Edited by:
Thomas Schröder

Julius Kühn-Institute (JKI)
Federal Research Centre for Cultivated Plants
Institute for National and International Plant Health

Pine Wilt Disease Conference 2013

15th to 18th Oct. 2013
Braunschweig / Germany

Scientific Conference
IUFRO unit 7.02.10 and
FP7 EU-Research Project REPHRAME

- Abstracts -

Berichte aus dem Julius Kühn-Institut

169

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Bundesforschungsinstitut für Kulturpflanzen

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Worldwide the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is one of the severest quarantine pests mainly in coniferous stands. In the concerned countries severe damage was caused by the nematode induced pine wilt disease (PWD).

Mainly the international trade of wood and wooden products led to an introduction of the pine wood nematode from its habitat in Northern America to Asia (Japan, China, Korea, Taiwan) and Europe (Portugal, Spain). Since then efforts were made in the infested areas to eradicate the nematode. Not only in the infested countries but also in many other countries new research approaches and conception plans were pursued in the previous years to manage the pine wilt disease.

Since the last IUFRO Symposium in Nanjing/China in 2009, several expert groups in the whole world - among other things - worked intensively on the following topics:

- Impact on the international trade as well as economic consequences in the infested areas including corresponding modeling of outbreak scenarios and pathways,
- Pathway analysis and modeling/predicting of pine wilt expression across eco-climatic zones taking account of latency ,
- Biology of *Bursaphelenchus xylophilus* and other *Bursaphelenchus* species including their interaction with bacteria and fungi and their impact on host trees,
- Diagnostic methods aimed to a fast and reliable determination of PWN in pure culture and in plant tissue as well as in laboratory and under field conditions,
- Examinations on the tree physiology and resistance characteristics of host trees,
- PWN and vector association, vector dispersal capacity, strategies for vector control,
- Behavior and population dynamics in infested trees,
- Non-vector transmission and treatment options for wood and wood products,
- Management strategies for PWD.

All research approaches contribute to enhance procedures on the eradication and the management of the PWN resp. the PWD and thus to minimize the economical and the ecological impact on concerned forests.

The aim of the symposium is to bundle the actual research progress and the management of the pine wood nematode and its vector beetles and to enhance the scientific exchange and thus to present the research results to a broad interested group of scientists, disease managers and decision makers.

This symposium, organized by the Julius Kühn-Institut (JKI), Braunschwig, Germany, is a joint action of the International Union of Forest Research Organizations (IUFRO) unit 7.02.10 Pine Wilt Disease (PWD) and the group of the EU-research project REPHRAME „Development of improved methods for detection, control and eradication of pine wood nematode“ in cooperation with the Deutsche Phytomedizinische Gesellschaft - German Scientific Society for Plant Protection and Plant Health (DPG).

Braunschweig, Oktober 2013
Thomas Schröder

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Andrea Hopf, Julius Kühn-Institut, Braunschweig, Germany

Program **Tuesday, 15th Oct. 2013 Opening Ceremony**		
08:45 - 10:00	**Registration; Forum of the Thünen-Institute**	
10:00 - 10:15	*T. Schröder*	Welcome by the coordinator of IUFRO unit 7.02.10 Pine Wilt Disease
10:15 - 10:35	*G. Backhaus*	Welcome and opening of the conference by the President of the Julius Kühn-Institut (JKI)
10:35 - 10:45	*F. Feldmann*	Welcome by the managing director of The German Scientific Society for Plant Protection and Plant Health
10:45 - 10:55	*H. Evans*	Welcome by the coordinator of the European Union Research Project "REPHRAME"
10:55 - 11:05	*T. Schröder*	Introduction in Conference and organizing issues
11:05 – 11:30		Group Photo and Coffee Break
Session 1: Insect Vector Chairperson: Thomas Schröder		
11:30 – 11:50	*Martin SCHROEDER*	Sampling strategies for *Monochamus sutor* – a potential vector of the pine wood nematode (103)
11:50 – 12:10	*Julien HARAN, Alain ROQUES, Géraldine ROUX*	Assessing potential expansion of the Pine Wood nematode (*Bursaphelenchus xylophilus*) from the spatial genetic structure of the vector (*Monochamus galloprovincialis*) (107)
12:10 – 12:30	*Gonzalo ÁLVAREZ, Estela SÁNCHEZ, Iñaki ETXEBESTE, Diego GALLEGO, Juan PAJARES*	Effective traps for live trapping of PWN vectors *Monochamus* spp. (134)
12:30 – 12:50	*Hervé JACTEL*	Effective attraction radius of pheromone traps for *Monochamus galloprovincialis* (172)
12:50 – 14:00	**Lunch**	

Continue Tuesday 15th Oct. 2013		
Session 1: Insect Vector Chairperson: Juan Pajares		
14:00 – 14:20	*Iñaki ETXEBESTE, Gonzalo ÁLVAREZ, Estela SÁNCHEZ, Juan PAJARES*	*Monochamus galloprovincialis* dispersal and the effective sampling area of operational traps (123)
14:20 – 14:40	*Guillaume DAVID, Hervé JACTEL, Dominique PIOU, Pedro NAVES, Edmundo de SOUSA*	Flight performances of *Monochamus galloprovincialis*, insect vector of the Pine Wood nematode (149)
14:40 – 15:00	*Christelle ROBINET, Guillaume DAVID, Dominique PIOU, Alain ROQUES, Hervé JACTEL*	Simulating the dispersal of *Monochamus galloprovinciallis* based on its flight mill performance and testing several management scenarios (110)
15:00 – 15:20	*H. MAS, R. HERNÁNDEZ, M. VILLAROYA, G. SÀNCHEZ et. al*	Dispersal behavior and long distance flight capacity of *Monochamus galloprovincialis* (Olivier 1795) (170)
15:20 – 15:40	*Fabio CHINELLATO, Mauro SIMONATO, Andrea BATTISTI, Massimo FACCOLI, Scott HARDWICK, Max SUCKLING*	Smart-traps combined with molecular on-site detection to monitor *Monochamus* spp. and associated pine wood nematode (152)
15:40 – 16:00	**Coffee Break**	
Session 1: Insect Vector Chairperson: Hervé Jactel		
16:00 – 16:20	*E. Sanchez HUSILLOS, Iñaki Etxebeste, G. Alvarez BAZ, Juan PAJARES*	Physiological development of *Monochamus galloprovincialis* immature adults through shoot feeding (133)
16:20 – 16:40	*Celia K BOONE, Jean-C. GRÉGOIRE, Nick BERKVENS, Hans CASTEELS, Nicole VIAENE*	Detection of exotic *Monochamus* spp. in Belgium - testing the tools in the areas of origin (130)
16:40 – 17:00	*Mehmet DAYI, Süleyman AKBULUT*	Preliminary results of potential vector species of *Bursaphelenchus* spp. (Nematoda:Parasitaphelenchidae) in Turkey (125)

Continue Tuesday 15th Oct. 2013 **Session 1: Insect Vector** Chairperson: Hervé Jactel		
17:00 – 17:20		Final Discussion Insect Vector
17:30		Bus transfer to hotel
19:00		Guided Tour Braunschweig Start from Tourist Information Address: Vor der Burg 1 (in front of the cathedral) Distance: 10 min from "Best Western Hotel"; 1 min from "Deutsches Haus"

Wednesday 16th October 2013 **Field trip National Park Harz**		
07:30		Departure Hotel by bus
09:00	*Sabine MANÉ*[1] *Christian LUX*[1] *Michael HABERMANN*[2] *Melanie KIRCHHÖFER*[2]	Field Trip National Park Harz • Silvicultural concept in the National Park • Forest Protection • Game Management
12:00 – 13:30		Lunch at „Bavaria Alm"
13:30 – 16:00	*Sabine MANÉ*[1] *Christian LUX*[1] *Michael HABERMANN* *Melanie KIRCHHÖFER*[2].	Continue Field Trip • "Strolling-through-the-forest"-path! • continental raised bog
16:00 – 17:00		Bus transfer to city of Goslar
17:00 – 18:30		Guided tour in the world heritage site city of Goslar
18:30 – 22:00		Dinner in the Kennel of Goslar

[1] Mangament Nationalpark Harz; **http://www.nationalpark-harz.de/en/**
[2] North-West-German Forest Research Center, Department Forest Protection (**http://www.nw-fva.de/**)

Thursday 17th Oct. 2013		
Session 2: Systematic and diagnostics		
Chairperson: Géraldine Roux		
09:00 – 09:20	*Anne-Marie CHAPPE, Geraldine ANTHOINE*	Validation of a real time PCR assay for the detection of *Bursaphelenchus xylophilus* in targeted matrices in the framework of national survey (114)
09:20 -09:40	*Renske LANDEWEERT, Paul MOOIJMAN, Winfried MULDER, Sven VAN DEN ELSEN, Johannes HELDER,*	Molecular detection of *B. xylophilus* in complex DNA backgrounds (112)
09:40 – 10:00	*Ye, WEIMIN, Robin M. GIBLIN-DAVIS*	Pine-Wood nematode assay and development of real-time PCR for species identification in North Carolina department of agriculture & consumer services (159)
10:00 – 10:20	*Isabel LEAL, Eric ALLEN, Jennifer ANEMA, Brett FOORD, Caralyn REISLE, Adnan UZUNOVIC, Aniko VARGA, Delano JAMES*	Development of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method to detect living pinewood nematode, *Bursaphelenchus xylophilus*, in wood (100)
10:20 – 10:40	Geraldine ANTHOINE	EUPHRESCO project - *Bursaphelenchus xylophilus*, early detection methods (115)
10:40 – 11:00		Coffee Break
Session 3: PWN in international trade, pathways and phytosanitary treatments		
Chairperson: Hugh Evans		
11:00 – 11:20	*Simone PROSPERO, Janina POLOMSKI, Daniel RIGLING*	Occurrence and distribution of *Bursaphelenchus* species in Switzerland (119)
11:20 – 11:40	*Luís FONSECA, José SANTOS, Hartmut NESTLER, Joaquim VERDASCA, Rui OLIVEIRA, Isabel ABRANTES, Clara SERRA*	Coniferous bark hot steam treatment for the elimination of the pinewood nematode (146)
11:40 – 12:00	*Andrea HOPE, Thomas SCHRÖDER*	Non vector spread of *Bursaphlenchus xylophilus* via wood chips (155)

Continue Thursday 17th Oct. 2013 **Session 3: PWN in international trade, pathways and phytosanitary treatments** Chairperson: Hugh Evans		
12:00 – 12:20	*L. BONIFÁCIO, M. L. INÁCIO, E. SOUSA, S. BUCKLEY E. M. THOMS*	Complementary studies to validate the proposed fumigation schedules of sulfuryl fluoride for inclusion in ISPM No. 15 for the eradication of pine wood nematode (*Bursaphelenchus xylophilus*) from wood packaging material (135)
12:20 – 12:40	*Thomas SCHRÖDER, Johannes WELLING, C. Aukamp-TIMMRECK*	Efficacy of kiln drying as phytosanitary treatment against wood borne nematodes (156)
12:40 – 13:40	Lunch	
Session 4: PWN interactions with bacteria Chairperson: Manuel Mota		
13:40 – 14:00	*O.A. KULINICH, E.N.ARBUZOVA, U.Sh. MAGOMEDOV, N.I.KOZYREVA, E.S. MAZURIN, M.S. KOLYCHIKHINA, A.Yu RYSS.*	Recent Research on Pine Wilt Disease in Russia (116)
14:00 – 14:20	*Jiajin TAN, Hongye Qu, Dejun HAO, Fengmao CHEN*	Inoculation Effects of *Pinus thunbergii* with *Bursaphelenchus xylophilus* and Two Bacterium Strains of *Bacillus firmus* (141)
14:20 – 14:40	*Cláudia S. L. VICENTE, Yoriko IKUYO, Manuel MOTA, Koichi HASEGAWA*	*Bursaphelenchus xylophilus* and associated bacteria under oxidative stress conditions (104)
14:40 – 15:00	*Paula V. MORAIS, Diogo Neves PROENÇA, Gabriel PAIVA, Romeu FRANCISCO, Paula VERISSIMO, Luís FONSECA Isabel M.O. ABRANTES*	Diversity and in vitro nematicidal activity of bacteria associated to pinewood nematode (161)

Continue Thursday 17th Oct. 2013 **Session 4: PWN interactions with bacteria** Chairperson: Manuel Mota		
15:00 – 15:20	*Cláudia S. L. VICENTE, F. X. NASCIMENTO, Margarida ESPADA, Pedro BARBOSA, Koichi HASEGAWA, Manuel MOTA, Solange OLIVEIRA*	Natural bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis* (105)
15:20 – 15:40		Final Discussion
15:40 – 16:00		Coffee break
Postersession and side events **Conference banquet**		
16:00 – 19:00		Poster session
16:00 – 17:00	*Thomas SCHRÖDER*	IUFRO unit PWD management meeting
17:00 – 18:00	*Geraldine ANTHOINE*	EUPHRESCO meeting
19:00 – 21:00		Conference banquet
21:10		Departure by bus to hotel

Friday 18th Oct. 2013 **Session 5: PWD management and contingency planning** Chairperson: Edmundo Sousa		
09:00 – 09:20	Katsunori NAKAMURA, Noritoshi MAEHARA, Takuya AIKAWA, Yu ICHIHARA, Hajime KOSAKA, Etsuko KAGAYA, Hisahsi SUGITA, Takashi MASAKI, Koki KIMURA, Jun-ichi KON, Tomonori KANEKO	A research project to develop strategic action plan in the pine-wilt-disease unaffected area in northern Japan (118)
09:20 - 09:40	Xu FUYUAN, Zheng HUAYING, Xu MING	Study on the techniques of sustainable control of pine wood nematode disease (*Bursaphelenchus xylophilus*) (136)
09:40 – 10:00	P. NAVES, M. VIEIRA, E. SOUSA	New Strategies for pine wilt disease (PWD) management in Portugal: preventive methods to reduce the spread of the disease to new areas (158)
Session 6: PWN Biology, Population dynamics, Epidemiology, Modelling Chairperson: Edmundo Sousa & Christer Magnusson		
10:00 – 10:20	Marek TOMALAK, Anna FILIPIAK	Inter-specific competition of *Bursaphelenchus xylophilus* with native populations of *B. mucronatus* in pine (121)
10:20 – 10:40	Han ZHENGMIN, Ben AILING, Guo YE, Cao DONGXIA	Interspecific hybridization between *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* (102)
10:40 – 11:00		Coffee break
11:00 – 11:20	Tetsuro KATO, Akira KANEKO, Ryoji SHINYA, Kazuyoshi FUTAI, Yuko TAKEUCHI	Phenotypic and genotypic traits of recombinant inbred lines of pine wood nematode, *Bursaphelenchus xylophilus* (113)
11:20 – 11:40	Lihua ZHU, Lin HUANG, Jianren YE	Pathogenicity, reproduction and survival of axenic *Bursaphelenchus xylophilus* (128)

Continue Friday 18th Oct. 2013 **Session 6: PWN Biology, Population dynamics, Epidemiology, Modeling** Chairperson: Christer Magnusson		
11:40 – 12:00	Rui-he GAO, Juan SHI, You-qing LUO	Influence of pine wood nematode invasion on typical Masson pine ecosystem in Three Gorges Reservoir Region of China (151)
12:00 – 12:20	Hannah GRUFFUDD, Hugh EVANS, Tom JENKINS	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 (124)
12:20 – 12:40	Ruifen HUANG, Juan SHI, Youqing LUO	Cold-tolerance and adaption of Pine wood nematode in China (153)
12:40 – 13:40	Lunch	
Chairperson: Gernot Hoch & Thomas Schröder		
13:40 – 14:00	Zheng HUAYING, Xu MING, Xu FUYUAN	A comparative proteomics analysis on resistant provenance of *Pinus massoniana* inoculated with *Bursaphelenchus xylophilus* (137)
14:00 – 14:20	Francisco LEISICO, Paula GOMES, Miguel PINHEIRO, Luís FONSECA, Isabel ABRANTES, Conceição EGAS	Comparative transcriptomics to understand the molecular basis of *Bursaphelenchus xylophilus* pathogenicity (154)
14:20 – 14:40	Lin HUANG, Minqi TIAN, Xiuwen QIU, Yi ZHANG, Xiaoqin WU, Jianren YE	The Function of Major Sperm Proteins (MSPs) in reproduction of pine wood nematode, *Bursaphelenchus xylophilus* (129)
14:40 – 15:00	Coffee break	
15:00 – 15:20	Claudia SL VICENTE, Yoriko IKUYO, Manuel MOTA, Koichi HASEGAWA	Exploring the relation between virulence and oxidative stress response of *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* (157)
15:20 – 17:00	Final Discussion and conclusions End of conference	
17:00	Departure to hotel by bus	

Oral Presentations

Session 1:

Insect Vector

Schroeder M, Sampling strategies for *Monochamus sutor* – a potential vector of the pine wood nematode. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 12, Braunschweig, ISSN: 1866-590X

Sampling strategies for *Monochamus sutor* – a potential vector of the pine wood nematode

Schroeder M

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According to the European Commission Decision 2012/535 all EU countries are required to conduct detection and delimitation (if detected) surveys for the pine wood nematode (PWN). In more northern areas, where development of pine wilt disease is unlikely, the two most efficient survey methods for PWN is to sample dispersing *Monochamus* beetles by traps (baited with attractants) and wood substrates colonized by *Monochamus* (easily identified due to characteristic larval galleries). The aim of this project was to develop sampling strategies, based on these two approaches, for *Monochamus sutor* in Sweden. In addition, an identification key based on male genitalia was developed for the European *Monochamus* species (Wallin *et al*. 2013). Trap catches of *M. sutor* were compared among three different stand types: fresh clear-cuts, old clear-cuts and pine stands. Catches were of the same magnitude on fresh and old clear-cuts, but 5 – 6 times higher on clear-cuts compared with in pine stands. Thus, clear-cuts should be used for trap locations if possible. Logging residues on clear-cuts and thinnings constitute the major breeding substrate for *M. sutor* in Sweden. Thus, surveys of average densities of colonized tops and branches on clear-cuts and thinnings provide a method to plan the number of samples required for achieving a certain statistical significance at which PWN can be stated to be absent. The identification key provides a reliable method for discerning between *M. sutor* and *M. galloprovincialis*. In addition, no difference between male genitalia of *M. sartor* and *M. urussovi* were found. Thus, we regard *M. urussovi* as a subspecies of *M. sartor*.

REFERENCES

Wallin H; Schroeder M; Kvamme T (2013). A review of the European species of *Monochamus* Dejean, 1821 (Coleoptera, Cerambycidae) – with a description of the genitalia characters. *Norwegian Journal of Entomology* 60, 11-38.

Haran J, Roques A, Roux G, Assessing potential expansion of the Pine Wood nematode (*Bursaphelenchus xylophilus*)from the spatial genetic structure of the vector (*Monochamus galloprovincialis*). In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 13, Braunschweig, ISSN: 1866-590X

Assessing potential expansion of the Pine Wood Nematode (*Bursaphelenchus xylophilus*) from the spatial genetic structure of the vector (*Monochamus galloprovincialis*)

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Monochamus galloprovincialis (Coleoptera, Cerambycidae) is the main factor involved in the natural spread of the Pine Wood Nematode (PWN), a serious pest for pine forests. Since its introduction in Portugal, the PWN has rapidly expanded its range to a large part of the country and will probably keep expanding to the rest of Europe. Estimation of dispersal abilities of *M. galloprovincialis* over various landscapes and across mountains is a key point to predict the invasion of the PWN in Europe, and will help to set up management for this pest.

Microsatellites are highly variable genetic markers. Their polymorphism provides information on genetic structure of organisms at a broad scale (phylogeography), but also at local scale (migration of individuals). We developed a set of 12 microsatellites loci specific to *M. galloprovincialis*. First assessments conducted on six populations along a European North-South transect reveal a reduction of the genetic diversity northward, with a maximum of allelic richness in Spain. This seems consistent with postglacial recolonization of Mediterranean species associated with Pines trees. We have also observed a significant differentiation between some Iberian populations.

Our perspectives are to use the microsatellites markers (i) to construct the European phylogeography of *M. galloprovincialis*, (ii) to estimate the effect of the Pyrenees and the landscape structure on dispersal abilities of this species, and finally, (iii) to look at the effect of PWN invasion on its genetic structure in Portugal.

Alvarez, G.; Etxebeste, I.; Sánchez, E.; Gallego, D.; Pajares J.A. Effective traps for live trapping of PWN vectors *Monochamus* spp. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 14-16, Braunschweig, ISSN: 1866-590X

Effective traps for live trapping of PWN vectors *Monochamus* spp.

Alvarez G, Etxebeste I; Sánchez E, Gallego D, Pajares JA

ABSTRACT

Pheromone-kairomone blend recently developed has shown high power to attract *Monochamus galloprovincialis* beetles to traps. This lure may be used for effective monitoring and even mass trapping of pine wood nematode vectors. However, for this to be true, an effective trap for trapping these species is required. *Monochamus* beetles are very agile and easily escape from most of traps if they are not killed. However, keeping the captured beetles alive is a key feature for monitoring nematode loads in the beetles and for obtaining other valuable information on the beetle population. Several designs of traps were tested to determine their suitability in maximizing *M. galloprovincialis* caught beetles and in keeping them alive. These included different modifications on conventional multiple-funnel traps and cross-vane traps, as one-way funnels, slippery coated inner surfaces, extended collector cups or wire screen bottoms. Experiments were carried out under different field conditions in Spain using randomized block designs. Traps were suspended from poles 2m height and baited with the kairomone/pheromone blend. Catches were sampled weekly during *M. galloprovincialis* flying period. Some experiments were replicated in other countries. Results of several experimental years showed that a slippery coat on the trap doubled catches, whereas the slippery coat and a tight wire screen on the collection cups bottom avoided escape of trapped live adults and increased their survival. These results have led to commercial development of two models of efficient traps.

INTRODUCTION

Insects of the genus *Monochamus* (Coleoptera: Cerambycidae) include species that colonize recently dead or heavily damaged conifers by factors as drought and attack by other organisms. Although they have been usually considered as secondary pests, their reported role as vectos of the pine wood nematode *Bursaphelenchus xylophilus*, has given them a main role in pest management. Since the introduction of the nematode in Europe, *M. galloprovincialis* has been confirmed as its only vector. A great deal of knowledge has been achieved in recent years on its chemical ecology leading to a pheromone-kairomone blend recently developed that has shown a high power to attract *Monochamus*

galloprovincialis beetles to traps. This lure may be used for effective monitoring and even mass trapping of pine wood nematode vectors. However, for this to be true, an effective trap for trapping these species is required. *Monochamus* beetles are very agile and easily escape from most of traps if they are not killed. However, keeping the captured beetles alive is a key feature for monitoring nematode loads in the beetles and for obtaining other valuable information on the beetle population.

MATERIAL AND METHODS

From 2010 to 2012, several designs of traps were tested in field assays to determine their suitability in maximizing *M. galloprovincialis* caught beetles and in keeping them alive. Assay in 2010 included multiple-funnel traps for 5 different treatments: 1) collecting cup provided with a small piece of DDVP (dimethyl 2,2-dichlorovinyl phosphate) insecticide strip (Econex S . L., M urcia, S pain) t o ki ll t rapped be etles; 2) collecting c up w ithout modifications or ins ecticide; 3) c ollecting cup internally coated with slippery s ubstance (Teflon); 4) bot h m ultiple-funnel t rap a nd c ollecting c up coated w ith T eflon a nd 5) collecting cup sheathed in a polystyrene cover and tight wire screen bottom with the aim of reduce internal temperature and improve insects survival. The assay carried out in 2011 compared the ef ficacy o f cat ching i nsects and mantaining t hem al ive of t hree di fferent designs o f traps: conventional mul tiple-funnel t rap, c ross-vane t rap and polytrap (a t ype of c ross-vane t rap). Finally i n 2012, effectiveness of ne w mul tiple-funnel t raps w ere compared with us ed m ultiple-funnel a nd c ross-vane t raps w ith the aim of eva luate t he suitability of t hese di fferent m odels a nd t he pos sible l oss of e fficacy of T eflon-coated traps t wo years af ter ha ving be en coated. All t hese e xperiments w ere c arried out us ing randomized bl ock de signs. T raps w ere s uspended from pol es 2m he ight and baited with the ka iromone/pheromone bl end. C atches were s ampled w eekly during *M. galloprovincialis* flying period.

DISCUSSION

Comparison be tween conventional mul tiple-funnel t rap a nd t his s ame pr ovided w ith insecticide showed t hat exist a pr oportion of c aught i nsects t hat e scape from c ollecting cup. T he num ber of i nsects obt ained w ith collecting cup i nternally coated w as not different from t hat us ing conventional c ollecting c up, but c ollecting cup covered w ith polystyrene got better results, due to better conditions inside the collecting cup, reducing temperature and ke eping i nsects qui et on t he background grid. However, the num ber of catches obt ained by both m ultiple-funnel t rap and c ollecting c up s lippery coated w as three times more than that by conventional multiple-funnel trap, suggesting that the key is not only to avoid escape of insects but maximize the number of attracted insects that fall into t he collecting c up. T he pr oportion of l iving i nsects w as not di fferent be tween treatments w ithout i nsecticide. C omparison be tween di fferent m odel of t raps i n 2011

showed no differences between cross-vane trap and multiple funnel trap, although polytraps showed clearly inferior (Figure 1). Finally, 2012 experiments showed no differences between old traps and new ones and confirmed no differences between cross-vane traps and multiple-funnel traps. Anyway, Teflón-coated traps have been not tested more than two years after being coated and long durability of Teflon coating under field conditions is unknown. The loss of effectiveness during time would have the double effect of not only escape of insects but a decrease in the number of catches. These results have led to the commercial development of both teflon-coated traps, ECONEX MULTIFUNNEL-12® and CROSSTRAP® (Econex S.L., Murcia, Spain), that are efficient enough to be recommended for monitoring and for mass trapping of *M. galloprovincialis* They are currently being used in Spain in the PWN eradication programs and live adult trapping is allowing the sampling for pine wood nematodes carried in the caught beetles within these programs.

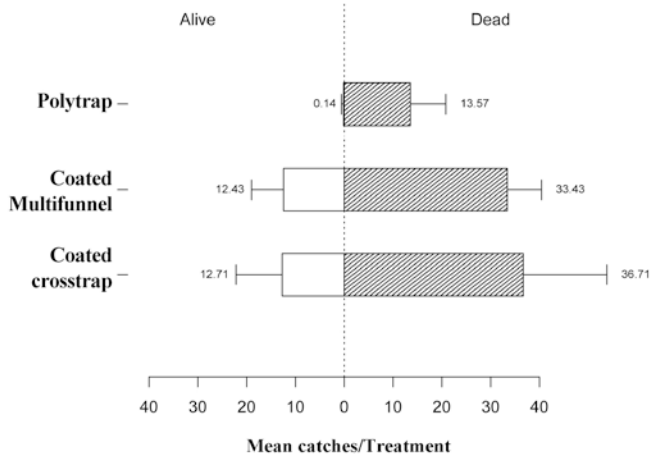

Fig. 1) Different type of traps tested in 2011 field assay.

Jactel H, Effective attraction radius of pheromone traps for *Monochmaus galloprovincialis*. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 17, Braunschweig, ISSN: 1866-590X

Effective attraction radius of pheromone traps for *Monochamus galloprovincialis*

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Pheromone trapping has been considered as a means to monitor populations of *Monochamus galloprovincialis*, the insect vector of the pine wood nematode (PWN) in southern Europe, since the identification and the synthesis of its aggregation pheromone, which shows excellent biological activity in the field. With the development of very effective interception traps, pheromone trapping is also envisaged for PWN management through mass-trapping. However little is known about the practical deployment of pheromone traps in the field, particularly about optimal density of traps in trapping networks. The concept of Effective Attraction Radius (EAR, Byers et al. 1989), which represents the radius of a passive "sticky" sphere that would intercept the same number of flying insects as the attractant, is of particular interest for optimizing the density of trapping network. In theory, capture efficiency is expected to reach an optimum for distance D_{Opt} between traps equal to twice EAR. We developed a "quick and cheap" experimental method to estimate EAR in the field, using pairs of pheromone traps at increasing distance from each other. Plotting the mean capture of *M. galloprovincialis* per trap against the distance between two traps we obtained an asymptotic curve that levelled out at D_{Opt} making it possible to estimate EAR.

Etxebeste I, Álvarez G, Sánchez-Husillos E & Pajares J, *Monochamus galloprovincialis* dispersal and the effective sampling area of operational traps. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 18-19, Braunschweig, ISSN: 1866-590X

Monochamus galloprovincialis dispersal and effective sampling area of operational traps

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ABSTRACT

The availability of an operational trapping system for *Monochamus galloprovincialis* (Olivier, Coleoptera: Cerambycidae), the European vector of the Pine Wood Nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer), has allowed the implementation of a reference tool for monitoring the presence and spread of the Pine Wilt Disease as well developing management methods based on mass-trapping of the vector. Furthermore, catch data gathered in traps can be potentially used in the study of *M. galloprovincialis* dispersal and the determination of absolute population densities. However, such applications require of the parameterization of dispersal models and detection functions that are in turn used to estimate values such as diffusion rates or the effective sampling area of traps (Turchin, 1998).

Two mark-release-recapture experiments carried out in 2009 and 2010 at two different pine stands in central-Spain were designed to describe such values. During the first trial, 174 unfed *M. galloprovincialis* imagoes were marked and released from the centre of a setup consisting of 28 traps distributed along four concentric rings at 50,100, 250 and 500m located on a natural *Pinus pinaster* stand (Figure 1A). A second study used a grid-based

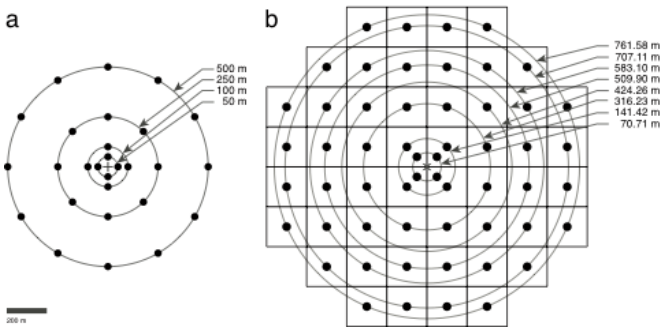

Figure 1. Experimental designs followed during *Monochamus galloprovincialis* dispersal studies in 2009 (a) and 2010 (b).

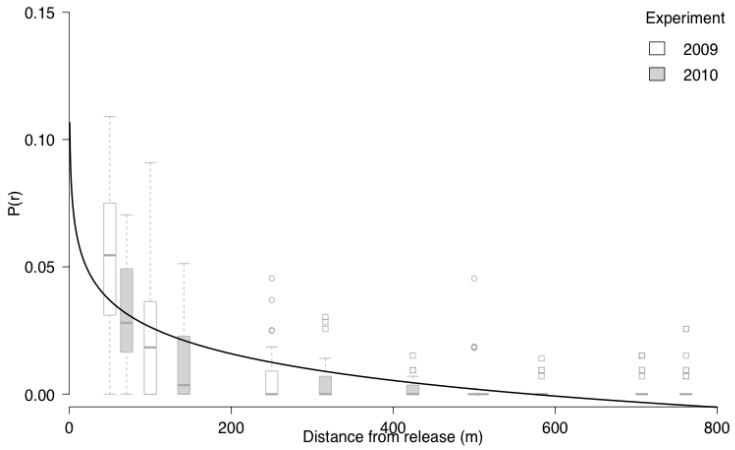

Figure 2. Proportions of recaptured *Monochamus galloprovincialis* [P(r)] at traps located a diverse distances from the release point (r) during field trials in 2009 and 2010 in Western Spain. The black line representes fitted linear regression for the estimated proportion of recaptures in relation to the common logarithm of the distance.

design with 56 traps that covered distances up to 761 m, to track the movement of 353 individuals (Figure 1B).

Up to 35 and 29% of released beetles were recaptured during 2009 and 2010 field experiments respectively. Values per each replicate during each experimental period were then modelled and the relationship between capture probability and distance to release point could be studied under different theoretical and empirical regression models (Turchin, 1998).

On the one hand, modeling of mean recaptures per distance under a simple diffusion model for time integrated data showed that about 50% of individuals do not dispers beyond 40 m. On the other hand, empirical fitting of proportion of recaptures allowed to set the effective sampling area for traps at ca. 0.77ha, while the seasonal sampling range at which the estimated proportion of the catch would be zero could be set to 570.44m (Figure 2; Ostrand & Anderbrant, 2003).

The variations in the estimates when using other modelling approaches are discussed, as well as the implications of the study of *M. galloprovincialis* dispersal on management decissions and the mmonitoring of its population density.

Keywords. Dispersal, Mark Release Recapture, Population Density, Baited Traps, Sampling Range

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David G, Hervé J, Piou D, Naves P, Sousa E, Flight performances of *Monochamus galloprovincialis*, insect vector of the Pine Wood nematode, In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 20, Braunschweig, ISSN: 1866-590X

Flight performances of *Monochamus galloprovincialis*, insect vector of the Pine Wood nematode

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The Pine Wood Nematode (PWN, *Bursaphelenchus xylophilus*) is the most important threat to pine plantation forests in Europe since its introduction from Asia and its establishment in pine forests of Portugal. It is currently spreading towards Spain and France. The natural transmission from tree to tree is done by insect vectors of the genus *Monochamus*. However until now little was known about the flight capacity of these vectors. To better evaluate their dispersal capacity under standardized conditions, we developed automatically recording flight mills. We found that *M. galloprovincialis* exhibits a wide array of flight capacities, with few beetles not flying at all, while others are able to fly over several tens of km when considering the cumulated flights through their life span. We also investigated the effect of several life traits on flight performances such as beetles' gender, age, maturation status, body size and also the impact of nematode load. We will discuss the implication of these findings for the development of PWN risk management methods such as precautionary clearcuts.

Robinet C, David G, Piou D, Roques A, Jactel H, Simulating the dispersal of *Monochamus galloprovinciallis* based on its flight mill performance and testing several management scenarios, In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 21, Braunschweig, ISSN: 1866-590X

Simulating the dispersal of *Monochamus galloprovinciallis* based on its flight mill performance and testing several management scenarios

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The potential spread of the pine wood nematode, *Bursaphelenchus xylophilus*, and its associated pine wilt disease are strongly associated with the flight capacity of the insect vector. Although some data were available from congeneric species in North America and Asia, the flight performances of the European vector, *Monochamus galloprovinciallis*, were largely unknown. They were assessed with flight mill experiments and used to fit a dispersal kernel. A stochastic individual-based model was then developed to simulate the trajectory of adults over one season. With this dispersal model and a transmission function of the nematode, the area where the vector can transmit the pine wood nematode can be determined and several forest management scenarios to contain its spread can be tested. Although this dispersal model is still at an exploratory stage, and beetle's dispersal capacity might be overestimated with flight mill, this approach contributes to a better understanding of vector's dispersal pattern and can be used to test the effects of several management scenarios. This dispersal model is a first step towards the development of a refined spread model of the nematode and the disease at larger spatial and temporal scales in Europe.

Mas et al., Dispersal behavior and long distance flight capacity of *Monochamus galloprovincialis* (Olivier 1795), In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 22, Braunschweig, ISSN: 1866-590X

Dispersal behavior and long distance flight capacity of *Monochamus galloprovincialis* (Olivier 1795)

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ABSTRACT

It is intended to evaluate the capacity of long distance natural spread of *Monochamus galloprovincialis* (Olivier 1795), vector of the pathogen Pine Wood Nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer, 1934).

Eight trials of trapping-marking-releasing-recapturing adult individuals have been held in different regions along the east of the Iberian Peninsula during 2009-2011. To catch the adults have been used three different types of traps: Lindgren funnel, Crosstrap and the Torre-LSF prototype, all of them baited with the specific kairomonal-pheromonal attractive of *M. galloprovincialis*. The traps were sited in the sampling areas at different distances from a central point where the insects have been released.

Results show that released mature adults of *M. galloprovincialis* are able to achieve long-distance spread, reaching maximum values of 13600 m and 22100 m. These distances, together with the high percentages of captures recorded above 3000 m (close to 2%) seem to show the low efficiency of quarantine belts (areas cleaned of possible host species) that are been used in the eradicating programmes and in the theoretical isolation of pest free areas.

Chinellato F et *al.*, Smart-traps combined with molecular on-site detection to monitor *Monochamus* spp. and associated pine wood nematode. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 23-25, Braunschweig, ISSN: 1866-590X

Smart-traps combined with molecular on-site detection to monitor *Monochamus* spp. and associated pine wood nematode

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INTRODUCTION

The pine sawyer beetles Monochamus spp. (Coleoptera Cerambycidae) are the main vectors of the Pine Wood Nematode (PWN), Bursaphelenchus xylophilus, the agent of pine wilt disease in various parts of the world (Mamiya 1983). In Europe, M. galloprovincialis (Olivier) gained importance as a vector after the finding of the PWN in Portugal in 1999 (Sousa et al. 2001). An effective monitoring method based on early detection of both vector insects and associated nematode is needed in order to adopt appropriate phytosanitary measures (Rassati et al. 2012 and 2013).

MATERIALS AND METHODS

The present study shows a new technology for the remote detection of beetle catch combined with on-site molecular detection of both vector and nematode identity. A multi-funnel trap, baited with either specific or generic blend, and equipped with a specifically modified security camera (BioCam, Mi5 Security, Auckland, New Zealand), composed by a wide-angle lens, 1 or 3 MegaPixel sensor, rechargeable battery pack and internal modem for General Packet Radio Service (GPRS) connection was used. The interval between images taken by the camera can be programmed and saved in a Secure Digital (SD) memory card. The images can be stored in the same SD card and simultaneously sent to a safe repository accessible through the web, from which they are downloadable. On the same repository it is possible to check the level of battery charge of each camera and the GPRS coverage as well.

When a target beetle is detected, an on-site visit is planned, during which a fragment of the thorax is analyzed using a Loop Mediated Isothermal Amplification (LAMP) portable

device (Genie II, Optigene, UK) to identify the trapped species of *Monochamus* spp. and to detect the PWN possibly vectored by the beetles. Currently, primers were deloped for the endemic *M. galloprovincialis* and *M. sutor,* and for the exotic *M. alternatus* and *M. carolinensis*. For the beetles identified as *Monochamus,* presence of PWN is also tested with the same device using slighty modified LAMP primers from Kikuchi *et al*. (2009), specific for the nematode ITS1 region. A positive control for the nematode is included in the test. The technique allows amplifying target DNA in a few minutes visualizing the results immediately.

RESULTS

Images obtained by cameras are definitely adequate to visually recognize large longhorn beetles such as *Monochamus* spp.. All the main morphological traits of the species are detectable (Fig. 1). The system works also under sub-optimal light conditions. LAMP primers designed to amplify the ITS2 region of *M. galloprovincialis, M. sutor, M. alternatus* and *M. carolinensis* show to be specific, giving a positive result only for these species after 10-15 minutes after the test start (Fig. 2). On the other hand, no positive insects for PWN have been detected until now.

CONCLUSIONS

Both technologies are designed for quick and cheap on-site analyses, and can be used by non-expert staff with a short training. In case of positive samples, they must be taken to the laboratory and analyzed more accurately with standard protocols for official confirmation.

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Figure 1 Picture taken by 3M P trap camera. One individual of *Monochamus* spp. is clearly recognizable on the left, together with several individuals of the longhorn beetle *Acanthocinus griseus*, one of the western seed bug *Leptoglossus occidentalis* (above) and several small bark beetles (right).

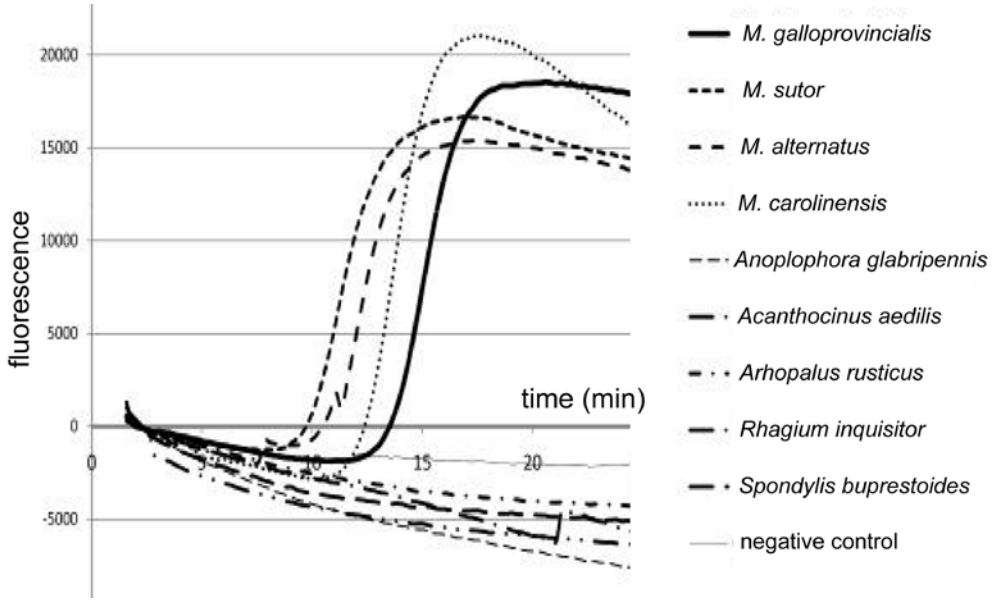

Figure 2. Amplification profile for the LAMP assay carried on *Monochamus* spp. and other cerambycid beetles. Positive curves are obtained in 10-15 minutes only for *M. galloprovincialis*, *M. sutor*, *M. alternatus* and *M. carolinensis*.

Sánchez-Husillos E, Etxebeste I Álvarez-Baz G, Pajares J, Physiological development of *Monochamus galloprovincialis* immature adults through shoot feeding. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 26-27, Braunschweig, ISSN: 1866-590X

Physiological development of *Monochamus galloprovincialis* immature adults through shoot feeding

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ABSTRACT

Adult shoot feeding is a vital feature of *Monochamus* life history playing a key role in the pine wilt disease infection cycle. After emergence, adults must feed on the phloem of healthy pine shoots thorough all their life for nutrition to sexm aturate, disperse, reproduce and survive. Furthermore, it is known to be required by some species as a necessary step for wing muscle development. Development of several physiological parameters and sexual maturation was studied on freshly emerged adults of the pine nematode vector *M. galloprovincialis* (Olivier, Col.: Cerambycidae) during one month of shoot feeding with the aim to gain knowledege on its dispersial behaviour.

Gonadic development was assessed on adults of both sexes (n=24) at 0, 4, 8, 14, 18 and> 18 day age intervals. Genitalia dissections served to track morphological changes during gonadic maturation as well as the presence of eggs or oocytes. Sex maturation could be established to occur after feeding for 8-14 days in males and 16 days in females.

Fat bodies of fed adults of both sexes (n=90) at previously mentioned age intevals were extracted as described by Anderbrandt (1988). *M. galloprovincialis* adults emerged with lipid content averaging 12.28% of their dry weight. This amount was decreasing during the first 4 days down to 9.7% , and then increasing to peak 14 days after feeding at 13.68% of their dry weight (Figure 1).

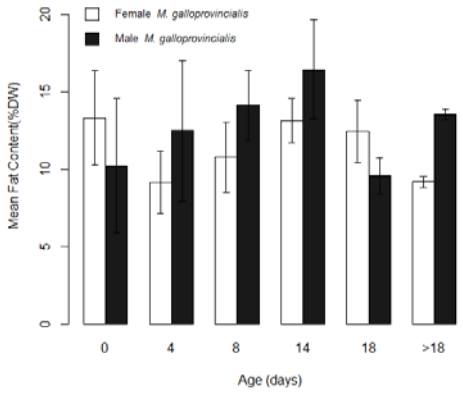

Figure 1:Histogram of mean fat content (%DW)

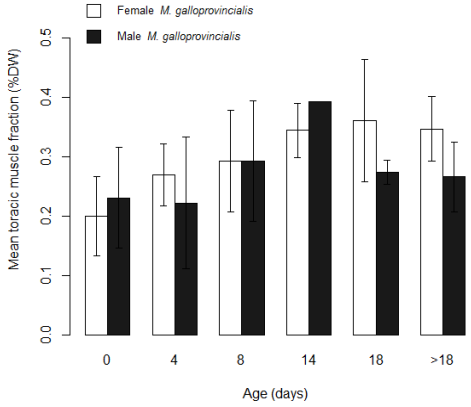

Figure 2:Histogram of mean toracic muscle fraction (%DW)

Up to 60 insects used in lipid extraction had their wing segement extracted and macerated in K OH. T he dr y weight di fference be tween l ipid-less and diggested s egments, i. e. without m uscle, a llowed t he de termination of a n approximated m uscle c ontent. R esults varied f rom 26.6% of d ry weight i n males and 20% i n females after emergence, to a maximum of 39% in males and 36% in females after 14-18 days of shoot feeding. Steady weight gain w as r ecorded i n f ed a dults t hrough t he first 16 da ys o f f eeding t hen be fore stabilizing. C onversely, weight l oss of un fed a dults m irrored gain w eight of f ed a dults (Figure 2).

Finnaly, 29 i ndividuals were fed with *Pinus pinaster* twigs until weight stabilization and 10 unf ed i nsects w ere k ept unt il de ath oc curred. S urvival of unf ed a dults a veraged 1 2 days and for a maximum of 20 days. Weight loss at the time of death of these beetles was 38% of dry weight.

These results show that freshly emerged, unfed, *Monochamus* adults have fat content and wing muscles enough to undertake sustained dispersal flight.

Key Words:

Bursaphelenchus xylophilus, pine wood nematode, gonads, fat content, wing muscles.

ACKNOWLEDGEMENTS

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Boone CK, Grégoire J-C, Berkvens N, Casteels H, Viaene N, Detection of exoctic *Monochamus* spp. in Belgium – testing the tools in the area of origin, In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 28, Braunschweig, ISSN: 1866-590X

Detection of exoctic *Monochamus* spp. in Belgium – testing the tools in the area of origin

Boone CK[1], Grégoire J-C[1], Berkvens N[2], Casteels H[2], Viaene N[2]

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ABSTRACT

In the European Union, Commission decision 2012-535-EC on emergency measures to prevent the spread within the Union of *Bursaphelenchus xylophilus* specifies that "*Member States shall annually conduct surveys for* Bursaphelenchus xylophilus *[...] on susceptible plants, susceptible wood and bark and on the vector, and determine whether there is any evidence of the presence of PWN in their territory in areas in which PWN was previously not known to occur*".

Bursaphelenchus xylophilus has not been detected in Belgium to date, however, potential native *B. xylophilus* vectors, *Monochamus galloprovincialis* and *M. sartor,* have been detected infrequently. To effectively monitor for potential vectors, both native and exotic, it is necessary to test the efficacy of traps and lures in both this country and the countries of origin of potential vectors. In 2013, Belgium initiated a two-fold experimental programme aiming to: 1) monitor native *Monochamus* species susceptible to vector the pinewood nematode; and 2) implement surveillance for exotic species that could enter the country via wood packaging material. European traps (Econex Crosstrap) and lures (Galloprotect Pack), which have been successfully tested in different EU countries, were deployed in various locations throughout Belgium, including ten points adjacent to companies importing goods in wood packaging material. In parallel, to validate the capacity of these traps and lures to capture exotic *Monochamus* species, traps were sent to several locations in North America known to harbour species that vector PWN. In the United States, traps and lures were sent to Arkansas targeting *M. caroliniensis* and *M. titillator*, and Utah targeting *M. scutellatus* and *M. clamator*. In Canada, they were sent to New Brunswick targeting *M. mutator* and *M. notatus*, and British Columbia targeting *M. obtusus*. Here we present the first results of this control experiment.

Dayi M, Akbulut S, Preliminary results of potential vector species of *Bursaphelenchus spp.* (Nematoda:Parasitaphelenchidae) in Turkey , In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 29, Braunschweig, ISSN: 1866-590X

Preliminary results of potential vector species of *Bursaphelenchus spp.* (Nematoda:Parasitaphelenchidae) in Turkey

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ABSTRACT

The detection of *Bursaphelenchus xylophilus* in 1999 in Europe prompted many European countries to carry out surveys to determine *B. xylophilus* and its insect vectors, and to prevent the pine wilt disease. As a result of these surveys, many *Bursaphelenchus* species isolated and reported from stressed, dying or newly dead conifer trees. In Turkey, several *Bursaphelenchus* species were found to be associated with dead or wilted conifer trees, but no records were available about insect vectors of these *Bursaphelenchus* species. For this purpose, several studies have been started in conifer forests in the Aegean and the Marmara regions of Turkey. In these studies, five trap trees, free from *Bursaphelenchus* species, were selected. These trees were cut and laid down in the same place to attract possible insect vector of *Bursaphelenchus* species reported from previous studies in the same regions. The trap trees were kept in the field between March and September to obtain oviposition of potential vector species in 2012 and 2013. The trap trees were checked periodically for insect and nematode presence. The wood chip samples were taken from each trap trees and controlled for the presence of *Bursaphelenchus spp.* In the lab. When the samples were positive for presence of *Bursaphelenchus* species, several log samples were taken from the trap trees. These logs were kept under constant conditions ($25\pm^{0}$C, 60-70 % RH) during the development of insects. *Orthotomicus erosus* and *Ips sexdentatus* emerged from *B. sexdentati* isolated *Pinus brutia* logs, *Monochamus galloprovincialis* emerged from *B. mucronatus* isolated *P. brutia* logs, *O. erosus* and *Acanthocinus griseus* emerged from *B. vallesianus* isolated *P. brutia* logs and *Pityokteines curvidens* and *Rhagium inquisitor* emerged from *B. hellenicus* isolated *Abies cilicica* logs.

Key words: Vector species, *Bursaphelenchus spp.*, Conifer

Session 2:

Systematics and Diagnostics

Anthoine G, Chappé AM, Validation of a real time PCR assay for the detection of *Bursaphelenchus xylophilus* in targeted matrices in the framework of national survey. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 31-32, Braunschweig, ISSN: 1866-590X

Validation of a real time PCR assay for the detection of *Bursaphelenchus xylophilus* in targeted matrices in the framework of national survey

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Surveys on *Bursaphelenchus xylophilus* need detection methods that are reliable, sensitive, that allow high throughput analysis and can be applied on any matrix tested, such as wood material or insect for a direct detection.

Many molecular tests, especially real time PCR tests, were recently published but none of them was fully validated to fulfill all the previous requirements.

From a selection of three real time PCR assays, François *et al*. (2007), Leal *et al*. (2007), Cao *et al*. (2005), a validation process was designed based on EPPO recommendation PM7/98 (EPPO, 2010) and applied for the evaluation of the following performance criteria: sensitivity, specificity, repeatability, reproducibility or robustness.

The three real time PCR assays tested proved to be very sensitive as they all detect one individual of *B. xylophilus*, even if the reaction profile is clearly different from one assay to another probably due to the target gene. They also are repeatable and reproducible.

The specificity of the three tests is different especially when analysing wood: some tests gave false positive results with routine wood samples. With some tests, false positives were obtained from non target Bursaphelenchus species, especially in case of large amount of DNA, with Ct values that could lead to confusion with *B. xylophilus*.

Based on its performance, the real-time PCR assay developed by François *et al*. (2007) was considered as the most adapted for our routine use. So, this test was coupled with universal primers and probe as internal control, targeting 18S gene which is present in plant and insects cells (Ioos *et al*., 2009). The performance of the *B. xylophilus* specific assay was not affected by the addition of this universal primer set, whatever the sample, wood or insect.This duplex real time PCR enabled the detection of one single *B. xylophilus*.

Consequently, the detection scheme applied for French national survey includes a first step of screening using real time PCR assay from François *et al*. (2007) for wood and insect samples. If any positive result is obtained for wood, it would be confirmed by morphological analysis and complementary molecular approach on a compulsory basis. For insect sample, any positive detection would lead to further investigations on site.

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Molecular detection of *B. xylophilus* in complex DNA backgrounds

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Dozens of *Bursaphelenchus* species are associated with pine trees (*Pinus spp.*), most of them being harmless, as they feed exclusively on fungi associated with stressed or dying pine trees. *Bursaphelenchus xylophilus* is exceptional as it is a facultative, mostly lethal parasite of vital pine trees. Morphological identification of *Bursaphelenchus* species is predominantly based on spicule characters and depends on the availability of adult males. Invariably, microscopic identification of *B. xylophilus* in nematode suspensions is a time-consuming specialist job, and this largely limits sample throughput.

To control the spreading of *B. xylophilus* through Europe, fast and high throughput detection tests are required. DNA-based screening assays allow for the sensitive screening of large numbers of nematode samples of any kind, and do not depend on the developmental stage of the nematodes under investigation. Several diagnostic methods for molecular detection of *B. xylophilus* have been published. Some of them are designed against a framework with representatives from most *Bursaphelenchus* groups (*e.g.* Burgermeister *et al.* 2009) but are laborious as they include enzymatic amplicon digestion followed by gel-based fragment analysis. On the other hand, a relatively fast satellite DNA-based TaqMan assay has been developed with verified contrast against a limited number (n=10) of congeneric species (Francois *et al.* 2007).

On the basis of a framework of ~ 2,800 full length nematode SSU rDNA sequences (Van Megen *et al.* 2009), we have developed a new molecular assay for the quantitative detection of *B. xylophilus*. Using this framework, we identified unique DNA motifs that enable 'blind' identification of *B. xylophilus* in complex DNA backgrounds. This *B. xylophilus* specific test was developed using 35 SSU rDNA sequences of *B. xylophilus* and 113 SSU rDNA sequences of 44 non-target *Bursaphelenchus* species (including 13 sequences of *B. mucronates*). SYBR Green-based detection assays (similar to Rybarczyk *et al.* 2012) allow for reliable and cost-effective molecular screening of large numbers of

nematode samples in any (inspection) laboratory. Results will be presented on the validation of this new test and include a comparison with the performance of the *B. xylophilus* test (Francois *et al.* 2007) from the EPPO Standard PM 7/4 (3).

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Pinewood Nematode Assay and Development of Real-Time PCR for Species Identification in North Carolina Department of Agriculture & Consumer Services

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Bursaphelenchus xylophilus, the pinewood nematode (PWN), is the causal agent of pine wilt disease, one of the most damaging emerging pest problems to forests around the world. PWN was introduced in Japan at the beginning of the 20th century (Yano 1913) and later in mainland China (Cheng *et al*. 1983), Taiwan (Chang & Lu 1996) and Korea (Yi *et al*. 1989), causing massive mortality of native pine trees. PWN was first recorded in Europe (Portugal) in 1999 (Mota *et al*. 1999); later on the Portuguese island of Madeira, 900 km SW of the European continent in 2010 (Fonseca *et al*. 2012); and more recently in three locations in Spain close to the Portuguese border (Robertson *et al*. 2011). The international spread of PWN occurs mainly through the movement of infested logs, untreated wood products and wood-packaging material. It is native to North America where it causes relatively minor damage to native conifers but is labeled an EPPO-A-2 pest and a quarantine nematode for many countries outside of the United States because of its potential for destruction to their native conifers. Exports of wood logs and commodities involving softwood packaging materials now require a lab test for the presence/absence of this regulated nematode species.

The Agronomic Division of the N.C. Department of Agriculture & Consumer Services operates a high-throughput and publicly operated nematode assay lab. Recently, due to more strict regulations on PWN, a large number of pine-wood samples were submitted to our lab (Table 1). In fiscal year 2013, 3,934 pine-wood samples were analyzed and 233 reports were generated for USDA/APHIS/PPQ in connection with the issuing of phytosanitary certificates for exported pine-wood logs to China; this workload represented a more than six-fold increase over the previous year. Although in the first two

months of fiscal year 2014, NCDA&CS assayed 1,139 samples for pinewood nematode—accounting for 55% of the sample total to date, July and August are during our non-busy season in receiving samples. PWN prevalence in pine-wood samples was 0.82%, 0.89% and 4.48% for fiscal year 2012, fiscal year 2013 and the first two months of fiscal year 2014, respectively. These results indicate the low presence of PWN in exported pine-wood logs in the USA and the importance of regulatory measures and laboratory testing 0.82% 0.89% 4.76%

Identification of these species using traditional morphology requires a high level of expertise and can be very time-consuming and inconclusive. However, rapid and accurate identification of PWN is required in order to comply with quarantine regulations and to prevent its movement between countries. Molecular diagnosis is potentially simple, rapid, sensitive and reliable and can be used with high precision to determine the presence of PWN in wood. We characterized the DNA sequences of the ribosomal DNA small subunit, large subunit D2/D3, internal transcribed spacer and mitochondrial DNA cytochrome oxidase subunit one of a large collection of Aphelenchid species. This allowed the development of a real-time-PCR method by either simplex (Figure 1) or duplex (Figure 2) for rapid and accurate identification of PWN targeting the ITS-1. A total of 97 nematode populations were used to evaluate the specificity and sensitivity of this assay, including 45 populations of *B. xylophilus* and 36 populations of 21 other species of *Bursaphelenchus*, which belong to the *abietinus*, *cocophilus*, *eggersi*, *fungivorus*, *hofmanni*, *kevini*, *leoni*, *sexdentati* and *xylophilus* groups and one unassigned group from a total of 13 groups in the genus *Bursaphelenchus*; 15 populations of *Aphelenchoides besseyi*, *A. fragariae*, *A.* spp. and *Aphelenchus avenae*; and one population of mixed nematode species from a soil sample. This assay proved to be specific to *B. xylophilus* only and was sensitive to a single nematode specimen regardless of the life stages present. This approach provides the rapid species identification necessary to comply with the zero-tolerance export regulations.

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Table 1. Pinewood nematode assay summary in NCDA&CS

	FY2012 (7/1/11-6/30/12)	FY2013 (7/1/12-6/30/13)	FY2014 (7/1/13-8/31/13)	Total
Total pine-wood samples	613	3934	1139	5686
Total pine-wood reports	31	234	80	344
Positive PWN samples	5	35	51	91
Positive PWN reports	3	16	16	35
Yearly nematode samples	34129	35012	2090	71231
Yearly nematode reports	4606	4744	279	9629
Pine-wood-sample percentage	1.80%	11.29%	54.50%	7.98%
Pine-wood-report percentage	0.67%	4.97%	28.67%	3.57%
PWN prevalence in pine-wood samples	0.82%	0.89%	4.48%	1.60%

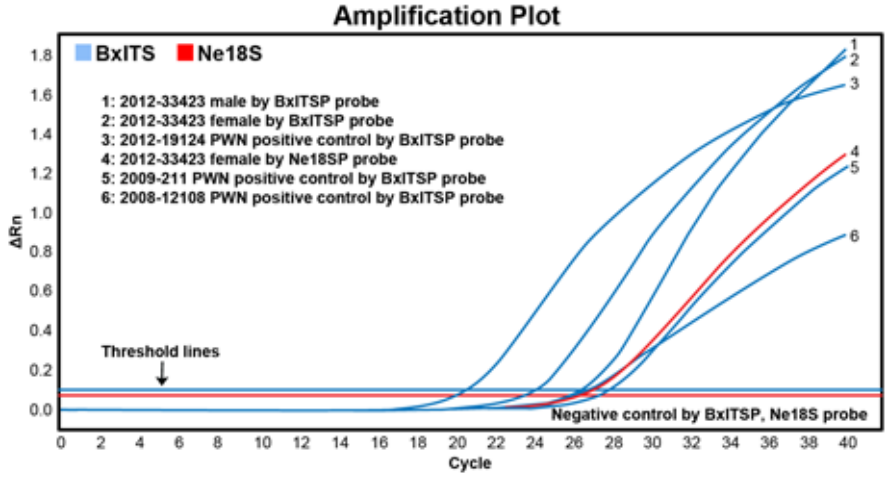

Figure 1. Example of a real-time-PCR result for testing sample 2013-33423 by Pinewood-nematode -specific and nematode-universal primer/probes.

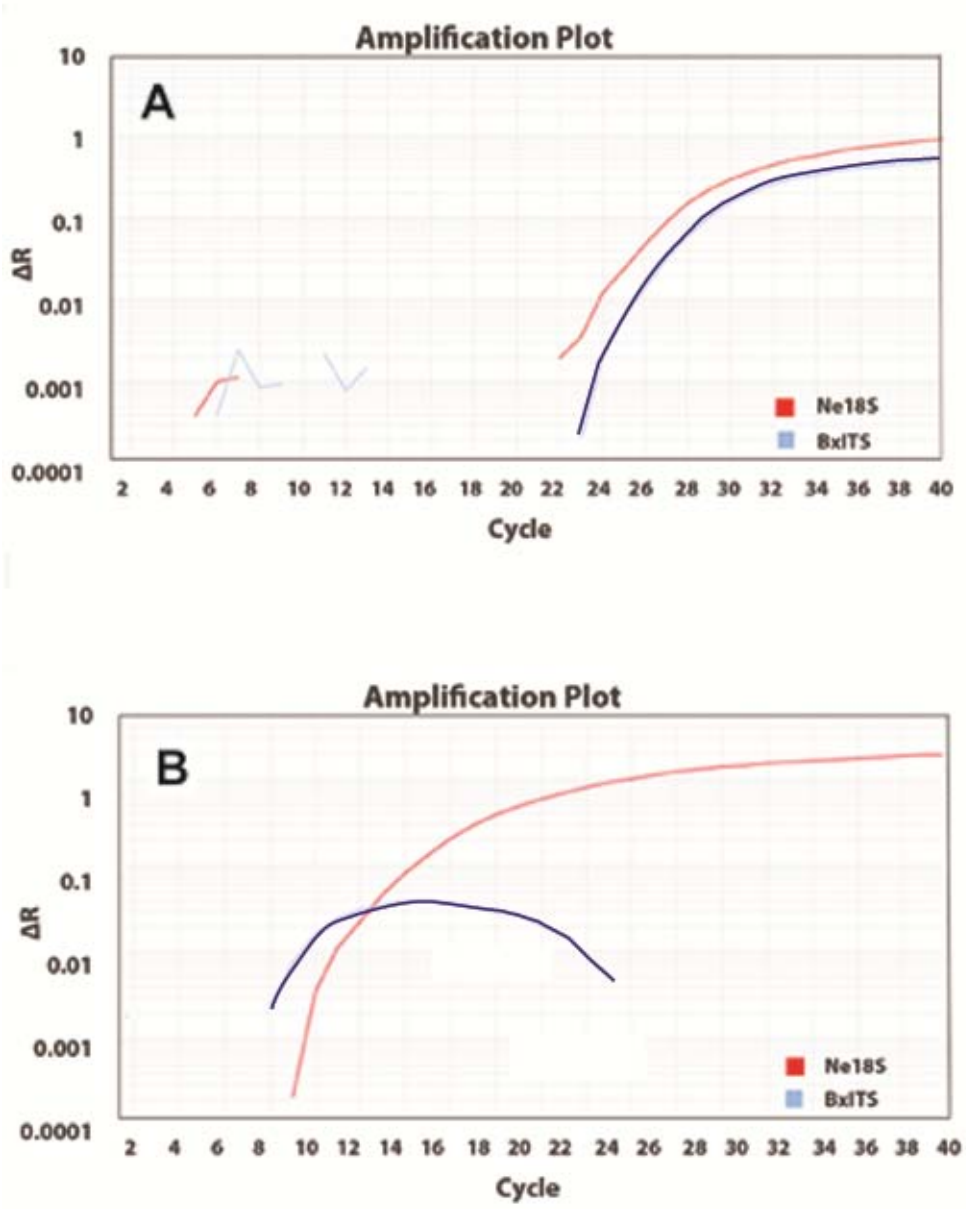

Figure 2. Duplex r eal-time-PCR r esult. A. A pos itive r esult w ith two sigmoid FAM (Pinewood-nematode-specific) a nd HEX (nematode-universal) curves in a mplification plot. B. A negative result with one sigmoid HEX curve, and nonincreased FAM curve.

Leal I, et al., Development of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method to detect living pinewood nematode, *Bursaphelenchus xylophilus*, in wood. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 39-40, Braunschweig, ISSN: 1866-590X

Development of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method to detect living pinewood nematode, *Bursaphelenchus xylophilus*, in wood.

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Current molecular techniques for the detection of PWN rely on the presence of genomic DNA and thus cannot differentiate between living and dead PWN. The detection of dead nematodes could lead to unnecessary trade disruption. We have developed a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay, which specifically identifies living PWN in wood by detecting the presence of expansin mRNA as a viability marker. This diagnostic method was found to be more sensitive, faster, more cost-effective, and allows for simpler visual detection as compared to PCR. We chose an expansin gene, because it had been sequenced for both PWN and its closest related species, *B. mucronatus,* (Kikuchi et al., 2009), and because it contains an intron, which is present only in genomic DNA. In order for an RT-LAMP method to be a reliable indicator of viability, it was important to ascertain that only cDNA transcribed from mRNA was amplified, and to eliminate the possibility that any genomic DNA (gDNA) could be amplified. We designed 6 LAMP primers that recognize 8 distinct regions in the target sequence (Notomi *et al.*, 2000). One of the primers was positioned at an exon-exon junction of the expansin gene, so that genomic DNA could not be amplified. When testing gDNA samples and cDNA from different *Bursaphelenchus* species, we found exclusive amplification of cDNA from PWN. Positive samples were detected with HNB (hydroxynaphthol blue) by a change of colour from violet to blue (Goto *et al*., 2009). The sensitivity of the RT-LAMP diagnostic to detect living PWN was higher than that obtained by a conventional PCR diagnostic method, and similar to a real time RT-PCR

assay. We modified an RNA extraction protocol (Chomczynski and Sacchi, 1986) in order to improve extraction quality from wood. We have optimized the RT-LAMP assay not only on nematodes from pure isolate cultures, but also on samples directly isolated from 4 g of PWN-infected wood. This assay was used to test the presence/absence of living PWN in wood that had been heat treated according to ISPM 15 (FAO 2009). From the results obtained, we found that all heat-treated wood samples were free of living PWN, and thus the heat treatment applied to these wood samples was an effective treatment to kill PWN. We will be using this method to verify the efficacy of other wood treatment such as sulfuryl fluoride and phosphine**.** This method will help resolve disputes over the detection of PWN by clarifying whether any PWN present in wood is alive or dead. It can also be used to evaluate the efficiency of wood treatment procedures.

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EUPHRESCO BURSA project: early detection methods of the pinewood nematode

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EUPHRESCO is an EU-funded ERA-NET (Coordination Action) which aims to increase cooperation and coordination of national phytosanitary (statutory plant health) research programmes at the EU level through networking of research funding activities.

EUPHRESCO-1 was funded by the EU 6th Framework Programme (FP6) from 2006-2010.

EUPHRESCO-2 is funded from the EU 7th Framework Programme (FP7) from 2011-2013. It is composed of 31 partners in 22 countries with 12 European Observer countries and 2 international Observers. Its partners are leading organisations involved with funding phytosanitary research in Europe.

EUPHRESCO aims to continue as a self-sustainable long-term network of European phytosanitary research funders after 2013.

The Eranet Euphresco initiates scientfic project on regulated or emerging pest.

In this regard, a specific project is about to start on *Bursaphelenchus xylophilus* and early methods of detection. The project would aim at validating published detection methods that could be used on many matrices and that could avoid nematodes extraction as such.

The PWD conference will be the best opportunity for a kick off meeting of this project including discussion with scientists and especially those involved in REPHRAME. This discussion would allow to avoid duplication of work.

Session 3:

PWN in International Trade, Pathways and Phytosanitary Treatments

Prospero S, Polomski J, Rigling D, Occurrence and distribution of *Bursaphelenchus* species in Switzerland, In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 43, Braunschweig, ISSN: 1866-590X

Occurrence and distribution of *Bursaphelenchus* species in Switzerland

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In Switzerland, pine forests cover about 43000 hectares and are dominated by Scots pine (*P. sylvestris)*. Most of these forests are located in the Alps where they protect human infrastructures against the impacts of natural hazards. Here, we present the results of a nation-wide survey on the occurrence of *Bursaphelenchus* species that was conducted between 2009 and 2011 in Swiss pine forests, including sites in proximity of risk areas (e.g. airport and sawmills), and in pine bark and solid wood packing material. In the pine forests, a total of eight *Bursaphelenchus* species were identified by morphological and molecular methods. The most frequent species were *B. vallesianus*, followed by *B. sexdentati*, *B. leoni*, *B. eggersi*, and *B. mucronatus*. *B. borealis*, *B. pinophilus*, and *B. polygraphi* were only rarely found. Although most species can probably be considered as saprotrophs, *B. vallesianus* and *B. mucronatus* may be involved in pine dieback observed in some areas. Five of these eight *Bursaphelenchus* species (including *B. mucronatus*) were also isolated from symptomatic pines at risk areas. In the bark of the sampled trees *Bursaphelenchus* nematodes were practically absent. The quarantine species *B. xylophilus* was only detected in imported pine bark from Portugal. This survey shows that *B. xylophilus* is not present in pine forests in Switzerland. However, the recovery of the closely related species *B. mucronatus* suggests that local climatic and ecologic conditions may be suitable for the establishment of *B. xylophilus*.

Fonseca L et al., Coniferous bark hot steam treatment for the elimination of the pinewood nematode. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 44-45, Braunschweig, ISSN: 1866-590X

Coniferous bark hot steam treatment for the elimination of the pinewood nematode

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ABSTRACT

In order to develop an artificial heat treatment to eliminate the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, from coniferous bark, an industrial equipment, based on hot steam was build up which enables continuous bark treatment for more than 30 min with temperatures above 80ºC. Biological assays were performed using experimental units (bags) with *Pinus pinaster* bark and wood chips containing more than 100 000 PWN (>60% third dispersal juvenile stage). The bags were heat treated for 30 min and the temperature inside monitored by temperature probes. The total number of live nematodes was quantified immediately after treatment and after incubation (25ºC for 15 days) and in both situations no nematodes were detected revealing efficacy in eliminating PWN from coniferous bark.

INTRODUCTION

The Food and Agriculture Organization of the United Nations, through the International Plant Protection Convention, adopted the International Standard for Phytosanitary Measures No. 15 (ISPM Nº 15) which defines guidelines for the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, elimination from wood products by heat treatment with a minimum temperature of 56ºC for 30 min (FAO, 2009). Since November 2012, pine bark that is traded from Portugal to other countries has to be heat treated by hot steam. This treatment is being used in six Portuguese companies and enables continuous bark treatment for more than 30 min with temperatures above 80ºC. The hot steam treatment is performed in industrial equipment composed by a bark feeder tank, a bark

inlet, a steam injection chamber and a heat storage chamber monitored by several temperature probes which permit real-time recording and storage of temperature data. In the present study, we have evaluated the efficacy of the hot steam treatment to eliminate PWN.

MATERIALS AND METHODS

In order to evaluate the efficacy of the hot steam treatment to eliminate PWN, biological assays were performed using six experimental units (bags) with bark and wood chips, from PWN infected *Pinus pinaster* trees, containing more than 100 000 PWN (>60% third dispersal juvenile stage) (Magnusson & Schröder 2009). Temperature probes were introduced in each bag. Then, the bags were placed into de hot steam equipment together with bark and heat treated for at leat 30 min. The number of nematodes, present in each bag was estimated before and after the treatment using the tray method (Whitehead & Hemming 1965). After the treatment, the bags were recovered. In three of them, the number of live nematodes was quantified immediately and the other three were incubated at 25ºC for 15 days to allow any live nematode present to breed and maximise the likelihood of detection (EPPO 2013). After the incubation period, the number of live nematodes was also quantified.

RESULTS

The temperature inside the bags exceeded the recommended by the ISPM Nº15 being higher than 80ºC. In both situations, after treatment and after incubation period, no nematodes were detected. The results confirmed that the continuous hot steam system go beyond the ISPM Nº15 and revealed effective in eliminating PWN from coniferous bark.

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Non vector spread of *Bursaphelenchus xylophilus* via wood chips

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ABSTRACT

The transmission of *Bursaphelenchus xylophilus* (PWN) to new host trees depends on its vector beetles belonging to the genus *Monochamus*. Nevertheless since the first interception of PWN in wood chips in the European Union in 1984 discussions on non vector transmission of PWN from wood chips to healthy trees takes place. Currently increasing interest in importing wood chips from North America for energy purpose or paper production can be observed. Within the EU research project REPHRAME we investigated under laboratory conditions using *Pinus sylvestris* saplings, whether PWN can spread from artificially infested *Pinus sylvestris* wood chips to pine trees.

MATERIALS AND METHODS

For this purpose pine logs (eight cm average diameter) were inoculated with PWN and incubated at 25 °C for 24 days. After stripping of the bark logs were processed to wood chips using a laboratory wood mill with a maximum size of 10 x 20 x 4 mm. At test start 100 g wood chips each were placed in three liter pots of 3-4 years old *Pinus sylvestris* saplings. Test temperatures were 15 °C and 25 °C respectively. Different combinations of tree conditions and wood chips were investigated. Except for the variant "healthy trees" the pines were either wounded on the stem, the roots or were cut above the root collar. The wood chips were mixed in the soil or were placed on the soil with direct contact or in a distance to the stem. The control variant did not include wood chips. In total 12 tree-wood chip combinations were investigated for each temperature. Four variants without wood chips but with different tree conditions served as controls. During 12 weeks the trees were evaluated concerning their development of wilt symptoms. Six wilt classes (0 to 5 = no wilt symptoms to death of the tree) were used for assessing the physiological condition of the trees. In this time trees with more than 75 % wilting needles but still alive (wilt class 4) were sampled for nematode extraction using the modified Baermann funnel

method. After 12 weeks all remaining trees were also sampled for nematodes irrespectively of their wilt class.

RESULTS

At 25 °C more saplings developed higher wilt classes and found to be PWN infested compared to the 15 °C variant. Stem injured pines with direct wood chip contact and root injured pines combined with wood chips in the soil at 25 °C showed the majority of trees with wilt class 4. PWN could be extracted from 47 of all 480 non-control trees. At 15 °C three trees in the variant with chips directly attached to stem wounded trees were affected by PWN. At 25 °C seven of 12 tree-wood chip-combinations with in total 44 trees showed PWN infestation.

CONCLUSIONS

The results of the current investigation indicate the possibility of non vector spread of PWN with wood chips to trees under laboratory conditions. The temperature, tree condition and wood chip location are influencing factors for this infestation pathway. The results need to be evaluated under outdoor conditions. For Pest Risk Assessment the changing end-use of wood chips as well as the increasing amounts in international wood chip trade needs to be considered.

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Bonifácio L, Inácio M L, Sousa E, Buckley S, Thoms E M, Complementary studies to validate the proposed fumigation schedules of sulfuryl fluoride for inclusion in ISPM No. 15 for the eradication of *Bursaphelenchus xylophilus* from wood packaging material, In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 48-50, Braunschweig, ISSN: 1866-590X

Complementary studies to validate the proposed fumigation schedules of sulfuryl fluoride for inclusion in ISPM No. 15 for the eradication of *Bursaphelenchus xylophilus* from wood packaging material

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INTRODUCTION

Sulfuryl fluoride (SF), a broad-spectrum fumigant, is under evaluation for inclusion in standard ISPM No. 15 - Guidelines for Regulating Wood Packaging Material in International Trade. The Technical Panel on Phytosanitary Treatments (TPPT) has evaluated the efficacy data submitted on SF against a range of insect species and pine wood nematode (*Bursaphelenchus xylophilus*) [PWN] to support the fumigant inclusion in the Standard. Following their evaluation, the TPPT considered that there were no further requirements for insects; however, additional information on PWN was requested. Several efficacy studies of SF on PWN were conducted and submitted: (Dwinell *et al*. 2005); (Flack *et al*. 2008) and (Bonifácio *et al.* 2013) but additional information on efficacy within 18 -29.9°C was requested. A new study was completed on PWN in Portugal in 2013 to validate a proposed treatment schedule for that temperature range.

MATERIALS AND METHODS

Boards of 45cm x 10cm x 5cm were prepared from PWN-contaminated pine trees (*Pinus pinaster*) felled in Portugal, where this pest is now naturalized. The boards were maintained in incubation chamber at 25°C 75 % rh to provide optimal conditions for reproduction of PWN. Boards were then placed in 1 m^3 chamber and fumigated with

commercial grade ProFume® gas fumigant (99.8% sulfuryl fluoride, Dow AgroSciences, Indianpolis, IN USA). A range of SF dosages and two exposure times were tested at 20°C as follows: 2,514-

4,263 g-h/m^3 at 24 h exposure and 2,459-3,216 g-h/m^3 at 48 h exposure. Each fumigation was monitored for temperature with a thermocouple and for SF concentration with an IR-specific monitoring device. Once target exposure time was achieved (24 or 48 h), chambers were aerated and wood boards placed in incubation chamber before counting PWN. Representative samples from both ends of boards were prepared by cutting them into wood cubes of ca. 1 cm^3. Live PWN were immediately extracted by total immersion of the wood cubes in water for 48 h, then sieving the water through a 38µm sieve to identify and count the nematodes under a microscope (Penas *et al.* 2002). Complementary genomic DNA identification of extracted nematodes was performed when no adult nematodes were found.

RESULTS AND CONCLUSION

The study conditions created a demanding, worst case scenario for PWN infestation. Initial populations of PWN ranged from 237 to 331 individuals per gram of wood, exceeding 3 million individuals per treatment, and further increased in the controls after fumigation of the treated samples. Infested wood contained a high proportion (53-90%) of the J_{III} juvenile dispersal stage. Wood moisture content was 43-61.4% before fumigation and decreased to 15.7-19.4% 21 days after fumigation. At 3 days after application, 100% control of the J_{III} juvenile dispersal stage was achieved with all SF dosages and exposure times. All dosages tested at 48 h exposure, compared to similar dosages at 24 h exposure, achieved better nematode control; 99.971-100% versus 99.617-99.998%, respectively. Survivors for 24 h exposure were mainly young larvae (J_2, J_3 stages), which would support the assumption that only eggs survived the SF treatment at this exposure. At 21 days after application, nematode control was 99.852-99.999% at 24 h exposure and 99.991-100% at 48 h exposure.

The dosage of 3,000 g-h/m^3 in 48 h achieved 99.999% to 100% control at T_3 and T_{21} and was selected for fumigation at 20°C-29.9°C. As a general rule, SF fumigant dosage decreases as temperature increases. A previous study verified this observation on PWN at 20 and 25°C (Flack *et al.* 2008). New proposed SF fumigation schedules, using the SF schedules developed in 2010 (Bonifácio *et al.* 2013) for temperatures below 20°C (3,200 g-h/m^3 with 24 h exposure) and above 30°C (1,400 g-h/m^3 with 24 h exposure) and adding 3,000 g-h/m^3 with 48 h exposure for 20ºC-29.9°C, were submitted to TPPT.

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Efficacy of kiln drying as phytosanitary treatment against wood borne nematodes

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INTRODUCTION

Kiln drying (KD) often is considered equivalent to a phytosanitary treatment, because it is believed that the reduction of the wood moisture content (MC) will inhibt the growth of harmful organisms and kill them. KD with a wood moisture content reduction to less than 20 % can be achieved using a wide range of process parameters normally defined in a drying schedule. In contrary to a phytosanitary measure the main aim of a KD process is moisture content reduction. Therefore, usually, a minimum drying temperature to be used is not defined – e.g. the EU quarantine legislation only refers to an "appropriate time/temperature schedule" (EU 2000). Therefore the well known lethal temperature (56°C for 30 minutes) is not in all cases reached in kiln drying operations.

Within the EUPHRESCO model project PEKID the influence of low temperature KD treatments on the survival of *Bursaphelenchus xylophilus* and *B. mucronatus* was investigated in comparison to KD treatments which included standard heat treatment conditions as described in ISPM No. 15 (FAO 2013).

MATERIALS AND METHODS

In a first step pre-trials concerning artificial infestion of pine wood with *B. xylophilus* and *B. mucronatus* as well as laboratory KD treatments were conducted. In a second step pilot-scale KD treatments were carried out to investigate the efficacy of KD with respect to phytisanitary aspects. Freshly cut logs (*Pinus sylvestris* mean diameter 29 cm and length 100 cm) were inoculated with *B. mucronatus*. After 59 days incubation time, 4 cm thick boards with a length of 100 cm and widths between 9 and 20 cm were sawn with a mobile band saw. Boards were stacked with 2.5 cm stickers to a final stack of 0.8 m x 1.0 m x 1.0 m (WxHxL). Each drying/phytosanitary treatment was carried out in a small pilot-scale kiln by using the following drying parameters (temperature kiln (T_{air}); equilibrium moisture content (EMC)), to reach the target wood moisture content (MC_{tg}) of 20 %:

1. Low temperature KD treatment: T_{air} = 35 °C and EMC = 13%,
2. KD treatment simulating conditions in a condensation kiln: T_{air} = 35 °C and EMC = 13 % until fibre saturation point (FSP) is reached followed by T_{air} = 50 °C until MC = < 20 %,
3. Low temperature pre-drying plus ISPM 15 treatment: T_{air} = 35 °C and EMC = 13 % until FSP is reached followed by T_{air} = 60 °C until 56 °C core temperature is reached for 30 minutes
4. KD treatment with parameters satisfying ISPM 15 requirments: T_{air} = 35 °C and EMC = 13 % until FSP is reached, T_{air} = 60 °C until MC =< 20 %

RESULTS

1. *B. mucronatus* survives a low temperature KD treatment (MC < 20 %) using a drying temperature of 35 °C.
2. *B. mucronatus* was effectively killed on Probit 9 level with a KD treatment (MC < 20 %) using a treatment temperature of 50 °C.
3. *B. mucronatus* does not survive a KD treatment (MC < 20 %) using a treatment temperature of 60 °C.
4. *B. mucronatus* does not survive an ISPM 15 treatment (56°C core temperature for 30 minutes, without drying the wood) using a treatment temperature of 60 °C.

CONCLUSIONS

A stand alone KD treatment using drying parameters that do not include threshold conditions (e.g. 56°C for 30 minutes throughout the whole wood profile [FAO 2013]), which are lethal to harmful organisms such as *B. xylophilus,* are not suitable to be used as phytosanitary treatment. Import regulations referring to KD therefore need to specify the minimum temperature as well as treatment times to make sure that phytosanitary requirements are met.

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Session 4:

PWN Interactions with Bacteria

Kulinich et al., Recent Research on Pine Wilt Disease in Russia, In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 54-55, Braunschweig, ISSN: 1866-590X

Recent Research on Pine Wilt Disease in Russia

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In early 1990 we initiated a survey in Russian conifer forests to determine if the pine wood nematode (PWN), *Bursaphelenchus xylophilus* occurred in Russia. Later, in 2010-2011 larger, more widely-distributed surveys were conducted for the PWN in conifer forests and in stored lumber in eleven regions of Russia. Based on the results of the surveys *B. xylophilus* has been not found in Russia, however, the closely related nematode species *B. mucronatus* has been found. Specificially in the 3718 samples analyzed *B. mucronatus* was found in 11.5% of the samples in 2010 and 5.6% in 2011. Inoclation experiments done in Russia with *B. mucronatus* isolates showed that sometimes these isolates killed pine (*Pinus sylvestris*) and larch (*Larix* olgensis) seedlings. Also recent research done in China and South Korea showed that PWD of conifers can occur following inoculations done using a complex of pathogenic bacteria and the PWN of *B.xylophilus* that carried them (Zhao *et al*. 2009; Kwon *et al*., 2010; Wu *et al*. 2013). As well, it has been shown that *B. mucronatus* species carry such bacteria and perhaps some *B. mucronatus* populations can vector such pathogenic bacteria. During our 2010-2012 survey twenty six isolates of the wood-inhabiting nematode *B. mucronatus* were extracted and propagated *in vitro* to determine, using sequencing techniques, the identity of the associated symbiotic bacteria..

Twenty species of bacteria belonging to the families Enterobacteriacea, Xanthomonadaceae, Pseudomonadaceae, Burkholderiaceae, Rhizobiaceae, Nocardiaceae, Flavobacteriaceae, Bacillaceae, Paenibacillaceae were isolated from the nematodes and identified as belonging to the genera *Achromobacter, Bacillus, Burkholderia, Enterobacter, Flavobacterium, Klebsiella, Pseudomonas, Rahnella, Rhodococcus, Stenotrophomonas, Pantoea, Paenibacillus,* and *Serratia* (Fig 1). The most frequently encountered bacterium belonged to the genus *Pseudomonas* (44%). Five species of this genus were identified: *P. lurida, P. brenneri, P. geniculata, P. fluorescens, Pseudomonas* sp. The bacterium *Pseudomonas fluorescens* was isolated from nine *B. mucronatus* isolates from the different regions. Two bacteria-associated species were found on the

dauerlarva stage of *B. mucronatus* nematodes were isolated from beetles identified as *Monochamus urussovi* Fisch. Four species of bacteria including *Pseudomonas fluorescens* species were isolated from the larva stage of a *B. mucronatus* isolate.

According to the results of Chinese researchers, *P. fluorescens* is an essential species of the nematode-bacterial complex that induces PWD in the pine forests of southern China (Zhao, 2008). It is assumed that *B. mucronatus* nematodes and symbiotic bacteria *P. fluorescens* can cause death of some Russian pine forests in areas where the mean air temperature during the summer months excedes 25^0C. The average monthly temperature in the Centre of European Russian in 2010 was 26.4 ° C in July and 25.5 ° C in August. Widespread death of *Pinus sylvestris* occurred there after 2010. *Bursaphelenchus mucronatus* nematodes and the symbiotic bacterium *Pseudomonas fluorescens* were isolated from some dead trees. Local foresters believe that the death of these trees was caused by drought, but we do not exclude the possibility that that PWD played some role in the death of these trees..

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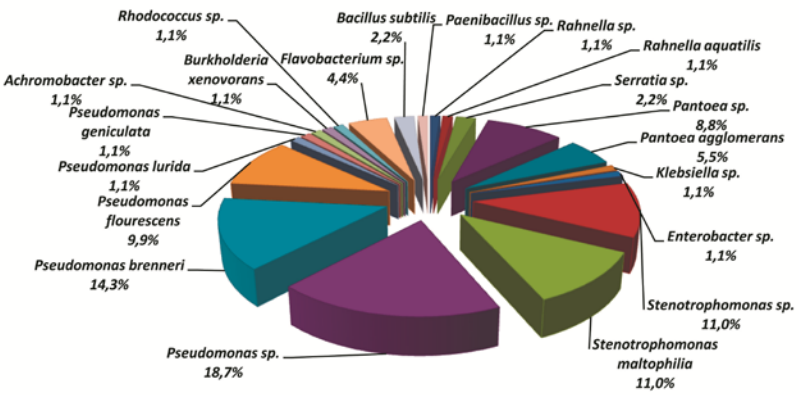

Fig 1. Symbiotic bacteria extracted from *Bursaphelenchus mucronatus* isolates from different regions of Russia

Tan J J, Qu H Y, Hao D J, Chen F M, Inoculation effects of *Pinus thunbergii* with *Bursaphelenchus xylophilus* and two strains of *Bacillus firmus*. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 56-57, Braunschweig, ISSN: 1866-590X

Inoculation Effects of *Pinus thunbergii* with *Bursaphelenchus xylophilus* and two strains of *Bacillus firmus*

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ABSTRACT

To clarify the role of bacterium in the pathogenesis of pine wilt disease, 3 years old Japanese black pine (*Pinus thunbergii*) were inoculated with disinfected *Bursaphelenchus xylophilus* (Bx), bacterium isolate GD1 of *Bacillus firmus* (isolated from the body of Bx)、GD2 of *B. firmus* (isolated from the healthy *P. massoniana*) and the mixture of the nematode and bacterium. The results showed that the pine seedlings were diseased when inoculated with the disinfected Bx and the mixture of Bx and bacterium, while not diseased when inoculated with *B. firmus* singly. Disease development of the pine seedlings was slower after inoculated with Bx singly than with the mixture of Bx and bacterium. The disease of pine seedlings was heavier when the inoculation concentration of bacterium was higher. After inoculated with Bx singly and the mixture of Bx and bacterium, the pith of pine seedlings browned, the process of pith browning was from lower part to upper part of the inoculated maim stem, while the pith of pine seedlings was normal after inoculated with *B. firmus* singly and the control. At early stage after inoculation with the mixture of Bx and *B. firmus*, the number of bacterium detected in the pine seedlings was larger. Therefore, it was concluded that the two bacterium strains enhanced the disease development of pine wilt disease.

Key words: *Pinus thunbergii*; *Bursaphelenchus xylophilus*; Bacterium; *Bacillus firmus*

INTRODUCTION

Up to now, the pathogenic mechanism of pine wilt disease keeps obscure. The role of bacterium in pathogenesis of the disease is still unclear (Oku *et al* 1980; Kawazu & Kaneko 1997; Zhao & Guo 2004; Zhu et al 2012).

MATERIALS AND METHODS

3 years old Japanese black pine (*P. thunbergii*) were inoculated with disinfected *Bursaphelenchus xylophilus* (Bx), bacterium isolate GD1 of *Bacillus firmus* (isolated from the body of Bx)、GD2 of *B. firmus* (isolated from the healthy *P. massoniana*) and the mixture of the nematode and different concentration of bacterium by bark inoculation method. The inoculation number of Bx and bacterium was 3500, 2.3×10^5CFU, 2.3×10^6CFU and 2.3×10^7CFU per seedling. At 5 days after inoculation, the number of bacterium in the inoculation main stem 3-4 cm above inoculation point was detected.

DISCUSSION

This inoculation expriment was conducted on 3 -year-old Japanese black pine seedlings, its results were similar to that on 1-2 years old excised branch of masson pine (*P. massoniana*) (Tan 2001). The bacterium strain GD2 was isolated from the healthy *P. massoniana*, it belongs to pine endophytic bacterium. The research on the relationship between pine endophytic bacterium and pine wilt disease is being carried out.

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Bursaphelenchus xylophilus and associated bacteria under oxidative stress conditions

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Plant pathogens have evolved a machinery of antioxidant enzymes and detoxyfying systems to reduce the plant oxidative burst impact upon invasion, and allow their successful colonization. Our study aimed to understand the contribution of *Bursaphelenchus xylophilus*-associated bacteria in interaction with the nematode, and as well independently, under oxidative stress conditions in an attempt to mimick their behaviour in the oxidative burst conditions of the host tree in the early stages of pine wilt disease (PWD). Thus, we begin by examining the oxidative stress resistance of three *B. xylophilus*-associated bacteria (*Serratia* spp. LCN-4, LCN-16 and PWN-146) (Vicente et al., 2011 and 2012), and as well *Escherichia coli* OP50 (control strain), in increasing concentrations of hydrogen peroxide (H_2O_2) ranging from 15 to 40 mM in a 24h-exposure period. We could see that all *Serratias* were able to tolerate the strong and prolonged H_2O_2 conditions, in contrast with control *E. coli*. Following, we checked the mortality of two isolates of *B. xylophilus* (virulent Ka4 and avirulent C14-5) in absence and presence of associated-*Serratia* and control strain in the same stressful conditions. Without bacteria (surface sterilized nematode), Ka4 and C14-5 presented significant differences in their ability to tolerate H_2O_2, being Ka4 clearly more resistant than C14-5. With *Serratia* spp., both Ka4 and C14-5 were able to survive at all H_2O_2 concentrations tested, with mortality rates lower than 10 %. In the presence of the *E. coli* OP50, mortality percentage of avirulent C14-5 was higher and closer to the values obtained in nematode alone conditions, with no statistical differences between treatments. These results indicate a beneficial and potential helper effect towards *B. xylophilus*, suggesting that these associated *Serratia* spp. are able to express several antioxidant enzymes and detoxifying systems, which explain their high tolerance to H_2O_2-mediated stress. Next, we focused on *B. xylophilus* catalase transcript levels to target H_2O_2. Two catalases were predicted in the *B. xylophilus* genome, BxyCTL-1 (BUX.s00579.159) and BxyCTL-2 (BUX.s01109.377), with a high protein similarity with other nematode catalases. Relative gene expression of catalase genes of *B. xylophilus* Ka4 and C14-5 in both absence and presence of *Serratia* spp. PWN-146 were studied under stress conditions (24h-exposure to 15mM H_2O_2) and

compared with non-stress condition. Bacterial effect was transversal to virulent and avirulent *B. xylophilus*. Relative gene expression of catalase genes of *B. xylophilus* show that, without bacteria, the virulent isolate K a4, for both non-secreted *Bxyctl-1* and secreted *Bxyctl-2* genes, presented a 1.5-fold difference to avirulent C 14-5. When in interaction with bacteria (*Serratia* spp. PWN-146), both virulent and a virulent *B. xylophilus* catalase levels decreased to levels comparable to normal conditions without oxidative stress, which is also in agreement with mortality test results.

Futher, we explored the bacterial interaction with *B. xylophilus*, namely the attachment to the nematode cuticle, an important characteristic in bacteria dissemination and that, to our knowledge, has not been studied before. We performed co-culturing of *B. xylophilus* and GFP-labelled bacteria in *Botrytis cinerea* plates. We observed that after 24-hour contact with *Serratia* spp. LCN-16, the density of nematode-attached bacteria was sparse, and no GFP fluorescence signal was detected from inside the nematode. From these results, adhesion of these bacteria to the nematode surface and organs seems to be weak and non-specific. Previously, Shinya *et al*. (2010) have shown, through scanning electron microscopy (SEM), the presence of few bacteria on the nematode cuticle even after the nematode was vigorously washed. *B. xylophilus*-associated bacteria are reported to be carried on the nematode's surface, and in average 290 were counted on the cuticle of PWN isolated from diseased trees (Zhao et al., 2003). If bacteria are not attached to the nematode surface, how can they be transported by *B. xylophilus* from and into a pine tree?

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

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Diversity and *in vitro* nematicidal activity of bacteria associated to pinewood nematode

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Bacteria have been suggested to play a role on pine wilt disease since they have been isolated associated with the pinewood nematode. The aim of this work was to evaluate the diversity of the nematode associated bacteria and their potential role in this disease by determining their in vitro nematicidal activity. The bacterial isolates, identified by 16S rRNA gene sequence, belonged to the families Microbacteriaceae, Oxalobacteriaceae, Burkholderiaceae, Enterobacteriaceae, Pseudomonadaceae and Xanthomonadaceae. The most nematicidal strain, Serratia sp. A88copa13, produced proteases in the supernatant.

INTRODUCTION

Bacteria have been suggested to play a role on pine wilt disease (PWD) since they have been found associated with the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. Therefore, PWN isolates from across the globe have been studied in order to understand whether these bacteria can produce toxins that could be involved in the development of PWD (Proença et *al*. 2010). The microbial community associated to PWN was accessed in nematodes from different recently affected areas in Portugal. The aim of this work was to evaluate the diversity of the nematode associated bacteria and their potential role in the PWD by determining their in vitro nematicidal activity.

MATERIALS AND METHODS

The microbial community associated to PWN was assessed isolating the strains on the track of nematodes from infected *Pinus pinaster* trees, from affected areas in Portugal. The bacterial isolates were identified by 16S rRNA gene sequence. Phylogenetic analysis were performed by using ARB software package and type strains from international databases. All isolates were screened for their ability to produce siderophores, lipases and

proteases (Proença et *al*. 2010). Strains were tested against *B. xylophilus* to evaluate their nematicidal activity assessed as the percentage of dead nematodes when incubated with bacteria supernatant during 24 h at 26ºC.

RESULTS

Strains isolated belonged to the families *Microbacteriaceae*, *Oxalobacteriaceae*, *Burkholderiaceae*, *Enterobacteriaceae*, *Pseudomonadaceae* and *Xanthomonadaceae*. Forty-seven strains were tested and 21 strains produced extracellular products with nematicidal activity (Figure 1). The most nematicidal strain, *Serratia* sp. A88copa13, produced proteases in the supernatant. Biological assays revealed differences in nematicidal activity of the proteases to different species of *Bursaphelenchus*.

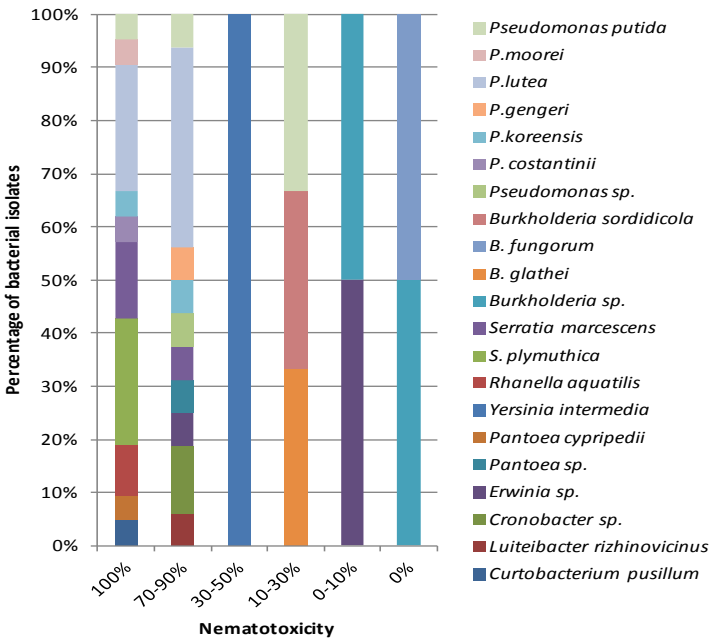

Figure 1 — Biochemical properties and nematicidal ability of bacteria associated with *Bursaphelenchus xylophilus*. Most strains produced siderophores and lipases.

CONCLUSIONS

In Portugal, strains belonging to the families *Enterobacteriaceae* and *Pseudomonadaceae* have be en isolated associated to the P WN a nd s ome ha ve pot ential to e liminate the nematode *in v itro*. In t his pr ocess, pr oteolytic e nzymes a nd l ipases, surfactants a nd possibly s iderophore, pr oduced b y t he ba cteria t o t he e xtracellular m edium, m ay b e involved, being the proteases (metalloproteinases and serine) the most relevant.

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Vicente et al., Natural bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis*. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 63-64, Braunschweig, ISSN: 1866-590X

Natural bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis*

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Most studies of cerambycids microbiota are related with gut-bacterial communities due to their importance in the insect's biology and ecology. Essentially, the research in this field showed that insect microbial communities are limited by specific niche characteristics. The present study investigates the natural bacterial communities of *Monochamus galloprovincialis* collected from Portuguese *Pinus pinaster* trees and *Bursaphelenchus xylophilus*-free, using a metagenomics approach. A total of 492 partial sequences (750-1200bp) of 16S rRNA gene were considered in this study. The rarefaction curves analyses showed 18 OTUs (operational taxonomic units) at genus-species level (95-97% sequence similarity). Bacterial communities associated with *M. galloprovinciallis* are mainly composed by Proteobacteria (78.5%), followed by Firmicutes (20.8%) and Bacteroidetes (<1%). From the phylum Proteobacteria, three classes were present: γ-proteobacteria (87.9%), β-proteobacteria (11.6%), and α-proteobacteria (0.5%). The most abundant genera of Proteobacteria were *Serratia* (76.4%), followed by *Janthinobacterium* (11.6%), *Rahnella* (5.0%), *Pseudomonas* (3.6%), and *Nevskia* (2.1%). Among Firmicutes, the genera found were: *Bacillus* (95%), *Paenibacillus* (3%), *Lactococcus* (1%), and *Lysinibacillus* (1%). From the phylum Bacteroidetes, representatives were identified as *Sphingobacterium* (n=1), *Sediminibacterium* (n=1) and uncultured *Bacteroidetes* (n=2). Our results show a predominance of γ-proteobacteria in *M. galloprovincialis*, which might be intrinsically related with their feeding diet and habitat characteristics. Interestling, a high-density population of *Serratia* spp. was found in *M. galloprovincialis*. The presence of *Serratia* in insects is well documented, both as symbiont or pathogen, and its related with their fitness ability to resist antibacterial substances ingested by the insect, as well as the powerful enzymatic cocktail produced (chitinases, lecithinases, and proteinases) (Grimmont and Grimmont, 2006). Previous culture-dependent studies have also reported isolation of *Serratia marcescens* in *M. alternatus* (Ma *et al*., 2009),

Aerobacter aerogenes and *Bacillus cereus* var. *mycoides* in *M. scutellatus*, *M. notatus* and *M. marmorator* (Soper and Olsen, 1963). Further studies are needed to understand their functional contribution to the bacterial community structure of *M. galloprovincialis*. In light of all knowledge regarding bacterial communities of *B. xylophilus* and the results here presented, it is tempting to establish the hypothesis that perhaps the *B. xylophilus* can harbour bacteria from the insect. An example is the predominance of *Serratia* in *M. galloprovincialis* and also *B. xylophilus* (Vicente *et al*., 2011). Vicente *et al*. (2012) described that some nematode-associated bacteria, including *Serratia* spp., were able to degrade cellulose, an advantage in the adaptation and colonization of wood tissues (Harakava and Gabriel, 2003). Although with this study it's not possible to establish a comparison between insect-vector and *B. xylophilus* bacterial communities, the results presented are useful and encourage future work in this subject (Vicente et al., 2013). Understanding the role of bacteria transmission in the PWD complex will bring important knowledge for future prospects in the disease management and control.

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Session 5:

PWD Management and Contingency Planning

Nakamura et al., A Research Project to Develop Strategic Action Plan in the Pine-wilt-disease Unaffected Area in Northern Japan. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 66, Braunschweig, ISSN: 1866-590X

A Research Project to Develop Strategic Action Plan in the Pine-wilt-disease Unaffected Area in Northern Japan

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Aomori is the northernmost prefecture in Honshu, the main island of Japan, that had not been affected by pine wilt disease (PWD). To develop an action plan to prevent from and be prepared for introduction of PWD into Aomori Prefecture, we tried to acquire critical information related to introduction, colonization and spread of the disease from the view point of presence/absence of the insect vectors and competitive substitution of *Bursaphelenchus* nematodes, as well as the tolerance of tree populations and forest communities to the loss of pine trees resulted form the disease.

Captures of adult *Monochamus alternatus* by attraction traps and genetical identification of the trapped adult using SSR markers indicated that accidental incoming of the vector insect occurred at the south-west border to the neighboring prefecture having severely damaged forests by PWD, but was effectively checked by the 2-km clear-cut zone of pine trees.

According to the whole tree investigations for subcortical insects in 124 dying and newly dead pine trees conducted in various locations in Aomori, it seemed that neither *M. alternatus* nor *B. mucronatus*, as substitutive species for *B. xylophilus*, was distributed in the prefecture.

The old-growth population of *Pinus densiflora* did not compensate the loss of forest canopy caused by PWD, and the broad-leaved trees mixed in *P. densiflora* or *P. thunbergii* dominated forests could not substitute the status and functions of pine trees in the original forests. Thus we concluded that the loss of pine trees by PWD epidemic would bring about severe degradation of the forests in the area.

On the basis of the irreplaceableness of the pine species in the forests, absence of effective vector and substitutive species of *B. xylophilus*, and limited entry route of the disease into the prefecture, we proposed regionally-specialized action plan against PWD in Aomori Prefecture.

Xu, F et al., Study on the techniques of sustainable control of pine wood nematode disease (*Bursaphelenchus xylophilus*). In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 67, Braunschweig, ISSN: 1866-590X

Study on the techniques of sustainable control of pine wood nematode disease (*Bursaphelenchus xylophilus*)

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ABSTRACT

Pine wood nematode (PWN) is a devastating global forest diseases and its spread is still increasing. According to the situation above we carried out research for more than 30 years. By the results we found that the sustainable control of PWN was the effective way to protect our afforestation and ecological security. Both by lab and field tests the tests results showed as following: 1. By use selected GD_5, GX_2 and GX_3 3 *Pinus massoniana* provenances resistant to pine wood nematode (PWN), monitoring their resistibility and large area of afforestation, resistant provenances selection provide technical support for breeding resistant stand. 2. Comprehensive development and utilization of the resources of natural enemy to control *Monochamus alternatus*. The technique developed the mass raising 15 million head of *Dastarcus helophoroides*, *Scleroderma guani* annually, and the technique of combined *D. helophoroides* with *S. guani* releasing to control *M. alternatus* larva were studied. Parasite rate to *M. alternatus* larva were more than 74.1%. Pine mortality rate at the beginning of the 25% fell to below 0.3% which were killed by PWN, has significant control effect in the test field.

KEY WORDS:

resistant provenances, the mass raise natural enemy, combined *Dastarcus helophoroides* with *Scleroderma guani* releasing, to control *Monochamus alternatus* larva, parasite rate, control effect

Naves P, Vieira M, Sousa E, New Strategies for pine wilt disease (PWD) management in Portugal: preventive methods to reduce the spread of the disease to new areas. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 68, Braunschweig, ISSN: 1866-590X

New Strategies for pine wilt disease (PWD) management in Portugal: preventive methods to reduce the spread of the disease to new areas

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Despite the importance of the pine wood nematode *Bursaphelenchus xylophilus*, the casual agent of pine wilt disease, and its insect vector *Monochamus galloprovincialis* in Portugal, there are few available options to control these organisms and to prevent the spread of the disease to new areas. Some new strategies were developed in Portugal: (i) Preventive trunk injection of Emamectin Benzoate (EB), (ii) Preventive dissemination of the disease by application of an insecticide net with alpha-cypermethrin to wood transport. Concerning the first trial (i), trunk-injections with EB were performed in a maritime pine (*Pinus pinaster*) forest in Portugal, testing three dose-rates: 0.032 g a.i./cm diameter at breast height (DBH), 0.064 g a.i./cm DBH and 0.128 g a.i./cm DBH, along with an untreated control plot. EB was successfully injected and translocated in pines, resulting in low mortality for the inoculated trees several months after inoculation, contrasting with much higher mortality of non-treated pines. Emamectin benzoate was successfully recovered in branches of treated pines during a period of more than three years. Concerning the second trial (ii), two studies were performed with the aim of studying the effectiveness and minimum exposure time of the insecticide-net to the vector and to test the net´s efficiency in relation to insects emerging from wood logs during a simulation of a truck transport. Results showed that exposure to the insecticide net proved fatal to *M. galloprovincialis* adults even at very short periods of contact with the net, of just 1 to 5 minutes. These two novel strategies to manage and control wilt disease in Europe offer new possibilities to prevent the spread of wilt disease natural and artificial spread of the disease by the vector.

Session 6:

PWN Biology, Population Dynamics, Epidemiology and Modelling

Tomalak M, Filipiak A, Inter-specific competition of *Bursaphelenchus xylophilus* with native populations of *B. mucronatus* in pine. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 70-71, Braunschweig, ISSN: 1866-590X

Inter-specific competiton of *Bursaphelenchus xylophilus* with native populations of *B. mucronatus* in pine

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Both the quarantine pest nematode, *Bursaphelenchus xylophilus* and native to Eurasia, unnharmful *B. mucronatus* are genetically closely related and present similar bionomics. They develop and reproduce in pine, can use the same insect vectors, and with contiuous colonization of new localities by *B. xylophilus*, the overlap of their geographic distribution continues to increase. The laboratory and field study in natural ecosystems of the Far East revealed a competitive displacement of the native *B. mucronatus* by the invasive *B. xylophilus* (Cheng *et al*., 2009), which could be attributed to faster population development in the later species (Futai 1980). Considering the observed genetic and phenotypic variation among European populations of *B. mucronatus* we have undertaken a research on interactions between selected strains of *B. xylophilus* (both European and Asiatic) and a series of native Polish isolates of *B. mucronatus* during concurrent invasion and development in the same host.

MATERIALS AND METHODS

In our quarantine glasshouse study, conducted on 2-3-year old seedlings and 20-cm-long logs of *P. silvestris*, reproduction of a single Chinese (Nanjing) and two Portuguese (Mad25c and Pt67OL) strains of *B. xylophilus*, five geographically distant isolates of *B. mucronatus* collected in Poland, and the recently constructed multi-strain intra-specific hybrid of the later species (MT-Rol-01) marked with a Roller *Bmrol-1(mt4)* mutation, were compared when reared separately or in two-species mixed populations. The seedlings were inoculated with a dose of 2500 or 5000 nematodes for single-species cultures, and of 2500 nematodes for each species in mixed populations. In logs the dose was reduced to 500 and 1000 nematodes, respectively. The nematodes were incubated for 1 month at 20 °C. Then, the wood was chopped and subjected to water extraction of nematodes. The nematodes were identified based on the shape of female tail terminus. The proportions of phenotypes present in the offspring were counted for each species/strain variant. Selected populations were also subjected to molecular (ITS-RFLP) analysis to confirm the taxonomic status of the offspring.

RESULTS AND DISCUSSION

Phenotypic examination of the nematode offspring revealed that in single-species populations of *B. xylophilus* the range of morphological variation of the female tail has increased when compared to nematodes of the parental populations, which were originally reared *in vitro*, on *Botritis cinerea*. This was particularly obvious in the Chinese strain (Nanjing), where in 28-42% individuals the tail terminus had a conical projection or a small mucro, compared to mostly broadly rounded terminus of *in vitro*-reared females. The shape of mucro was, however, distinctive from that in *B. mucronatus*. In *B. mucronatus* the female tail was similar in both the nematodes reared *in vitro* and in wood.

In the offspring of two-species mixed populations the proportions of females with *B. xylophilus*- and *B. mucronatus*-like tail generally drifted to one of the parental phenotypes. Interestingly, not only *B. xylophilus* but also *B. mucronatus* could dominate and contribute to significant reduction of the second species. Among five isolates of *B. mucronatus* examined in logs, one (Mdz-1) proved to dominate *B. xylophilus* (Nanjing and P t67 O L) in 73 and 63% of replicates, respectively, while the isolate Maz-02 dominated these strains in almost 50% of replicates. *B. xylophilus* (Mad 25c) dominated in all experimental variants, however, in individual seedlings or logs these nematodes could also be outperformed by local isolates of *B. mucronatus*.

Insertion of the Roller mutation into the intra-specific hybrid population of *B. mucronatus* (MT-Rol-01) clearly simplified the process of phenotypic examination of the offspring in two-species mixed cultures. By producing the unique phenotype in all developmental stages of *B. mucronatus* the Roller mutation seems to be a very handy marker for any intra- and inter-specific hybridization and controlled rearing of this nematode.

In similar environmental conditions the results of the inter-specific competition in the host differed among species / strain combinations and were apparently related with variation in innate characteristics of the nematode populations. We speculate that the observed phenomenon may have some retarding effect on early success of *B. xylophilus* in colonization of new regions where native populations of *B. mucronatus* are present. These observations need, however, further substantiation in field experiments.

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Zhengmin H, Ye G, Ailing B, Dongxia C, Interspecific hybridization between *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 72-73, Braunschweig, ISSN: 1866-590X

Interspecific hybridization between *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*

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ABSTRACT:

Pine wood nematode (*Bursaphelenchus xylophilus*) is the pathogen of pine wilt disease that causes pine wilt or death. *Bursaphelenchus mucronatus*, belonging to same genus with *B. xylophilus,* was previously considered as no-virulence or weak pathogenicity. Because *B. mucronatus* exists widely in the pine forests of Eurasia, there has been a lot of interest in the hybridization between B. *mucronatus* and *B. xylophilus* and many investigations could be found in the literature. However, the investigations were inconclusive and many issues remained unresolved and unaddressed. For example, the enhance pathogenicity of *B. mucronatu* and the indistinctive classification characteristic of the *Bursaphelenchus mucronatus* and *B. xylophilus* and so on. Gaining new insights on the issues will contribute not only to *Bursaphelenchus* classification, but also to the quarantine and control of the pine wilt disease.

We conducted indoor hybridization using the nematode isolates of *B. xylophilus* from China, Japan and the nematode isolates of *B. mucronatus* from China, Japan and France. Our objectives were to examine the two species in mating ability, hybrid offspring survival and fecundity. The study results would provide experimental evidences on the hybridization of the two species and insights on whether the two species could be merged. Our study has important implications for the classification of genus *Bursaphelenchus* and pine disease quarantine and control.

Male and female adults of the isolates of *B. xylophilus* and *B.mucronatus*, were orthogonal or reverse crossed, with the intraspecific self mated and single female adult cultured as control, cultivated 7 days to observe the F1 generation. All the combinations were able to cross and had the ability to generate F1 progeny, although the offspring counts varied from tube to tube in a range of 17- 44. Compared with intraspecific self mating, the hybrid of *B. xylophilus* and *B.mucronatus* produced a smaller number of F1 generation offspring. In the intraspecific mating, the number of offspring produced each

tube is around 100. In the interspecific mating, however, the number of F1 generation offspring is around 50, only half of what is in the intraspecific mating.

For the research of F2 generation nematodes production , each F1 generation of 10 larva was selected and in *Botrytis cinerea* slope, 25 ℃ cultured 7 day, observed under microscope , and recorded the results. From the results we can see that all the combination has F2 generation produced, just differ in nematodes total quantity, larva rate and nematodes vigor.

In order to understand the reproductive capacity of hybrid offspring, we obtained the hybrid offspring of Chinese combinations (BxZJ×BmCHN). The result indicated that the hybrid Chinese combination could produce up to 22 generations. The average number of larvae increased while the average number of adults decreased with an increasing generation. In addition, nematode mortality increased. But sex ratio was stable from generation to generation.

The backcross outcomes of different cross combinations with their parents were also researched ,and the combination of F1 generation of female adults and their parents were mated. The result indicated that all F1 hybrids could backcross with their parents and produce offspring. Based on these study findings, we propose to merge *B. xylophilus* and *B. mucronatus* into one "species" and each belongs to a ".pathogenic type" under the "species".

Keywords: *Bursaphelenchus xylophilus*, *Bursaphelenchus mucronatus*, interspecific hybridization

Kato T, Akira K, Ryoji S, Futai K, Takeuchi Y, Phenotypic and genotypic traits of recombinant inbred lines of pine wood nematode, *Bursaphelenchus xylophilus.* In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 74-78, Braunschweig, ISSN: 1866-590X

Phenotypic and genotypic traits of recombinant inbred lines of pine wood nematode, *Bursaphelenchus xylophilus*

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ABSTRACT

Pine wood nematode, *Bursaphelenchus xylophilus*, exhibits a wide range of intraspecific variation in several biological traits. Among them virulence (degree of pathogenicity), reproductive ability and boarding ability on the vector beetle are important pathogenicity-related traits, although their molecular basis has not been determined. In this study we generated a set of recombinant inbred lines (RILs) of *B. xylophilus* from two inbred lines, F7 and P9, which greatly differ in the degree of pathogenicity. In addition, we conducted bioassays to estimate above-mentioned three traits in the newly obtained 17 RILs and two parental inbred lines. As a result, RILs showed various virulences and reproductions along a continuum and two distinct transmission abilities. This indicates that virulence and reproduction may be quantitative, polygenic trait, while transmission ability is a qualitative trait which is controlled by a single or few genes.

1. INTRODUCTION

Pine wilt is a disease of pine caused by the pine wood nematode, *Bursaphelenchus xylophilus*, transmitted by vector beetle of the genus *Monochamus*. In recent years, molecular biological approach have been vigorously conducted for comprehension of disease mechanism (e.g. Jones *et al.* 2008), although the pathogenic factor has not yet determined. In this study we applied a classical genetics to address this matter by using newly conducted recombinant inbred lines (RILs) of *B. xylophilus*.

2. MATERIALS AND METHODS

2.1. Construction of RILs

Two parental lines, a virulent inbred strain 'P9' and an avirulent inbred strain 'F7' of *B. xylophilus* (Shinya *et al*. 2012), served consecutive full-sib mating (brother-sister mating) to yield a set of RILs. One unmated virgin female of one strain was transferred into a breeding plate containing one adult male of the other strain (P9 female for F7 male, and vice versa) to let them cross. Unmated adult nematodes of F1 generation thus obtained served crossing to obtain the F2 generation. Each couple of nematodes of F2 generation was used as ancestral RIL for subsequent full-sib mating that was repeated 20 times.

2.2. SSR marker-based characterization of RILs

Genomic data of *B. xylophilus* (sequence data ver1.2.) downloaded from the GeneDB website (http://www.genedb.org/Homepage) was used to find out the repeated sequences and for each SSR a unique pair of primers were designed. For genotyping a set of RILs, PCR amplification of candidate SSR markers was carried out using genomic DNA of them as template. PCR amplicons were then separated by electrophoresis and compared.

2.3. Reproductive ability of RILs on grey mould

Bursaphelenchus xylophilus of 19 test population including 17 RILs and 2 progenitors, the virulent P9 and avirulent F7, was examined for reproduction. The food source fungus *Botrytis cinerea* was initially cultured on PDA medium in a Petri dish. A nematode suspension containing 100 individuals was inoculated on the fungal mat and incubated at 25ºC. Twelve days after inoculation nematodes were extracted and counted under a stereomicroscope.

2.4. Estimation of Virulence of RILs against Japanese Black Pine Seedlings by Inoculation Test

To determine the virulence of each of the RIL populations of *B. xylophilus* 3-month-old seedlings of a highly susceptible Japanese black pine, *Pinus thunbergii*, served as experimental host plants to be challenged. After making a lengthwise slit that reached cambium on main stem, nematode suspension containing 500 individuals was inoculated into the incision. Twenty seedlings were challenged with each test population and 20 other seedlings were inoculated with an equal volume of distilled water as control. This experiment was repeated 4 times. Seedlings inoculated were incubated for 2 months with weekly health checks.

2.5. Boarding Ability of RILs on Vector Beetle

To create a culture vessel, barley and woodchip of Japanese red pine (*Pinus densiflora*) were added in this order to a glass tube and plugged. The tube was inoculated with the

blue-stain fungus (*Ophiostoma minus*). After incubation, 100 *B. xylophilus* individuals of each test population was inoculated to the tube and incubated for another 2 weeks. Finally a larva of *Monochamus alternatus*, obtained from naturally-infected pine trees, was introduced to the tube, incubated and monitored at the same hour every day. Eclosed beetle was taken from the tube and nematodes were extracted and counted both from the beetle and from the medium in the tube.

3. RESULTS AND DISCUSSION

3.1. Construction and SSR marker-based characterization of RILs

Using two separate inbred lines of *B. xylophilus*, P9 and F7, a set of 17 RILs derived from 17 couples of F_2 generation has been generated. Among them was eight RILs descended from F7 female and P9 male, and nine RILs descended from P9 female and F7 male. A search of the genomic data of *B. xylophilus* permitted 16 SSRs primer design in different scaffolds. These SSRs PCR-amplified with unique primer pairs showed polymorphism across the two progenitors, i.e. P9 and F7, and they were therefore used in genotyping of RILs as SSR marker. As a result, 17 RILs showed unique genotype different from each other with high degree of homozygosity ranging from 0.88 to 1.00 (0.99 in average).

3.2. Reproductive Ability of RILs on Grey Mould

Change in the number of *B. xylophilus* grown on the fungus is shown in Figure 1. Reproductive ability shown by the newly obtained RILs were intermediate between those shown by the two progenitors; that is, no RILs showed a significantly larger or a significantly smaller population than P9 or F7, respectively. Thus the resultant RIL populations showed a continuously varying distribution of reproductive ability, which can be explained by quantitative inheritance controlled by polygene.

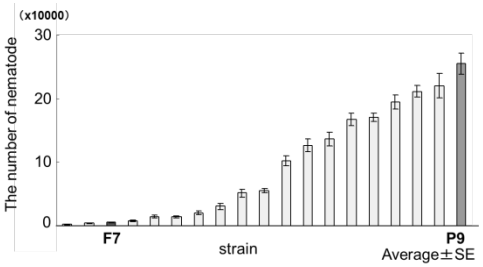

Figure 1. The number of each RIL population grown on *B. cinerea* for 12 days.

3.3. Estimation of Virulence of RILs against Japanese Black Pine Seedlings by Inoculation Test

Figure 2 shows increase of the number of dead *P. thunbergii* seedlings after inoculation of *B. xylophilus*. The seedling mortality, which indicates virulence of the *B. xylophilus* isolate inoculated, widely varied, from 0% to 58%. The progenitor F7 caused 1% mortality, while the other progenitor P9 caused 49% mortality. Distribution of mortality

resulted from the 17 RIL populations varied along a continuum between those from the progenitors, with two exceptions. This suggests that virulence of *B. xylophilus* is a quantitative trait to which a combination of several genes contributes.

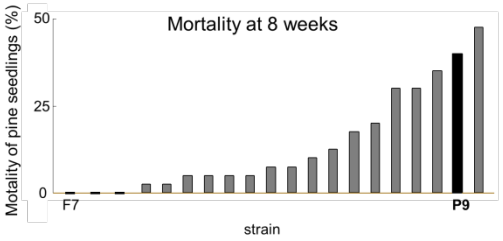

Figure 2. Mortality of pine seedlings after inoculation with each of the RIL populations

3.4. Boarding Ability of RILs on Vector Beetle

Data obtained by coculture of *B. xylophilus* with its vector beetle is summarized in Figure 3. Three RILs were omitted since no nematode offspring was extracted from either of beetle body and medium in culture vessels. All RILs showed a largely similar fluctuation pattern in number of total nematodes recovered from the beetle body and medium decreased with time. Multiple comparisons demonstrated that the progenitor P9 had a significantly higher value than the other progenitor F7. RILs were divided into two groups; one gave extremely low scores in the similar manner to F7, and the other generated a large number of boarded nematode that got aboard in the similar manner to P9. Thus the pattern of inheritance for boarding ability seems monogenic, suggesting that this trait is influenced by one or few genes with a major effect.

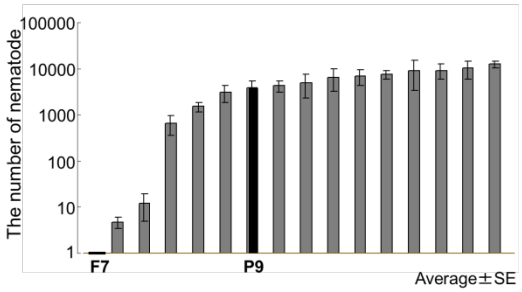

Figure 3. the number of Jiv nematode boarding on *M. alternatus* in each of RILs populations

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Pathogenicity, reproduction and survival of axenic *Bursaphelenchus xylophilus*

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Pine wilt disease (PWD) is the most serious tree epidemic which causes vast catastrophic damage to pine forests. For a long time, the pine wood nematode (PWN), *Bursaphelenchus xylophilus* was believed to be the only pathogenic agent causing the disease. More recently, it has been hypothesized that some bacteria associated with *B. xylophilus* may play a crucial role on pine wilt. The role of nematodes and associated bacteria in PWD development remains to be further studied. Here, we focused on the biology of axenic *B. xylophilus*.

The pathogenicity of axenic *B. xylophilus* was tested by inoculating greenhouse-grown 4-year-old seedlings and 6-month-old axenic microcuttings of *P. densiflora* with aseptic PWNs and non-aseptic PWNs. Seedlings were inoculated with 5,000 PWNs. Microcuttings were inoculated under axenic conditions with 200 nematodes. After 20 days, the microcuttings inoculated with aseptic PWNs and non-aseptic PWNs wilted, and the wilting ratios were 90% and 80%, respectively. The average numbers of recovered nematodes were (364 $\pm$355) and (66$\pm$52) per microcutting, respectively. Similarly, after 38 days, both of aseptic PWNs and non-aseptic PWNs wilted 80% of greenhouse-grown seedlings, with (34733 $\pm$ 34162) and (25057 $\pm$ 21410) nematodes per seedling, respectively. To compare the reproduction of aseptic PWNs and non-aseptic PWNs, 100 nematodes were transferred into a PDA plate with *Botrytis cinerea* and cultured at 25°C. One week later, the nematodes were isolated from the plate and aseptic and non-aseptic nematodes were counted. The results showed that there was no significant difference in the number between them. Furthermore, the survival of aseptic PWNs and non-aseptic PWNs under axenic conditions was studied. Five thousand PWNs were maintained in flasks containing 2 ml sterile water and incubated at 25°C. After 36 days, the survival rate of non-aseptic PWNs was lower than 5%, on the other hand, the survival rate of aseptic PWNs was about 50%.

Based on our research, it can be concluded that aseptic *B. xylophilus* does not lost its pathogenicity character. Also, it was amazing to see them live longer than non-aseptic one under axenic condition.

Gao RH , Shi J, Luo YQ. Influence of pine wood nematode invasion on typical Masson pine ecosystem in Three Gorges Reservoir Region of China. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 80-81, Braunschweig, ISSN: 1866-590X

Influence of pine wood nematode invasion on typical Masson pine ecosystem in Three Gorges Reservoir Region of China

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ABSTRACT:

Due to complex terrain and biological diversity, the Three Gorges Reservoir Region is becoming hot spot region for researches from China to the world. However in recent years, the invasion of *Bursaphelenchus xylophilus* (pine wood nematode ,PWN) caused a devastating impact on Masson pine stand ecosystem and terrible effects on water quality of Yangtze River as well as the ecological safety of Three Gorges Dam.The purpose of this research is to serve for protecting the ecological safety of Three Gorges Dam and pine resources in Three Gorges Reservoir region.Based on "sample plots setting" and "the measurement of all individual trees" methods, we analyzed the species composition , diversity changes and the dynamic changes of structure and function of Masson pine communities after attacked by PWN with different years （0year,1year,3year,5year and 7year） in 2012. Results indicated, for the pine stand ecosystem infected by PWN, pure Masson pine forest had evolved into coniferous and broad-leaved mixed forest. Moreover, the Masson pine was ranked as the dominant species meanwhile some broad-leaved trees, such as *Cinnamomum camphora*, *Quercus aliena*, *Quercus variabilis Blume* and *Loropetalum chinensis*, were ranked as the subdominant species. As for the indicators that reflect healthy status of Masson pine's structure and function, the healthy pine stand ecosystem was higher than infected pine one. With the increasing of infected years, each indicator showed a trend of decreasing. Through analyzing the relationship between pine wilt disease and stand structure in infected pine stand ecosystem, results indicated that the invasion of PWN had great influence on biological diversity of arbor, shrub and herb. In general, the relationship between species diversity indicators and infected years followed the "Mid-altitude bulge" theory. Specifically, both one-year infected and seven-year infected Masson pine forest would have the decline of plant species diversity in certain degree. Various practices could be carried out to prevent the further spread of PWN, to

improve the simple structure of Masson pine forest into a complex one for increasing the pine forest resistant ability in Three Gorges Reservoir region.

Key words: Three Gorges Reservoir region; pine wilt disease; plant community; Masson Pine; ecosystem

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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

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ABSTRACT

Pine Wilt Disease (PWD) is a xylem restricting disease of pine trees, caused by the Pine Wood Nematode (PWN) *Bursaphelenchus xylophilus*. The nematode is carried from host tree to host tree by vector longhorn beetles in the genus *Monochamus*. The interaction between the nematode and beetle is crucial in the establishment and spread of the disease. PWN, a native of North America where it does not kill pine trees, has spread internationally killing trees in Japan, China, Korea, Taiwan and, from 1999, Portugal. Based on the locations where tree mortality has been recorded, it appears that pine trees growing in hot, dry conditions are more susceptible to the nematode, resulting in pine wilt disease. Results in the literature show that the growth and development of PWN is temperature-dependent and that there is a temperature range, outside which nematode development is restricted. In this paper we describe the ETPN model, an evapo-transpiration model (previously developed by Forest Research), which has been modified to incorporate the presence of PWN inside a tree, to predict the regions of Europe that are likely to succumb to pine wilt disease. ETPN acts independently of the vector; hence we predict the likelihood of PWD, assuming that a tree in a particular region has been infested by the pine wood nematode.

We have run the ETPN model for various locations in Japan, where PWD has been killing pine trees for over a century. The results of the ETPN model are in good agreement with observations in Japan and provide strong validation for the model.

We have also considered different regions in Europe: Portugal; where PWD has been found, France; where we would not currently expect to see PWD, but a region that might become suitable under future climates and Sweden; where we would not expect to see PWD. We demonstrate how the different climates of these three regions give very different results. Finally, we consider various climate change scenarios to demonstrate how PWD is likely to affect different regions in Europe in the future, especially in areas where there might be a shift from nil to low likelihood of PWD to a higher likelihood of tree mortality.

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Cold-tolerance and adaption of Pine wood nematode in China

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ABSTRACT:

Bursaphelenchus xylophilus (pine wood nematode, PWN) is a kind of plants parasitic nematode which survival in tropical and temperate zone, temperature is an important environmental factor affecting its spread areas. From 2012 to 2013, we collected PWN species from the different location which represent separately is the most north (Shannxi province), the most south(Guangdong province), and the middle part (Zhejiang and Hubei province) of distribution area of PWN in China to explore the influence of temperature on different geographical populations.Results showed that PWN are depressed by - 5℃ for 24 h, there exists difference in survival of the PWN among different regions .The survival rate of Shannxi, Zhejiang, Hubei and Guangdong strain are 39.10%, 37.48%, 48.51% and 29.21% separately. In addition, the survival of pine wood nematode after cultivation of 20 d at 15℃ was obviously higher than that cultivation of 20d at 25℃. In general, there exists some cold tolerance and adaption ability of PWN in China, which improved the survival of PWN in China, the deep reason that how low temperature cultivation improve the PWN survival would be further discussed in the future.

Key words: pine wood nematode, cold-tolerance, cultivation.

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A comparative proteomics analysis on resistant provenance of *Pinus massoniana* inoculated with *Bursaphelenchus xylophilus*

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ABSTRACT:

Pine wilt disease caused by *B. xylophilus*, also known as the pine wood nematode (PWN), is the most devastating disease of pine trees. From different geographical provenance of *P. massoniana* were inoculated with nematodes, test results selected the provenance GD_5 which has strongly resistance to PWN. This article used resistant provenance GD_5 as the experimental material, and sensitive provenance SX_1 as comparison. Total proteins were extracted by using 2-DE and MALDI-TOF/TOF technology from the provenances pine needles respectively. Differentially expressed proteins in the provenance before and two weeks after inoculated with PWN, were analyzed. At last, 89 differentially expression proteins were successfully identified by MALDI-TOF–TOF. The test result also fund that there were five proteins involved in hydrogen peroxide scavenging capacity and protecting the redox homeostasis system from damaged. Their up-regulation may be the main cause of the provenance GD_5 resistant to PWN.

Keywords: *Pinus massoniana*, pine wilt disease, resistant provenance, proteomic, 2-DE, MALDI-TOF/TOF

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Comparative transcriptomics to understand the molecular basis of *Bursaphelenchus xylophilus* pathogenicity

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Pinewood nematode (PWN) biology and ecology are strictly associated to pine wilt disease (PWD) and have been extensively investigated. However, the disease molecular mechanism has not yet been established. Aiming to unravel the mechanism of pathogenicity we used a transcriptomics approach to study the gene expression of *Bursaphelenchus xylophilus* and the closely related *Bursaphelenchus mucronatus*. Furthermore, we sequenced the transcriptomes of *B. xylophilus* males, females and dispersal juveniles J_{III}. We then built a transcriptomics platform to carry out educated searches on differential gene expression to highlight the molecular basis of PWN pathogenicity or discover new targets with high interest for nematode control.

The five transcriptomes were sequenced in the 454 platform (Roche). Pyrosequencing generated on average 455,000 reads and 8,500 transcripts per transcriptome; more than 60% of these corresponded to InterPro terms (Table 1). Nucleotide and aminoacid sequences and corresponding annotation were organized in a web-based database. The huge amount of data generated represents an important opportunity to increase the available scientific knowledge on the nematode and to carry out comparative analysis.

Table 1. Summary of sequencing, assembly and annotation data. *B. xylophilus* and *B. mucronatus* mixed stages were collected from fungal cultures, while *B. xylophilus* males, females and JIII were collected from infected pines. Total RNA was isolated from each nematode isolate, and cDNA synthesized according to the SMART technology. Transcript assembly and annotation were performed as described in Bettencourt (2010).

	B. xylophilus (fungi)	*B. mucronatus (fungi)*	*B. xylophilus (pine)*		
			male	female	J_{III}
# Reads	647,641	465,256	407,835	227,307	531,049
# Transcripts	11,006	8,822	6,724	5,760	10,608
# Amino acid sequences	12,038	9,231	6,897	6,013	11,444
# Amino acid sequences assigned to InterPro	7,321	5,547	4,148	4,135	7,120

The platform was queried for three main comparisons: *B. xylophilus* versus *B. mucronatus* grown in fungi; PWN males versus females versus J_{III} grown on pine and PWN grown on fungi versus growth in pine. The transcriptomes were compared for genes exclusively present in each condition according to different annotation strategies such as KEGG and Gene Ontology, and also based on sequences similarity using the CD-HIT program (Huang 2010). In addition, we also studied the gene expression of potential nematode parasitism effetors directly on the database using Myrna as described in Santos (2012). The comparisons identified more than 30 genes potentially involved in PWD parasitism that are being experimentally validated by RT-PCR.

Here, we focus on the comparative gene expression of oxidative response genes, ubiquitination-related genes and a potential secreted purple acid phosphatase (Figure 1). The gene expression experimental validation indicated an overexpression in the oxidative response in males and J_{III} when compared to females grown in pine and also an overexpression in *B. mucronatus* when compared to *B. xylophilus* grown on fungi. Interestly, only *B. xylophilus* males showed an overexpression of 4HPPD (4-hydroxyphenylpyruvate dioxygenase) and an underexpression of HGD (homogentisic acid oxidase), indicating that also homogentisate can be a potentially important nematode oxidative detoxification mechanism in pine (Martin and Batkoff, 1987; Arias-Barrau, 2004). Ubiquitination-related genes were overexpressed in females when compared to the other *B. xylophilus* samples, suggesting an important regulation of internal proteolytic activity, probably to reach normal homeostasis required for reproduction (Comyn, 2013). The marked overexpression of secreted PAP (purple acid phosphatase) in females and J_{III} when compared to *B. xylophilus* grown in fungi and males grown in pine may represent a potential role in the nematode interaction with pine due to the relation of PAPs to a wide range of actions such as reactive oxygen species generation (Schenk, 2013).

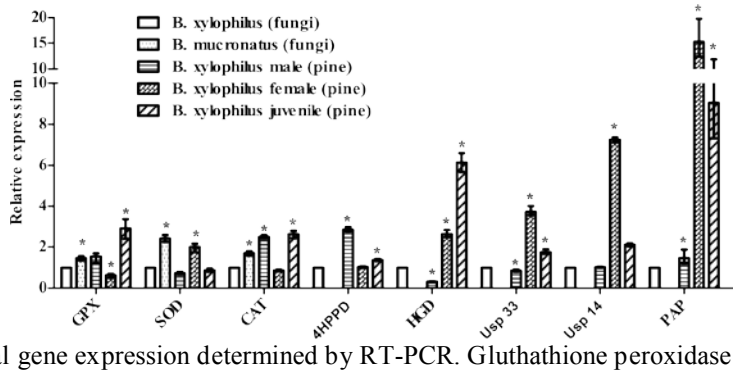

Figure 1. Differential gene expression determined by RT-PCR. Gluthathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) were studied in the five conditions, while 4-hydroxyphenylpyruvate dioxygenase (4HPPD), homogentisic acid oxidase (HGD), Ubiquitin-specific-processing protease 33 (Usp 33), Ubiquitin-specific-processing protease 14 (Usp 14) and purple acid phosphatase (PAP) were studied in the four *B. xylophilus* samples. Data is presented under the form mean±SD (standard deviation). * indicates significative differential expression with $p < 0.05$ as determined with the REST software. The cell division control protein 42 was the endogenous control and *B. xylophilus* grown in fungi the control sample.

Additional differentialy expressed genes corresponded to peptidases and respective inhibitors, carbohydrate-active enzymes, genes involved in oxidative detoxification, phenolic compound degradation or host mimicking. These genes showed higher expression levels for the nematode grown in pine, and also differences between the 3 developmental stages. These results suggest differences between males, females and J_{III} while growing in pine and may elucidate the contribution of the different stages to the PWN pathogenicity. Results and discussion of these genes will be presented at the meeting.

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The Function of Major Sperm Proteins (MSPs) in reproduction of pine wood nematode

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Abstract

BxMSP1, BxMSP2 and BxMSP3 of *Bursaphelenchus xylophilus* were cloned in this study. The senior structure of these proteins was rich of β sheets, which was highly conserved in MSP members of the nematode species. In situ hybridization showed that these genes were specifically expressed in the seminal vesicle tissue of the male adults. The reproduction ability of *B. xylophilus* decreased when the nematodes were soaked by the dsRNA of BxMSP1 and BxMSP2. qPCR analysis showed lower transcript abundance of the targeted mRNAs when the nematodes were soaked by the dsRNA of the MSPs. These results indicated that BxMSP1 and BxMSP2 were required for reproduction of *B. xylophilus*.

1 Introduction

The major sperm protein (MSP) is a nematode specific protein. MSP has first been identified in *Caenorhabditis elegans*. It is the most abundant protein present in nematode sperm, MSP is the key player in the motility machinery of nematodes that propels the crawling movement of nematode sperm in *C. elegans*. But the function of MSP in the plant nematode is still very limited known. In this paper, three MSPs were cloned form *B. xylophilus* and gene function were identified by RNAi method.

2 Materials and methods

BxMSP1, BxMSP2 and BxMSP3 were cloned by the methods of transcriptomic sequencing and rapid amplification of cDNA ends (RACE). In situ hybridization was used to locate the gene expressed tissue site. Nematodes were treated 48h by the dsRNA of BxMSP1, BxMSP2 and BxMSP3 respectively. Then these nematodes were fed on the *Botrytis cinea* to evaluate the reproduction ability. qPCR was used to detect the gene expressed level.

3 Results

3.1 Gene cloning of BxMSP1, BxMSP2 and BxMSP3

Genomic sequence analysis indicated that BxMSP1, BxMSP2 and BxMSP3 contained a intron respectively. Mobile-Sperm domain was contained in these MSPs. These proteins was rich of β sheets, which was highly conserved in MSP members of the nematode species.

3.2 Tissue expression site of MSPs

In situ hybridization showed that three MSPs were specifically expressed in the seminal vesicle tissue of the male adults. There is no hybridization signal in the females and larvas.

3.3 RNAi of MSPs

dsRNA of BxMSP1 and BxMSP2 significantly suppressed the reproduction ability of *B. xylophilus,* and significantly decreased the yield and the hatching rate of eggs. But dsRNA of BxMSP3 had no significantly effect on the nematode reproduction.

3.4 qPCR of gene expressed level of MSPs

qPCR indicated that dsRNA of BxMSP1 and BxMSP2 significantly decreased the expression level of BxMSP1 and BxMSP2. But the dsRNA of BxMSP3 had no significantly effect on the expression level of BxMSP3. These results indicated BxMSP1 and BxMSP2 had important roles of regulating the reproduction of *B. xylophilus*.

4 DISCUSSION

The pine wood nematode is a disastrous pathogen of the pine forests in East Asia and European. But because of limited understanding of its pathogenic mechanism, there is no efficient measures to control this nematode. In this study, BxMSP1 and BxMSP2 were required for reproduction of *B. xylophilus*. But how these genes regulate the pine wood nematode is still unknown. The interactional proteins and regulation network of BxMSP1 and BxMSP2 in the reproduction process need to be illuminated in the future. These work will help us to understand the molecular mechanism of nematode sperm development and reproduction. It is useful for screening the potential target gene for control this nematode.

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Exploring the relation between virulence and oxidative stress response of *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*

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Tolerance to host-mediated oxidative stress (OS) conditions is an essential characteristic of plant-parasitic organisms. Susceptible *Pinus thunbergii* reacts to *Bursaphelenchus xylophilus* invasion with a strong oxidative burst (Hirao *et al*., 2012), which may indicate that virulent *B. xylophilus* must possess an efficient antioxidant system to cope with these conditions. Previous studies have suggested that PRX (2-cysteine peroxiredoxin), GST (glutathione S-transferase) and GAPDH, all localized in the surface coat, are potential scanvengers of *B. xyophilus* to plant reactive oxygen species (ROS) (Shinya *et al*., 2010; Li *et al*., 2011). More recently, 12 anti-oxidant proteins were identified in the *B. xylophilus* secreotome after plant extracts timuli, emphazing the ir importance in the control of global oxidative stress of *B. xylophilus* (Shinya et al., 2013). In this work, our main concern was to study of OS tolerance of *B. xylophilus* isolates and *B. mucronatus* and the relation with their pathogenicity (virulence level) to susceptible pine species. Previous results (Vicente *et al*., *submitted*) have already suggested a relation virulence-OS tolerance among *B. xylophilus* isolates virulent Ka4 and avirulent C14-5. So, firstly, three *B. xylophilus* isolates, Ka4 and T4 (virulent) and C14-5 (avirulent), and one *B. mucronatus* (avirulent) were tested for OS tolerance using hydrogen peroxide as oxidative agent, in concentrations ranging from 0-40 mM H_2O_2. After 24h-exposure to this oxidant agent, nematode survival was checked. A clear difference between virulent and avirulent isolates was recorded in OS conditions, even in the lowest H_2O_2 concentration. The virulent isolates (Ka4 and T4) presented lower mortality percentage in all concentrations than avirulent ones (C14-5 and *B. mucronatus*). Statistical differences between Ka4 and T4 were also found until 30mM H_2O_2 treatment, being Ka4 the most resistant isolate. Concerning a virulent isolates, mortality percentage was higher than 90% in all concentrations, with no statistical differences found between C14-5 and *B. mucronatus*. Next, we assessed transcription levels of 5 main antioxidant and detoxifying enzymes during the OS conditions (15mM H_2O_2, 24h-exposure), and compared with normal conditions (no stress applied) by qRT-PCR. The following enzyme genes were analysed:

CTL (catalases, *Bxy-ctl-1* and *Bxy-ctl-2*), SOD (superoxide dismutase, *Bxy-sod-1*, *Bxy-sod-2* and *Bxy-sod-3*), GXP (glutathione peroxidase, *Bxy-gxp-1*, *Bxy-gxp-2* and *Bxy-gxp-3*), GST (glutathione S-transferase, *Bxy-gst-1* and *Bxy-gst-3*) and PRDX (peroxiredoxin, *Bxy-prdx-2*). In the case of *B. mucronatus*, this analysis was not possible to conduct since no information is available about its genome. From the selected enzymes, only *Bxy-ctl-1* and *-ctl-2* were significantly upregulated ($P<0.05$) in virulent isolates Ka4 and T4. In the case of C14-5, only *Bxy-ctl-2* was significantly downregulated ($P<0.05$) in comparison with normal conditions. For SODs and GPXs, there were no statistical differences between isolates, although we could assess that *Bxy-sod-1* and *-2* were nearly 1-fold upregulated for T4; *Bxy-sod-3*, *Bxy-gxp-2* and *Bxy-gxp-3* for Ka4 and T4 were expressed at the same level than normal conditions; and that *Bxy-sod-2* and *-3*, and *Bxy-gxp-2* and *-3* were downregulated for avirulent C14-5. Concerning the detoxifying enzymes GST and PRDX: *Bxy-gst-1* of isolates Ka4 and T4 were, respectively, downregulated and unchanged under OS conditions, and that expression of *Bxy-prdx-2* for both virulent isolates was suppressed in stress conditions. In contrast, *Bxy-gst-1* of avirulent C14-5 was upregulated in OS conditions and *Bxy-prdx-2* remained unaltered. GST-3 was not detected in all isolates. Following, we will analyse gene expression of these enzymes in *in vivo* conditions for all *B. xylophilus* isolates to ascertain the global oxidative status of the nematode as result of natural oxidative stress conditions. We were able check 100 % sequence similarity of coding sequences of CTLs, SODs and GXPs for Ka4, T4 and C14-5, suggesting that if different enzymatic activities are presented may be due to posttranslational modifications.

Based in these results, we hypothese a possible positive correlation between the level of OS tolerance and the level of virulence of *B. xylophilus*, which can be further investigated as a virulence marker.

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Poster Presentations

Sarniguet C et al., *Bursaphelenchus xylophilus* identification, from literature to routine analysis: how to make morphological analysis reliable. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 96-97, Braunschweig, ISSN: 1866-590X

(101) *Bursaphelenchus xylophilus* identification, from literature to routine analysis: how to make morphological analysis reliable.

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Official surveys on *Bursaphelenchus xylophilus* are conducted in a compulsory manner since 2000 in European Union including in France. Considering the Portuguese situation and the dispersal of *Bursaphelenchus xylophilus* throughout its territory and recent occurrences in Spain, the reliability of sampling and analysis undertaken is a critical issue to early detect any further dispersal.

The implementation of quality assurance for official analysis also led to evaluate the reliability of identification methods, based on morphology or on molecular principles.

Published morphological identification keys to *xylophilus* group (Braasch *et al*. 2009) and to *B. xylophilus* species levels (EPPO 2009) were considered for evaluation. The keys were submitted to a panel of slides including *B. xylophilus* individuals, males and females. Conclusions were drawn about the possibility to observe specific criteria, such as lateral lines number, shape of spicules, number of caudal papillae and presence of vulval flap for the group level (Table 1), and the position of excretory pore for species level (Table 2). Some criteria were shown not to be reliable for routine use: number of caudal papillae and excretory pore position.

Consequently, reliable identification keys for *B. xylophilus* were designed (Table 3) and validated according to a standardized process and taking into account recommendations from EPPO protocol (EPPO 2010).

Additional results are available in Sarniguet *et al*. (2013).

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Table 1. Results of evaluation of the *xylophilus* group key according to Braasch *et al*. (2009): number of conform observation/total number of observations

Observed criteria	Females	Males
Lateral lines	23/60	41/60
Spicules	NA	60/60
Caudal papillae	NA	0/60
Vulval flap	46/60	NA

NA: not applicable

Table 2. Results of evaluation of the EPPO (2009) *B. xylophilus* identification key: number of times the criteria is observed/ total number of observations

Features to be observed	Results
Excretory pore not observed	29/60
Excretory pore not at expected place	11/60
Position of the excretory pore conform to *B. xylophilus*	20/60

Table 3. Key designed for *Bursaphelenchus xylophilus* species identification from female individuals

1	Female with conical or slender tail with or without mucro	not *B. xylophilus*
	Female with sub-cylindrical tail ……	**2**
2	Female with sub-cylindrical tail, rounded end without mucro	*B. xylophilus*
	Female with sub-cylindrical tail with a terminal mucro	not *B. xylophilus* or *B. xylophilus* mucronate form (1)

(1) molecular identification needed

Akiba et al., Genetic diversity of the pinewood neamtode, *Bursaphelencus xylophilus* after 100 years of invasion in Japan. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 98-99, Braunschweig, ISSN: 1866-590X

(106) Genetic diversity of the pinewood nematode, *Bursaphelenchus xylophilus* after 100 years of invasion in Japan

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The pinewood nematode *Bursaphelenchus xylophilus* is a pathogen that causes pine wilt disease. The nematode is thought to have been introduced into southwestern Japan (Nagasaki Prefecture in 1905 and Hyogo Prefecture in 1921) from North America followed by dispersion throughout Japan, except to Hokkaido, the northernmost island. However, details on the origins of the invading nematodes and the routes of dispersal in Japan are unknown. We collected 223 nematode isolates from dead trees in affected areas covering almost all damaged areas in Japan and analyzed their nuclear ribosomal DNA (ITS1-5.8SRNA-ITS2) and mitochondrial DNA (cytochrome oxidase subunit I [COI]) sequences. Three SNPs in the ITS1 region, 2 SNPs and 2 indels of two bases in the ITS2 region, and 6 haplotypes (R1–R6) were detected in the nuclear DNA sequence data. Five percent of isolates showed heterogeneity of two haplotypes. Haplotype diversity was 0.625 ± 0.019 and nucleotide diversity was 0.0016 ± 0.0001 among all isolates. R1 and R2 were the dominant haplotypes (38.2% and 47.2%, respectively) and only these two haplotypes occurred in northeastern Japan, where disease expanded after the 1970s. In southwestern Japan where the history of pine wilt disease is older, four other haplotypes (R3–6) were detected. Twenty-eight variable sites were detected within the 658-bp mitochondrial DNA sequence and the isolates separated into 11 haplotypes (4 haplotype groups). Haplotype diversity was 0.596 ± 0.032 and nucleotide diversity was 0.0109 ± 0.0005. The dominant haplotypes in all areas of Japan were C1a (60.1%) and C4a (18.8%). The C3 haplotype was detected only on Okinawa Island, possibly due to the founder effect of introduced nematodes. Differences in both nuclear and mitochondrial DNA in each haplotype may indicate different geographic origins of *B. xylophilus*, and therefore, multiple invasions of nematodes from different native locations. The higher diversity of haplotypes in southwestern Japan may reflect multiple invasions in that region followed by expansion of several haplotypes to northeastern Japan. The ITS haplotype pattern did not correspond to that of COI. Considering the different inheritance

modes of nuclear and mitochondrial DNA, hybridization between nematodes of different origins must have occurred after introduction to Japan. Comparison of Japanese haplotypes with sequences registered in public DNA databases showed that the R3 and R4 ITS haplotypes and some COI haplotypes were unique. Almost all *B. xylophilus* isolates from Portugal were consistent with the R2 ITS haplotype and the C1a COI haplotype, which were dominant haplotypes in Japan. The diversity of *B. xylophilus* in Japan was higher than in Portugal where nematode invasion occurred in 1999. This study shows that multiple invasions are likely to have occurred in Japan, but the origins of the invading nematodes are unknown. To address this gap in our knowledge, extensive sampling of nematodes in native locations (USA and Canada) and more detailed analysis using molecular markers with high resolution such as SSRs or SNPs are necessary.

Kozlovsky M, *Bursaphelenchus mucronatus* as a cause of dying a secondary fir forests in Ukrainian Carpathians. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 100, Braunschweig, ISSN: 1866-590X

(109) *Bursaphelenchus mucronatus* as a cause of dying a secondary fir forests in Ukrainian Carpathians

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Natural range of the stem nematode Bursaphelenchus mucronatus (Mamya & Enda, 1979) in the Ukrainian Carpathians reach upper tree-line. In fir-wood belt (1000-1400 m above sea level) there were found some dead and dying fir trees with a few stem nematodes. No mass infection of wood was revealed.

At lower altitude (400-900 m) in zone of beech forest formations the secondary fir woods are exposed noticeably more on infection (from 50% to 100% of inspected trees of age 40 – 100 years). Trees at the age 60-110 years are infected much more and often, compare to 40 years old fir trees. Stem nematodes were found in trees of different healthiness categories – dead, dying as well as trees without signs of disease.

A lot of trees are infected in severe parts of trunk. Entirely infected fir tree, from butt to top of crown, there was not found. Mostly, stem nematodes were found on upper part of crown and lower or middle part of trunk, that indicates frequentative infection of trees.

In beech-forest belt the infectiousness of fir trees by stem nematodes counts in the range from dozens to 300 nematodes in 1 g of dry wood. Perhaps, stem nematodes expansion is stimulated here by warmer climate.

On some trees were found stem nematodes in space between yellow top and green low part of crown; damages of roots by mushrooms and trunk by xylophagous insects were not revealed. Therefore, we assumed that stem nematodes indeed causes dying of crown top of fir.

Diameter of body of Bursaphelenchus mucronatus averages for female 20-24,6, for male - 25-30 µ ; morphological parameters with ♀: L = 648,2±78,5; a = 30,2±2,4; b = 10,5±0,9; c = 16,2±1,2; V = 69,1±0,8%; St = 13,7±0,1. ♂: L = 714,2±35,9; a = 30,9±8,0; b = 11,0±0,7; c = 18,6±1,3; Sp = 24,3±0,6; St = 13,7±0,1.

In our opinion, in zone of beech forest the stem nematodes are the one of the main reason of fir wood dying.

Takefumi Ikeda, Keisuke Kobayashi, Shoji Naoe, Growth properties of pine trees died from pine wilt disease In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 101-102, Braunschweig, ISSN: 1866-590X

(111) Growth properties of pine trees died from pine wilt disease

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ABSTRACT

Are there any traits in appearance of dead trees in the early stage of massive pine death caused by pine wilt disease? In other words, what is the landmark for *Monochamus* species as a vector choosing pine trees for after-ripening? The study was conducted in *P. thunbergii* community of Amonohashidate, Kyoto, Japan. Pine trees with larger diameter were easy to die in comparison with those with smaller diameter. Trees with larger diameter seem to have a larger tree crown. This might show that *Monochamus alternatus* can easily access to pine trees with a larger crown and do not come flying to declined pine trees.

INTRODUCTION

What is the landmark to *Monochamus* species as a vector choosing pine trees for after-ripening? Researchers on pine wilt disease almost certainly know this. It is important to know to which pine tree *Monochamus* comes flying in order to manage control programs. This study tried to evaluate a relationship between tree growth properties such as tree height and diameter at breast height (dbh) and pine death caused by pine wilt disease.

STUDY AREA

The study was conducted in *P. thunbergii* community of Amanohashidate, Kyoto, Japan (35°34'N, 135°11 'E). Amanohashidate has been best known for its coastline of white sands in Japan. Death of pine tree is of crucial significant to its landscape maintenance. The pine community of Amanohashidate does not connect with the pine forests of surrounding mountainous area and is on a flatland.

TESTED TREES AND ANALYSIS

1. Tree height and dbh: 36 pine trees died in 2002 and 154 live pine trees surrounded dead pines were selected in September 2003. Relationship between dead or not in pine trees as objective variables, and height or dbh of pine trees as explaining variables was analyzed using the Generalized Linear Mixed Model (GLMM). Tree height and dbh have been measured in March 2002.
2. Annual ring analysis: 5 dead pine trees and 10 live pine trees selected among above pine trees were selected. Width of annual ring on increment core collected by an increment borer was measured.

RESULTS AND DISCUSSION

1. Tree height and dbh: There was significant correlation between death and dbh ($p=0.06$, RIV=0.78). This shows that pine trees with larger diameter were easy to die in comparison with those with smaller diameter. *M. alternatus* just after emerging makes choice of healthy pine tree in random manner and then feeds younger shoots (Togashi 2006). *M. alternatus* can easily access to pine trees with a larger crown because of larger target.

2. Annual ring analysis: In both dead pines and live pines the width of annual ring in 2001 was narrower than that in 2000. This seems to be due to low rainfall in early summer of 2001. Dead pine trees did not suffer severe stresses as live pine trees. That's not to say that *M. alternatus* did not target at declined trees for after- ripening.

CONCLUSION

M. alternatus can easily access to pine trees with a larger tree and do not come flying to declined pine trees.

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Hoch et al., Testing attractants for trapping *Monochamus sartor* and *Monochamus sutor*. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 103-104, Braunschweig, ISSN: 1866-590X

(117) Testing attractants for trapping *Monochamus sartor* and *Monochamus sutor*

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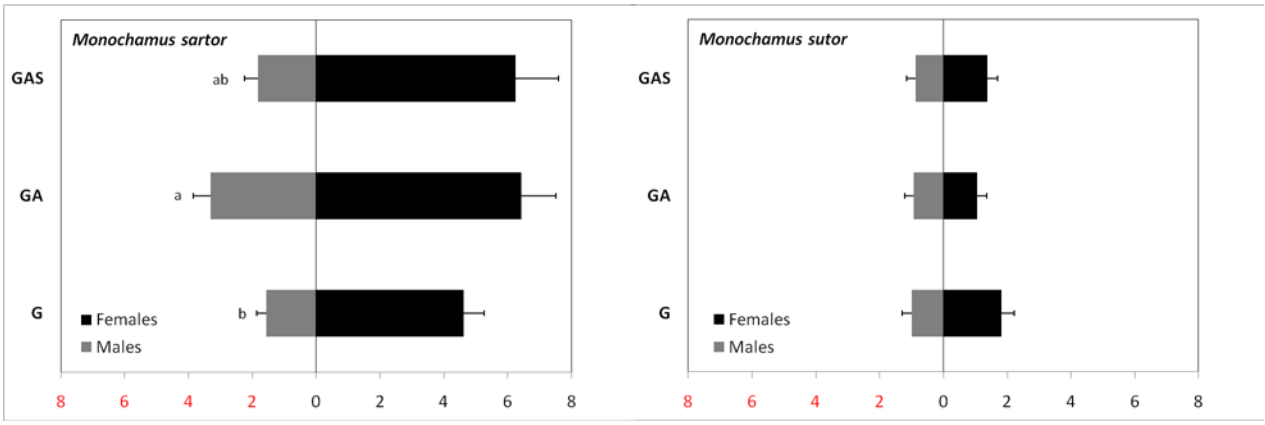
ABSTRACT

Trapping vectors is one important measure for monitoring and control of pine wilt disease. Lures consisting of bark beetle pheromone components and a *Monochamus* pheromone compound have been developed for *Monochamus galloprovincialis*, the main pine wood nematode vector in Europe. The *Monochamus* pheromone compound 2-undecyloxy-1-ethanol has been shown to be attractive for other species in the genus, such as *M. alternatus*. We tested the response of *M. sartor* and *M. sutor* to lures known to attract *M. galloprovinicialis*. These two species are important colonizers of weakened or freshly killed Norway spruce in Central Europe and have the potential to become important vectors should the pine wood nematode be introduced in this area.

The experiment was set up in a mountainous mixed spruce forest in a wilderness area in Lower Austria. No forest sanitation measures had been carried out following attacks of spruce by bark beetles as well after major damage by an avalanche allowing build-up of populations of *M. sartor* and *M. sutor* as well as other phloeo-xylophagous insects. Teflon coated 12-funnel traps (ECONEX, Spain) with three different combinations of attractants were deployed in four randomized blocks. The following lures were tested: (1) the commercially available Galloprotect-2D (SEDQ, Spain) consisting of 2-undecyloxy-1-ethanol, ipsenol, and 2-methyl-3-buten-2-ol, (2) Galloprotect-2D plus α-pinene (SEDQ, Spain), and (3) Galloprotect-2D plus α-pinene plus a blend of smoke volatiles (produced in D.R.H.'s laboratory at the Univ. Greenwich). Positions of lures were re-randomized every 10 days; the experiment lasted fom 10 July to 20 August 2012. Traps were emptied every 3 or 4 days.

Traps baited with Galloprotect-2D caught 4.6 ± 0.6 female and 1.6 ± 0.3 male *M. sartor* on average per 10-day trapping period (Figure 1). Highest catches were attained when the host tree volatile α-pinene was added (6.4 ± 1.0 females and 3.3 ± 0.5 males); the increase in males was statistically significant. Further addition of smoke volatiles did not enhance captures. Due to lower *M. sutor* catch, no significant differences in response to the lures



were established. In total, our traps caught 277 *M. sartor* females and 107 males as well as 68 *M. sutor* females and 45 males over the entire trapping period.

Figure 1. Numbers of *Monochamus sartor* and *M. sutor* beetles caught per trap per 10-d period (means + SE, n = 16). G = Galloprotect-2D, GA = G plus α-pinene, GAS = G plus α-pinene plus smoke volatiles. Different letters indicate significant differences (Mann-Whitney U tests (corrected α = 0.017) following up Kruskal-Wallis H tests).

Catches of *M. sartor* and *M. sutor* were significantly correlated with mean air temperature (Kendall's $\tau = 0.626$ and $\tau = 0.657$, respectively). No beetles were caught when mean temperatures were below 15° C. Traps caught high numbers of other phloeo- or xylophagous insects, such as other cerambycids and buprestids (total of 95 and 24 specimens). Most frequent species were *Acanthocinus griseus*, *Arhopalus rusticus*, *Spondylus buprestoides*, and *Leptura rubra*. Moreover, 136 specimens of the bark beetle predator *Thanasimus formicarius* were caught during the total 40-d trapping period. Generally, bycatch was highest in traps additionally baited with α-pinene. Woodwasps were only caught in traps containing this host tree volatile.

This experiment gave first insight into flight activity of two potential pine wood nematode vectors in mountainous Austria and their attraction to volatiles. The results indicate that *M. sartor* and *M. sutor* respond to the pheromone compound 2-undecyloxy-1-ethanol (monochamol). Attractants developed for *M. galloprovincialis* appear suitable for monitoring these potential pine wood nematode vectors.

Nakamura et al., Inhabitation of the Pinewood Nematode and Its Vectors in the Tsunami-damaged *Pinus thunbergii* and *P. densiflora* trees. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 105, Braunschweig, ISSN: 1866-590X

(120) Inhabitation of the Pinewood Nematode and Its Vectors in the Tsunami-damaged *Pinus thunbergii* and *P. densiflora* trees

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The tsunami (tidal wave) following the Great East Japan Earthquake on March 11, 2011, devastated vast areas of seacoast forest, mainly composed of the Japanese black pine (*Pinus thunbergii*), and red pine (*P. densiflora*). Needle chlorosis occurred not only in severely damaged trees but also in those without conspicuous external damage. We investigated presence/absence of *Monochamus alternatus* and *Bursaphelenchus xylophilus* in the pine trees with discolored foliage in the tsunami-damaged seacoast forests, to evaluate the potential of the trees as the source of infection of pine wilt disease (PWD).

B. xylophilus was rarely detected in the wood samples collected from the dead trees in November 2011, except for the trees that was considered to have been latent infected and died after the tsunami. *P. thunbergii* trees grown along the shoreline were severely damaged by the tsunami and most of them died promptly. In the stands behind the frontline forests, small *P. thunbergii* trees suppressed by the canopy trees and then flooded with sea water from tsunami tended to die shortly after the disaster. Those trees were hardly attacked by *M. alternatus*. In contrast, most of *P. densiflora* trees became declined after the tsunami and kept stressed condition through the summer. Such trees were, resultingly, infested by *M. alternatus*, when there were PWD damaged trees from which adult sawyers emerge in the vicinity.

Emerging adults of *M. alternatus* from the infested trees in the following year often carried *B. xylophilus*, though we could not detect it in those trees in the November survey. Since the *P. densiflora* trees seemed to be debilitated by sea water flooding, not because of *B. xylophilus* infection, it is unlikely that the trees harbored the nematode before *M. alternatus* adults' oviposition. The nematode was possibly transmitted to the tsunami-damaged trees when *M. alternatus* adults laid their eggs onto them. Consequently, tsunami-induced damage in seacoast pine forest may facilitate the spread of PWD epidemic when the forest is composed of *P. densiflora* and PWD damaged trees have already been there.

Kato T, Futai K, Takeuchi Y, Bacterial flora and its association with the pine wood nematode (*Bursaphelenchus xylophilus*). In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 106-109, Braunschweig, ISSN: 1866-590X

(126) Bacterial flora and its association with the pine wood nematode (*Bursaphelenchus xylophilus*)

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ABSTRACT

In recent years a hypothesis was proposed that not pine wood nematode, but its accompanying bacteria is responsible for the symptom development in pine wilt disease. To ascertain this, we investigated the bacterial flora associated with the nematode and its possible roles in the disease by means of molecular biological techniques. As a result, the dominant bacterial species were different from those in past researches and none of them showed a significant pathogenicity against susceptible pine seedlings. On the other hand, one of the dominant species was frequently detected in seedlings inoculated with bacteria-free nematodes and in vector beetle-associated samples, indicating its possible involvement in the disease.

1. INTRODUCTION

Pine wilt disease is caused by the pathogen, pine wood nematode (PWN; *Bursaphelenchus xylophilus*). Recently, it is suggested that PWN needs bacteria inhibiting its body surface in host infection to cause wilt symptoms (Han *et al*. 2003), although this hypothesis still remains a matter of debate. At this time we have no promising method for effectively controlling pine wilt disease, partly because of lack of definitive causal therapy, and it is necessary to identify the pathogenic factor(s) precisely. In this study, in order to clarify the significance of PWN-associated bacteria in this disease, we described the bacterial flora on the PWN body and determined the potential pathogenicity of isolated bacteria to the host pine trees.

2. MATERIALS AND METHODS

2.1. Bacteria Accompanied by PWN in Naturally Infected Pine Stands

We investigated the bacterial flora on the body surface of PWN isolated from naturally-infected pine trees. Woody tissues were taken from dead pine trees during the infection season in 2011 at two pine stands; one of Japanese black pine, *Pinus thunbergii*, located at Arid Land Research Center, Tottori University, Tottori, Japan, and the other of Japanese red pine, *Pinus densiflora*, on Mt. Ogura, Ukyo, Kyoto, Japan. Samples were used for nematode extraction with Baermann funnels to obtain *B. xylophilus*, from which accompanying bacteria were isolated on a plate of R2A medium. All bacterial isolates served DNA extraction for species identification based on the nucleotide sequence of 16S rRNA.

2.2. Pathogenicity of Accompanying Bacteria

To examine the interaction between host plant (pine) and PWN-accompanying bacteria, we test the pathogenicity of two bacteria which were frequently isolated in experiment 2.1, namely, *Serratia proteamaculans* and *Erwinia mallotivora*. Seedlings of 4-month old *P. thunbergii* which were grown under axenic condition were challenged with 1) bacteria-free PWN (virulent isolate, Ka4), 2) 1) mixed with *S. proteamaculans*, 3) 1) mixed with *E. mallotivora*, 4) *S. proteamaculans* alone, 5) *E. mallotivora* alone, and 6) sterilized water as control and the symptom development was monitored.

2.3. Succession of Bacterial Flora on the Body Surface of PWN during Symptom Development

Potted seedlings of *P. thunbergii* were artificially inoculated with bacteria-free cultured PWN (virulent isolate, Ka4) in the open air to monitor the transition of bacterial flora accompanied by PWN inside host plant. One, 2, 4, and 6 weeks from inoculation, woody tissues of the seedlings were sampled to serve Baermann funnels extraction, nematode thus obtained were used as bacterial source, and the cultured bacterial mixture served molecular characterization by t-RFLP techniques with a set of universal primers specific to bacteria.

2.4. Interaction of Accompanying Bacteria and the Vector Beetle

The result of experiment 2.3 showed that *S. proteamaculans* was frequently accompanied by PWN. Considering the past report that *S. proteamaculans* was symbiotic to a kind of beetles (Morales-Jimenez *et al*. 2009), we examined the interaction between this bacteria and the vector beetle of PWN. Sampling of dead pine log that harbored the beetle larvae were conducted at Mt. Ogura in January 2013. By chopping the log with an ax, larvae of Japanese pine sawyer beetle, *Monochamus alternatus* and its pupal chambers were taken. DNA samples extracted from the larvae gut and from bacterial bodies scraped from the chamber were used for molecular characterization by t-RFLP in a same manner as experiment 2.3.

3. RESULTUS AND DISCUSSION

3.1. Bacteria Accompanied by PWN in Naturally Infected Pine Stands

In the analysis of bacteria accompanied by PWN isolated from pine forests naturally infected with pine wilt, neither *Pseudomonas* nor *Bacillus* were frequently detected and in especially *Pseudomonas fluorescens* was never detected (Figure 1), all which were suggested to be associated with pathogenicity of pine wilt (Kawazu & Kaneko 1997; Zhao *et al*. 2003). Dominant species in both pine stands were *Serratia proteamaculans* and *Erwinia mallotivora*, which is consistent with the previous study in Portugal (Vicente *et al.* 2011).

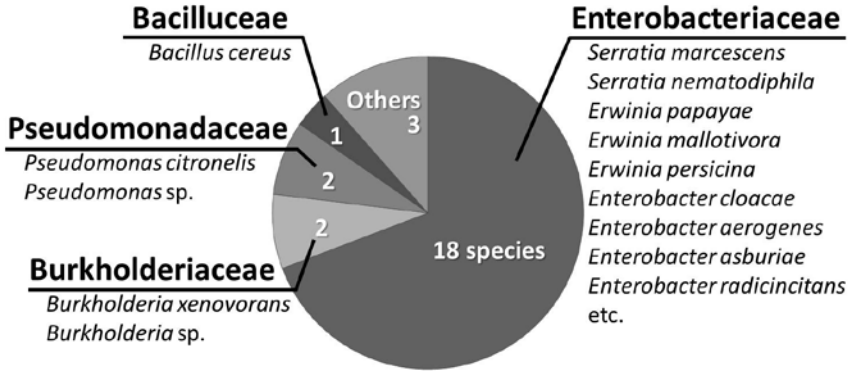

Figure 1. Bacterial flora on the body surface of pine wood nematode isolated from naturally infected pine stands in Japan

3.2. Pathogenicity of Accompanying Bacteria

In pathogenicity test using axenic pine seedlings, PWN inoculation caused a significant mortality regardless of whether and which bacteria was inoculated (Figure 2). Also, no bacteria was detected in the seedlings killed by PWN inoculation. All these suggest that PWN-accompanying bacteria is not necessary for the pathogenicity of pine wilt disease.

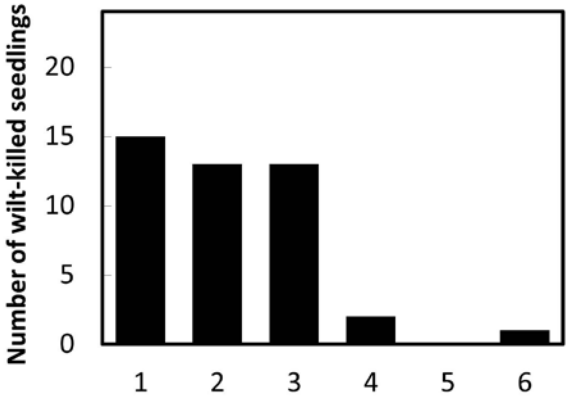

Figure 2. Mortality of pine seedlings after inoculation with (1) bacteria-free PWN, (2) PWN mixed with *S. proteamaculans*, (3) PWN mixed with of *E. mallotivora*, (4) *S. proteamaculans* alone, (5) *E. mallotivora* alone, and (6) sterilized water is shown. Values are averaged number of dead seedlings in two experiments (n=24 for each).

3.3. Succession of Bacterial Flora on the Body Surface of PWN during Symptom Development

Bacterial flora accompanied by PWN after inoculation into host pine was monitored by means of t-RFLP. In all samples a clear peak of 366 bp was detected, which was identical to that of *S. proteamaculans*. It suggests that *S. proteamaculans* get infected into pine plant by any way to establish a specific relation with PWN.

3.4. Interaction of Accompanying Bacteria and the Vector Beetle

Bacterial flora in the pupal chamber and the gut of vector beetle of PWN was characterized by means of t-RFLP. In the pupal chamber, more species of bacteria were detected and the dominance of *S. proteamaculans* (detected as a peak at 366 bp) was lower than in the gut. Thus *S. proteamaculans* may be accompanied by the body surface of PWN which is infecting host plant, propagate inside the plant (on the surface of pupal chamber), and then preferentially colonize the beetle gut to encounter PWN.

4. CONCLUSION

Our results strongly suggest a lack of pathogenicity of PWN-accompanying bacteria, although it is highly possible that certain species of bacteria inhabit on the body surface of PWN. Further studies need to determine the ecological significance of such bacterial species in association with pine wilt disease.

5. ACKNOWLEDGEMENTS

Authors thank Asahi Glass Foundation for financial support.

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Akami et al., Embolism development observed with a compact MRI in Japanese black pine clones resistant to pine wilt disease. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 110-111, Braunschweig, ISSN: 1866-590X

(127) Embolism development observed with a compact MRI in Japanese black pine clones resistant to pine wilt disease

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1. INTRODUCTION

The symptom development of pine wilt disease is divided into two stages, the early and advanced stages. In the early stage, pinewood nematodes (*Bursaphelenchus xylophilus*) mainly disperse in cortical and xylem resin canals, then induce parenchyma denaturation which cause xylem embolism partially. In the advanced stage, nematode population drastically increase, which coincides with cambial destruction and embolism development in the whole stem. Symptoms observed in resistant pines, which were selected in national projects, don't progress to the advanced stage in general. However, their resistance shows great variation among families or clones, which have been ranked from grade 1 (low) to 5 (high). In this study, to clarify the symptom development in detail, we examined embolism development in resistant pine clones inoculated with nematodes.

2. MATERIALS AND METHODS

Potted Japanese black pine (*Pinus thunbergii*) clones, two resistant (Tosashimizu 63 (grade 4), Oita 8 (grade 1)) and two susceptible (Kashima 2, Futaba 1) varieties, were used in this study. Virulent (S10) or avirulent (C14-5) isolates of pinewood nematodes reared on the fungus (*Botrytis cinerea*) were inoculated into 1-year-old stem of each seedling. Embolism development was observed in multi-cross-sectional slices taken with a compact MRI. At 16 positions along the stem at 1 cm intervals from 5 cm above to 10 cm below the inoculation point (0 cm), embolisms were monitored periodically.

3. RESULT

Xylem embolism only occurred around inoculation site in all clones inoculated with avirulent nematodes. On the other hand, in the seedlings inoculated with the virulent nematodes, embolism occurred beyond the inoculation site even in the resistant clones,

but it enlarged more moderately in the resistant clones than in the susceptible ones. The pattern of the embolism development in the highly resistant clone (Tosashimizu 63) spread from outer to inner xylem along ray tissues regardless of distance from the inoculation site, while in the lower resistant (Oita 8) and the susceptible (Kashima 2, Futaba 1) clones, xylem embolism was mainly enlarged as a cluster from the inoculation site verticality and horizontally (Figure 1).

4. DISCUSSION

Avirulent nematodes did not induce embolisms in xylem except the inoculation sites even in susceptible clones, while virulent isolate induced embolism in resistant clones. This indicates that some numbers of virulent nematodes can migrate in xylem resin canals even in highly resistant clones. However, in the highly resistant clone, nematodes did not cause a mass embolism around the inoculation site. This may suggest that proliferation of nematodes around the inoculation site was inihibited in these seedlings.

In conclusion, restricted development of mass embolisms corresponded to the suppression of symptoms development to the advanced stage in resistant pine clones.

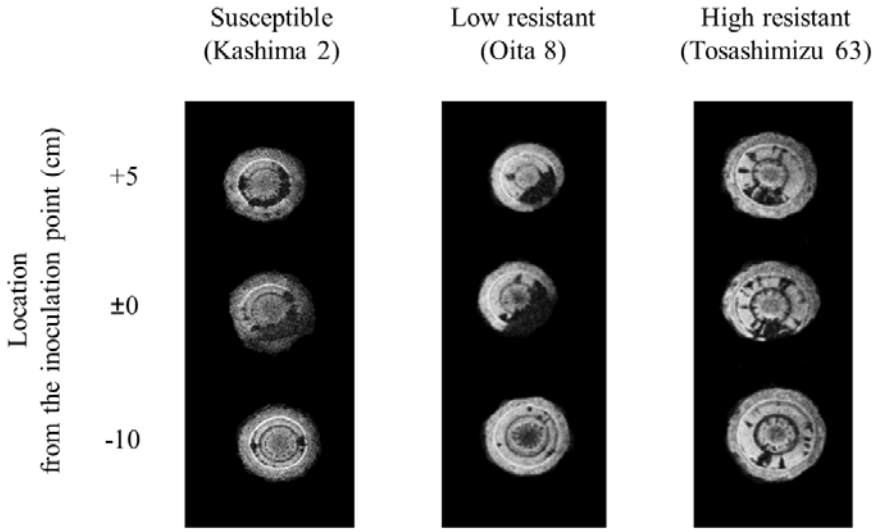

Figure 1. Embolism pattern in pine clones observed with a compact MRI

Liu F Y, Chen F M, Xie L Y, Ye J R, Tan J J, Analysis of genetic diversity of *Bursaphelenchus mucronatus* and *B. xylophilus* isolates based on ISSR markers. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 112-114, Braunschweig, ISSN: 1866-590X

(131) Analysis of genetic diversity of *Bursaphelenchus mucronatus* and *B. xylophilus* isolates based on ISSR markers

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ABSTRACT

Nine of 130 primers were collected by using PCR screening. The nine primers were used to amplify the genomic DNA of 11 *Bursaphelenchus mucronatus* parasitic groups from six provinces and of 10 *B.xylophilus* groups from 10 provinces. A total of 1111 clear strips were amplified with 1069 polymorphic strips, which reached a percentage as high as 96.2%. Primer PTY1424 amplified the maximum number of bands, whereas primer PTY888 amplified the least bands. The results showed that the genetic similarity coefficient range of the 21 nematodes was from 0.4014085 to 0.9436620. The DNA molecular dendrogram of these 21 groups were established through the un-weighted pair group method with arithmetic average (UPGMA) cluster analysis. The 11 *B.mucronatus* groups were gathered as one category when the similarity coefficient value was 0.715, whereas the 10 *B.xylophilus* groups were gathered as one when the coefficient was 0.755. However, the 21 groups divided into two species when the similarity coefficient was 0.52. No obvious difference was observed in the geographic relationship among the clustering results. The results of the inter simple sequence repeat (ISSR) markers can be used to effectively distinguish the genetic relationship between *B.mucronatus* and *B.xylophilus* groups. Based on the result of PCR, ISSR produced higher polymorphism on *B.mucronatus* as compared with *B.xylophilus* groups. The reason may be that compared with *B.xylophilus*, *B.mucronatus* are native species that have undergone geographical isolation for a long time. Further study is required to explain whether the genetic diversity of *B.mucronatus* is related to their pathogenic differentiation.

Key words: *Bursaphelenchus mucronatus*, *B.xylophilus*, genetic diversity, ISSR

INTRUDUCTION

Recent research supported that *B.mucronatus* has pathogenicity to pine trees, especially to those with adversity stress(Chen *et al* 2010; Zhang *et al* 2002, 2004). In this study, ISSR

was used to explore the genetic diversity between different *B.mucronatus* and *B.xylophilus* groups.

MATERIALS AND METHODS

Nematode sources: 11 *B.mucronatus* groups from 6 provinces and of 10 *B.xylophilus* groups from 10 infected areas of China.

PCR amplification primer: 100 primers (UBC800-UBC900) and 30 PTY primers.

Detection of PCR products: QIAxcel was used to detect PCR products.

CONCLUSION AND DISCUSSION

11 *B.mucronatus* groups and 10 *B.xylophilus* groups were analyzed by using the nine primers of ISSR. The results obtained 1111 amplified DNA fragments with the size ranging from 200 to 2000 bp (Fig.1). The band spectrum of these primers could clearly distinguish the geographical population of the 21 nematodes. Compared with *B.xylophilus* groups, *B.mucronatus* groups have more varieties. Considering the origin of these two kinds of nematode, *B.mucronatus* are widely distributed in Europe and Asia, whereas *B.xylophilus* originated in North America. *B.xylophilus* began to spread in Japan in the early 20th century, and to China in the 1980s. In China, *B.mucronatus* are native species, which have undergone geographical isolation for a long time. Such long isolation may be proven by their larger genetic variation. By contrast, *B.xylophilus* have spread to China for a short time, leading to higher genetic similarity between groups.

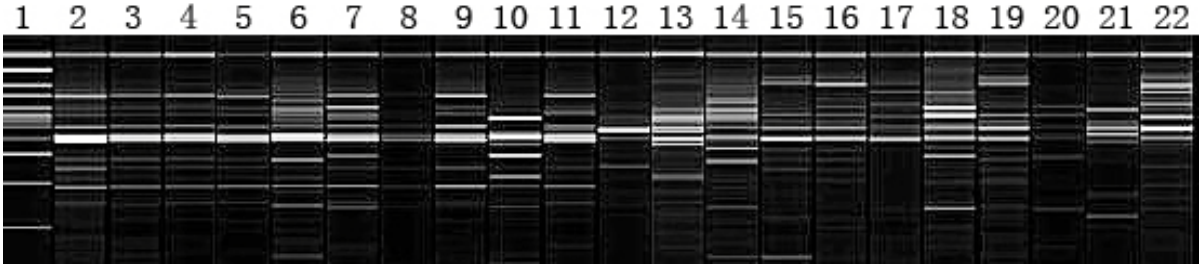

Fig.1 PCR-amplifiedfragments of 21isolated nematodes with primer PTY1425
1：marker；2-11:*B.xylophilus* groups, 12-22: *B. mucronatus* groups

ACKNOWLEDGEMENT

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(132) Studies on non-vector transmission of Bursaphelenchus xylophilus on Pinus pinaster.

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Since the pinewood nematode (PWN), *Buraphelenchus xylophilus* was regulated as a quarantine pest questions have been raised concerning the possibility of nematode spread with means other than the vector beetles *Monochamus* spp. In the REPHRAME-project these concerns are addressed in Work Package 5. In Portugal experiments on non-vector transmission of PWN to maritime pine *Pinus pinaster* have been started. Studies on root transmission in the field are carried out in the Lisbon area and are located at Herdade da Comporta, Companhia das Lezirias and Mata da Machada. Here, PWN transmission from nematode-inoculated adult trees (50 000 ind/tree) to covered undergrowth trees will be studied. In an outdoor greenhouse facility root transmission of PWN is studied on 7-years-old trees. The experiment includes 60 trees potted in pairs in 30 containers, where 1 tree in each pair is inoculated with 6 000 PWN and the spread of PWN from one tree to its neighbor is followed. Transmission of PWN from boards to trees is studied in the field. Infested boards (n=5) and nematode-free boards (n=5) will be tied to trees with intact bark, to trees with exposed cambium and to trees with exposed xylem. Transmission from infested chips to trees will be studied in the outdoor greenhouse facility on 7-year-old potted trees, with chips placed on top of soil or in contact with roots. In both situations there will be a treatment with intact and a treatment with artificially wounded roots.

Keywords: Non-vector transmission, *Bursaphelenchus xylophilus*, *Pinus pinaster*, Portugal.

Berkvens N et al., *Bursaphelenchus xylophilus* does not occur in Belgium, but what about its vectors, the *Monochamus* spp.? In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 116-117, Braunschweig, ISSN: 1866-590X

(138) *Bursaphelenchus xylophilus* does not occur in Belgium, but what about its vectors, the *Monochamus* spp.?

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INTRODUCTION

It is unclear if pine wilt disease (PWD) threatens the conifer forests of Belgium. Entry and presence of *Bursaphelenchus xylophilus* (PWN) are monitored by the Belgian NPPO (the Federal Agency for the Safety of the Food Chain, FASFC), but the nematode was never found. However, knowledge about the presence of its vector, *Monochamus* spp., in Belgium is lacking. Single specimens of *M. galloprovincialis* and *M. sartor* were reported in Belgium on 7 occasions (Anonymous, 2013). The origin of these beetles (endemic or imported) is unknown. It is essential to gather more information about *Monochamus* spp. in Belgium to assess the risk of PWD.

1. MATERIALS AND METHODS

From 2000 until today, FASFC collected suspicious samples during phytosanitary controls and national surveys in pine stands, public green areas, and logging and wood processing facilities. Imported packaging material suspected of insect attack were sampled also. An average of 170 wood and bark samples were analysed yearly at the Institute for Agricultural and Fisheries Research (ILVO). All procedures were according to the EPPO Standard PM 9/1(5) (EPPO 2012).

1. RESULTS AND DISCUSSION

No specimen of *B. xylophilus* were detected in the 72 and 108 samples analysed in 2000 and 2001, respectively (De Wael *et al*. 2002), nor in the following years (Table 1). Only two living *Monochamus* individuals (adult and larva) were intercepted.

Table 2. Results of phytosanitary controls and national surveys in Belgium for *Monochamus* spp. (packaging wood from import) and *Bursaphelenchus xylophilus*; n.d.: not determined

	2004	2005	2006	2007	2008	2009	2010	2011	2012
Nematodes									
Total number of samples	106	90	123	239	251	213	200	178	143
(samples of imported materials)					(25)	(55)	(51)	(96)	(96)
Samples with *B. xylophilus*	0	0	0	0	0	0	0	0	0
Import with *Laimaphelenchus* spp.	n.d.	n.d.	n.d.	n.d.	1	0	0	4	1
Import with *Aphelenchoides* spp.	n.d.	n.d.	n.d.	n.d.	0	0	3	17	42
Import with other saprophytic nem.	n.d.	n.d.	n.d.	n.d.	16	49	43	68	45
Insects									
Total number of samples	13	2	9	1	11	4	7	6	8
Samples containing *Monochamus* spp.	0	0	0	0	0	1	1	0	0
Samples containing other longhorn spp.	2	0	0	0	1	1	0	0	0
Samples containing other insects	2	1	2	1	2	0	0	0	4

However, live saprophytic nematodes, including genera closely related to PWN (*Aphelenchoides* spp. and *Laimaphelenchus* spp.), as well as some live beetles belonging to the Bostrichidae and Cerambycidae (*Anoplophora glabripennis, Xylotrechus rufilius* and *Phoracantha semipunctata*) were detected on several occasions on imported materials.Their presence in imported wood and bark can indicate insufficient treatment of imported wood material using heat or fumigation (EPPO Standards PM 10/6 and10/7). The consequences of an introduction of PWN are unclear due to a lack of knowledge about the *Monochamus* spp. in Belgium. This essential information will be gathered in a three-year project of ILVO and the Biological Control and Spatial Ecology Lab (LUBIES), in cooperation with FASFC.

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Li Sheng Nan et al., Construction of Engineering Bacterium Expressing Flagellin of *Pseudomonas fluorescens* and its Toxicity to *Pinus thunbergii* in Vivo. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 118-119, Braunschweig, ISSN: 1866-590X

(139) Construction of Engineering Bacterium Expressing Flagellin of *Pseudomonas fluorescens* and its Toxicity to *Pinus thunbergii* in Vivo

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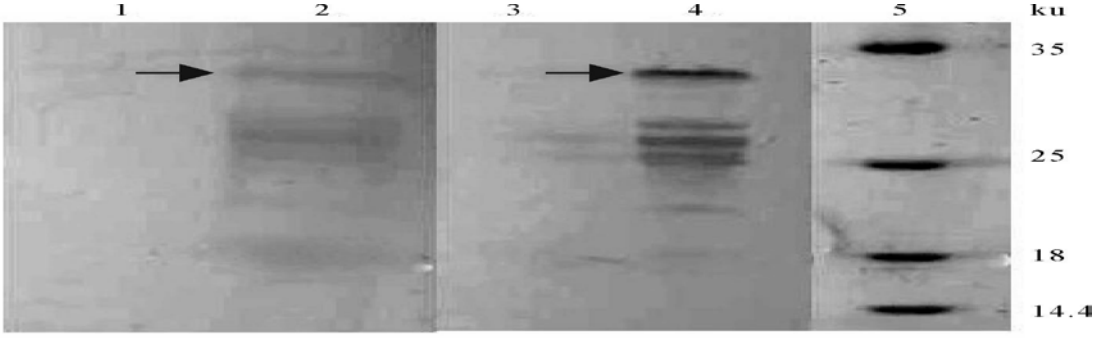
A constitute and secreting expressing plasmid pUC18ompA was constructed. The gene fliC encoding flagellin of *Pseudomonas fluorescens* Pf-5 was cloned into this plasmid to construct pUC18ompA-fliC. The plasmid was transformed into *E. col i* BL21 (DE3) to construct engineered bacteria.



1 supernatant of *E. c oli* BL21 (DE3); 2 supernatant of the engineered bacteria ; 3 Proteins of *E. c ol i* BL21 (DE3); 4 Proteins of the engineered bacteria; 5 Marks of the standard proteins

Fig. 1 Western blotting of the proteins in the engineered bacteria

Bacterium-free seedlings of P inus t hunbergii were i noculated wit h a mixture of t he engineered bacteria and the aseptic pine wood nematodes (*Bursaphelenchus xylophilus*) to determine it s pathogenicity. The results of inoculation showed that inoculation with a mixture of engineered bacteria and aseptic pine wood nematodes also caused wilt of pine seedlings to some extent . T he important role of flagellin played in vivo in pathological process was further verified.

Table 1. The wilting rate of the inoculated seedlings and re-isolation of the namatodes and bacteria

Treamtment	wilted seedlings within 7 days	Re-isolation of the inoculated bacteria		Re-isolation of the inoculated nematodes	
		Frenquence	Species	Frenquence	Species
Bx	20/ 20	20/ 20	*P. fluorescens* etc.	20/ 20	*B. xylophilus*
ABx	0/ 20	0/ 20	–	20/ 20	*B. xylophilus*
ABx+ *E. coli*	0/ 20	20/ 20	*E. coli* BL21(DE3)	16/ 20	*B. xylophilus*
ABx + EB	12/ 20	20/ 20	the engineered bacteria	20/ 20	*B. xylophilus*
ABx+ Pf	16/ 20	16/ 20	*P. fluorescens*	16/ 20	*B. xylophilus*
E. coli	0/ 20	0/ 20	–	0/ 20	–
Pf	0/ 20	0/ 20	–	0/ 20	–
EB	0/ 20	0/ 20	–	0/ 20	–
CK	0/ 20	0/ 20	–	0/ 20	–

Bx: non-sterilized nematodes; ABx: Sterilized nematodes; *E. coli*: *E. coli* BL.21 (DE3); EB: the engineered bacteria; ABx+ *E. coli:* the mixture of sterilized nematodes and *E. coli* BL.21 (DE3); ABx+EB: the mixture of sterilized nematodes and the engineered bacteria; Pf: *Pseudomonas fluorescens*; ABx+Pf: the mixture of sterilized nematodes and *Pseudomonas fluorescens:* Ck: sterilized water control; "-": neither bacteria no nematodes were re-iosolated.

Cardoso JMS et al., Gene silencing in *Bursaphelenchus xylophilus*: knock down of a calponin gene and its effect on nematodes movement. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 120-122, Braunschweig, ISSN: 1866-590X

(140) Gene silencing in *Bursaphelenchus xylophilus*: knock down of a calponin gene and its effect on nematodes movement

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INTRODUCTION

Post-transcriptional gene silencing by RNA interference (RNAi) was first discribed in Caenorhabditis *elegans* (Fire *et al*. 1998) and occurs when double-stranded RNA (dsRNA) is recognized by an organism as foreign, trigering a chain of processes in which both ds RNA and its mRNA homolog are degraded preventing the synthesis of the encoded protein. RNAi became an established experimental technique to investigate the function of different genes and its application and efficiency in the pinewood nematode, *Bursaphelenchus xylophilus*, function genomics has also been studied (Park *et al*. 2008; Cheng *et al*. 2010; Kang *et al*. 2011; Li *et al*. 2011; Ma *et al*. 2011; Kang *et al*. 2012; Wang *et al*. 2012). There are also some evidences that RNAi can be used to confer resistance to engineered host plants that express dsRNA to target and silence specific nematode genes (Lilley *et al*. 2012).

New ways for the manegement of *B. xylophilus* are needed and gene silencing by RNAi is a potencial strategy. The calponin gene (*unc-87*), in *C. elegans*, is required to maintain the structure of myofilaments in muscle cells of the body wall (Kranewitter *et al*. 2001). In the present study, the knock down of the calponin homolog *Bx-unc-87* was performed by RNAi to evaluate the role of this gene in *B. xylophilus* and the applicability of *Bx-unc-87* silencing as a control strategy for this nematode.

MATERIALS AND METHODS

The knock down of the *B. xylophilus* calponin homolog gene was carried out by soaking the nematodes in a solution containing dsRNA of the *Bx-unc-87* gene during 24 h. Afterwards, the phenotype of the nematodes was estimated by mobility and nematodes reproduction. The relative *Bx-unc-87* transcript abundance, after dsRNA treatment, was assessed by RT-PCR with SybrGreen using the ABI PRISM 7900 HT Fast System (Applied Biosystems) and the Comparative C_T ($\Delta\Delta C_T$) method.

RESULTS

The dsRNA treated nematodes revealed some paralysis and uncoordinated movement in contrast to the regular and sinusoidal movement of the non-treated nematodes and reproduction was lower in treated nematodes. The reduction in the *Bx-unc-87* transcript abundance confirmed the effectiveness of *Bx-unc-87* gene knock down. Further studies are being conducted in order to improve the efficiency of the silencing effect.

ACKNOWLEDGMENTS

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(142) Comparative analysis of MspI satellite repeats of the pinewood nematode, *Bursaphelenchus xylophilus*, at different geographic scales

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The MspI satellite DNA (satDNA) family of the pinewood nematode Bursaphelenchus xylophilus is herein analyzed in an attempt to understand the intraspecific variability at different geographic scales. A total of 425 MspI monomer units, either PCR-amplified from isolates of local (Peninsula of Setúbal, Portugal) or worldwide origin, or retrieved from the B. xylophilus genome sequence, were characterized and compared. Whatever their origin, sliding window analysis of sequence variability patterns among monomers revealed low, moderate and highly variant domains, indicating that variable levels of evolutionary constraint may act upon the entire monomers. The phylogenetic inference based on the different sets of MspI satDNA family for this species shows a broad polymorphism of the individual monomers, which were distributed into four main clusters. However, such clustering appeared independent from the geographic origin of the nematodes, and could not discriminate isolates or groups of geographically close isolates. Rather, the formation of different phylogenetic groups within this satDNA family suggests an a priori embodying of a set of diverging repeats from a common ancestor satDNA library, which have been differently amplified along the evolutionary pathway of this species. The present work improves knowledge on the evolutionary dynamics of satDNA at the intraspecific level, and provides new information on satDNA sequence variability among natural populations sampled at a local geographic scale.

Abrantes I et al., Physiological responses to water stress and temperature on the pine wilt disease development in *Pinus* spp. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 124-126, Braunschweig, ISSN: 1866-590X

(143) Physiological responses to water stress and temperature on the pine wilt disease development in *Pinus* spp.

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ABSTRACT

Four to five-years-old *Pinus pinaster*, *P. pinea* and *P. radiata* trees were inoculated with 6000 pinewood nematodes (PWN) and symptoms evolution, trees physiological responses and PWN population densities were assessed under high and low water availability conditions, at 25 and 30ºC. Pine wilt disease symptoms were observed in all infected *P. pinaster* and *P. radiata* leading to a decrease in photosynthetic activity and water potential values, under the highest temperature and low water availability. *Pinus pinea* did not develops symptoms and no significant changes in physiological status were detected. Nematodes were found in higher numbers, in high temperatures and low water availability, in *P. pinaster* followed by *P. radiata* and *P. pinea*.

INTRODUCTION

After entering into the host tree, the pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, multiply intensively and migrate throughout the plant. During this process, the blockage of xylem water conduction by tracheid embolisms causes high decrease in leaf water potential and cessation of photosynthesis (Kuroda 2008). To develop proper control methods, based on early diagnosis of the disease, is important to understand the physiological responses and internal changes of infected hosts pecies. The main objectives of this study were to understand the effect of water stress and temperature on PWN development and to evaluate the photosynthetic activity and water potential values of PWN infected *P. pinaster*, *P. pinea* and *P. radiata*.

MATERIALS AND METHODS

A total of 120 four to five-years-old trees (40 trees/*Pinus* species) were grown in a greenhouse under high and low water availability conditions at 25 and 30ºC. Trees were inoculated with 6000 PWN and trees inoculated with sterilized water were used as controls. Predawn xylem pressure potential of needles was measured using a Scholander pressure chamber and photosynthetic and transpiration rates of needles were taken, twice a week, using a portable infra-red gas analyzer (GFS-3000, Walz) equipped with a red led light source. Symptoms development was followed for 50 days and classified in six stages based on the wilting and consequent discoloration of the needles according to Proença *et al*. (2010). At the end of the experiment, the trees were cut and the final PWN population was estimated, in each tree, at the branches, trunk, roots and soil.

RESULTS

Pine wilt disease symptoms (PWD) were observed in all infected *P. pinaster* and *P. radiata* leading to a decrease in photosynthetic activity and water potential values, under the highest temperature and low water availability. *Pinus pinea* did not develop symptoms and no significant changes in physiological status were detected. Nematodes were found, in higher numbers, in the highest temperature and low water availability, in *P. pinaster* followed by *P. radiata* and *P. pinea*. In *P. pinaster* and *P. radiata*, nematodes were detected in all PWN inoculated trees, at the branches, trunk and roots while in *P. pinea* they were detected only in four trees, at the branches and trunk. Pine species reacted differently to PWN, water stress conditions and temperature, enhancing the development of the PWD, which may have implications on the enlargement of infected area and on the shortening period of PWD development under climate change scenarios.

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(144) Improved key to the species of the *xylophilus* group of the genus *Bursaphelenchus* Fuchs, 1937

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A correct determination of the pine wood nematode becomes more difficult, the more related or similar species are described. In view of the fact that a further spread of the nematode should be considered and continuously new *Bursaphelenchus* species are detected, identification keys must be adjusted accordingly. Since the publication of a dichotome morphological key for species of the *xylophilus* group of the genus *Bursaphelenchus* (Braasch, 2008), several new species of this group have been described. These species are *B. macromucronatus* Gu, Zheng, Braasch & Burgermeister, 2008; *B. populi* Tomalak & Filipiak, 2010; *B. firmae* Kanzaki, Maehara, Aikawa & Matsumoto, 2011; *B. paraluxuriosae* Gu, Wang, Braasch, Burgermeister & Schröder, 2012; *B. koreanus* Gu, Wang & Chen, 2013 and *B. gillanii* Schönfeld, Braasch, Riedel & Gu, 2013. Based on the key published by Braasch (2008) and the revised intrageneric grouping of *Bursaphelenchus* by Braasch *et al.* (2009), an improved dichotome key including in the meantime newly described species is presented. The decision-making is supported by pictorial representation of important features.

Two species found in aspen partially share characters with the *xylophilus* group and are not considered in the key: *B. trypophloei* Tomalak & Filipiak 2011 and *B. masseyi* Tomalak, Worrall & Filipiak, 2013, which can easily be separated from the *xylophilus* group species by spicule morphology, having shorter condylus and rostrum.

Characters useful for identification of *xylophilus* group species are female tail shape (cylindrical, subcylindrical or conoid, round-tailed or digitate, with or without mucro), shape and size of spicules, several measurements and ratios (a, c') and position of excretory pore with a certain variation of the last character.

The recognition of the harmful quarantine pest *B. xylophilus* using morphological characteristics in laboratories of the National Plant Protection Services is facilitated by an adapted and simplified key, which separates species clearly distinguishable from *B. xylophilus* without species identification and includes only two very similar species. Molecular methods are additionally advisable to confirm a morphological diagnosis.

Fonseca L *et al.*, Vacuum pressure impregnation for the elimination of the pinewood nematode from pine wood. In: Schröder, T. (ed.), Pine Wilt Disease Conference 2013, pp. 129-130, Braunschweig, ISSN: 1866-590X

(147) Vacuum pressure impregnation for the elimination of the pinewood nematode from pine wood

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ABSTRACT

In order to evaluate the efficiency of the vacuum pressure impregnation with chemical preservatives in the elimination of the pinewood nematode (PWN) from wood, naturally PWN infected *Pinus pinaster* sections were introduced into a wood treating autoclave and exposed to the vacuum and pressure impregnation with the wood preservative TANALITH® E NB. After treatment, wood sections were removed and left at room temperature to dry and the total number of nematodes was quantified after incubation at 25ºC for 15 and 30 days. No nematodes were detected in the treated wood sections after the incubation periods. These preliminary results revealed a potential use of this process to eliminate PWN from wood.

INTRODUCTION

The introduction of several invasive alien plant pests into non-native areas led to the development of appropriate phytosanitary measures against the introduction and spread of these species. Since wood material was recognized as one of the most important pathways for introductions of forest-related pests, the International Plant Protection Convention adopted the International Standard for Phytosanitary Measures No. 15, which serves as a guideline for the regulation of wood packaging material used in international trade (FAO 2009). The use of pinewood material for agricultural commodities and industrial items, has increased significantly as global trade has developed over recent decades. This has resulted in an increased risk of movement of non-native pest species on wood used in international transport. This is the case of the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. In this study, we have evaluated the efficacy of vacuum pressure impregnation with chemical preservatives to eliminate PWN from wood.

MATERIALS AND METHODS

Pinewood nematode infected *Pinus pinaster* trees were felled and trunk sections were cut. Initial nematode population was estimated by cutting 5 cm segments of each end of the trunk sections. Nematodes were extracted using the tray method (Whitehead & Hemming 1965) and the total PWN number was estimated. Wood moisture content (WMC) was measured using a digital wood moisture meter. Trunk sections with less than 25 % WMC and containing more than 100 000 PWN (›60% third dispersal juvenile stage) (Magnusson & Schröder 2009) were selected and introduced into a wood treating autoclave and exposed to the vacuum and pressure impregnation with the wood preservative TANALITH® E NB plus the additive AC 3744 following the subsequent treatment scheme: i) initial vacuum with -950 mbar for 25 min; ii) autoclave filling with the preservative solution at 12500 mbar for 75 min; iii) emptying of the remaining solution to the autoclave support tank and iv) final vacuum to remove the excess preservative solution on the surface of the wood at -800 mbar for 8 min. After treatment, wood sections were removed and left at room temperature to dry and then, incubated at 25ºC for 15 and 30 days to allow any live nematode present to breed and maximise the likelihood of detection (EPPO 2013). Nematodes extraction was performed by the tray method.

RESULTS

In the treated trunk sections, 100% nematode mortality was achieved in all post-treatment assessment incubation periods (15 and 30 days). These preliminary results revealed a potential use of this process to eliminate PWN from wood.

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(150) Pine wood nematodes - as a factor of pine decline in Croatia

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In recent years significant decline of pine trees of different species, age, size and position in the forests of Northern Dalmatia (Croatia) has been recorded.

Climatic extremes, especially drought, can be considered the basic adverse factor causing stress and physiological weakening of pine trees and simultaneously improving the conditions for attacks of various types of pests. Analyzing several biotic factors associated with climate extremes shows presents of Pine Processionary Moth, longhorn beetles, bark beetles, needle cast disease caused by fungus but also pine wood nematodes.

So far it is not possible to determine the scope of impact of wood pathogenic nematodes in the chain of pine die back without further studies. 20 samples of *Pinus nigra*, *Pinus pinaster* and *Pinus halepensis* were collected at 6 locations along the coast of Northern Dalmatia. The first results indicate the presents of several groups of nematodes that leave in wood *Aphelenchoides* spp., *Laimaphelenchus* spp., *Ditylenchus* spp., saprofitic nematodes *Cephalobus* spp., *Rhabditis* spp. and *Plectus* spp., nematodes on insects in order *Neotylenchidae* and *Diplogasteride,* as well as species of genus *Bursaphelenchus*: *B. mucronatus*, *B. sexdentati*, *B. eggersi*, *B. minutus* of witch two first ones are considers as pathogenic. In addition, nematode vector *Monochamus gal loprovincialis* has been determined which may play an important role in possible occurrence of quarantine species of *Bursaphelenchus xylophilus*.

Keywords: pine decline, climate extremes, bark beetles, pine wood nematodes, *Bursaphelenchus* spp.

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(162) The use of PCR-HRM technique for detection of the quarantine nematode *Bursaphelenchus xylophilus*

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INTRODUCTION

High Resolution Melting Analysis (PCR-HRM) is a recently developed technique for fast, high-throughput post-PCR analysis of genetic mutations or variance in nucleic acid sequences. It enables researchers to detect rapidly and categorize genetic mutations, identify new genetic variants without sequencing, or determine the genetic variation in a population prior to sequencing (Pasay *et al*., 2008).

We have examined this technique for detection and distinguishing of the quarantine pest, *B. xylophilus* from the morphologically and genetically most similar nematode *B. mucronatus.*

MATERIALS AND METHODS

Genomic DNA of *B. xylophilus* (isolate China) and *B. mucronatus* (isolate Wro-01) (about 100 nematodes) was isolated with QIAamp DNA Micro Kit (QIAGEN) according to the protocol provided by the manufacturer. For each species the isolation was conducted separately. The specific primers were designed from the ITS-1 region (forward: 5’ - CGTGCAACGGTAAAGTCTGGGTTT-3’ and reverse 5’-AATCCTACGCTCGCCAGAACGAAT-3’) (Fig. 1). The PCR product was expected to be 112 bp in length. The PCR-HRM assays were performed with the use of Rotor–Gene Q T hermocycler (Qiagen). The obtained data was analysed according to the manufacturer’s instructions.

A

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            ....|....| ....|....| ....|....| ....|....| ....|....|
                   160        170        180        190        200
BmWro-01    CGCACGGAAG CCGAGAGGTG ACCGTGCAAC GGTAAAGTCT GGGTTTCTAT
Bx          CGCATGGAAG CCGAGAGGCG ACCGTGCAAC GGTGAAGTCT GGGTTTCTAC
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B

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            ....|....| ....|....| ....|....| ....|....| ....|....|
                  260        270        280        290        300
BmWro-01    GCTGCTTGCC GATTCGTTCT GGCGAGCGTA GGATTGAAAA GCCCGAGCGG
Bx          GCTGCTTGCC GATTCGTTCT GGCGAGCGTA GGATTGAAAA GCCCGAGAGG
```

Fig. 1. Diagrams indicating differences in sequence alignment of the forward primer (A) and reverse primer (B) between *B. xylophilus* and *B. mucronatus*.

RESULTS AND DISCUSSION

The study conducted with the designed primers allowed us to distinguish and identify two morphologically similar nematode species, *i.e. B. xylophilus* and *B. mucronatus*. Melting curve analysis of the reaction products showed the presence of a single amplification product for each of the performed reactions (Fig. 2). The graph shows mean values from four replicates of each reaction.

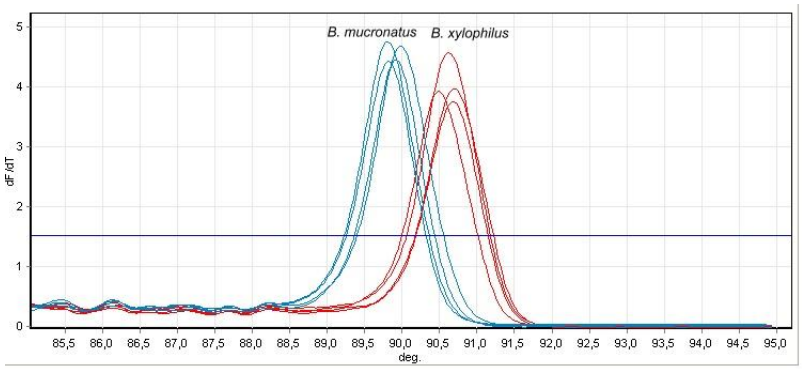

Fig. 2. Melting curves of PCR-HRM products*: B. xylophilus* and *B. mucronatus*. The vertical axis shows the fluorescence value (dF/dT), and the horizontal axis the value of the melting temperature (Tm) (°C).

The normalized DNA melting curves obtained in the HRM analysis differed from each other in denaturation temperature, as evidenced by a substantial shift of these curves in relation to each other. This indicates the differences between examined species in the composition of nucleotides within the amplified region of the genome. The analogous differences are also evident in the normalized differentiating graph (Fig. 3).

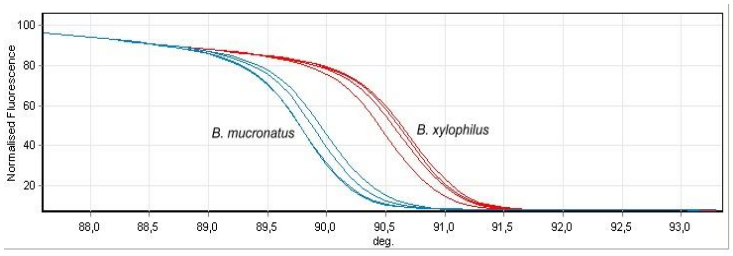

Fig. 3. Normalized differentiating graph of PCR-HRM products for: *B. xylophilus* and *B. mucronatus*.

Conducted study confirmed the high efficiency of the designed primers to distinguish the quarantine nematode *B. xylophilus* from the most closely related *B. mucronatus*. Since the PCR-HRM reaction allows for detection of single changes in nucleotides in tested of PCR products, it seems to be a very promising method for supporting identification decisions in the case of closely related species and isolates which could give ambiguous results in the real-time PCR reaction. Compared to the other molecular methods, PCR-HRM technique can be much simpler and less expensive way to identify the quarantine nematode *B. xylophilus.*

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(164) Modelling of potential spread of Pine Wood Nematode by natural means in Slovenia at present climate conditions and in light of predicted climate changes

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ABSTRACT

Pine wood nematode (PWN), *Bursaphelenchus xylophilus*, threatens economy and biodiversity of Slovenian forest. A model was developed to simulate natural means of spread of PWN from three (3) potential entry points to Slovenia. The stochastic model of natural spread was developed on a 1 × 1 km grid. The model is based on real data, such as host tree density, susceptibility of each conifer species to PWN infestation, suitable temperature for PWN development, and appearance of the drought stress. Modelling is performed on the assumption that *Monochamus* spp. vectors are present in all Pine tree growing areas and that their maximum annual spread is 3 km. Simulation results show that if no containment measures are applied, at present climate conditions PWN would be spread almost over the entire Pine forest-covered and temperature appropriate territory of Slovenia in average of approximately 200 years. The extent and speed of PWN spread differ depending on entry point and climate conditions. The modeling was simulated also using a medium scenario of predicted climate changes for two periods: 2021–2050 and 2061–2090. At present environmental conditions, 8.954.000 m^3 of *Pinus* spp. growing stock is endangered. The model clearly demonstrated that the spreading of PWN is relatively slow by the natural means. The human factor is the most critical for rapid PWN spreading.

INTRODUCTION

PWN represents a serious threat to susceptible conifer trees by causing Pine wilt disease. By natural means the PWN is transmitted by vectors – beetles of genus *Monochamus*. The maximum annual distance of beetle movement is up to 3.3 km (Kobayashi *et al*., 1984). On long distance PWN is dispersed by human activity which increases the speed

of spread considerably. On the other hand it is difficult to predict such long distance jumps (Robinet *et al.* 2011). We modelled the spread of PWN in Slovenia by natural means only, considering different influencing factors, such as host tree density, susceptibility of each conifer species to PWN infestation, suitable temperature for PWN development, and appearance of the drought stress. International trade is the most likely pathway of PWN potential introduction so modelling was done for 3 different entry points: sea port, airport and a saw-mill.

MATERIAL AND METHODS

Spread of PWN was modeled in 1 x 1 km grid with cellular automata using rule of extended Moor's neighborhood. Probability for establishment of PWN in the cell was defined with temperature and drought stress while speed of dispersal was determined with maximal annual distance of beetle movement (3 km) and host tree density and susceptibility. Each factor was classified and their influence in the rule of PWN spread was defined. The drought stress in the model was defined as a point in time, when soil moisture decreases below 50% of plant available water and last at least for two consecutive months. The climate changes were simulated with general circulation models (Bergant 2007). The modelling simulations were performed in 300 replicates.

RESULTS AND DISCUSSION

Spread of PWN by natural means is relatively slow. The modeling results show that appropriate space for PWN would be infested in 200 years at present climate condition. The modeling was also simulated using a medium scenario of predicted climate changes (Bergant, 2007) for two periods, 2021–2050 and 2061–2090. At present environmental conditions, 8.954.000 m^3 of *Pinus* spp. growing stock is endangered. With climate changes endangered quantities of growing stock would increase to 14.464.000 and 18.577.000 m^3 for periods of 2021–2050 and 2061–2090, respectively.

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(167) Analysis of Transcriptional Expression Variation of Pine Wood Nematode (*Bursaphelenchus xylophilus*) using EST-based Simple Sequence Repeats

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ABSTRACT

EST-SSR marker was used to study the transcription variation of Pine Wood Nematode (PWN) *Bursaphelenchus xylophilus*. The results imply that there is very low transcriptional expression variation level of *B. xylophilus* in China, and Chinese *B. xylophilus* were more likely to be introduced from Japan.

INTRODUCTION

Pine wilt disease was first detected in Nanjing in 1982 and then it has spread to 176 counties of 15 provinces (SFA 2013) in China. The EST-SSR marker could reveal the population genetics of individual species based on exon sequence. Thus, EST-SSR was used to identify the differential expressed genes of *B. xylophilus* from different areas aiming at addressing the relationship between various genetic population and spread route in *B. xylophilus*.

MATERIALS AND METHODS

138 *B. xylophilus* DNA samples assorted randomly and mixed equally to 3 DNA pooling samples, which from 59 infected areas in 12 provinces of China. And 2 DNA pooling samples consist of 6 DNA samples from Japan and 3 from America respectively and one *B. mucronatus* DNA pooling sample including 63 DNA samples from different areas of China. ESTs of *B. xylophilus* which 13357 from NCBI and 884 from our laboratory were preprocessed by EST-trimmer, removed vector by cross-match, spliced by CAP 3, and searched SSR loci by MISA (Hu 2005). All the EST-SSRs were designed primers by Primer 5.0, and pre-screened with one Chinese *B. xylophlus* DNA pooling for PCR, after

that, re-screened with 6 DNA poolings. The result of products detected by QIAxcel automatic gel electrophoresis analysis system (Wang 2009), and NTSYS-pc 2.10e was used for UPGMA cluster.

DISCUSSION

A total of 14241 ESTs of *B. xylophilus* were spliced, and 6945 Uingenes (non-redundant ESTs) were obtained. 237 Unigenes contained 265 SSR loci, comprising 76 types of repeat motif, which account for 3.41% of all Unigenes. The average distance between SSRs was 12.14 kb. In the SSRs, the tri-nucleotide repeat motifs were the most abundant (64.53%), followed by tetra-nucleotide repeat nucleotide motifs (20.38%). Further, 189 primers were designed and synthesized based on the above motif types, then verified with 5 DNA pooling samples of *B. xylophilus* and one *B. mucronatus* DNA pooling sample for PCR. The result of products showed that the products of 130 primers are clear and effective in *B. xylophilus*, and 120 primers are versatile in *B. mucronatus*. All of EST-SSRs showed no polymorphism in *B. xylophilus* from China, including 101 homozygous loci and 29 heterozygous loci, which indicate the SSRs developed from the exons have no obvious differentiation. However, there are 8 pleomorphic EST-SSRs developed in *B. xylophilus* from America and Japan, and *B. mucronatus* from China respectively, which showed higher polymorphic content in *B. mucronatus*. Above all, transcriptional expression variation level of *B. xylophilus* is very low, after invading China 30 years. The result of UPGMA dendrogram supports the view that *B. xylophilus* was introduced into China from Japan.

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(171) The Dutch approach to Pine Wilt Disease (PWD): longhorn beetles and nematodes under surveillance

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Climatic conditions in the Netherlands are similar to those of the area of pine wood nematode (PWN) distribution in North America. Environmental conditions in North America are generally unsuitable for pine wilt expression (Evans *et al*. 1996). According to this study, below the summer isotherm of 20 degrees expression of pine wilt disease (PWD) is not expected. It is, therefore, presumed that infested trees in the Netherlands will not express symptoms. The absence of symptoms makes it difficult to design a survey on basis of early detection without analysing a large number of samples. We explored experiences and investigations of other European countries without conditions for PWD expression (Magnusson *et al*, 2007; Magnusson, 2009; Schröder *et al*, 2009), to clarify the possibilities for a survey strategy in the Netherlands.

Since the Netherlands are a major importer and exporter of products, the establishment potential of PWN (*Bursaphelenchus xylophilus*) is foremost determined by the possible introduction of infested longhorn beetles *Monochamus* in wood or wood packaging material (WPM). Therefore, the Netherlands opted for a similar strategy as other European countries where PWD symptomes are not to be expected. The monitoring of PWN and *Monochamus* in the Netherlands is mainly based on surveys at locations were wood and/or wooden products (bark, wood chips, lumber, packaging), originating in countries were PWN occurs, are imported or stored. Additionally surveys are carried out for the vector in the rest of the Netherlands.

Each year, our survey plan for the Netherlands consists of 7 parts, which are as follows:

1. On high risk locations *Monochamus* pheromone traps are used at the site itself. When longhorn beetles are found, they are analysed for the presence of nematodes. Dead or dying trees at a distance of 50 to 100 m are inspected for beetle activity. In collaboration with the European Invertebrate Survey (EIS-NL) at 10 selected locations with 3 pheromone baited traps each, the presence of *Monochamus* populations is monitored in nearby forests or groups of host plants (beyond 100 m).
2. Random survey of forest areas for the presence of *Bursaphelenchus* species in dead or dying trees.
3. Monitoring programme of wood packaging material originating in countries where PWN is known to occur.
4. Sampling and testing of coniferous wood (including chips, particles, shavings, etc.) originating in countries where PWN is known to occur.
5. Sampling and testing of coniferous bark originating in Portugal.
6. Monitoring and sampling of potted plants with coniferous bark used as mulch coverage originating in Portugal.
7. Inspection and sampling of plants for planting of *Chamaecyparis, Pinus* and *Juniperus* originating in Japan and South Korea, with reference to Commission Decisions 2002/887/EC and 2002/499/EC.

Results of our surveys from the last five years will be presented. PWN, *Bursaphelenchus xylophilus*, has not been found in the Netherlands so far, although it has been recorded once from imported wood. In imported bark only *B. fungivorus*, *B. minutus* and *B. sexdentati* have been found. Its vector, *Monochamus spp.*, has been found in imported wood package material or other wooden products. Surveys performed in The Netherlands show that a small population of *M. galloprovincialis* is persistent in a small pine wood forest near the coast (3x5 km), whereas it is absent in all other parts of the Netherlands.

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