. This review is an up-to-date comprehensive perspective and brings new insights on the role of PWN-associated bacteria in PWD. (Figure 2.6)

Catalases induction in high virulence pinewood nematode *Bursaphelenchus xylophilus* under hydrogen peroxide-induced stress Cláudia S. L. Vicente, Yoriko Ikuyo, Ryoji Shinya, Manuel Mota, Koichi Hasegawa
PlosOne (re-submitted in December 2014 after major revisions)

Considered an EPPO A2 quarantine pest, *Bursaphelenchus xylophilus* is the causal agent of the pine wilt disease and the most devastating plant parasitic nematode attacking coniferous trees in the world. In the early stages of invasion, this nematode has to manage host defence mechanisms, such as strong oxidative stress. Only successful virulent (level of pathogenicity) nematodes are able to tolerate the basal plant defences, and further migrate and proliferate in number inside of the host tree. In this work, our main objective was to understand to what extent *B. xylophilus* catalases are involved in their tolerance to oxidative stress, as well as in their virulence, using as oxidant agent the reactive oxygen species hydrogen peroxide (H2O2). After 24-h of exposure, high virulence isolates of *B. xylophilus* could withstand higher H2O2 concentrations in comparison with low virulence B. xylophilus and *B. mucronatus*, corroborating the up-regulation of catalases (Bxy-ctl-1 and Bxy-

ctl-2) observed in the same experimental conditions. Both catalases are expressed throughout the nematode intestine. In addition, transgenic strains of *Caenorhabditis elegans* overexpressing *B. xylophilus* catalases were constructed and evaluated for survival under similar conditions as previous. Our results suggest that catalases of high virulence *B. xylophilus* were crucial for the nematode survival in prolonged exposure to in vitro oxidative stress, highlighting their adaptive response, which could contribute for their success in host conditions. (Figure 2.7, Figure 2.8)

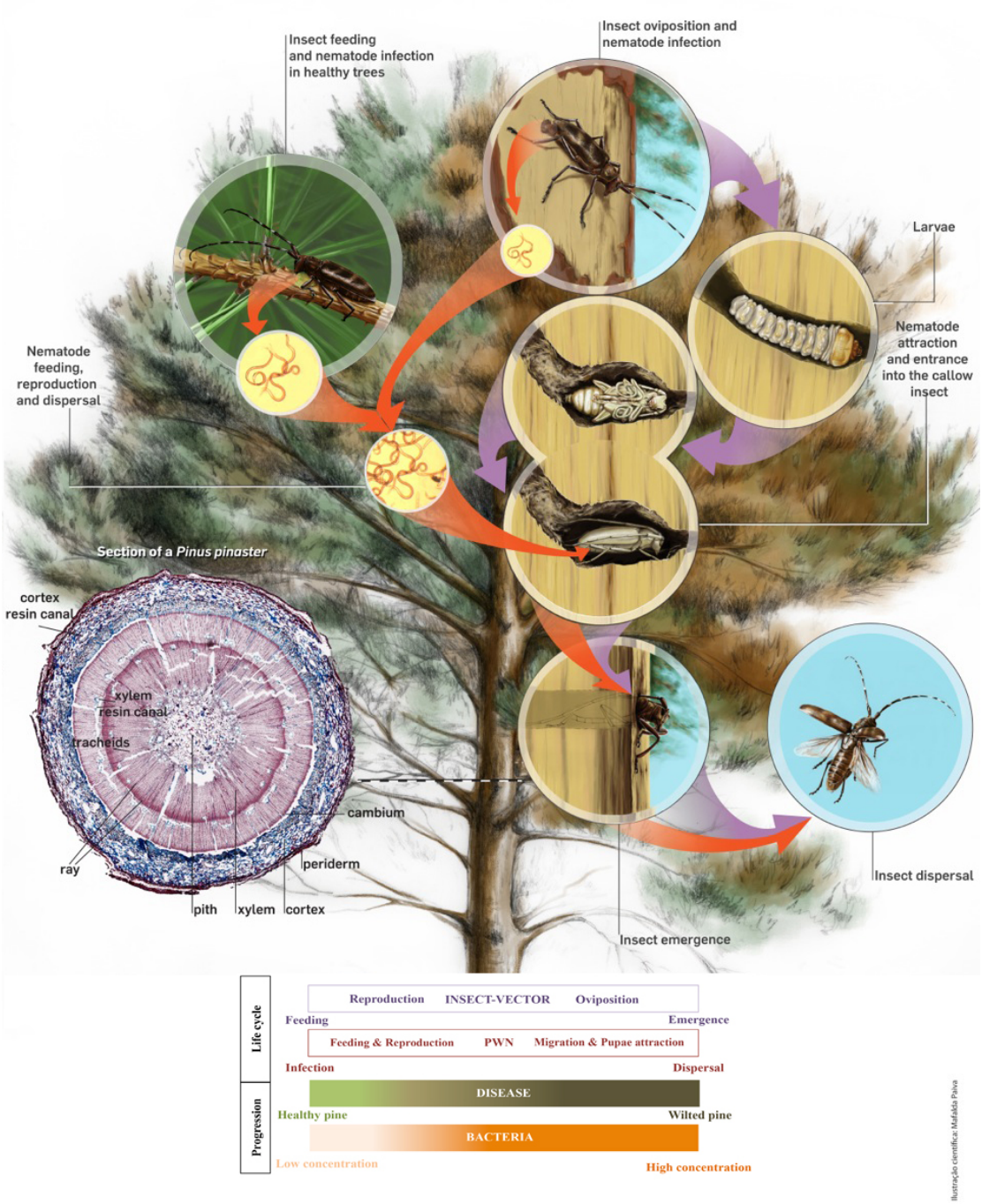


Figure 2.6 Pine wilt disease – interaction *of Bursaphelenchus xylophilus* (red arrow) and the insect-vector *Monochamus* spp. (purple arrow) in healthy and declining *Pinus* sp.

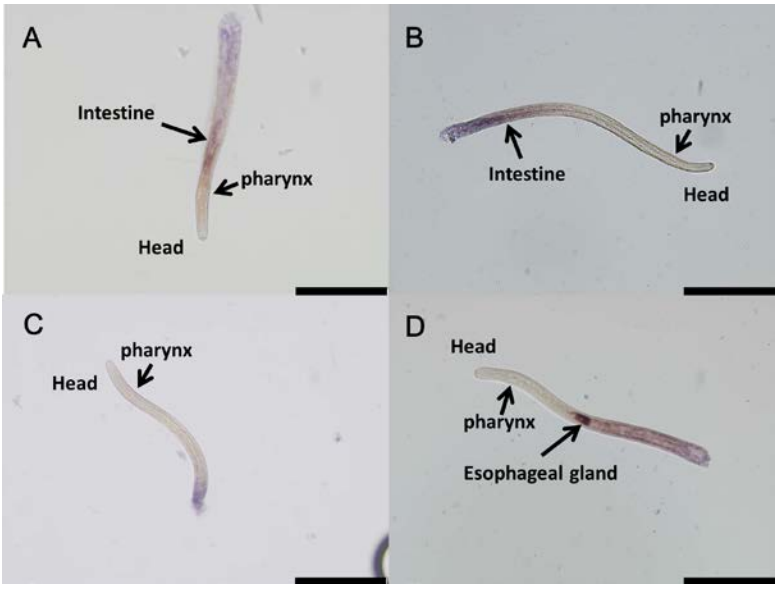


Figure 2.7 mRNA expression patterns of Bxy-ctl-1 (A), Bxy-ctl-2 (B), and Bxy-eng-1 (D) in *B. xylophilus* Ka4. No expression signal was observed with Bxy-ctl-1 sense probe (C). Light microscope images of *B. xylophilus* head region. Scale bars, 100 µm.

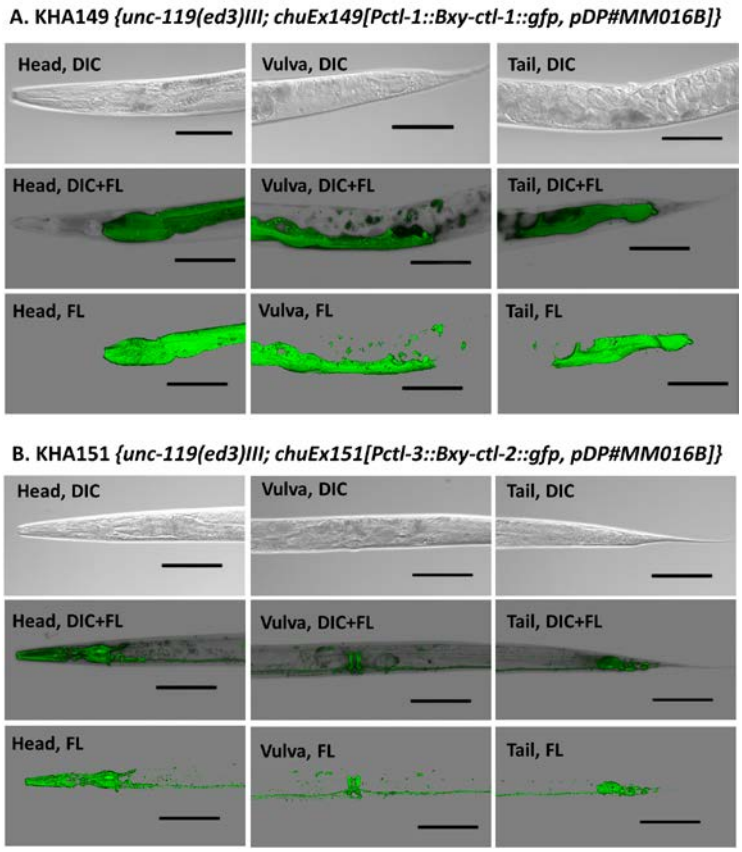


Figure 2.8 Expression patterns of Bxy-ctl-1::gfp (A) and Bxy-ctl-2::gfp (B) in, respectively, transgenic *C. elegans* KHA149 and KHA151. Differential interference contrast (DIC) microscope images and, DIC and fluorescence-merged images (DIC+FL) of C. elegans head, vulva and tail region. Scale bars, 100 µm.

Task 2.3: Investigation of movement of PWN inside the host (B7, leader)

D 2.3: Pathogenicity of PWN in host tree species (30-11-2014)

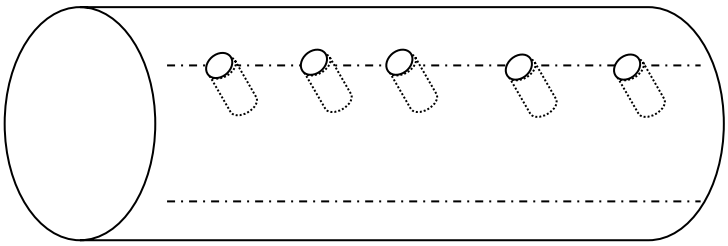
Assessment of sampling techniques in the framework of PWN monitoring

In the framework of monitoring activities either in affected countries or during routine monitoring according to the EU-implementing decision 2012/535/EC trees or wood are sampled to detect *Bursaphelenchus xylophilus.* In most cases drills or chain saws are used to create shavings to be analyzed in the laboratory. The current investigation was carried out to analyze whether different sampling techniques lead to different amounts of isolated nematodes per gram dry matter (g_{dm}).

Material and methods

A 80 year old *Pinus sylvestris* tree was cut into logs 1m long. The cut ends were sealed with hot paraffin (Sigma-Aldrich, Germany, congealing range 45 °C– 50 °C).
In each upright standing log four rows of 5 inoculation holes were drilled (diameter 10 mm, angle 45°, depth approx. 55mm) (Figure 2.9). In each hole a nematode suspension using 4000 *B. mucronatus* per ml were inoculated using a glass pipette. The holes were sealed with a wooden plug.

Figure 2.9 log with inoculation holes.



Nematodes were incubated at 25°C and 80%rF for 10 weeks.

Following incubation each log was divided into four discs between the inoculation holes. Each disc was divided in four identical segments. Each segment was associated with a sampling technique and the techniques were moved one segment clockwise for the next disc. The segment used for sampling by cutting with secateurs in addition was used to cut wood samples to be chipped in a wood mill.

The following sampling techniques were tested:

1. wood drill (20 mm)
2. centre bit (20 mm)
3. chain saw
4. secateurs
5. laboratory wood mill

All drills, chains etc. were new and without signs of wear. In total per log five repetitions for five methods of extraction were carried out.

Results:

Though it is known that nematodes are distributed quite unevenly within wood samples, this could not explain the isolated number of nematodes between the tested variants. The number of isolated *B. mucronatus* per gram dry matter is shown in Figure 2.10. It seems that from variants with less mechanical destruction of the wood more nematodes can be extracted

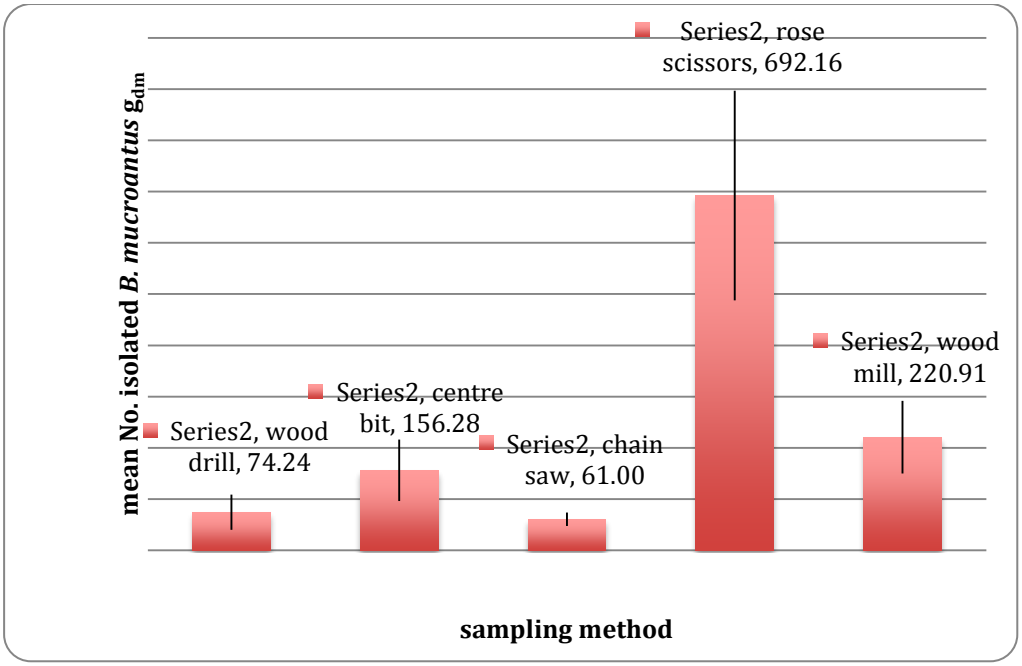


Figure 2.10 Number of isolated nematodes per gram dry matter wood depending of the sampling technique. Error bars show the standard deviation. (n=20)

Movement of PWN inside host-tree - Relationship between pine tolerance and susceptibility and their anatomical structure.
These results are related to Task 6.3 “Breeding for Resistance to PWN Disease”, comparing the same species used in establishing a breeding programme.

Objectives: Comparison of the anatomical differences between susceptible and tolerant species that can be related to a possible nematode tolerance mechanism.

Results:
Histological studies were performed in order to observe possible anatomical differences between *Pinus pinaster* and *Pinus halepensis*.

Anatomical differences that could help understand tolerance mechanisms:

- It should be noted that there are differences in the cortex of *P. pinaster* resin canals that are greater in number than in *P. halepensis* (Figure 2.11);
- Distances between cortex resin canals of *P. pinaster* are smaller than in *P. halepensis* (Figure 2.11);
- *P. pinaster* xylem resin canals density by surface is greater than in *P. halepensis* (Figure 2.11).

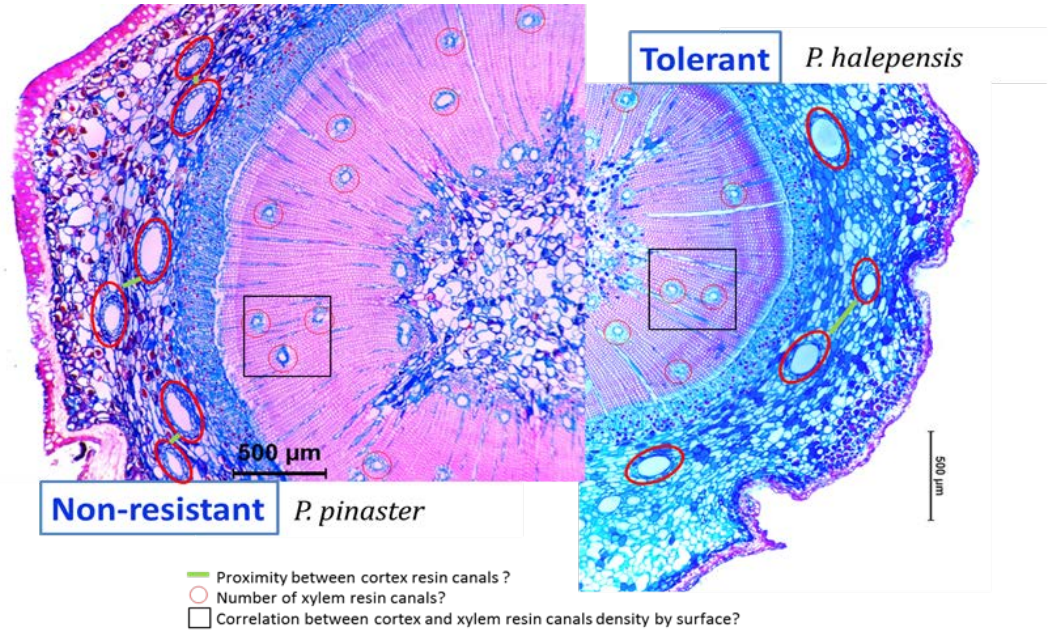


Figure 2.11 Transverse histological sections of non-inoculated *Pinus pinaster* and *Pinus halepensis*.

The two species after inoculation with Bursaphelenchus xylophilus also showed different behaviours. The following differences are worth highlighting:

- 48h after infection there is noticeable tissue damage caused by nematodes on *Pinus pinaster* (Figure 2.12 and Figure 2.13);
  - Damage in cortex parenchyma cells and xylem parenchyma cells of rays are already noticeable
  - Cortex and xylem resin canals degradation
  - Thickening of the xylem cell walls
  - Damage of pit cells
- By contrast in *P. halepensis* there is no visible extensive damage at the cellular level, however it is noted (Figure 2.14):
  - Intact structures
  - Intact pit cells
  - Part of resin canals have been plugged by tylosoid formation

Conclusions

- **Number and size** of cortex and xylem **resin canals** are **different** in the two species
- After 48h there is degradation of cortex and xylem resin canals, parenchyma cells and tracheids (pit cells) caused by nematodes in ***Pinus pinaster***
- Part of resin canals in xylem have been plugged by **tylosoid** formation in **Pinus halepensis**
- **Thickening** of the xylem **cell walls** were observed in both species
-

Further evidence in tolerant species was that among the parenchyma cells there are clear tannin idioblasts, showing that plant defence mechanisms, including production of secondary metabolites, were activated,**.** Furthermore, the lumen of the resin canals in the cortex is delimited by two layers of epithelial cells. These cells are rich in cytoplasm with secretory function where biosynthesis of terpenes takes place.

Biochemical response **together** with **anatomical** analysis could be an answer to the resistance of tree species to nematodes.

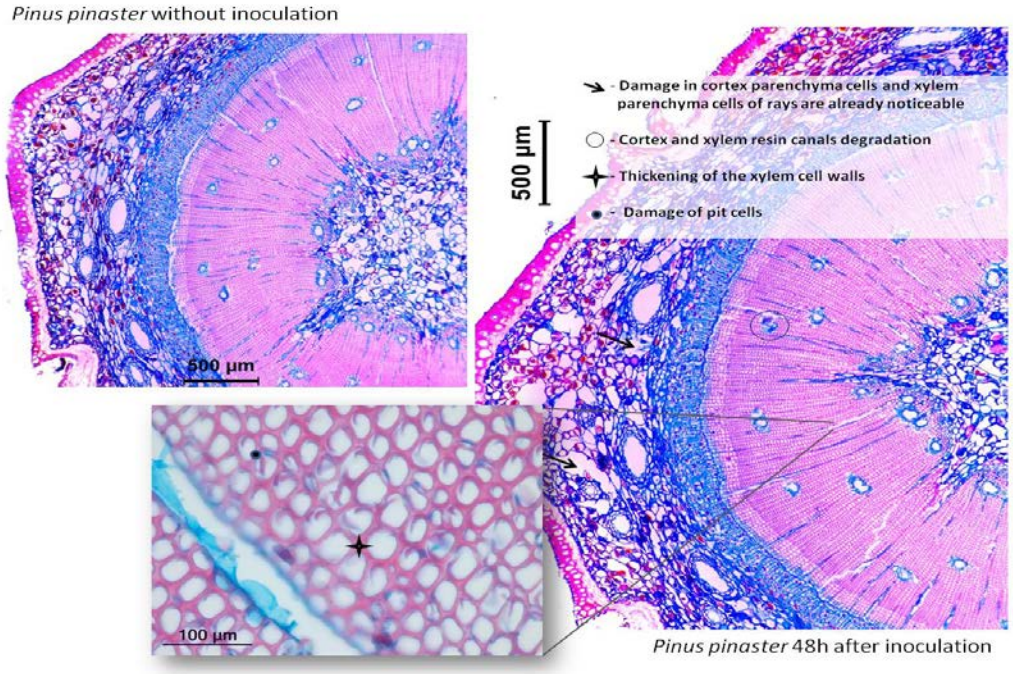


Figure 2.12 *Pinus pinaster* transversal histological sections of not inoculated and 48h after inoculation showing some details of the damage caused by the presence of *Bursaphelenchus xylophilus*

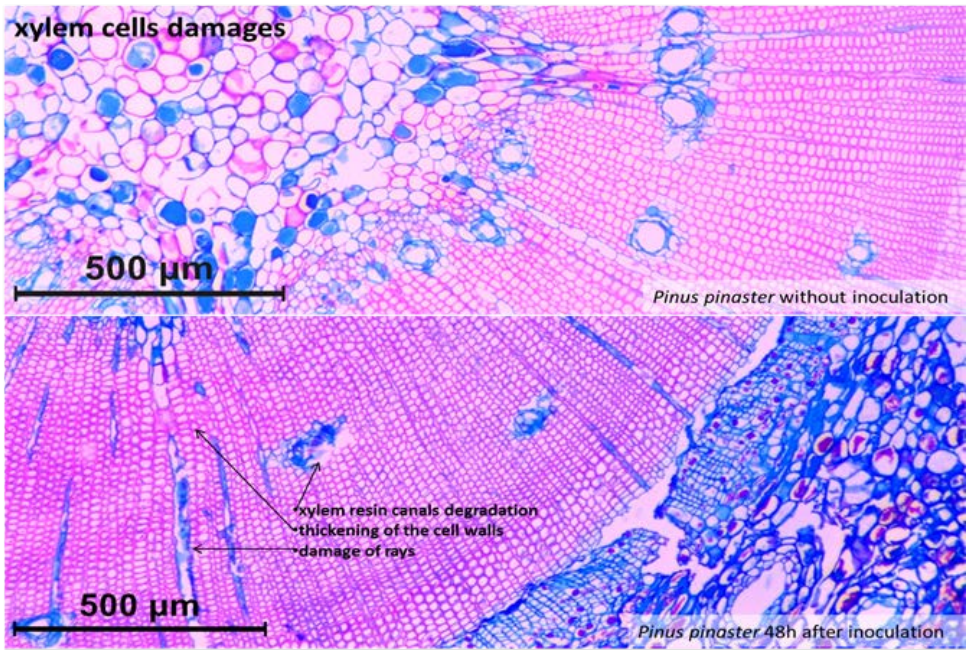


Figure 2.13 *Pinus pinaster* transversal histological sections of not inoculated and 48h after inoculation showing some details of xylem cell damage caused by the presence of *Bursaphelenchus xylophilus*

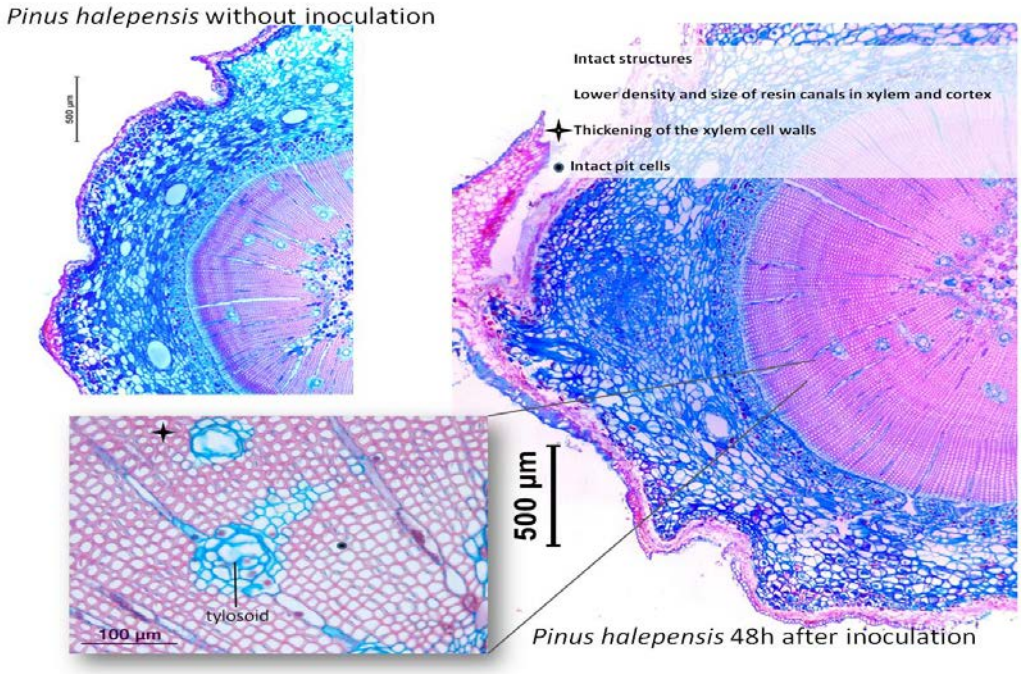


Figure 2.14 *Pinus halepensis* transversal histological sections of not inoculated and 48h after inoculation with *Bursaphelenchus xylophilus*.

Bacterial work: "clarification of the role of bacterial associates in pathogenesis"
Task 1 – Sampling Procedure

In 2013, three PWD-affected locations were chosen for sample collection based upon their PWD history – Mortágua (Penacova), Carvalhal (Comporta), and Prazeres (Calheta, Madeira) (Figure 2.15). Although private owners (Penacova and Comporta) and forestry authorities were contacted prior to the beginning of the project, unexpected tree logging in Penacova and Comporta restricted sampling collection (Table 2.4). Logs and softwood of non-symptomatic and symptomatic *Pinus pinaster* were sampled carefully. Tree trunks were visually inspected for points-of-entry of the insect-vector, and the selected ones were cut and brought to the laboratory.

In 2014, three locations were selected for sampling collection – Mata da Oitava (Góis), Carvalhal (Comporta) and Prazeres (Madeira)

Table 2.4– Sampling collection in 2013 and 2014.

Year	Locale		Samples	N	Description
Spring 2013	Penacova	Mortágua	Healthy	1	Approximately 15-20 year *P. pinaster*. Softwood collected.
			Symptomatic	4	Approximately 15-20 year *P. pinaster*. Detected secondary insects attack.
	Comporta	Carvalhal	Healthy	1	Approximately 15-20 year *P. pinaster*. Core sections sampled.
		Carvalhal	Symptomatic	1	Core sections sampled. Detected *M. galloprovincialis* entry points.
Spring 2014	Mata da Oitava	Góis	Healthy/Symptomatic	7	Approximately 19-35 years *P. pinaster.* Core sections samples (top, middle and base). Collected trunk for *M. galloprovincialis* captured.
	Madeira	Prazeres		7	
	Comporta	Carvalhal		7	
Autumn 2014	Mata da Oitava	Góis	Healthy/Symptomatic	7	Approximately 15-25 years *P. pinaster.* Core sections samples (top and middle).
	Madeira	Prazeres		7	

2013, Spring collection - Coimbra, Penacova, Mortágua

2013, Spring collection - Setubal, Comporta

2014 Spring collection - Prazeres, Calheta, Madeira

2014 Autumn collection – Prazeres, Calheta, Madeira

Figure 2.15 Sampling locations in 2013 and 2014

Task 2 – Isolation Procedure and Identification of Culturable Bacteria from wood

Wood samples were processed into small cubes, and divided according to the following procedures. Samples for non-culturable analysis were stored at -20ºC until use. Samples for culturable analysis were sub-divided for microbiological and nematology analysis. In the case of the insect-vector, trunks were maintained in sealed boxes until adult emergence. Emerged insects were stored at -20ºC until analysis. For the detection of *Bursaphelenchus xylophilus*, 60 grams of wood were maintained for 48h in trays with water. The water was passed through a 38µM mesh and concentrated in low volume for binocular visualization.

Wood cubes were superficially sterilized with 70% EtOH (v/v) for 30 sec, followed by several rinses with sterilized distilled water. These cubes were suspended in sterile 0.8% NaCl (w/v) and incubated overnight at room temperature. Afterwards, 100 µl of wood suspension were inoculated in three different culture media (TSA, trypticase soy agar; NA, nutrient agar; King'B medium). Bacterial colonies with different morphological characteristics were isolated and incubated in the respective medium. Plates were incubated at 28°C for 1 week and the isolates subsequently streaked onto fresh medium to obtain pure cultures. Each bacterial isolate was maintained at 4°C for routine use and also stored long-term in 20% glycerol (v/v) at -80°C.

Bacterial isolates were identified taxonomically using 16S ribosomal RNA gene. Genomic DNA was extracted with Wizard® Genomic DNA Purification kit (Promega). Amplification of 16S rRNA gene was conducted using universal primers (63F and 1387R) (Marchesi et al., 1998). PCR reactions (25 µl) contained 40ng of DNA, 1x buffer *Taq* polymerase (Fermentas), 0.2 mmol/l from each dNTP and 0.5 µM from each primer. The amplification program was 94ºC for 1 min; 30 cycles of 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 1.5 min; and final extension for 7 min at 72ºC. PCR products were purified using Fast Gene Gel/PCR extraction kit (Nippon Genetics Co. Ltd., Japan) following the manufacturer's protocol, and sequenced at Macrogen (Korea) and GATC (Germany).

Different bacterial isolates were phenotypically characterized in terms of: fitness (tolerance to oxidative stress, antibiotic resistance, antifungal activity), metabolic (protease production; phosphate solubilisation, siderophores production, IAA production and biofilm formation) and pathogenicity features (nematicidal activity and gnotic root elongation). All experiments were repeated three times and described as Vicente et al. (2011 and 2013).

Tolerance to oxidative stress

Briefly, bacterial isolates were grown until OD600 of 1.00 (approximately 10^7-10^8 CFU/ml) and inoculated (10 µl) in 96-well microtiter plate containing per well 100 µl LB supplemented with H_2O_2. The concentrations of H_2O_2 were 15, 20, 30 and 40 mM. Plates were incubated for 24h at 30ºC. Tolerance to H_2O_2 was assessed by OD_{600} reading in a multi-spectrophotometer.

Antibiotic resistance

Antibiotic resistance was checked for the following antibiotics and concentrations: ampicillin (Ap) (100 µg/ml), kanamycin (Km) (50-100 µg/ml), rifampicin (Rif) (25-50 µg/ml) and tetracycline (Tet) (5-15 µg/ml). Bacterial isolates were grown overnight, adjusted to OD600 of 1.00, and inoculates in 96-well microtiter plates containing LB medium supplemented with each antibiotic. Plates were incubated for 24h at 30ºC, and bacterial growth was checked by OD_{600} reading in a multi-spectrophotometer.

Protease production
Protease production was assessed according to Zhou et al. (2009). Culture medium was prepared with 0.2% yeast extract, 0.5% gelatin, 1.5% agar supplemented with 0.5% (w/w) casein. Bacterial isolates were grown overnight, adjusted to OD600 of 1.00, and spot-inoculated into the medium. Plates were incubated at 30ºC for 5 days. The production of protease was indicated by the formation of hydrolytic zone.

Biofilm formation
Bacterial isolates were grown overnight, adjusted to OD600 of 0.02, and inoculated in 96-well microtiter polystyrene plate containing LB medium. Plates were incubated for 48 h at 30ºC without shaking. After incubation, wells were washed gently with 150 µl of 0.9% (w/v) NaCl solution, and dried at 30ºC for 30 min. Wells were stained with 0.1% crystal violet solution (150 µl), incubated for 20 min., and washed with 0.9% (w/v) NaCl solution. Next, 180 µl ethanol (96%) was added to each well and incubated more 20 min. Biofilm formation was checked by measuring OD_{590}. OD_{590} values over 1 were considered as good biofilm formers.

Cellulase activity
For each bacterial isolate, CMC (carboxymethylcellulose) plates were spot-inoculated (5 µl) with overnight-culture, and incubated at 28ºC for 48 h. Cellulase activity was observed by the formation of a zone of clearance around the bacterial colony, after flooding the CMC plates with Gram's iodine solution (2.0 g KI and 1.0 g iodine in 300 ml distilled water) for 5 minutes. The production of cellulase was determined by comparing the diameter of the zone of clearance between the isolates. Testing for cellulase activity was repeated three times for each bacterial isolate.

Siderophore production
Siderophores produced by bacteria take up iron from a complex with the dye chrome azurol S (CAS) and a positive reaction is indicated by a colour change of the CAS reagent from blue to orange. About 5 µl of an overnight bacterial culture grown in King's B medium was spotted onto a CAS agar plate and incubated at 30ºC for 2 days.

Phosphate solubilization
Bacterial phosphate solubilization activity was screened in PDYA-CaP medium (potato-dextrose yeast extract agar supplemented with calcium phosphate CaHPO4). For each isolate, three plates of PDYA-CaP were spot-inoculated with 5 µl of overnight culture in TSB medium, and incubated at 28ºC for 14 days. This procedure was repeated three times for each bacterial isolate. Phosphate solubilization activity was determined by measuring the clearance zone (area of solubilization) developed around the colony.

Gnotobiotic Root Elongation
Each bacterial isolate was prepared as follows: overnight cultures were centrifuged at 8000 g for 10 minutes at 4ºC. The cell pellet was washed twice, and diluted with 0.03M MgSO4 to a final OD600 of 0.5. Tomato seeds, previously surface sterilized with 70% (v/v) ethanol (1 minute) and 1% (v/v) sodium hypochlorite (10 minutes) were soaked in the bacterial inoculum for 1 hour. After incubation, 15 pre-treated seeds were sown in soft- water agar and grown at room temperature. Root elongation was measured and further compared with negative control (only water).

Conclusions: The bacterial communities associated with the system (nematode-pine-insect) are highly diverse and seem to have different roles in the ecology of pine wilt. However we have been able to demonstrate the role of some species in providing protection to PWN from the oxidative stress created by the plant. On the

other hand, we have been able to also demonstrate that some bacterial mutants (Pseudomonas putida UW-4) may be helpful in protecting the plant against the invasion of the pinewood nematode.

Results

A total of 276 bacteria were isolated in both 2013-2014 collections, from which only 98 were characterized following the methodology previously described (Table 2.5).

Table 2.5 Number of bacterial isolates obtained in each collection (H-healthy trees; S- symptomatic trees)..

MicroNema			**Isolation**			**Characterization**		
Year	**Collection**	**Site**	**Total N Bac**			**Total N Bac**		
2013	Spring	Comporta	84	H	35	26	H	9
				S	49		S	17
2014	Spring	Comporta	57	H	20	29	H	8
				S	38		S	21
		Góis	23	H	8	23	H	7
				S	15		S	16
		Madeira	31	H	14	20	H	10
				S	17		S	10

Phenotypic characterization of 98 bacterial isolates from all collections sites is summarized in Table 2.6, Table 2.7, Table 2.8 and Table 2.9.

Publications:

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* (doi: 10.1111/1758-2229.12202. [Epub ahead of print]).

Vicente, V., Yoriko Ikuyo; Ryoji Shinya; Manuel Mota; Koichi Hasegawa. 2014. Catalases induction in high virulence pinewood nematode *Bursaphelenchus xylophilus* under hydrogen peroxide-induced stress. *PloS ONE* (submitted, Sep. 2014).

Vicente, C., F. Nascimento, P. Cock, I. Toth, J. Jones and M. Mota. 2014. Genomic sequencing of the pinewood nematode-associated bacteria Serratia proteomaculans, LCN-4 and LCN-16 (in preparation).

Zhao, L. M. Mota, P. Vieira, RA. Butcher, and J. Sun. 2014. Interspecific communication between pinewood nematode, its insect vector, and associated microbes *Trends in Parasitology*, 2014: 1-10.

Table 2.6 Phenotypic characterization of Comporta 2013 bacterial collection.

Collection 2013			Antibiotics						OS	Prot	PO4	CEL	Actividade		Biofim	Gnotic root elong		SIR	IAA
			Rif50	Cm50	kan 50	Te15	Amp50	Strep50					Nema	Fungo		Control	Bacteria		
SYMPT	C1	Pseudomonas sp.	-	-	+	-	+	+	-	-	-	-	-	-	+	1,950	0,213	++	++
SYMPT	C7	Klebsiella sp.	-	-	-	-	-	-	-	-	-	-	-	-	++	1,925	0,213	+	-
SYMPT	C10		-	-	+	+	+	+	+	-	-	-	-	+	++	2,500	0,213	-	-
SYMPT	C14b	Pseudomonas sp.	-	+	+	+	+	+	-	-	-	-	-	-	+	0,788	0,213	++	-
SYMPT	C18	Bacillus sp.	-	-	-	-	-	+	-	-	-	-	-	-	+	1,613	0,213	-	-
SYMPT	C20		-	+	+	+	+	+	-	-	+	-	-	-	-			-	-
SYMPT	C21	Xenophilus sp.	+	-	+	-	+	+	-	-	-	-	-	-	-			-	+
SYMPT	C23	Pseudomonas sp.	-	+	-	-	+	+	-	-	-	+	-	-	+	1,350	0,213	++	+
SYMPT	C25	Enterobacter sp.	-	-	-	-	-	-	-	-	-	-	-	-	-			-	++
SYMPT	C26	Enterobacter sp.	-	+	-	-	+	+	+	-	++	-	-	-	-	4,263	0,484	+	++
SYMPT	C27	Chryseobacterium sp.	-	-	+	+	+	+	-	+	-	+	-	-	-	3,188	0,484	+	-
SYMPT	C31	Pseudomonas sp.	-	+	-	-	+	+	+	-	-	-	-	-	+	1,875	0,213	++	+
SYMPT	C32	Ochrobactrum haematophilum	-	-	+	-	+	+	-	-	-	-	-	-	+	1,613	0,213	-	++
SYMPT	C33	Pseudomonas sp.	-	-	-	-	-	-	-	-	+	-	-	-	-	4,763	0,484	+	++
SYMPT	C36	Rahnella	-	+	-	-	+	-	+	-	-	-	-	-	++	1,175	0,213	-	++
SYMPT	C40	Micrococcus sp.	-	-	+	+	-	+	-	-	-	-	-	-	-	2,171	0,227	-	-
SYMPT	C41	Devosia sp.	-	-	+	-	+	-	+	-	-	-	-	-	-			-	-
NON-SYMPT	C57	Pantoea sp.	-	-	-	-	-	-	-	-	+	-	-	-	-	3,463	0,484	-	++
NON-SYMPT	C60	Pantoea sp.	-	-	-	-	-	-	-	+	-	-	-	-	-			-	+
NON-SYMPT	C65	Rhodanobacter sp.	-	-	+	+	-	+	-	-	+	-	-	-	-	4,175	0,484	-	+++
NON-SYMPT	C67	Paenibacillus sp.	-	+	+	-	+	+	+	-	-	-	-	-	-	3,850	0,484	-	++
NON-SYMPT	C68	Ochrobactrum sp.	-	-	-	-	-	-	-	-	-	+	-	-	-			-	+
NON-SYMPT	C69	Burkholderia sp.	-	-	-	-	+	-	-	-	-	-	-	-	+	1,450	0,213	-	+
NON-SYMPT	C72	Shinella sp.	-	-	+	-	+	+	-	-	-	-	-	+	++	1,938	0,213	-	+
NON-SYMPT	C74	Kaistia sp.	-	+	-	-	+	+	+	-	+	-	-	-	++	1,425	0,213	++	++
NON-SYMPT	C58	Pseudomonas putida	-	-	-	-	+	+	-	-	++	-	-	-	-			-	-
NON-SYMPT	C79	Pantoea sp.	+	-	-	-	+	-	+	-	-	-	-	-	-	1,938	0,213	-	++

Table 2.7 Phenotypic characterization of Comporta 2014 bacterial collection.

Collection 2014			Antibiotics						OS	Prot	PO4	CEL	Actividade		Biofim	Gnotic root elong		SIR	IAA
			Rif50	Cm50	kan 50	Te15	Amp50	Strep50					Nema	Fungo		Mean	St.Error		
NON-SYMPT	14-C1	Pseudomonas sp.	-	+	-	+	+	+	-	-	-	++	-	-	+	3,08	0,48	+++	++
NON-SYMPT	14-C2	Pseudomonas sp.	-	+	-	-	+	+	+	-	-	+	-	-	+	1,64	0,29	+++	++
NON-SYMPT	14-C5		-	+	-	-	+	+	-	-	+	++	-	++	+	0,76	0,29	+++	++
NON-SYMPT	14-C7	Pseudomonas sp.	-	+	-	-	+	+	-	-	+	+	-	-	+	1,91	0,29	++	++
NON-SYMPT	14-C8		-	-	-	-	+	+	-	-	-	-	-	+++	-	0,88	0,29	-	+++
NON-SYMPT	14-C12	Pseudomonas sp.	-	+	-	-	+	+	-	-	++	+	-	-	-	0,91	0,29	+++	++
NON-SYMPT	14-C15	Rahnella sp.	-	-	-	-	+	+	+	-	-	-	-	-	+	1,61	0,29	-	++
NON-SYMPT	14-C57	Pseudomonas sp.	+	+	+	+	+	+	-	-	+++	-	-	+	-	1,90	0,29	-	-
SYMPT	14-C20		-	-	-	-	+	+	-	-	++	-	-	-	-	1,21	0,29	+	++
SYMPT	14-C21		-	+	-	-	+	+	+	-	++	-	-	-	-	1,11	0,29	-	++
SYMPT	14-C23		-	-	-	-	+	-	+	-	++	-	-	-	-	0,93	0,29	+	++
SYMPT	14-C24	Enterobacter sp.	-	-	-	-	+	+	-	+	++	-	-	-	+	1,65	0,29	++	+
SYMPT	14-C25	Enterobacter sp.	+	+	+	+	+	+	-	-	+	-	-	-	-	1,11	0,29	++	+++
SYMPT	14-C26	Bacillus sp.	-	-	-	-	+	-	-	-	-	+++	-	+++	-	1,03	0,29	-	-
SYMPT	14-C27	Klebsiella/Raoutella sp.	+	-	+	+	+	+	-	-	+	-	-	-	-	1,41	0,29	+	++
SYMPT	14-C30		-	-	-	-	+	-	+	-	+++	-	-	-	-	1,08	0,29	-	++
SYMPT	14-C32	Rahnella sp.	-	-	-	-	+	-	+	-	++	-	-	-	+	1,61	0,29	+	++
SYMPT	14-C36	Rahnella sp.	+	+	+	-	+	+	+	-	+	-	-	-	+	4,20	0,48	-	+++
SYMPT	14-C38	Chryseobacterium sp.	-	+	+	+	+	+	+	+	++	++	-	-	-	1,58	0,22	-	+
SYMPT	14-C40		+	+	+	+	+	+	+	-	+	-	-	-	+	1,33	0,29	++	++
SYMPT	14-C42		+	-	+	-	+	+	-	-	++	-	-	-	-	1,16	0,29	+	++
SYMPT	14-C44		+	-	-	-	+	+	-	-	+	-	-	-	+	1,60	0,33	+	+
SYMPT	14-C45	Chryseobacterium sp.	-	+	+	-	+	+	-	+	-	+++	-	-	-	2,37	0,21	+	+
SYMPT	14-C47		+	+	+	+	+	+	-	-	+	-	-	-	-	0,65	0,29	++	+
SYMPT	14-C49		-	+	+	-	+	+	+	+	-	+++	-	-	++	2,48	0,21	+	-
SYMPT	14-C50		+	+	+	+	+	+	-	-	++	+	-	+++	-	4,55	0,48	-	-
SYMPT	14-C51	Micrococcus sp.	+	+	+	+	+	+	+	-	-	++	-	+++	-	0,57	0,31	-	-
SYMPT	14-C53	Pseudomonas sp.	-	+	-	+	+	+	+	-	+++	+	-	+	+	1,94	0,29	+++	++
SYMPT	14-C54	Pseudomonas sp.	-	-	-	-	+	+	-	-	+++	-	-	-	-	0,89	0,29	-	+

Table 2.8 Phenotypic characterization of Góis 2014 bacterial collection.

Collection 2014			Antibiotics						OS	Prot	PO4	CEL	Actividade		Biofim	Gnotic root elong		SIR	IAA
			Rif50	Cm50	kan 50	Te15	Amp50	Strep50					Nema	Fungo		Rootsize	SD		S/T
NON-SYMPT	14-G1	Stenotrophomonas sp.	-	-	-	-	+	-	-	-	+	-	-	-	-	3,09	0,48	-	+
NON-SYMPT	14-G2	Ochrobactrum haematophilum	-	-	-	-	+	-	-	-	-	-	-	-	+	4,91	0,48	+	+
NON-SYMPT	14-G3	Stenotrophomonas sp.	-	-	-	-	+	-	-	+++	-	-	-	-	-	5,01	0,48	+	+
NON-SYMPT	14-G4	Achromobacter sp.	-	-	-	-	+	-	-	+++	-	++	-	-	++	5,64	0,48	+	+
NON-SYMPT	14-G5	Achromobacter sp.	-	-	-	-	+	-	+	-	+	-	-	+	+	5,00	0,48	-	-
NON-SYMPT	14-G6	Pseudomonas sp.	-	-	-	-	+	-	-	-	-	-	-	-	+	6,94	0,48	-	-
NON-SYMPT	14-G7	Pseudomonas sp.	-	-	-	-	+	-	-	-	-	++	-	++	+	4,68	0,48	++	+
SYMPT	14-G8	Stenotrophomonas sp.	-	-	-	-	+	-	-	+++	-	-	-	-	+	4,58	0,48	+	+
SYMPT	14-G9		-	-	-	-	+	-	-	++	+	++	-	-	+	3,88	0,48	-	-
SYMPT	14-G10	Rahnella sp.	-	-	-	-	+	-	-	-	-	-	-	-	+	4,63	0,48	-	-
SYMPT	14-G11		-	-	-	-	+	-	-	-	+	-	-	-	+++	3,85	0,48	-	-
SYMPT	14-G12	Pseudomonas sp.	-	-	-	-	+	-	+	-	-	-	-	-	+	4,21	0,48	+	+
SYMPT	14-G13	Stenotrophomonas sp.	-	-	-	-	+	-	-	+++	-	-	-	-	-	3,48	0,48	+	+
SYMPT	14-G14	Pseudomonas sp.	-	-	-	-	+	-	+	-	-	-	-	-	-	3,50	0,48	+	+
SYMPT	14-G15	Rahnella sp.	-	-	-	-	-	-	+	-	-	+	-	-	+	5,16	0,48	-	++
SYMPT	14-G16	Stenotrophomonas sp.	+	-	-	+	+	-	-	+++	-	-	-	-	+	3,64	0,48	-	+
SYMPT	14-G17	Pantoea sp.	-	-	-	-	-	-	-	-	++	+	-	-	+	4,83	0,48	-	+
SYMPT	14-G18	Enterobacter sp.	-	-	-	-	-	-	-	-	++	+	-	-	+	4,03	0,48	-	+
SYMPT	14-G19		-	-	-	-	-	-	-	-	-	-	-	-	++	2,60	0,48	-	+
SYMPT	14-G20	Rahnella sp.	-	-	-	-	+	-	+	-	+	-	-	-	+	3,80	0,48	-	+
SYMPT	14-G21	Rahnella sp.	-	-	-	-	-	-	+	-	-	-	-	-	++	4,01	0,48	-	+
SYMPT	14-G22	Pseudomonas sp.	-	+	-	-	+	-	+	-	-	+	-	-	++	3,25	0,48	+++	+
SYMPT	14-G23	Ewingella sp.	-	-	-	-	+	-	+	-	-	-	-	+	++	3,11	0,48	-	+

Table 2.9 Phenotypic characterization of Madeira 2014 bacterial collection.

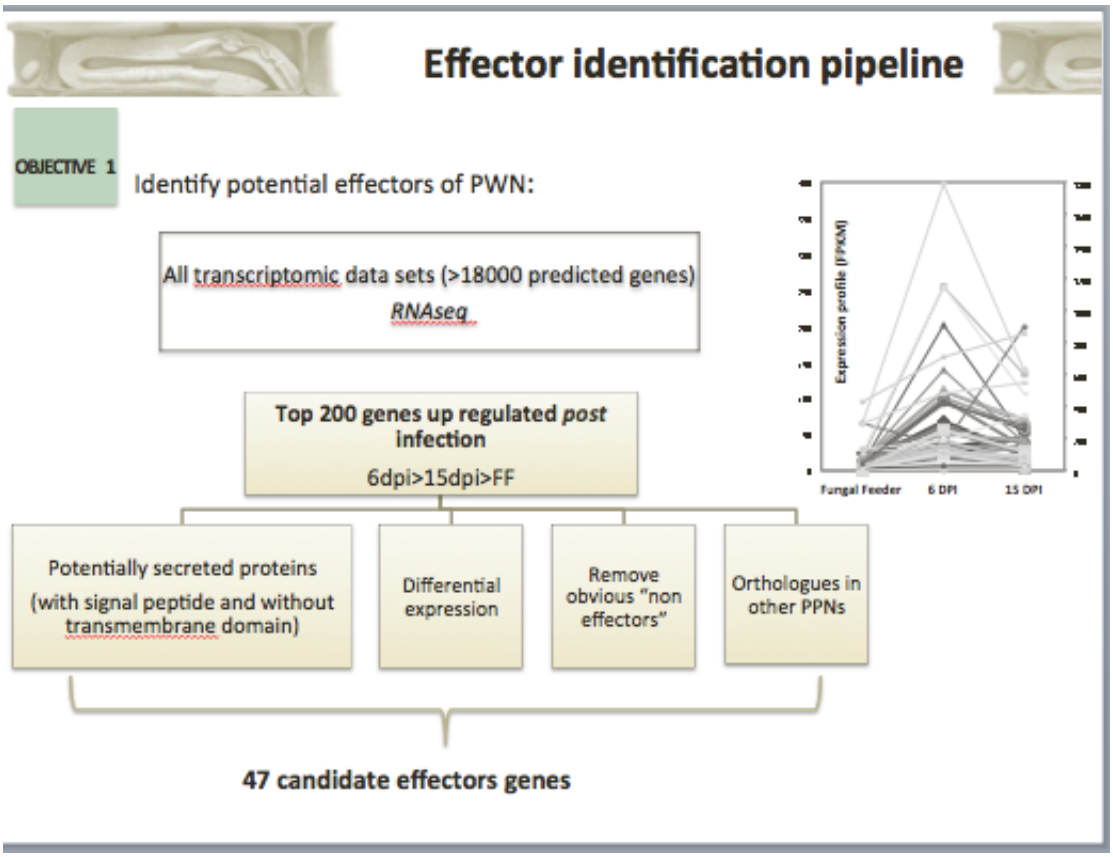
Collection 2014			Antibiotics						OS	Prot	PO4	CEL	Actividade		Biofim	Gnotic root elong		SIR	IAA
			Rif50	Cm50	kan 50	Te15	Amp50	Strep50					Nema	Fungo		Mean	Stdev		
NON-SYMPT	14-M2	Staphylococcus sp.	+	+	+	+	+	+	-	-	-	-	-	+	++	3,188	0,354	-	+
NON-SYMPT	14-M3	Bacillus sp.	-	-	-	-	+	+	-	+++	-	++	-	+++	+++	2,700	0,354	+	-
NON-SYMPT	14-M4		-	-	-	-	-	-	-	-	+	-	-	-	-	2,713	0,354	-	-
NON-SYMPT	14-M5	Leclercia sp.	-	-	-	-	+	-	-	-	++	+	-	-	+	3,287	0,480	+	+++
NON-SYMPT	14-M7	Staphylococcus sp.	-	-	-	-	+	-	+	-	-	-	-	-	-	2,813	0,354	-	+
NON-SYMPT	14-M8	Pantoea sp.	-	-	-	-	-	-	-	-	-	-	-	-	+	3,025	0,354	+	+
NON-SYMPT	14-M9	Enterobacter sp.	-	-	-	-	-	-	+	-	+	-	-	+	+	2,025	0,354	+	+
NON-SYMPT	14-M11	Staphylococcus sp.	-	-	-	-	-	-	-	-	+	-	-	-	+	2,475	0,354	-	+
NON-SYMPT	14-M13		-	-	-	-	-	-	-	-	+	-	-	-	+	1,900	0,354	-	+
NON-SYMPT	14-M14	Brevibacterium sp.	-	-	-	-	-	-	-	-	-	-	-	+	+	2,588	0,354	-	+
SYMPT	14-M15		-	-	-	-	-	-	-	-	-	+	-	+	+	1,250	0,354	-	+
SYMPT	14-M17	Rahnella sp.	-	-	-	-	-	-	+	-	-	-	-	-	++	2,975	0,354	-	+
SYMPT	14-M18	Serratia sp.	-	-	-	-	+	+	+	++	-	+	-	+	+++	1,000	0,354	+	+
SYMPT	14-M21	Staphylococcus sp.	-	-	-	-	+	+	-	-	+	-	-	-	-	2,363	0,354	-	+
SYMPT	14-M22	Staphylococcus sp.	-	-	-	-	-	-	-	-	+	-	-	+	-	2,038	0,354	-	-
SYMPT	14-M24		-	+	+	+	+	+	-	-	+	-	-	+	++	1,163	0,354	+	+
SYMPT	14-M25	Pseudomonas sp,	-	+	-	+	+	+	-	-	+	-	-	-	++	1,313	0,354	+	+
SYMPT	14-M26	Staphylococcus sp.	-	-	+	+	+	+	-	-	+	-	-	-	-	2,063	0,354	-	-
SYMPT	14-M27		-	-	-	-	+	-	-	-	+	-	-	-	-	2,000	0,354	-	-
SYMPT	14-M29		-	-	-	-	+	-	-	-	+	-	-	-	++	2,113	0,354	-	-

Task 2.4: <u>Development of early detection methods for PWN in trees</u>

2.4.2. <u>Investigation of DNA detection</u>

Task 2.4.2. *"…Novel methods for detecting gene products, resulting from the pathogenic action of the nematode will also be investigated byB7…"; "understand(…)factors governing expression or latency of wilt expression…"*

Research on the functional characterization of putative **<u>effectors</u>** in pinewood nematode, *Bursaphelenchus xylophilus*, has been ongoing during this period. Main goal: to exploit recent advances in genomics of plant parasitic nematodes to identify effector proteins of *B. xylophilus* and to determine their function in the host-parasite interaction.



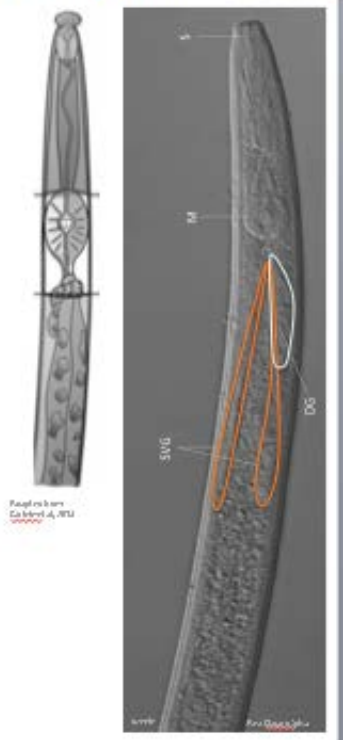
- **RNAseq** *(HI-Seq Illumina<u>)</u>*
- Three Conditions: <u>Fungal Feeder</u> (**FF**) vs. <u>*Post* infection</u> (**6dpi** and **15dpi**)
- Three biological replicates for each condition
- Mixed life stages; Portuguese isolate; Maritime pine
- Reference genome (BUX v1.2), (Japanese isolate)

- Few differentially expressed genes – only 29 genes (0.16%)
- High variability in the clusters
- Influence of environmental conditions?

Candidate genes

OBJECTIVE 1 List of 47 candidate genes:

Predicted function	
PROTEASES	Peptidase A1, M13, C1A, C46 Cathepsin Cysteine proteases Serine-type protease
FATTY ACID TRANSPORT	Fatty acid retinoid binding proteins
DETOXIFICATION OF XENOBIOTICS COMPOUNDS	FMO (flavin monooxygenase) UDP-glucuronosyl transferase Multicopper putative acid oxidase Glutathione S-transferase Cytochrome P450 Acid phosphatase
UNKNOWN PROTEINS DOMAIN (PIONEERS)	Unknown protein with toxin domains (Metridin-like Sht toxin) Putative allergen V5/TPx1 Several novel genes
LYSOZYME	Lysozyme 7,8
CAZymes	GH29 (a-L-fuco domain) GH30



- A **RNAseq dataset** from preinvasive nematodes and two stages of parasitic nematodes has been generated and analysed.
- Used the RNAseq dataset as a neutral approach to identify a panel of novel candidate effectors on the basis of the presence of a signal peptide and significant upregulation in parasitic stages.
- *In situ* hybridization has been used to identify genes from this candidate list that are expressed in the gland cells
- This study identified **10 new effectors secreted in the gland cells, potentially involved in parasitism.**
- The most interesting results include **novel effectors** and sequences.

In summary:

1. A fully replicated RNAseq dataset from preinvasive nematodes and two stages of parasitic nematodes has been generated and analysed.
2. The RNAseq dataset has been used to identify a panel of candidate effectors on the basis of the presence of a signal peptide and significant upregulation in parasitic stages.
3. *In situ* hybridization has been used to identify genes from this candidate list that are expressed in the gland cells and are therefore validated effectors. This is the first study that has identified new effectors from this nematode. Novel effectors and sequences similar to detoxification enzymes are included in this dataset.

Publication:

Espada, M., Ana Cláudia Silva, Sebastian Eves-van den Akker, Peter J.A. Cock, Manuel Mota & John T. Jones. 2014. Identification and characterization of novel effectors from the pinewood nematode *Bursaphelenchus xylophilus* (in preparation).

2.4.3. **Heat field deformation** (**B6**, leader)

It was not possible to carry out any work on this topic due to prioritization for other tasks within the project.

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

As indicated in this and earlier reports, the great majority of tasks were achieved within WP2 but some changes in emphasis and prioritization were made. Some work was carried out on task 2.2 but this was not completed as a result of re-prioritisation of staff time to more productive outcomes. A relatively minor component concerning heat field deformation (2.4.3) was not carried out after consideration of timing of work and in assessing priorities for use of resources in the final phase of the project. Since this was a relatively minor component of the planned work, it did not have a significant impact on overall deliverables for WP2.

Statement on the use of resources

Resources were diverted from task 2.4.3 to other tasks in WP2.4, notably the excellent progress in determining .the fully replicated RNAseq dataset which is a new finding.

WP 3 Assessing phenology and flight capacity of PWN vectors

Work carried out by B3, B4, B6 and B9

Objectives for the period
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 land molecular techniques

Deliverables for the period
D 3.1: Vector flight capacity related to physiology (30-11-2013)
D 3.2: Vector dispersal related to forest condition (30-11-2013)
D 3.3: Vector dispersal related to population genetics (30-11-2014)
D 3.4: Climate influences on vector dispersal (30-11-2013)

D 3.1: Vector flight capacity related to physiology

Work carried out by partner B9 (UVA)
In 2014, the experiments initiated in 2012 and in 2013 to study adult development from emergence through shoot feeding were continued.

Fat content (Experiment1)

Material & Methods
Fat content of 52 *M. galloprovincialis* adults after 0, 4, 8, 14, and 40 days of feeding from emergence was measured in 2014. Total studied insects in 2012-14 are shown in Table 3.1.

Table 3.1 Insects analyzed for fat content

Sex/Days feeding	0	4	8	14	18	40		Total
Females	24	18	16	38	14	11	121	
Males	17	13	18	28	13	12	101	
Total	**41**	**31**	**34**	**66**	**27**	**23**	**222**	

Flight muscle content (Experiment 2)

Material & Methods

Flight muscle content of 52 *M. galloprovincialis* adults after 0, 4, 8, 14, and 40 days of feeding from emergence was measured in 2014. Total studied insects in 2012-14 are shown in Table 3.2.

Table 3.2 Insects analyzed for flight muscle content

Sex/Days feeding	0	4	8	14	18	40	Total
Females	12	16	12	28	14	5	87
Males	15	13	10	22	13	5	78
Total	**27**	**29**	**22**	**50**	**27**	**10**	**165**

Flight muscle content of unfed fight exercised adults (Experiment 3)

Material & Methods

Six *M. galloprovincialis* (3 ♀♀ and 3 ♂♂) unfed adults, were exercised for various flying distances in a wind mill during 7 days. Flight muscle content was determined afterwards.

Results

Analysis of the physiological status of the immature beetles from emergence to trough maturation feeding revealed that:

Adults emerged with an average lipid content of 12.3% of their Dry Weight. This value remained fairly constant during the initial 20 days of feeding (Figure 3.1). Females emerged with 20% DW muscle content in their thorax (i. e. wing muscle), whereas males had 26.6% DW. These values increased to 36-39% DW after 18 days of feeding under lab-conditions (Figure 3.2).

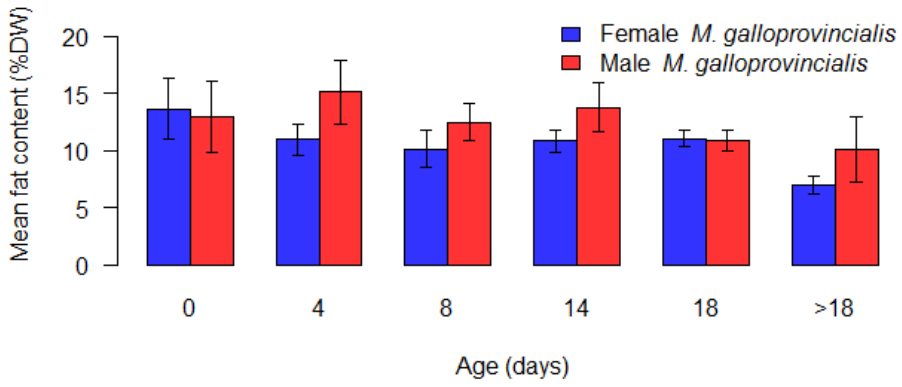


Figure 3.1 Mean Fat content (% Dry weight) of immature *M. galloprovincialis* adults

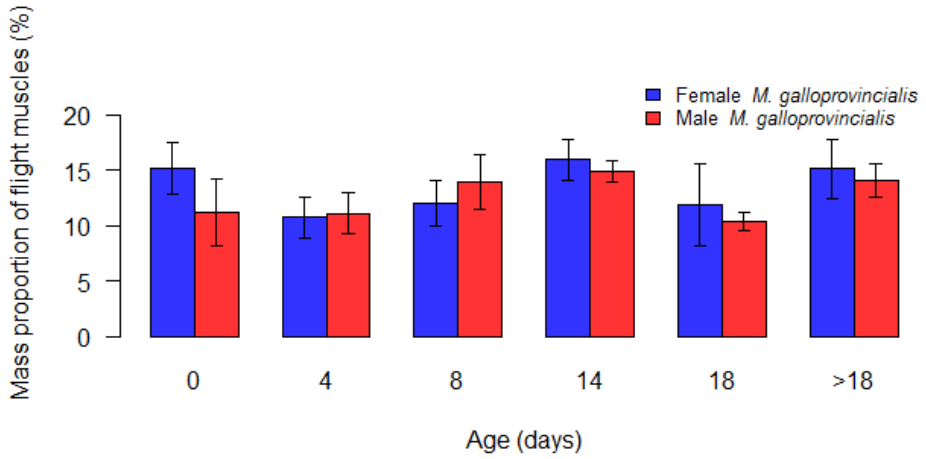


Figure 3.2 Mean Flight Muscle content (% Dry weight) of immature *M. galloprovincialis* adults

Thus, recently emerged, unfed, *M. galloprovincialis* adults seem to be well suited for sustained dispersal flights. However, immature, unfed, beetles did not readily initiate flight at laboratory flight-mills. Those that did showed varying muscular fractions (5-30% DW), and with no apparent link to the distances flown.

Longest survival of emerged beetles that were kept unfed was 14 days. Average survival of beetles kept unfed was of 12 days (Figure 3.3). Fed insects increased their weight during the first 16 days to some 40% in females and 20% in males. Females had more weight than males. Unfed immature adults lost to 60% of their weight to death (Figure 3.4).

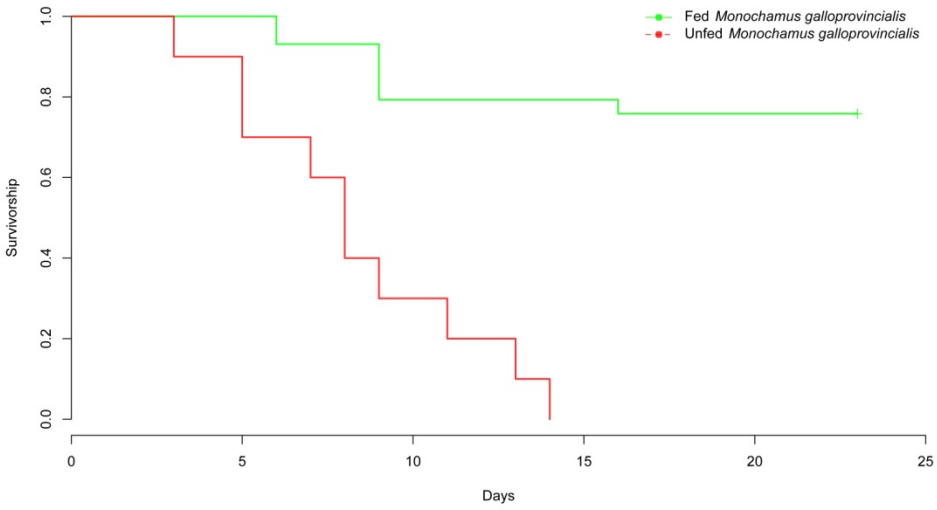


Figure 3.3 Survival of fed and unfed immature *M. galloprovincialis* adults

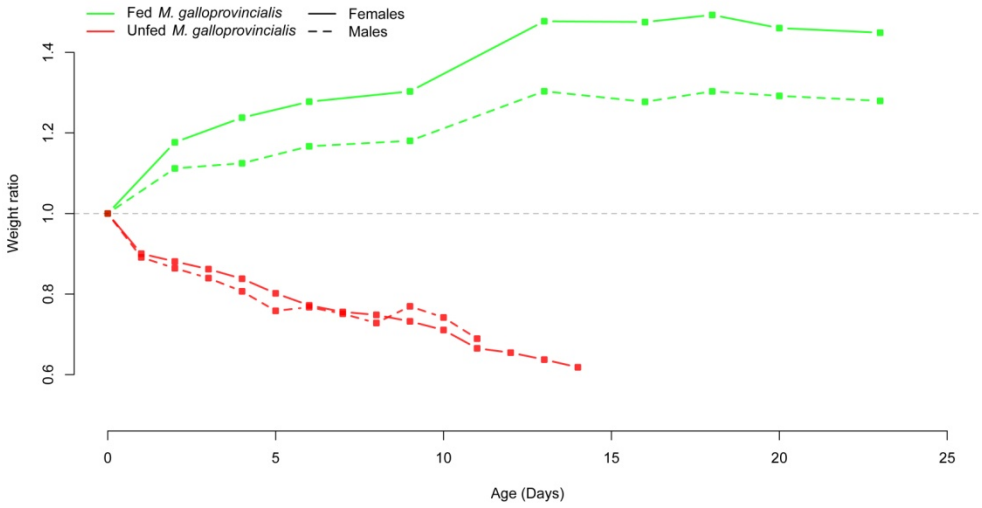


Figure 3.4 Weight gains and losses of fed and unfed immature *M. galloprovincialis* adults

Work carried out by partner B3 (BFW)
Flight mill experiments with *M. sartor*

Material & Methods

Flight capacity and behaviour of *M. sartor* was studied using flight mills. A total of 23 laboratory reared *M. sartor* was tested throughout the adult lifespan. Additionally, 13 field collected beetles were used.

Results

Ten of the laboratory reared and 12 of the field collected *M. sartor* exhibited extended flights longer than 5 min at least once. Overall, 85 and 41 of such individual flight events of female and male beetles were recorded. No differences occurred in individual flights between laboratory and field beetles; covered distances ranged from 273 m to 1455 m (means are given in Table 3.4). The maximum lifetime distance of 8.5 km was travelled by one female in a total of 12 flight sessions within 38 days.

Table 3.3 Distance, time and speed of flight (mean ± SE) of beetles that exhibited extended flight > 5 min on the flight mill.

		Distance (m)	Time (min)	Speed (m/s)
M. sartor	Males	810.4 ± 97.3	16.2 ± 1.3	0.88 ± 0.12
	Females	688.8 ± 81.7	15.9 ± 1.4	0.79 ± 0.07

Main findings

Among the eight tested artificial diets for *M. galloprovincialis*, 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.

The 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.

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.

Distances of individual flights of *M. sartor* were shorter than the reported for *M. sutor* and *M. galloprovincialis*, which flew more than 1000 m (see previous periodic report). Also the flight speed of the large *M. sartor* was slower. Nevertheless, they are also able to travel more than 500 m in individual flights.

Work carried out by partner B3 (BFW)
Flight mill experiments with *M. sartor*

Material & Methods
Flight capacity and behavior of *M. sartor* was studied using flight mills as described in the previous periodic report for *M. sutor*. A total of 23 laboratory reared *M. sartor* was tested throughout the adult lifespan. Additionally, 13 field collected beetles were used.

Results
Ten of the laboratory reared and 12 of the field collected *M. sartor* exhibited extended flights longer than 5 min at least once. Overall, 85 and 41 of such individual flight events of female and male beetles were recorded. No differences occurred in individual flights between laboratory and field beetles; covered distances ranged from 273 m to 1455 m (means are given in Table 3.4). The maximum lifetime distance of 8.5 km was travelled by one female in a total of 12 flight sessions within 38 days.

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Conclusions

Distances of individual flights of *M. sartor* were shorter than the reported for *M. sutor* and *M. galloprovincialis*, which flew more than 1000 m (see previous periodic report). Also the flight speed of the large *M. sartor* was slower. Nevertheless, they are also able to travel more than 500 m in individual flights.

Task 3.2 Life time audit of vector flight behaviour and physiology

Activities carried by B4 (INRA Bordeaux):
Assessing flight activity during adult life span

In 2012 we conducted a study to assess the flight capacity of adult *Monochamus galloprovincialis* beetles (35 males, 26 females from 30 days since emergence until death). This study has been published:

David, G., Giffard, B., Piou, D., Jactel, H. 2014. Dispersal capacity of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae), European vector of the pine wood nematode, on flight mills. *Journal of Applied Entomology*, 138, 566–576.
Using computer-linked flight mills, we evaluated for each beetle, with sessions of 2h once a week, the distance flown, the flight probability and the flight speed throughout adulthood, and investigated the effects of age, sex and body weight on these flight performances which are proxies for dispersal capacity.

The proportion of *M. galloprovincialis* adults classified as fliers on the flight mill was 77% (47 flyers out of 61 tested insects), with no significant difference between sexes (Table 3.5). The total distance flown during the entire adult lifespan (99.5 ± 4 days since emergence) was 15.6 km, on average, for males, and 16.3 km for females (no significant difference between sexes), with a maximum flight distance of 62.7 km recorded for a male. Half of the tested population covered total flight distances exceeding 11.4 km (Figure 3.5). The average speed was similar in males and females, at about 1.4 m/s or 5 km/h.

Table 3.5 General flight parameters

	Total cumulated flights for flyers		**Cumulated flights / recording session**		**Individual flights**	
	Females n = 20	**Males n = 27**	**Females n = 169**	**Males n = 197**	**Females n = 325**	**Males n = 316**
Total distance (km) mean (± SE)	16.26 ± 2.70	15.59 ± 2.90	1.93 ± 0.12	2.14 ± 0.14	1.00 ± 0.05	1.33 ± 0.08
median	12.37	62.72	1.53	8.54	0.72	0.81
maximum	44.85		6.80		4.33	8.54
Flight speed (m/s) mean (± SE)	1.43 ± 0.06	1.35 ± 0.05	1.36 ± 0.03	1.36 ± 0.03	1.33 ± 0.02	1.37 ± 0.02

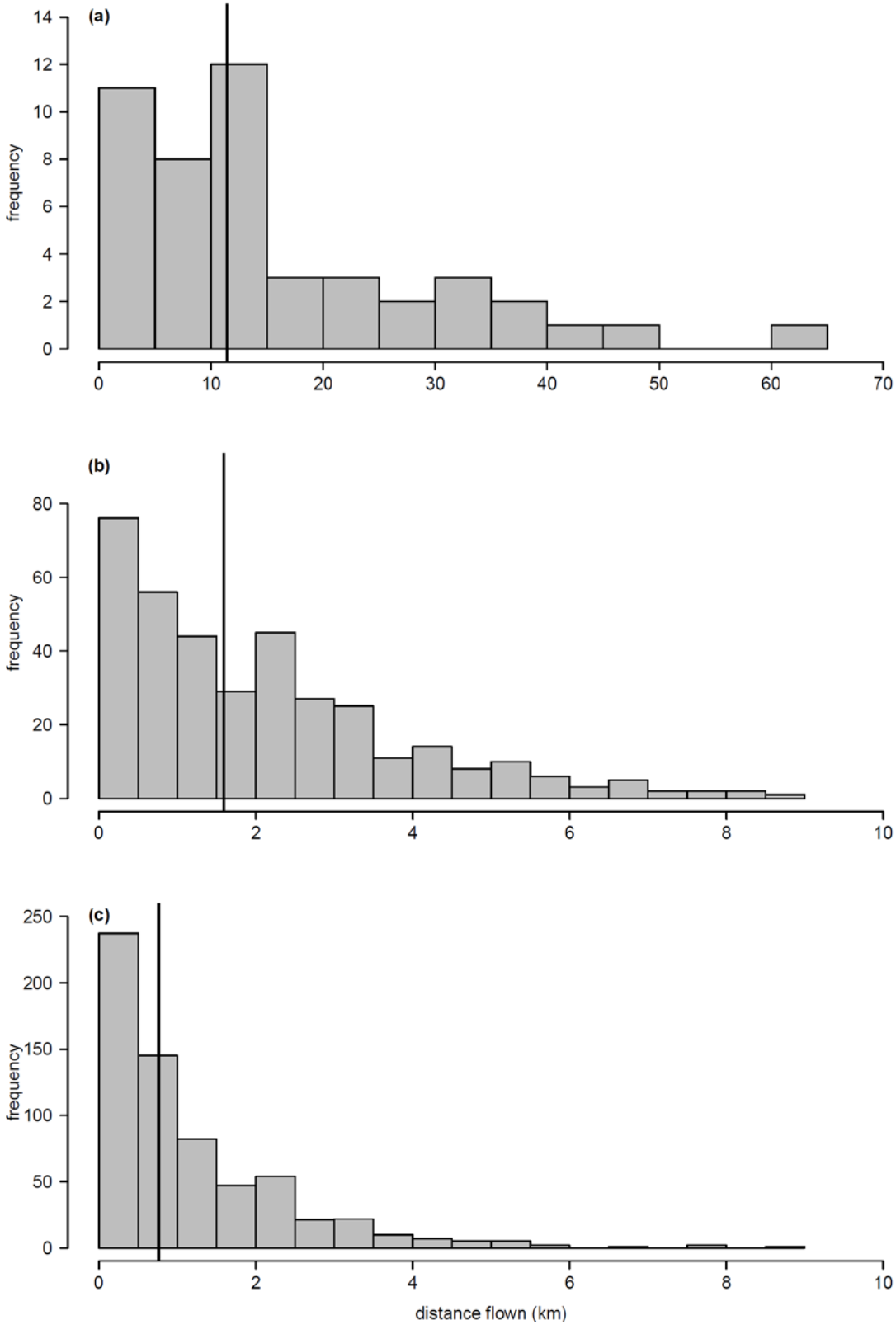


Figure 3.5 Histogram of the frequency of distances flown for **(a)** total lifespan, **(b)** each flying session, and **(c)** each individual flight. The black line shows the median value: 11.4 km for **(a)**, 1.6 km for **(b)**, 0.8 km for **(c)**.

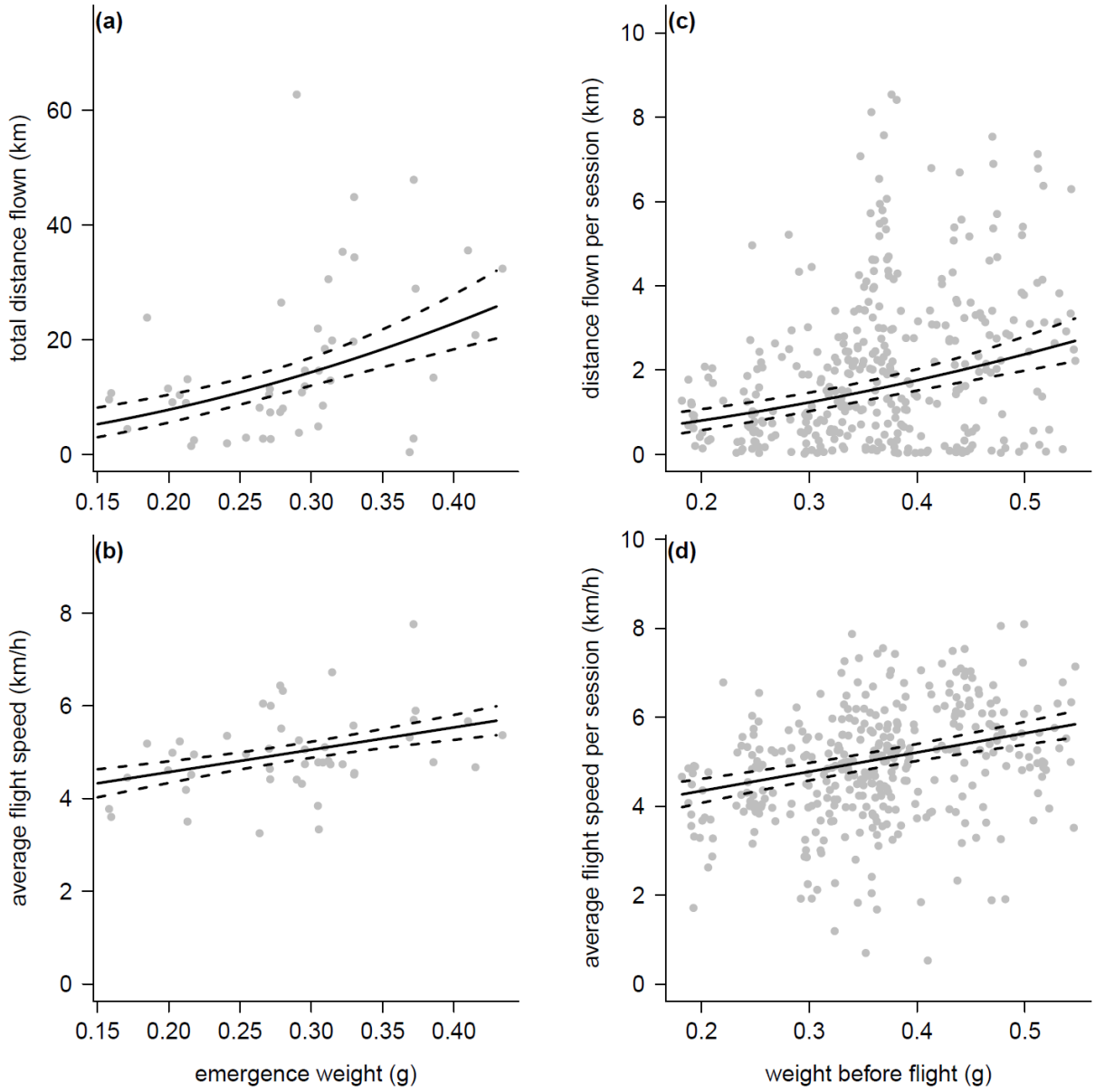


Figure 3.6 Effects of emergence weight (W0) on **(a)** total distance flown (total distance flown = $(9.95 \times W0 + 0.90)^2$) and **(b)** average flight speed (average flight speed = $1.34 \times W0 + 1.04$) during its entire lifespan, and of weights at the start of each flying session (W) on **(c)** distance flown (distance flown per session = $(0.15 \times W + 0.36)^2$) and **(d)** average flight speed (average flight speed per session = $1.10^{-3} \times W + 0.86$) per session. The solid lines correspond to the model predictions and the dotted lines indicate the standard errors.

The probability of an insect flying was not affected by its body weight. For both *M*. *galloprovincialis* flying males and females, the total distance flown increased significantly with body weight at emergence (Figure 3.6 a), but was not affected by relative weight gain over this 30-day period.

For *M*. *galloprovincialis* flying males, the total distance flown increased significantly with body weight at 30 days but not for *M*. *galloprovincialis* flying females. For both males and females, the flight speed significantly increased with *M*. *galloprovincialis* body weight at emergence (Figure 3.6 b) and at 30 days.

Beetle age had a significant negative effect on the number of individual flights per recording session (Figure 3.7 a). However the average distance covered per individual flight per session also significantly increased with beetle age (Figure 3.7 b). As a result, the insects flew on average the same distance per recording session (of 2h) irrespective of their age; they performed fewer but longer individual flights.
Individual flight characteristics were an average of 1.2 km (distance) and 5 km/h (speed). They did not differ between males and females (Table 3.5). Half of the individual flights covered distances of more than 750 m (Table 3.5, Figure 3.7 c). Furthermore, flight rank within a session had a significant negative effect on the distance flown and on the flight speed, consistent with a process of exhaustion.

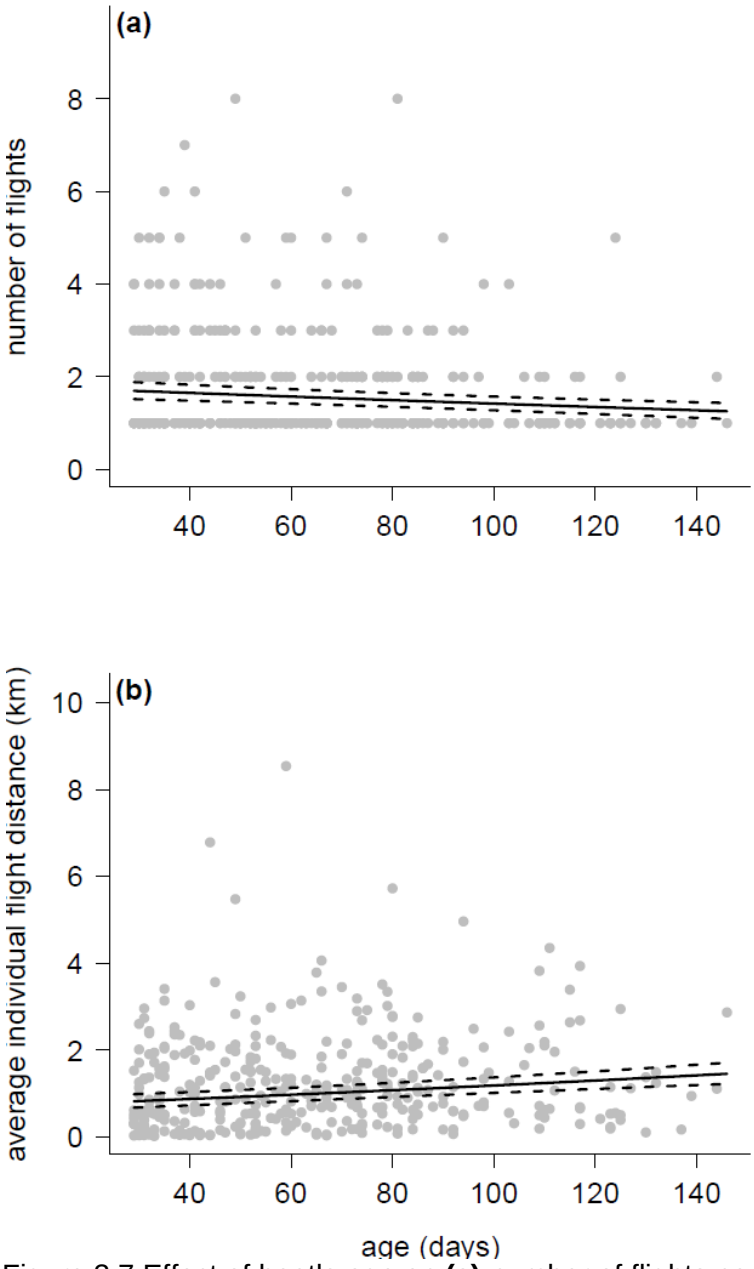


Figure 3.7 Effect of beetle age on **(a)** number of flights per session, (number of flights = $(-2.10^{-3} \times \text{age} + 1.29)^2$) and **(b)** average individual flight distance per session, (average individual flight distance = $(3.10^{-3} \times \text{age} + 0.14)^2$)**.** The solid line corresponds to the model prediction and the dotted lines indicate the standard errors.

Assessing flight activity in function of nematode load
In 2013, in collaboration with B6, we conducted in Portugal an experiment to determine the impact of the nematode load on the flight capacities of immature *M. galloprovincialis*.

Dead wood was collected near Setubal to provide beetles with a range of nematode loads. At emergence, beetles were tested on a flight mill for a 2h session (David et al., 2014). Just after the flight test, insects were crushed in water to extract the nematodes which were then counted under a microscope.

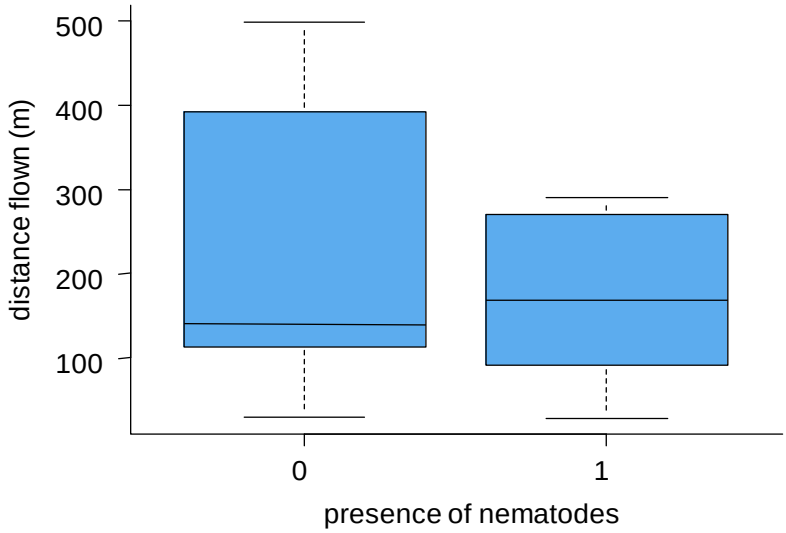


Figure 3.8 Distance flown as a function of nematode load

We tested 98 insects and did not find any significant difference in distance flown between the beetles with and without nematodes (Figure 3.8). However, only 15 beetles were carrying nematodes and with very small loads. Thus we were unable to correctly test the effect of nematode load on the flight capacities of *M. galloprovincialis.*

Assessing the initiation of flight activity in the insect vector
In 2012, we carried out an experiment to investigate the flight activity of immature beetles (from the day of emergence until day 30). This work is a part of an article submitted to *Ecological Entomology* entitled "Energy allocation during the maturation of a long-lived insect: implications for dispersal and reproduction" (by G. David, B. Giffard, I. van Halder, D. Piou, H. Jactel.)

With flight mill tests of 10 min we measured the probability of flying and the distance flown by beetles of 0, 5, 10, 20, 30 days old.

We showed that 45% of immature beetles are fliers and that the probability of flying increased significantly with the weight at emergence but was not affected by sex or age. The distance flown on the flight mill increased significantly with increasing age (Figure 3.9). There was no difference in distance flown between sexes and no effect of weight at emergence.

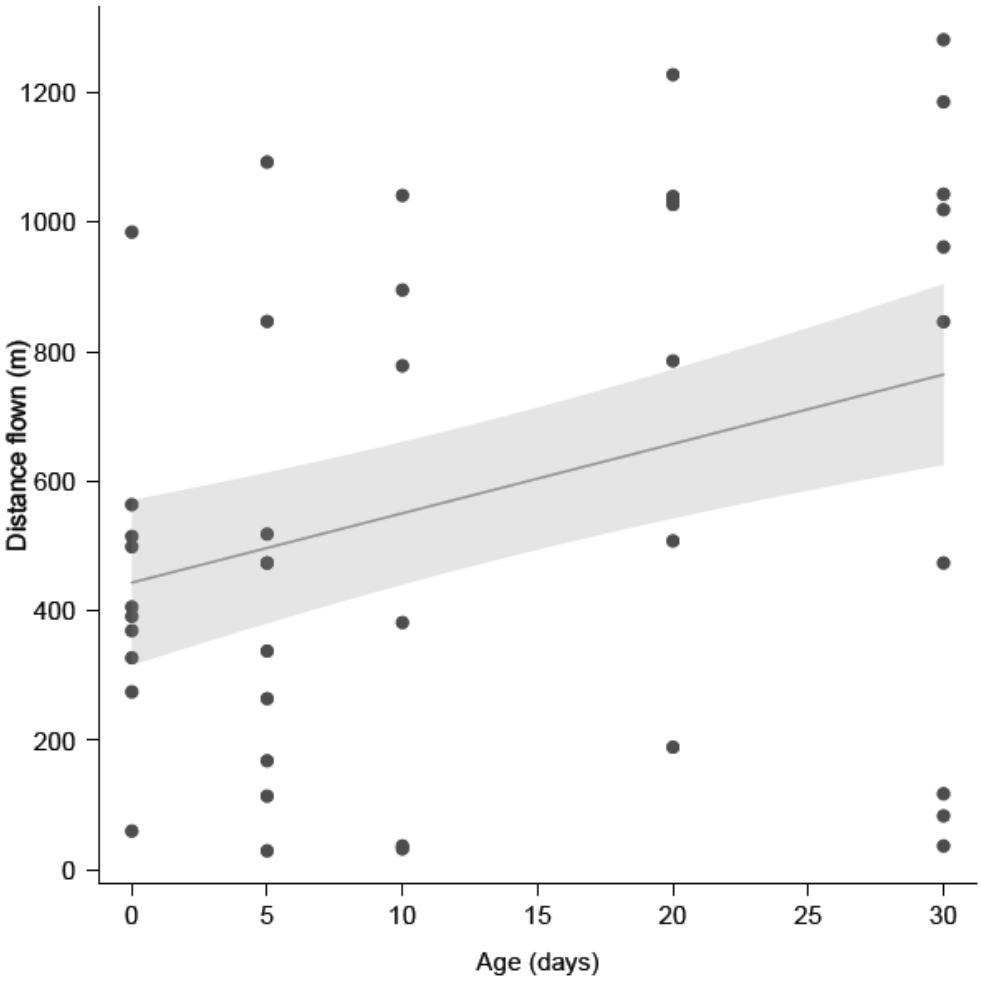


Figure 3.9 Effect of beetle age on distance flown by *M. galloprovincialis*. The line corresponds to the model prediction, and the shaded area to the standard error. Distance flown (m) = 10.71 × Age (days) + 200.16

Then with basic biochemical analysis we explored the link between energy allocation and dispersal. We demonstrated that the distance flown on flight mills significantly increased with increasing proportion of energy reserves in the thorax, but not with proportion of muscle mass in the thorax (Figure 3.10). This might be explained by an accumulation of energy reserves during insect maturation, which is essential to sustain flight activity.

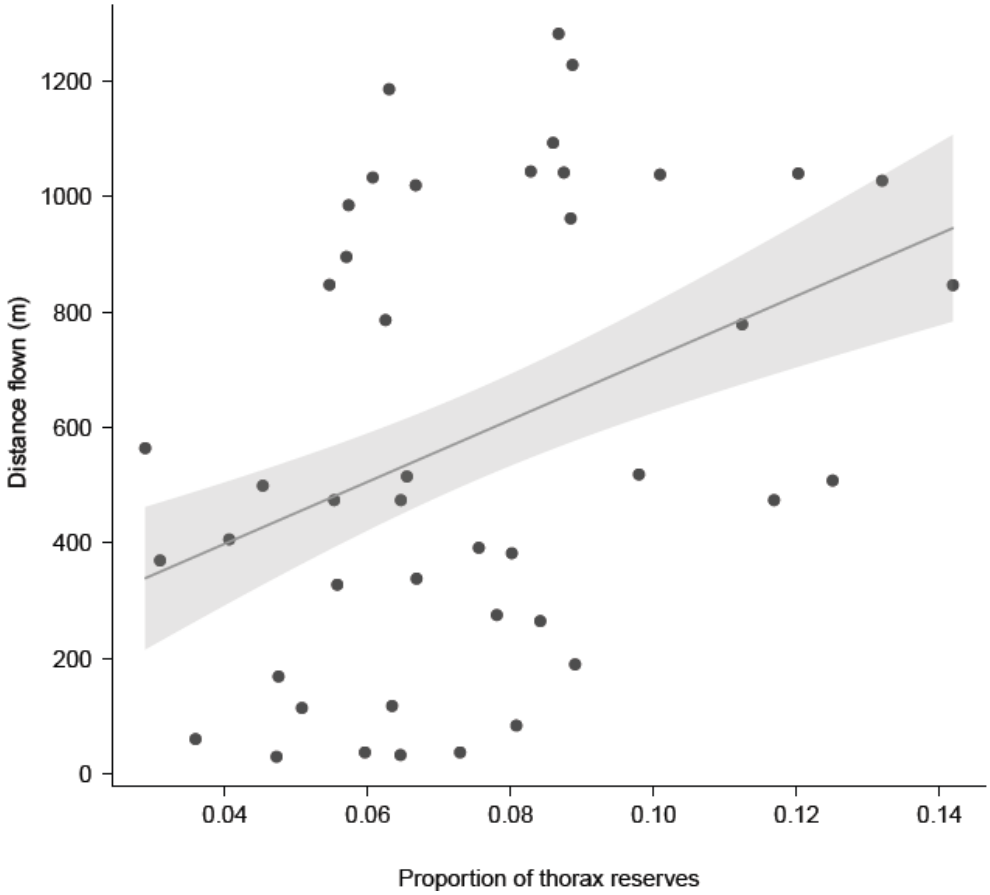


Figure 3.10 Effect of the proportion of thorax reserves (%thres) on distance flown per session. The line corresponds to the model prediction, and the shaded area to the standard error. Distance flown = 53.7 × %thres+ 117.9

Summary:

Mean performances:

- 2 km/2h session
- 16 km for entire lifespan

No effect of sex

Effect of age only for immature beetles:

- Distance increase with age

Effect of weight:

- better physical characteristics (larger wing span)
- more energy allocated to sustain flight

D 3.2: Vector dispersal related to forest condition

Work carried out by partner B6 (INIAV)

The study planned for 2014 attempted to understand the movement of the PWN insect vector in the absence of pine host trees, and so an area without forest (covered with rice, sunflower plantations and, pastures) was selected. The closest conifer trees were isolated pines in the border of a road over 10km from the release of the marked insects.

Material & Methods

The experiment was carried out at Porto Alto, Samora Correia County, Portugal, from the 21th July until the 26th August. A total of 192 marked *M. galloprovincialis* (87 immature adult beetles less than 9 days after emergence and 105 mature beetles older than 15 days) were released during the afternoon in the centre of three concentric circles (1km, 2km and 3km radius) in which the traps were placed (multifunnel traps with Galloprotect 2D-plus and containers loaded with fresh cut maritime pine logs covered with a net with Temocid (TM) glue (Figure 3.11).

The traps were visited weekly and the captured insects were collected and transported to Oeiras INIAV lab where they were identified and counted.

Results

Most of the *Monochamus* beetles started to fly within 30 minutes after release. No *Monochamus* was recaptured and no other cerambycids were caught in the traps. Almost all insects found in the traps were Diptera, Lepidoptera, Odonata and very few Coleoptera (ladybugs - Coccinellidae).

After the removal of the traps from the site, the wood placed in the containers was debarked and no bark or wood boring beetle colonized it, confirming the absence of these insects in the assay area and the inefficiency in recapturing the marked *M. galloprovincialis*.

The placement of the traps on the floor (containers) and at 2 meters height (multifunnel) seems to be incompatible with the observed flight pattern of the *Monochamus* adults, since the released insects flew at a higher altitude and probably spread out of the range of the traps' odour emissions. It is also possible that in the absence of a tree silhouette the insects had no visual stimuli to stop the flight (Figure 3.12).

Some birds that feed on insects were also common on the area may have fed on the insects, although this it wasn't possible to verify this possibility.

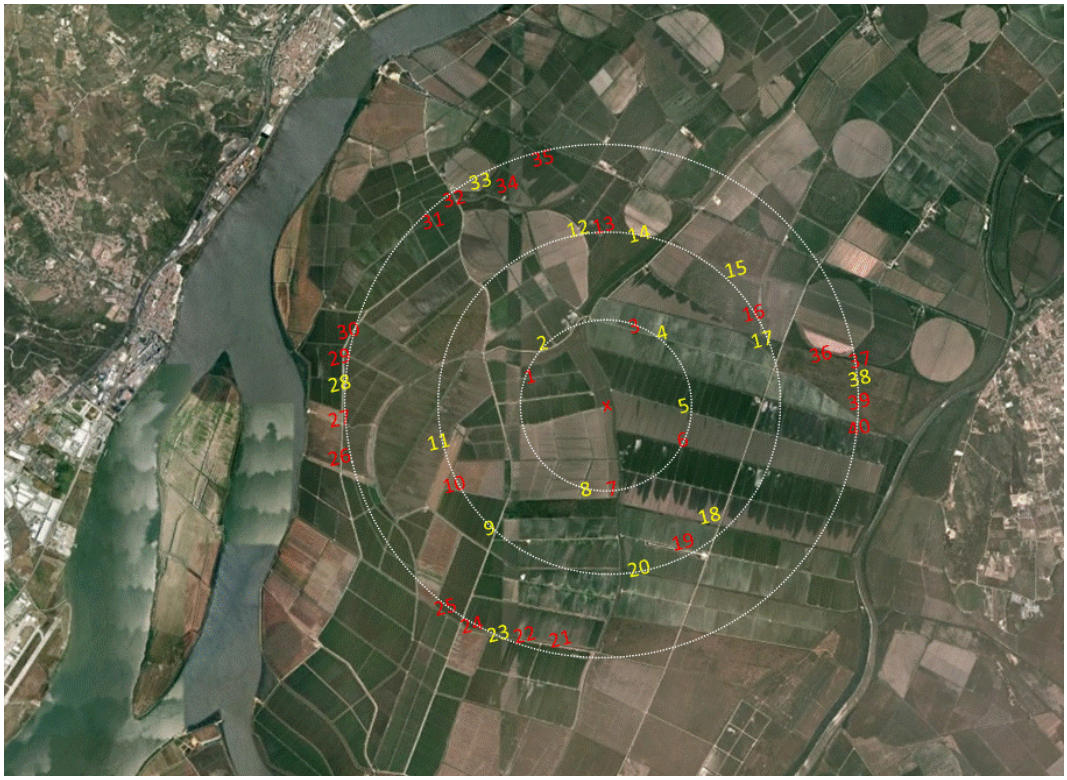


Figure 3.11 Placement of the traps for the *M. galloprovincialis* dispersal assay conducted in Porto Alto: yellow number - container with fresh maritime pine logs; red number - multifunnel trap with Galloprotect 2D-plus lure. X - release point of marked insects.

Figure 3.12 Traps used for the *Monochamus galloprovincialis* dispersal assay conducted in Porto Alto, Portugal from the 21th July until the 26th August. (a) container with fresh maritime pine logs; (b) multifunnel trap with Galloprotect 2D-plus lure.

Work carried out by partner B3 (BFW)

Materials & Methods

Monitoring of *Monochamus* flight using multifunnel traps (Econex, Spain) baited with Galloprotect-2D (SEDQ, Spain) was continued in 2014. Traps 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 sides of the eastern Alps spanning typical areas for *M. galloprovincialis*, *M. sutor*, and *M. sartor*. In addition to sites used in 2014, two new traps were placed in lowland Scots pine forests in southern Austria.

Results

Capture of *Monochamus* spp.: Traps in the Austrian pine forests Baden and Scots pine forests Höhenbergen and Mittlern caught predominantly *M. galloprovincialis*. One specimen of *M. saltuarius* was additionally caught on the latter two sites. 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 3.6, Figure 3.13).

Table 3.6 Total trap catches of *Monochamus* spp. from May to October 2014

	M. gallo.	*M. sutor*	*M. sartor*	*M. salt.*
Baden I	106	0	0	0
Baden II	20	0	0	0
Naßwald Waldeben	0	138	35	0
Naßwald Rainerboden	0	134	35	0
St. Oswald	0	131	0	0
Pöls	1	49	0	0
Gerlitzen	0	59	0	0
Höhenbergen	21	0	0	1
Mittlern	105	0	0	1

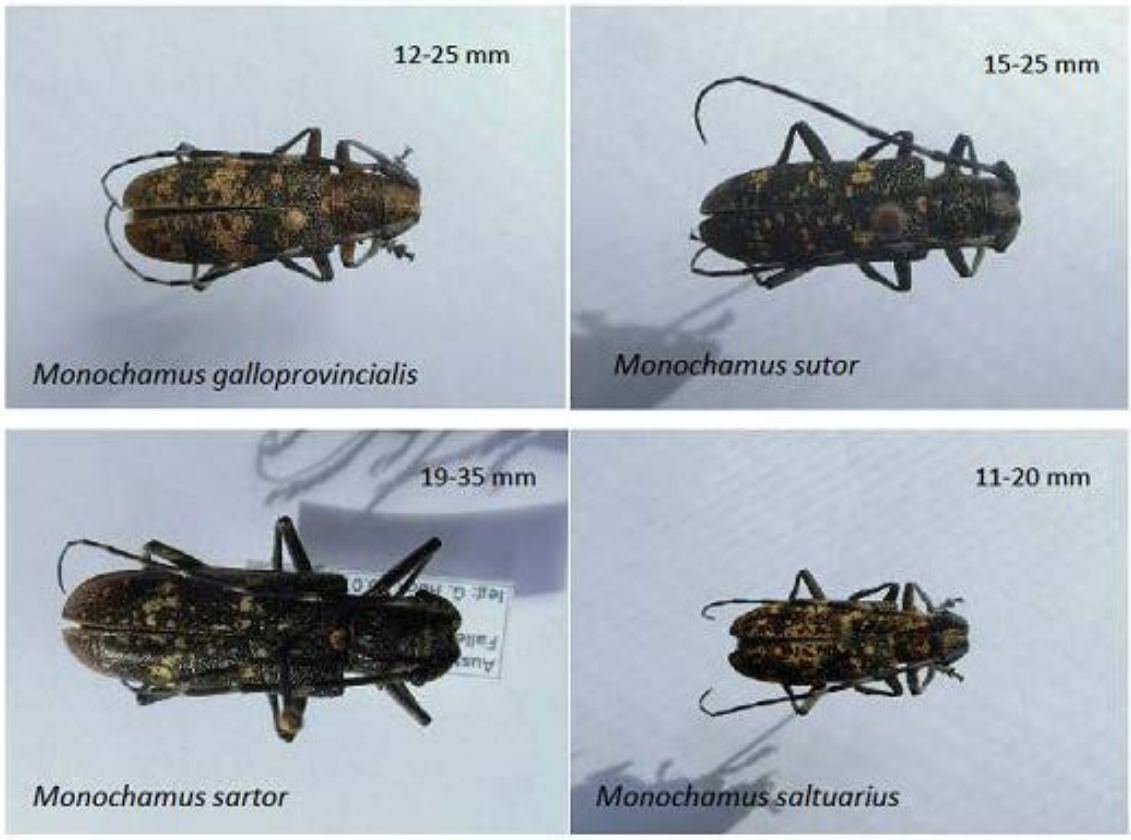


Figure 3.13 *Monochamus* species found in Austria

The first *M. galloprovincialis* were caught on May 21 in Baden, first *M. sutor* on May 26 in Pöls and first *M. sartor* on June 6. Once flight had started it continued until October. No clear flight peaks became apparent during this period. Weeks with no or low catch, particularly from end of August to early September were characterized by

low temperature and high precipitation. Generally, trap catches were positively correlated with air temperature (Table 3.7) although correlations were weaker than in 2013.

Table 3.7 Correlation between weekly mean air temperature and number of trapped beetles (n = 17…23)

Species	Location	Kendall's τ-b
M. galloprovincialis	Baden I	0,306
	Baden II	0,501
	Höhenbergen	-0,001
	Mittlern	0,317
M. sartor	Naßwald-R.	0,293
	Naßwald-W.	0,189
M. sutor	Naßwald-R.	0,311
	Naßwald-W.	0,483
	Pöls	0,729
	St. Oswald	0,520
	Gerlitzen	0,599

Conclusions

Our trap network in various regions of Austria provided important information on flight phenology and occurrence of the four *Monochamus* spp. 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 from 2013 and 2014 show a continuous flight season from June to October with short episodes of no flight due to low temperatures (often together with high precipitation).

D 3.4: Climate influences on vector dispersal

Work carried out by partner B6 (INIAV)
Thermal requirements for development of post-dormancy larvae

Developmental thresholds and thermal requirements for development of post-dormancy larvae of *M. galloprovincialis* were studied in the PHRAME project, resulting in a publication in a scientific journal presenting the results (Naves & Sousa, 2009). It was found that the lower threshold for larval development was 12,2 ± 0.8ºC, and an average of 822 degree-days (DD) above that value was required to obtain 50% adult emergence under laboratory conditions. By using a modified sine wave model the adult emergences could be accurately predicted, with errors below 10% of actual observed emergences. Nevertheless, the model was only tested for beetles emerging from wood exposed to ambient temperatures (in the shade), while in the terrain the wood is normally exposed to direct sun which can make the larvae accumulate more degree-days and accelerate their development.

Material & Methods

To test and validate the model´s estimation of emergences for sun-exposed wood, an experiment was carried out in Portugal to study and compare the emergence of *M. galloprovincialis* from both shaded and sun-exposed pine bolts. Pine bolts were obtained from *Pinus pinaster* dead trees, felled in February 2014 in Tróia, Portugal. The pine material with *M. galloprovincialis* larvae was divided into small bolts (80cm

long) and taken to the INIAV laboratory in Oeiras, where it was placed in wooden box frames (1.5mx0.75m) with metal wire meshes in the sides. Half of the material was kept at ambient temperatures in a shaded location, and the remaining material, in a sun-exposed location receiving between eight and ten daily hours of direct sun. Hourly ambient temperature was recorded at both locations with EL-USB-2 data Loggers. Degree-day (DD) accumulations were calculated using the sine wave method available at University of California's IPM online degree-day models web-page (http://www.ipm.ucdavis.edu/WEATHER/index.html), inputting daily minimum and maximum air temperatures. DD accumulations were calculated from 1 March onwards, as suggested by Naves & Sousa (2009). An upper intermediate cut-off was selected to slow heat accumulation at temperatures above the upper developmental threshold specified (33,0ºC). The emergence dates predicted by the model were compared with observed cumulative emergences at five percentile dates with a t-test.

Results

A total of 373 adults emerged from the pine bolts, with 226 collected from the shaded bolts and 147 from the sun exposed wood. The emergence pattern was similar for the shaded and sun-exposed wood ($F= 0.055$; $df= 1$; $p< 0.0001$), with the first beetles appearing in mid-May and the last ones in mid-August on both treatments, well within the normal pattern of *M. galloprovincialis* emergence described for Portugal (Naves *et al*, 2008). The peak of emergence took place throughout the months of July and August, as can be seen in Figure 3.14.

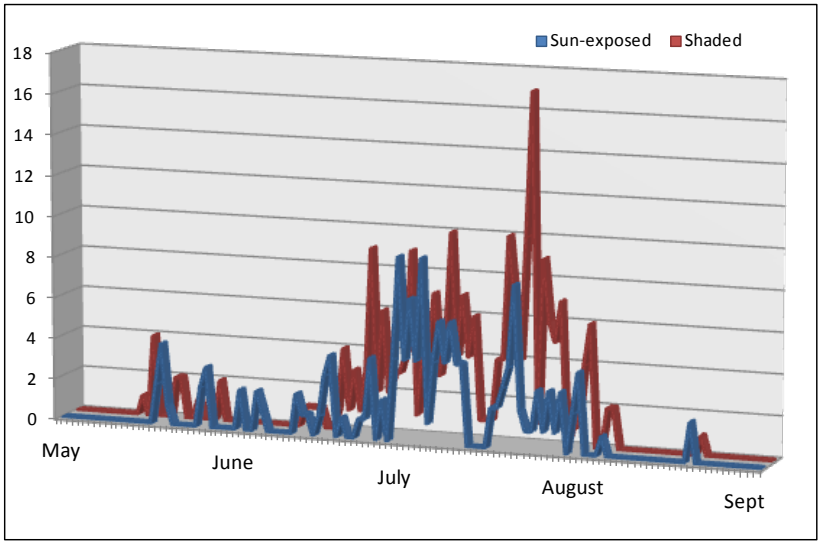


Figure 3.14 Emergence of *M. galloprovincialis* from sun-exposed and shaded wood bolts during 2014.

The estimation of the sine wave method generally agreed well with the patterns of adult emergence observed, and differences between estimated and observed DD values were not significant for the shaded or the exposed wood (*t*-test, $p = 0.01$). The DD estimation for the median (50%) cumulative emergence corroborated well for the sun-exposed bolts (difference of three days), while for the shaded bolts a slightly higher difference of eight days was obtained (Table 3.7).

Table 3.8 Emergences (in Julian days) for *M. galloprovincialis* adults at five cumulative periods (1-99%) for sun-exposed and shaded wood bolts, considering degree-day (DD) requirements observed and predicted by the sine wave model.

Cumulative percentile emergence	DD estimate*	Sun-exposed bolts (Julian days)			Shaded bolts (Julian days)		
		observed	predicted	Mean deviation	observed	predicted	Mean deviation
1 %	**450**	138	150	+12	135	146	+11
10 %	**608**	156	166	+10	171	164	-7
50 %	**822**	185	188	+3	191	183	-8
90 %	**1029**	207	206	-1	207	200	-7
99 %	**1146**	230	218	-12	216	210	-6
				+2.4 ± 8.6**			-3.4 ± 7.2**

*From Naves & Sousa (2009); **Mean ± SD between predicted and observed emergence (Julian days).

Conclusions

The model tended to underestimate larval development for the sun-exposed bolts, while for the shaded wood the development was generally overestimated. Nonetheless, in both cases the model systematically underestimated the emergence of the very first insects by up to 12 days, resulting in less accurate predictions for the beginning of the season.

Overall, the sine wave method forecasted the emergence pattern of *M. galloprovincialis* with relatively high accuracy, resulting in similar deviations for the shaded and sun-exposed bolts. Apparently, the accumulation of heat by the sun-exposed wood does not result in a significantly anticipated emergence period, and therefore the model can be employed to forecast the emergence pattern of the pine sawyer from natural populations of sun-exposed trees in the terrain, without losing its accuracy.

References

Naves P & Sousa E (2009). Threshold temperatures and degree-day estimates for development of post-dormancy larvae of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae). Journal of Pest Science 82: 1-6.

Naves P, Sousa E & Rodrigues JM (2008). Biology of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae) in the Pine Wilt Disease affected zone, Southern Portugal. Silva Lusitana 16: 132-147.

Work carried out by partner B3 (BFW)

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

Material & Methods

Breeding experiments were initiated in the first full field season of the project 2012 and have been going on continuously (see previous reports for details). Breeding experiments were continued in the summer of 2013. Emergence from all logs was monitored in 2014; logs infested in 2012 were dissected and analyzed in August 2014.

Results

First beetles emerged from logs incubated under outdoor conditions on June 12, 2014 when daily mean temperatures reached more than 20°C for five days following

12 days of lower temperature. A first period of days with mean temperature above 20°C from May 22 to 25 did not trigger emergence. Beetles emerged from logs that had been infested in 2012 as well as in 2013. All beetles emerged in the short period until June 23. Dissection of logs infested in 2012 showed cadavers of larvae and callow adults in most logs as well as individual living larvae in 22% of the logs. Of beetles successfully emerging from logs infested in 2012, 60% exhibited a one-year and 40% a two-year life cycle.

Effective temperature sums (in degree days) were calculated by tentatively using the 12.2°C developmental threshold of *M. galloprovincialis*. After the cold period, beetles accumulated 265 to 346 DD prior to emergence from the logs in 2014.

Conclusions

When kept at room temperature, *M. sartor* was able to complete development from oviposition to adult emergence in approximately 6 months (see previous periodic report). Under outdoor temperatures, this period was significantly prolonged to a minimum of 11 months or 23 months. All *M. sartor* from the experimental rearing emerged within two weeks in June from either one- or two-year development periods in the logs. This corroborates results from the previous two years, when all beetles emerged in June through to early July after the first consecutive days with mean temperatures above 20°C.

Work carried out by partner B4 (INRA)

Two main questions:

i) How vector populations are structured among the whole geographical range of the species?
ii) What are the factors affecting the dispersal of the vector?

Microsatellite analysis (tasks 3.4.1 and 3.4.2) was postponed due to a delay in sampling the first year of the project and the necessity to complete data sets for phylogeographic and landscape analysis.

Work performed during the last 9 months enabled field sampling to be completed (populations from Pyrenean chain and East-European countries) and to analyse final data sets.

This work is validated by the publication of two papers presenting molecular tools to study genetic diversity and structure within populations of *Monochamus galloprovincialis*:

P1. J. Haran & G. Roux-Morabito (2014). Development of 12 microsatellite loci for the longhorn beetle *Monochamus galloprovincialis* (Coleoptera Cerambycidae), vector of the Pine Wood Nematode in Europe. *Conservation Genetics Resources* DOI: 10.1007/s12686-014-0262-0

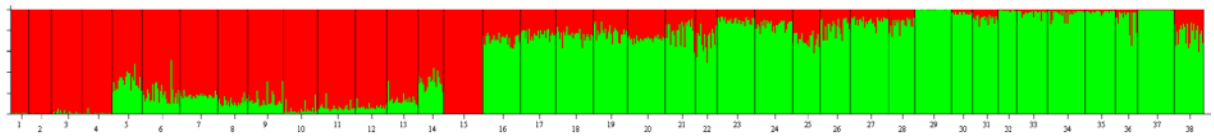
P2. J. Haran, F. Koutroumpa, E. Magnoux, A. Roques & G. Roux. Ghost mtDNA haplotypes generated by fortuitous NUMTs can deeply disturb infraspecific genetic diversity and phylogeographic pattern. (accepted in *Journal of Zoological Systematics and Evolutionary Research*)

In addition, the analysis of the genetic structure and differentiation patterns of the beetle populations throughout the Pyrenean area was combined with a spread model simulating the potential movements of nematode infested-beetles across this mountain range. The results are presented in the following manuscript:

P3. J. Haran, A. Roques, A. Bernard, C. Robinet & G. Roux, Altitudinal barrier to the spread of invasive species: Could the Pyrenean chain slow the natural spread of the pine wood nematode? submitted at PlosOne.

Results:

- Population genetic study, by the way of **development of highly polymorphic molecular markers** (12 microsatellites) in the vector (P1), offered an alternative for obtaining information about individual movements and effective dispersal over geographic and temporal scales.
  - ✓ Estimation of natural dispersal of the vector = **define barriers and corridors**
  - ✓ Characterization of long distance human-mediated transportation = **provide valuable diagnostic tools for tracing movements of individuals** (frontier interception – trapping survey)
- **Phylogeography at large scale** analysis revealed that *M. galloprovincialis* populations could be grouped in two main clusters. Bayesian assignment with STRUCTURE indicated that K=2 is the best for describing the genetic structure of these populations (Figure 3.15). 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.



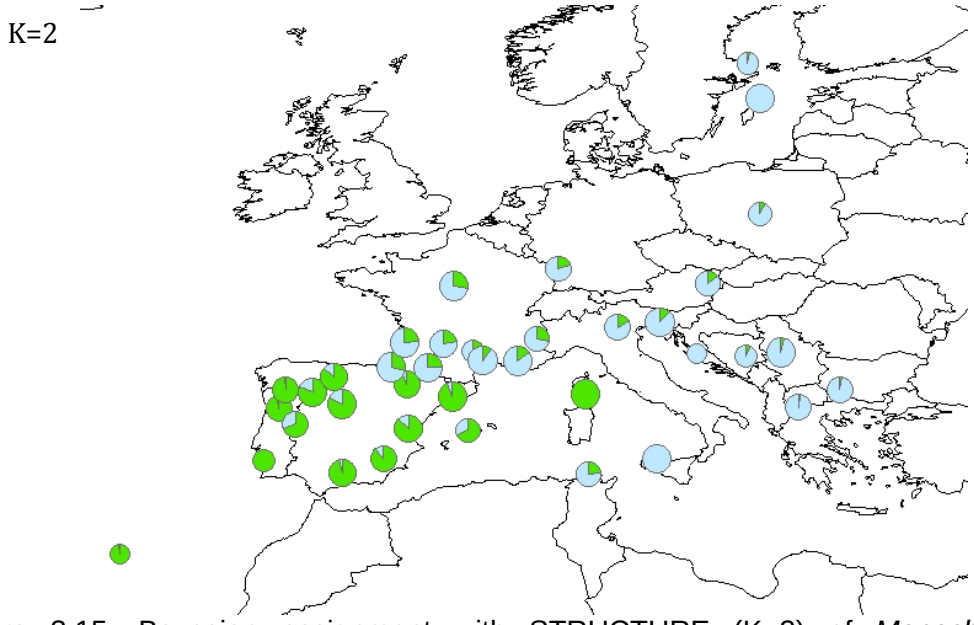


Figure 3.15: Bayesian assignment with STRUCTURE (K=2) of *Monochamus galloprovincialis* populations in Europe

Nevertheless, geographical structuration obtained for K=6 (Figure 3.16) indicated that four main lineages occurred in Eastern and Western part of the Iberian Peninsula, in Central and Eastern Europe (islands not included).

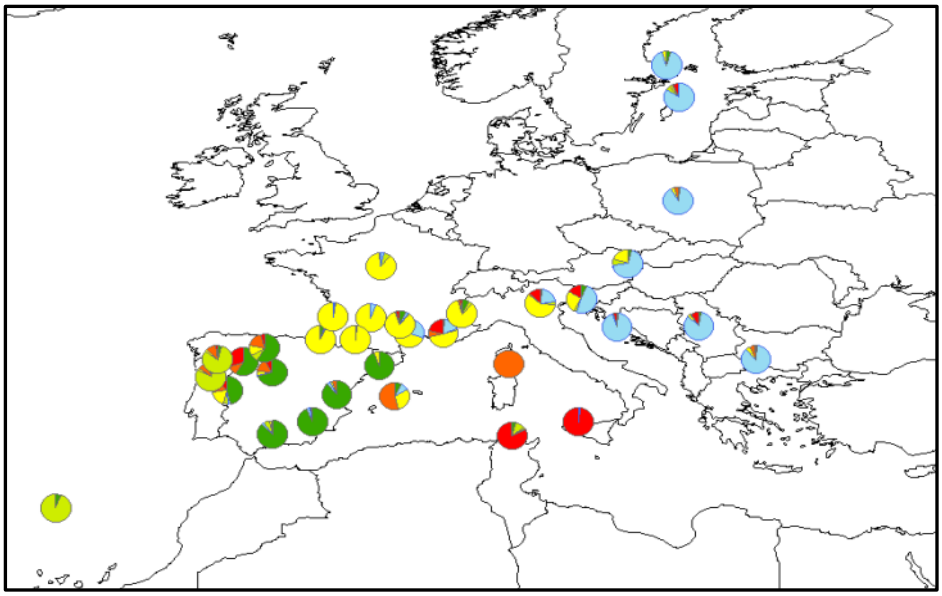


Figure 3.16 Bayesian assignment with STRUCTURE (K=6) of *Monochamus galloprovincialis* populations in Europe

At fine analysis, we are able to detect patchy genetic structuration that could reveal zones of population admixture and/or long distance movements of the vector (Figure 3.17).

- **Identification of the corridors and barriers to natural dispersal of the vector**: Estimation of gene flow across the Pyrénées (P3).
    - The Bayesian clustering method clearly identified two genetic strains in the **northern** and **southern** side, forming a suture zone along the ridgeline of the mountain range.
    - Signature of migration along the Atlantic coast and to a lesser extent, along the Mediterranean one.
    - The admixture in the western hillside of the mountain shows an asymmetric migration of individuals, from France to Spain in the Western side of the Pyrenees.
    - The low genetic differentiation within hillsides suggests important gene flow and subsequently high dispersal abilities for *M. galloprovincialis*
    - Existence of long dispersers in quite high proportion.

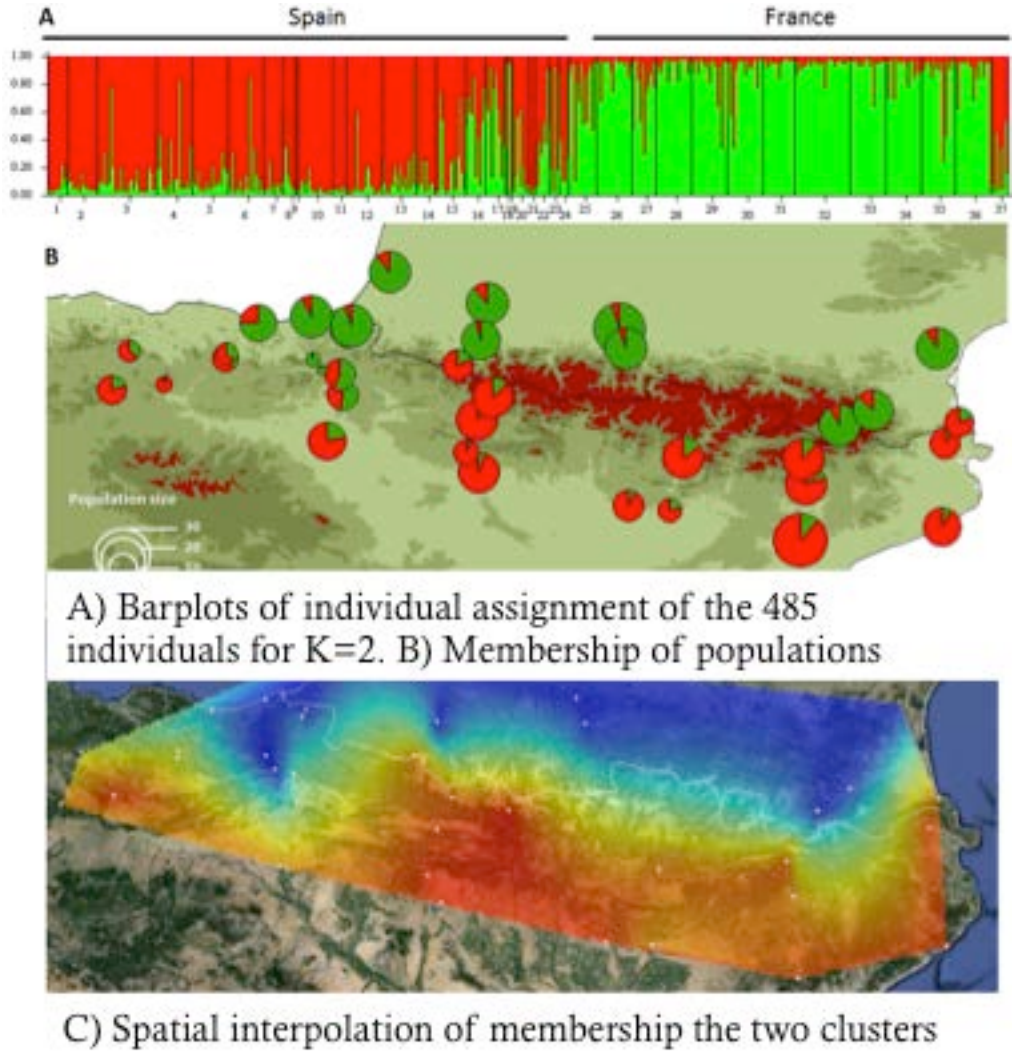


Figure 3.17 Cluster analysis of *Monochamus galloprovincialis* populations related to the Pyrenean chain as a barrier

Take home message: Populations of the vectors highly structured at European scale reflecting the existence of natural barriers (mountains). Important movements of individuals reflecting high dispersal abilities of the vectors at fine scale.

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

The objectives and outcomes from this Work Package have added to the knowledge base planned in the DOW. Virtually all objectives were achieved in the time available for the project and there were no significant deviations from the planned work.

Statement on the use of resources
There were no significant departures from the planned use of resources.

WP 4. Development of new methods for monitoring and control of *Monochamus spp* and PWN based on vector trapping

Deliverables
D 4.1: Lure for *M. galloprovincialis.* Month 24
D 4.2: 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 36

Progress March-November 2014

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

Previous work within REPHRAME had 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). Drawback: attracts many non-target beetles
- Smoke volatiles may also significantly increase attraction to the pheromone-kairomone lure and can replace terpene as synergist to the G2D bait.
- Freshly emerged, immature, adults do not respond to any lure tested until they have fed for about 10 days.
- (+) Limonene did not show repellent effect (i.e. reduced attraction to G2D)

Deliverable D 4.1: Lure for *M. galloprovincialis* was thus provided.

However, further lure improvement could be pursued:

- Continue testing potential for smoke volatiles to effectively replace a-pinene as synergist of G2D (cheaper and easier to release, more target specific).
- To integrate G2D dispenser into one G1D dispenser lure (cheaper, easier to use)
- Test if (-) limonene, naturally occurring in *P. pinea*, has a repellent effect (i.e. can reduce attraction to G2D)

Activities by B2 and B9

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

Experiment 1/14:

One experiment was carried out from July 7th to August 13th to continue evaluating the synergistic effect of smoke volatiles compared to α -pinene in the standard lure. 6 tested treatments were: G2D (standard lure); G2D + α (Galloprotect Pack); G2D + S1 (Smoke volatile 1); G1D (integrated dispenser); G1D + α; G2D + S1. Smoke dispensers and G1D were provided by SEDQ, (Barcelona, Spain).

Results:

No significant differences were found between any of the treatments. However, catches when smoke compound S1 was added were 20-27% higher compared to catches in the standard or in the integrated lure respectively (Figure 4.1).

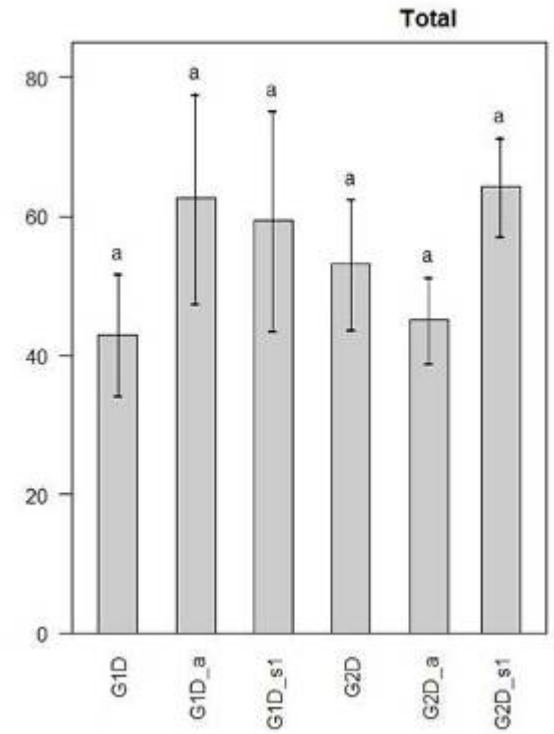


Figure 4.1 Results of experiment 1/14 on smoke volatiles in Spain (2014)

Experiment 2/14

As results of the previous experiment were inconclusive, it was decided to continue it during the remaining flight season period, from August 13th to .September 26th. Besides, three new integrated dispenser prototypes: G1Db, G1Dc and G1Dd were also tested. Dispensers were provided as in experiment 1.

Results:

As in experiment 1, there were no significant differences among treatments (Figure 4.2). This time, none of the treatments resulted in higher catches than the standard lures G2D or G2D+ α pinene (G:Pack).

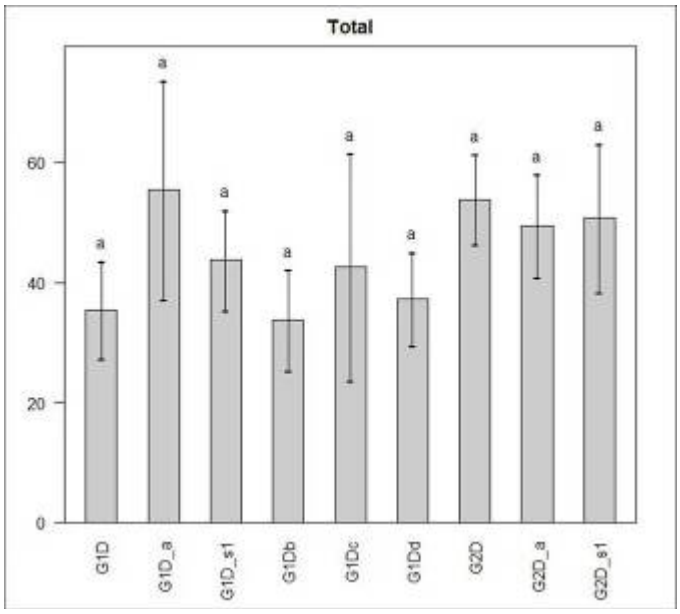


Figure 4.2 Results of experiment 2/14 on Smoke volatiles in Spain (2014)

Experiment 3/14

Previous assays showed that (+)-limonene isomer was not effective as a repellent to reduce attraction by *M. galloprovincialis* to attractive lures (G2D). An experiment was conducted from July 7th to August 13th to test such a potential effect in the (-)-limonene isomer, naturally occurring in *P. pinea*. Thus tested treatments were: G2D; G2D + α pinene, G2D+ (-) limonene; G2D + α pinene + (-) limonene and G2D + (+)-limonene.

Results:

There were no significant differences among treatments (Figure 4.3). None of the limonene isomers reduced *M. galloprovincialis* catches when added to the standard lure baited traps.

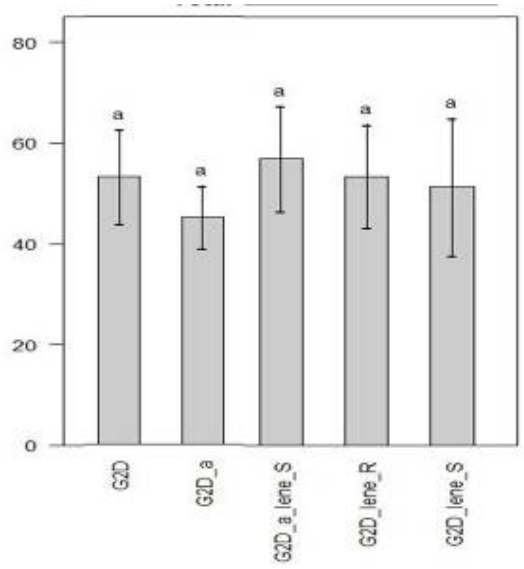


Figure 4.3 Results of experiment 4/14 on the limonene effect in Spain (2104)

Contrary to earlier results in 2011, these, and results in 2012 and 2013, suggest that none of the limonene isomers causes a significant repellent effect to beetles being attracted to pheromone-kairomone baited traps, thus making practical use of this terpene for protective aims unlikely.

Activities of B5

Experiment 4/14

An experiment was carried 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* could distinguish between the VOCs of the different treated hosts. In addition, assessment of the potential for early detection of infestation with the help of VOCs was assessed as a possible early-warning tool.

Studied trees were: controls (inoculated with tap water) (5 trees), PWN-inoculated (5 trees), drought-stressed (inoculated with tap water) (5 trees) and double controls (without any inoculation and cutting of an inoculation slit) (1 tree). VOCs were collected using the CLSA method (Figure 4.4) on two 2-3 year old main side shoots of 7-8 year old *P. sylvestris*. These 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 4.4 Collection of VOCs with CLSA method on 7-8 year old *P. sylvestris*

Results:

Results (Table 4.1) show similarities but also differences of many chemical compounds between the tested pine groups: controls, drought stressed and PWN-infested trees and between the three collection days. The same was found for the peak area of the compounds in the chromatograms, which indicate different concentrations.

Table 4.1 Chemical compounds with CAS no., retention time [min] and peak area per collection date (1: four, 2: eight and 3: nine weeks after test start) and pine group (controls, drought stressed, PWN inoculated), n=1

			Peak area								
			Collection date 1			Collection date 2			Collection date 3		
Compounds	**CAS**	**Retention time** [min]	Control	Drought stressed	PWN infested	Control	Drought stressed	PWN infested	Control	Drought stressed	PWN infested
β-Pinene	127-91-3	6.961	<1			1.15	6.08	14.72		1.22	
(-)-β-Pinene	18172-67-3	6.979		2.32	<1				<1		2.72
3-Carene	13466-78-9	7.685	14.72	10.45	6.56	<1	15.64	37.95	3.83	4.71	32.56
β-Myrcene	123-35-3	7.898	5.39	17.99	5.32		5.84	6.95	1.88		6.83
β-Terpinen	99-84-3	7.995	2.00							<1	
m-Cymene	535-77-3	8.208	<1	1.55	<1			4.22	<1	<1	1.65
D-Limonene	5989-27-5	8.591	<1	5.69	1.76	<1	3.3	2.89	<1	1.87	2.14
β-Phellandrene	555-10-2	8.750	7.97	5.99			4.8	3.43	<1	2.68	2.59
Eucalyptol	470-82-6	8.823		9.15	7.7	2.68			3.83		<1
o-Cymene	527-84-4	9.899		1.84	<1					<1	
Terpinolen	586-62-9	10.100	2.04	1.59			3.85	<1	<1	<1	1.07
3-Hexen-1-ol, acetate, (E)-	3681-82-1	10.666	1.2	1.12	3.3	<1			<1	<1	
Dihydroisophoron	873-94-9	11.858	<1	1.82	2.65	<1	2.13	<1	1.04	<1	<1
Nonanal	124-19-6	12.047	<1	<1	<1		<1	<1	<1	<1	<1
α,4-Dimethylstyrene	1195-32-0	12.862	<1	1.5				1.19	<1	<1	2.78
trans-2-Caren-4-	4017-82-7	13.069		<1							2.36
Copaene	3856-25-5	13.811	1.24						1.03	<1	
L-camphor	464-48-2	14.298	<1		1.37				<1		
Benzaldehyde	100-52-7	14.389	<1	<1	<1			1.05	<1	<1	2.5
Pinocarvone	30460-92-5	15.150		<1	<1			<1		<1	2.53
Bornyl acetate	76-49-3	15.229	3.68		2.36	<1			<1		
2-Undecanone	112-12-9	15.399	2.74		2.07	<1			1.29		
L-4-terpineol	20126-76-5	15.509	<1			<1			<1	<1	1.02
Caryophyllene	87-44-5	15.575	3.31	<1	2.99		<1	<1	2.58	<1	<1
Methyl benzoate	93-58-3	15.941			<1			<1		<1	1.85
L-pinocarveol	547-61-5	16.373	<1	1.73			<1	<1		<1	2.02
(Z)-β-Farnesene	28973-97-9	16.427	1.67	8.75	22.75	78.3	34.72		19.83	40.97	1.13
cis-Verbenol	18881-04-4	16.664						<1		<1	1.65
Carveol	99-48-9	16.677		1.29						<1	
α-Caryophyllene	6753-98-6	16.719	1.36		1.32		1.76		1.47	3.11	
Crypton	500-02-7	16.750								1.59	
Terpineol	98-55-5	16.932		<1	<1	1.48		<1			<1
γ-Muurolene	30021-74-0	16.944							1.68	<1	
Borneol	10385-78-1	17.042	<1		1.4			<1			<1
3-Cyclohexene-1-methanol, 5-hydroxy-α,α,4-trimethyl-, (1S-trans)-	38235-58-4	17.236						<1		<1	1.08
(-)-Verbenone	1196-01-6	17.297									1.41
Germacrene D	23986-74-5	17.297			1.91	<1			1.31		
2-Pinen-4-one	80-57-9	17.303		1.56			<1	<1			
β-Cubebene	13744-15-5	17.309	1.94								
α-Guaiene	3691-12-1	17.467			1.2						
Eremophilene	10219-75-7	17.473							3.34		
β-Selinene	17066-67-0	17.474	2.74	<1			<1	<1		<1	<1
Benzyl acetate	140-11-4	17.522				1.85					
α-Selinene	473-13-2	17.541	1.62					<1	1.36	<1	<1
α-Farnesene	502-61-4	17.668			7.12	5.68	8.7		16.44	16.631	
Elixene	3242-08-8	17.680	2.93								
1,1-Cyclohexanedimethanol	2658-60-8	17.741						1.11			
Cadinene	523-47-7	17.948	7.31		2.58		<1		7.19		
δ-Cadinene	483-76-1	17.960		2.18				<1		1.59	
γ-Cadinene	39029-41-9	18.021	7.34	1.49	2.25	<1		<1	6.31	1.23	<1
cis-α-Bisabolene	29837-07-8	18.112			<1	1.53			1.58		

			Peak area								
			Collection date 1			Collection date 2			Collection date 3		
Compounds	**CAS**	**Retention time** [min]	Control	Drought stressed	PWN infested	Control	Drought stressed	PWN infested	Control	Drought stressed	PWN infested
Methyl salicylate	119-36-8	18.343			7.82	<1	1.34		3.27	5.5	<1
2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	460-01-5	18.654	<1		1.19					<1	
2,6,9,11-Dodecatetraenal, 2,6,10-trimethyl-	4955-32-2	18.982					1.01				
p-Cymen-8-ol	1197-01-9	19.049	1.73	3.12	1.23	1.02	1.11	1.27	1.34	<1	3.63
p-Cymen-8-ol	1197-01-9	19.140	1.02	1.77	<1	<1		<1	<1	<1	1.67
Phenol	108-95-2	20.996					1.14				
±-trans-Nerolidol	40716-66-3	21.616							4		
γ-Muurolene	30021-74-0	21.939	2.83	<1	4.91						
7-Tetracyclo[6.2.1.0(3.8)0(3.9)]undecanol, 4,4,11,11-tetramethyl-	74842-43-6	24.804	<1		<1	1.08			1.5	1.53	

Chemical compounds for which antennal reactions of *M. galloprovincialis* could be observed and which could give hints for pine group differentiation were tested using serial dilutions. Electroantennography results of VOC samples containing these compounds as well as of serial dilutions are illustrated in Table 4.2. Reactions for

- benzyl acetate (listed in block 1) was found only for control samples,
- eucalyptol (block 2) for controls, drought stress and PWN infested samples,
- compounds of block 3 for controls, but also or mainly for drought stressed and PWN infested samples,
- hexyl acetate (block 4) for controls and drought stressed samples,
- compounds of block 5 only for drought stressed samples and
- methyl benzoate (block 6) for drought stressed and PWN infested samples.

Table 4.2 Results of chemical compounds (+ CAS no.) tested with *Monochamus galloprovincialis* using electroantennography for number of observed antennal reactions to VOC samples per collection date (1: four, 2: eight and 3: nine weeks after test start) and pine group (controls, drought stressed, PWN inoculated) and for concentration at significant reaction start (serial dilutions starting from 10^{-7}), n per variant see in brackets

			No. observed antenna reactions to VOC samples									
			Collection date 1			**Collection date 2**			**Collection date 3**			
	Compounds	**CAS**	Controls (5)	Drought stressed (6)	PWN infested (4)	Controls (5)	Drought stressed (10)	PWN infested (4)	Controls (3)	Drought stressed (9)	PWN infested (8)	**Concentration significant reaction start**
1	Benzyl acetate	140-11-4	1			1						10^{-2}
2	Eucalyptol	470-82-6	2		2	1	1					10^{-7}
	Bornyl acetate	76-49-3	4	1	2	2		1	1		1	10^{-3}
	D-Limonene	5989-27-5	2				2	1	1	4	1	10^{-3}
3	L-pinocarveol	547-61-5	1				2	2			3	10^{-3}
	β-Myrcene	123-35-3	3	4	3	1	7	2	1			10^{-6}
	Terpinolen	586-62-9	3	1		1	5	1				10^{-7}
4	Hexyl acetate	142-92-7	2		3	1						10^{-7}
	6-Methyl-5-heptene-2-one	110-93-0					1			1		10^{-2}
5	(Z)-Hex-3-en-1-ol	928-96-1								3		10^{-3}
	Nonanal	124-19-6					2			1		10^{-3}
	Ethylhexanol	104-76-7					2			1		10^{-3}
6	Methyl benzoate	93-58-3					2	1		1	1	10^{-3}

Figure 4.5 illustrates an example of *M. galloprovincialis* antennal reactions to a VOC sample of a PWN infested tree. L-pinocarveol could be identified.

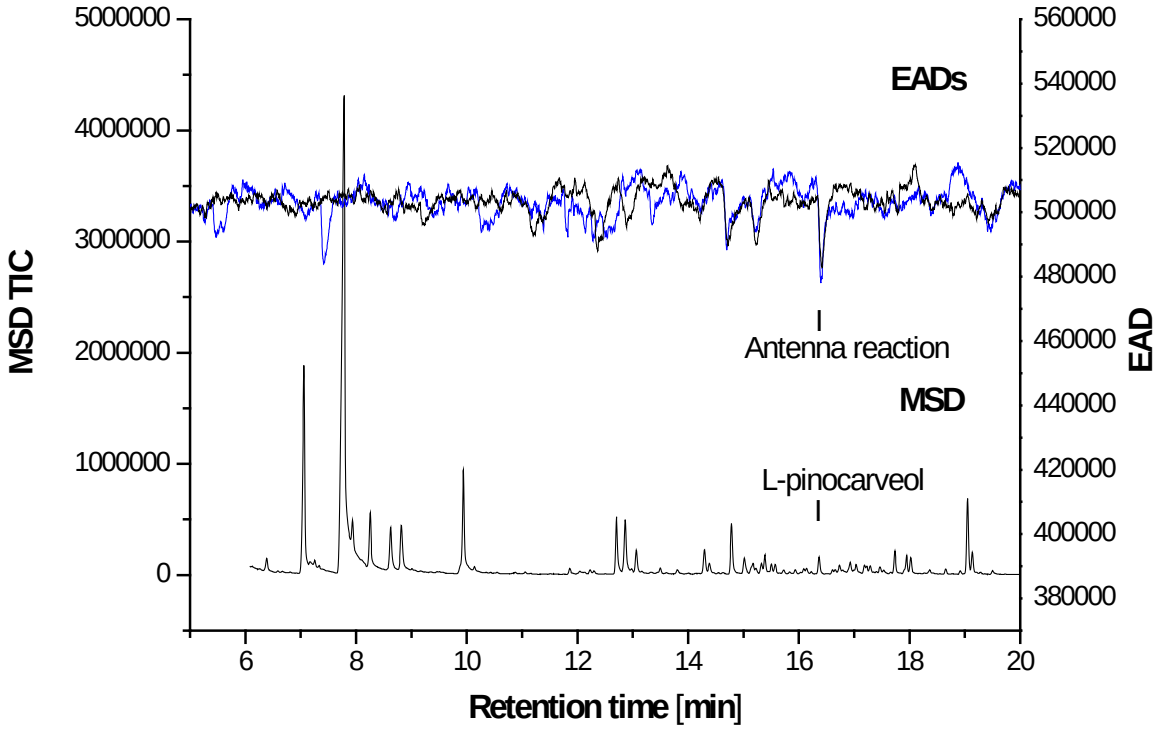


Figure 4.5 GC-MS/EAD recording of odour samples from PWN-infested pine trees using antennae of *Monochamus galloprovincialis*; identification of L-pinocarveol

Highlights of results obtained in Task 4.1

1) Smoke volatile 1 could replace α-pinene as a more suitable synergist of the standard lure. However, since results were not very consistent, further testing should is recommended to confirm this finding.

2) The integrated dispenser can replace the G2D dispenser. Again, more tests are needed to operationally replace the G2D well-established effectiveness.

3) Limonene isomers seems not to be practical for use as repellents against *M. galloprovincialis*

4) No VOCs that are antennally detected by *M. galloprovincialis* have been found exclusively associated with PWN-infested pines. Some compounds associated with both stressed and PWN-infested but not to control trees would be worth field testing

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

Highlights of results obtained in Task 4.2

Activities for this task were carried out in 2011 and 2012. As proposed objective were accomplished, no further activities have been undertaken in 2014.
The results of these studies have just been published:

Álvarez G, Etxebeste I, Gallego D, David G, Bonifacio L, Jactel H, Sousa E, Pajares JA 2014. Optimization of traps for live trapping of Pine Wood Nematode vector Monochamus galloprovincialis. J. Appl. Entomol. on line.

As was already outlined, Deliverable 4.2: Development of traps for monitoring & control has been accomplished.

At least two commercially available Teflon-coated, extended collector, traps are efficient enough to be recommended for monitoring and for control of *M. galloprovincialis* and for live trapping of adults:
Econex SL (Murcia, Spain):

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

However, the unknown durability of the Teflon treatments over time must be taken in consideration. Tests are underway by the manufacturer with the aim of providing users with an easy way to re coat the traps with Teflon.
It is likely that new designs that would appear in the future will require field testing.

Task 4.3. Assessing of potential mass-trapping of vectors

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.

Activities by B9

A mass trapping experiment was carried out in Spain in 2013 (see 2013 mid-term report). Preliminary analysis of results was carried out in 2013 and formed the basis of further work in 2014. Determining beetle population density is a key feature in order to determine the proportion of beetles that are removed by the mass trapping effort. For that, we initially used the model approach "Spatially Explicit Capture Recapture" by M.Efford (SECR; R library), which is based in the capture-mark-release and recapture of native beetles. This method of analysis is however to be applied only in "closed type" populations which may not have applied in our case. Eventually, during 2014, the Jolly Seber (JS) approach to calculate density for "open" populations was applied, using the same data from the Capture-Mark-Recapture experiment.

The JS method quantifies beetle population abundance and, to transform this into density, a determination of the corresponding area is required. Several methods were assessed: seasonal sampling range (561.76 ha), the mean maximum distance

covered by the beetles between trapping events (517.43 ha) and the defined study area (260 ha). The later was finally chosen, so that it offered the most conservative estimates, that is, the higher beetle density and thus the lower beetle proportion removed by mass trapping.

Results were highly relevant to the task objective, and a significant relationship between trapping density and proportion of population removal was fitted (Figure 4.6). Even within this conservative approach, a fairly high proportion of beetles can be removed within a single mass trapping season (i.e. 60% using a trap density of 0.44 traps/ha), for an estimated moderate beetle density of 82 beetles/ha.

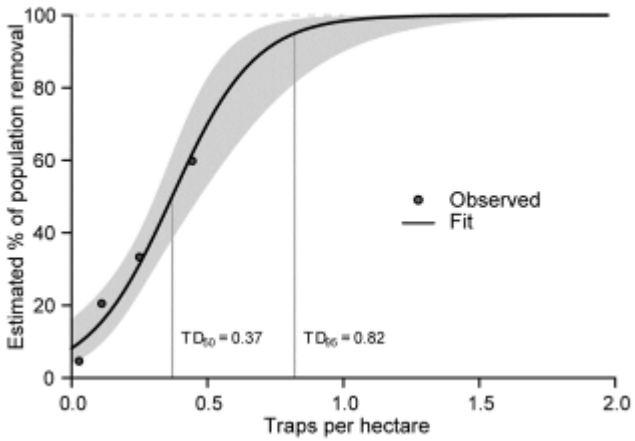


Figure 4.6 Relationship between trap density and population removed after an experiment in Spain (2013)

These results have been submitted for publication:

Sánchez-Husillos E., Etxebeste I., and Pajares J.A. 2014. Effectiveness of mass trapping in the reduction of *Monochamus galloprovincialis* Olivier (Col.: Cerambycidae) populations. J. Applied Entomology (submitted).

Experiment 5/14

The above relationship was obtained for a moderate beetle density. It is to be expected that it would be affected by beetle density, so in 2014 a similar experiment was carried out in Pino del Río (Palencia, Spain) within a *P.sylvestris* reforested stand where the local *M. galloprovincialis* population was estimated to be low.

Nine mass trapping 300x300 m plots, testing 3 trap densities (distances), were distributed within the stand in a randomized design of 3 blocks (Figure 4.7). Adjacent plots were separated by a 100m wide buffer and blocks by at least 200m. Trap densities (distances) tested were: 0.11 trap/ha (300m), 0.44 trap/ha (150m) and 1 trap/ha (100m).

For determining beetle density by Capture-Mark-Recapture, 15 traps were set at 150m distance on a regular grid within a 35 ha adjacent stand. Native trapped adults were marked weekly and released near the place of capture. For extra information, 217 laboratory-obtained immature adults were also released within the mass trapping (10 in each of the 12 plots) and the density (127) blocks. In total, 57 traps were checked weekly from June 13th to October 22th.

Figure 4.7 Layout of experiment 5/14 on mass trapping in Spain (2014)

Results

A total of 631 *M. galloprovincialis* individuals were caught in all blocks. Native beetles captured twice totalled 78. With respect to the laboratory reared beetles that were released, 62 individuals were recaptured twice and 12 were recaptured three times. Results are currently being analysed.

Highlights of results obtained in Task 4.3

Results to date confirm the potential of the trapping system (lures and trap) for mass trapping and population reduction of PWN vectors in particular areas.

1) Experiments in Spain have enabled the fitting of a curve relating population removal to trap density. This relationship, for a moderate-high beetle density indicates that a single mass trapping campaign might be able to remove a high proportion of the beetle population.

2) A Similar relationship is being worked out for low beetle densities.

Thus, Deliverable D 4.3: Effectiveness of mass trapping for vector control, has been achieved. Further analysis of the experiment during 2014 would complete the picture, extending the range to other beetle densities. This provides managers with a tool to determine the suitable trap density to be used in mass trapping programmes related to their specific objectives and resources.

Task 4.4 Development of attractants for other European *Monochamus*

The aim of this task was to develop traps and lures for *M. sutor* and *M. sartor*, the other European *Monochamus* species considered likely to be vectors of PWN.

It has been already determined during the project, and published, that *M. sutor* releases the same aggregation pheromone as *M. galloprovincialis*, and responds to the same kairomonal compounds, so that both sexes of this species can be efficiently caught by the standard Galloprotect 2D lure which was the best attractive blend tested for *M. sutor*.

Activities by B2 and B9

Earlier work had shown that *M. sutor* males rely on a contact sex pheromone present on the female cuticle for discriminating mates. In particular, the presence of two male specific peaks, also present in *M. galloprovincialis* males was demonstrated. Results from experiments carried out in 2103 to explore the role of these male specific compounds of these species suggested that these male specific peaks are key in *M. sutor* male mate discrimination (see 2013 mid-term report).

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

Experiment 6/14

As only a low number of beetles could be tested in previous years, bioassays were continued in 2014. Several male and female cuticular fractions were applied to decoys (glass rods) and offered to males as proxies for mates (Figure 4.8). At the same time chemical analysis was carried out to identify the male peaks.

Figure 4.8 Bioassay testing of cuticular fractions in relation to *M. sutor* male mate attempts (2014)

Mate bioassays showed that M. *galloprovincialis* and *M. sutor* recognise conspecifics by species-specific suites of cuticular hydrocarbons. However, they distinguish sex by presence of male-specific, not necessarily species-specific, compounds in the cuticle.

The male-specific compounds have been identified and synthesised and shown to be ethers, possibly related biosynthetically to components of the long-range aggregation pheromone. This is the first time male-specific components in the cuticular hydrocarbons have been found to be responsible for sex discrimination in cerambycid beetles, and significant progress has been made in understanding close-range mating behaviour of *Monochamus* spp.

Activities by B3

It was previously found that *M. sartor* produced the same aggregation pheromone as *M. galloprovincialis* and *M. sutor*, and that it was attracted to the same standard combination of pheromone and kairomones (see 2013 mid-term report).

Experiment 7/14

In 2014 one experiment was performed in Austria to corroborate results on attractiveness of pheromone plus kairomones. The experiment was set up in a mountainous mixed spruce managed forest at 1040-1220 m elevation. Three treatments were set up in five blocks (replicates): (1) the pheromone 2-undecyloxy-1-ethanol (= P), (2) the bark beetle kairomones ipsenol plus 2-methyl-3-buten-2-ol (= IM), and (3) pheromone plus ipsenol and 2-methyl-3-buten-2-ol, that is the standard G2D lure (= IMP). Traps were checked weekly throughout the 6 weeks trapping period in July-August 2014.

Results

The experiment confirmed the attractiveness of 2-undecyloxy-1-ethanol for *M. sutor*; when the pheromone was added to bark beetle kairomones. Average trap catches were 3.9 times and significantly higher than with kairomones alone (Figure 4.9).

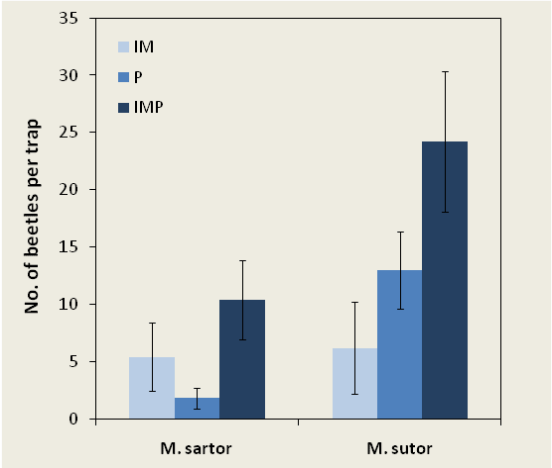


Figure 4.9 Mean trap catches during six weeks period (means ± SE) in traps baited with (1) the pheromone (P), (2) the kairomones (IM), and (3) pheromone plus kairomones (IMP).

Catches of *M. sartor* were lower in this managed forest than in the unmanaged conservation areas (2013 mid-term report); the 1.9-fold increase in average catches in traps with pheromone plus kairomones compared to kairomones alone was not statistically significant. The experiments in 2013 and 2014 unequivocally demonstrated the field attractiveness of the pheromone 2-undecyloxy-1-ethanol for *M. sutor* in spruce forests in the eastern Alps. Results were not significant for *M. sartor* in 2014 due to overall low capture of this species. However, the indicated trend of higher catches in pheromone baited traps is in agreement with experiments from 2013, where a significant 4.8-fold increase in catches in traps was evidenced (previous periodic report).

Highlights of results obtained in Task 4.4

1) Male cuticle specific peaks are involved in *M. sutor* mate discrimination, as in *M. galloprovincialis.*

2) As for *M. sutor*, the standard pheromone plus bark beetles kairomone blend described for *M. galloprovincialis* can be efficiently used for *M. sartor* monitoring and control.

Thus Deliverable D 4.4 Development of lures for other *Monochamus spp*. (due M 35) has been achieved. *M. sutor* and *M. sartor* pheromones have been identified and, consequently, there is an effective lure available already for monitoring, and even control, of these species (Galloprotect 2D).
Progress in our understanding the role of specific peaks on mate discrimination of *M. galloprovincialis* and *M. sutor* has been made.

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

No deviations from Annex I and from critical objectives have occurred during this extension period.

Statement on the use of resources

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

WP 5 Determine risk of non-vector spread of PWN through various pathways to healthy forests

Objectives for the period

The objectives of this WP are:

- 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 for the period

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 (30-11-2013).

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 (30-11-2014).

D5.3: Direct tree to tree transmission of PWN: Assessment of transmission from tree to neighbouring tree through the soil or by root contact (30-11-2013).

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 (30-11-2014).

D5.6: PWN genetic diversity as indicators of invasion history: Assessment of genetic diversity of European PWN populations in relation to invasions (30-11-2014).

Progress during reporting period 01.03.2014 - 30.11.2014

Overall comment for WP 5: As already stated in the previous reports some parts of the work have been done by B6 and B1 during a research project outside REPHRAME covering similar questions as raised in WP 5 of the REPHRAME proposal. The results have been 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.) 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 shifted

within WP 5 to allow other tasks to be investigated in more detail or with a higher sample size than originally planned.

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

5.1.1 and 5.1.2 see previous reports

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

Work carried out by B5 (JKI):

Supplement to 2nd report.

Materials and methods:

Breeding and identification of fungi:

Subsequent to the long-term survival test of PWN in infested *P. sylvestris* wood chips from 2012 until 2013 (see Second Periodic Report) fungi occurred in wood chips were isolated and tested for PWN survival and reproduction. PWN in wood chips with open storage at 15 °C survived nearly one year and PWN in wood chips sealed in plastic bags at 15 °C as well as 25 °C (Figure 5.1) survived for more than one year. Therefore four wood chips of each of the five samples of these three variants of the last extraction date (after 369 days) were individually placed on 1.5 % malt extract agar plates (Figure 5.2). Chlortetracycline (antibiotic) in a concentration of 500 mg/l was diluted in 70 % ethanol (2-3 ml), sieved (sterile filter) and added to the malt extract agar after autoclaving and cooling down according to Oberwinkler *et al*. (1995). Two of these four wood chips per sample were surface sterilized. For the surface sterilisation the wood chips were placed in 70 % ethanol for 30 sec., followed by 3 % natrium hypochloride for 2 min and 96 % ethanol for 30 sec. and were two times rinsed in sterile water referring to Schöder (1999). The plates were covered and stored for incubation at room temperature (approx. 20 °C). On a daily basis the fungi plates were checked to isolate all developing fungi from each other and from the wood and to transfer them to fresh agar plates. The fungi genera were morphological identified using identification keys of BARNETT AND HUNTER (1998) and WATANABE (2002). The dominant fungus species was additional molecular identified with the help of Dr. Benjamin Pickel (Department of Molecular Wood Biotechnology and Technical Mycology, Buesgen-Institute, Georg-August-University Göttingen, Germany) as well as of Dr. Wolfgang Maier (Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institut Braunschweig, Germany) and multiplied for testing of PWN survival and reproduction.

Figure 5.1 Sealed *P. sylvestris* wood chips

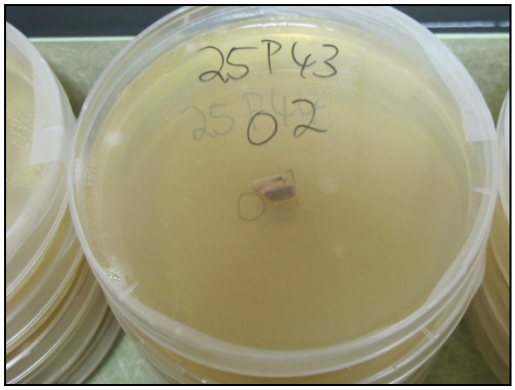


Figure 5.2 *P. sylvestris* wood chip on malt extract agar

PWN reproduction:

Extracted PWN from wood located in Portugal in the year 2013 (provided by Dr. E. Sousa – Instituto National de Recursos Biologicos) were reared on non-sporulating *Botrytis cinerea* on 1.5 % malt extract agar medium at approx. 20 °C. The most often from wood chips isolated fungus species was reared on 25 ml agar plates (using a tilt automat). On 9th April 2014 a suspension of 100 *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) in 50 µl water was pipetted on each of ten fungi plates (two weeks old, without antibiotic). The plates were stored for incubation at 24 °C in an incubator for three weeks. After nematode extraction a second trial cycle using the same inoculum size, but nematodes of the single plates of the first cycle, was planned to enhance the significance of the later gained result. Living nematodes were extracted using the Baermann-funnel technique according to BAERMANN (1917) modified for plant parasitic nematodes according to DECKER (1969). The nematodes were counted. A differentiation between males, females and juveniles was planned.

Results:

The isolated fungi genera of all tested PWN infested wood chip variants after a storage time of more than one year is listed in Table 5.1.

Table 5.1 Isolated fungi genera and number per PWN infested wood chip variant after a storage time of more than one year, n=10

Variant	Fungi genera	No.
15 °C - open storage - surface sterilised	Sterile wood chip	10
15 °C - open storage - not surface sterilised	*Trichoderma* spp.	10
	Rhizopus spp.	1
	Penicillium spp.	1
15 °C - sealing - surface sterilised	*Trichoderma* spp.	9
	Sterile wood chip	1
15 °C - sealing - not surface sterilised	*Trichoderma* spp.	10
25 °C - sealing - surface sterilised	*Trichoderma* spp.	10
25 °C - sealing - not surface sterilised	*Trichoderma* spp.	10
	Graphium spp.	1
	Botrytis spp.	1

Trichoderma spp. was most often isolated and therefore additionally molecular identified as *Trichoderma atroviride* (Figure 5.3).

Figure 5.3 *Trichoderma atroviride* on malt extract agar

The test for survival and reproduction of PWN on *T. atroviride* was negative. After an incubation time of three weeks no PWN could be extracted. The test was repeated and came to the same statement.

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; see also comment in 2nd report.

Materials and methods:

Experimental set-up:

Petri dishes (ø 5.5 cm) were filled with 10 ml 1.5 % malt extract agar using a bottle topper dispenser (Dispensette® ISO 9001 CERTIFIED, Brand) for breeding of sporulating *Botrytis cinerea*. The plates were covered and stored for incubation at room temperature at approx. 20 °C for two weeks. On 08th August 2014 in the middle of each plate a suspension of 400 PWN (PT-7 (w)) and 100 µl tap water was pipetted. The plates were sealed with parafilm and stored for incubation at 25 °C in a climate chamber (Figure 5.4) until PWN reproduced and emptied nearly all plate. The Petri dishes were stored in eight different climate chambers at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C or 60 °C for a time span of 24 h, 48 h, 72 h, 96 h or 120 h. The previous incubation temperature of 25 °C for reproduction was used for the control samples. Per variant 10 replicates were tested.

Figure 5.4 PWN suspension on sporulating *Botrytis cinerea* after six days incubation at 25 °C

Nematode survival according to temperature and time span:

At the end of the different test periods PWN were extracted by cutting the PWN infested fungi plates in pieces using a sterile spatula. Per sample all agar pieces were placed inside two filter papers (kitchen roll paper) plus the rinsed nematodes, which remained on the Petri dish. The closed filter papers were completely covered with water inside a funnel (Baermann-funnel technique according to BAERMANN (1917) modified for plant parasitic nematodes according to DECKER (1969)). During 24 h all living nematodes could be extracted. For recording of the percentage distribution of males, females and juveniles at test start and after first incubation at 25 °C for PWN reproduction 40 suspension drops and 40 plates were sampled. PWN were identified, fixated and counted.

Results:

The sporulating *Botrytis cinerea* agar plates were nearly emptied by PWN after 14 days. The start inoculums consisted of 5 % males, 11 % females and 84 % juveniles. The same result was gained for the distribution of males, females and juveniles after first incubation at 25 °C for PWN reproduction.

After storage of the samples at different test temperatures and time spans the extraction of nematodes showed the following results illustrated in Table 5.2.

This investigation showed that not all temperatures occurring in wood chip piles are lethal for PWN. The temperature and period had an influence on the survival. PWN did not survive temperatures of 50 °C, 55 °C and 60 °C for 24 h. In contrast at 25 °C, 30 °C, 35 °C and 40 °C living PWN could still be found after 5 days in a dimension of thousands of nematodes. At 45 °C a time dependency for lethality during these 5 days could be found. From a median of 25200 of extracted nematodes after 24 h only one nematode on three plates could be observed after 48 h, which the median in Table 5.2 does not show. After 72 h no PWN could be extracted. Therefore with this 5 °C- and 24 h-incremental study with temperatures occurring in wood chip piles temperature borders for PWN survival tested with *B. cinerea* plates were found at 45 °C after 72 h and at 50 °C after 24 h.

Table 5.2 Extracted living PWN from sporulating *Botrytis cinerea* plates after different incubation temperatures and time spans (medians), n=10

	Time [h]				
Temperature [°C]	24	48	72	96	120
25	71000	70500	99000	44000	42500
30	111000	79000	89000	68750	71500
35	83667	105500	47500	92000	53500
40	112500	94000	62000	51250	23250
45	25200	0	0	0	0
50	0	0	0	0	0
55	0	0	0	0	0
60	0	0	0	0	0

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Work carried out by partner B6 (INIAV) and B11 (Bioforsk)

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

Proposals concerning potential outdoor trials have been exchanged with the Canadian Forest Service in Victoria/Canada as well as personal exchanges of views during meetings such as International Forestry Quarantine Research Group etc. While the on the scientific side the proposed research was agreed to be interesting, the main problem was to find ways to carry out the investigations on site. Over the time it became clear that carrying out the relevant trials including inoculating the trees, supervise them over months and finally assess them in detail would need more personal and financial input than expected. In addition financial limitations of the Canadian partners made it impossible for them to fully support the investigations from their own budget. Parallel money of the REPHRAME project was also limited as it would have been possible that a scientist of the core REPHRAME team went to Canada to carry out the trials there. Though quite intensive negotiations have been carried out it was not possible to figure out a feasible way to carry out the planned corresponding outdoor trials.

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 (Figure 5.5). Chips were produced from PWN inoculated logs. Trees were planted in sand mixed with 2l of chips containing 300 nematodes per gram (fw) wood. In controls sand was mixed with fresh chips. In all treatments n =12. The plants were kept under observation for wilting symptoms and by the end of the experiment (14 months) were all sampled for PWN infestation regardless of the symptoms. Periodically, in the first 90 days of the experiment, the nematode community in the soil was analysed.

Figure 5.5 Chip transmission experiment with *Bursaphelenchus xylophilus*: a) artificially damaged roots of *Pinus pinaster* plants; b) addition of pine chips to the potted plants; c) experimental set-up.

Results

By the end of the experiment, 33% of the trees that received PWN-infested chips were dead, with nematode densities ranging from 0 to 16200 per 100 g stem wood (fw). One of the plants in the treatment with nematode-infested chips and one of the

control plants with intact roots died although PWN was not detected in the stem wood (Table 5.3). PWN was not detected in any of the symptomless plants in the four experimental treatments that were analysed.

Table 5.3 Result of PWN analysis in dead *Pinus pinaster* plants of the chip transmission experiment

TREATMENT *300 PWN per gram wood chips (fw)	PLANT NO.	PWN per 100 g stem wood (fw)
Control* intact root	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	47	1 128
Infested chips* damaged root	48	16 200

In the soil, saprophagous nematodes in the order Rhabditida dominated the material, followed by the miscellaneous feeders (omnivores) in the family Dorylaimidae. Fungal feeders, i.e. genera *Aphelenchus*, *Aphelenchoides*, *Boleodorus* and species in *Tylenchus sensu lato* were noted less frequently. Strict root-parasites (i.e. genera *Tylenchorhynchus*, *Pratylenchus*, *Rotylenchus* and *Helicotylenchus*) occurred in low frequencies. Strict predators in the family Monochidae were rare. The pinewood nematode *Bursaphelenchus xylophilus* was absent in the material.

Table 5.4 Nematode community in soil samples taken in the wood chip transmission experiment. Relative occurrence (%) of nematodes.

[1)] Four soil samples were taken from each of the four treatments, i.e. “nematode-free chips intact root”, “nematode-free chips damaged root”, “nematode-infested chips intact root” and “nematode-infested chips damaged root”; [2)] Samples taken from all pots in the treatments with nematode-infested chips.

Ecological group	Order	Family/Genus/ Species	Sampling, days post-inoculation					
			0[1)]	5[2)]	10[2)]	30[2)]	60[2)]	90[2)]
			n=16	n=24	n=24	n=24	n=24	n=24
Saprophages	Rhabditida	Rhabditidae	100	96	96	92	100	100
Plant nematodes	Aphelenchida	*Aphelenchus*	50	13	4	4	21	21
		Aphelenchoides	19	17	21	8	21	13
		Bursaphelenchus xylophilus	0	0	0	0	0	0
	Tylenchida	*Tylenchus sensu lato*	44	0	8	13	29	17
		Ditylenchus	0	0	4	0	4	8
		Boleodorus	19	0	4	4	4	25
		Tylenchorhynchus	19	0	4	0	8	0
		Pratylenchus	0	0	8	0	21	0
		Rotylenchus	6	4	13	4	4	0

		Helicotylenchus	6	0	13	4	4	0
Omnivores	Dorylaimida	Dorylaimidae	44	63	54	58	38	54
Predators		Mononchidae	6	0	0	0	4	0

Air and soil temperatures were monitored during the experiment (Figure 5.6), indicating that the conditions for the development of PWN were met.

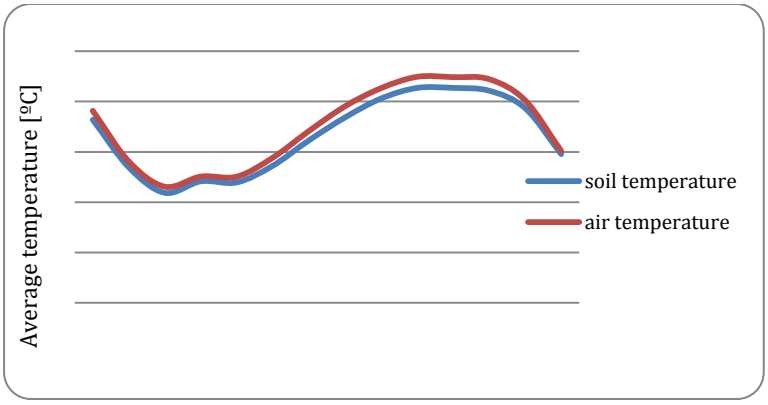

Figure 5.6 Average soil and air temperature in the greenhouse experiments.

Discussion

In the literature dealing with *B. xylophilus*, studies on the nematode transmission from infested wood to living trees are uncommon. In most experiments wounded roots have been a prerequisite for successful transmission from nematode infested soil (Mamiya & Shoji 1989) or chips (Halik & Bergdahl 1992) to roots. Our experiment also demonstrated that PWN can infest small trees of *P. pinaster* with artificially damaged roots. Four out of 12 trees with damaged roots became infested by the nematode.

Nematode densities in infested trees were significant, ranging from 420-16,200 individuals per 100 gram fresh stem wood. Nematode infestations of plants with damaged roots planted in soil-chips mixtures have previously been reported in 3-5 year-old plants of *Pinus sylvestris*, *P. strobus,* and *P. resinosa* (Halik & Bergdahl 1992). Early experiments in Japan demonstrated that PWN infestation of trees could be established from nematode-infested wood discs buried in soil around roots on 14-year-old *P. thunbergii*, however in this case the roots were reported as undamaged. In our study 33 % of the trees were infested by PWN. Kiyohara & Tokushige (1971) reported an infestation frequency of 50 %, while Halik and Bergdahl (1992) recorded frequencies of 50 % in *P. sylvestris* and 8 % in *P. strobus* and *P. resinosa*. The present study is the first study of soil transmission of PWN to *P. pinaster.*

In previous studies the occurrence of *B. xylophilus* in soil during the infestation process has not been studied in detail, but it has been noted that the nematode can survive in soil for 48 hours to 1 month (Mamiya & Shoji 1989). In the present study we were unable to detect PWN in soil samples taken regularly up to 90 days post-infestation. The nematode fauna recorded in this experiment showed a normal composition and occurrence of nematode groups and genera.

Test of the non-vector spread of PWN from artificially infested *P. pinaster* wood boards to *P. pinaster* trees in the field

Materials & Methods

Transmission studies from infested boards to adult *P. pinaster* trees in natural stands were performed in spring 2014. Fresh pine boards were inoculated with PWN under controlled conditions, yielding infested boards averaging 100 nematodes per gram (fw) wood. The boards were tied to healthy pines (27-41 cm DBH) in three different arrangements: (1) board tied to tree on intact bark, (2) board tied to tree with exposed cambium and (3) board tied to tree with exposed inner wood (Figure 5.7). Fresh pine boards were used as controls. In all treatments n =5. The trial took place before the period of flight of the insect vector in order to ensure that no insect-vectored nematode infestation of the pines occurred. Forty-five days after the establishment of the experiment in the field the trees were felled and both the boards and the trunk adjacent to the board were sampled for PWN infestation.

Figure 5.7 Board transmission experiment with *Bursaphelenchus xylophilus*: a) board tied to tree on intact bark; b) board tied to tree exposed cambium; c) board tied to tree inner wood.

Results

Both in the treatment with boards attached to the exposed cambium and for boards attached to the inner wood, nematode transmission occurred in one of five trees. In both cases the nematode densities in the adjacent trunk wood were 100-300 per 100 g wood (fw) (Table 5.5). All boards initially inoculated with nematodes were still infested at the end of the trial.

Table 5.5 Result of the PWN board transmission experiment to *Pinus pinaster*

TREATMENT *100 PWN per gram wood boards (fw)	Number of trees with Bx	
	infested board (n=5)	Control (n=5)
Board tied to tree on intact bark	0	0
Board tied to tree exposed cambium	1	0
Board tied to tree inner wood	1	0

Discussion

The results demonstrate the capacity of *B. xylophilus* to infest living trees through wood contact. This is a unique study never done before and revealed that infestations can take place in situations when nematode-infested lumber comes into contact with exposed cambium or xylem of a living tree. One out of five trees (20 %) was infested in this experiment. For technical reasons the trial had to be restricted to 45 days only. If the experiment had been continued for a longer period of time, there is a possibility that the frequency of infestation could have been higher. Although, *B. xylophilus* is known to be capable of moving over bark surfaces (Arakawa & Togashi 2002), infestation of the tree did not take place through intact bark. The presence of bark in this case seems to have protected the tree from nematode infestation. It could be that the steeper moisture gradient established between donor wood and the cambium/xylem of the tree has stimulated the nematode transmission. Infestation of trees as a result of contact transmission has previously been observed in dense natural stands of *P. sylvestris* where branches come in close physical contact (Malek & Appleby 1982).

D 5.3: Direct tree to tree transmission of PWN: Assessment of transmission from tree to neighbouring tree through the soil or by root contact

Green house experiments

Materials & Methods

Root transmission of PWN was studied on 5-years-old *P. pinaster*. The trees were potted in pairs in 30 plastic 80 L boxes. In the nematode treatment one tree in each pair was inoculated with 6 000 PWN at 6 points along the stem. For controls, one tree in each pair was inoculated with water (Figure 5.8). In all treatments n=15. The plants were kept under observation for wilting symptoms and were sampled.

Figure 5.8 Root transmission experiment in paired plants with *Bursaphelenchus xylophilus*: a-b) pine inoculation; c) inoculated plants, d) experimental set-up.

Results

The experiment with paired trees of *P. pinaster* has been running for 16 months. 69% of the inoculated pines were dead with nematode densities ranging from 500 to over 5000 per 100 g stem wood (fw). None of the neighbouring trees (recipients) of the inoculated trees showed symptoms (Figure 5.9). Mortality occurred in both donor and recipient control plants, but no PWN was detected (Table 5.6). The regular observation of the pine trees for wilt symptoms and sampling is still in progress. As shown in Figure 5.6, air and soil temperature conditions favourable for the development of PWN were present.

Table 5.6 Result of the root transmission experiment in paired plants

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

Figure 5.9 Progression in the root transmission experiment with *Bursaphelenchus xylophilus* in paired trees of *Pinus pinaster* showing inoculated plants with wilting symptoms and recipient plants still green.

Discussion

No mortality was observed in the recipient-plants in spite of the fact that 11 out of 16 donor-plants died from the nematode infestation with high nematode densities in the stem wood. One possible route for infestation between infested trees and healthy neighbour trees would be through root grafts. Root grafts occur in at least 26 *Pinus* spp. (Gleason & Fulling 1966). Root grafting is important for within-stand spread of pathogens like *Armillaria mellea*, *Phellinus chrysoloma*, *Ceratocystis ulmi* and *Heterobasidium annosum* (Gleason & Fulling 1966, Johansson & Unestam 1988).

However, the frequency of root grafting may vary between species of pine. Roof grafts have not been detected in maritime pine *P. pinaster* in Portugal (PHRAME 2007). Mamiya and Shoji (1989) reported that *B. xylophilus* normally needs to be inoculated at a distance shorter than 1 cm from wounded roots of *P. thunbergii* and *P. densiflora* to infest successfully. The kind of root contacts occurring in our experiment may not have been physically close enough to allow for nematode transmission.

Field experiments

Materials & Methods

The experiments on root transmission in the field were carried out in the Lisbon area and were located at Companhia das Lezírias and Mata da Machada. A third plot was established in Herdade da Comporta; however due to constraints on access to the property, it was not possible to continue the experiment in this location.

Each experimental plot was selected based on the presence of healthy trees with ca. 40 cm DBH with smaller trees nearby. The absence of *B. xylophilus* on those trees was confirmed by laboratory analysis. The plots were georeferenced and the surrounding small trees were covered with net structures to prevent access by vector insects. The healthy trees were ring barked to cause weakening stress and were inoculated at the base with 50 000 PWN (10 x 5 ml of a suspension of 1000 nematodes/ ml) using Arbojet Quick-Jet kit (Figure 5.10).

Figure 5.10 Root transmission experiment with *Bursaphelenchus xylophilus* in the field: a) net coverage of small pines; b) pine inoculation; c) inoculated pine cut in ring to cause weakening stress.

The trees inoculated with *B. xylophilus* were sampled one month after inoculation to confirm the effectiveness of this process. The covered pines are being observed regularly for wilt symptoms detection and sampling.

Results

Four months after the start of the experiment, the inoculated pines exhibited wilting symptoms (Figure 5.11). In Mata da Machada settlement, one small covered tree died but no *B. xylophilus* was detected. Observation of the covered trees is still in progress.

Figure 5.11 Pine inoculated with *Bursaphelenchus xylophilus* in Mata da Machada presenting wilting symptoms.

Discussion

This experiment is the first of its kind both regarding the host tree *P. pinaster* and the design of the experiment, involving mature trees and their undergrowth in forest sites.

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(5.4.2) Importance of infection 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.

This study had to be omitted by B 11 due to lack of facilities and the uncertainty of whether the extension to the project would be approved. The results from the wood to wood transfer experiments, reported in the EPPO Bulletin, also suggested that the work was not essential in relation to possible movement of nematodes from moist sources to moist sinks.

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

Work of P4: INRA UMR 1355 / University of Nice Sophia Antipolis / CNRS UMR7254
Institut Sophia Agrobiotech
400, route des ChaWppes, BP167, 06903 Sophia Antipolis cedex, France
E-mail : philippe.castagnone@sophia.inra.fr

• List of P4 scientific and technical personnel involved:

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:

P7: University of Evora, Portugal (Dr. Manuel Mota, Dr. Paulo Vieira, Margarida Espada).

Specific sub-objectives of B4:

- to develop a set of microsatellite genetic markers for use in PWN genetic characterisation (deliverable D5.5 achieved)

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

Work progress and achievements during the project

Our main objective in the project was to characterize the genetic diversity of pinewood nematode populations from both the native and the invaded areas, and to use this information to infer the worldwide routes of invasion of the PWN based on population genetics approaches.

For that purpose, our first goal was to develop a set of molecular markers that could be used for population genetics studies, i.e., that could reveal genetic polymorphism at the individual level. Therefore, a set of 15 polymorphic loci has been characterized and validated; these are usable on single *B. xylophilus* nematodes in three complementary multiplex reactions (deliverable D5.5 achieved; Mallez et al., 2013).

Classical population genetics methods inferred inconclusive results, since they alternatively suggested either an American or a Japanese origin for all the Portuguese samples tested, without any statistical confidence evaluation for inferences of the source population (see previous report). Given the limitations of classical and Bayesian methods, we further used of a recent model-based method, the approximate Bayesian computation (ABC). ABC offers several advantages that may be crucial in studies of PWN: (i) it can take complex scenarios into account, (ii) it can manage incomplete sampling by providing the possibility of considering unsampled “ghost” populations and, most importantly, (iii) it makes it possible to evaluate quantitatively and to compare statistically the various competing scenarios, through the calculation of posterior probabilities (Guillemaud et al., 2010).

The analyses have been performed on a wide range of PWN natural samples (no laboratory multiplication on fungi) originating from both the native and the invaded areas:

- USA: 28 geographic samples (554 individual nematodes)

- Japan: 7 geographic samples (210 individual nematodes)

- China: 4 geographic samples (147 individual nematodes)

- Portugal (including Madeira): 9 geographic samples (169 individual nematodes).

Thus, a total of 1080 individual nematodes have been genotyped using the 15 microsatellite markers previously characterized (see report 1).

The ABC analyses have been performed using the DIYABC v2.0.4 software (Cornuet et al., 2014).

Thanks to this intense sampling effort from several international collaborators in infested pine forests in the native (USA) and invaded (Japan, China, Portugal) areas, we then used these 15 microsatellites to genotype more than 1,000 individual nematodes. The samples from the USA displayed significant genetic differences, highlighting the existence of a strong spatial genetic make-up of the nematode in its

native area. Spatial differentiation was detected over a very short scale, with PWN populations from neighbouring trees differentiated significantly. This suggests that PWN dispersal, whether active or passive, can be spatially limited even over a short distance and that genetic drift may play an important role at a local scale. In some cases, different genetic clusters were identified within a single tree, suggesting that different beetles carrying genetically differentiated nematode populations infested a single tree. Furthermore, some nematodes sampled in different US States were assigned to a unique genetic cluster despite the large geographical distance between them (more than 500 km). This result is in agreement with the potentially important role of the human-induced long-distance dispersal of the PWN. Conversely, we found very low levels of polymorphism in the studied invaded areas (Japan, China and Portugal, including Madeira island), suggesting single introduction events together with intense demographic and genetic bottlenecks. In the European context, these findings firmly suggest that the second outbreak detected in the centre of mainland Portugal in 2008 resulted from expansion of the first outbreak detected close to Lisbon in 1999. Our results also suggest that the PWN populations on Madeira originated from mainland Portugal, given the near identity of the populations from Madeira and mainland Portugal and the first detection of PWN outbreak on Madeira 10 years after the first outbreak in mainland Portugal (Mallez et al., 2015).

Classical population genetics methods were then applied to identify the origin of the Portuguese populations. However, these analyses inferred inconclusive results, since they alternatively suggested either an American or a Japanese origin for all the Portuguese samples tested. Given the limitations of these methods, we further used on the same data set a recently developed model-based method, the approximate Bayesian computation.

The results of this study (deliverable D5.6) can be summarized as follows:

1. At least three independent introduction events occurred from the USA into the invaded areas at a worldwide scale.

2. The origin of the Japanese PWN invasive populations is the USA. However, a second introduction event probably occurred, whose origin remains to be elucidated.

3. Two independent introductions occurred in China, one from the USA and one from Japan.

4. The most probable origin of the invasive PWN populations in Portugal is the native area of the nematode (USA).

5. The origin of the Madeira outbreak is continental Portugal.

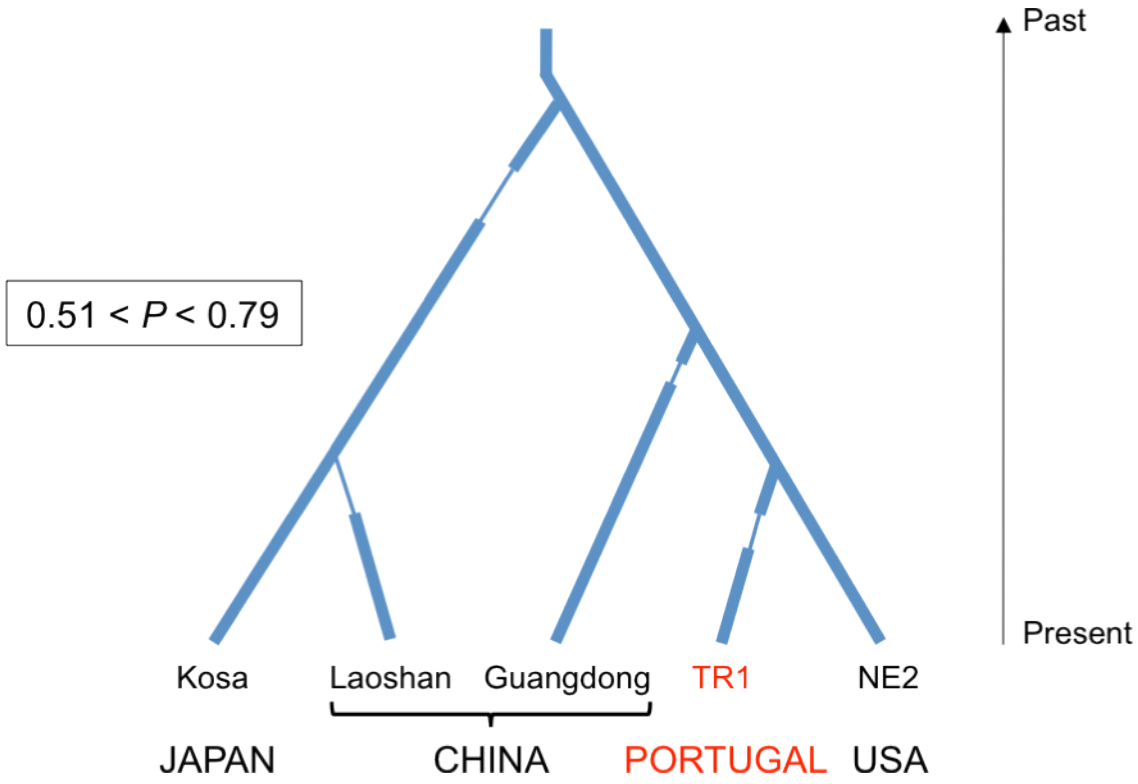

Fig. 5.12. The most probable scenario describing the invasion routes of the pinewood nematode as inferred from ABC analyses.

Clearly, these results challenge previous reports that suggested an Asian origin of the Portuguese outbreaks (e.g., Vieira et al., 2007; Metge and Burgermeister, 2008; Valadas et al., 2012; Figueirido et al., 2013). However, in our opinion, these reports suffered a number of experimental and/or analytical flaws, including the use of collection PWN strains, the analysis of bulk samples instead of individual nematodes, the low resolution of the molecular markers used and the lack of true population genetics analyses. These new data, obtained here with more adequate sampling and methodologies, are of direct practical importance for the implementation of efficient phytosanitary measures for quarantine regulation.

Publications:

Mallez S., Castagnone C., Espada M., Vieira P., Eisenback J.D., Harrell M., Mota M., Aikawa T., Akiba M., Kosaka H., Castagnone-Sereno P. and Guillemaud T. (2015). Worldwide invasion routes of the pinewood nematode: what can we infer from population genetics analyses? *Biological Invasions*. DOI 10.1007/s10530-014-0788-9.

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family of the pinewood nematode *Bursaphelenchus xylophilus* at different geographic scales. *Molecular Phylogenetics and Evolution* 70 : 120-129.

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Mallez S. (2014). Invasion du nématode du pin *Bursaphelenchus xylophilus.* Thèse de Doctorat en Sciences de l'Université de Nice Sophia-Antipolis, France. 182 pp. (PhD defence on 2nd December 2014).

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

The great majority of planned tasks were completed as planned, other than the wood to wood transfer experiments that were carried out independently of REPHRAME and therefore would have been unnecessarily duplicated if carried out again. As indicated, the sub-task 5.4.2 was also eliminated for the reasons given above.

Statement on the use of resources

Resources were used to the full extent planned but some deviation in use of those resources, as described above, took place.

WP 6 Host tree resistance to PWN and its vectors for future planting
Work carried out by B4, B5, B6, B7 and B8

Objectives for the period

The objectives of this WP are:

- 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 for the period

D6.1: Susceptibility of *Pinus sylvestris* provenances to PWN (30-11-2013)
D6.3: Identification of PWN resistance genes in pines (30-11-2014)
D6.4: Resistance of pines to feeding by *Monochamus* (30-11-2013)
D6.5: Host preferences for *Monochamus* oviposition (30-11-2014)
D6.6: Hybrid progenies with different tolerance/resistance to the PWN (30-11-2013)
D6.7: Tree species mosaics to reduce PWN impact (30-11-2014)

As recommended in the mid-term review by Dr Ilaria Pertot, Task 6.2 Testing of resistance of Pinus spp to PWN insect vectors, with sub-tasks 6.2.1 Evaluation of resistance level and identification of resistance markers during the maturation feeding phase by Monochamus spp. on cut shoots and 6.2.2 Evaluation of resistance level and identification of resistance markers during the maturation feeding phase by Monochamus spp. on seedling trees, have been eliminated. The precise recommendation was: “Major organizational changes in B6 have taken place in 2011/2012 with significant consequences on expected working capacity. Therefore for Volatile organic compounds and bark extracts the only feasible possibility is to appoint a new sub-contractor with the necessary skills to all these Tasks. The recommendation is to delete task 6.2 and reallocate the person months to other WPs”

For these reasons, the whole of 6.2 was eliminated and resources re-allocated within WP6.

Task 6.6

D6.1: Susceptibility of *Pinus sylvestris* provenances to PWN

Work carried out by partner B5 (JKI)

<u>Symptom development (Supplement)</u>

Evaluation of the pathogenicity of non-sporulating *Botrytis cinerea* towards *Pinus sylvestris*

For PWN inoculation tests *B. xylophilus* were reared on grey mould rot fungus, *Botrytis cinerea* (FR.) PERS. (anamorph). This fungus causes the grey mould disease on conifer species at all stages of production in the greenhouses and tree nurseries.

On killed needles the fungus colonizes, sporulates and enters healthy stem tissues. The tree can die especially under additional environmental stress factors (BAKER 1946, BAKER 1972, PETERSON 1974, PETERSON ET AL. 1988, SMITH 1900, SUTHERLAND 1977). To exclude this pathogenic effect of *B. cinerea* the non-sporulating form was chosen. Additionally, this trial was conducted to prove the absence of a pathogenic influence of non-sporulating *B. cinerea* in the filtrate produced in preparation of the nematode suspension as well as *B. cinerea* infested agar pieces as worst case scenario.

Materials and methods

Non-sporulating *B. cinerea* was reared on 1.5 % malt extract agar medium at approx. 20°C. *P. sylvestris* saplings of the provenance HkG 851 04 (Middle and East German Lowland) eight to nine years old and *P. sylvestris* saplings of the provenances HkG 851 14 and 15 three to four years old were tested. The trees were planted in 1 or 3 l pots and were pre-air-conditioned in a greenhouse for two weeks. For the provenance 851 04 20 controls and 20 saplings in contact with non-sporulating *Botrytis cinerea* infested agar pieces as well as for each of the provenances 851 14 and 851 15 ten controls and ten with non-sporulating *Botrytis cinerea* filtrate inoculated pines were tested. The test was run at 25°C and an average 74 % rH.

All saplings were randomized and watered as required. A non-sporulating *Botrytis cinerea* filtrate from 20 plates according to the necessary nematode plate number for Task 6.1.1 (Symptom development) was produced. Two week old *B. cinerea* plates were divided into pieces and stored on cotton paper in a funnel completely under water for 24 h according to the Baermann-funnel technique (BAERMANN 1917) modified for plant parasitic nematodes (DECKER 1969) used for preparation of suspensions for PWN trials. On 6st June 2014 300 µl of this fungus filtrate or tap water in the case of controls were inoculated in the main shoot of the previous year below the youngest whorl of the three to four year old pines (Figure 6.1).

A 1-2 cm long I-shaped slit was cut into the bark and a 9 x 1 cm cotton strip with one side fixed inside this slit. The suspension was pipetted on the folded cotton strip, which was sealed with a plastic strip and adhesive tape on both ends. Instead of a bark slit an approx. 0.5 x 0.5 cm wide square of bark was removed from the eight to nine year old pines using a scalpel to attach a non-sporulating *B. cinerea* infested agar piece (the same size) or uninfested agar piece in the case of control trees on the stem approx. 2 cm above the soil (Figure 6.2). The agar piece was covered and fixed with parafilm, a plastic strip and adhesive tape on both ends.

Figure 6.1 Inoculation of *Botrytis cinerea* filtrate/ tap water in the bark slit of the pine shoot of the previous year below the youngest whorl

Figure 6.2 Attaching of *Botrytis cinerea* infested agar/ uninfested agar piece on a bark free square on the pine stem above the soil

Assessment of pine wilt:
A rating scheme of wilt classes (Table 6.1) according to DAUB (2008) was used for visual evaluation of pine wilt symptoms weekly for 12 weeks. 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

Results

The younger saplings of the provenances 851 14 and 851 15 had a similar quality and showed no significantly different results and were therefore summarized.

Table 6.2 shows the distribution of observed wilt classes (with 0: 0 %; 1: 1-25 %; 2: 26-50 % tree coverage of needle discoloration) of the non-sporulating *B. cinerea* inoculated or attached to pines and their controls in week 12.

Table 6.2 Distribution of wilt classes of 3-4 years old trees inoculated with non-sporulating *B. cinerea* filtrate/ tap water (controls) and 8-9 years old trees stem-attached with non-sporulating *B. cinerea* infested agar/ uninfested agar (controls) at test end, n=20.

Variant + Medium	Wilt class 0	Wilt class 1	Wilt class 2
B. cinerea inoculum	17	3	0
Control inoculum	19	1	0
B. cinerea infested agar	5	14	1
Control agar	12	7	1

No pines died. The younger inoculated trees showed for the fungus filtrate inoculation as well as for the control variant similar results with 85-95 % wilt class 0 until the end of the test. The rest developed wilt class 1 starting in week 12. All older trees attached with the fungus or uninfested agar piece showed wilt class 0 at 100 % until week eight. Starting in week 9 (August) wilt class 1 developed. Until week 12, 35 % of the controls and 70 % of the fungus attached trees represented wilt class 1, which could be attributed to needle cast. Wilt class 2 appeared only in the last week for both variants with 5 %. At the end of the test no significant differences between all four test variants neither for wilt class 0 nor for wilt class 1 were found using the Chi^2 test.

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D6.3: Identification of PWN resistance genes in pines

Work carried out by partner B6 (INIAV)

Material & Methods

Isabel Carrasquinho carried out the work reported in this section as part of a Masters programme (see dissemination). Ninety-six half-sib progenies were obtained from 120 plus trees selected for tolerance/resistance, at Comporta, and sowed at Obidos nursery (Quinta do Furadouro) in November 2012. In April 2014, all the plants were transferred to Oeiras nursery.

The experiment to estimate genetic parameters (genetic variability, heritability) for pinewood nematode tolerance/resistance was established in a greenhouse equipped with a cooling system (Figure 6.3).

Figure 6.3 Plants in the greenhouse equipped with a cooling system.

A randomized complete block design was applied for the 96 half-sib families, with 4 blocks and 15 plants per family. All the 5760 plants were measured for total height and diameter at the base and the inoculation tests were done in August/September 2014 using 500 µl/tree, of a suspension of 1000 PWN/ml.

The symptom evaluation began 27 days after the inoculation. Three more evaluations were done during the course of the trial (27, 38, 57 and 156 days after inoculation). Four symptom levels were considered: 1-healthy or less than 25% of dead needles; 2- 25%-50% of dead needles; 3- 51-75% of dead needles; 4- More than 75% of dead needles or dead. Plants classified with “1” or “2” and with “3” or “4” were considered alive and dead, respectively. Survival was evaluated for each family in each block.

Statistical analysis followed the mixed models theory. For survival data (response variable with binomial distribution), a generalized linear mixed models was fitted for each of the four evaluation dates. Two link functions (logit and probit) were considered.

For the evaluation of the family performance, the empirical best linear unbiased predictors (EBLUP) of the family effects were obtained. These EBLUPs were ranked to identify the families with higher and lower effects in the survival. Additionally, the predicted survival percentages for each family in each block ($\hat{\pi}_{ij}$) were calculated from the inverse of the logit link function. For the interpretation of the results, each family mean predicted survival percentage over the four blocks was ranked.

Results

The results indicated genetic variability for survival (Table 6.3 and Table 6.4). Table 6.3 shows that plant survival is influenced by the overall height and diameter of the stem base.

Table 6.3: Family variance estimates (

estimated survival, it was possible to identify the top 10 resistant/tolerant families to pine wilt disease, at 27, 38, 56 and 157 days after inoculation (DAI). The results are presented in Table 6.5.

Table 6.5: Family mean survival (

D6.5: Host preferences for *Monochamus* oviposition

Work carried out by partner B6 (INIAV)

The study to determine the preference of *M. galloprovincialis* to lay eggs on wood with and without the pine wood nematode (PWN) under laboratory conditions could not be completed because the inoculation of the pine wood nematode into the wood logs was not successful for unidentified reasons.

A second experiment was done to determine the preferred age of the wood for breeding by *M. galloprovincialis*. *Monochamus* beetles are known to breed in weakened or recently-dead pines, being dependent on biotic or abiotic agents, such as bark beetles, droughts, fires or PWN infection, to kill or damage the pines and make them suitable for laying eggs. Felled wood is also a preferred breeding substrate, and can serve to maintain and augment the inset´s populations in the field. But the preferred conditions of the wood for breeding are unknown.

Material & Methods

In this experiment we studied the preference of *M. galloprovincialis* to lay eggs on *P. pinaster* bolts from healthy trees at 4 different times after being cut (7, 31, 74 and 115 days) to simulate different wood conditions. Wood was kept at room temperature (≈21ºC) and protected from the sun and rain until used in the experiment. In July of 2014, two adult insect couples (ca. 25 days old) were randomly chosen and placed in a 0.5 m^3 screened wooden box along with the four pine bolts (≈50 cm long). There were a total of 15 replicates, rotating the bolt´s position on each occasion (Table 6.3).

Table 6.6 Characteristics of the pine bolts

	DBH		Bark thickness	
Nb days after cut	Mean	Std	Mean	Std
7	80,72	13,20	2,95	0,69
31	91,41	18,75	3,42	0,78
74	69,93	11,68	3,07	1,07
115	83,82	9,16	3,07	0,70

After 48 hours the insects were removed and the bolts stored at room temperature for a period of 70 days, after which they were dissected to count and weigh live larvae.

Results

Overall, a total of 100 eggs were laid. Female *M. galloprovincialis* laid eggs on 28 out of 60 available wood logs (47%), with significant differences (Kruskal–Wallis test) according to the wood´s condition. The logs that were 7 and 31 days after cutting presented a preference for oviposition (Table 6.4, Figure 6.4).

It is important to notice that there were no significant differences for the bark thickness of different aged-wood (F= 0.9053, d.f.=3, P=0.4444), which is relevant because bark thickness is one of the main aspects conditioning the insect´s preference and choice of substrate to lay eggs.

Table 6.7 Oviposition preferences for the pine bolts at different times after cutting

Time after	% with	Number of eggs (mean ±	Number of eggs/surface 10cm^2

cut (days)	eggs	SE)	(mean ± SE)
7	60	2,2 ± 0,6	0,019 ± 0,005
31	93	3,9 ± 0,7	0,028 ± 0,006
74	20	0,4 ± 0,2	0,004 ± 0,002
115	13	0,1 ± 0,1	0,001 ± 0,001

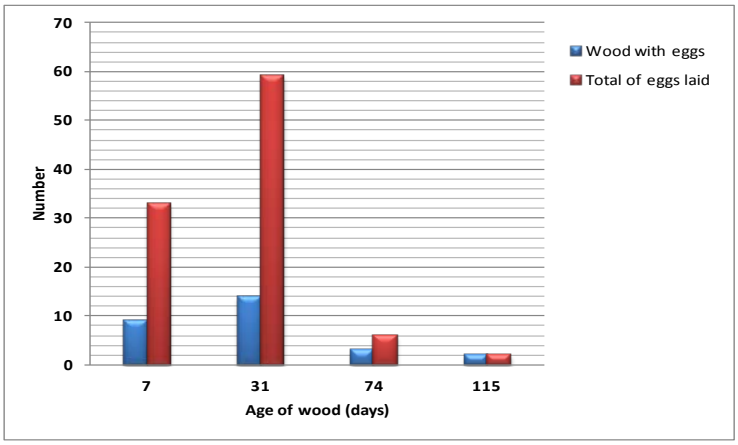

Figure 6.4 Total number of wood replicates with eggs laid and total number of eggs laid on each wood-age.

Additionally females also favoured laying a high number of eggs on wood previously felled at 7 and 31 days (Total 92 eggs), while conversely the older wood cut for 74 or 115 days was least preferred by the insects (8 eggs). The same pattern can be seen for the number of eggs per surface, considering simultaneously the choice of bolts and number of eggs laid (Table 6.4, Figure 6.5).

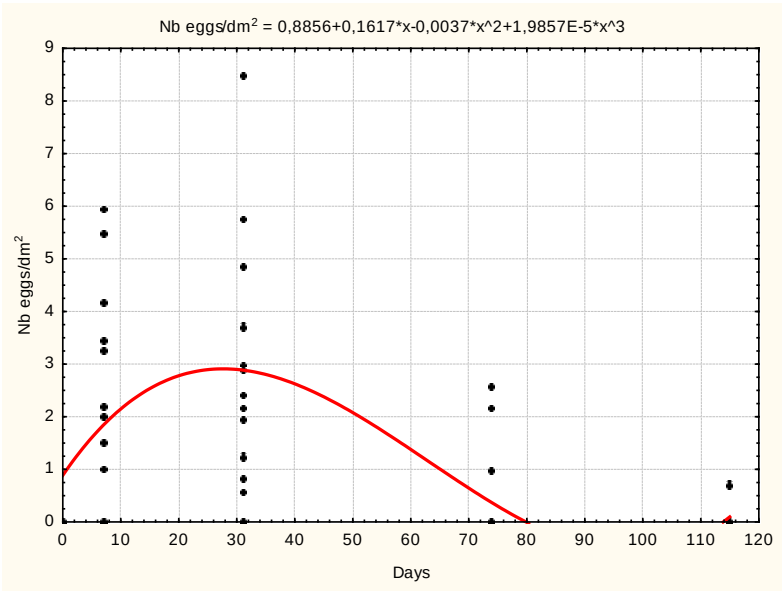

Figure 6.5 Number of eggs/ dm^2 laid by female *M. galloprovincialis* on wood with different ages. A polynomial curve was fit to the data.

The weight of the larvae also differed according to the age of the wood. In fact, larvae collected from the wood cut at 31 days were significantly heavier (Kruskal–Wallis test) than the remaining larvae (Figure 6.6), which may indicate a more adequate nutritional content of the phloem/inner bark interface were the larvae fed.

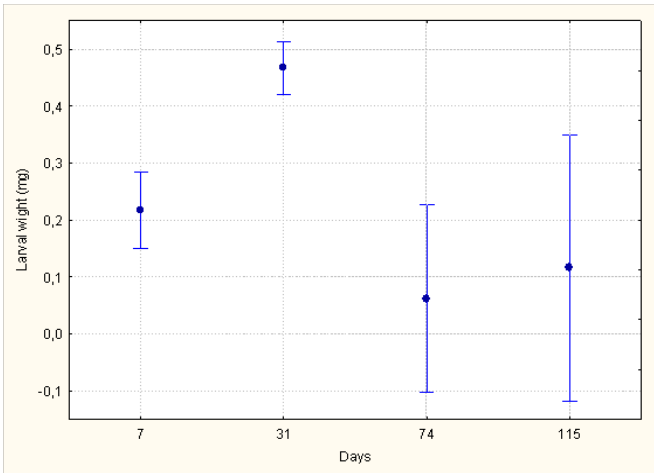

Figure 6.6 Mean ± SE weight (in mg) of *M. galloprovincialis* larvae from wood with different ages.

Conclusions

Overall, results confirm *M. galloprovincialis* to be associated with dead and declining pine wood, requiring relatively fresh material to lay its eggs. Fresh wood around one month after being felled /dead was found to have the highest attractiveness for the females, resulting in the highest risk for oviposition, while older wood more than two months after cutting appeared to be less attractive to the females (at least when more suitable material is available), and has therefore lower risk of being colonized by the insects.

This knowledge has implications for the silvicultural management of the pine sawyer populations, because in relation to beetle breeding there is a lower risk for wood that is more than two months post felling to be colonized by the insects. Therefore, there is a prolonged period from mid-autumn to early spring when healthy trees can be felled and remain in the field for the subsequent months, because when the beetles emerge in the following spring this wood will have a low attractiveness for the breeding *Monochamus* females.

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

Work carried out by B4, B5, B6, B7 and B8

Work carried out by partner B6 (INIAV)

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

Material & Methods

The hybridization between *P. pinaster* x *P. halepensis* occurred in 2012 at Quinta do Marquês located at Oeiras, Portugal (Figure 6.7). The trees that were selected as female and male parents were young and medium age, parasite free, vigorous and with abundant flowers.

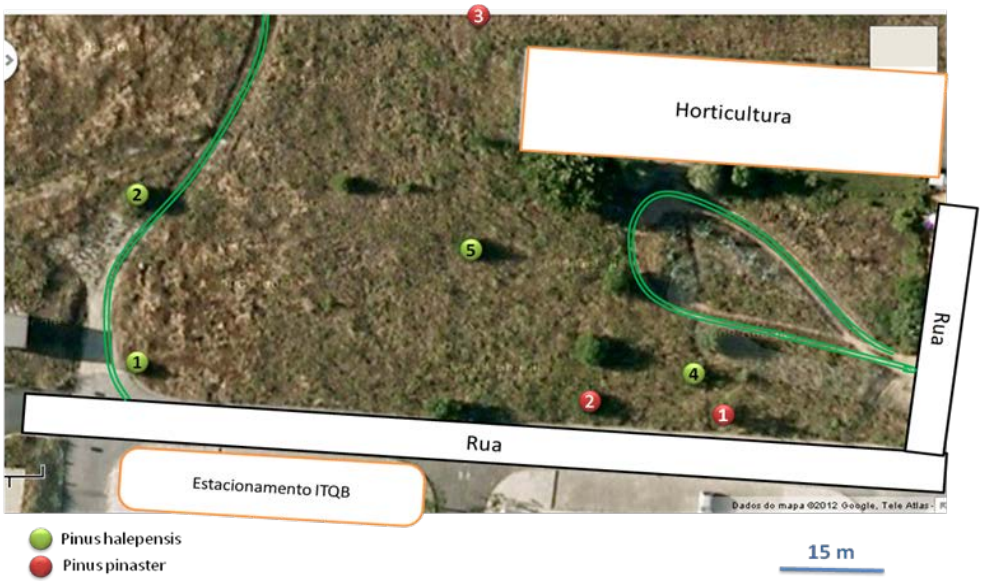

Figure 6.7 Location of selected trees for artificial pollination *Pinus pinaster* x *Pinus halepensis* at Quinta do Marquê.

Male flowers of *P. halepensis* and female flowers of *P. pinaster* occurred simultaneously and during female receptivity while pollen of *P. pinaster* was not yet dehiscent (Figure 6.8).

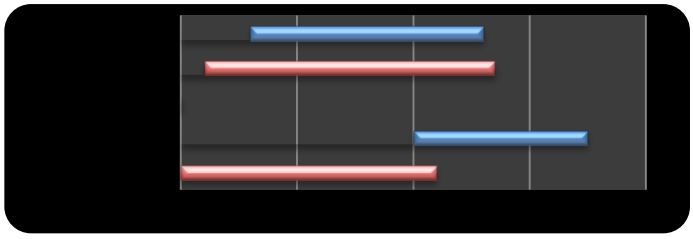

Figure 6.8 *Pinus pinaster* and *P. halepensis* phenology (Quinta do Marquês 2012)

Therefore, in order to perform crosses between *P. pinaster* X *P. halepensis* next year (2013) *P. pinaster'*s pollen has been collected, processed and stored. *P. halepensis* male strobili were removed from trees for pollen collection. In the laboratory the pollen was filtered and dried at 27ºC for 20% moisture. Pollen was stored into labelled vials at -80ºC to be used in future pollinations (Figure 6.9).

Female flowering cones were bagged before female flower receptivity (Figure 6.10 A and B), then were pollinated (Figure 6.10 C and D) when first signs of receptivity

were visible through the bag window. The pollination bags were removed after three weeks.

Figure 6.9 Pollen handling at laboratory, pollen collection, filtrating, drying and -80ºC storing

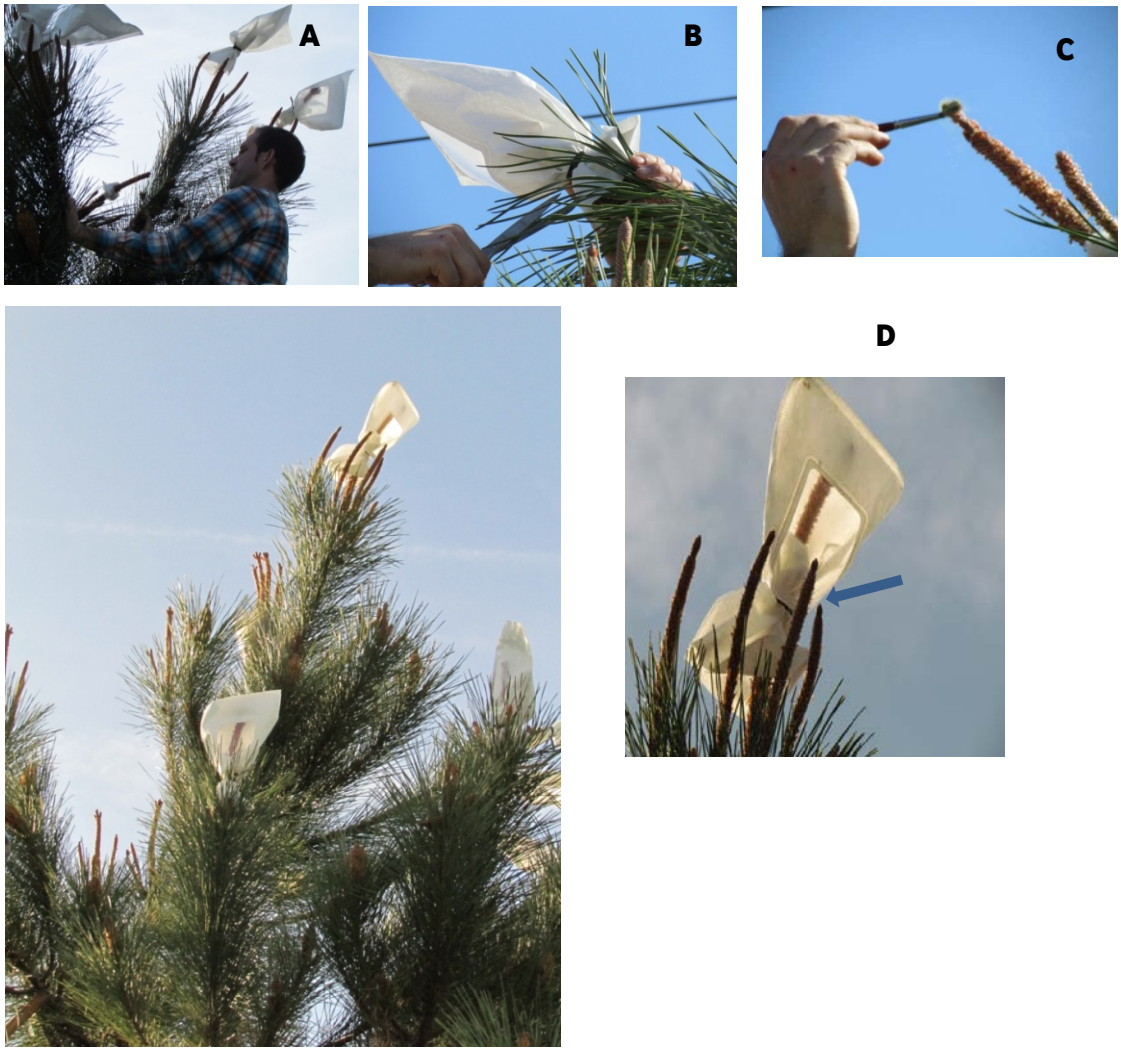

Figure 6.10 A) and B) Bagging *Pinus pinaster* female flowers; C); Pollination; D) Female flowers after pollination with pollination bags.

Results

The observations on controlled pollination of *Pinus pinaster* x *Pinus halepensis* on trees by August 2013 (number of cones, measurements and photos) are presented in Table 6.5, Table 6.6 and Table 6.7.

Table 6.8 Documentation: Pollination Form (Tree Nº 1)

Tree name	*Pinus pinaster* Aiton Quinta do Marquês
Bag Date	7th March 2012
Pollination date	14th March 2012
Male (pollen) Parent	Pollen from *Pinus halepensis* Quinta do Marquês tree Nº5
Dried or fresh?	The pollen is fresh
Bags	3 bags placed, (how many recovered during harvest)

Table 6.9 Documentation: Pollination Form (Tree Nº 2)

Tree name	*Pinus pinaster* Aiton Quinta do Marquês
Bag Date	3th March 2012
Pollination date	7th March 2012
Male (pollen) Parent	Pollen from *Pinus halepensis* Quinta do Marquês tree Nº4
Dried or fresh?	The pollen is fresh
Bags	17 bags placed

Table 6.10 Observations on controlled pollination *Pinus pinaster* x *Pinus halepensis* on trees at Quinta do Marquês, Oeiras by August 2013.

***Pinus pinaster* tree Nº 1**	**Number of cones by Aug 2013**	**Size (cm)**
Terminal shoot	1	12
Lateral shoot W	1	15
Lateral shoot E	1	15
***Pinus pinaster* tree Nº 2**		
Terminal shoot	0	-
Lateral shoot S	5	15;16;14;12;12
Lateral shoot W	6	10;11;16;15;13;14
Lateral shoot N	3	14;14;13

Lateral shoot E	5	Measurements not possible but size is similar

Seeds from 2012 artificial hybridization between *Pinus pinaster* x *Pinus halepensis* were collected by November 2013. *Pinus halepensis* x *Pinus pinaster* crosses performed by February 2014 will be mature by November 2015.

Results from 2012 crosses *P. pinaster x P. halepensis* are described in

Table **6.8** concerning the number of viable and empty seeds, and germination percentages.

Table 6.11 Germination data from *P. pinaster* x *P. halepensis* hybrid seeds

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

A total of 1332 hybrid seeds from 19 fully mature seed cones from *P. pinaster x P. halepensis* crosses performed in 2012 at Quinta do Marquês were obtained. Seeds were manually extracted, examined, and a buoyancy test was used to discard empty seeds (497 empty seeds were discarded). A total of 835 seeds were sowed in containers from January 30 - February 4, 2014 (Figure 6.11).

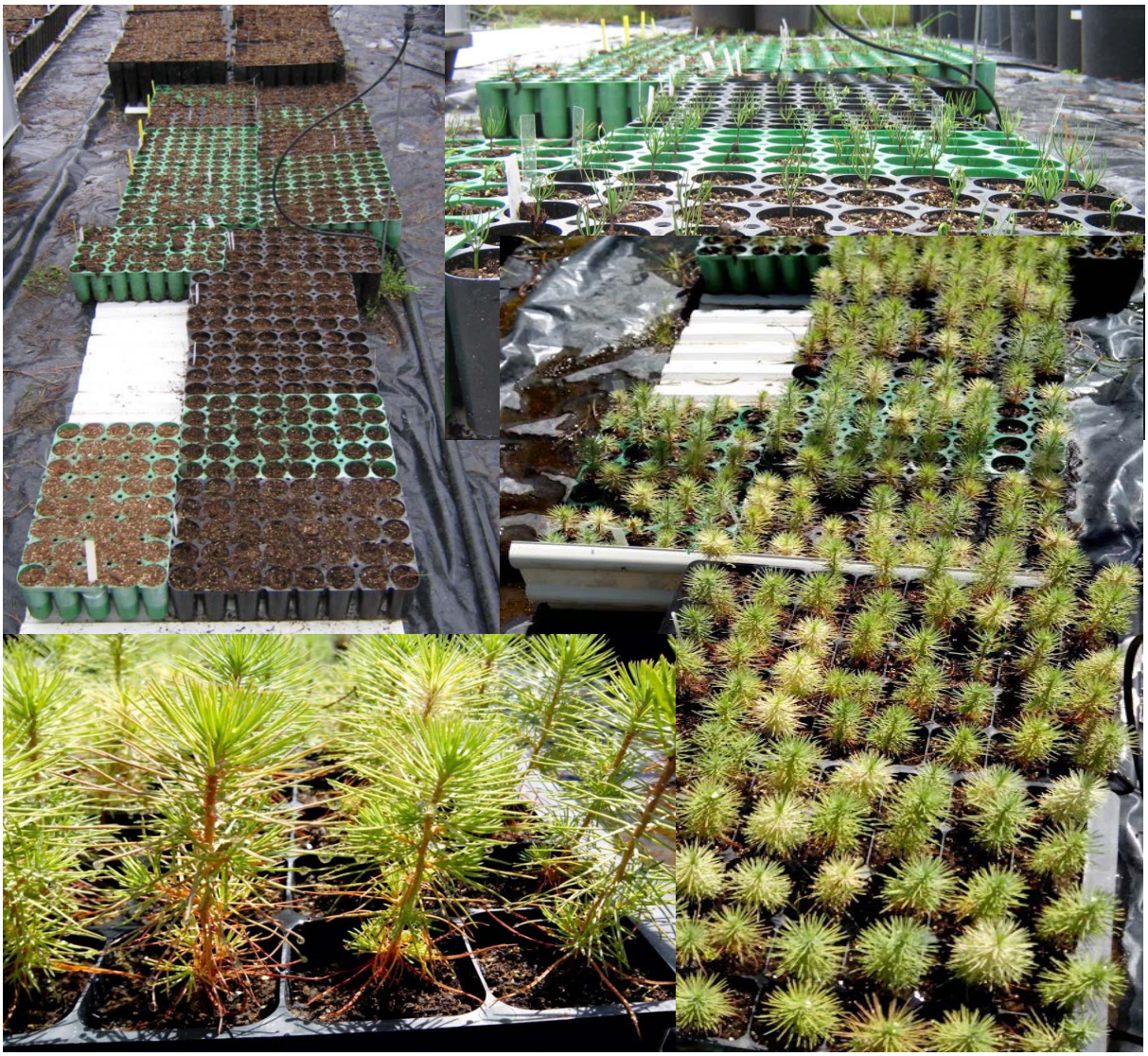

Figure 6.11 Evolution of 835 hybrids from sowing (January) until September 2014

D6.7: Tree species mosaics to reduce PWN impact

Work carried out by B4, B5, B6, B7 and B8

Work carried out by partner B6 (INIAV)

Material & Methods

In 2012 two maritime pine plots with 1 ha were set up on Madeira Island to evaluate the spread of the PWD in these specific ecological and climatic conditions (warmer than in Continental Portugal). Of the two maritime pine plots delimited, the one located at Gaula (eastern part) was completely burned by the huge forest fire that occurred during summer 2012, such that only 2 trees near the road survived (Figure 6.12).

Figure 6.12 Dead burned maritime pine trees of Gaula plot at Madeira Island, after the forest fire of summer 2012.

The survey carried out in 2014 at Prazeres plot, in the west of Madeira Island, enabled identification of many dead and symptomatic trees among the 355 maritime pine tree that were alive when the plot was delimited. In fact, after the 36 pines dead in 2012 (9,2% mortality) (Figure 6.13a), in 2014 survey 181 dead pines were found (Figure 6.13b), representing 51,0% indicating that the mortality rate increased enormously during these two years (Figure 6.14) and the overall appearance of the plot was changed dramatically (Figure 6.15).

There were dead pines all over the plot; in some areas there were clusters of more than 10 dead pines of all sizes. In fact, mortality affected pines of all breast height diameter classes (Figure 6.16) and after 2014, 90% of smaller trees (less than 7cm DBH) were dead, while in the other dimensions mortality ranged between 47% (DBN 20-35 cm) and 68% (DBH 14-20).

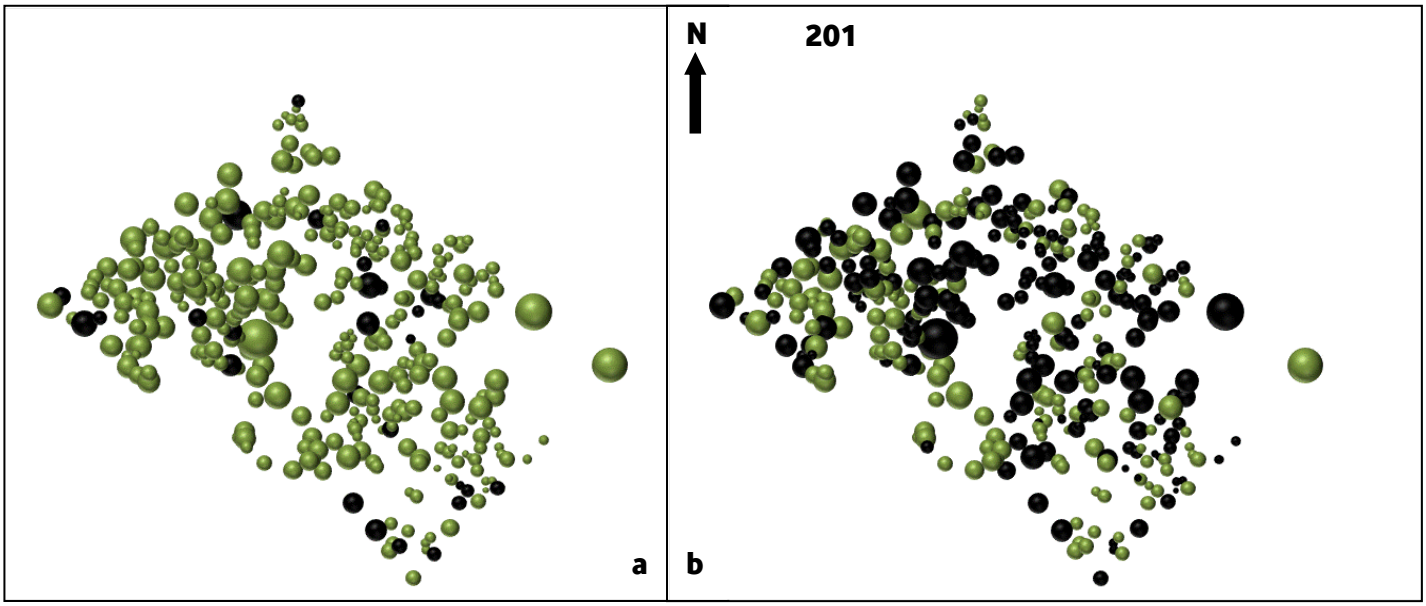

Figure 6.13 Plot delimited in a maritime pine stand at Prazeres, Madeira Island in 2012. Size of bubbles reflect diameter at breast-height of pines. Black bubbles represent dead pines when plot was delimited in 2012 (a) and after the survey of 2014 (b).

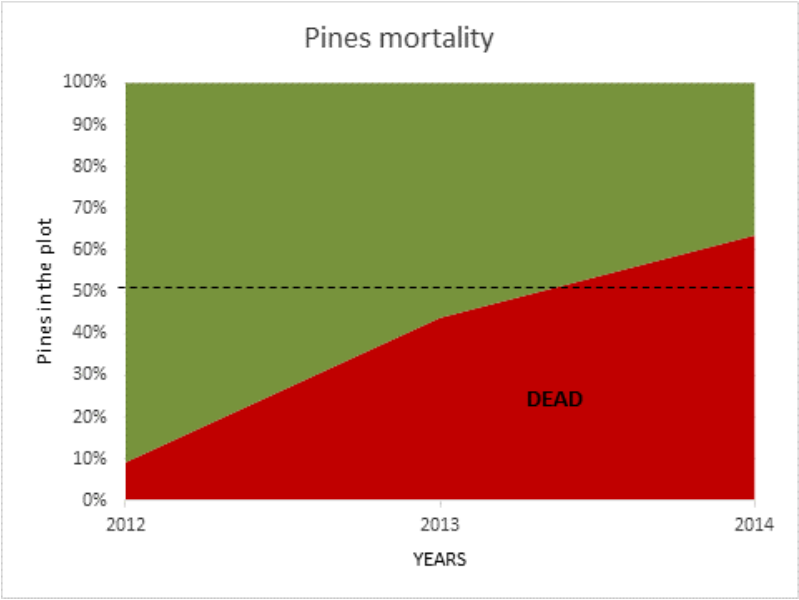

Figure 6.14 Evolution of the maritime pines mortality pines Madeira Island plot.

Figure 6.15 Mortality registered in 2014 at the plot delimited in a maritime pine stand at Prazeres, Madeira Island.

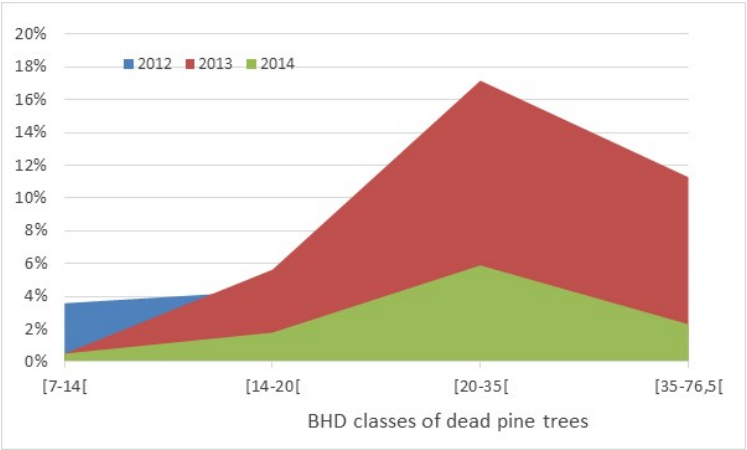

Figure 6.16 Distribution of mortality in Madeira Island plot according to dimension of the maritime pine trees (Breast Height Diameter).

The wood of all dead pines was sampled and analysed in INIAV laboratory at Oeiras for the presence of the pine wood nematode and 63% were infested, confirming a high spread of the nematode and pine wilt expression.

The infestation by the PWN occurred in trees of all DBHs with increasing incidence as trees in size (from 40% infestation of pines thinner than 20 cm, 63% in intermediate size pines and 88% in thicker pines, over 35cm).

The colonization of these pines by the PWN vector *M. galloprovincialis* was very high (87%) and occurred independently of the pine dimensions.

The pine shoot beetle (*Tomicus* sp.) was the bark beetle more frequent on the surveyed dead pines occurring in about one third of the pines (37%) while *Orthotomicus erosus*, the most common in Continental Portugal colonized only 20%.

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

This Work Package was difficult to carry out due to the problems of obtaining and analyzing potentially resistant sources of pine species and varieties. This resulted in changes of direction which were particularly highlighted following the mid-term review carried out by Dr Ilaria Pertot. Specifically, she recommended:

“Major organizational changes in B6 have taken place in 2011/2012 with significant consequences on expected working capacity. Therefore for Volatile organic compounds and bark extracts the only feasible possibility is to appoint a new sub-contractor with the necessary skills to all these Tasks. The recommendation is to delete task 6.2 and reallocate the person months to other WPs”

For these reasons, the whole of 6.2 and the related deliverable 6.4 which would have depended on the results were eliminated and resources re-allocated within WP6.

Statement on the use of resources

Resources were re-allocated within WP6 as recommended in the mid-term review. This placed more emphasis on sub-tasks other than 6.2 and 6.4 which were eliminated.

WP 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 – Completed**
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. **Month 45: Field experiment was not possible but verification was done in Japan using data from Japan and results from literature.**
D7.3: Latency sub-model: Development of a sub-model to account for latency in wilt expression. **Month 44 – Completed**
D7.4: Analysis of PWN history in Portugal: Analysis of the history of infestation and wilt expression in Portugal. **Month 42 – Completed**
D7.5: PWN spread model: Development of a PWN spread model taking into account human influences. **Month 44 – Completed**.

Work carried out by B1, B4, B7 with some inputs of B5, B6, B10

Progress

All the tasks of WP7 have been completed during this final reporting period (Mar-Nov 2014). A description of the work done is given hereafter for each deliverable.

D7.1: Refinement of the core model (M44)

Work carried out by B1
This section is structured as follows:

- Modifications to Core model,
- Model Validation (addressed within this section as it is an important step prior to producing model output and sensitivity analysis. D7.2 (Field verification of process model) was not possible and this is briefly mentioned in this section and again under D7.2),
- Sensitivity analysis,
- Model output, including risk maps,
- Development of user-friendly simplified model,
- Small section on the key results of the model/simplified model and
- Future risk of PWD due to climate change

Since the last reporting period significant progress has been made in refining the core model. In the previous report we discussed the key changes that were made to the original ETp (Evapo-Transpiration) model, developed by Forest Research (Evans *et al.*, 2003), to incorporate the presence of the pine wood nematodes. The new model was called the ETpN (Evapo-Transpiration + Nematodes) model. Three main elements were added to the model: a nematode element, which calculated the number of nematodes inside the tree, with a temperature dependent growth rate, a photosynthesis element, which related the number of nematodes inside the tree to the cavitations inside the tree, and hence, how much photosynthesis was being restricted. Finally an energy element was developed to model the trees ability to use some of its available energy to defend itself against the nematodes.

Since the last reporting period, some further modifications have been made to the nematode element of the model in order to better reflect what happens to nematode numbers inside the tree when temperatures become unfavourable over the winter months. In addition to this, following discussions at the REPHRAME workshop on 30th Sept – 2nd Oct held in Madrid, Spain and Coimbra, Portugal, it was decided that the term “tree resistance” used in the ETpN model was misleading and that it would be better to refer to this parameter as a type of tolerance that a tree has towards the nematodes. In fact it is impossible to quantify what we mean by “a trees tolerance to PWD” at least at this stage where there is uncertainty in the mechanisms causing resistance in pine trees. As a result, we fix the tolerance level in the model and assume that when we run the model we are dealing with susceptible species. We discuss these changes in detail below.

Nematode Population Element

In the previous report we introduced a simple equation (exponential growth with restrictions on the population that survives to breed (Murray, 1989)) for calculating 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. For large nematode numbers, this equation modelled the numbers well; however, when nematode numbers became low, as a result of cold temperatures over winter months, nematode numbers would become less than one, which is unrealistic. As a result we have changed the equation used to calculate nematode number on day i.

The PWN has two different modes in its life cycle; a propagative mode and a dispersal mode. The propagative mode refers to reproduction of the PWN, which usually occurs in living trees. The dispersal mode refers to the transfer (via its vector the sawyer beetle) to new host trees. The first two stages in the PWN life cycle are the same for both modes. There are two different third juvenile stages, depending on whether or not the conditions inside the tree are favourable for nematode growth. If the conditions are not right, i.e. lack of food or low temperatures, the second stage moults into a resistant third stage which can survive for long periods at cold temperatures. Experiments with PWN's saw them surviving over a month when kept in a fridge (Bockgard, 2011). A significantly larger number of juveniles than adults survived deep freezing at -180°C. These juveniles were then used to initiate new cultures, which reproduced rapidly over several generations. Furthermore, these nematodes were as pathogenic as untreated nematodes (Riga & Webster, 1991). Nematodes in the resistance juvenile stage have been demonstrated to survive storage at -17°C for 5 months suffering a minimal degree of mortality (Kondo *et al.*, 1982), so winter temperatures seem to have little or no effect on third stage resistant Juveniles. If the tree is still alive and the temperature is still favourable, the third stage will quickly develop into adults.

Results from the literature give detail on the development and reproduction of PWN at different temperatures. Results of experiments, where PWN are maintained on fungal cultures (EPPO, 1986), show that the PWN reproduces in 12 days at 15°C, 6 days at 20°C and 3 days at 30°C. Furthermore, egg-laying starts on the 4th day after hatching and the eggs hatch in 26-32 hours at 25°C. The temperature threshold for development is 9.5°C. Similar results (Stokes, 1979) show that *Bursaphelenchus lignicolus* (*B. xylophilus* is synonymous with *B. lignicolus*) may complete its life cycle in 4-5 days at 25°C.

B. xylophilus has a mean life span of approximately 15 days and a maximum life span of approximately 30 days when grown at 25°C (Oh *et al.* 2009). Female PWN cultured on *Botrytis cinerea* (fungus) produced an average of 79 eggs during their adult life span of 15 days at 25°C (Dropkin *et al.,* 1981). Laboratory studies show that reproduction of *B. xylophilus* can progress from egg to adult in four to five days in the laboratory, feeding on fungi at 25°C; however, generation time is temperature-dependent (Donald *et al.*, 2012). Temperatures between 20-25°C are ideal for nematode development (Sathyapala, 2004).

Temperatures above 28°C affect *B. xylophilus* development, and its embryonic development is disrupted at 35°C (Wang et al., 2012). From experiments with different isolates of *B. xylophilus* (Rutherford *et al.,* 1992), the severity of the disease (PWD) is correlated with the number of nematodes inoculated in that the number of cells disrupted correlates well with the number of nematodes present in a tree. Furthermore, *B. xylophilus* move faster at higher temperatures. Six out of the seven *B. xylophilus* isolates that were tested produced more offspring at 27.5°C or greater. In addition to this, the mean numbers of offspring produced per day by different isolates of B. xylophilus are given and we use these values, along with the thresholds we mentioned above, to calculate an average number of offspring per day at different temperatures. We fit the following curve to the data points, (the same curve we introduced in the previous periodic report), to give a (temperature dependent) birth rate per nematode (per day) of:

$$r_i(t_i) = 0.425 * (40 - t_i)^{2.32} \exp\left\{\frac{-(40 - t_i)}{3.3}\right\} \qquad \textbf{(7.1)}$$

In order to accurately model the development of the nematodes at low temperatures, we split the nematodes into two groups; juveniles and adults. When temperatures become too low for nematode development the juveniles and nematodes will enter a growth arrested stage and remain at relatively constant numbers (with the adult nematodes being more unstable than the juveniles and having greater mortality) until the temperature becomes favourable again (Zhao *et al.*, 2007).

We have seen evidence in the literature that the pine wood nematode takes around four days to develop from egg to adult and that an adult has an average life span of 15 days. Combining the above, we calculate the number of juveniles J_i and adults N_i in a tree on day i by the following equations:

$$N_i = N_{i-1} + r_{i-3}N_{i-4} - r_{i-19}N_{i-20}$$

$$J_i = J_{i-1} + r_i N_{i-1} - r_{i-3}N_{i-4}$$

(7.2)

where $r_i N_{i-1}$ represents the number of nematodes (juveniles) born on day *i*. So, for example, $r_{i-19}N_{i-20}$ represents the number of nematodes born on day *i*-19, and since egg to adult takes 4 days and an adult has a life span of 15 days, $r_{i-19}N_{i-20}$ is also the number of nematodes that we would expect to die on day *i*.

The birth rates of nematodes that were obtained from experiments are the result of nematodes being reared on food sources at a constant temperature without any biotic or abiotic factors affecting them, whereas inside a tree, the situation would be very different and we would not expect to get such high numbers of nematodes.

As a result we assume that a small proportion of nematodes are killed daily while inside the tree, due to some level of tolerance (which we refer to as σ) that a tree has to PWD. If this proportion is too high the nematodes will die out and if it is too low the nematodes will multiply to unrealistic numbers. Using results from the literature on disease development (Kuroda *et al.*, 1988 and Zhao *et al.*, 2008), we can deduce that σ needs to be around 0.17 to give realistic nematode numbers. For example, at locations where we would expect wilt, when we fix σ at 0.17, 3 weeks after infestation the model gives values for nematode numbers at around 10 times the amount that was inoculated, which is what is reported (part IV, Zhao *et al.*, 2008).

For trees that are resistant (e.g. American pine species), we can increase σ to 0.3 and nematode numbers will quickly disappear, even in areas where we have seen high amounts of wilt, e.g. Nagasaki in Japan. Alternatively, if we know we are dealing with particularly weak, susceptible trees we can reduce the value of σ to 0.1, ensuring that for most locations (with relatively warm summers), the model will predict PWD.

In reality, it is almost impossible to quantify what we mean by a trees resistance/tolerance to PWD and so we fix this value as 0.17, which gives the most realistic output.

The set of equations labelled 7.2 that are used to calculate the number of adult and juvenile nematodes inside the tree on day *i*, are multiplied by 0.17 at each time step.

Figure 7.1 shows the total nematode numbers (adult + juvenile) calculated by the model for Lisbon, Portugal (a location where PWD has been reported) and Amsterdam, Netherlands (where we would not currently expect to see PWD). 500 nematodes are inoculated on the 1st July and the model is run for 365 days. By the 12th of August there are almost 90 times more nematodes in a tree at Lisbon compared with Amsterdam. We note that eventually, the nematode numbers peak at around 4×10^{11} at Lisbon, while at Amsterdam the numbers peak at around 57000.

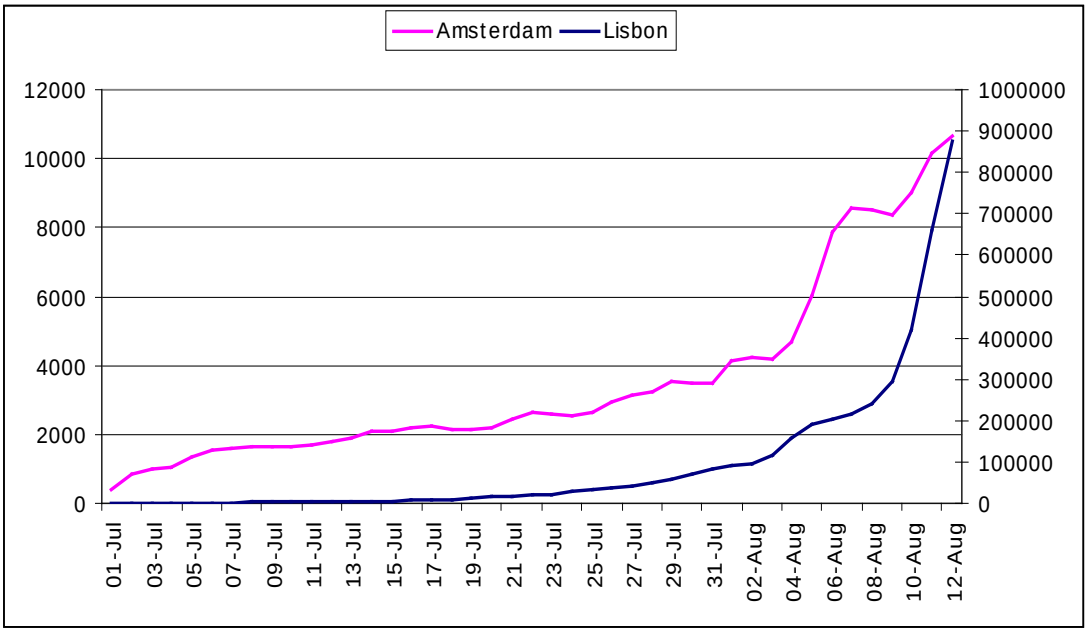

Figure 7.1 Nematode numbers at Lisbon and Amsterdam. Left hand axis refers to numbers at Amsterdam and right hand axis for Lisbon.

The significant difference in nematode numbers between locations explains in part why we see PWD in Lisbon but would not expect to in Amsterdam.

The photosynthesis restricting element introduced in the previous periodic report has not been modified. We use a logistic curve to relate the number of nematodes inside the tree to the amount of photosynthesis that can take place.

The available energy element that was introduced in the previous periodic report has been modified very slightly in that the equation that calculates the available energy on day *i* no longer contains an expression for tree resistance. The energy used in defence is governed purely by a logistic curve (which is dependent on nematode number). For details, see previous periodic report.

Model validation

Deliverable D7.2 was not possible due to uncertainty of getting an extension and also not being able to get permission to carry out inoculation work in Canada. The model has been verified using climate data from Japan and data from the literature of PWD in Japan.

As mentioned in the previous periodic report, the model is validated using data for Japan where PWD has caused devastating damage through most of Japan with the exception of the northernmost island Hokkaido (Zhao *et al.*, 2008). Due to the modifications in the nematode element of the model it is crucial to re-run the model for Japan to ensure that the predictions are still representative of the observed wilt expression in Japan.

We run the model for 12 locations in Japan (Table 7.1).

Table 7.1 Locations (with latitude and longitude) in Japan used to validate the model.

Location	Latitude	Longitude
Aburatsu	31.58	131.4
Nagasaki	32.73	129.87
Kyoto	35.02	135.73
Hiroshima	34.4	132.47
Tokyo	35.68	139.77
Yamagata	38.25	140.35
Akita	39.72	140.1
Morioka	39.7	141.7
Aomori	40.82	140.77
Hakodate	41.82	140.75
Tomakomai	42.62	141.55
Abashiri	44.02	144.28

Model outputs are shown, colour coded, in Figure 7.2. The model predicts no wilt at Hakodate, Tomakomai and Abashiri, all on the island of Hokkaido. The model predicts PWD in the year of infestation at Aburatsu, Nagasaki, Hiroshima, Tokyo, Kyoto, Yamagata and Akita. At Morioka and Aomori, the model predicts wilt in the year following infestation.

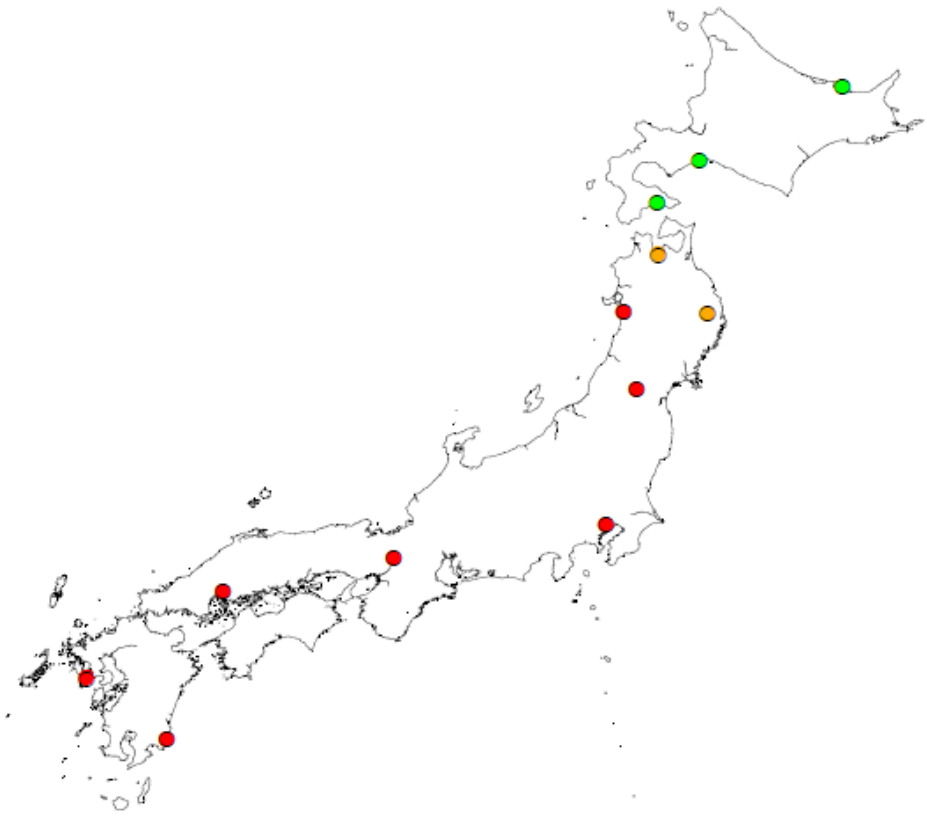

Figure 7.2 Model output for 12 locations in Japan. Locations coloured red are where the model predicts wilt in year of infestation. Locations coloured amber are where the model predicts wilt in the year following infestation and the locations coloured green are where the model predicts no wilt.

Sensitivity Analysis

We use GEM-SA (Gaussian Emulation Machine for Sensitivity Analysis) to run a sensitivity analysis. GEM-SA is a GSA software based on BACCO approach and was developed by Centre for Terrestrial Carbon Dynamics (CTCD) (Kennedy and O'Hagan, 2006).

We performed two sensitivity analyses; one with regards to climatic and local data (i.e. using multiple locations) and a second with regards to model parameters (i.e. we use one location only so that climatic variables are fixed).

With regards to climatic data, nearly 55% of the variance in output is accounted for by temperature parameters (Table 7.2). 11% of the variance in model output is accounted for by location parameters, e.g. latitude, longitude and altitude. Mean summer temperature is an important model input, which has the largest effect on model output.

Table 7.2 Results of Sensitivity Analysis: percentage variance in output caused by climate data.

Input	**Range**	**Percentage of Variance in output**
Altitude (m)	-23 – 2504	4.84
Latitude (degrees)	32.7 – 68.97	4.60
Longitude (degrees)	-21.9 – 60.63	1.26
Max Temperature (°C)	22.87 – 42.33	0.12
Min Temperature (°C)	-37.17 – 3.7	6.94
Mean Summer Temperature	7.04 – 28.82	24.02
Mean Winter Temperature (°C)	-13.14 – 13.27	5.72
Total Annual Precipitation (mm)	256.12 – 2581.4	0.14
Mean Summer Precipitation (mm)	2.29 – 1060.45	1.15
Mean Winter Precipitation (mm)	49.95 – 2094.99	0.10
Mean Annual Temperature (°C)	0.46 – 18.81	17.83

With regards to model parameters, the tree tolerance has the greatest effect on model output (accounting for 23.41% of the variance). Initial nematode number is the second highest, accounting for 18.13% of the variance in model output (Table 7.3). This is in agreement with results in the literature where severity of the disease correlates with the number of nematodes inoculated (Rutherford *et al*., 1992). Inoculation day also has a significant effect on model output.

Table 7.3 Results of Sensitivity Analysis: percentage variance in output caused by model parameters

Input	**Range**	**Percentage of Variance caused in output**
Initial Nematode Number	10 – 10000	18.13
Inoculation (Julian Day)	30 – 280	12.13
Tree Tolerance (proportion)	0.05 – 0.3	23.41
Initial Energy (Joules)	10 – 1000000	8.66
Age (years)	19 – 60	4.57
DBH (cm)	11.47 – 40	1.11

Model Output

The full ETpN model had been developed and validated. We use daily climatic data (2009-2011) for locations all over Europe to run the model. We set initial nematode number to 500 nematodes (it is suggested that around 300-1000 nematodes successfully invade tissue in inoculation trials, (Zhao *et al.*, 2008)). Infestation/inoculation day is set as Julian day 182 (1st July), which, for current climate, is when we can begin to see beetle emergence for most locations in Europe where we would expect wilt (data from maps produced by B4). Tree tolerance is fixed at 0.17, as discussed in previous section. Initial storage energy is fixed at 1000 Joules; this parameter does not have a big effect on model output. The trees' ability to make energy from photosynthesis is more important than the value of energy it has on day zero. Finally, tree age and DBH are fixed at 30 (years) and 18 (cm) respectively.

We run the model for over 400 locations and the output is evaluated to determine whether or not a location can expect to have PWD given the presence of PWN.
Below are examples of model output for Braganca, Portugal, a location where the model predicts wilt and Llanwddyn, Wales, a location where the model predicts no wilt.

In the following figures the units for photosynthesis are $\mu molm^2s^{-1}$, energy is in Joules (scaled by dividing by 1000) and mean temperature is in °C. The right hand axis refers to nematode numbers, while the left hand axis gives values for the other three outputs.

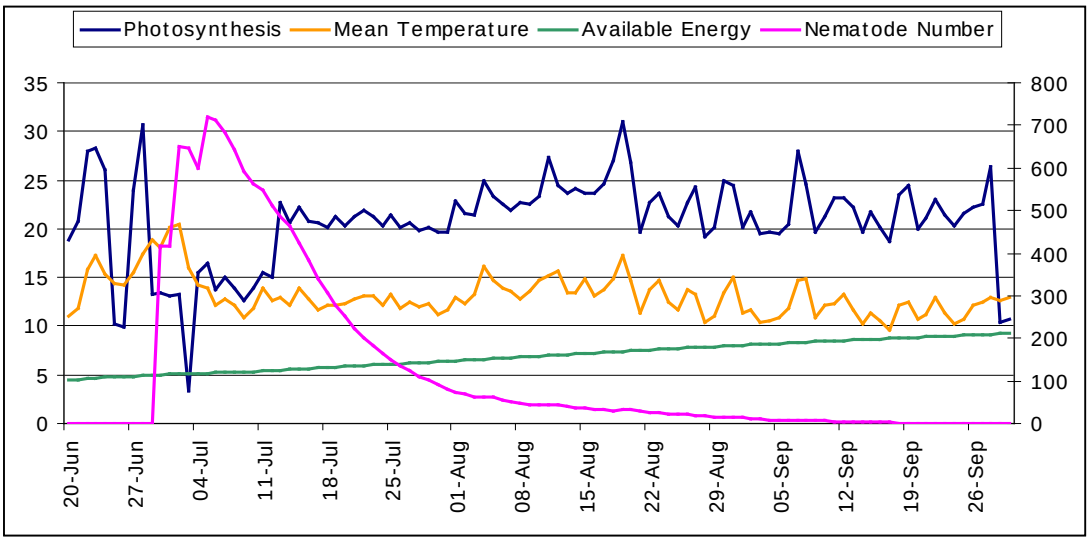

Figure 7.3 Model output for Llanwddyn in Wales.

At Llanwddyn, there is an initial peak in nematode number following infestation which corresponds to a peak in temperature (which reaches over 20°C) (Figure 7.3). Following this peak, the mean temperature only gets above 15°C on a few occasions during the summer. The nematode numbers gradually decrease over the summer as temperatures are not hot enough. Eventually, nematode numbers become zero (by mid-September). Photosynthesis and available energy recover quickly after the initial peak in nematode numbers and tree remains healthy.

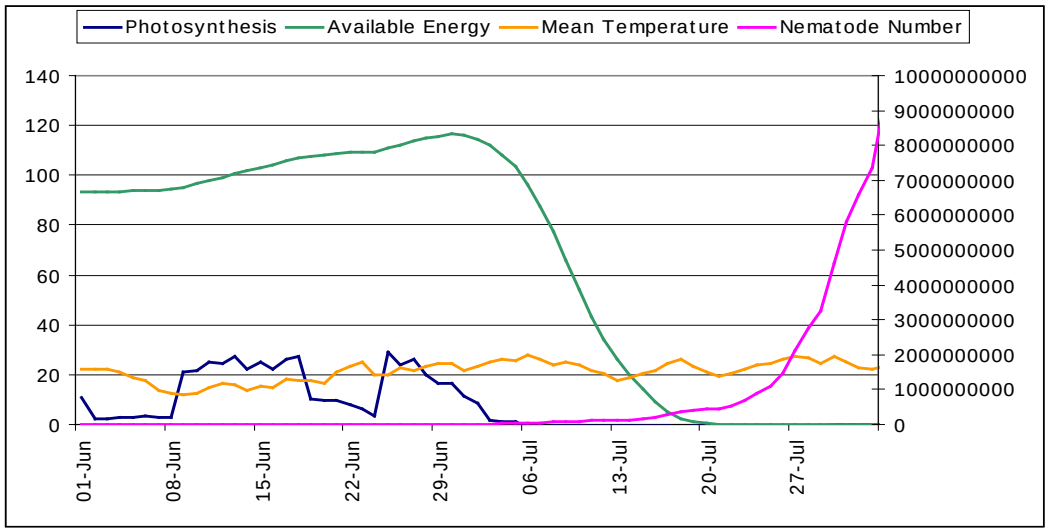

Figure 7.4 Model output for Braganca in Portugal.

At Braganca, the tree does not die from PWD until the year following infestation. Figure 7.4 shows model output for the summer following infestation. Nematode numbers are significantly higher than numbers seen in simulations for Llanwddyn, even before tree death. Photosynthesis ceases around 6th of July and the available energy decreases rapidly following this. Tree death occurs at around 20th July, after which we see a huge increase in nematode numbers. After June 22nd, the mean temperature remains above 20°C until tree death.

There is a significant difference in output between the two locations. Photosynthesis, nematode number and available energy are the key outputs in determining whether or not a tree succumbs to PWD.

We have run the model for all locations for the same set of parameters. The results for Europe are given in Figure 7.5.

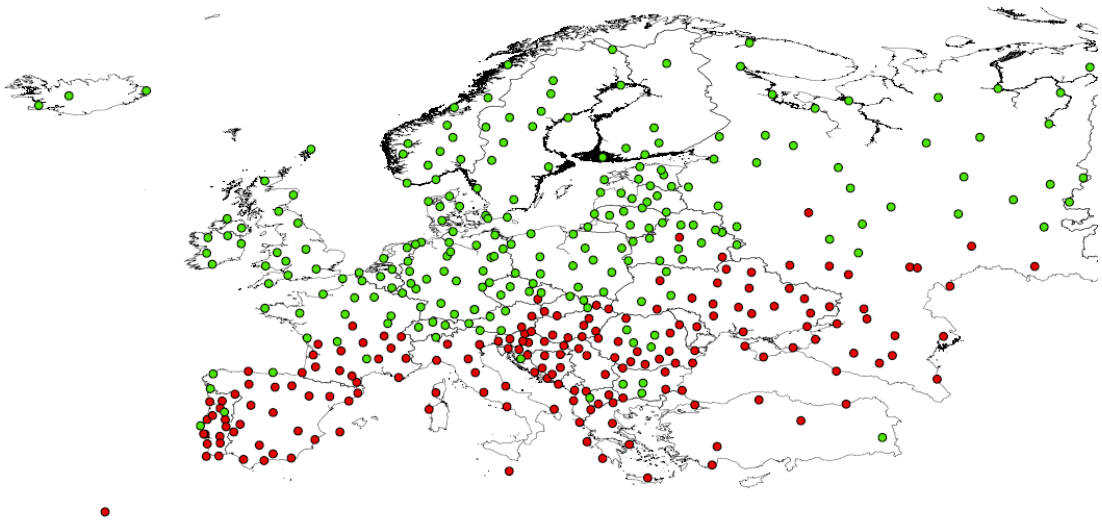

Figure 7.5 Map of model output for locations across Europe. Red points represent locations for which the model predicts wilt and green points represent locations for which the model predicts no wilt.

Note that in Figure 7.5 we have not differentiated between the year when wilt will express. Furthermore, we note that the day of infestation is particularly early for most of Europe, so this can be considered a worst case scenario for current climate in Europe.

We have re-run the model by varying inoculation day from 160-240. For some inoculation days (late in the year), the nematode population is not able to increase to sufficient numbers before the decrease in temperature over winter and nematode numbers die out. For locations where we only see wilt for some inoculation days we assign these a medium risk of PWD, which we colour as amber in Figure 7.6.

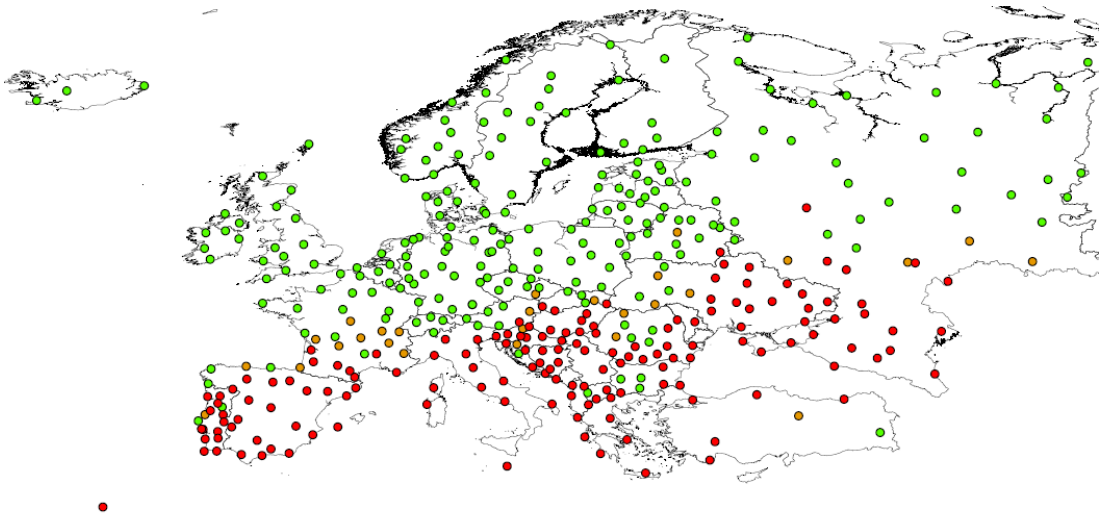

Figure 7.6 Map of model output for locations across Europe. Red points represent locations for which the model predicts wilt, amber points represent locations for which the model predicts wilt under certain conditions and green points represent locations for which the model predicts no wilt.

User-Friendly Sub-model

As part of the refinement of the core model D7.1 also includes work to develop a user-friendly “light” version of the model. Sensitivity analysis has allowed us to determine the key variables and parameters that are driving the model which is a first step towards developing a user-friendly model.

The core model is complex and requires climatic data as well as other parameters. A general user may not have such data and so it is important that a simplified model is developed to allow users to determine the risk of PWD at a particular location.

We begin by comparing the climatic data between locations where the model predicts wilt and locations where the model predicts no wilt. The following figures and statistical analysis were created/performed using the software R (R Core Team, (2014)).

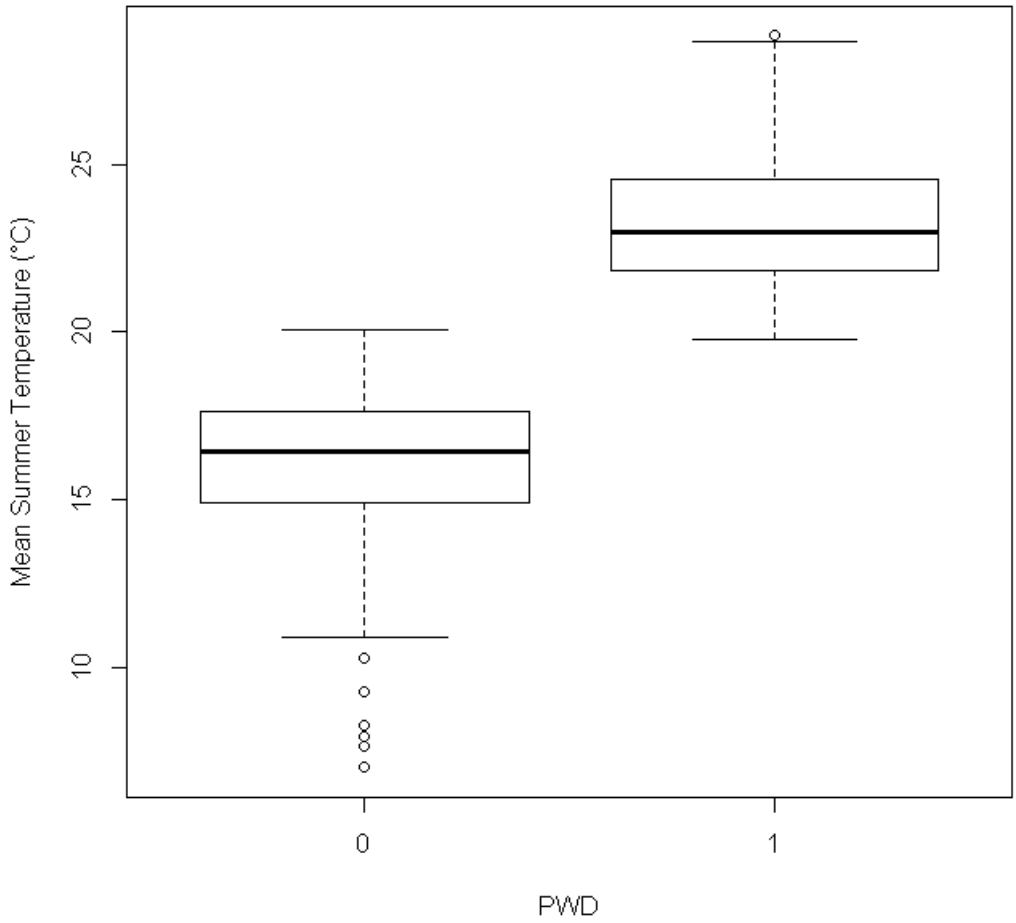

Figure 7.7 Box plot of Mean Summer Temperature (MST) in °C against PWD (0 corresponds to no wilt and 1 corresponds to PWD)

A Welch Two Sample t-test comparing the MST between locations where the model predicts PWD and locations where the model predicts no wilt gives a p-value < 2.2e-16 (Figure 7.7). There is strong evidence to suggest that the means between the two groups are different.

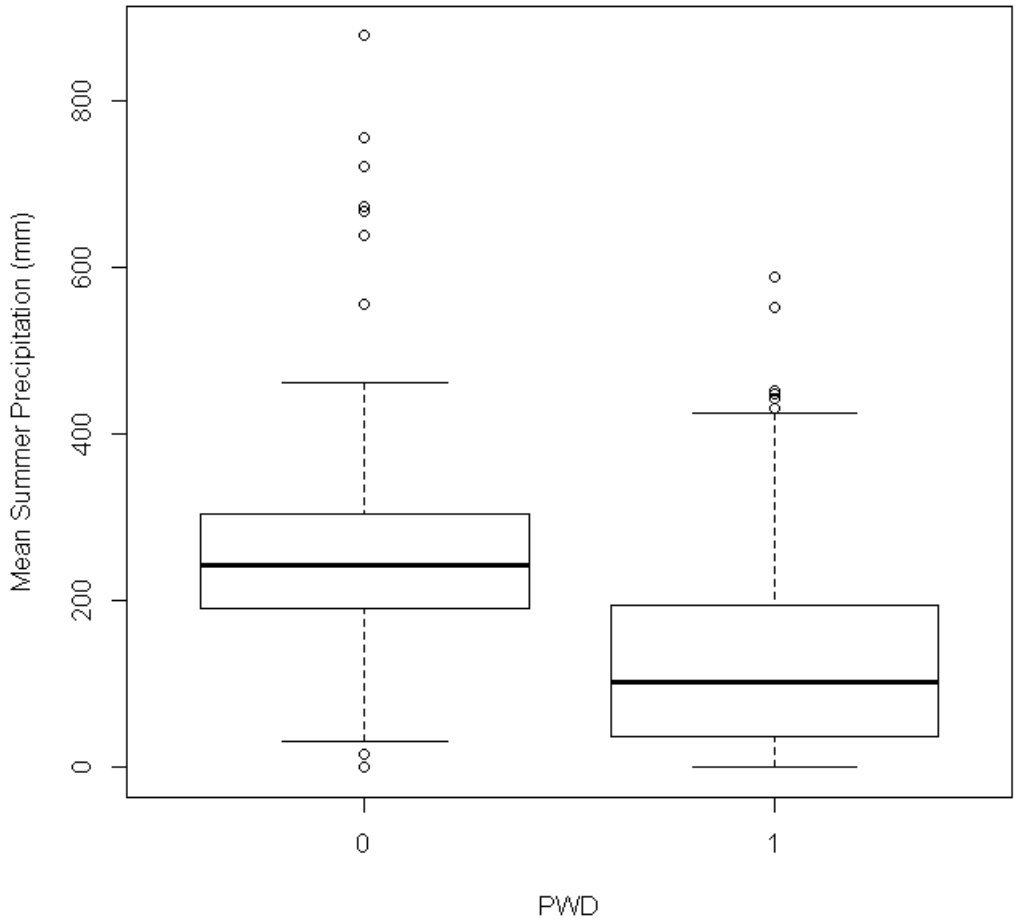

Figure 7.8 Box plot of Mean Summer Precipitation (MSP) in mm against PWD (0 corresponds to no wilt and 1 corresponds to PWD)

A Welch Two Sample t-test comparing the MSP between locations where the model predicts PWD and locations where the model predicts no wilt gives a p-value < 2.2e-16 (Figure 7.8). There is strong evidence to suggest that the means between the two groups are different.

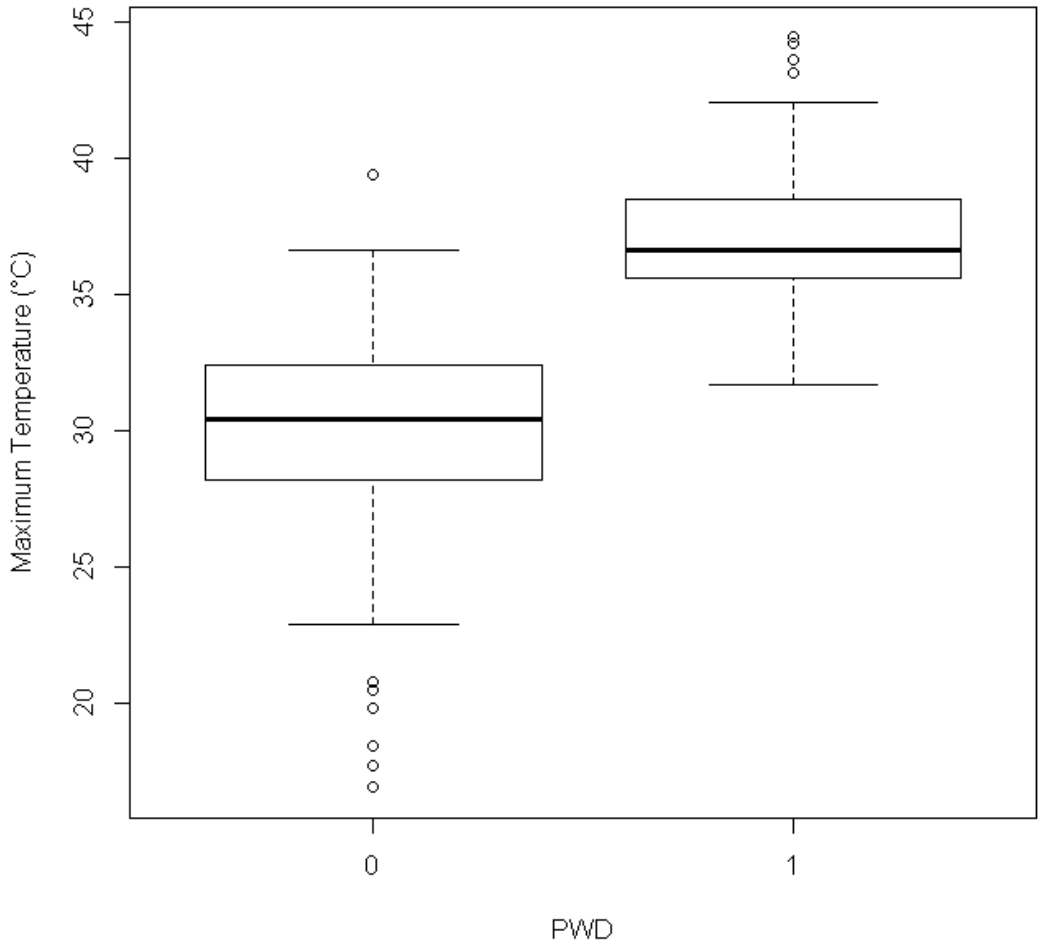

Figure 7.9 Box plot of Maximum Temperature (MaxT) in °C against PWD (0 corresponds to no wilt and 1 corresponds to PWD)

A Welch Two Sample t-test comparing the MaxT between locations where the model predicts PWD and locations where the model predicts no wilt gives a p-value < 2.2e-16 (Figure 7.9). There is strong evidence to suggest that the means between the two groups are different.

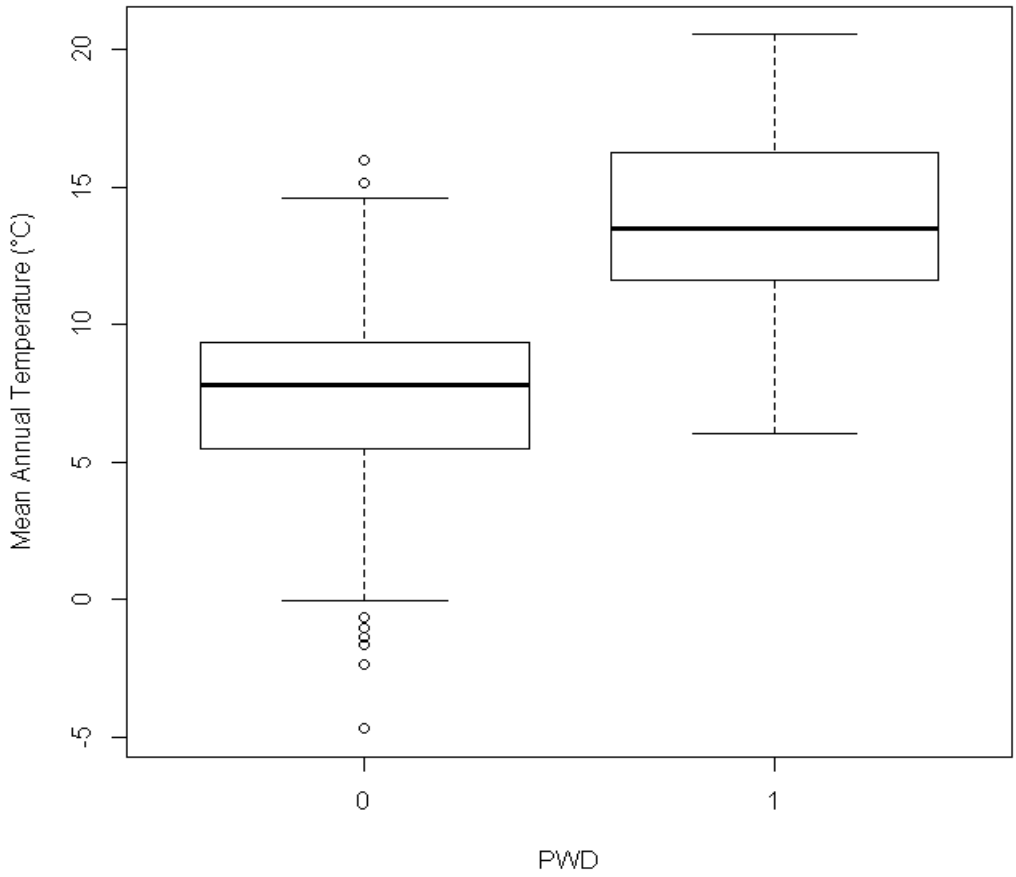

Figure 7.10 Box plot of Mean Annual Temperature (MAT) in °C against PWD (0 corresponds to no wilt and 1 corresponds to PWD)

A Welch Two Sample t-test comparing the MAT between locations where the model predicts PWD and locations where the model predicts no wilt gives a p-value < 2.2e-16 (Figure 7.10). There is strong evidence to suggest that the means between the two groups are different.

Combining the above, we can say that there is clearly evidence that average temperatures and precipitation amounts differ between wilt locations and non-wilt locations. The most obvious one being MST, where we can see in Figure 7.7 that there is very little overlap between box plots. This suggests that MST is split between wilt and non-wilt locations.

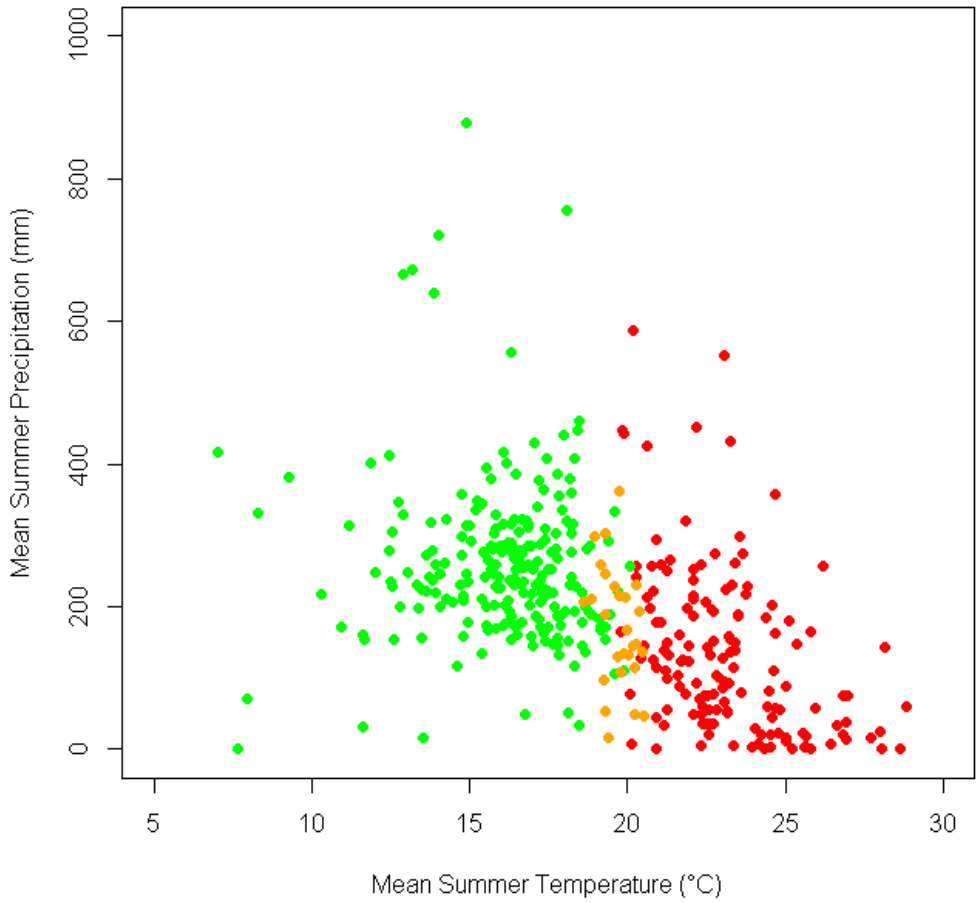

Figure 7.11 Plot of MSP against MST for all locations. Red points represent locations where ETpN predicts wilt; amber points represent locations where the model predicts some wilt (under certain conditions), while green points represent locations where no wilt is predicted.

Figure 7.11 shows a clear split of wilt and no-wilt points at around MST = 20°C, while the amber points are all very close to MST = 20°C. In addition, there is some evidence that precipitation is higher at “no-wilt” locations.

The ETpN model predicts wilt for 99% of locations with MST $\geq$20°C.
The ETpN model predicts no wilt for 100% of locations with MST < 19.31°C.
The ETpN model predicts some wilt (under certain conditions) for 83% of locations with 19.31°C $\leq$ MST < 20°C.

We deduce that we can use MST as a good indicator of wilt which is “user-friendly” and requires little knowledge of climate at a particular location and no knowledge of inoculation dates, nematode densities, tree tolerance etc.

In relation to further simplifying the model, we have posed the question: if a user knows no more than their location, can we provide a relatively accurate measure of risk of PWD at their location?

We have already seen from the maps in Figure 7.5 and Figure 7.6, that PWD is predicted for locations with latitude below a certain threshold.

Consequently, we have plotted latitude against longitude for all locations.

In Figure 7.12, we have used different colours to represent model output.
Green - locations where model predicts no wilt.
Red - locations where model predicts PWD.
Amber - possible locations for PWD or delayed expression of wilt.
Blue - locations with altitude $\geq$ 800m and where model predicts no wilt.

Due to the mountain ranges across Europe, fitting a straight line is not very accurate. Instead, we fit a polynomial (13th order) to the data which takes into account the mountain ranges and potential spread into France.

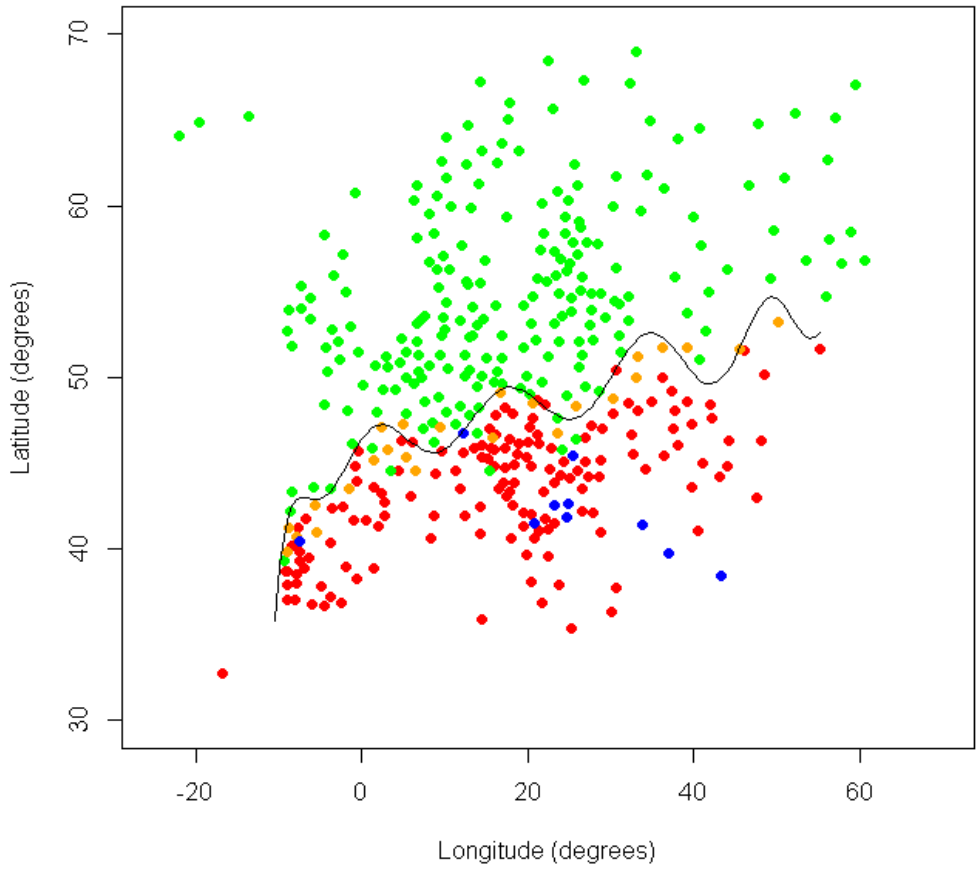

Figure 7.12 Plot of latitude against longitude for all locations in Europe with a polynomial fitted to the edge of wilt locations.

The curve, fitted to some of the northern-most locations where the model predicts wilt, gives us an upper limit for the extent of wilt in Europe, where we expect wilt when:

$y \leq f(x)$

with

$$f(x) = a_{13}x^{13} + a_{12}x^{12} + a_{11}x^{11} + a_{10}x^{10} + a_9x^9 + a_8x^8 + a_7x^7 + a_6x^6 + a_5x^5 + a_4x^4 + a_3x^3 + a_2x^2 + a_1x + a_0$$

where x and y represent longitude and latitude respectively.

We predict a high risk of wilt for locations below this curve and some risk of wilt for locations where $y \leq f(x)+1.2$. If $y \leq f(x)$ and altitude is $\geq$ 800m then the risk is reduced from high to medium.

The coefficients of the curve are:

$a_{13} = -4.2716235E-17$	$a_6 = -2.0946235E-5$
$a_{12} = 1.2184542E-14$	$a_5 = 0.000069825976$
$a_{11} = -1.4265794E-12$	$a_4 = 0.0024502893$
$a_{10} = 8.6199246E-11$	$a_3 = -0.014679433$
$a_9 = -2.6591771E-9$	$a_2 = -0.1129682$
$a_8 = 2.5733313E-8$	$a_1 = 0.68441653$
$a_7 = 7.3633335E-7$	$a_0 = 46.391586$

We note that this location model applies only to locations where -9 $\leq$ Longitude $\leq$ 55 degrees.

For Iceland we note that the model predicts no wilt and for Madeira, Portugal, the model predicts PWD.

Key results for D7.1

The core model has been modified slightly since the last reporting period; however, these changes have made the model more realistic. The model has been validated and model predictions are accurate. The core model has been used to produce European PWD risk maps.

Following a sensitivity analysis and statistical analysis, we have been able to determine the key climatic variables and parameters that are driving the model. Although some of the parameters do have a significant effect on the model output, the climate data is the key driving variable.

A User-friendly sub-model has been developed, using two different approaches. The first, and most accurate method, is to use MST (Mean Summer Temperature) to predict the likelihood of wilt at a particular location, where:

- Locations with MST $\geq$20°C are at a high risk of PWD
- Locations with MST < 19.31°C are at a low risk of PWD, and
- Locations with 19.31°C $\leq$ MST < 20°C are at risk of some PWD.

When a user does not have any information about climate, a simple location model can be used (for -9 $\leq$ Longitude $\leq$ 55 degrees), where we predict:

- a high risk of PWD if $y \leq f(x)$,
- a medium risk of PWD if $f(x) < y \leq f(x)+1.2$,
- a medium risk of PWD if $y \leq f(x)$ and altitude is $\geq$800m, and
- a low risk of PWD if $y > f(x)+1.2$,

where

$$f(x) = a_{13}x^{13} + a_{12}x^{12} + a_{11}x^{11} + a_{10}x^{10} + a_9x^9 + a_8x^8 + a_7x^7 + a_6x^6 + a_5x^5 + a_4x^4 + a_3x^3 + a_2x^2 + a_1x + a_0$$

And

$a_{13} = -4.2716235E-17$ $a_6 = -2.0946235E-5$

$a_{12} = 1.2184542E-14$ $a_5 = 0.000069825976$

$a_{11} = -1.4265794E-12$ $a_4 = 0.0024502893$

$a_{10} = 8.6199246E-11$ $a_3 = -0.014679433$

$a_9 = -2.6591771E-9$ $a_2 = -0.1129682$

$a_8 = 2.5733313E-8$ $a_1 = 0.68441653$

$a_7 = 7.3633335E-7$ $a_0 = 46.391586$

Distribution of PWD given future climate scenarios

We have run the ETPN model for 3 contrasting regions in Europe:

1. Lisbon in Portugal – an area that has suffered with PWD.
2. Bourges in France – a marginal area that has a small risk of PWD under certain conditions and may be an area of high risk in the future.
3. Junsele in Sweden – an area not expected to suffer with PWD.

For each location we consider a **best-case**, **intermediate (typical) case** and **worst-case** scenario, where we vary tree tolerance, inoculation day and inoculation number (Table 7.4).

Table 7.4 Parameter combinations for best, worst and medium (typical) case scenarios

Parameter	**Value**
Number of Nematodes inoculated	10/100/1000
Day of inoculation	180/200/220
Tree Tolerance	0.1/0.17/0.3

The best case and worst case scenarios are at two extremes. The chances of having either very weak trees (low tree tolerance) in a stand of trees (in a well-managed forest weak/unhealthy trees are less likely to occur or are potentially removed during forest operations), or tiny amounts of nematodes being introduced into a tree, are low and so these cases are shown for interest but are highly unlikely.

We consider 2 climate change scenarios for 2070-2100:

1. A1B Scenario, a **medium-high emissions scenario** (Special Report on Emissions Scenarios - The Intergovernmental Panel on Climate Change), which predicts climate projections for a future world of rapid economic growth, new and more efficient technologies, and convergence between regions. The A1B scenario adopts a balance across all energy sources, i.e. fossil and renewable, for the technological change in the energy system.

2. E1 Scenario. This scenario was constructed as a **mitigation scenario** aimed at achieving the EU's 2-degree goal. The E1 scenario leads to long-term stabilisation at 450ppm of CO_2. It is estimated to limit global warming to less than 2 degrees.

For current climate (we use daily data over 3 years: 2009-2011) the ETpN model does not predict wilt at Junsele for any of the three parameter combinations. At Bourges, the model predicts PWD for the worst case scenario only, while at Lisbon, the model predicts PWD for both the worst case and medium case scenarios.

E1 Scenario

We run the ETpN model using E1 climate scenario climate projections.

For the best case scenario (inoculation day is Julian day 220 with 10 nematodes inoculated and tree tolerance of 0.3) the model does not predict PWD at any of the three locations. For the worst case scenario (inoculation day is Julian day 180 with 1000 nematodes inoculated and tree tolerance of 0.1) the model predicts PWD at Lisbon and Bourges, but not at Junsele. For the medium (typical) case scenario (inoculation day is Julian day 200 with 100 nematodes inoculated and tree tolerance of 0.17) the model output is shown in Figure 7.13.

In the following figures the units for photosynthesis are $\mu molm^2s^{-1}$, energy is in Joules (scaled in each graph) and mean temperature is in °C. The right hand axis refers to nematode numbers, while the left hand axis gives values for the other three outputs.

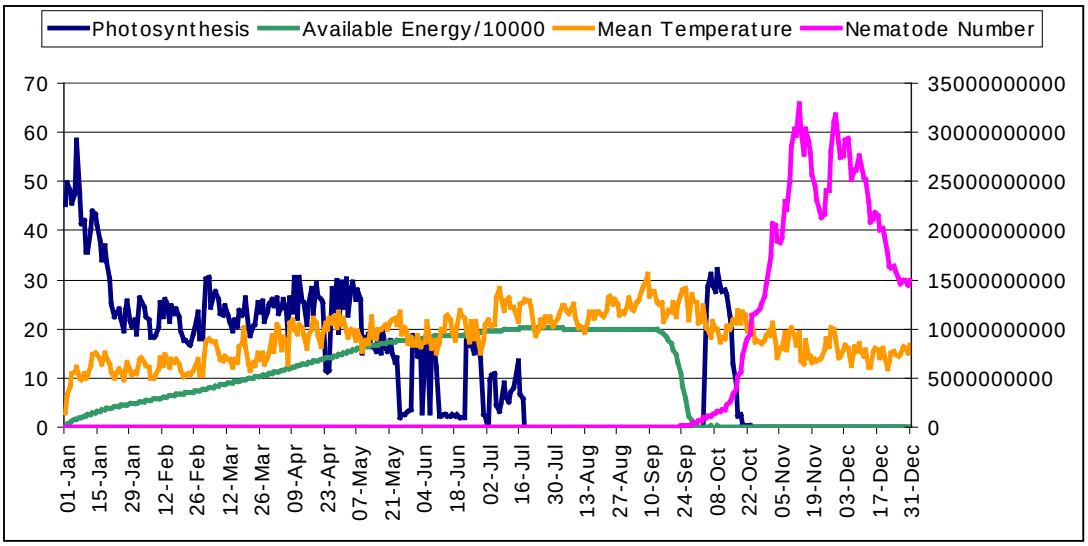

Figure 7.13 Model output for Lisbon, Portugal – medium (typical) case scenario and E1 climate scenario.

At Lisbon, Portugal we run the model for 100 nematodes inoculated on day 200 (19th July) (Figure 7.13). The net photosynthesis becomes zero before this date due to hot, dry conditions at Lisbon. The photosynthesis recovers for a short period in October, however by this point the available energy is almost zero and the tree cannot survive. Tree death occurs on October 22nd which is followed by a huge increase in nematode numbers.

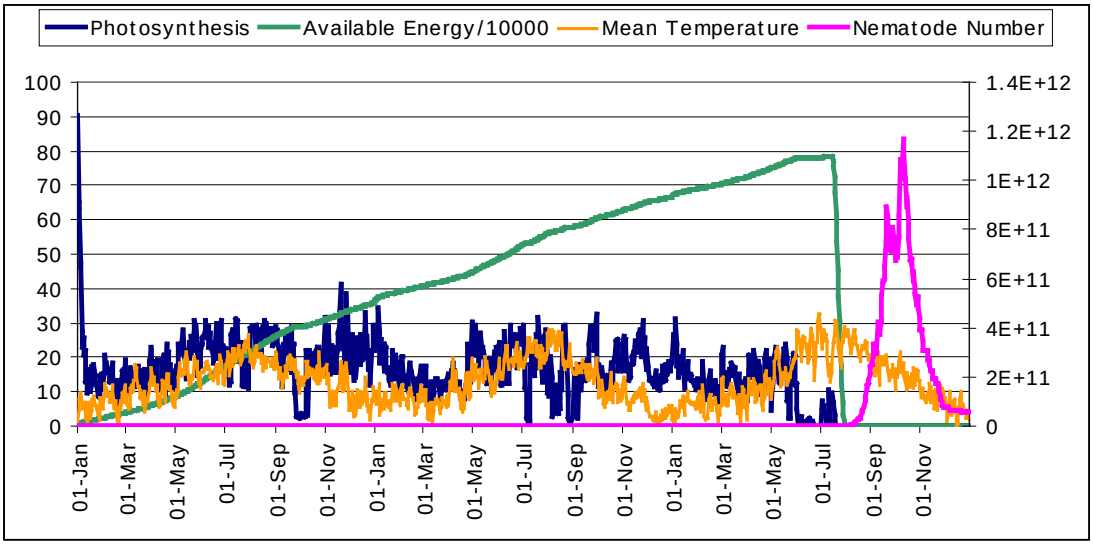

Figure 7.14 Model output for Bourges, France – medium (typical) case scenario and E1 climate scenario.

For the E1 scenario, we see PWD two years after infestation at Bourges in France (Figure 7.14). We can see that the mean temperature gets above 20°C for a very short period only in the year of infestation. In the year following infestation the mean temperature gets above 20°C for a slightly longer period than in the first year, while in the third year, the mean temperature gets above 20°C for a much longer period allowing the nematode numbers to increase to large enough quantities to cause PWD. The photosynthesis and available energy (and hence tree death) become zero at the beginning of August in the third year.

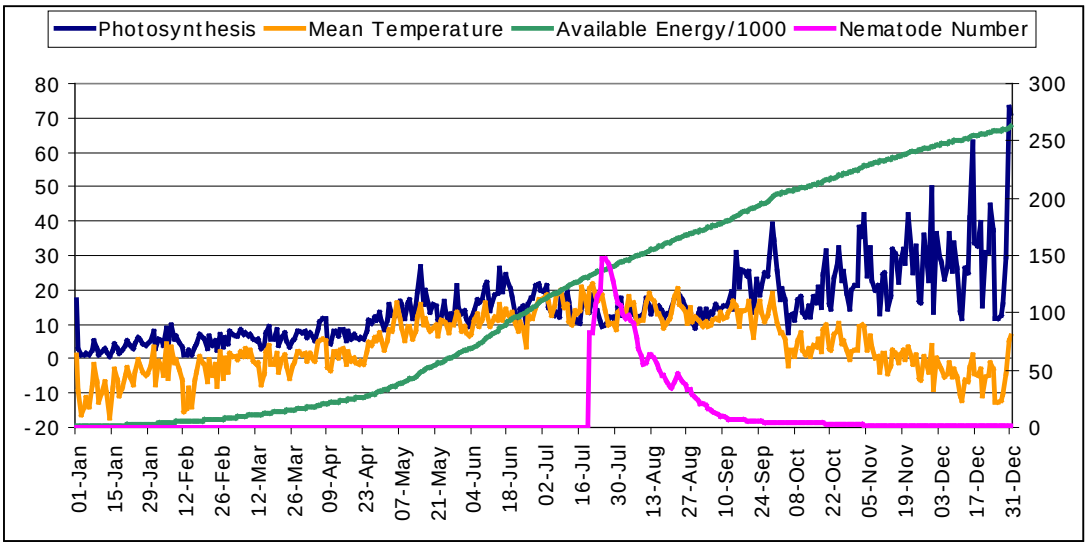

Figure 7.15 Model output for Junsele, Sweden – medium (typical) case scenario and E1 climate scenario.

At Junsele, Sweden, we see an initial peak in Nematode numbers for a few days following the day of infestation (Figure 7.15). Mean temperature gets above 20°C for a few days around the time of infestation, however following this, the mean temperature remains below 20°C for the remainder of the year. The nematode numbers gradually decrease until they become zero around the beginning of November. The tree is not affected by the nematodes and remains healthy.

A1B Scenario

For the best case scenario (inoculation day is Julian day 220 with 10 nematodes inoculated, and tree tolerance of 0.3) the model does not predict PWD at either Junsele or Bourges, but does predict PWD at Lisbon in the year following infestation. For the worst case scenario (inoculation day is Julian day 180 with 1000 nematodes inoculated and tree tolerance of 0.1) the model predicts PWD at Lisbon and Bourges in the year of infestation and at Junsele in the year following infestation.

For the medium case scenario (inoculation day is Julian day 200 with 100 nematodes inoculated and tree tolerance of 0.17) the model output is shown in Figure **7.16**.

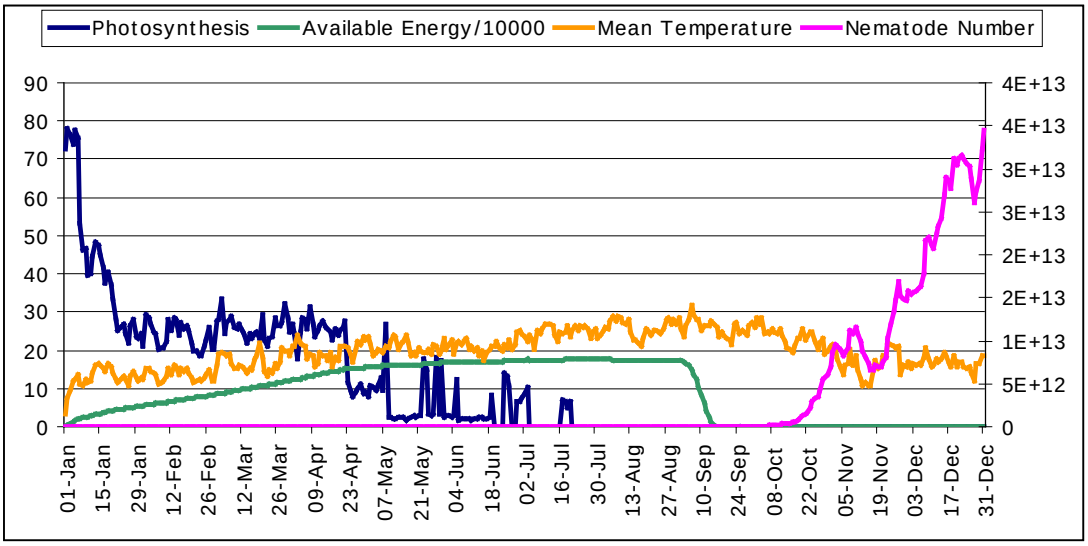

Figure 7.16 Model output for Lisbon, Portugal – medium (typical) case scenario and A1B climate scenario.

At Lisbon, Portugal we run the model for 100 nematodes inoculated on day 200 (19th July). The net photosynthesis becomes zero before this date due to hot, dry conditions at Lisbon. Unlike the output form the E1 scenario, the photosynthesis does not recover later in the year. Tree death occurs on the 19th September (over a month earlier than what was predicted for the E1 scenario), followed by a huge increase in nematode numbers.

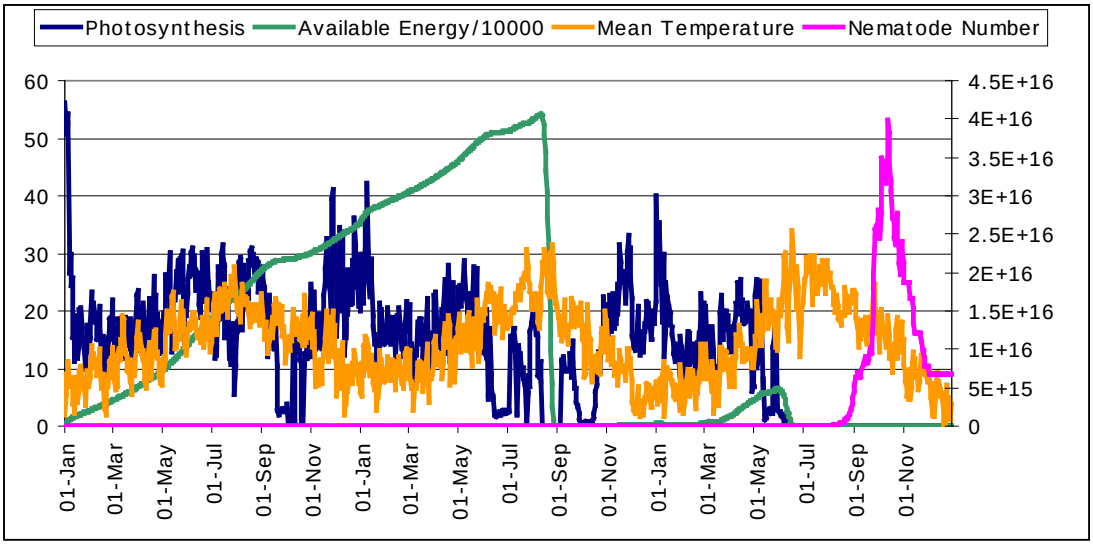

Figure 7.17 Model output for Bourges, France – medium (typical) case scenario and A1B climate scenario.

For the A1B scenario, we see PWD two years after infestation at Bourges in France (Figure **7.17**). However, note that the tree comes very close to death in the year following infestation where the available energy and photosynthesis reaches zero for short periods. As with the output from the E1 scenario for Bourges, we can see that the mean temperature only gets above 20°C for a very short period in the first year, while in the second and third year the mean temperature gets above 20°C for longer periods. The photosynthesis and available energy become permanently zero at the end of May in the third year (over two months earlier than for the E1 scenario).

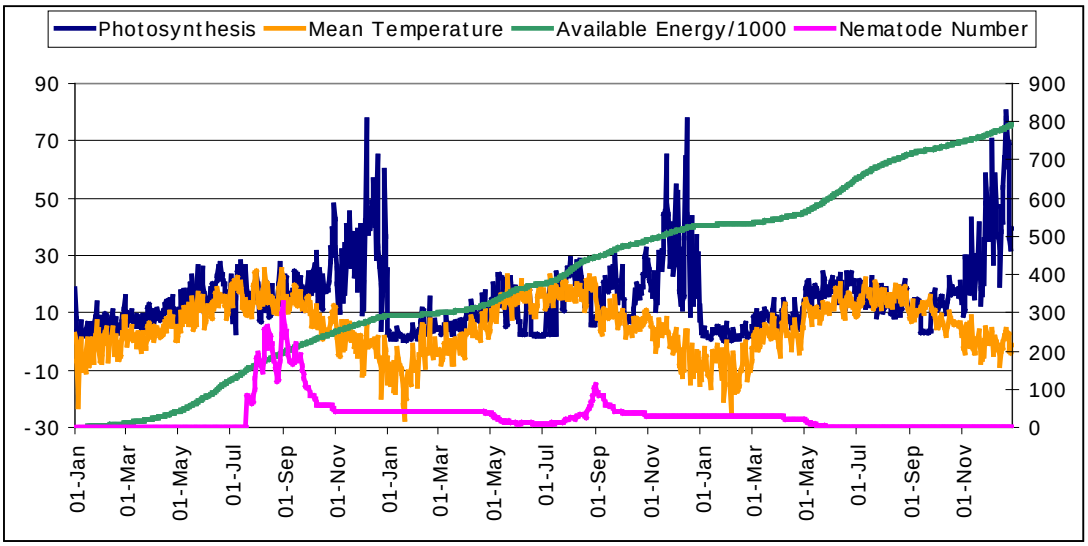

Figure 7.18 Model output for Junsele, Sweden – medium (typical) case scenario and A1B climate scenario.

For the A1B scenario, at Junsele, we see a peak in Nematode numbers for over a month after infestation (
Figure **7.18**). The numbers reduce over winter but unlike the output for the E1 scenario, the nematodes do not completely disappear until year three. The very low

nematode numbers to not seem to have any effect on the health of the tree, where photosynthesis continues as normal and available energy continues to increase over the three years.

Conclusions

- For current climate (2009-2011 data), and the most realistic set of parameters, we see PWD symptoms (following inoculation by nematodes) in Lisbon, Portugal but not in Bourges, France or Junsele in Sweden. In Lisbon we see death in the year following inoculation.
- For E1 2070-2100 climate projections we see PWD symptoms and death in Lisbon 3 months after inoculation. Model output for Bourges shows PWD symptoms two years after inoculation. The temperature gets above 20°C for very short periods in the first two years, while in the third year; the mean temperature gets above 20°C for a much longer period allowing the nematode numbers to increase to large enough quantities to cause PWD. We do not observe PWD symptoms in Junsele.
- For the A1B 2070-2100 climate projections we observe PWD symptoms in Lisbon a month earlier than for the E1 climate projection. In Bourges, we observe PWD symptoms over two months earlier than for the E1 scenario (in third year), however there are signs of PWD in the second year. We do not observe PWD symptoms in Junsele.

Risk maps for future climate

Using the user-friendly sub-model, where:

1. High risk of PWD is predicted for locations with MST (Mean Summer Temperature) $\geq$ 20°C
2. Medium risk of PWD is predicted for locations with 19.31°C $\leq$ MST < 20°C
3. Low risk of PWD is predicted for locations with MST < 19.31°C,

we have created risk maps for current climate (taking averages of MST over 3 years; 2009-2011) and also for 2009-2011 averages +1°C, 2°C and 3°C.

PWD is likely to have a southern limit as well as a Northern limit. Low temperatures are necessary for the formation of dispersal stages of *Bursaphelenchus xylophilus* (Zhao *et al*., 2007), where temperatures of 0°C and 4°C were required for the development of growth arrested stages. The development of the dispersal stage is crucial for the survival of the nematode, to ensure they can survive harsh conditions (low temperatures and lack of food) and also to be able to enter the immature beetles before they emerge, for transmission to a new host tree. Currently, the temperatures across Europe do drop below 4°C and so it is not an issue. However in the risk maps below, increases to MST have been considered only. It may be that, in future, the winter temperatures in some of these locations will become too warm to allow the PWN to enter a dispersal/growth restricted stage. Furthermore, temperatures above 28°C affect *B. xylophilus* development and embryonic development is disrupted at 35°C (Wang *et al*., 2012). Again, this is not currently an issue for most of Europe but could be in the future.

The maps below (Figure 7.19 to Figure 7.22) show the (user-friendly) model output across Europe with increasing average temperatures relative to MST for 2009-2011.

Risk maps for future climate

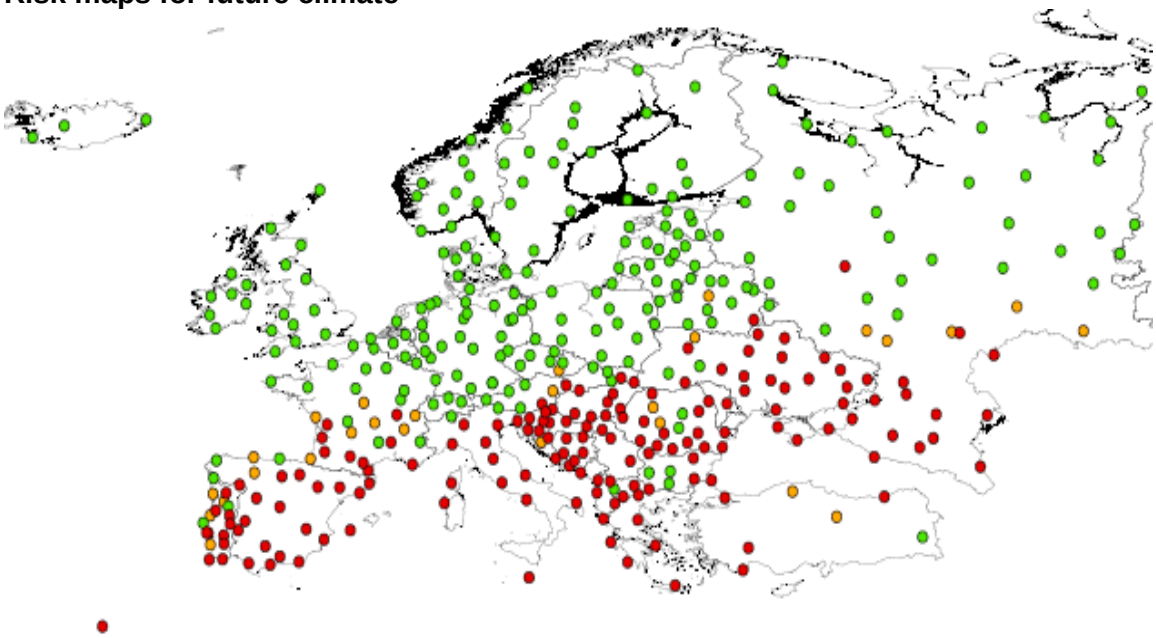

Figure 7.19 PWD risk map for Europe - average MST over 2009-2011

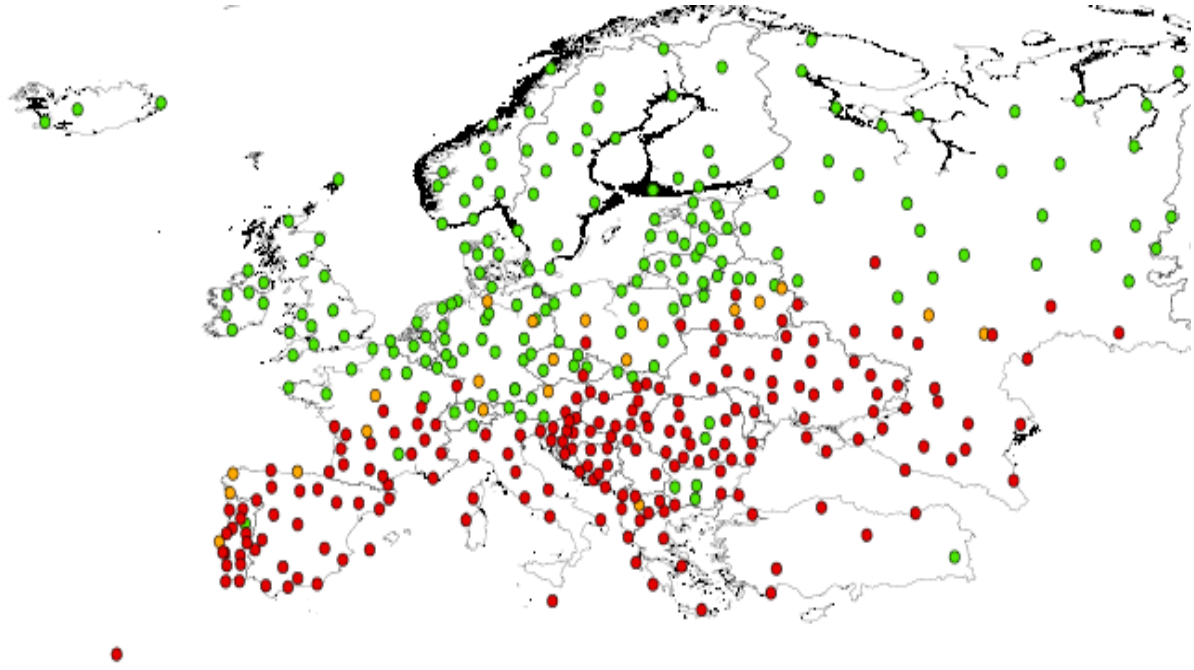

Figure 7.20 PWD risk map for Europe - average MST (over 2009-2011) +1°C

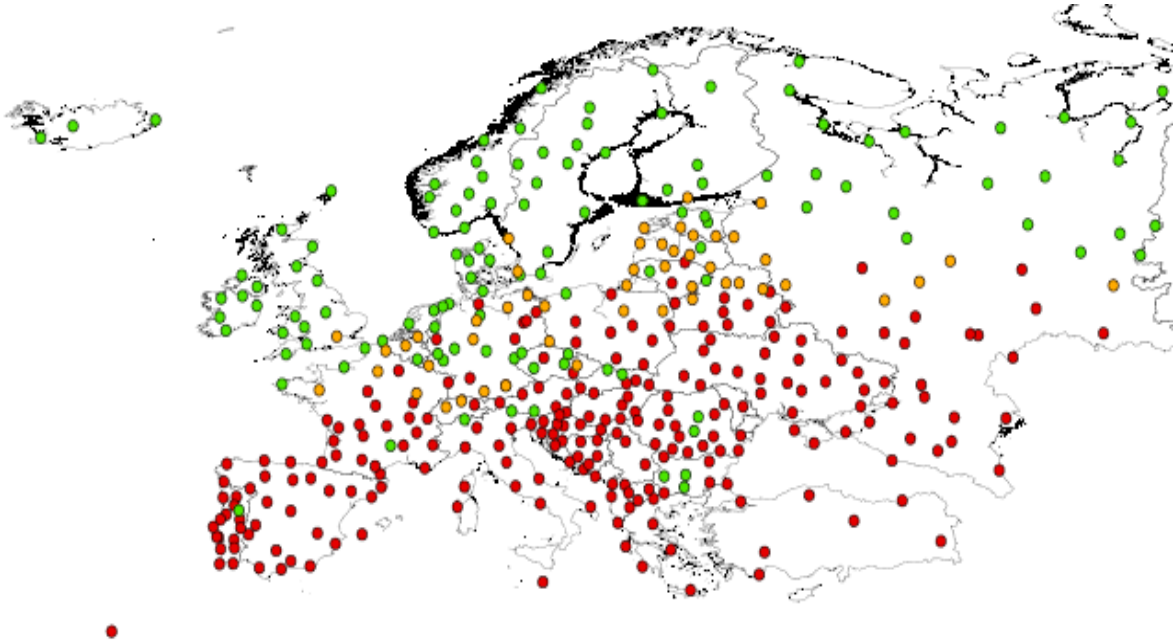

Figure 7.21 PWD risk map for Europe - average MST (over 2009-2011) +2°C

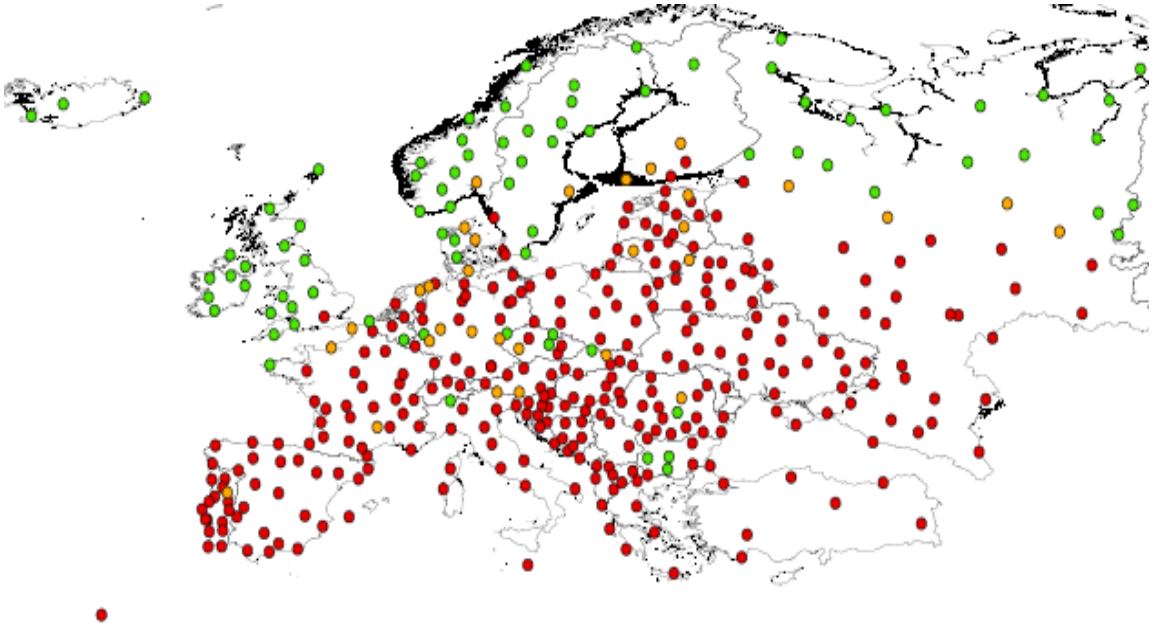

Figure 7.22 PWD risk map for Europe - average MST (over 2009-2011) +3°C

D7.2 Field experimentation of process model (M45)

Work carried out by B1

Deliverable D7.2 was not possible due to uncertainty of getting an extension and also not being able to get permission to carry out inoculation work in Canada. The model has been verified using climate data from Japan and also data from the literature on the disease development. See Model validation section above for more details.

D7.3 Latency sub-model (M44)

Work carried out by B1

Latency is "biologically realistic", where *B. xylophilus* can persist in living *Pinus sylvestris* without inciting pine wilt disease (Chapter 12, Zhao *et al.*, 2008). Furthermore, inoculation experiments, where wilt is controlled by reducing the temperature and then reactivated again when temperatures are increased, provides evidence that delayed disease development of naturally infested pine trees in cool areas can be attributed to the effect of low temperature (Mamiya, 1988).

Latency in wilt expression is a particularly important feature of PWD and understanding what causes latency in symptom development is crucial in tracking infestation rates and potentially eradicating PWD from a stand. Dead and unhealthy trees would be removed from a stand at the end of a growing season in order to stop the spread of both vector and nematode. If symptoms do not develop until the following year then PWN infested trees could be left in a stand of healthy trees. These may then be colonized by *Monochamus* vectors early in the following spring or even in the autumn of nematode inoculation to the tree, even though no visible symptoms are apparent.

We have developed a latency sub-model to relate latency to climate and model parameters in order to predict where there is likely to be latency in disease symptoms. The figures and statistical tests in this section are produced using the software R (R Core Team, (2014)).

We have seen from the sensitivity analysis in the previous section that the main parameters affecting the model output are: initial nematode number, infestation day and tree tolerance. We note that since tree tolerance is difficult to quantify it will be impossible to use this parameter to quantify the likelihood of latency and so we do not address this in detail. It is true that if we increase the level of tolerance in the model it will increase the time it takes for a tree to die from PWD. We conclude that a healthy tree is likely to take longer to die from PWD than stressed/unhealthy tree, but quantifying "how healthy" a tree is, is almost impossible.

With regards to inoculation day, which can be estimated from data on beetle emergence, the model output changes as we increase the day of infestation. We have run the model for all locations that have some chance of PWD and we vary the inoculation day between Julian day 160 and Julian day 240 to reflect the main period of adult vector activity. Initial nematode number is fixed at 500. The results are displayed in Figure 7.23.

Figure 7.23

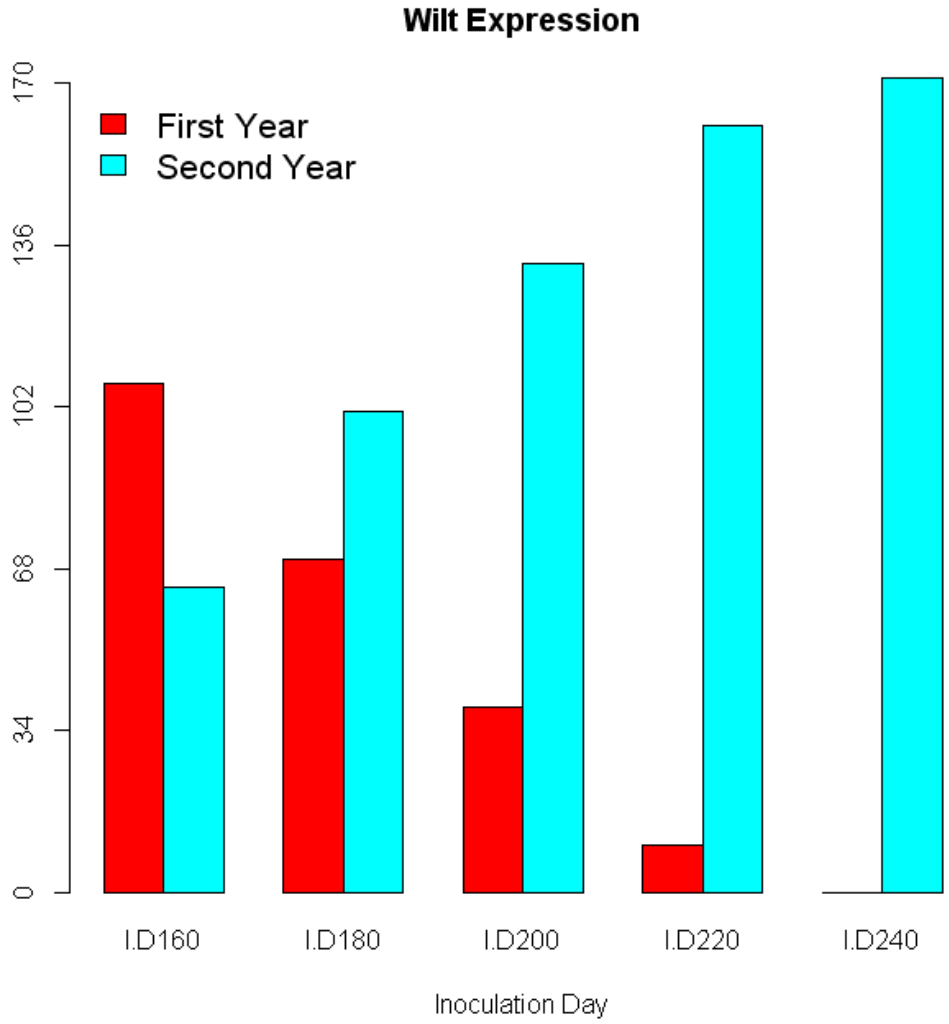

Figure 7.23 Bar chart of wilt symptoms for 5 different inoculation days for 171 locations.

Figure 7.23 shows the shift of wilt symptoms to the year following infestation, as inoculation day is increased. Julian day 160 is very early for beetle emergence, few locations will actually have beetles emerging this early, and even then we have over a third of locations where trees are expected to die a year after infestation. For inoculation day 240, the model predicts wilt in the year following infestation at all locations.

We perform a Chi squared test for the data.

```
> mydata
       I.D160 I.D180 I.D200 I.D220 I.D240
First     107     70     39     10      0
Second     64    101    132    161    171

> chisq.test(mydata)
        Pearson's Chi-squared test

data:  mydata
X-squared = 233.2103, df = 4, p-value < 2.2e-16
```

Pearson Chi-Square statistic, $\chi^2 = 233.2103$, and $p < 0.001$;
i.e., a very small probability of the observed data under the null hypothesis of no relationship. The null hypothesis is rejected, since $p < 0.05$ (in fact $p < 0.001$).

We conclude that there is a relationship between infestation day and year of wilt expression, however, some of these infestation days may not be realistic for some of the locations.

Next, we consider the effect of the number of nematodes inoculated on when wilt expression is predicted. Inoculation day is fixed at 180.

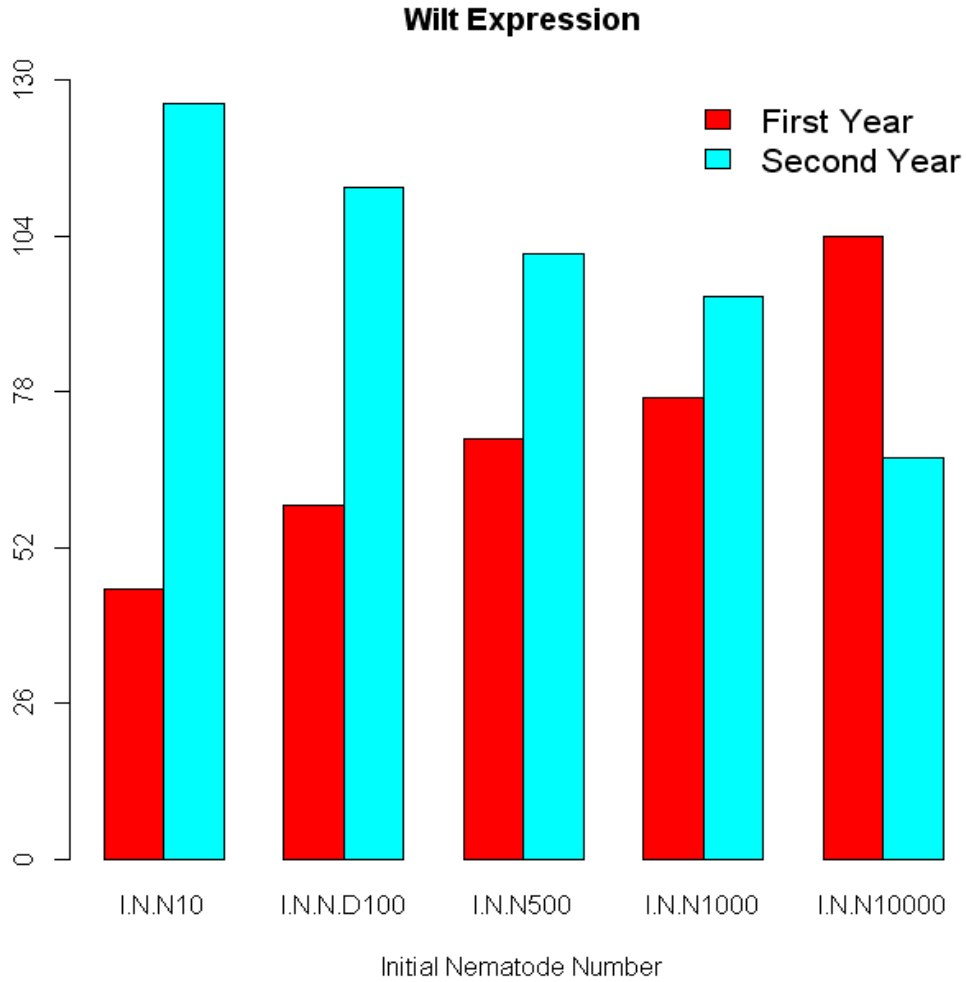

Figure 7.24 Bar chart of wilt symptoms for 5 different initial nematode numbers for 171 locations.

Figure 7.24 shows the shift of wilt symptoms to the year following infestation, as the initial nematode number is increased. An initial nematode number of 10 is quite unlikely, but it is interesting to see how many locations show wilt in the year of infestation given this low initial number.

We perform a Chi squared test for the data.

```
       I.N.N10 I.N.N.D100 I.N.N500 I.N.N1000 I.N.N10000
First       45        59       70        77        104
Second     126       112      101        94         67
> chisq.test(mydata)

        Pearson's Chi-squared test
data:  mydata
X-squared = 46.8685, df = 4, p-value = 1.624e-09
```

Pearson Chi-Square statistic, $\chi^2 = 46.8685$, and p = 1.624e-09;
i.e. a very small probability of the observed data under the null hypothesis of no relationship. The null hypothesis is rejected, since $p < 0.05$ (in fact $p < 0.001$).

We split the locations into three groups:

1. locations where we expect PWD symptoms in the **year following infestation** - where the model predicts wilt in the year following infestation, for at least 8 out of the 10 parameter combinations,
2. locations where we expect PWD symptoms in the **year of infestation** - where the model predicts wilt in the year of infestation, for at least 6 out of the 10 parameter combinations and
3. locations where the year in which we see PWD symptoms vary depending on parameter values.

For these first two groups we compare climatic data to test for a relationship (Figure 7.25).

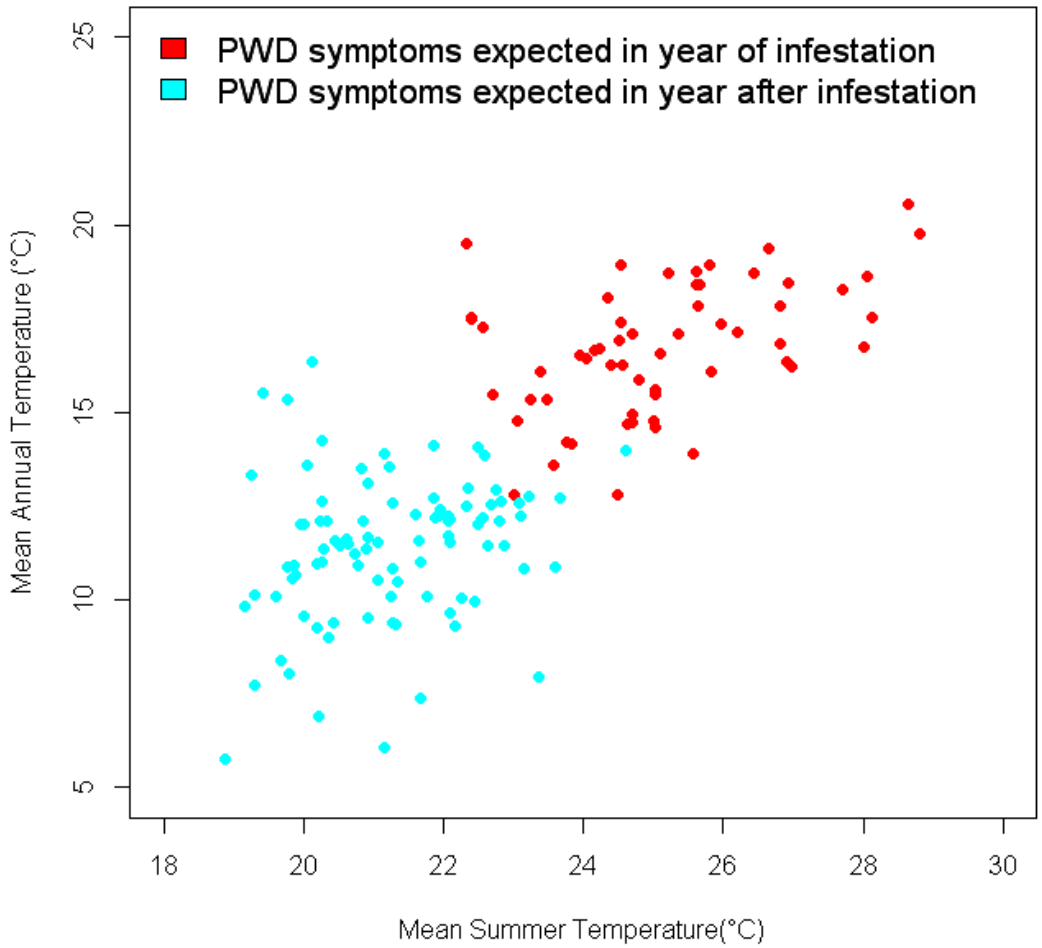

Figure 7.25 A plot of Mean Annual Temperature (°C) against Mean Summer Temperature (°C), split between locations where PWD symptoms are expected in year of infestation and locations where PWD symptoms are expected in year after infestation.

We perform a Welch Two Sample t-test to compare the MST between the two groups. For all three pairs we have a p-value < 2.2e-16, which is evidence to reject the null hypothesis that the true difference in means is zero. We deduce that there is a significant difference in the MST between the two groups. The average MST for locations where PWD symptoms are expected in year of infestation and locations where PWD symptoms are expected in year after infestation are: 25.09°C and 21.27°C respectively.

There is a significant difference between the average MAT between the two groups also (p-value < 2.2e-16), and from Figure 7.25 there appears to be a split point around 14°C.

Mean summer temperature and mean annual temperature are good indicators of latency, where there is a high chance of latency at locations where MST <23°C and MAT<14°C. As indicated earlier, we do not expect PWD at locations where MST< 19.31°C.

With regards to precipitation, we performed two Welch Two Sample t-tests to compare precipitation between the two groups: locations where PWD symptoms are expected in year of infestation and locations where PWD symptoms are expected in year after infestation. The first test compares the average TAP (total annual

precipitation - mm) between the two groups and the second test compares the average MSP (mean summer precipitation - mm) between the two groups.

For the TAP we have a p-value of 0.1729, i.e. there is not sufficient evidence to reject the null hypothesis; that the two means are equal. For the MSP we have a p-value<1.516e-05. There is evidence that the MSP between the two groups are different. The average MSP for locations where PWD symptoms are expected in year of infestation and locations where PWD symptoms are expected in year after infestation are 82.34mm and 164.63mm respectively.

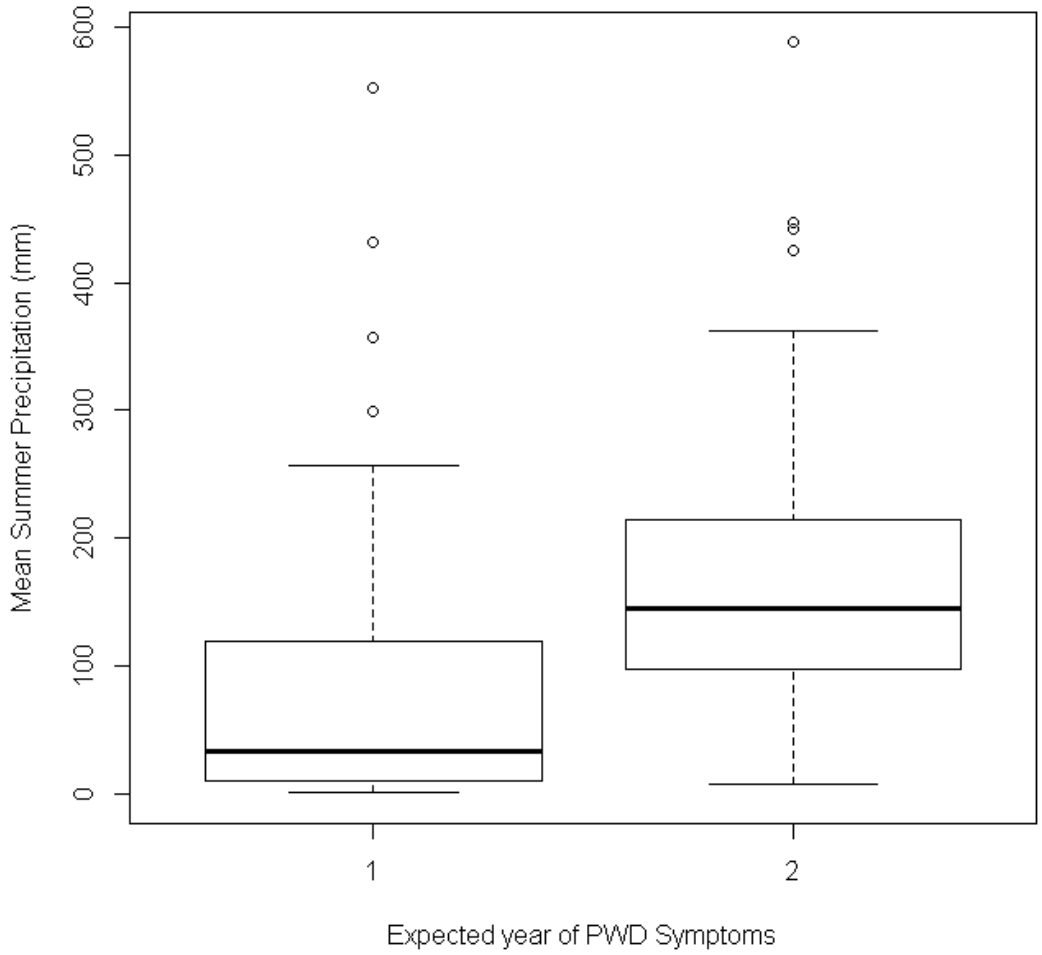

Figure 7.26 Boxplot of Mean summer precipitation (mm) for locations with a high risk of PWD in year of infestation, locations with a high risk of PWD in year after infestation.

Figure 7.26 shows the difference in average MSP between locations where PWD symptoms are expected in year of infestation and locations where PWD symptoms are expected in year after infestation. Mean summer precipitation is higher at locations where we expect latency, but as we can see in Figure 7.27, we cannot deduce a precipitation threshold for latency.

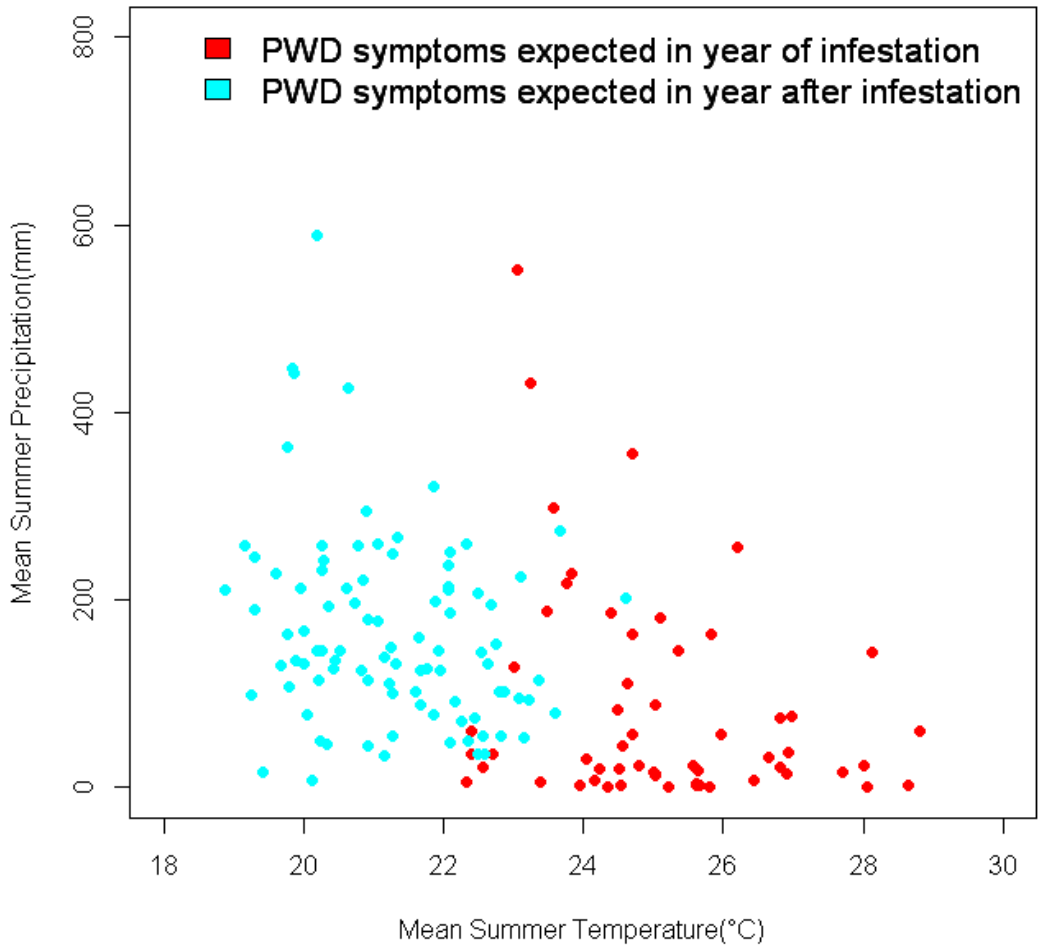

Figure 7.27 Plot of Mean Summer Precipitation (mm) against Mean Summer Temperature (°C), for locations where PWD symptoms are expected in year of infestation and locations where PWD symptoms are expected in year after infestation.

Average summer precipitation is higher at latency locations but we cannot say much more than this.

Finally, we consider the relationship between latency and location. We compare the average altitude, latitude and longitude between the two groups. Results of Welch Two Sample t-tests give p-values as follow: p-value = 0.000964, p-value < 2.2e-16 and p-value = 4.703e-05 for altitude, latitude and longitude respectively. All averages are significantly different, with latitude being the most significantly different.

Key results of D7.3: Latency sub-model

We have demonstrated in this section that model parameters, location parameters and climate have an effect on latency. The key model parameters that affect when we see PWD symptoms occurring are inoculation day, initial nematode number and tree tolerance, where latency is more likely for later inoculation dates, lower initial nematode numbers and higher tree tolerance. Quantifying what we mean by some of these is difficult, especially when we talk about tree tolerance; where at most we can say that healthy trees are likely to be more tolerant than weak/unhealthy trees and hence less likely to succumb to PWD in the year of infestation.

For initial nematode numbers, we considered the values: 10, 100, 500, 1000 and 10000. The model predicts PWD symptoms in the year of infestation for an increasing number of locations as initial nematode numbers are increased. It is difficult to use this fact to predict locations that are likely to exhibit latency in PWD symptoms as it is not known how many nematodes successfully enter a tree during maturation feeding. Nematode numbers in the range 3000-10,000 are used in inoculation experiments as only about 10% successfully invade the tree (Zhao *et al*., 2008). Furthermore, there are differences in the numbers of nematodes carried by the beetles, depending on the trees from which they emerged; where higher numbers of nematodes were carried by beetles they had emerged from trees with intense blue-stain fungi on the walls of the pupal chambers (Maehara *et al*., 2005). Predicting likely inoculation numbers is therefore very difficult.

With regards to inoculation/infestation day, this will depend on beetle emergence, which varies due to climatic factors. Beetle emergence can span up to 4 months at a particular location, and the beetles can transmit nematodes for most of their adult life which makes it difficult to predict an accurate infestation day at a location. It is possible to predict (using a degree day model (Naves *et al*., 2009)) when we are likely to see 50% emergence of beetles and we can use this to estimate the chances of latency at a location. The model predicts PWD in the **year of infestation** at 62% of locations when inoculation day is Julian day 160 (9th June), while for Julian day 200 (19th July), the model predicts PWD in the **year after infestation** for over 75% of locations and when inoculation day is Julian day 240 (28th August), the model predicts PWD in the **year after infestation** for 100% of locations.

For Europe, using a degree day model, for 50% beetle emergence (Naves *et al*., 2009), we estimate where we are likely to have a zone where latency is likely (Figure 7.28).

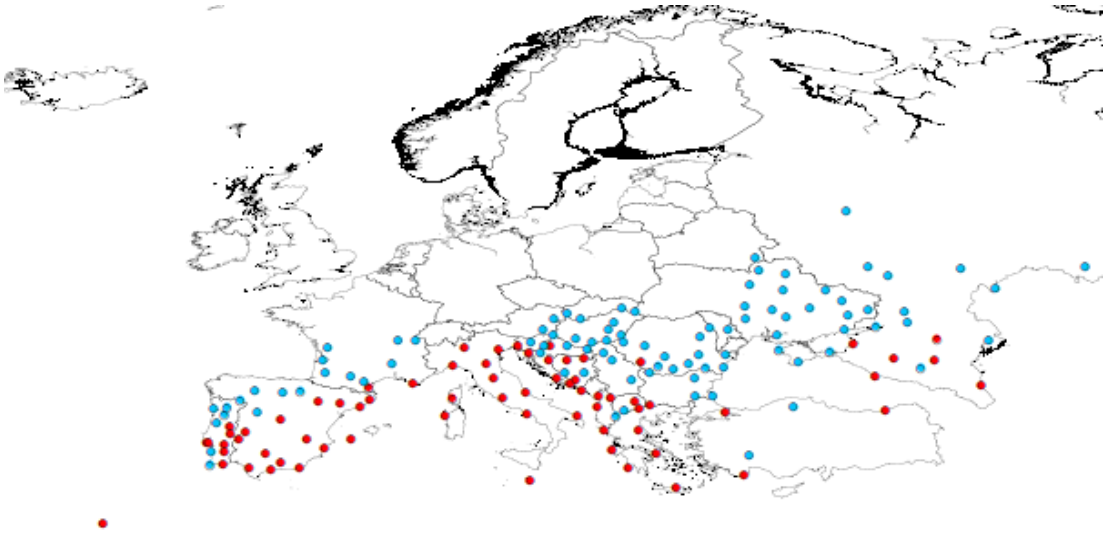

Figure 7.28 Map of Europe with locations where a degree of latency in PWD symptoms is likely. Red points are locations with wilt in year of infestation and blue points are locations with wilt in year after infestation

Finally, with regards to climate and location, there is evidence that location (latitude, longitude and altitude) has an effect on latency. However, climate variables have the greatest effect on latency, where mean summer temperature and mean annual temperature are good indicators of latency.

There is a high chance of latency at locations where MST <23°C and MAT<14°C. From the model approach, we do not expect PWD at locations where MST< 19.31°C.

Interactive Simplified ETpN model

The sub-models developed from the simplified ETpN model will be available on the REPHRAME website as interactive models. A user will be able to specify either their location or temperature variables at a particular location to determine a risk of PWD.
In addition to this we have developed models to predict the time (in weeks or months) it takes for a tree infested with PWN to succumb to PWD.

The interactive model uses the following results:
MST (Mean Summer Temperature) is used to predict the likelihood of wilt at a particular location, where:

- Locations with MST $\geq$20°C are at a high risk of PWD
- Locations with MST < 19.31°C are at a low risk of PWD, and
- Locations with 19.31°C $\leq$ MST < 20°C are at risk of some PWD.

When a user does not have any information about climate, a simple location model can be used (for -9 $\leq$ Longitude $\leq$ 55 degrees), where we predict:

- a high risk of PWD if $y \leq f(x)$,
- a medium risk of PWD if $f(x) < y \leq f(x)+1.2$,
- a medium risk of PWD if $y \leq f(x)$ and altitude is $\geq$800m, and
- a low risk of PWD if $y > f(x)+1.2$,

where

$$f(x) = a_{13}x^{13} + a_{12}x^{12} + a_{11}x^{11} + a_{10}x^{10} + a_9x^9 + a_8x^8 + a_7x^7 + a_6x^6 + a_5x^5 + a_4x^4 + a_3x^3 + a_2x^2 + a_1x + a_0$$

And

$a_{13} = -4.2716235E-17$	$a_6 = -2.0946235E-5$
$a_{12} = 1.2184542E-14$	$a_5 = 0.000069825976$
$a_{11} = -1.4265794E-12$	$a_4 = 0.0024502893$
$a_{10} = 8.6199246E-11$	$a_3 = -0.014679433$
$a_9 = -2.6591771E-9$	$a_2 = -0.1129682$
$a_8 = 2.5733313E-8$	$a_1 = 0.68441653$
$a_7 = 7.3633335E-7$	$a_0 = 46.391586$

To be able to predict a time scale for death following infestation we require a range of output from the full ETpN model; particularity with regards to the time it takes a tree to succumb to PWD given different inoculation days.

In the interactive model we will give the user a choice of inoculation days linking to look-up results for those days, since allowing a user to specify any date will make calculations much more difficult.

We have run the model for 175 locations (locations where the model predicts wilt for at least some parameter combinations) for 5 different inoculation dates: 1st May, 1st June, 1st July, 1st August and 1st September.

For each inoculation date we split the locations into two groups; those locations where wilt is predicted in the year of infestation and those locations where wilt is predicted in the year following infestation. For each location and set of inoculation dates we record the number of days it takes the tree to die from PWD.

We plot MST – mean summer temperature (°C) against the number of days until death and fit curves to these points. Furthermore, we use MST and MAT – mean annual temperature (°C) to predict the year we expect wilt in order to know which curve to use for days.
We demonstrate below using data for inoculation date 1st June (day 152) (Figure 7.29).

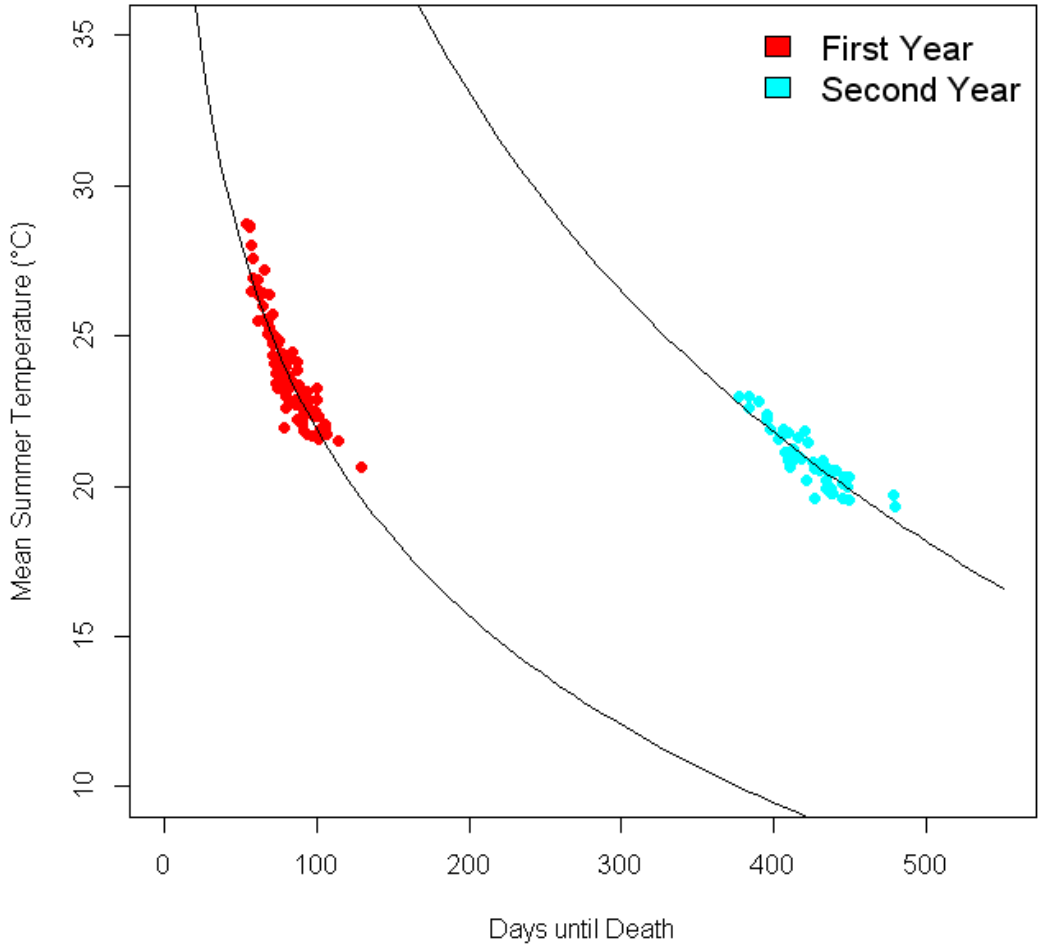

Figure 7.29 Plot of MST (°C) against days until death for locations split between year of infestation and year following infestation.

We fit the following curves to the data:

$$D_1 = \exp\left\{\frac{63.314723 - MST}{8.989318}\right\}$$

$$D_2 = \exp\left\{\frac{119.59916 - MST}{16.325108}\right\}$$

where D_1 and D_2 are the expected number of days until death for locations where trees are expected to die in the year of infestation and for locations where trees are expected to die in the year following infestation respectively.

The fitted curves D_1 and D_2 have R^2 values of 0.8476 and 0.8051 respectively.
In order to determine which of these curves to use to calculate the number of days from infestation to death we fit a probit model, using the software R (R Core Team, (2014)), to the data using MST and MAT as independent variables to predict the expected year of wilt.
We repeat the above for all 5 inoculation dates and use the results to estimate the number of weeks (if death is expected in year of infestation) or months (if death is expected in year following infestation) it will take a tree to succumb to PWD given MST and MAT values.
The models will be available on the REPHRAME website.

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D7.4: Analysis of the PWN history in Portugal: Analysis of the history of infestation and wilt expression in Portugal (M42)

Task 7.1.4 Analysis of the history of infestation and wilt expression in Portugal

Work carried out by B7

National analysis

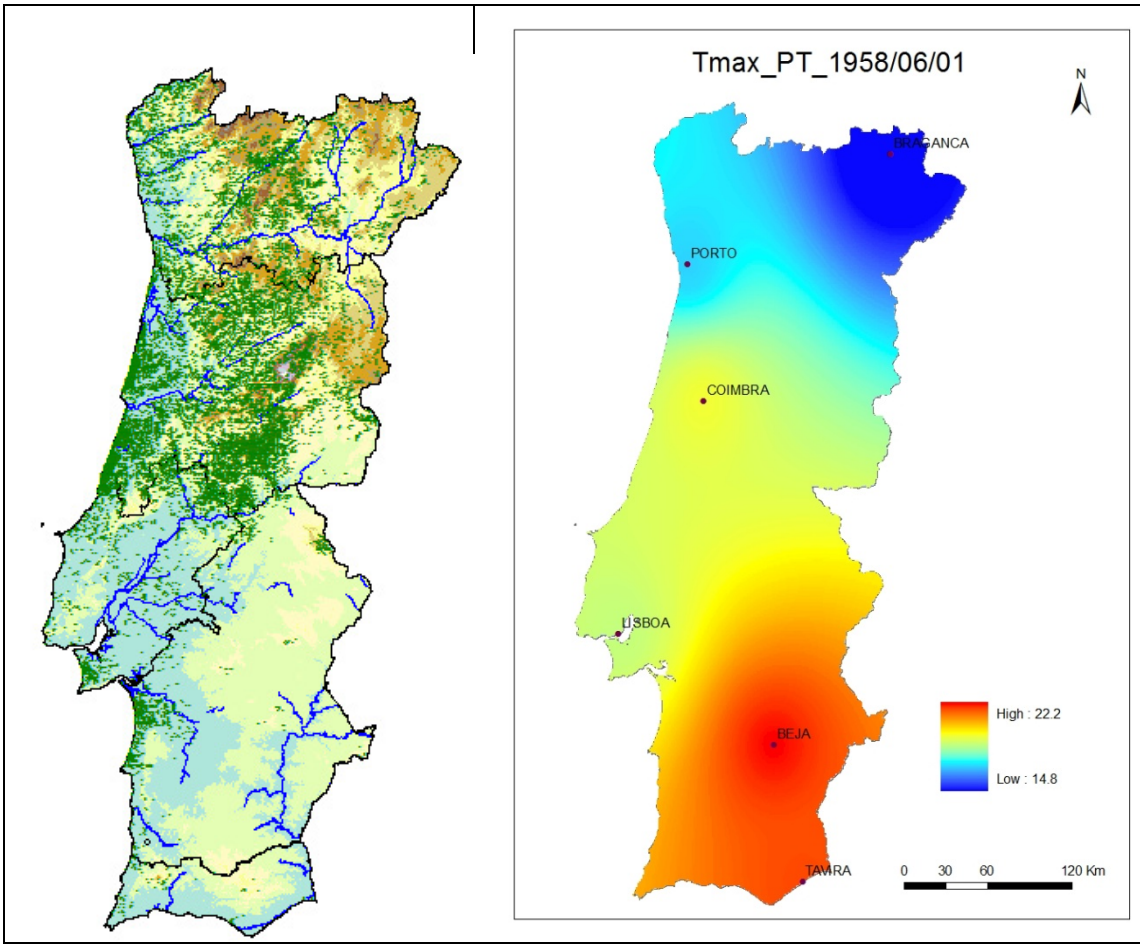

Figure 7.30 – *Pinus pinaster* presence (left) and derived climatic variables (right).

Pinus pinaster presence and temperature were some of the data gathered to try to understand PWD in Portugal and Iberia (Figure 7.30).

From previous data, we modelled the presence of trees killed by PWD with ICNF data, as it is the only confirmed PWD field presence available for the moment. Our field data (2nd periodic report) were a powerful tool to confirm modelled data.

The regression tree (methodology applied and explained in previous reports) had percentage of PWD trees as dependent variable and 5 explanatory variables: distance to the coast, continentality (temperature difference between the hottest and the coldest month), Summer precipitation, temperature of the hottest month and altimetry

1) root 669 116622.30000 5.1001490
2) Distance to the coast>=3474250 439 25718.77000 2.3280180
4) Continentality>=24.66332 284 1333.61600 0.3908451 *
5) Continentality < 24.66332 155 21366.67000 5.8774190
10) Summer precipitation>=89.39443 103 1572.71800 1.2038830 *
11) Summer precipitation < 89.39443 52 13088.06000 15.1346200
22) Summer precipitation < 71.11137 22 1511.86400 4.7727270 *
23) Summer precipitation >=71.11137 30 7481.86700 22.7333300
46) Distance to the coast >=9897021 14 1359.21400 11.3571400 *
47) Distance to the coast < 9897021 16 2725.43800 32.6875000 *
3) Distance to the coast 3474250 230 81090.78000 10.3913000
6) Temperature hottest month>=30.64481 26 337.84620 0.9230769 *
7) Temperature hottest month < 30.64481 204 78125.04000 11.5980400
14) Continentality < 25.02617 168 54283.14000 9.4285710
28) Summer precipitation >=24.56881 116 23659.21000 6.8448280
56) Altimetry< 62.96527 49 4905.95900 2.7959180
112) Continentality < 23.98093 40 95.10000 0.3500000 *
113) Continentality >=23.98093 9 3508.00000 13.6666700 *
57) Altimetry >=62.96527 67 17362.48000 9.8059700
114) Temperature hottest month >=26.68528 28 1284.96400 3.5357140 *
115) Temperature hottest month < 26.68528 39 14186.31000 14.3076900
230) Continentality < 21.31688 32 9863.50000 11.6250000
460) Distance to the coast >=1850161 11 314.00000 3.0000000 *
461) Distance to the coast < 1850161 21 8302.57100 16.1428600
922) Continentality < 18.95981 7 192.85710 2.1428570 *
923) Continentality >=18.95981 14 6051.71400 23.1428600 *
231) Continentality >=21.31688 7 3039.71400 26.5714300 *
29) Summer precipitation < 24.56881 52 28122.08000 15.1923100
58) Summer precipitation < 21.33931 30 9814.96700 6.3666670
116) Temperature hottest month >=26.26735 21 60.95238 0.3809524 *
117) Temperature hottest month < 26.26735 9 7246.00000 20.3333300 *
59) Summer precipitation >=21.33931 22 12783.86000 27.2272700
118) Temperature hottest month >=27.85765 12 4254.91700 15.4166700 *
119) Temperature hottest month < 27.85765 10 4846.40000 41.4000000 *
15) Continentality 25.02617 36 19361.22000 21.7222200
30) Continentality >=25.62334 25 8607.44000 14.6800000
60) Summer precipitation >=44.01871 8 552.87500 4.1250000 *
61) Summer precipitation < 44.01871 17 6743.88200 19.6470600 *
31) Continentality < 25.62334 11 6696.18200 37.7272700 *

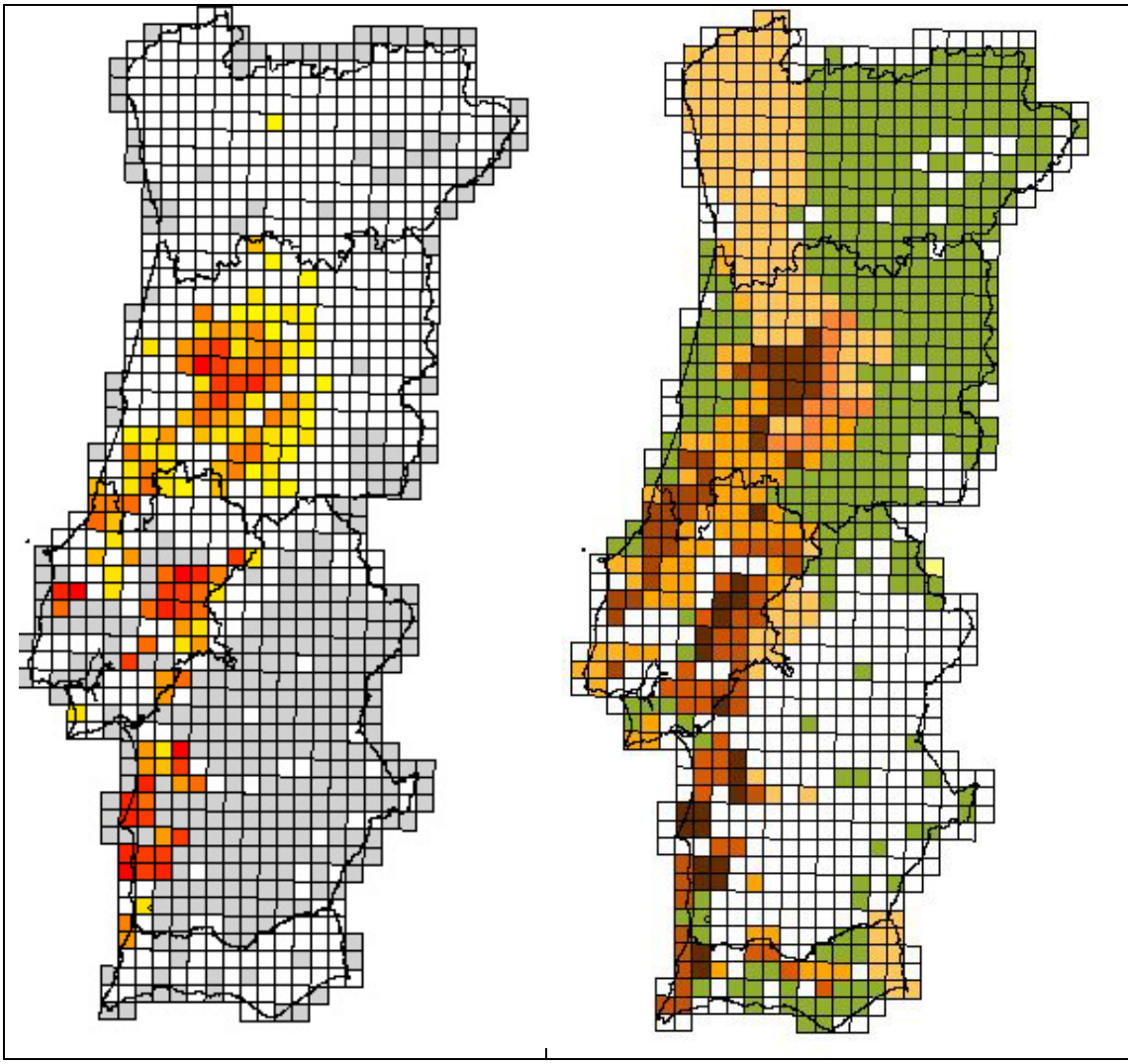

Figure 7.31 PWD observed (left) and PWD predicted by the model (right). On the left, data from ICNF (percentage of infested trees, from white – not infested, to yellow – infested and red severely infested). On the right, brown is more than 21%, orange is around 5%, light cream is 1% and green 0%. White is *Pinus pinaster* absence.

Figure 7.31 shows PWD distribution in Portugal, both for observed and confirmed data (left) and with a climatic model (right).

As we can see, the model fits well to the observed pattern and extrapolates the present boundaries in south and in north. From our own field data and remote sensing analyses (previous reports), the model fits well with the observed patterns, in particular in central Portugal, around Sines, and Nazaré.

It is also interesting to note that the model predicts a light PWD infestation in North Portugal. In fact, in the frontier near Serra do Caramulo and Serra da Freita, although there are PWD some years ago, it seems not to produce a high likelihood of PWD compared with the situation around Coimbra. If the climatic envelope is the main determinant in PWD spreading and severity, thus it is possible to assume that there will be different degrees of PWD in continental Portugal.

PWD seems to be associated with low summer precipitation and high temperatures, but the fitted model is complex, and some combinations are yet uncertain.

Nevertheless, the fitted model adjusts well to the ICNF data and gives clues to the possible PWD spreading. The model also adjusted well to field survey reported in first report, as we can see in Figure 7.32.

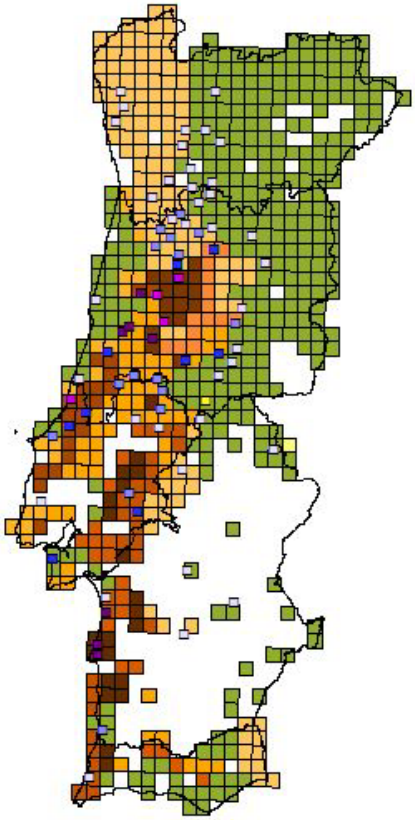

Figure 7.32 Field evaluation of PWD (symptomatic trees (small squares), from no PWD (white), to 1% (light blue), 5% (blue), 15% (light purple) and more than 25% (strong purple).

Regional analysis

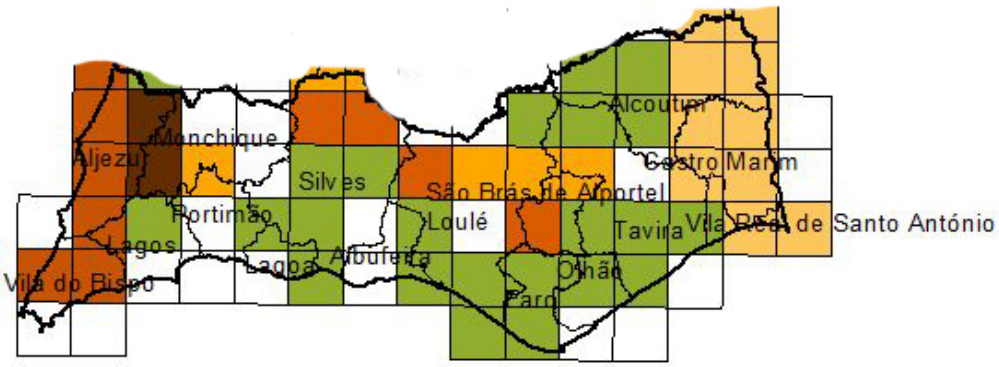

Figure 7.33 PWD in Algarve region predicted by the model. Brown is more than 21%, orange is around 5%, light cream is 1% and green 0%. White is *Pinus pinaster* absence.

In Algarve (Figure 7.33), the model predicts severe PWD around Monchique and a less severe PWD near the coast and in Caldeirão. It also predicts the 1% PWD on the frontier with Spain. In the south of Algarve the model predicts 0% PWD.

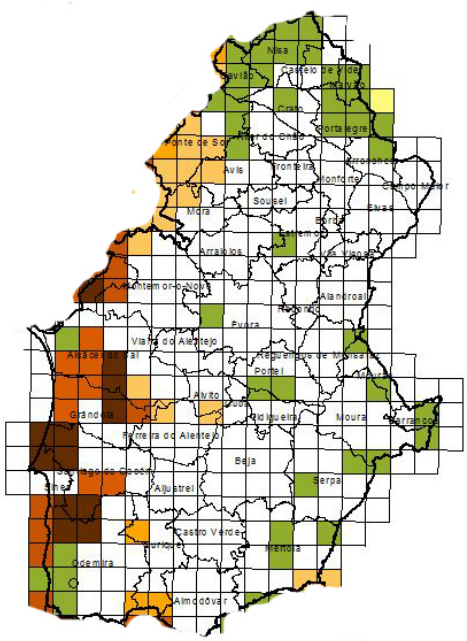

Figure 7.34 PWD in Alentejo region predicted by the model. Brown is more than 21%, orange is around 5%, light cream is 1% and green 0%. White is *Pinus pinaster* absence.

In Alentejo (Figure 7.34), the model predicts high PWD near the coast. In fact, as we confirmed in the field and with remote sensing, the region around Sines has 50% of death trees, most probably caused by PWD. As we can see in the following imagery, from 2004 to 2011 (Figure 7.35), there is at least 50% mortality around Santo André, in the region were the model predicts 40% of PWD.

2004 satelitte image

2011 satelite image

Figure 7.35 2004 -2011 evolution of PWD in Lagoa de Santo André.

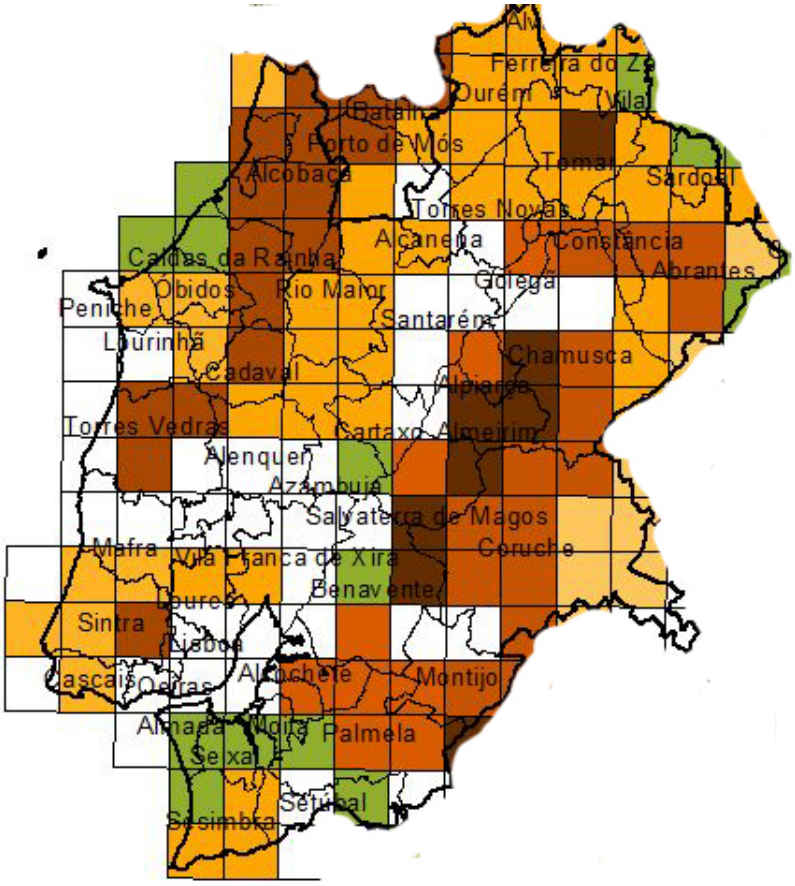

Figure 7.36 PWD in LVT region predicted by the model. Brown is more than 21%, orange is around 5%, light cream is 1% and green 0%. White is *Pinus pinaster* absence.

In LVT region (Figure 7.36) the model predicts middle to high percentage of PWD, but this region has very low cover of *Pinus pinaster* forest (Figure 7.30). Nazaré region (left to Alcobaça) has a high percentage of PWD that was also confirmed with remote sensing (Figure 7.35).

2006 2009

Figure 7.37 PWD in single stand *P. pinaster* forest, near Nazaré.

The model predicts 23% PWD in Nazaré, and between 2006 and 2009, we can see at least 30% of death trees in this region (Figure 7.37).

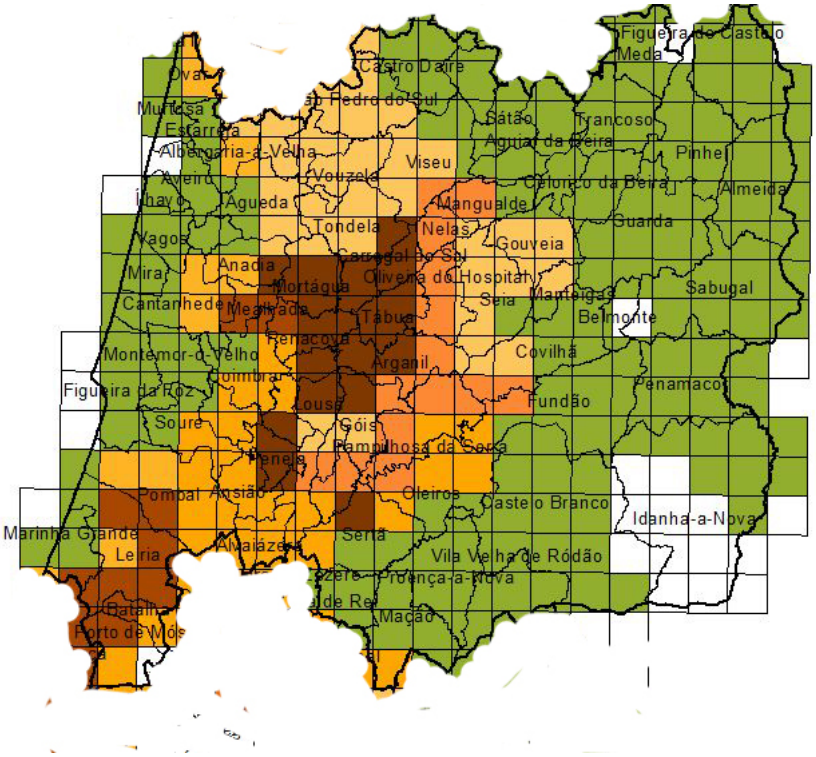

Figure 7.38 PWD in centre region predicted by the model. Brown is more than 21%, orange is around 5%, light cream is 1% and green 0%. White is *Pinus pinaster* absence.

Centre region (Figure 7.38) is very affected by PWD and the model predicts high percentages around Penacova. The interior of the region has 0% of probability to have PWD with the data that we have at this moment. From direct observation in the field, we can confirm that in Vouzela and Oliveira de Frades, PWD is present but in low percentage (around 1%).

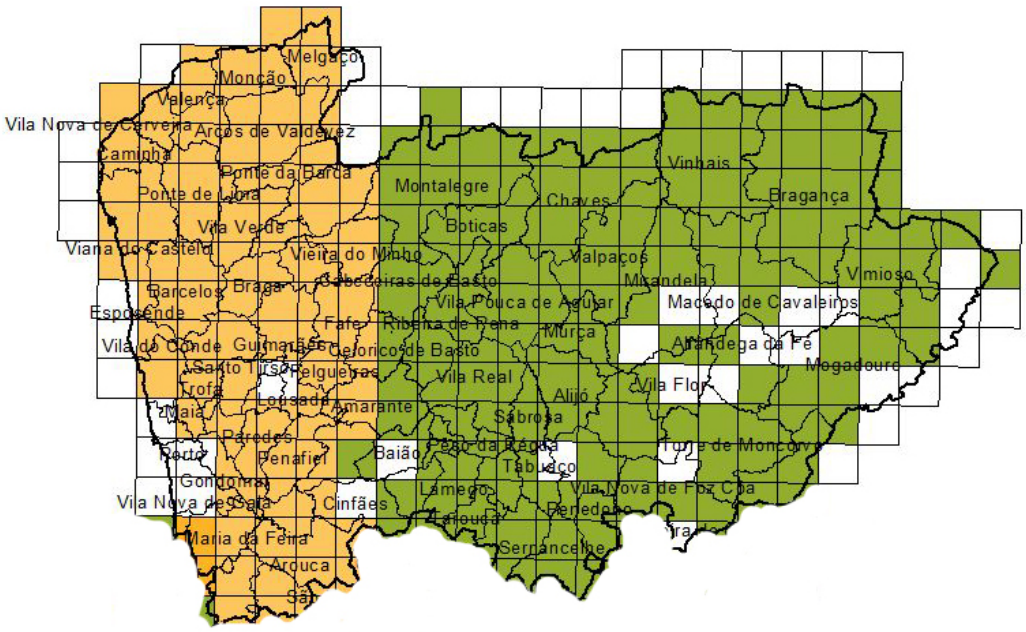

Figure 7.39 PWD in north region predicted by the model. Light cream is 1% and green 0%. White is *Pinus pinaster* absence.

Finally, the model predicts that PWD will be present in North region (Figure 7.39), but only 1% and concentrated in Minho region. In Trás os Montes, the model predicts 0% of PWD. The model predicts PWD in this region, and even if it is not yet present, it is already at the border, near Arouca region (Figure 7.32).

Iberian analysis

We prepared GIS data for Iberia with more than 50 explanatory variables and we used the same ICNF data (percentage of PWD infested trees) to predict the PWD in Iberia.

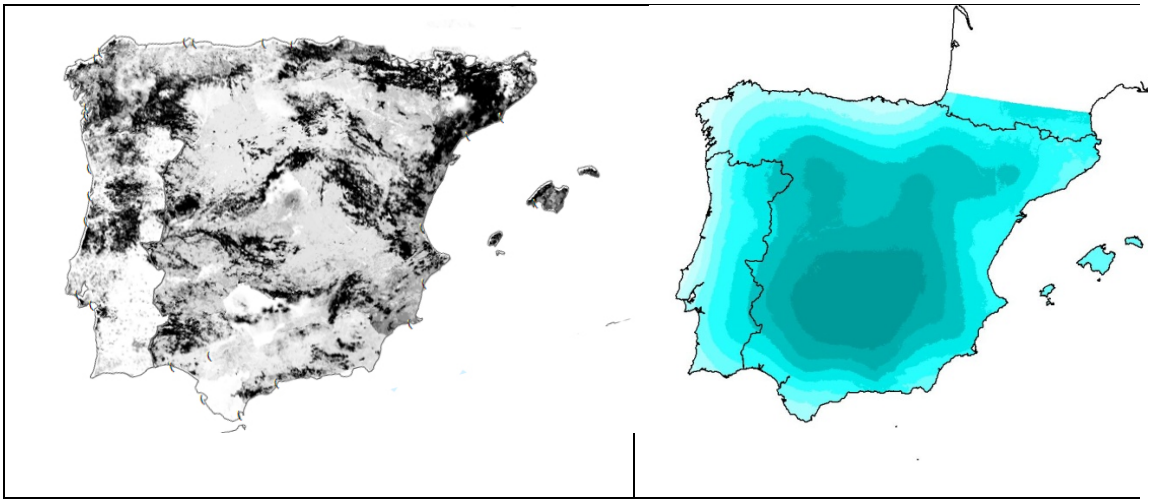

Figure 7.40 On the left, *Pinus sp*. presence in Iberia and on the right, continentality explanatory variable.

The explanatory variables (Figure 7.40, right) used for this model are less accurate than in the previous one and thus the model outputs are slightly different. With a similar model, we predict PWD for Iberia, with some added variables (total precipitation, minimum coldest month temperature and winter precipitation) and without distance to coast. The result is shown in Figure 7.41.

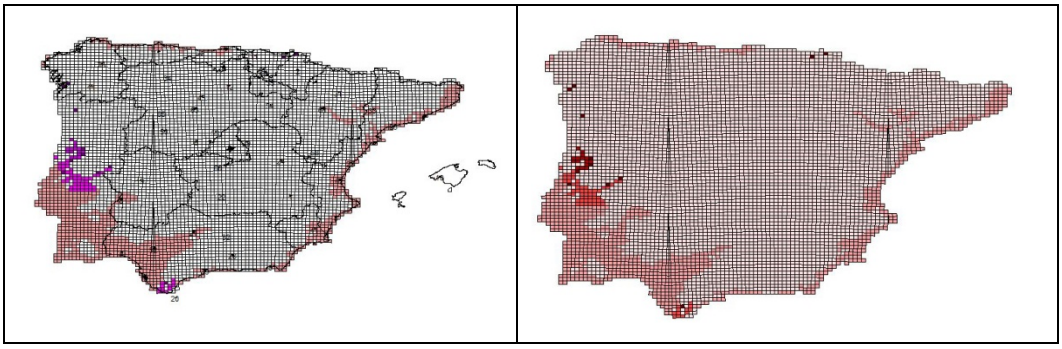

Figure 7.41 Predicted PWD in Iberia. Strong colours correspond to high percentages of PWD. On the left, there are political boundaries and main population concentrations in Spain.

As we can see, the model predicts PWD in Spain, with low percentage except for the region around Cadiz, near Vigo and in the Basque country (around 20-25% of PWD). With around 1%, it predicts PWD near the coast in almost all Iberia. The Vigo

location and Extremadura are consistent with the observed initial entry points in Spain.

Final considerations

The model is based on climatic driven variables and thus is a simplistic approach. Nevertheless, it fits remarkably well the present PWD distribution knowledge, both from ICNF data, direct field observation and remote sensing data. It also gave us provides extrapolation of the PWD hypotheses that can help us to concentrate future efforts in potential critical spots. The possibility of having low impact zones of PWD in Portugal is open, but further studies are needed to better understand the evolution of PWD in the already infested zones. Remote sensing proved to be a useful tool in spotting PWD evolution and spreading over time and this should be essential in future studies. Finally, this analysis indicated possible future spreading of PWD both in Portugal and Spain. The proximity to France in Catalunya and Basque country show that it very likely that PWD can be observed in this country.

The outputs fit closely with the ETpN model predictions and provide confidence that the dual modelling approach provides accurate representation of current and future risks of PWD in Europe.

D7.5: PWN spread model (M35)

Task 7.2 Development of a PWN spread model taking into account human influences

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 has been developed to describe more precisely the potential spread of the pine wood nematode and wilt disease in Europe.
- 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

The spread model comprises several sub-models describing various mechanisms involved in the spread of the pine wood nematode (Figure 7.42) and calibrated to the spread mechanism observed in Europe. In this reporting period, all these sub-models were connected and we did some simulations of spread at small scale (Iberian Peninsula) to validate the model followed by some future projections at the European scale.

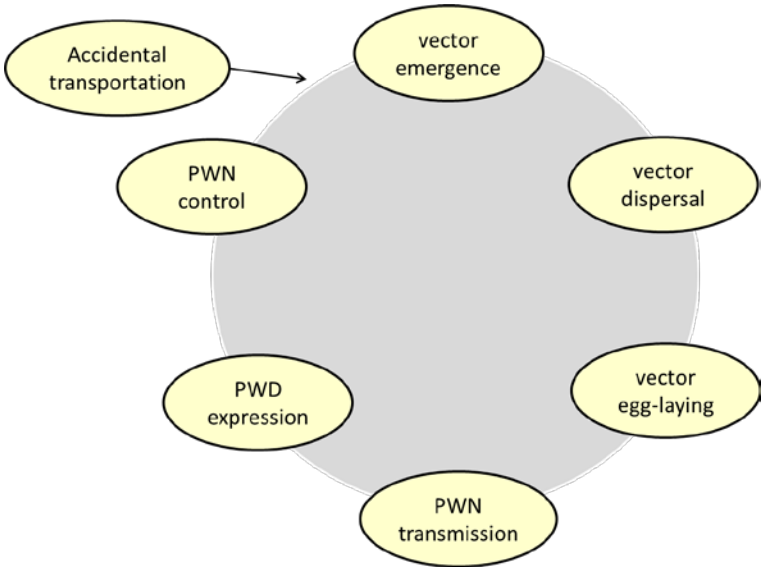

Figure 7.42 General flow chart of the spread model

Description of the model

1- Vector dispersal
A dispersal kernel was fitted to the data recorded on the flight mill for immature and mature beetles (Figure 7.43; David et al. 2013; David 2014). Due to the uncertainty about the daily flight capability, we considered three scenarios for mature beetles:

- scenario D ("daily"): the mean daily dispersal distance is 2268 m
- scenario M ("moderate"): the mean daily dispersal distance is 1296 m
- scenario W ("weekly"): the mean daily dispersal distance is 324 m

We converted the results of the individual-based model at a population level over a grid of 1 km x 1 km over the Iberian Peninsula (for validation) and over a grid of 10 km x 10 km over Europe (for projections).

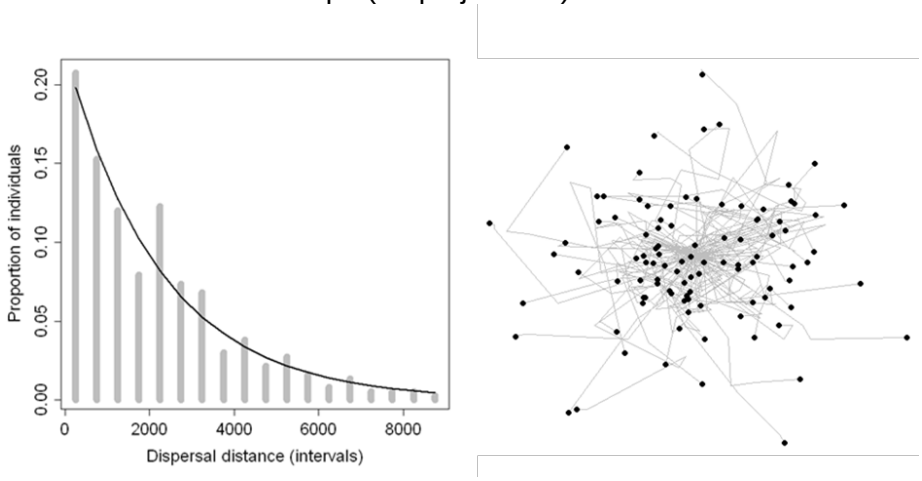

Figure 7.43 Dispersal kernel of mature beetles (on the left) and simulated trajectory based on this kernel (on the right). The black dot indicates the location of the beetles and the grey lines represent their trajectory.

2- Vector egg-laying

Half of the adult beetles are presumed to be females. Their fecundity likely depends on the pine species on which they live. From literature, we found that:

- on *Pinus pinaster*: they lay 67 eggs during 30-44 days and there is sexual maturation at 20 days (Naves et al. 2006)
  => we considered that each female lays 2 eggs every day from day 20 to 53
- on *Pinus sylvestris*: they lay 126 eggs and the adult life span is 48 days (Akbulut 2009)
  => we considered that each female lays 4 eggs every day from day 17 to 48
- on *Pinus nigra*: they lay 57 eggs and the adult life span is 33 days (Akbulut 2009)
  => we considered that each female lays 3 eggs every day from day 15 to 33 on other pine species than *P. pinaster* and *P. sylvestris*.

The number of eggs laid is spatially simulated by the individual-based model, and then applied at the population level taking into account the proportion of each pine species (*P. pinaster*, *P. sylvestris* and other *Pinus*).

3- Pine wood nematode transmission

We assume that the pine wood nematode can be transmitted during maturation feeding on healthy trees (main transmission) or during oviposition on declining trees (secondary transmission). We consider the transmission rate during maturation feeding depends on the age of the beetle (day since emergence of the adult) (Naves et al. 2007). This transmission is simulated from the individual-based model and then applied at the population level taking into account the proportion of healthy trees relative to declining trees.

4- Pine wilt disease expression

A detailed mechanistic model can simulate the disease development at the tree scale (see D7.1). However, at the spatial scale of this study, we need a simplified condition to apply over a grid over Europe. From D7.1, it appears that wilt occurs mostly when the mean temperature in summer (June-July-August) is above 20°C. Therefore, we considered this threshold in the spread model (Figure 7.44).

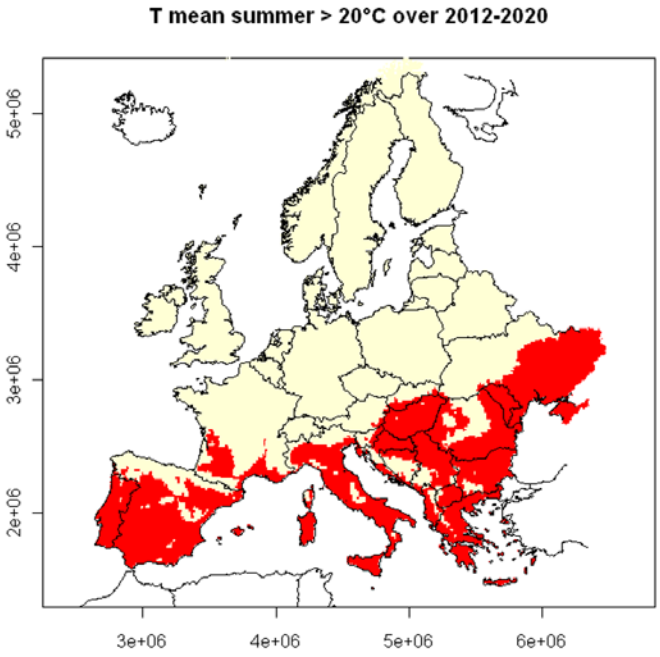

Figure 7.44 Area (in red) where the mean temperature in summer (June-July-August) is expected to be above 20°C between 2012 and 2020 from the climate change scenario B1 (Climate Local Model, CLM_B1_mm_T_2M_AV_2012-2020; provided by Partner 7).

5- Pine wood nematode control

To validate the model, it was necessary to account for the effect of PWN control measures at large scales. Given the detection effort on symptomatic and non-symptomatic trees, given the radius of the clear cut belt where host trees are removed (from 0 to 3000 m tested), we calculate the transmission rate of the pine wood nematode. According to the dispersal scenario (D, M or W), the efficiency of the control varies considerably (Figure 7.45).

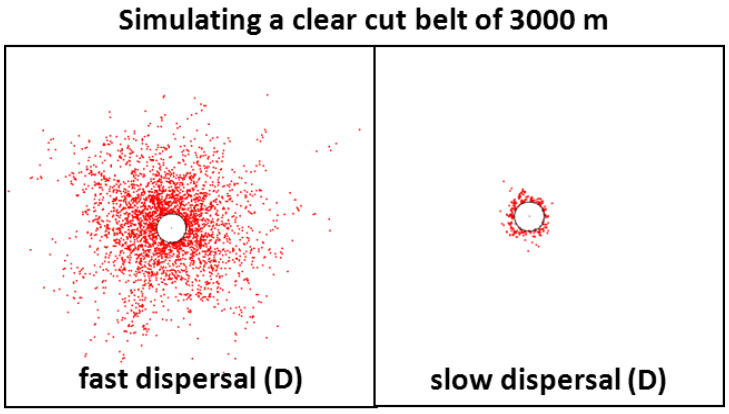

Figure 7.45 Simulating a clear cut belt of 3000 m radius. The black circle indicates the clear cut zone and the red dots indicate the locations where the beetles have stopped and transmitted the nematode. Removing trees over 3000 m radius does not eliminate the nematode, especially if the beetles have high dispersal capabilities.

6- Accidental transportation

Due to the lack of data about actual human-mediated dispersal, we consider a combination of three factors that are assumed to play a role in the nematode dispersal over long distances: human population density, road density and wood factory density. To combine these three factors, we obtain two different ratings (Figure 7.46). The resulting maps show the areas where the probability of introducing the pine wood nematode is particularly high. In the model projection for the future, we therefore test several scenarios: the nematode only spreads from Portugal and Spain from current infested areas, or it can spread from there and from another location where it is assumed to arrive following these maps. We selected an arbitrary number of 12 points in these areas at risk and we tested a hypothetical introduction at each of these points.

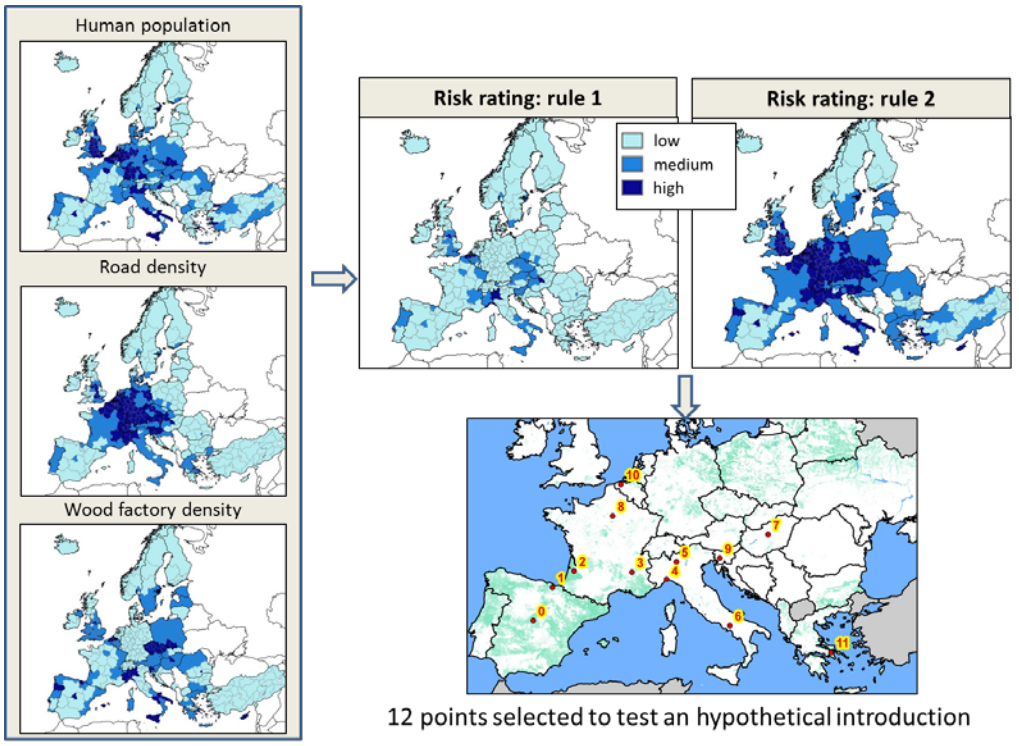

Figure 7.46 Combining the factors potentially involved in long distance dispersal of the nematode. A total of 12 points were selected in areas where the probability to introduce the nematode was particularly high. We simulated the nematode spread from infested areas in Portugal and Spain, and also from these points (considered separately).

7- Vector emergence

We consider the degree-day model (Naves et al. 2009) to calculate the emergence time and thus the proportion of the beetle population that could make its development within one year versus the one that needs two years to complete development. Results in Portugal (Lisbon), France (Orléans) and Finland (Tuusula) were consistent with observations with nearly 100% of the population able to make a development within one year in Lisbon, around 80-90% in Orléans and around 0-20% in Tuusula (Figure 7.47). In addition, in the model, we consider that the beetle emerging from an infested tree has a given probability to be infested (baseline value = 33%).

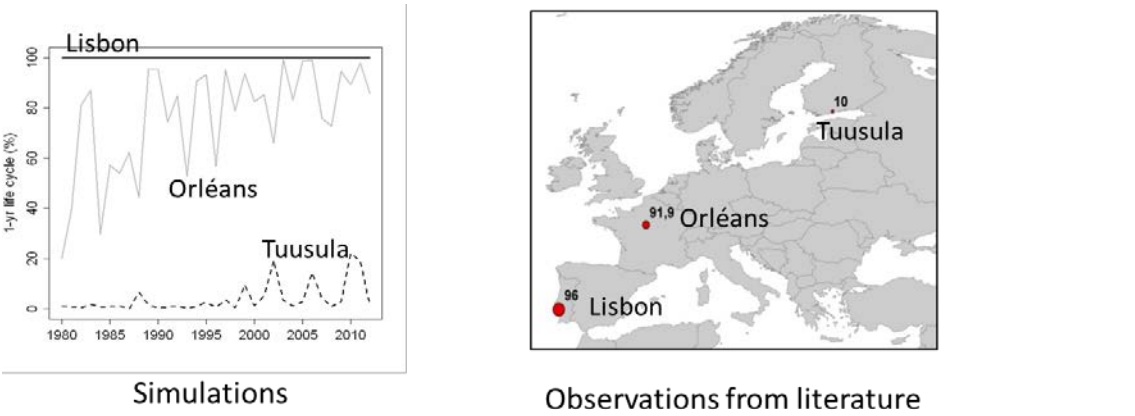

Figure 7.47 Proportion of beetle population able to make its development within one year (simulations on the left and observations on the right).

Model validation

Validation of the model was done at fine scale (southwestern Europe, 1999-2011, 1 km x 1 km grid resolution) based on the data recorded in Portugal in 2008-2011 (provided by Partner 7; Figure 7.48). In the model, we considered that the nematode could spread from the two locations initially found near Setubal. In addition, we tested whether the infestation in Coimbra resulted from a "natural" dispersal of the vector or from an additional introduction. We tested different years of possible introduction (2002 to 2008).

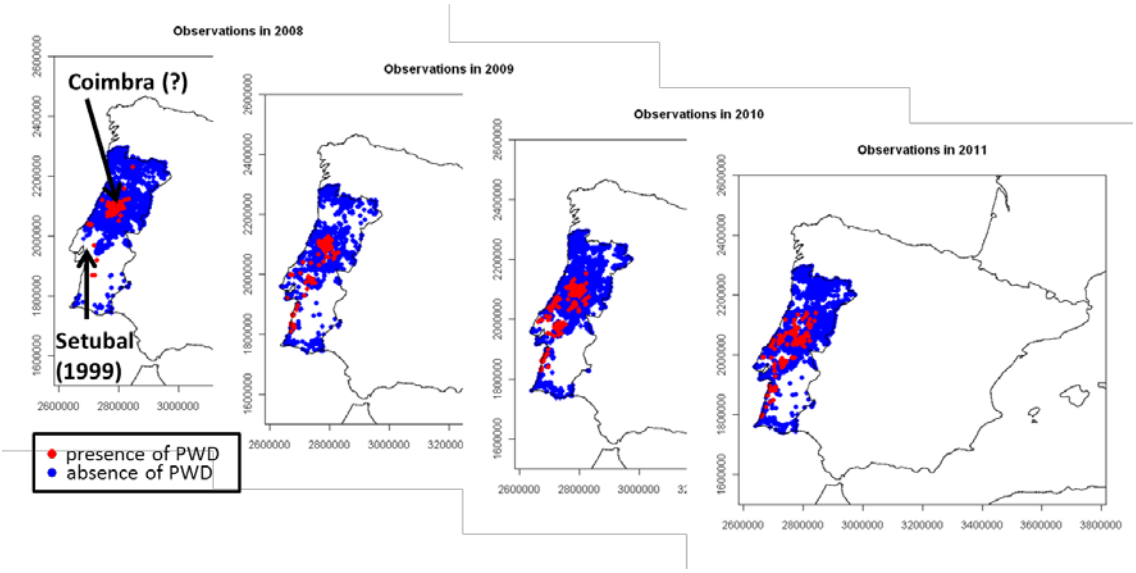

Figure 7.48 Data about the history of invasion in Portugal between 2008 and 2011.

Results show that the fast dispersal scenario ("D") better predicts the presence points whereas the slow dispersal scenario ("W") better predicts the absence points. The infested area near Coimbra could result from an introduction in 2002 (or even before) under the slow dispersal scenario ("W") whereas it could result from a "natural" range expansion from Setubal under the fast dispersal scenario ("D"). In the absence of more precise data about dispersal capabilities and, while there are still some uncertainties about observation data (for instance: does data recorded as "absence" really indicate an absence of the nematode in the whole 1 km x 1 km corresponding cell?), it is difficult to choose the most likely dispersal scenario. Therefore, in the model predictions, we test all three scenarios.

Model projections

The potential spread was simulated in the future at large scale (Europe, 2011-2020, 10 km x 10 km grid resolution). It seems that the pine wilt disease could expand in northern Portugal and even in Central Spain under fast dispersal (Figure 7.49).

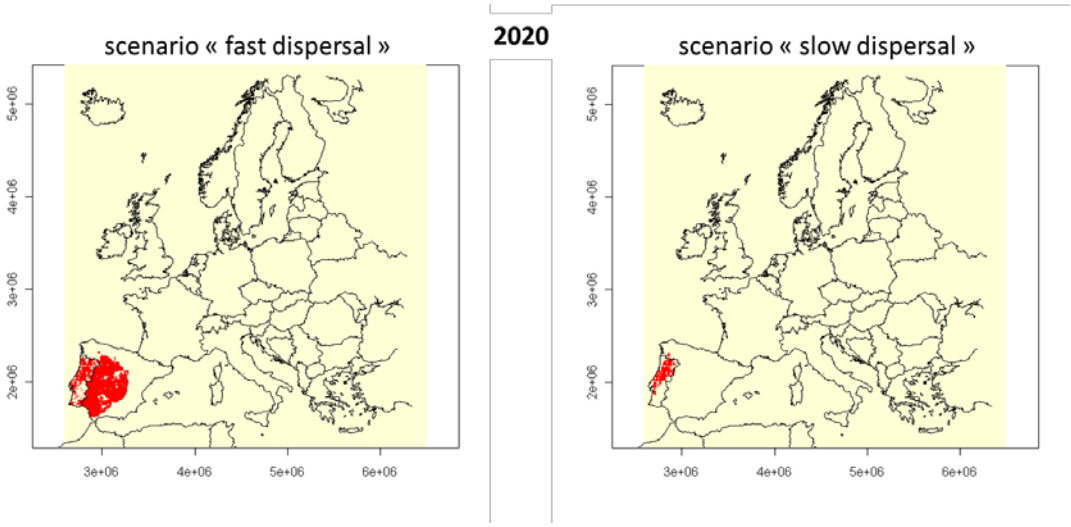

Figure 7.49 Simulated spread from Portugal in 2020 (red indicates where pine wilt disease is expected) under the fast dispersal scenario (« D »; on the left) and under the slow dispersal scenario (“W”; on the right).

An introduction in Hungary or Slovenia could result in fast spread of the pine wilt disease across Europe whereas an introduction in Belgium will not result in the disease expression there (Figure 7.50). Therefore Hungary and Slovenia are countries that should be carefully surveyed as both the risk of introduction and the risk of spread from there are very high. In colder regions, such as Belgium, the introduction of the pine wood nematode can be high but the disease may not develop due to the local climate conditions. However, in case of climate warming (+2°C or more), an introduction in this area could result in the disease spread. If temperatures are warmer than expected, the pine wilt disease could spread more rapidly.

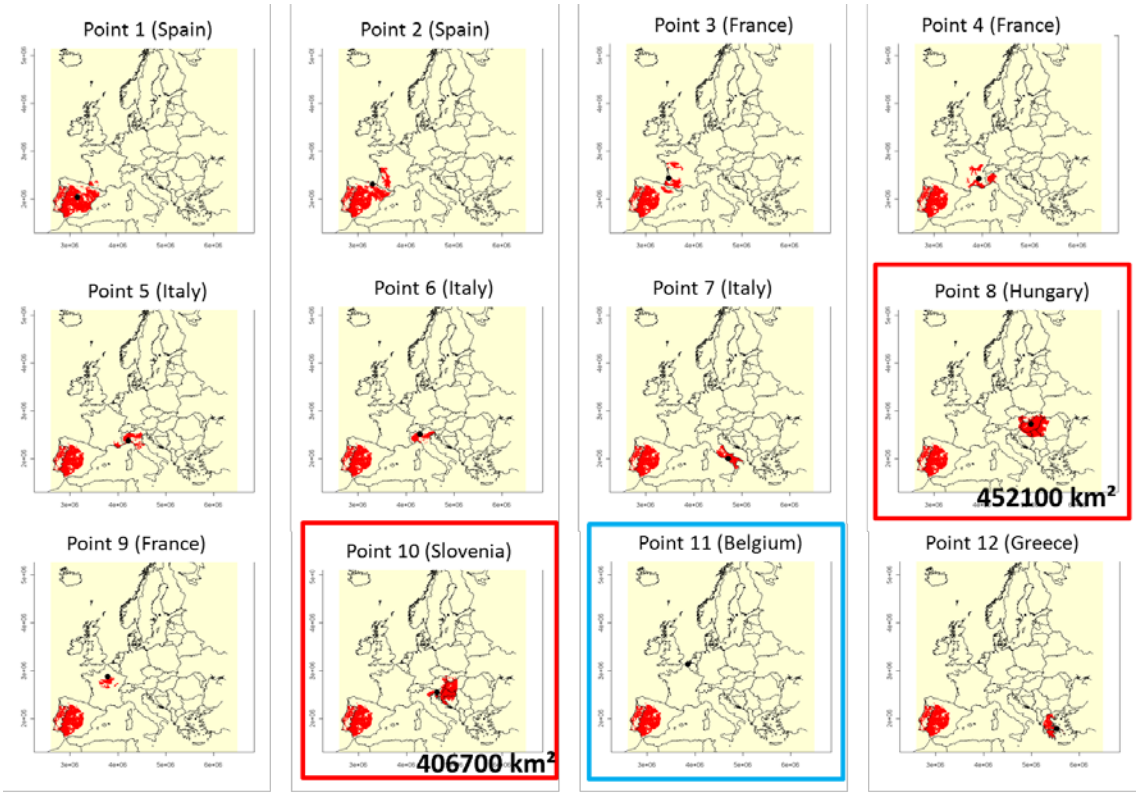

Figure 7.50 Simulated spread in the future in case of an additional introduction. Introduction points were selected in areas where the risk to introduce the nematode was the highest based on accidental transportation ratings. Area in red indicates the area where the pine wilt disease is expected by 2020.

Summary and conclusions of the model results

- pine wilt disease could spread from Portugal even under control measures (at least, under current conditions and measures and at the spatial scale of the simulations)
- it should however not invade all Europe in the following years
- only accidental introduction by humans could threaten European countries outside the Iberian Peninsula by 2020.
- it is important to :
  - improve containment measures to avoid the range expansion of the pine wood nematode
  - avoid any additional introduction in Europe

Uncertainties and possible improvements

- although we have now estimates of the dispersal capability of the European vector, we have to :
  - estimate them more accurately (i.e., choose the most likely scenario among “D”, “M”, and “W”) and cross-validate these estimates (based on field data);
  - refine the estimate of other parameters (e.g., proportion of infested insects emerging from an infested tree)
- it is not very appropriate to test management measures at this scale (the cell size being 10 km x 10 km whereas clear cut belts have been done over a maximal radius of 3 km until now). So a refined small-scale model is necessary to assess more accurately the effects of various control options (task not expected within this project).

PART 2:

This second study is part of a PhD thesis (Julien HARAN, INRA – Partner 4) aiming at exploring the role of various factors in the spread of the vector. This study combines genetic analysis of the vector populations across the Pyrenees and a simulation model to explore the potential spread across this mountainous area. The objective is to assess whether the Pyrenees could play the role of a barrier to the spread into the rest of Europe, and notably France. Genetic results are described in Deliverable 3.3.

The model developed here is based on a reaction-diffusion model similar to the one developed for the nematode spread in China (Robinet et al. 2009, 2012) but refined to take account of mutual effects of nematode and vector in the spread mechanism. We differentiate resident beetle populations from infested beetle populations that carry and transmit the nematode.

We modified the model to account for the 4 chronological steps in the invasion process of the nematode:

- before the nematode arrives: there is only resident population and no infested populations
- soon after nematode arrival: the nematode is transmitted and the number of declining trees increases (offering a higher carrying capacity to the beetle)
- the year after nematode arrival: the next generation of the beetle is infested and there is the recruitment of the resident population
- after: the whole population is presumed to be infested

This very simple scheme does not aim to provide an accurate prediction of the spread rate of the nematode invasion but, instead, to account for the main processes involved and notably the close relationship between the nematode and its vector. This model explicitly describes that each partner of this PWN-beetle association is affected by the dynamics of the other one.

The results show that the Pyrenean Mountains, due to high elevation and low temperatures, could effectively play the role of a partial barrier to dispersal of infested beetles. These results are consistent with genetic data showing that M. *galloprovincialis* meets a barrier to dispersal in higher altitude areas, but can cross the Pyrenean Mountains on the western and eastern sides, where the elevation is lower. Results also show that a moderate climate change may affect significantly the range expansion of the pine wilt disease by reducing the extent of potential barriers.

A manuscript will be submitted very soon to PLoS ONE to publish these results (end of 2014 / early 2015).

2- Oral communications and posters related to Deliverable 7.5

Haran J, Roques A, Robinet C, Roux-Morabito G (2014) Etude des processus de dispersion de *Monochamus galloprovincialis*, vecteur du nématode du pin : rôle potentiel de la chaine pyrénéenne. GDR Invasions Biologiques, Rennes, 20-22 Octobre 2014. (Poster) http://ecobio.univ-rennes1.fr/Invabio/

Haran J, Robinet C, Bernard A, Roques A & Roux G. Etat des connaissances sur la dispersion de *Monochamus galloprovincialis*, vecteur du nématode du pin en Europe. GEFF, Lucelle (France), 15-18 Sept 2014,

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)

3- References cited in Deliverable 7.5

Akbulut S (2009) Comparison of the reproductive potential of *Monochamus galloprovincialis* on two pine species under laboratory conditions. Phytoparasitica, 37:125-135.

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

David G (2014) Etude des capacités de dispersion de *Monochamus galloprovincialis* vecteur du nematode du pin *Bursaphelenchus xylophilus*. PhD dissertation, Université de Bordeaux (France). 173 pp.

Naves P, de Sousa E, Quartau JA (2006) Reproductive traits of *Monochamus galloprovincialis* (Coleoptera : Cerambycidae) under laboratory conditions. Bulletin of Entomological Research, 96 : 289-294.

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.

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

All work planned for this WP has been carried out and progress has been excellent for all sub-tasks.

Statement on the use of resources

There have been no significant deviations from the planned use of resources.

WP 8 EU and international cooperation and collaboration

Reporting period: 1st March to 30th November 2014

Deliveries

D8.2 Interaction with EU/International projects

Milestones

MS13 Sharing knowledge with international research community
MS14 Sharing staff resources by exchange internationally

Task 8.2: Synthesis and interaction with current EU projects

Within the **REPHRAME** consortium, collaborative research and exchange of expertise has continued among partners during this extended period.

This has been done mainly by contributions to specific tasks in WPs led by these partners, but also by expanding the collaborative networks. In addition to the established objectives, the exchange and transfer of knowledge among different partner members was carried out in the following way:

B4 (INRA-Orleans) and B10 (IOZ, Beijing):

The collaboration between B4 and B10 led to the publication of a joint book chapter synthesizing our present knowledge about the relationships between carrier beetles, PWN, and climate change:

Roques A., Zhao L.L., Sun J.H., Robinet C., 2015. *Pine wood nematode, pine wilt disease, vector beetle and pine tree: how a multiplayer system could reply to climate change?* In: BJÖRKMAN C. & NIEMELÄ P. (Eds), *Climate Change and Insect Pests. CABI Editions, in press.*

B7 (UÉvora) and B5 (INRA, Sophia Antipolis):

These two partners have maintained a continuing collaboration regarding PWN population genetic studies as support for understanding the movement and establishment of the nematode in Europe.

B7 (UÉvora) and B10 (IOZ, Beijing):

These two partners have established cooperative research in 2014, which has resulted in a major review paper on the complex interactions among PWD actors:

Zhao, L. M. Mota, P. Vieira, RA. Butcher, and J. Sun. 2014. Interspecific communication between pinewood nematode, its insect vector, and associated microbes *Trends in Parasitology*, 2014: 1-10.

List of **publications** produced resulting from activities within this task:

Papers

Haran J & Roux-Morabito G (2014) Development of 12 microsatellite loci for the longhorn beetle *Monochamus galloprovincialis* (Coleoptera Cerambycidae), vector of

the Pine Wood Nematode in Europe. *Conservation Genetics Resources* DOI: 10.1007/s12686-014-0262-0

Haran J., Koutroumpa F., Magnoux E., Roques A., Roux- Morabito G., 2015. Ghost mtDNA haplotypes generated by fortuitous NUMTs can deeply disturb infraspecific genetic diversity and phylogeographic pattern. *Journal of Zoological Systematics and Evolutionary Research* (accepted, minor revision).

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? *Biological Invasions*, doi: 10.1007/s10530-014-0788-9 (published online, 28 September 2014).

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 *Trends in Parasitology*, 2014: 1-10.

International Conferences:

B4:

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. (*Oral 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, USA.(*Oral presentation)*

Haran J., 2014. Landscape genetics of *Monochamus galloprovincialis*, vector of the pine wood nematode in Europe. *International Conference on Insect Invasions*. Le Studium, Orléans, France, 17- 19 December 2014 (*invited talk*).

Robinet C., 2014. Assessing the invasion probability of the pine wood nematode with imported wood. *International Conference on Insect Invasions*. Le Studium, Orléans, France, 17- 19 December 2014 (*invited talk*).

B7:

Mota, M. 2014. Pine wilt disease, and the pinewood nematode: a worldwide issue, a nematological challenge. 6th International Congress of Nematology, Capetown, S. Africa, May 5-10, 2014.

Nascimento, FX, Cláudia S.L. Vicente, Pedro Barbosa, Margarida Espada, Paulo Vieira, Koichi Hasegawa and Manuel Mota. 2014. Ecological role of bacteria associated with the pine wilt disease system. 6th International Congress of Nematology, Capetown, S. Africa, May 5-10, 2014.

Vicente, CV, Yoriko Ikuyo, Ryoji Shinya, Manuel Mota, Koichi Hasegawa. 2014. Virulence and oxidative stress response of the pine wood nematode *Bursaphelenchus xylophilus*. 6th International Congress of Nematology, Capetown, S. Africa, May 5-10, 2014.

Vieira, P., Natalia Rodiuc, Lieven De Veylder, Gilbert Engler and Pierre Abad, Janice de Almeida Engler. 2014. Ectopic expression of cell cycle inhibitor genes effectively interferes with root-knot nematode feeding site development. 6th International Congress of Nematology, Capetown, S. Africa, May 5-10, 2014.

Mota, M., Francisco X. Nascimento, Cláudia S.L. Vicente, Joana Henriques, Pedro Barbosa, Margarida Espada, Paulo Vieira, and Koichi Hasegawa. 2014. The pinewood nematode, *Bursaphelenchus xylophilus*, and pine wilt disease: a serious forest threat to Turkey and Europe. 2nd Symp. Turkey Forest Entomology and Pathology. Antalya, Turkey, April 7-9, 2014, and IUFRO Joint Meeting Antalya (Turkey) 9-14 April, 2014.

Dayi, M., Henriques, J., Alves, M., Henriques, I., Mota, M., Akbulut, S., Correia, A. 2014. Preliminary genetic analysis of the bacteria associated with *Monochamus galloprovincialis* from Turkey. 2nd Symp. Turkey Forest Entomology and Pathology. Antalya, Turkey, April 7-9, 2014, and IUFRO Joint Meeting Antalya (Turkey) 9-14 April, 2014.

National Conferences :

B4:

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. Réunion du Groupe des Entomologistes Forestiers Francophones (GEFF), Bedoin (France) 22-24 Oct 2013. (*Oral presentation)*

Robinet C (2013) Expansion potentielle du nématode du pin : vers une modélisation calibrée sur l'Europe. Réunion du Groupe des Entomologistes Forestiers Francophones (GEFF), Bedoin (France) 22-24 Oct 2013. (*Oral presentation)*

B7:

Vieira da Silva, I., Barbosa, P., Mota, M. and Ascensão, L. 2014. Histological changes in stems of *Pinus sylvestris* seedlings infected with a virulent isolate of the pinewood nematode *Bursaphelenchus xylophilus*. International Conference on Microscopy and Microanalysis XLVIII Congress of the Portuguese Microscopy Society, Oporto, Portugal, 2014.

Barbosa' P., Ana M. Rodrigues, Jorge MS Faria, Luís S. Dias, Luís G. Pedro, José G. Barroso, Ana C. Figueiredo and Manuel Mota. 2014. Nematotoxic activity from essential oils and own hydrocarbons and oxygen-containing molecules fractions against the pinewood nematode *Bursaphelenchus xylophilus.* 62nd Int. Cong. and Ann. Meeting, Soc. Med. Plant and Natural Prod. Res, Guimarães, Portugal, 31 August- 4 September, 2014.

Rodrigues, AM., Pedro M. Barbosa, Ana S. Lima, Luísa Mota, José G. Barroso, Luis G. Pedro, Lia M. Ascensão, Manuel Mota, A. Cristina Figueiredo. 2014. Volatile response of *Pinus halepensis* and *Pinus sylvestris* after inoculation with the pinewood nematode *Bursaphelenchus xylophilus* . XVIII Congresso da Sociedade Portuguesa de Bioquímica, Coimbra, Portugal, 17- 20 December, 2014.

Barbosa, Ana M. Rodrigues, Jorge M.S. Faria, Marta D. Mendes, Ana S. Lima, Luís S. DiaS, P. Vieira, MT Tinoco, Richard N. Bennett, Luis G. Pedro, José G. Barroso, A. Cristina Figueiredo and Manuel Mota. 2014. Plant volatile compounds to control the pinewood nematode in Portugal. 1º Simpósio SCAP "Novos desafios na protecção das plantas" e 7º Congresso SPF, Oeiras, Portugal, 20-21 Novembro, 2014.

Espada, M, C. Vicente, F. Nascimento, M. Mota. 2014. An integrative approach on Pine wilt disease. 1º Simpósio SCAP "Novos desafios na protecção das plantas" e 7º Congresso SPF, Oeiras, Portugal, 20-21 Novembro, 2014.

Mota, M. 2014. A doença do nemátode da madeira do pinheiro, em Portugal e na Europa: reflexões e perspectivas futuras. 1º Simpósio SCAP "Novos desafios na protecção das plantas" e 7º Congresso SPF, Oeiras, Portugal, 20-21 Novembro, 2014.

Vieira da Silva, L. Mota, P. Barbosa, M. Mota, and L. Ascensão. 2014. Histopathology of *Bursaphelenchus xylophilus*-infected seedlings of two pine species grown in Portugal. 1º Simpósio SCAP "Novos desafios na protecção das plantas" e 7º Congresso SPF, Oeiras, Portugal, 20-21 Novembro, 2014.

Task 8.3: Interaction with international phytosanitary organizations, relevant to PWD

Contacts of interest have been maintained with several European and international quarantine laboratories, on both scientific and technical issues related with the pinewood nematode and pine wilt disease (see Table 3 from previous report).

B1 (FR, UK)

Continuing contributions to the EPPO Forest Quarantine Panel and also to the newly established EU Expert Group on PWN.

B3 (BFW, Austria):

Nothing new to report after periodic report in February, but ongoing activity in Task 8.3.: EPPO panel and function as experts of forest health issues (H. Krehan)

B7 (UE, Portugal):

M. Mota has been invited by the French phytosanitary agency ANSES as external expert on pine wilt disease, to provide advice for the French government.

List of **publications** produced resulting from activities within this task:

Calin, M., C. Costache, H. Braasch, M. Zaulet, L. Buburuzan, V. Petrovan, M. Dumitru, M. Mota and P. Vieira. 2014. New reports of *Bursaphelenchus* species associated with conifer trees in Romania. *Forest Pathology*, doi: 10.1111/efp.12163

(accepted: 24.11.2014).

Mota. 2014. First detection of Bursaphelenchus xylophilus associated with Pinus nigra in Portugal and in Europe. *Forest Pathology*, doi: 10.1111/ efp.12162 (accepted: 15.11.2014)

Čermák, V., P. Vieira, M. Čudejková, V. Gaar, K. Tománková, K. Mikušková, JD. Eisenback and M. Mota. 2014. *Bursaphelenchus hofmanni* Braasch, 1998 associated with peat growth substrate in hops nurseries in the Czech Republic. *Nematology*, 16: 739-742

Čerevková, A., Mota, M., and Vieira, P. 2014. *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle 1970 – háďatko borovicové: hrozba pre európske lesy / *Bursaphelenchus xylophilus (Steiner & Buhrer, 1934) Nickle 1970 – pinewood nematode: a threat to European forests*. *Lesnicky Casopis For. J*. 60 (2014) 125–129

Bonifácio, LF, E. Sousa, P. Naves, ML Inácio, J. Henriques, M. Mota, P. Barbosa, MJ Drinkall and S. Buckley. 2014. Efficacy of sulfuryl fluoride against the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchidae), in *Pinus pinaster* boards. *Pest Management Science* 70:6–13.

Task 8.4: Interaction with the EUMAINE MSc programme in Nematology, based in Ghent

During 2014, a group of international students under this prestigious European MSc programme EUMAINE (http://www.eumaine.ugent.be/index.asp), coordinated by Gent University, Belgium, have become aware of the PWD issue in Europe and worldwide. As a partner of this MSc program, B7 has supplied technical and practical training to several EUMAINE students, including: field trips to pine areas affected by the PWN, as well as standard methodologies for extraction of PWN from wood samples, and morphological identification of this species. Short laboratory mini-projects, involving molecular genetic analysis and nematode-bacteria interactions have taken place. A significant action of sensitizing these students to the PWD issue has taken place and will continue beyond the scope and time frame of this project. These students, upon return to their home countries in Asia, Africa, Europe and South America, will assume relevant job positions in nematological, plant protection and quarantine institutions.

As a result, one MSc thesis (Ana Cláudia Silva), in combination with an ongoing PhD thesis (Margarida Espada) has been produced, and a joint paper is in its final preparation and should be submitted early 2015:

Silva, Ana Cláudia. 2014. Identification of novel effectors from the pinewood nematode *Bursaphelenchus xylophilus.* MSc thesis, EUMAINE program, Univ. Gent.

Espada, M., Ana Cláudia Silva, Sebastian Eves-van den Akker, Peter J.A. Cock, Manuel Mota & John T. Jones. 2014. Identification and characterization of novel effectors from the pinewood nematode *Bursaphelenchus xylophilus* (in preparation).

Task 8.5: International and collaboration with international PWN research groups

Contacts of interest have been continued with several laboratories, in different areas of expertise related to the PWN. Beside the exchange in terms of nematode material and/or information related to the field, different collaborations resulted in reciprocal training and generation of new biological data concerning the mechanisms of pine wilt disease.

Several members of REPHRAME continue to contribute to international phytosanitary initiatives:

IUFRO
7.02.10 Pine Wilt Disease: Chaired by B5
7.03.05 Ecology and Management of Bark and Wood Boring Insects
7.03.12 Alien Invasive Species and International Trade: Deputy Chair B1

There will be a joint meeting between 7.03.05 and 7.03.12 in Bariloche, Argentina in September 2015.

International Forestry Quarantine Research Group (IFQRG)
Several members of REPHRAME have contributed to this important group over many years. Regular reports on the work of REPHRAME have been provided to the annual meetings of IFQRG.

European Food Safety Authority (EFSA)

Contributions to European phytosanitary development have been done as experts working within EFSA groups. Recent contributions by REPHRAME members have been on PWN, especially the roles of vectors.

List of **publications** produced resulting from activities within this task:

Espada, M., Ana Cláudia Silva, Sebastian Eves-van den Akker, Peter J.A. Cock, Manuel Mota & John T. Jones. 2014. Identification and characterization of novel effectors from the pinewood nematode *Bursaphelenchus xylophilus* (in preparation).
Vicente, C., F. Nascimento, P. Cock, I. Toth, J. Jones and M. Mota. 2014. Genomic sequencing of the pinewood nematode-associated bacteria Serratia proteomaculans, LCN-4 and LCN-16 (in preparation).
Vicente, V., Yoriko Ikuyo; Ryoji Shinya; Manuel Mota; Koichi Hasegawa. 2014. Catalases induction in high virulence pinewood nematode *Bursaphelenchus xylophilus* under hydrogen peroxide-induced stress. *PloS ONE* (submitted, Sep. 2014).
d'Errico, G., B Carletti, T Schröder, M Mota, P Vieira and PF Roversi. 2014. An update on the occurrence of nematodes belonging to the genus *Bursaphelenchus* in Mediterranean area (submitted to *Forestry*, Nov. 2014).
Faria, JMS, I Sena, I Vieira da Silva, B Ribeiro, P Barbosa, L Ascensão, R Bennett, M Mota, ACS Figueiredo. 2014. *In vitro* co-cultures of *Pinus pinaster* with *Bursaphelenchus xylophilus*: a biotechnological approach to study Pine Wilt Disease (submitted to *Planta*, Nov. 2014).

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* (doi: 10.1111/1758-2229.12202. [Epub ahead of print]).

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

All work planned for this WP has been carried out and there has been excellent interaction both between Beneficiaries and with the wider scientific and end-user communities.

Statement on the use of resources

There have been no significant deviations from the planned use of resources.

WP 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

Developments on the PTK structure and interface

The outline structure of the PTK has been under intense development during the final 9 months of the project and has been improved by interaction with Stakeholder Observer Group (SOG) (see WP10) and directly with end-users at the end of project Workshops (Portugal/Spain and Brussels) and by interaction with the newly formed EU Task Force on PWN. This has enabled 9.2 to be refined (see below).

9.2 Synthesis of results and construction of PTK modules.

The current version of the PTK content, which will form the basis for the on-line system, is shown in Figure 9.1. As indicated in previous reports and in 9.1, this was developed by interaction with the beneficiaries within REPHRAME and the end of project dissemination events as well as the ongoing outputs from the other Work Packages.

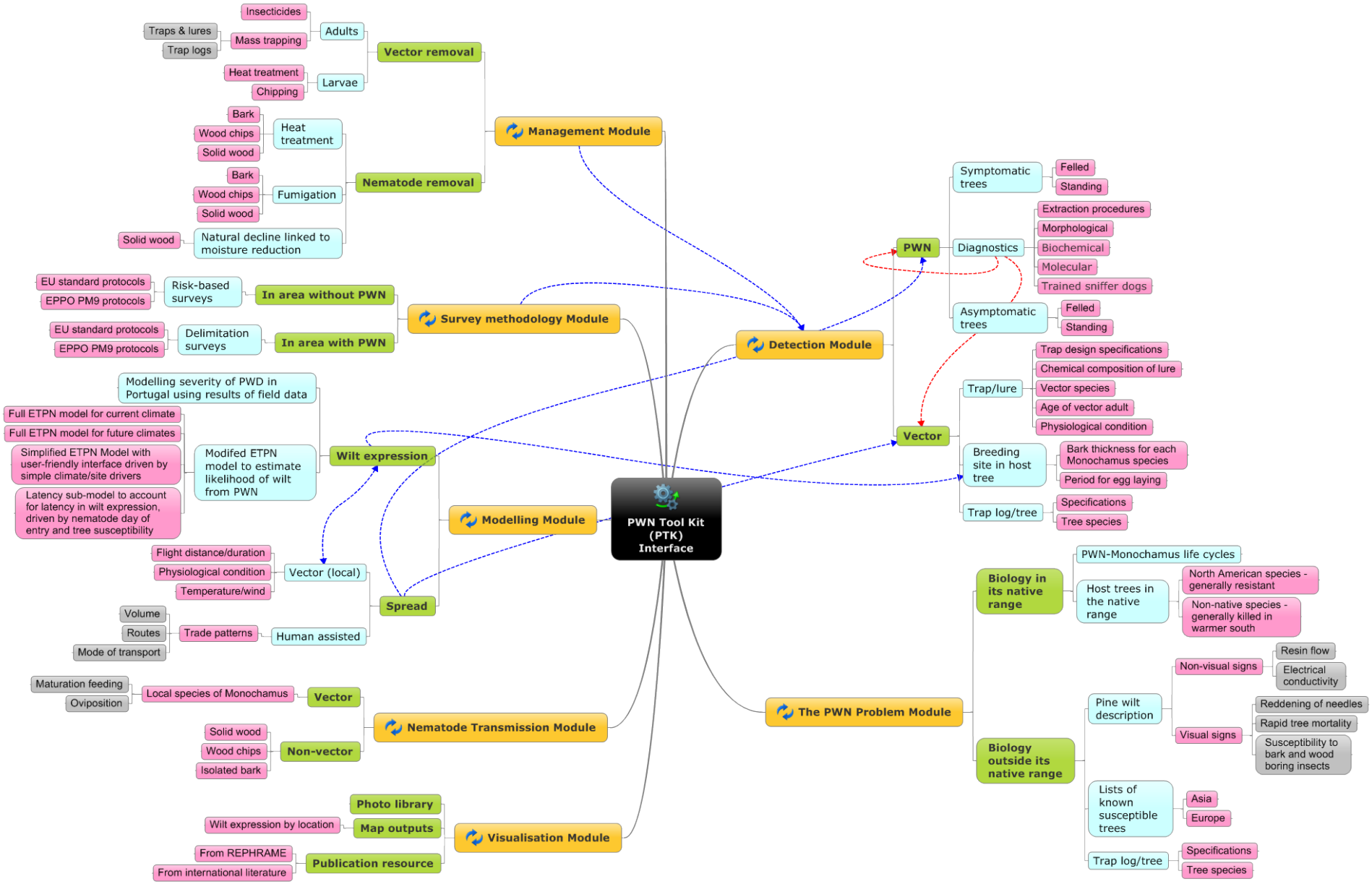

Figure 9.1: Structure of the PTK following analysis of progress in Work Packages and SOG engagement.

The aim of the PTK is to provide a relatively simple top-level interface to give summary information for the particular topics and then to provide links to enable users to find more detailed information should they wish to. The high level summaries are now available on-line in the project website (www.rephrame.eu) and this has only been possible at the very end of the project when the reports from all Beneficiaries for each Work Package were completed and collated. The aim is to keep the website live and to update at regular intervals, even though the project has reached its formal end date.

Draft versions of the PTK were presented at the REPHRAME final Workshop held in Madrid on 30 September and also at the EU Seminar held in Brussels on 13 November. It was also presented and discussed at the meeting of the EU Task Force on PWN held in Spain in September 2014.

9.3 Full public launch of the PTK

Building on the interactions with stakeholders during 2014 and using the final information from the project delivered during November 2014 – January 2015, the PTK was launched in January 2015 on www.rephrame.eu.

Particular highlights from the various modules include:

- Advice and specifications for monitoring vector populations using a refined lure (Galloprotect 2D) and Teflon-coated traps.
- New information on vector flight capacity indicates that individual flights can be over several kilometres and life-time flight can be up to 40 km. This has major implications for felling regimes in relation to management of PWN.
- Molecular analysis of PWN populations that have indicated the origin of the Portuguese population is likely to have been directly from North America (the native range of the pest).
- Non-vector transmission can take place from nematode-infested wood (either sawn wood or wood chips) to living trees, although there needs to be some damage to the tree to facilitate transfer. This needs to be accounted for in management of the end use of potentially infested wood and wood products.
- Models of wilt expression indicate those areas of Europe that can be expected to suffer from pine wilt under both current and future climates. The process model indicates that delayed expression of wilt (latency) can take place, which must be accounted for in carrying out surveys.
- A set of user-friendly models has been developed to enable end users to assess the likelihood of wilt in their own regions.
- A parallel modelling exercise in Portugal, using data on known PWN distribution, confirms the important role of temperature, precipitation and proximity to the coast as driving variables for wilt.
- Dispersal modelling using both vector and human-assisted routes of transfer has provided valuable predictions of the highest risk areas of Europe in relation to potential movement of PWN from the current foci in Portugal. The model has considerable value in determining both local and regional management strategies against PWN.

- Dissemination events have enabled the project to deliver the outcomes as they have been produced, thus keeping end users well informed of the latest developments.

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

All work planned for this WP has been carried out and progress has been excellent for all sub-tasks.

Statement on the use of resources

There have been no significant deviations from the planned use of resources.

WP 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 an additional 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

Objective: Production of a website/ project leaflets/ reports/ scientific publications

The website is available at www.rephrame.eu and the leaflet has been widely disseminated and is also available for download.

An information leaflet concerning PWN and PWD to inform interested stakeholders and the public in Germany was produced:
http://www.etracker.de/lnkcnt.php?et=dQsHU3&url=http://www.jki.bund.de/fileadmin/dam_uploads/_veroeff/faltblaetter/kiefernholznematode_.pdf&lnkname=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 *Msp*I 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)

10.2 Stakeholder Observer Group

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.

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.

The PTK structure and Stakeholder Interaction was publicised at the EU Task Force (Expert Group) meeting held in San Martin de Trevejo, Spain on 17-18 September. This was the first opportunity to present the consolidated findings from REPHRAME and the information was well received by those present. Several contributors to REPHRAME are members of the Task Force and presented their particular findings to the meeting. Prof Hugh Evans, Coordinator of REPHRAME, provided an overview of the entire project through the PTK structure and was subsequently asked to contribute to the Task Force itself (done by correspondence so far).

10.3 Themed Workshops

Two workshops, a webinar and a seminar (at the request of the EU Science Officer for REPHRAME) were organised during the extension period in 2014.

REPHRAME Final Workshops in Spain and Portugal 30 September – 2 October 2014

The programme for the back to back Workshops is shown below:

Programme for the scientific presentations on 30 September

This will commence at 14.30 at the Natural History Museum, Calle José Gutiérrez Abascal, 2, 28006 Madrid.

Programme:

Time	Speaker	Title/subject of presentation
14:30	H Evans	Welcome and introductions (REPHRAME team and participants)
14:45	H Evans and H Gruffudd	Description of REPHRAME and introduction to the PWN Took Kit (PTK) and model outputs to predict pine wilt expression
15:20	T Schroeder	Population dynamics of PWN in *Pinus sylvestris* and research into non-vector transmission of PWN to susceptible host trees.
16:00	Break	
16:20	G Hoch	European *Monochamus* spp as actual and potential vectors for PWN dispersal
16:50	E Sousa and P Naves	Studies on host tree resistance to PWN and its *Monochamus* vectors
17:20	L Robertson and J Pajares	Studies on PWN with emphasis on molecular techniques for assessing species and variation. Details on the chemical composition of *Monochamus* lures (as introduction to the field visit on 1 October).
18:10	M Mota	Interactions between REPHRAME and scientists/stakeholders involved or interested in PWN. Including description of the successful international conference held in Germany in 2013
18:40	H Evans, J Pajares, E Sousa	Short guided discussion and description of the field visits on 1 and 2 October.
19:10		Depart for Hotel Zurbano for joint dinner and any further small-group discussions.

Timetable and programme of the field trip October 1and 2

Activity	**Time**
October 1st	
Depart Madrid Hotel	7:30
Coffee break on route	9:15-9:45
Sancti-Spiritus eradication area	11:00-12:10
Lunch in Ciudad Rodrigo	12:30-14:00
Mass trapping area (C.Rodrigo)	14:30-16:00
Depart for Coimbra	16:00
Arrival at Coimbra	19:00 Sp time 18.00 Pt time
Activity	**Time**
October 2nd	
Depart Vila Galé Coimbra Hotel	7:30
Arrive at Tábua County	09:30
Visit to infested pine stands.	10:00-12:00
Lunch at Carapinha	13:00- 14:00
Visit to Resimadeiras	14:30 – 15:30
Departure from Resimadeira	15:30 Pt time
Dinner at Ávila	20:45-22:00 Sp time
Arrival at Madrid hotel	23:30

Activities during the field trip visit in Spain:

Sancti Spiritus

Stop at point 1 (remain on bus): view of the focus/eradication area. Video on eradication operations (20')

Stop at point 2: short walk (200 m) through the felled area (path). On site explanation by responsible managers on the eradication operations carried out. Explanation of a Multifunnel trap system to monitor for *Monochamus* spp (50 ')

Mass trapping area (C.Rodrigo)

Stop at point 1: short walk through the area (400 m dirt road). On site explanation of: Cross vane trap, mass trapping operations being carried out (by responsible managers). Explanation of *M. galloprovincialis* Scots pine colonized logs at different life cycle stages (egg lying wounds, larval burrowing, pupal entrances, emergence holes) (90')

Activities during the field trip visit in Portugal:

Visit to infested pine stands (Tábua County with FNAP, Federação Nacional dos Produtores Florestais)

Work in progress (surveys, identification & eradication of symptomatic trees; destruction of wood residues; *Monochamus galloprovincialis* traps; wood sampling for analysis)

Visit to Resimadeiras

Continuous treatment system using hot steam to disinfest isolated pine bark

The workshops were publicised through the EU Standing Committee on Plant Health and the EPPO Forest Quarantine Panel as well as by scientific interactions with

potential attendee organisations. Although successful, there was a rather disappointing turnout from Member States other than those in close proximity to the PWN problem. Also, some attendees came just for the field visits rather than to the indoor presentations in Madrid. The initial attendance on day 1 was 14 people (1 Belgium, 1 Estonia, 1 France, 1 Germany, 2 Malta, 1 Netherlands, 4 Portugal, 3 Spain). Nevertheless, the feedback from the attendees was very positive and further discussion and interaction took place over the next two days in the field. We were fortunate to have input from local staff and forest managers at the various stops on the field trips, culminating with a total of 37 people during the visit to Portugal. Special thanks are due to Mr José Vasco de Campos, Director General of Federação Nacional das Associações de Proprietários Florestais and his staff who were generous in their time in showing management processes to combat PWN in Portugal. As part of the dissemination, Hugh Evans was interviewed by the local TV station Centro TV and there was press coverage of the event.

Webinar entitled The Future of European Forests on 4 November 2014.

This was a plant health orientated webinar featuring two EU FP7 projects – ISEFOR and REPHRAME. A summary is available on the media company website - http://www.projectsmagazine.eu.com/news/the_future_of_europes_forests_webinar. The webinar presentations are available by registration on the website on this link: http://view6.workcast.net/?pak=3315733587423686.

Feedback from the organisers and some delegates who attended the webinar indicated that it was successful. Overall there were 123 pre-registered participants of which 54 of the pre-registered attended. An additional 64 viewers who hadn't pre-registered, joined the session on the day. This gave a total of 118 registered attendees. In total, however, there were 315 different sessions (views by different people) during the event. Not all stayed for the whole event, but most logged in and out for different parts of the webinar. On average, there were between 50 and 60 viewers logged in at any one time throughout the whole webinar.

These are good statistics. A good average of pre-registrations turning up for a webinar like this is 30-40% and the webinar more than matched that, while casual, on-the-day, viewing figures were better than average.

Seminar to invited audience in Brussels, 13 November 2014

The science officer for REPHRAME, Dr Juli Mylona, requested a final dissemination seminar to be held in Brussels and 9 scientists from the project provided summary updates on the latest work carried out. Although there was a relatively small audience, the work was appreciated and provided a good opportunity for the project to provide a 'summing up' of the key findings from REPHRAME. The programme included:

- Biology of the Pine Wood Nematode (PWN) -Vector-Tree relationship and development of the PWN Tool Kit (PTK) **Hugh Evans** 20 min
- Population dynamics of PWN in trees **Thomas Schröder and Andrea Hopf-Biziks** 20 min
- Studies on host tree resistance to PWN and its *Monochamus* vectors **Edmundo Sousa and Luis Bonifácio** 15 min
- Molecular detection methods including new analysis of the origin of the Portuguese PWN population **Manuel Mota and Philippe Castagnone** 20 min

- Monitoring and potential management of the *Monochamus* vectors **Juan Pajares and Gernot Hoch** 25 min
- Molecular tools for tracing *Monochamus* vector dispersal **Geraldine Roux and Julien Haran** 15 min
- Non-vector transmission of PWN **Thomas Schröder, Andrea Hopf-Biziks, Edmundo Sousa and Luis Bonifácio** 20 min
- Modelling wilt expression **Hannah Gruffudd and Hugh Evans** 15 min
- Modelling potential spread of PWN and PWD in Europe **Christelle Robinet** 15 min
- Interactions between REPHRAME and scientists/ stakeholders involved or interested in PWN. Dissemination of our findings. **Manuel Mota** 15 min

In summarising the key outcomes, Hugh Evans highlighted the following:

1. Effective monitoring of the *Monochamus* vectors is now well developed with good lures and traps.
2. New information on flight capacities of *Monochamus* spp indicates that short flights are common in dense woodland but in more open areas, long distance flight is likely. Distances of >2 km are common and up to 40 km is possible during the lifetime of a beetle.
3. Molecular diagnostic techniques have improved and this has enabled a new analysis of the origin of PWN in Europe to be carried out, suggesting that the source is directly from the native range in North America.
4. Non-vector transmission from nematode-infested wood chips through the roots of trees has been demonstrated, especially if there is any wounding of the roots. Similarly, direct transfer from infested sawn wood to living trees is possible if the wood makes direct contact to under-bark exposed tissues of the recipient tree. This needs to be accounted for in defining end use of potentially infested wood and wood products.
5. Modelling of the likelihood of pine wilt occurring has provided risk maps of Europe under current and future climates. In addition, a simplified model has been produced that will enable end users to estimate the potential for wilt in a given area or region. Latent expression of wilt (delayed by one or two years) has also been modelled and has important implications for carrying out surveys based on symptoms of affected trees.
6. A parallel modelling approach in Portugal confirms the important role of temperature and site in determining wilt expression, with the additional factor of maritime (coastal) influences on likelihood of wilt.
7. Dispersal of PWN and its vectors through Europe has been modelled and accounts for both vector-driven (local) dispersal and human-assisted (long distance) spread of the nematode. Linked to the wilt expression models, this provides a risk profile of likely spread across Europe.
8. The main results from the project, as well as links to world literature on PWN and its vectors, have been brought together in the PWN Tool Kit which is an on-line resource to guide end-users on the main topics that affect the PWN-vector-tree-environment relationship. This is available at www.rephrame.eu.

10.4 International Conference on PWN and its vectors.

Objective: Organization of the International Conference on PWN and publishing of the conference proceedings

Information on the final conference was provided in the second periodic report. It was held from 15th to 18th October 2013 and titled “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).

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.

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.

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

All work planned for this WP has been carried out and progress has been excellent for all sub-tasks. An additional Workshop, at the request of the EU Science Officer, was included which exceeded the original planned outputs.

Statement on the use of resources

There have been no significant deviations from the planned use of resources.

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 and second periodic reports, 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 has enabled virtually all the original planned programme to be delivered by the new end date of 30 November 2014.

As is apparent from this scientific report, which only covers the 9 month extension period, there has been excellent and significant progress in most of the tasks planned for REPHRAME and these have been extensively disseminated in various events 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.

6. Discussions at the back to back Workshops held in Spain and Portugal during 30 September-2 October 2014.

7. Final meeting of the Consortium held in Brussels on 12 November 2014. Discussions were mainly on planning for preparation and delivery of the third periodic and final report.

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 were managed through the project extension period.

There were workshops for Portuguese and Spanish end users during the late summer of 2014 and, at the request of the EU Science Officer an additional (not planned originally) final workshop was held in Brussels on 13 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, FR (Beneficiary 1) had to carry out a further procurement exercise to keep the external website going and this was only completed in late summer 2014. The main focus in developing the site was to structure and populate the PWN Tool Kit (PTK) which, of necessity, could only be done at the end of the project to be able to include the latest information form the work in 2014.

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.