IUFRO 2009

Official meeting of the IUFRO

Unit 7.02.10 Pine Wilt Disease

20 – 23 July 2009, Nanjing Forestry University, P.R. China

Conference Proceedings

Introduction

The International Symposium on Pine Wilt Disease, the official meeting of the Working Party 7.02.10, IUFRO took place on 20 - 23 July, 2009 in Nanjing Forestry University, China. The goal of the meeting was to promote the global exchange of information on research and control of the disease and to cope with its worldwide impact on environment and international trade.

In the beginning of the meeting three scientists, Dr. K. Futai, the vice coordinator of the Working Party 7.02.10, IUFRO, Dr. Bo Guang Zhao, the Chair of the meeting, and Dr. M. Mota, Associate Professor of University of Evora, were invited to give a Keynote speech. Furthermore 38 scientists who came from around the world gave talk on: Economic and International Trade, Impacts of Pine Wilt Disease, PWN: Systematics, diagnostics, PWN: Interactions with Other Microbes, Insect Vectors: Biology, Ecology and Modeling, Etiology and Epidemiology, Disease Management and Control respectively. 11 posters were exhibited during the meeting. 30 scientists came from Canada, Portugal, Germany, Russia, Korea and Finland and 43 from China. During the meeting a business meeting attended by the representatives from the main countries that suffer from the disease elected the new coordinators of the IUFRO unit Pine Wilt Disease: Dr. Thomas Schroeder (Germany), Dr. Shin Sang Chul (Korea); Dr. Christer Magnusson (Norway) and Dr. Katsunori Nakamura (Japan). The next PWD unit meeting is intended to be held in Europe and probably Germany.

Official meeting of the IUFRO Unit 7.02.10 Pine Wilt Disease 20 – 23 July 2009, Nanjing Forestry University, P.R. China

Conference Proceedings

Session 1 (papers)
Economical and international trade impacts of pine wilt disease

Session 2 (papers)
Systematics, diagnostics

Session 3 (posters)

Session 4

PWN: Interactions with other microbes

Session 1 (papers): afternoon, July 21:

Economic and international trade impacts of pine wilt disease

Current research and management of pine wilt disease in Korea

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Since pine wilt disease was first reported from Korea in 1988 it has spread throughout most of South Korea and caused major damage in Korean forests. Damage increased dramatically from 2000 onward and peaked in 2006 with 7,871 ha being affected. The total number of trees removed reached 1,851,225 between 2000 and 2008. The annual cost for pine wilt management is more than 10 million dollars and this has increased annually. However, the damaged area has slowly decreased after 2006 and amounted to 6,015 ha in 2008. By the end of 2008 the disease had spread to 54 districts, counties, and cities in 11 provinces. In Korea, pine (*Pinus* spp.), the most susceptible hosts of the disease, occupies 23.5% of the total forest area and 15.1% of the national land. In December, 2006, pine wilt was newly reported on Korean pine (*Pinus koraiensis*) at Gwangju in Gyeonggi province and we found that a new insect vector, *Monochamus saltuarius*, was involved. The management strategies are 1) continuous monitoring, 2) early detection of affected trees, 3) pruning off infected wood which harbours the vectors and 4) cutting, burning, or fumigation of the wood. For prevention, a trunk injection has been selected especially for protection of pines designated as cultural treasures or those in mature, healthy forests. Aerial spraying is also a way of prevention and it is done from May to July when the adult pine sawyer beetles emerge. The Korea Forest Service has established “a special law for pine wilt disease” to restrict the movement of infected trees and recently made the “Clean area selection system” which applies to those areas where there is no further report of infected trees for 3 years after the first occurrence. Various approaches for the management of pine wilt disease have contributed to diminishing the area affected by pine wilt disease in Korea.

Portuguese national action plan for pinewood nematode control: strategy, actions and results

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Since the detection in 1999 of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle et al. (PWN), in Portugal, in declining maritime pines (*Pinus pinaster*), that a set of measures aiming to control and eradicate the PWN and to control its vector, *Monochamus galloprovincialis* (Oliv.), have been implemented and have been incorporated into the National Eradication Programme to Control the Pinewood Nematode (PROLUNP).

Diverse strategic action plans were prepared and implemented which focus on diverse actions, namely surveying, eradication, monitoring, insect-vector control and inspection and control of forest operations, particularly the ones related to coniferous trees forestry operations.

Given the detection in 2008 of PWN in new pine forest areas, located in the Centre Region, considered before as a 'Free Zone', the continental territory survey activities were intensified and the territorial coverage increased. The results of this monitoring plan showed the presence of PWN in several Municipalities.

A new strategy was adopted and a new Action Plan was set up, in line with the EU emergency measures against PWN, adapted to the characteristics of the new affected areas and which takes into account the up to date related scientific knowledge to define adequate phytosanitary measures to control the PWN.

The current scientific knowledge supports the present control strategy employed in Portugal if implemented with efficiency and with the correct timings. Nevertheless, the existence of limitations to control this disease suggest that a more efficient management and control can only be achieved following new scientific studies and discoveries, which should be conducted not only in Portugal, but all over World.

A brief overview of the results will be presented.

Significance of the Pine Wood Nematode (*Bursaphelenchus xylophilus*) in international phytosanitary policy

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The pinewood nematode, *Bursaphelenchus xylophilus*, is identified by some countries as a quarantine pest in recognition of the serious pest risk that it presents to certain forest ecosystems. In response to concerns regarding the pest moving in association with wood products in international trade, work on risk mitigation measures has been ongoing since the mid-1980s. Research on heat treatment carried out in North America resulted in the recognition of the treatment schedule of 56˚ C for 30 minutes (wood core temperature) being accepted as a phytosanitary measure for pinewood nematode in wood products traded between North America and Europe. Subsequently this schedule formed the basis for heat treatment recommendations in ISPM No. 15, the International Plant Protection Convention (IPPC) standard, *Guidelines for regulating wood packaging material in international trade.* Heat treatment, as well as other treatments being evaluated by the IPPC is expected to reduce the risk from a wide range of pests moving with wood commodities. A number or representative bark and wood–inhabiting pests including *B. xylophilus* are being considered as test organisms to evaluate the effectiveness of treatment protocols. Treatment efficacy must be determined, but complete sterilization is not always necessary as systems approaches that include several phytosanitary measures can reduce pest risk to acceptable levels as defined by individual countries.

Studies on risk assessment, epidemic analysis, pathogen detection and host resistance to *Bursaphelenchus xylophilus*

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Pine wilt disease is listed as the fourth most serious forest disease in the world and it is also one of the most serious forest pests in China. The pine wilt pathogen, the pine wood nematode (PWN), *Bursaphelenchus xylophilus,* is an important international quarantine pest. The disease was first found in Japan in 1905 and in 1982 it was detected on the mainland of China at the Zhongshan Mausoleum, Nanjing, Jiangsu Province. So far the disease had been found affecting some 80 thousand hectares in 13 provinces. Besides affecting 3.33 millions hectares of pine trees there is an enormous ecological effect of the disease are great. The work reported here relates to risk assessment, epidemic situation analysis, pathogen detection and host resistance to the PWN using the new pine wilt epidemic in Hunan province as a model.

The main results and conclusions of the research are:

1. Pest risk analysis of the PWN in Hunan

A risk analysis for the PWN has been undertaken on the basis of the rule of PRA No. 11 [International Standards for Pest Measurements (ISPM)] and use of a pest assessment system and a synthesis index calculation measure. By qualitative and quantitative analysis, the value of the risk synthesis index for the PWN in Hunan is 2.56 and it has reached the most dangerous level for a quarantine pest in China. The strategy of the risk management for the PWN was also evaluated in this work.

2. The pine wilt disease was first found in Hunan

The epidemic situation for pine wilt in Hunan was initially based on the morphology of the PWN and molecular biology. The results of a survey showed the range and damage of the disease in six prefectures, and 12 counties.

3. Determination of the source of the PWN using bio-molecular techniques

The analysis results of rDNA-RFLP and DNA sequence for the PWN has shown that the pathogen is highly homogenous between the new occurrence area and old occurrence areas. As well, molecular biological evidence was initially provided for the spread of pine wilt disease in China.

4. Studies on the chemical inspection technology for pine wilt disease show:

Three quick-inspection methods for the PWN were studied by comparing wood containing PWNs and healthy pine wood. The results of the cellulase activity inspection showed that there were conspicuous differences between diseased and healthy wood. A method of inspection using benzoic acid revealed that light

absorption values did not differ between diseased and healthy wood. The results were inconsistent. The color reagent results have shown conspicuous differences between disease and healthy wood. The color of diseased wood with *B. xylophilus* varies from crimson to shallow red. Healthy wood remains a natural color while dead wood with *B. mucronatus* is uncolored or shallow white. The color reagent method was one of the best methods for detecting the PWN because it is simple to use and quick.

5. Studies on the detection technology of real-time fluorescent PCR for the PWN

Studies on the real time fluorescent PCR(ATF-PCR) method using a TaqMan probe (designed within rDNA-ITS2) were made to detect the PWN. The results of detecting mass amounts of DNA and single PWNs showed that a fluorescent signal was present for the PWN, but none for *B. mucronatus*. The BXP probe was highly specific for the PWN. The lowest concentration detected by the TaqMan probe was 1 pg/μl. The results of the DNA sequence and ATF-PCR detection were in agreement. This research provided a new detection method for the PWN.

6. Made a survey on the cause of wilt in a seed orchard of resistant pine and research on second generation resistance pine

In assembling material for a seed orchard of pine resistant to the PWN in Yamaguchi prefecture, Japan, the pine wilt rate of 25 clones of Japanese red pine and 16 clones of Japanese back pine were found to be 20.4% and 39.5% respectively. The PWN was the main cause of the wilt. The research showed that resistance of red pine was stronger than that of back pine and the different clones varied in their resistance. The death rate of susceptible Japanese black pine was 91.1% 3 months following inoculation with the PWN. The death rate of second generation, resistant Japanese black pine and red pine inoculated with different concentrations of PWNs was 0 and 4.46%, respectively. The results showed that resistant pine had a higher resistance to the PWN.

Session 2 (papers): afternoon continued, July 21:

Systematics, diagnostics

Spread of the genus *Bursaphelenchus* as a part of the order Aphelenchida distribution

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Using the secondary Brooks parsimony approach a model was developed (sBPA). for determining genus dispersal based on the data for all *Bursaphelenchus* records in natural biotopes. I then analyzed the established previously species groups on the basis of morphological data and the biotic relationships with the host plants and insect vectors.

A modified sBPA was used to determine the specific features of the spread of these nematodes and their relationship with their host plants. In the studies by Brooks *et al*., matrices and dendrograms were obtained by the parsimony method using the well-grounded phylogenies of plant and animal taxa. Thus, dendrograms of the evolution for the taxa and fauna of the areas under consideration, accurate to species level, were developed. In this study, the matrices of plants and large regions were developed on the basis of the species groups that are of high prognostic value, rather than on the phylogenetic branches of the nematodes.

Since no detailed phylogenetic model is available for *Bursaphelenchus* species the Brooks approach is applicable only in the modification used here. As a result, the accuracy of historical interpretation is changed: As such, no conclusions can be made on the dispersal history or changes in host specificity of each species. However, the major trends in historical development of the genus as a whole and of prognostic species groups can be followed. The dendrogram was constructed using PAUP ver. 4.0b10 software.

The genus *Bursaphelenchus* may have originated in the center of Pangea, at the interface of the continental plates (Carboniferous period); the *hunti* group is presumably of American origin, the *xylophilus* group appeared in the northern Laurasian shield of Pangea (during the Carboniferous period) in the area of the present day East Asia. *B. xylophilus*, a species of world quarantine importance, developed in North America and afterwards expanded into the coniferous forests of East Asia due to similarity with the resident *Bursaphelenchus* species with respect to adaptations in East Asia, other species of the *xylophilus* group are distributed, and suitable hosts and vectors (longhorn beetles) are available.

According to the distribution of the aphelenchids belonging to different phylogenetic lines, the following concept may be drawn. The origin of the

superfamily Aphelenchoidoidea and the order Aphelenchida as a whole seem to be linked with the east areas of the former paleocontinent Gondwana (India, Indo-Malaya, Australia, Antarctica), presumably in the Devonian period. From these areas at fusing of Gondwana and Laurasia in the Pangea paleocontinent, the aphelenchids spread in the Laurasian part of Pangea during the Carboniferous period. Endemism of the advanced ectoparasitic family Acugutteridae indicates that the secondary aphelenchids speciation center as being in the Carribean area. The success of the Aphelenchida expansion into the cold areas of the Holarctic region was facilitated by the formation of the anhydrobiotic stages in the nematode life cycles. Another important adaptation was the formation of the specialized, duerlarva stage for insect vector dispersion.

Development of a new technique to detect mRNA by reverse transcription and PCR as an indicator of viability in the pinewood nematode

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Phytosanitary regulations imposed in response to the presence of dead nematodes found in internationally traded wood products may result in unnecessary trade disruption. Therefore, accurate techniques for the detection and differentiation between live and dead PWN are critical.

Molecular techniques such as conventional PCR and Real-Time PCR, used as molecular diagnostic methods for PWN, are based on the detection of DNA and therefore fail to differentiate between dead and live nematodes. In order to address this issue, we are developing a new reverse transcriptase PCR assay based on the use of heat shock protein 70A mRNA as a viability marker on the basis of its rapid degradation compared to DNA in dead organisms. Preliminary results will be presented.

Morphology and molecular characterization of *Bursaphelenchus* (Nematoda: Aphelenchoididae) spp. in Korea

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The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, causes pine wilt disease (PWD) which affects many species of pines. PWD was first found in Korea in 1988, but damage from the disease has increased dramatically since 2000. Recently, PWD has been reported as affecting Korean pine tree (*Pinus koraiensis*) and thus PWD is considered to be one of the most important forest diseases in Korea. In this study 15 isolates of *B. xylophilus*, 3 isolates of *B. mucronatus*, and 5 unidentified *Bursaphelenchus spp*. were collected from different geographical locations and hosts, and were characterized by ITS and D2D3 rDNA sequence analysis. Template DNA was prepared by DNA extraction from single female nematodes. ITS and D2D3 region were amplified by PCR and followed by cloning and sequencing. The results showed that all the sequences of ITS and D2D3 from the *B. xylophilus* isolates were identical and there is no intraspecific variation. However, two genotypes of *B. mucronatus* were found with one from *P. thunbergii* being the East Asia type and the other one from *P. koraiensis* was the European type. The data showed that five unknown species of *Bursaphelenchus* spp. were closed to *B. tusciae*, *B. lini*, *B. thailandae*, *B. doui*, and *B. hylobianum*, respectively, which were also supported by morphological characteristics. The ITS-RFLP data also allowed us to distinguish different species and genotypes by using five enzymes of Hinf I, Alu I, Msp I, Hae III, Rsa I, and each PCR product was cloned and sequenced. *Bursaphelenchus conicaudatus* was used as the control species for this experiment, and informative nucleotide sequences of *Bursaphelenchus* were downloaded from GenBank in NCBI. All DNA sequence data were aligned by using Clustal W program and molecular phylogenetic analysis was performed by PAUP*4.0.

Detection of the pine wood nematode, *Bursaphelenchus xylophilus* using Padlock probes

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Padlock probes are single-strand oligonucleotides whose ends are complementary to adjacent target sequences. The link sequence of the padlock probe includes the universal amplification primers and a unique sequence identifier, the so-called Zipcode, which can hybridize to the oligonucleotides immobilized on supporter. Our object here was to develop a new, fast and accurate hyperbranched rolling circle amplification (HRCA) detection system for diagnosing *Bursaphelenchus xylophilus* based on the padlock probe and HRCA techniques.

The Padlock probe was designed to target the ITS of genome DNA from *B. xylophilus*, and the universal amplification primers were designed according to the linking part. The ligation system and procedure, the digest system and rolling circle amplification system were all optimized; the amplified products were analyzed by agarose gel electrophoresis. Then the HRCA detection system was established: ligation with terminal concentration of 10 pmol/L padlock probe using thermal cycling method, digestion with 20U Exo- Ⅰ for 4 hours, and isothermal amplification at 64.5℃. The efficiency of ligation was increased after the inhibitory factors caused by the linear probe were removed. The specificity and sensitivity of the HRCA detection system for *B. xylophilus* were tested by comparing the results with conventional PCR. The detection sensitivity of this method was 438.5 fg/μL for the cloned plasmid, which was 10 fold higher than the sensitivity of conventional PCR. Using this detection system, *B. xylophilus* can be detected specifically while other nematodes are not, according to 16 nematode samples collected from different countries and regions, including populations of *B. xylophilus, B. mucronatus, B. doui* and so on.

According to the design strategy of padlock probe, it is expected that the HRCA detection system can be applied to multiplex detection of plant pathogens in conjunction with southern blot and micro arrays.

Detection of pine wilt-damaged trees using near-infrared color photograph

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The failure to detect damaged trees harboring pine wilt disease (PWD) insect vectors is one of the main reasons for lack of success in the control of PWD. Aerial surveys to detect wilt-affected trees, i.e. those with discolored foliage, has proven to be a reliable disease survey technique and as such many governments in Japan have already adopted such methods. The conventional methods of aerial survey and following the treatment to detect trees, however, suffer from some technical problems such as insufficient flight time for a detailed survey, the ambiguity of foliage coloration, and the imprecise location of the spotted trees causes difficulties in locating them from the ground.

To overcome these problems we introduced aerial photography using near-infrared color films. Recent progress in such technology enables us to readily obtain an ortho-rectified aerial photograph that can provide the exact location (latitude/altitude) of any point. Properly processed near-infrared photos enhance foliage discoloration and make it more distinct than that in natural color photographs. With the photographs, we can carefully search for the discolored trees in the office without flight-time restrictions. Recognition of trees with foliage discoloration is highly accurate and can be done by personnel with minimal training; however, detection is not 100% because of some problems such as the invisibility of shaded trees from above.

The photographs may be electronically reproduced and displayed on a computer monitor. We are developing software with which we can designate the target trees and obtain their coordinates on an image file shown on the monitor. The location data for the target trees with the background image of the aerial photograph can be transferred to a palmtop computer with a built-in GPS receiver and the other original software will produce a graphical navigation system for ground approach of the target trees. Using the palmtop computer we can check and edit the data of the target trees on site, and the updated data can be transferred back to the host computer.

A consecutive system from detecting the trees to be treated to easy access for disposal and management of the individual tree data will be provided by making good use of the latest technology on aerial photography. This will bring an extensive improvement in control operations against PWD.

Simple diagnosis of Pine Wilt Disease using loop-mediated isothermal amplification

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Pine wilt disease (PWD), a serious threat to pine forests in East Asia and Europe, is caused by the pinewood nematode, *Bursaphelenchus xylophilus*. Because the nematodes are transmitted by insect vectors from dead to healthy trees, PWD spreads extremely rapidly. Accurate and rapid diagnosis and then complete removal of the nematode-infected trees from pine forests are thus very important for control of PWD. To diagnose PWD, *B. xylophilus* must be detected in infected trees. Generally, the nematode is extracted from wood tissues using the Baermann funnel technique and is then identified under stereo and light microscopes. However, this method requires technical knowledge of nematode morphology and expensive equipment such as microscopes. In addition, nematode extraction takes a long time (usually 1 or 2 days), and identification of the nematode is possible only when adults (both male and female) are extracted. Several DNA-based protocols for the identification of *B. xylophilus* have recently been developed. Although these molecular techniques are sensitive and independent of the life stage of the nematode, they require both the use of a Baermann funnel to collect the nematodes from the wood tissues and expensive equipment such as a thermocycler or real-time PCR apparatus to amplify the DNA. We developed a simple and low-cost method of determining the presence of PWD by using loop-mediated isothermal amplification (LAMP). This method consists of the following three processes: i) DNA extraction. The DNA of *B. xylophilus* is directly extracted from small wood chips in DNA extraction buffer in a micro test tube for 30 min; ii) DNA amplification. Part of the extracted DNA solution is transferred to a reaction mixture in a 0.2-ml micro test tube, and then the *B. xylophilus* DNA is specifically amplified in the tube under a constant temperature for 60 min; iii) Judgment. Amplification of the target DNA is confirmed by the colour of reacted solution. The only piece of equipment that LAMP diagnosis requires is an incubator for maintaining a constant temperature, and the procedure is completed in only 90 min. Because this new method of diagnosing PWD is simpler and cheaper than the traditional one, its use is likely to become widespread.

Aphelenchoides dalianensis. Nov. (Nematoda, Aphelenchoidae) from *Pinus thunbergii* in China

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A new species of nematode, *Aphelenchoides dalianensis* n. sp. was extracted from a pine wilt-affected black pine (*Pinus thunbergii*) from Laotieshan (38.8° N, 121.2°E), Dalian City, China. *A. dalianensis* is characterized by its length (female: 571.5-658μm; male: 436.8-520μm); slender stylet (female: 10-12.7μm; male: 9.2-11.8μm) with basal knobs; and four lateral lines. Female vulva post median, at 60%-75% of the body length; female tail with a special mucronate structure which has a divarication, forming an antennae-like terminus; male tail strongly hooked ventrally to from the characteristic 'walking-stick' form, tapering to a single terminus; small spicule (10-12.9μm); three pairs of caudal papillae and bursa absent. *A. macronucleatus* resembles the new species, especially in male spicule, but differs from female tail terminus and the shape of male after heat-killed. The PCR-ITS-RFLP pattern and its rDNA sequence also provided further evidence that this isolate is a new species. *Aphelenchoides dalianensis* sp. nov. is named after the name of the city, Dalian, where it was found.

Session 3 (posters):
afternoon (17:00-18:00) July 21, repeated 17:30-18:00, July 22:

Taxonomic aspects and intra-generic grouping of *Bursaphelenchus*

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The number of known *Bursaphelenchus* species (Aphelenchoididae, Parasitaphelenchinae) has more than doubled since the first publication of a key for 37 species of the genus by Tarjan & Aragon (1982). At present, almost 100 species are known exhibiting considerable morphological differences. A common character is the presence of a small bursa on the male tail terminus. Other genera with small terminal bursa are *Parasitaphelenchus* and *Deviburs*a*phelenchus*. The latter genus was re-established by Braasch (2009), and five former *Bursaphelenchus* species (*B. typographi*, *B. lini*, *B. eproctatus*, *B. teratospicularis*, *B. hunanensis*) were combined with it. Characters for differentiaton of *Bursaphelenchus*, *Parasitaphelenchus* and *Deviburs*a*phelenchus* are listed in this contribution. Several authors divided the genus *Bursaphelenchus* into species groups, mainly by using spicule morphology (Giblin & Kaya, 1983; Ryss *et al*., 2005) or by considering character sets for the groups (Braasch, 2001). Molecular techniques for systematic analysis, particularly DNA sequencing, allowed the estimation of phylogenetic relationships of species and confirmed the existence of species groups (Metge *et al*., 2006; Ye *et al*., 2007; Kanzaki, 2008). Different group names have been given by different authors, and this is clearly confusing to others. To resolve this confusion, it is proposed that, henceforth, authors should use the terminology given here. Based on a complex of characters, in particular the number of incisures in the lateral field, the arrangement of male caudal papillae, presence or absence of a vulval flap, spicule shape, position of excretory pore and length of post-vulval uterine branch, the genus is divided into seven groups with four incisures in the lateral field (*xylophilus, okinawaensis, africanus, fungivorus, cocophilus, kevini* and *sexdentati* groups), four groups with three incisures

(*eggersi, eremus, hofmanni* and *leoni* groups) and two groups with two incisures (*abietinus* and *sinensis* group). Assignment of 73 species to the groups is listed, 24 species could not be grouped due to lack of detailed morphological information. The most important group characters (number of lateral incisures, position of caudal papillae and spicule shape) are presented in two tables. The shape of bursa cannot be used for group differentiation. A molecular tree for 39 *Bursaphelenchus* species, inferred from sequences of near–full length SSU of ribosomal DNA, shows the subdivision of *Bursaphelenchus* spp. into groups. A second molecular tree, inferred from sequences of the D2/D3 region of LSU from 28 species of the subfamilies Parasitaphelenchinae, Aphelenchoidinae and Ektaphelenchinae, demonstrates the systematic relations within the Aphelenchoididae, for those species for which sequences are available.

Topic of presentation: Poster for 2. PWN: Systematics, diagnostics

Suppression of Pine Wilt Disease by an antibacterial agent oxolinic acid

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Pine wilt disease is a very complex disease and has been reported to be caused by pine wood nematode, *Bursaphelenchus xylophilus* and its accompanying bacteria. However, the role of bacteria in the disease is still debatable. In order to elucidate the hypothesis, we selected one antibacterial agent, oxolinic acid, showing potent antibacterial activity and then evaluated its control efficacy against pine wilt disease in greenhouse and field conditions. Among 6 antibiotics tested, oxolinic acid showed the strongest antibacterial activity against 5 bacteria isolated from three strains of pine wood nematode. In an *in vivo* assay, it effectively suppressed the development of pine wilt disease in 3-year old seedlings of *Pinus densiflora*; it showed 71% control value when injected 3 mg per seedling. The mixture of oxolinic acid and a nematicidal agent abamectin showed higher disease control efficacy against pine wilt disease than either oxolinic acid or abamectin alone. In addition, it alone and the mixture of oxolinic acid and abamectin also controlled pine wilt disease in approximately 20-year old pine trees under field conditions. These results strongly indicate that the bacteria associated with the pine wood nematode are necessary for the occurrence of pine wilt disease and pine wilt disease could be controlled by antibacterial antibiotics alone or a combination of antibacterial and nematicidal agents.

Nematicidal activity and composition of the essential oils from the Portuguese aromatic flora against the pinewood nematode (*Bursaphelenchus xylophilus*)

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One of the most important pests and pathogens of conifers forests worldwide, the pinewood nematode (PWN) *Bursaphelenchus xylophilus* was detected for the first time in 1999, in Portugal and Europe, from the Setúbal Peninsula, in dead *Pinus pinaster*. In Portugal, the PWN has found the ideal requirements for spreading (temperature above 20ºC, suitable host tree and presence of an insect vector). Despite the phytosanitary measures implemented by the Portuguese government, there is nowadays an extensive *P. pinaster* infected and damaged area, which has reached the center of the country. In 2008 the nematode was also detected from a single tree in Spain (Cáceres). The use of synthetic pesticides, as well as attractants to control the insect vector, heat treatment of wood, or the cut and removal of infected trees are the most commonly control practices. Essential oils may provide an alternative to PWN control, since they are a rich source of bioactive chemicals and, simultaneously, are more environment-friendly. In the present study, essential oils from 27 species of 11 families of the Portuguese flora were evaluated for their nematicidal activity against *B. xylophilus*. Essential oils were isolated by hydrodistillation and analysed by GC-FID and GC-MS. Nematicidal activity was evaluated using previously described methods. Good nematicidal activity was obtained with *Thymbra capitata* = *Thymus caespititius* > *Satureja montana* > *Cymbopogum citratus* essential oils, all of which caused >95% mortality at 1mg.ml^{-1}. *T. capitata* and *Th. caespititius* oils contained mainly carvacrol (65% and 75%, respectively). The main components of *Satureja montana* and *Cymbopogum citratus* essential oils were carvacrol (47%) and geranial (42%), respectively. The oils from *Thymbra capitata* and *Satureja montana* represent the first report of the nematicidal activity of these plants against the PWN. Given the present results, essential oils from Portuguese aromatic flora deserve further study as potential natural nematicides against the PWN.

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The Finnish contingency plan for the pine wood nematode

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Pine wood nematode has never been found in the forests in Finland. However, because it is frequently intercepted, especially in wood packaging material, the risk of invasion is considered noteworthy. Finland has had a contingency plan for the pine wood nematode since 2002. The plan focuses mainly on the administration of the situation, i.e. defining the responsible bodies and their division of labour in case of a pine wood nematode invasion. Recently the contingency plan was complemented by an action plan that describes the actual eradication measures and the measures aiming to subsequent spread of the pest.

The core of the action plan constitutes of setting up a clear-cut zone and a quarantine zone. The clear-cut zone is a 3 km zone and the quarantine zone is a 6 km zone around the infested area. In case of small infestations the clear-cut and the quarantine zones are set up directly around the affected trees. For wider infestations the clear-cut and the quarantine zones are set up around the edges of the infested area. In the clear-cut zone all coniferous trees, i.e. Scotch pine (*Pinus sylvestris*) and spruce (*Picea abies*) are felled. From the quarantine zone wood can be transported only with specified restrictions that aim to eliminate the risk of spreading of the pest. The zones will be maintained for 15 years after the last record of pine wood nematode.

Last April a contingency practice was arranged to test the contingency and action plans and the legislation in force. The practise highlighted the immense scale of both financial and human resources needed to carry out the eradication measures. It also stressed the need for further research to better specify the survey and eradication measures needed. Moreover, the practise demonstrated the need for an even more practical contingency plan. The experience gained in the practice will be utilised when updating the Finnish contingency plan for the pine wood nematode later this year.

Dispersal of the pine wood nematode in resistant pine trees and host cell response to nematode invasion

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Pine wilt disease (PWD) caused by pine wood nematode (PWN) is the most serious pine forest disease in East Asia and Europe. The disease has caused great damage to the forest resources, natural landscape and ecological environment in China. Great advances in selective breeding against the PWN have been achieved to date. One-year- old resistant pines (*Pinus thunbergii)* inoculated with the PWN were selected to study nematode dispersal and distribution in pine stems. And variety of water potential, cells and tissues has been tested.

Quantity variation of the nematodes in stems showed that the number of nematodes in stems was less，the dispersal speed of the nematode was slower and the activity of them were weaker at the initial stage. The nematodes in the resistant *Pinus thunbergii* mainly moved downwards and the number of nematodes was obviously smaller than that in the susceptible individuals, while nematodes moved upwards as well as downwards in the sensitive *Pinus thunbergii*. And the quantity and moving-speed downwards were obviously larger than upwards. In later stage, nematodes started to propagate and improve the dispersal ability. The results suggested that the seedlings' symptom was related to the nematode's quantity.

Changes in water potential had been tested in the study and the result showed that water potential changed at first in the sensitive pine, but both the sensitive pine trees and the resistant ones had the same trend that it rose at first, then got down, and rose again finally.

Variation of quantities and speed of cells and pathological tissues seedlings was related to nematodes moving and spreading. After inoculation, parenchyma cells which were far away from the inoculation point had less apparent pathological changes and its quantities were only a few. Pathological changes occurred in the cortex parenchyma within the 0-0.5 cm below the inoculation point of the resistant pine after inoculation for 12 hours. However this change occurred on the sensitive ones at the same position 6 hours earlier. In addition，the cambium of the sensitive pines appeared the above changes relatively earlier. With the continuous increase of

cell death of the seedlings, the longer inoculation time taken and the more extensive and severe browning and death of tissue cells appeared. Browning and death of the cortical layer, the phloem and the parenchyma cells of the xylem were very commonly distributed at the beginning of the symptom. The tissue browning of the resistant pines first appear to the cortical after inoculation for 72 hours, but that of the sensitive one can be observed easily just after inoculation for 24 hours. Degrees of browning were related to the distance to the inoculation point, but not to the inoculation point directly. The farther away from the point, the lighter degree of browning appeared vice versa. The lower part was more obvious than the upper one. The resin canal was broken, the parenchyma cells of axial and radial died with time passing. At this time, nematodes were in the prosperously multiplying stage, and the host appeared symptom.

PWN, *Bursaphelenchus xylophilus*, survival in stumps and roots

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The infection possibility of adjacent healthy tree from the roots of PWN-infected trees was investigated. Under outdoor conditions in Korea an infection test made on 7-year-old *Pinus thunbergii*. The results showed that PWN caused a high mortality of inoculated pine trees. On the other hand, the PWN was extracted from 18.2% of the trees that were not inoculated with PWNs. In the natural infection region of PWN, the rate of PWN extraction from stumps was 54.1% at 2 years after infection. The results suggest that root infection of PWN is possible in case of root callus

Distribution of *Bursaphelenchus* spp. transmitted by *Monochamus alternatus* and *M. saltuarius* in Korea

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The pine wood nematode, *Bursaphelenchus xylophilus*, causes pine wilt disease of many species of pines. Until 2006, *Monochamus alternatus* was the only known insect vector of the pine wood nematode in Korea where the disease was thought to affect only *Pinus densiflora* (Japanese red pine) and *P. thunbergii* (Japanese black pine). However, in 2006 pine wilt disease was also reported from Korean pine (*Pinus koraiensis*) and we found that another insect vector, *M. saltuarius*, also transmitted the nematode. Both *Monochamus* species were confirmed to transfer pine wood nematode to their hosts, but there is no detailed information about other nematodes which may also be transmitted. Of special interest was *Bursaphelenchus mucronatus* which is commonly transmitted by *Monochamus* species and which is morphologically similar to *B. xylophilus*. Moreover *B. mucronatus* has two genotypes; one the East Asian type and the other the European type. Both genotypes of *B. mucronatus* have been found in Korea, but the host and vector information related to the genotypes of *B. mucronatus* were not clear.

To answer the above question, *Monochamus saltuarius* beetles were collected from three different geographical locations and the nematodes were extracted and identified. For the identification of the juveniles, nematode DNA was extracted and ITS-RFLP analysis was done by PCR and gel electrophoresis. The selected enzymes were Hinf I, Alu I, Msp I, Hae III, Rsa I. Most of *Bursaphelenchus* species carried by *M. saltuarius*, which was collected from a pine wilt disease-free area, was determined as the European type of *B. mucronatus*. We will compare the nematode species and genotypes carried by *M. alternatus* and *M. saltuarius*. As well, the numbers of nematode carrying insects and the average number of nematodes per single insect will be determined and compared.

Effects of wood-rotting fungi on *Bursaphelenchus xylophilus* populations

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Pine wilt disease caused by pine wood nematode (*Bursaphelenchus xylophilus*, PWN) is the most serious forest disease in China, and the population of PWN in the stomps of diseased and dead trees is one of the infection origins. It is desirable that the stomps with pine wood nematode be treated.

In this study, twenty-three strains of thirteen species of wood-rotting fungi were chosen to evaluate their relation with PWN, the ability to decompose the wood specimens of *Pinus massoniana* and the stumps of dead pine trees caused by PWN. The results showed that all tested wood-rotting fungi have certain nematicidal effects on PWN except *Schizophyllum commune*. Among the wood-rotting fungi, PWN could not survive on the strains of W10, W11(*Fomitopsis pinicola*), 5452, 6600(*Laetiporus sulphureus*), 6221(*Pleurotus ostreatus*), 6320(*Tremellodon gelatinosum*), 6284(*Poria cocos*), 6501(*Ganoderma lucidum*) absolutely. The strains of W10，W11(*Fomitopsis pinicola*)，6600(*Laetiporus sulphureus*)，6923(*Coriolus versicolor*) and 6284(*Poria cocos*) have greater ability to decompose *P. massoniana* specimens. Consequently, stump inoculations were made with strains 6600, 6923, W11, 6284, 6221. After 70days the results showed that 6600 strain (*Laetiporus sulphureus*) had the greatest ability to decompose the stumps and also it had certain nematicidal effects on PWN. This strain has very good effects on dealing with the stumps of dead wood caused by PWN. A correlation analysis indicated that the number of PWN was significantly and positively correlated with the weight of the samples from stumps. The strains could be used to treat stumps of dead wood caused by PWN in the future.

Occurrence of nematode species associated with pine wood in Jiangsu, Zhejiang and Gansu Provinces of China

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A survey was carried out in various localities of Jiangsu, Zhejiang and Gansu provinces of China for investigating and identifying nematode species associated with pines. A total of 122 wood samples were collected from healthy and dead or dying pine trees viz., *Pinus massoniana*, *P. thunbergii*, *P. elliottii*, *P. densiflora*, *P. armendii* from May to November, 2008. Nematodes were extracted from wood chips by a modified Baermann funnel technique and propagated on *Botrytis cinerea* grown on potato Dextrose Agar medium (PDA) at 25°C. Nematodes were heat-relaxed (65°C, 2 min) and fixed in 4% formaldehyde, processed to anhydrous glycerine by slow method (Seinhorst's method, 1959). Measurements were taken using a Leica DMLB2 light microscope with QWin image analysis and processing software. Scanning electron microscopy (SEM) examination of some nematode species was done according to Wergin (1981).

Ten species of nematodes comprising five genera viz., *Bursaphelenchus xylophilus*, *B. mucronatus*, *B. aberrans*, *B. hofmanni*, *B.* sp., *Aphelenchoides macronucleatus*, *A. vaughani*, *A. bicaudatus*, *Seinura wuae*, *Ektaphelenchoides pini* and *Laimaphelenchus* sp. were recovered during the faunistic survey of Jiangsu, Zhejiang and Gansu provinces of China. Species of nematodes were recovered from 40% of the samples, most from dead or dying pine trees and least from healthy trees. *Aphelenchoides bicaudatus* was the only species recovered from the wood of healthy *P. thunbergii*. Species of *Bursaphelenchus* were recovered from 83% of the dead or dying trees, but were never found in healthy trees. Based on the information gained from the survey it is observed that among ten nematode species, the pine wood nematode was found to be the dominant species and its occurrence would lead to a significant weakness in nematode diversity within pine wood. As pine wilt disease is epidemic in China, further studies on the predatory lifestyle and biological control potential of *Seinura* species to pine wood nematode need further studies in China (Huang & Ye, 2006). An undescribed species of *Laimaphelenchus* was found under the bark of *Pinus armendii* Franch. This is the first record of the genus from China and *P. thunberghii* Parl. reported as the new host record for *Seinura wuae*. The project is supported by Natural Science Foundation of China (30771728).

Direct control measures against the pine wood nematode *Bursaphelenchus xylophilus*. The portuguese case.

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Pine wilt disease, caused by the pine wood nematode *Bursaphelenchus xylophilus,* was first reported in Portugal in 1999. Since its detection in Portugal, direct control against the pine wood nematode has been done mainly by heat treatment (HT), fumigation with methyl bromide (MB), as well as removing and burning the affected trees. Recently, a new official publication (Norma Portuguesa NP 4487/2009) referring to the Heat Treatment Control Measures defined the requirements to certify the control against *B. xylophilus* in softwood sawn timber, pallets and other packages. The same publication also refers to the requirements for affixing the efficacy of treatment by the traders. At the moment there are 175 registered companies for the HT use. Fumigations are made with MB and there are four companies registered to do it. Following the ratification of the Montreal Protocol by the Portuguese Government, in 17/10/1988, and the EU reevaluation of the active substances (Directive 91/414/EEC) the MB can only be used until 19/9/2010. Now, the MB is classified as a “high risk” active substance, and can only be used in quarantine and pre-shipment situations. Sulfuryl fluoride, which is already in the EU market and with its status under Directive 91/414/EEC “pending”, could be a technical alternative to MB. Emamectin benzoate and milbemectin, both in the EU market, but not yet authorized in Portugal are two other possible options to considerer as trunk-injection treatments. Milbemectin has already been reevaluated and “included” in the Annex I (Directive 91/414/EEC) while emamectine benzoate status under the same Directive is “pending”.

Session 4: morning, July 22:

PWN: Interactions with other microbes

Endophytic bacteria isolated from pine wilt-affected *Pinus pinaster* trees

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The characteristic rapid death of maritime pine, *Pinus pinaster*, after infection by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, suggests the involvement of other organisms in pine wilt disease (PWD). Endophytic bacteria are ubiquitous in nature and colonize a broad spectrum of plant species. They colonize plants internally without harming them and although they have been reported to promote plant growth and stimulate plant defence mechanisms. Although such bacteria may be involved in improving plant health, they have also been seen as a potential cause of the low regeneration capacity of mature trees. The bacteria carried out by the PWN throughout pine trees may also contribute to the rapid wilting of the maritime pine trees. This study was made to assess the endophytic microbial community structure of pine wilt affected and non-affected *P. pinaster*, based on culture isolates, amplified ribosomal DNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE) profiling, and to relate the results to a possible association between the characteristics and bacteria associated with the PWN. The microbial community from trees and PWNs included strains from *Beta* and *Gammaproteobacteria*, but *Alphaproteobacteria* and *Bacteroidetes* were only found in the endophytic community whereas *Actinobacteria* and *Firmicutes* were only found to be associated with the nematode. ARDRA and DGGE profiles showed differences in the diversity of the endophytic microbial community that may be related to the presence of the PWN. Those differences seem to be associated with the number of strains of the genera *Burkholderia*, *Pseudomonas* and *Luteibacter*. Isolates belonging to the species *Janthinobacterium agaricidamnosum*, *P. lutea* and *Dyella yeojuensis* were isolated from trees and were associated with the PWN. This research documents the presence of bacteria associated with the PWN by molecular methods and the impact of the nematode in the plant endophytic microbial community structure.

Populations originating from hybrids between *Bursaphelenchus xylophilus* and *B. mucronatus*, their virulence to *Pinus thunbergii* and their boarding ability onto *Monochamus alternatus* adults

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After the invasion of Japan by *Bursaphelenchus xylophilus* in the early 1900s, its range extended in western Japan until 1947 when the spread ceased between 1948 and 1958. After that, the range of *B. xylophilus* extended to eastern Japan and high-altitude areas in western Japan. During the lull in spread it is thought that the virulent nematode *B. xylophilus* coexisted with the avirulent *B. mucronatus* which is native to Japan and the two nematode species crossed there. Introgression of *B. mucronatus* genes to *B. xylophilus* genome may have made it possible for *B. xylophilus* populations to spread to eastern Japan. To test the hypothesis, using virgin individuals, Taga and Togahi (2008) hybridized the two species in the laboratory using conditions where the F_1 hybrids were allowed to backcross with the parents. As a result, we obtained "hybrid" isolates with *B. mucronatus* nuclear genome and *B. xylophilus* cytoplasm, those with *B. mucronatus* nuclear genome and *B. mucronatus* cytoplasm, and those with *B. xylophilus* nuclear genome and *B. xylophilus* cytoplasm. The "hybrid" isolates persisted for 3 years in our laboratory. Here, we compared the two traits necessary for persistence of "hybrid" isolates among "hybrid" and parent isolates.

To compare the virulence among eight "hybrid" and five parent isolates, we inoculated 2-year-old *Pinus thunbergii* seedlings of two pine-wilt resistant families, Yoshida #2 and Ei #425, with 5,000 nematodes per seedling. About 90 seedlings of Yoshida #2 and ca 30 seedlings of Ei #425 were used for the inoculation test of each nematode isolate. The inoculations were done on 19 July and 10 August, 2007. Dead seedlings were counted on 6 November, 2007. Seedling mortality differed depending upon the nuclear genome. Mortality resulting from the inoculations with the two *B. xylophilus* isolates and the two "hybrid" isolates with *B. xylophilus* nuclear genome were between 36 and 85 %, whereas those done with three *B. mucronatus* isolates and six "hybrid" isolates with *B. mucronatus* nuclear genomes were 0 %. Two of the six "hybrid" isolates with *B. mucronatus* nuclear genome had *B. xylophilus* cytoplasm because they were originated from crossing of *B. xylophilus* females and *B.*

mucronatus males. This suggests that the gene(s) related to the virulence were in the *B. xylophilus* nuclear genome. This experiment showed that "hybrid" isolates with *B. xylophilus* nuclear genome could kill pine trees and reproduce in them.

To compare the boarding ability onto vectors among one "hybrid" and its parent isolates, a hole, 1.1 cm in diameter and 5 cm deep, was drilled into each *Pinus densiflora* bolts, 7.5 cm long and 4.6 cm in diameter. The "hybrid" isolate used originated from crossing of virgin *B. xylophilus* females and virgin *B. mucronatus* males, and had *B. xylophilus* nuclear genome. Two weeks after the blue stain fungus *Ophiostoma minus* was inoculated on the autoclaved pine bolts, 3,000 nematodes and one *Monochamus alternatus* larva were placed in each hole, and were incubated at 25 °C. There was no difference in time required for adult emergence. Mean (± SE) of the initial nematode loads on beetles was heavier for the "hybrid" (1125.5 ± 480.1) and *B. xylophilus* (990.4 ± 485.6) isolates than the *B. mucronatus* isolate (39.0 ± 34.7). Consequently, it was not considered that the "hybrid" populations was selected out at the time of transfer from trees to insect vectors.

Session 5 (papers): morning, July 22

Insect vectors: Biology, ecology and modeling

Interactions of the pine wood nematode *Bursaphelenchus xylophilus* with its vector in Portugal

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The most important aspects of the interactions between the pine wood nematode (PWN) *Bursaphelenchus xylophilus* and its local insect vector, the pine sawyer *Monochamus galloprovincialis* have been recently studied for the first time in Portugal and Europe. Inside the dead pines the pinewood nematode was found to associate with the callow adults in the pupal chambers, just before the insects' emergence. The nematodes are more abundant on the thoracic region of the insects, namely on the meta-thorax segment. After emergence, transmission of the PWN into new hosts occurs by the feeding activity of the beetles, being more frequent during the six weeks after emergence. Nematode transmission also occurs by the oviposition activity of the female beetles, although with less frequency and success.

Overall, the main aspects of the multiple interactions between *B. xylophilus* and *M. galloprovincialis* (recently studied for the first time in Europe) were found to be extremely similar to other well-known interactions of the PWN with other *Monochamus* vectors worldwide, namely with *M. alternatus* in Asia and *M. carolinensis* in North America. The factors that regulate the most important aspects of the nematode-vector phoresis probably result from a complex interaction of endogenous and exogenous factors such as chemical odours and signals, which are discussed in this paper. Such factors apparently exert their effect independently of the pine host involved and the *Monochamus* species vectoring the PWN locally. Nevertheless, despite their importance these mechanisms and stimuli are mostly unknown and, in general, very poorly understood. A better understanding of such mechanisms could lead to the development of innovative perspectives to disrupt the key moments of the interaction between the two organisms, therefore creating innovative approaches to control the pine wilt disease.

General aspects of the relationships between *Monochamus galloprovincialis* (Oliv.) and *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle in Portugal

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Increased international transport of plants or plant-products has resulted in greater associated risks of the dissemination of pests between countries and ecosystems. This was also the case in 1999 with the detection for the first time in Portugal and in Europe of the pine wood nematode (PWN) *B. xylophilus* in dead Maritime Pines (*Pinus pinaster*). This organism is the causal agent of the Pine Wilt Disease and a quarantine organism within the European Union. Being native to North America it has been disseminated internationally and can also be found in Japan, Korea, Taiwan and Mainland China.

A key element of the nematode's life history and the initial determinant of its ultimate impact on living potential host trees is its transmission by insect vectors, namely longhorn beetles of the genus *Monochamus* (Coleoptera: Cerambycidae). Consequently, pine wilt disease is the result of an interaction of three distinct agents (the pine wood nematode, an insect vector and a tree host), although only the first is the fixed element whenever the disease is present, as the vector and the host can vary. Soon after the nematode's introduction into Portugal, various studies on the local specificities and regional variations of this disease were conducted, namely on the specificities of the interactions between the nematode-vector (the efficiency of vectoring and spread) and the nematode-host (the aggressiveness of the nematode as a mortality agent), of which a global synthesis is presented here.

The sole vector of the PWN in Portugal is the pine sawyer *Monochamus galloprovincialis*. Before *B. xylophilus* was introduced this insect was considered a secondary xylophagous and its biology and ecology had never been studied. Recent studies recently showed that the pine sawyer has one generation per year in Portugal, with beetles emerging from May to August. Biological parameters such as longevity, sexual maturation, fecundity and oviposition rates were studied under laboratory conditions. Adult beetles live for about two months and begin laying eggs at about 20 days, mainly in the upper trunk and branches of mature *P. pinaster* trees. Under laboratory conditions female beetles laid eggs on other hosts such as *P. sylvestris*, *P. pinaster, P. halepensis*, *P. radiata*, *P. pinea*, and *Pseudotsuga menziesii*, but insects only completed their life-cycle on the first four pines.

The PWN can be transmitted into new hosts by both the maturation feeding and the oviposition activity of the adult beetles, although transmission by the feeding appears to be more efficient. Nematode-infected trees develop wilt symptoms which are not specific to the infection by the PWN. Overall, our results suggest that the most important aspects of the *B. xylophilus*/*M. galloprovincialis* interaction are similar to other well-established nematode-vector associations from distinct geographic locations.

Evaluation criteria and an indicator system for determining resistance and resilience of pine forest ecosystems to *Bursaphelenchus xylophilus* invasion

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Bursaphelenchus xylophilus (Steiner et Buhrer) Nickle [the pine wood nematode, (PWN)], is an invasive, alien species in China where it causes the extremely destructive pine wilt disease, especially for Masson pine *Pinus massoniana*. In the work reported here Masson pine characteristics were analyzed in five sample plots in Zhejiang Province, China, where *B. xylophilus,* carried by the vector beetle *Monochamus alternatus,* predominately attacks Masson pine in the lower diameter classes. Among the 10 tree characteristics examined, mean crown width, percentage of bole with crown, 5-year cumulative diameter growth, and resin amount showed significant variation between successfully attacked and non-attacked trees. Attacked trees had a lower percentage of the bole covered with tree crown, lower crown width, lower radial growth in the last 5 years, and produced less induced resinosis than non-attacked trees.

In the a second study, an indicator system of resistance and resilience of the pine forest ecosystem against pest invasion was created for Zhejiang province using two indexes of resistance and resilience which describe the anti-disturbance ability of the forest system, with mathematical research of Interpretative Structural Modeling (ISM), an evaluation criteria. The results provided an initial illustration of the resistance and resilience mechanism of the pine ecosystem invaded by *B. xylophilus*. As well, two experimental plots were used to verify the precision of the evaluation criteria and indicator system (devised by author). The field investigation showed that the disease incidence rate in plot 1 was lower (4.3%) than in plot 2 (18.6%). That is, the infestation was weak in plot 1 and heavy in plot 2, indicating the higher resistance and resilience of plot 1 than for plot 2. At same time, when we used the evaluation criteria and indicator system, we obtained the following results: plot 1 was characterized as having a high resistance system to *B. xylophilus* while plot 2 had weak resistance system. This shows that, the present results agree with our model analysis results and field investigations and as such verified the precision of our resistant and resilience system.

The biogeography and economic geography of the pine wood nematode in China

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Following its discovery in Jiangsu Province in 1982 the pine wood nematode (PWN) has been found in 113 counties (or cities) in twelve provinces in mainland China, in addition in Taiwan and Hong Kong. As such China has been confronted with an enormous threat to its pine forests. The work reported here re-traces the historical progress of the nematode's occurrence and the associated pine wilt epidemic in China based on the principles of biogeography and economic geography. The elevation-distribution areas for the PWN in China mostly occur below 1000m altitude and damage is most severe in the low altitude regions. That is the same situation as has occurred in Japan and other countries and it corresponds to the distributive characteristic of vertical zonality for the PWN. The regions damaged most severely are in the middle-lower Yangtze River area (East China area), the Pearl River delta area (South China area), where the average elevation is lowest in China. However, the disease is more severe in the flatter, wider Yangtze River area than in the latter area. Damage is mid-severity in the regions in the middle Yangtze River area, the middle Pearl River. As well, areas in Central China with their higher elevation and hilly-gully landform suffer mid-level damage. Disease-affected regions at about 1000m altitude, such as the borderlands of Yunnan-Guizhou plateau, are fragmentary in distribution and only suffer slight damage.

The PWN mainly occurs in the main river drainage areas of China, particularly the Yangtze River drainage area and this illustrates another biogeographical characteristic about PWN spread along river drainages. Within the main river drainage the dispersal of the PWN has occurred from east to west, and from coastal areas to upcountry areas in China.

As one of alien pest species with strong invasion specialty, PWN's occurrence, epidemic and dispersal are always closely related to human economic activities. While the world economy is at the beginning of a new round of economic globalization, and Chinese economy is at the beginning of opening to the outside world in the early 1980s, the occurrence of PWN's invasion into China indicate that its pandemic infection is coming within the scope of world. At the same time, the earliest infecting

regions of PWN in China would inevitably occur in the front line of Chinese economy opening to the outside world, and most closely related to those countries where PWN is present. The dispersal process of PWN would inevitably spread along Yangtze River drainage area, that is the greatest arterial of the Chinese economy and its main dispersal pathway in China, with Chinese economy developing from the coastal areas to upcountry areas.

Therefore, the Chinese geographic, topographic characteristics, and the regional patterns of Chinese economic development, and the interaction among them, determine the situations about PWN's occurrence, epidemic and dispersal in China.

Predicting potential habitat for the pine wood nematode based on an ecological niche model

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Timely gathering of information on exotic forest diseases and pests, early detection of the space distribution of alien species, and preventing them from entering suitable habitats are the preconditions for devising management measures. The pine wood nematode *Bursaphelenchus xylophilus* occurrence points plus 31 environmental variables were gathered，four modeling methods of Classification and Regression Trees(CART)，Genetic Algorithm for Rule-set prediction(GARP)，maximum entropy method (Maxent)，and Logistic Regression(LR) were introduced to generate potential geographic distributions for the invading pine wood nematode in Jiangsu province, China. Then three statistical criteria of the area under the Receiver Operating Characteristic Curve(AUC), correlation(COR) and Kappa were calculated to evaluate the performance of the models, followed by analyses of major contribution variables. The results showed that in terms of three statistical criteria, except the prediction results of the CART, other ecological niche models were either excellent or outstanding, in which Maxent outperformed others in (i) three aspects of predicting current distribution habitat, (ii) selecting of major contribution factors, and (iii) quantifying the influence of environmental variables on habitat. GARP outperformed others in predicting the potential habitat for pine wood nematode. Elevation, annual precipitation, precipitation seasonality and temperature annual range were the four forcing environmental factors.

Session 6 (papers): morning, July 22:

Etiology and epidemiology

Toxins secreted by *Pseudomonas fluorescens* GcM5-1A carried by pine wood nematode and their toxicities to Japanese black pine

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Pseudomonas fluorescens GcM5-1A, isolated from the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, was cultured in Luria Broth medium (LB). One protein of 50 kDa was purified through ammonium sulfate precipitation, DEAE-Sepharose FF ion exchange chromatography and a Superdex-75 column. N-terminal sequence of the protein was ALSVNTNITS, which showed high homology to the flagellin of *P. fluorescens* strain Pf-5. The protein could react with antibodies raised against recombinant flagellin of *P. fluorescens* strain Pf-5, which was identified as flagellin. Meanwhile, two cyclic dipeptides were also purified from clarified culture by extraction with ethyl acetate and chromatography a silica gel (200 - 400 mesh) column. The chemical structures of the cyclic dipeptides were identified as cyclo(-Pro-Val-) and cyclo(-Pro-Tyr-), respectively, by MS, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY spectra. Bioassay results showed that both flagellin and the two compounds were toxic to both suspension cells and seedlings of *Pinus thunbergii*.

To study the possible roles of flagellin in pine wilt disease, interaction between flagellin and suspension cells of *P. thunbergii* as well as the toxicity mode of flagellin to pine cells were investigated. The immunofluoresces assay showed that there exited a direct interaction between suspension cells of *P. thunbergii* and flagellin. Trypan blue staining result indicated that the suspension cell of *P. thunbergii* treated with

flagellin had little tinting strength, its cell membrane was shrunken and the cytoplasm was concentrated. The Giemsa staining indicated that the tinting strength of the treated cells was strengthened which implied an increase in the permeability of suspension cell membrane. Quantitative analysis showed that compared to the controls, the conductivity of the cells treated with flagellin for 24h increased to some extent. Acridine orange staining result showed that the nuclears of the treated cells were divided into many micronuclears and cytoplasmic RNA was degraded.

Electrophoresis analysis of genomic DNA confirmed that DNA breakage happened in the treated cells, but no evident DNA ladder was formed. We supposed that the toxicity mode of *P. thunbergii* cells caused by flagellin is atypical apoptosis.

Pathogenicity of *Bursaphelenchus mucronatus* in Russia

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Pine wilt disease, caused by the pinewood nematode (PWN) *Bursaphelenchus xylophilus,* has severely damaged susceptible conifers in Asian and European forests. Although the pathogen has the potential to cause pine wilt disease of conifer species in a large part of Russia the disease has, to date, not been found there. Where surveys have been conducted the results show that only the closely-related nematode *B. mucronatus* is widespread in Russia. *B. mucronatus* and *B. xylophilus* are closely related species and can mate, and they have similar biologies, but their pathogenicity differs in that in general of the two only *B. xylophilus* is pathogenic. Here we tried to determine if *B. mucronatus* isolates are pathogenic to conifers and what factors affect this process. Too, we review the pathogenicity experiments with *B. mucronatus* that have been done in Russia.

Of all the *B. mucronatus* isolates used in the pathogenicity tests most emphasis has been placed on isolate BmRFE from the Russian Far East (Primorski region). The nematodes (isolate) were originally extracted from the trunk of dead, naturally-affected plantation-grown, *Pinus koraiensis* where the population density was determined to be 20000 nematodes per gram of wood. (Kulinich *et al.,* 1994). Subsequent pathogenicity tests with this isolate, conducted in a greenhouse, showed that nematode-inoculated, 5-year-old *Pinus koraiensis* and larch, *Larix olgensis,* died following inoculation while 70% of the inoculated *P. sylvestris* and *P. densiflora* survived. Some other conifer species were resisted to the nematode (Eroshenko *et al.,* 1996).

Another inoculation experiment was conducted out of doors in the Central European part of Russia using 2 year-old *Pinus sylvestris* seedlings inoculated with the same *B. mucronatus* isolate (BmRFE) and isolates from the Komy, Republic of Russia (BmKOMY) and from China (BmCh). One year later after inoculation it was found that all nematodes died in the seedlings except the BmRFE isolate where the population density in *P. sylvestris* was 11 nematodes per gram of fresh wood (Kulinich, Orlinski, 2001). In a third experiment conducted in a locality south of the above site the 4 year-old *P. sylvestris* seedlings were inoculated with a Russian isolate of *B .mucronatus* (BmKOMY)) and also a French isolate (BmFr), and their hybrids. Nearly all isolates survived in seedlings 1 year after inoculation, but only minor symptoms of pine wilt disease occurred in only trees inoculated with the French isolate.

What factors affect to pathogenicity of *B. xyloiphilus* and *B. mucronatus* and can *B. mucronatus* populations induce pine wilt disease, i.e. as occurs for *B. xyloiphilus*? The most likely factors affect*ing B. xylophilus* pathogenicity include: biological characters of the nematode species, the host plant and climate. Our final investigation showed that pine wilt disease is caused by a complex of both the nematodes and pathogenic bacteria that they carry (Zhao *et al.,* 2003) and the

severity of this disease depends upon the phytotoxicity of the bacterial strains. Studies of three *B. mucronatus* isolates from different regions of Russia showed that BmRFE isolates 'carried' the most phytotoxicity bacteria. It is proposed that this fact explains why this isolate has shown greater comparative virulence than other nematode isolates that have been studied.

Surface coat proteins of the pine wood nematode, *Bursaphelenchus xylophilus*

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The nematode surface coat (SC) has various crucial roles in host–parasite interactions. For example, some multi functions of the SC include adhesion, lubrication, elicitor of the host defence responses and modulation to help counter host defence responses. However, little is known about the detailed characters of the nematode SC due to all difficulties (e.g., small amount, heavily glycosylation) involved in dealing. In pine wilt disease, it is quite likely that the SC of the pine wood nematode (PWN, *Bursaphelenchus xylophilus*) is relevant to the infection process and host symptom development as it lies at the interface where the PWN and the host first come into contact. To verify this hypothesis, it is essential to characterize the SC molecules at the host-pathogen interface. We considered that the variability of SC protein should be especially important in performing multi functions, and therefore tested several kinds of lectins for their binding property to the SC of the PWN to detect the stage- and isolate-specific changes of the SC proteins. Also, the alteration of the SC of PWN proteins due to invasion into the host pine was then investigated. Finally, for further detailed characterization, the SC proteins extracted were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by lectin blotting. The lectin-binding study using individual PWNs demonstrated the stage-specific characters of SC in binding to one of the lectins used, i.e. wheat germ agglutinin (WGA). WGA-binding was observed only to the outer surfaces of 3rd-stage propagative juveniles and to egg shells, and this occurred more frequently in virulent than in avirulent PWN isolates. A greater variety of lectins bound to eggs than to any other life stage. As for the PWN after infection into the host pine tree, WGA-binding to the body surface was observed at higher frequency irrespective of nematode stage. The molecular analysis of the SC proteins extracted showed that the carbohydrate and protein patterns of the SC of PWN changed during nematode development and in infecting host pine. All these results clearly indicate that the SC proteins of PWN should be of importance in pine–PWN interactions. In this presentation, we will also show other characteristics of the SC proteins of PWN and propose the significance of the SC itself and its variability in pine-PWN interactions.

Non-virulent strains and mutation breeding of a toxin producing *Pseudomonas* strain carried by *Bursaphelenchus xylophilus*

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A *Pseudomonas* strain carried by *Bursaphelenchus xylophilus* was used as the starting strain for screening of non-virulent strains of a *Pseudomonas* sp. by using chemical mutation of HNO_2 and UV light. Two new mutant strains, SP-A and SP-B, were obtained after four repeated treatments with the HNO_2 and UV light. A bioassay using non-cell filtrates from the fermentation of the two strains showed their low toxicity to the callus cells of *Pinus thumbergii*, the surviving rate of which were 2.62％, 25.78％ and 26.67％, respectively for the original strains, stains SP-A and SP-B by fluorescent microscope observation. We conclude that the ability of toxin production by the two mutant strains decreased compared to that of the original strain.

Partial purification and characterization of extracellular lignin peroxidase from *Pseudomonas fluorescens* GcM5-1A

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Pseudomonas fluorescens GcM5-1A carried by *Bursaphelenchus Xylophilus* was found to secrete extracellular lignin peroxidase which was involved in the biodegradation of lignin. The liquid fermentation of GcM5-1A was precipitated with ammonium sulfate precipitation and used to analyze the toxicity to suspension cells of *Pinus thunbergii*. Ammonium sulfate fractionation between 50% and 70% showed relatively strong toxicity, which also gave maximal specific lignin peroxidase activity. The present study was also undertaken to investigate the enzyme characteristics of partially purified lignin peroxidase(LiP) from *Pseudomonas fluorescens* GcM5-1A. The GcM5-1A LiP-catalyzed veratryl alcohol oxidase activity showed that the optimum pH and temperature of the peroxidase activity were about 4.0 and 35 ℃, respectively. The enzyme activity was stable in the pH range of 6.0 to 9.0, and at temperatures 15 - 35 ℃. The activity was enhanced by Ca^{2+}, Mn^{2+} and Fe^{3+} but was moderately inhibited by Hg^{2+} and Zn^{2+}. The typical peroxidase inhibitors KCN and NH2OH · HCl inhibited 57 % and 88 % of the enzyme activity, respectively, while NaN_3 was found to have low inhibitory effect of only 15%. The absorption spectrum of the native enzyme showed a Soret peak at 406 nm. Reduction by 1mmol/L $Na_2S_2O_4$ resulted in a marked decrease in absorbance, while shift of Soret peak was not observed.

Genetic diversity in pathogenicity among isolates of the pine wood nematode, *Bursaphelenchus xylophilus*

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Within *Bursaphelenchus xylophilus*, the pine wood nematode (PWN), isolates differ widely virulence. To fully understand the pathogenic mechanism of pine wilt disease it is important to identify the hidden key that is involved in the PWN virulence. Until now, although many reports had been given on the physiological characteristics of the PWN, little is known about the critical determinant of virulence and also the associated genetic conditions. Here, using molecular techniques, we focused on the genetic characteristics of the PWN and estimated the genetic diversity among and within isolates of the nematode. In molecular biological studies on the PWN, genetically uniform isolates, so-called 'the pure-lines', are required because of their uniform characteristics and ease of genetic analysis. To acquire basic information on the genetic status of the PWN, we first prepared and investigated pure-lines of the PWN produced by repeatedly inbreeding each of the virulent isolate (S10) and the avirulent isolate (OKD-1), obtaining three pure-lines and two pure-lines, respectively. The virulence potential of the resulting pure-lines was estimated by doing an inoculation test using susceptible *Pinus thunbergii* seedlings and then by comparing the resulting host mortality with that resulting from inoculations done using the original nematode isolates. As a result, PWN pure lines were obtained which differed in virulence compared to the original isolates. To determine the basic physiological ability of each pure-line, the population of nematodes cultured on the fungus *Botrytis cinerea*, was investigated. The reproductive ability of the pure-lines differed among one another, and also from the original isolates. Thus, the newly-obtained pure-lines were different from the original isolates both in virulence and reproductive ability, which strongly suggests that both original isolates, S10 and OKD-1, are genetically heterogeneous. In addition, these two characteristics were not consistent; the isolates which caused high mortality of the pine seedlings did not necessarily reproduce well on *B. cinerea*. This may indicate that reproductive ability is not the crucial determinant of virulence in the PWN. To further clarify the genetic diversity within each PWN isolate, we applied the DNA marker, amplified fragment length polymorphism (AFLP) technique. Resulting from its high polymorphism and high reproducibility the AFLP assay is useful for evaluating genetic diversity. In our

presentation we introduce the methodology we developed and discuss the genetic diversity among and within PWN isolates.

Pathogenicity of *Bursaphelenchus xylophilus* from natural and *in vitro* sources to *Pinus thunbergii* seedlings and saplings in Nanjing, China

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The most effective control for pine wilt disease, caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is replanting with resistant *Pinus* species or other conifers. An important factor in screening for pine wilt resistance is the aggressiveness of the nematode populations used and their pathogenicity to plants. The variations in our results obtained from previous pathogenicity tests were criticized by McNamara (2004). Therefore, recent pathogenicity experiments were designed to evaluate the factors that might affect the pathogenicity of *B. xylophilus* to Japanese black pine, *Pinus thumbergii*. These included simulating natural nematode infection via the beetle vector *Monochamus alternatus* versus inoculation using dispersal juveniles collected from beetles.

The results obtained in 2 years (2004-2005) of experiments showed the impracticality of using nematode-carrying beetles, i.e. simulating natural transmission, as inoculum in pathogenicity tests. Artificial inoculation is the only practical way to investigate the pathogenicity of *B. xylophilus* on *Pinus* species, but many other factors need to be considered. For example, inoculation density has important effects on the expression of the pathogenicity of *B. xylophilus* to *P. thunbergii*. In artificial (*in vitro* produced inoculum) inoculations with 200 nematodes per plant was found to be the appropriate density, which is close to the maximum number (350) of nematodes transmitted by one beetle on one day under natural conditions (Li *et al*., 2007). Plants inoculated with 200, non-surface sterilised nematodes originating from beetles or nematodes cultured for one life cycle on the fungus *Botrytis cinerea* showed faster symptom development and higher mortality rates compared to those inoculated with surface sterilized nematodes from beetles or diseased logs and cultured for one or multiple generations on the fungus. Wilt symptoms developed faster on 3-4-year old *P. thunbergii* seedlings than on 7-9-year old plants; seedlings were more sensitive than mature plants.

A comparative study on the pathogenicity of different pine wood nematode isolates to *Cedrus deodar*

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In order to determine why *Cedrus deodara* has not been affected by pine wilt disease in China, three isolates of pine wood nematode from Japan and China were used to inoculate *Cedrus deodara*, *Pinus thundergii* and *P. massoniana*. The results showed that the three isolates of the nematode did no differ in their pathogenicity to *P. thundergii* and *P. massoniana*, but differed markedly regarding their pathogenicity to *Cedrus deodara* . When inoculated with the nematode isolates from Japan *Cedrus deodara* wilted or died (mortality rate 50%), but no *Cedrus deodara* wilted or died when inoculated with the nematodes from China. The results show that the reason for *Cedrus deodara* surviving in China is that the pathogenicity of *Bursaphelenchus xylophilus* has changed after the nematode was introduced into China.

Post-inoculation population dynamics of *Bursaphelenchus xylophilus* and associated bacteria in Pine Wilt Disease of *Pinus thunbergii*

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Population dynamics of the pine wood nematode *Bursaphelenchus xylophilus* (PWN) and its accompanying bacteria in non-inoculated twigs along with the process of the disease was observed in Japanese black pine, *Pinus thunbergii* inoculated with PWN. In the non-inoculated twigs, bacteria could be detected when only a few pine needles became yellow. Once most needles had turned yellow or brown, the nematode began to appear and the bacterial populations increased. At the late stages of the disease when the inoculated pine was dying and the needles were totally wilted, the populations of both nematode and bacteria started to increase rapidly. Only a few bacterial species were found at the early stages. As the disease process advanced, the bacterial populations increased rapidly in both population size and variety of the species. However, *Pseudomonas fluorescens*, *P.* sp., *Pantoea* sp. and *Sphimgomenas pancimobilis*, remained dominant.

Study on the selective toxicity of disinfectants to *Bursaphelenchus xylophilus* at low temperature

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Because of the complex nature of the organisms associated with pine wilt disease, caused by the pine wood nematode (*Bursaphelenchus xylophilus*) (PWN) and associated bacteria, axenic cultures of the PWN are useful for researching the pathogenic mechanism of the disease. Disinfectants have selective toxicity to the PWN and its associated bacteria. This research was done to determine the usefulness of room temperatures and low temperatures to increase the processing time and enhance the effectiveness and reduce the toxicity of surface sterilants to the PWN. The surface sterilants tested for ridding the PWN of bacteria were: (i) 3％hydrogen peroxide, 4％sodium hypochlorite and 0.1％mercuric chloride using 22－25 ℃ (room temperature) and low 4－8 ℃ (low temperature). Following treatment the nematodes were washed with sterile water and then added to bouillon culture-medium. The effectiveness of the treatments was determined on whether or not the culture-medium become cloudy and the time required for this to occur. The survival of nematodes treated with disinfectants was also observed at the same time.

The results showed that：

1. The three disinfectants completely killed the PWN-associated bacteria after 5min at both the low and room temperatures and that they can sterilize PWN homogenate in 30min at low temperatures. No shorter periods have been tested.

2. The three disinfectants do not completely sterilize the PWNs at either test temperature unless the treatments are applied for 4.5h at the low temperature and for 25 min at room temperature. There was no indication that extending the treatment time would improve the sterilization effect.

3. 3% hydrogen peroxide was the best sterilant, followed by 0.1% mercuric chloride, and 4% sodium hypochlorite was the poorest.

4. The survival time of the PWN when treated with the three disinfectants at the

two temperature treatments (low temperature data followed by room temperature data in parentheses) 3% hydrogen peroxide, 23h (for more than 3.5h); 0.1% mercuric chloride, 8.5h (3h); and 4％ sodium hypochlorite for 3.5h(1h).

The results suggest that some associated bacteria may live in inaccessible areas on the PWN's body

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Preliminary partition of the toxins of a strain of *Pseudomonas fluorescens* associated with *Busaphelenchus xylophilus*

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To explore the mechanisms of pine wilt disease, *in vitro* toxin production of a bacterium strain, GcM5-1A (*Pseudomonas fluorescens*) isolated and identified from *Bursaphelenchus xylophilus*, was examined. Toxicity of the cell free filtrate of the liquid medium after culturing the bacterium was assayed by the fluorescence microscopic bioassay. Results of the bioassay showed that toxicity of the cell free filtrate of the liquid medium was increased with culturing days. Toxicity of the cell free filtrate reached a relatively stable phase from the 4th day during a 7 day experiment. Therefore, 4 days can be the duration of the culturing time, when the toxins of the strain are going to be isolated and identified. The cell free filtrate of the liquid medium after 4 days' culturing was dialyzed with DM-36 dialyzed into two parts, the interior part of the dialyzer and the exterior part. Bioassay results indicated that toxicity existed in both the interior and the exterior parts, because there were significant differences between the interior, the exterior parts and the control, respectively in the toxicity of their cell free filtrates by the test. The results demonstrated that a single compound did not cause toxicity of GcM5-1A strain, rather than multi components, in which there were both macro molecules, like proteins, peptides, enzymes, and small organic molecules.

Session 7 (papers): afternoon, July 22 and morning, July 23:

Disease management and control

Application of *Esteya vermicola*, an endoparasitic fungus of the pinewood nematode, for controlling pine wilt disease

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Esteya vermicola, the first endoparasitic fungus found to be associated with the pinewood nematode (PWN), shows great potential as a biological control agent for combating the devastating pine wilt disease. Recently, new *E. vermicola* strains have been isolated from soil in Korea. These straits have two different types of conidiogenous cells and conidia. However, only the lunate conidia are adhesive and can attach to the cuticle of the PWN and cause subsequent infection. *In vitro*, *E. vermicola* kills and colonizes almost all the tested pinewood nematode isolates within 4-5 days and is highly infective. However, to determine its potential as a biocontrol agent for the PWN, studies are needed on the feasibility of using the fungus. Isolation results and the taxonomic status of *E. vermicola* suggest that it is associated with vector beetles and affected trees. Consequently, greenhouse tests were done using the fungus to control the PWN and during the last 2 years field tests have been done in mountainous areas. Three application methods were used in the experiments： suspension spraying, trunk injection into trees, and applying infected nematodes onto trees. The results showed that *E. vermicola* grew into pine seedlings and successfully infected PWNs and *E. vermicola* mycelium were observed within pine trees.

These results indicate that *E. vermicola* could help pine trees survive PWN infection. Presently, studies are being made to evaluate the application and formulation of *E. vermicola* as a commercial biocontrol agent.

Esteya vermicola, an endoparasitic fungus with high infectivity to pinewood nematode with

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The pinewood nematode (PWN) causes pine wilt disease which results in catostrophic economic losses to the forest industry. Many nematophagous organisms have been tested, including nematophagous fungi, for controling the PWN. In 2006, a rare hyphomycete fungus (isolate CNU 120806) was isolated from infected nematodes found in pine-forest soil in South Korea. This fungus is characterized by its ability to produce two types of conidiogenous cells and conidia. However, only the lunate conidia are adhesive and can attach to the cuticle of PWNs and cause subsequent infection. With the consumption of the dead nematode's body, *E. vermicola* gradually grows out from the cadavers and then produces new conidia for the next infection cycle. Based on phenotypic characteristics and molecular phylogenetic analyses, we identified the fungus as *Esteya vermicola*, the first recorded endoparasitic fungus of the PWN. Resulting from its high infectivity *in vitro E. vermicola* shows great promise as a biological control agent for combating the devastating pine wilting. In subsequent studies, our isolate has been described and

compared with three other isolates (ATCC 74485, CBS 115803, and CBS 100821) and their detailed morphological and molecular characteristics have been determined as have their infectivity against the PWN.

The results show that *E. vermicola* CBS 115803 kills and colonizes almost all the tested pinewood nematode isolates within 4-5 days, i.e. it is highly infectious which has been attributed to its relatively high proportion of lunate conidia. The factors that influence the infectivity of *E. vermicola* against the PWN and the interaction between *E. vermicola* and the nematode were also studied in detail. The results show the high potencial of applicating certain formulations of the fungus as a commercial biocontrol agent against the PWN.

Isolation and identification of actinomycetes from soil in Yunnan and Inner Mongolia, and their possible anti-*Bursphelenchus xylophilus* (Steiner & Buhrer) activity

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Some 121 actinomycetes were isolated from soil in Yuanjiang, Yunnan Province, and Ordos, Inner Mongolia. Their anti-nematode activity was tested using a fermentation product assay. The activity screening results showed that five strains of the actinomycetes had anti-nematode activity. According to their morphological and physiological characteristics and phylogenetic analysis based on 16S rRNA gene sequences the strains were identified as Streptomyces spp. The fermentation product of *Streptomyces* sp. LM-21 showed a high anti-nematode activity, the broth of the strain was extracted gradually by chloroform, ethyl acetate and n-butanol, then a fraction from the extracts was isolated by high-speed counter-current chromatography (HSCCC). Five compounds were obtained, and YC-2 had a high anti-nematode activity.

Preliminary studies on *Metarhizium anisopliae* and its carrier, *Scleroderma guani*, to control *Monochamus alternatus*

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Twenty-one strains of *Metarhizium anisopliae* were collected and their basic biology characters and virulence were studied. The results showed that the virulence of these strains varied. An experiment to determine the possibility of controlling *M. alternatus* larva was done with Ma_{789} carried by *Scleroderma guani*. The main results were:

1. When the virulence of different *M. anisopliae* strains to *M. alternatus* adults was tested by direct spraying the results showed that strain *M. anisopliae*$_{789}$ (Ma_{789}) was the most virulent, followed by Ma_{788}, while strain Ma_{42} was the least virulent.

2. When the virulence of different strains carried by *S. guani* to control *M. alternatus* larva was tested the results showed that strain Ma_{789} was the most virulent. The LC_{50} for *M. alternatus* larva was 3.2×10^5 conidia per mL ($C=10^5$~10^8 conidia per mL). When every *S. guani* carried 1.0×10^7 conidia of Ma_{789}, and one *M. alternatus* larva was inoculated with 1, 2, or 3 *S. guani*, the ratios of parasitism resulting in death were 56.7, 70, and 100%, respectively. The results showed that the death rate of *M. alternatus* larva increased when the number of *S. guani* carried Ma_{789}.

3. Mass rearing of *S. guani* in the laboratory resulted in 13 million female adults of *S. guani* and 0.3 million larvae of *M. alternatus* annually from 2004–2009. *S. guani* was released to control the larvae of *M. alternatus* in the same and other areas damaged by *Bursaphelenchus xylophilus*. The numbers of dead pine trees were significantly reduced in the test area.

The sustainable control techniques and their effects on Pine Wilt Disease

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Bursaphelenchus xylophilus was first discovered in Xiamen City, The People's Republic of China, in 2002 and initially the extent of the disease was quite large. Pine is difficult to replace with other trees because it is a major component in the shore district, therefore, it is necessary to take effective measures to control the disease in order to retain the pine forests there. Beginning in 2002 a series of sustainable management strategies for the pine wilt disease (caused by the pine wood nematode, *Bursaphelenchus xylophilus)* have been conducted in Xiamen City, Fujian Province. Included among the strategies are epidemic surveys, removal of dying branches and trees, setting out beetle traps, setting vertical induced wood and biological control. The results showed that the damaged pine forest area has decreased from 30000 Mu since first being discovered to 642 Mu in 2007, and the number of dead pine trees has been reduced from 136968 in 2002 to just six in 2007. As well, the average numbers of trapped *Monochamus alternatus* by individual traps has diminished from 72.7 in 2002 to 1.8 in 2007, and the control related costs per year has averaged 40.9 Yuan per Mu. Proof of the effectively of the measures is that healthy pine forests still exist in Xiamen even though *Bursaphelenchus xylophilus* was discovered there 7 years ago.

Releasing *Scleroderma guani* to control *Monochamus alternatus* in the pine forest of Fujian

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The ant-like bethylid wasp *Scleroderma guani* Xiao et Wu is an important natural enemy in the biocontrol of the vector beetle, *Monochamus alternatus* Hope, of the pinewood nematode. *S. guani* has been released into pine forests in Xiamen for controlling *M. alternatus*. The results show that it is better to release *S. guani* on every tree or every other tree, which resulted in corrected parasitism rates of 31.27% and 30.99% respectively. These values are significantly higher than releasing *S. guani* into the center of pine forest which resulted in a corrected 11.36% parasitism rate. The best time for releasing *S. guani* is June and July, when the temperature in the forest is around 27 ℃ and *M. alternatus* is in the larval instar stage of development, and the optimal parasitoid-host ratio was 3:1. The high parasitism rate for releasing *S. guani* into a 266 ha pine forest for biocontrol of *M. alternatus* proved that the control of *M. alternatus* larvae by releasing *S. guani* into the forest was feasible, and resulted in an average parasitism rate of over 20%.

Occurrence and coping strategies of Pine Wilt Disease Caused by Bursaphelenchus xylophilus in Guangxi, China

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Since pine wood nematode was found in 1982 in Nanjing, Jiangsu Province, P. R. China, pine wilt disease has spread to 14 provinces; and it has become a serious threat to forests, tourism and the land ecological security. Since 1996 the Animal and Plant Quarantine Bureau of Liuzhou has intercepted pine wood nematode from Japanese pine wood packaging material, and quarantine officers of the Animal and Plant Quarantine Bureau of Fangchenggang and Entry-exit Inspection & Quarantine Bureau of Guigang have intercepted pine wood nematode in wood packing material from the USA, Japan and Germany in 1997, 1999, 2000, and 2003. To prevent pine wilt disease from spreading in Guangxi, the following measures are presently being taken:

1) Rationally dividing the prevention and eradiaction regions

According to the occurrence and spreading law for pine wilt disease, the distribution and regional location of pine resources and other factors, all of Guangxi was divided into three region types, i.e. the region of disease where the disease occurs, another region of general prevention and a third key prevention region.

2) Adopting super-conventional measures to purposefully curb the spread of pine wilt disease.

Guangxi province mainly uses the following powerful measures for pine wilt disease prevention and cure. First, preventive measures which are taken rapidly to control and extinguish the epidemic. Second, a system of setting deadlines for removal work to be carried out. The Forestry Bureau of Guangxi province has established clear mission and completion times for prevention and cure work in every city and county, and ordered that all new outbreak areas must eradicate the disease within a required timeframe. Third, prompt removal of pine wilt killed the trees. In the outbreak area, monthly inspections are a must. Professional teams have to quickly clean up the dead trees. Fourth, execute clear cutting. Base on the work of quickly removing pine wilt-killed trees ,forest compartments where the disease occurs must be clear cut in a planned way. Only this can ensure that the hosts of pine wilt disease are

thoroughly removed.

3) Conscientiously carry out surveys to promptly detect the disease

Surveys are the foundation of thoroughly eradicating pine wilt disease. Therefore, Guangxi province gives priority to information provided by national and autonomous region-level forecasting regional stations, and overall improvement in monitoring and early-warning systems. As well, the Guangxi provincial government provides 1.2 million yuan of financial assistance per year for carrying out surveys in 60 regional forecasting stations across the province. All levels of the various forestry departments have also set up a sound monitoring system for the epidemic, and are concise about the goals in regions which focus on monitoring. Also, annual fixed point surveillance and periodic surveys in strict accordance with the technical requirements are being done.

4) Strengthen the management of pine wood nematode infested wood, and enforce strict quarantine and inspection

The lack of accountability regarding the movement of pine wood nematode infested wood is the main reason for the spread of pine wilt disease. In the process of harvesting, transporting, processing and utilizing such wood some personnel should be specially assigned to: (i) supervising the entire process, and preventing unrestricted distribution of infested wood;(ii) intensifying quarantine and inspection in the outbreak area, (iiii) enforcing a strict quarantine blockade, and (iv) prohibiting the illegal management and transportation of infected wood and its products. Towards achieving these goals plant quarantine checkpoints have been established along the main traffic routes of Guangxi. At these check points there is strict inspection and quarantine of forest plants and their products passing through the checkpoints with the goal of preventing artificial spread of the epidemic. As a result of increased infested wood management, the whole province has prevented an epidemic from occurring via infested wood.