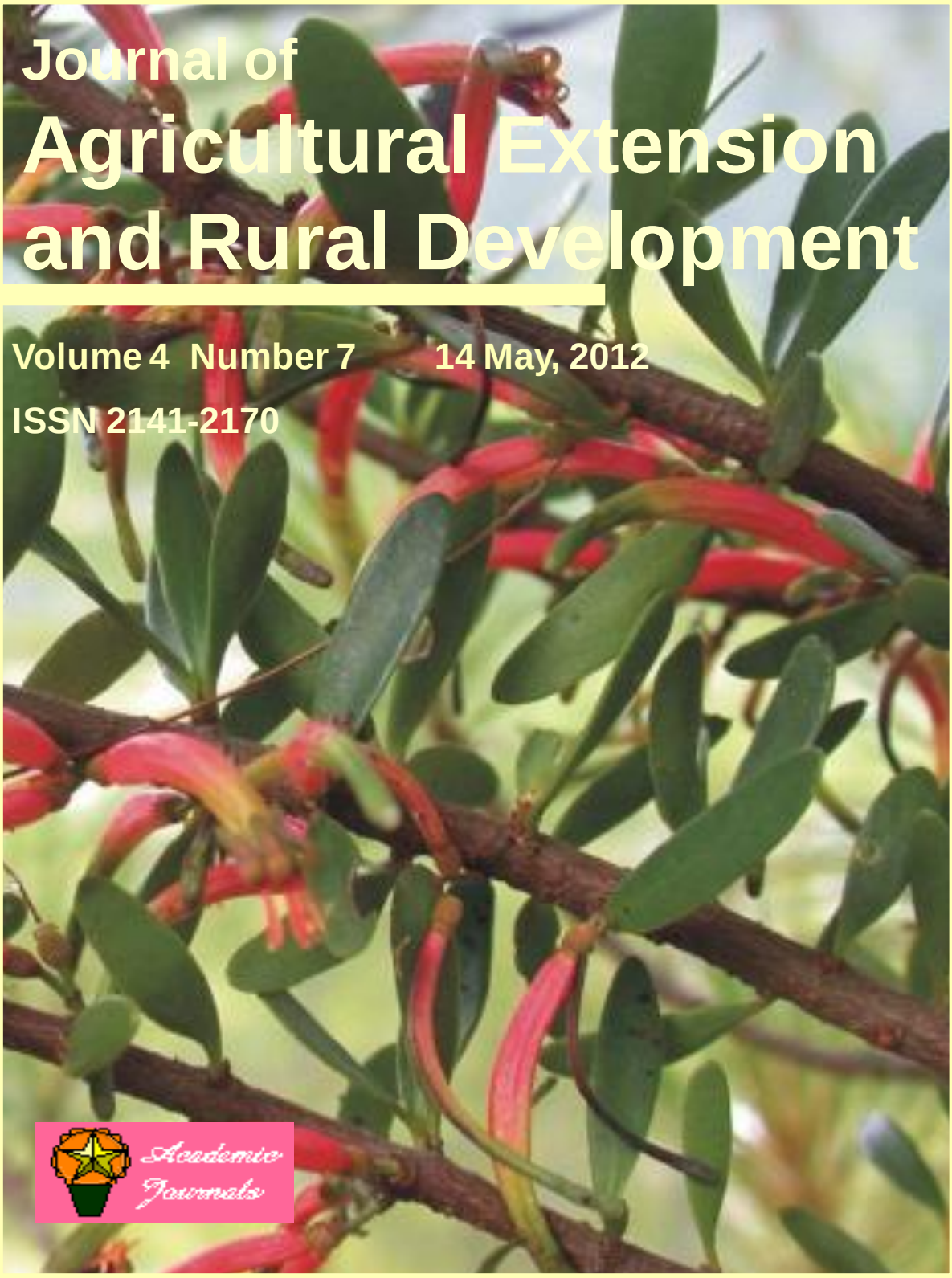

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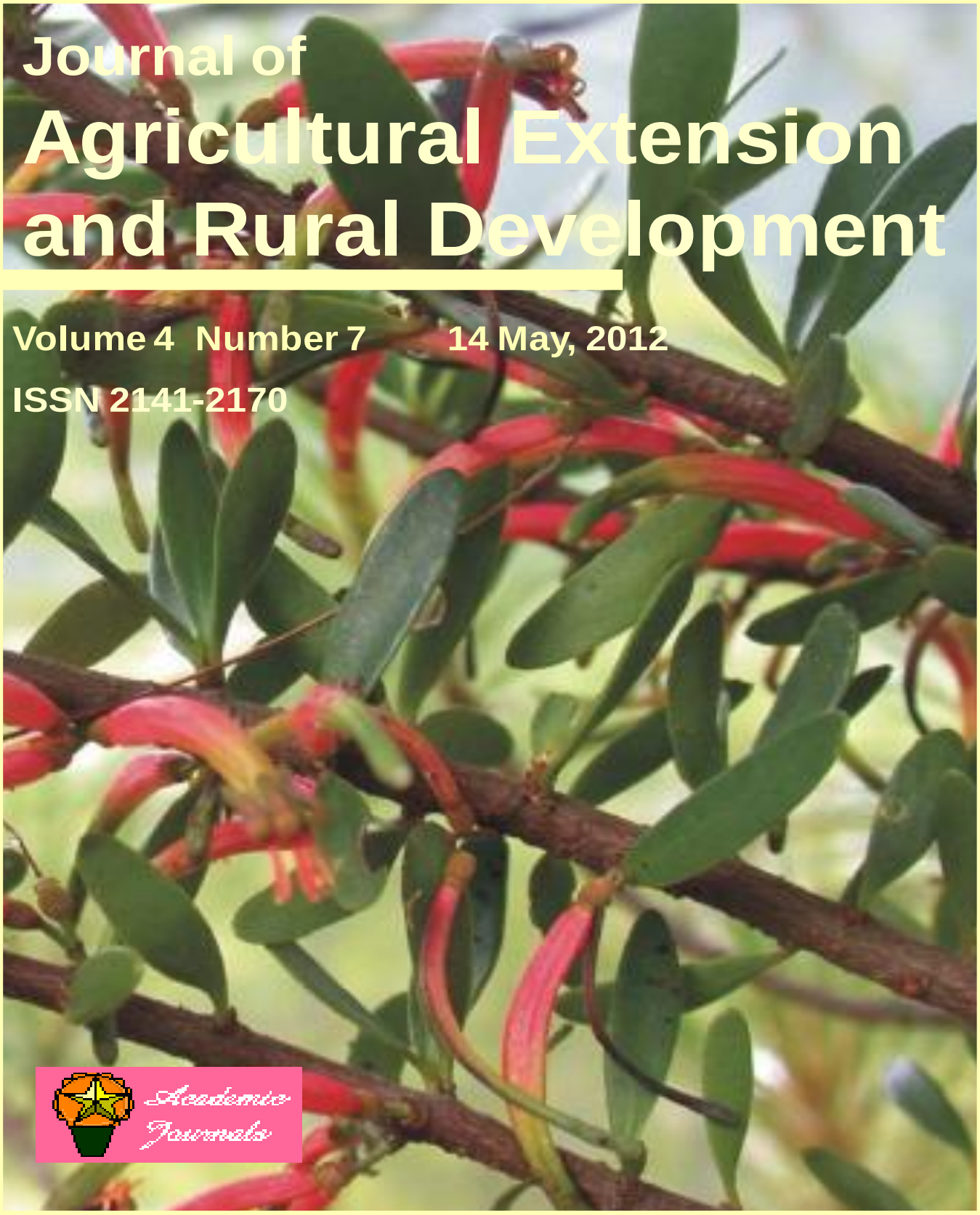

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Frequency of virus in some *Diplodia pinea* and *Gremmeniella abietina* isolates originated from Turkey

Aday A. G.[1]*, Lehtijarvi A.[2] and Doğmuş- Lehtijarvi H.T.[2]

[1]Yenişar Bademli Vocational School, Suleyman Demirel University, Isparta- Turkey.
[2]Faculty of Forestry, Süleyman Demirel Üniversity, 32260 Isparta- Turkey.

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***Diplodia pinea* and *Gremmeniella abietina* are common pathogens causing shoot blight and dieback of pine all over the world. *D. pinea* is one of the main causal agents of shoot blight of Calabrian pines in the Mediterranean countries including Turkey. *G. abietina* has been recently observed on saplings and seedlings of *Pinus nigra*, which remain under snow cover during winter dormancy in Dedegül Mountain in the Mediterranean Region of Turkey. The presence of viruses in fungi has been known for many years. An accumulating number of cloned and sequenced viral genomes have enabled us to detect virus in increasing number of fungal species in the recent years. *D. pinea* and *G. abietina* are known to contain members of the virus families *Narnaviridae*, *Totiviridae* and *Partitiviridae,* which can infect single fungal isolates. Viral dispersal in fungi mainly occurs via anastomosis. Some *Diplodia* and *G. abietina* isolates have different characteristics, such as reduced virulence and growth rate, lack of pigmentation, altered colony morphology, and reduction in conidial production due to presence of viral particles. In this study, 18 *D. pinea* and 6 *G. abietina* isolates were investigated for the presence of dsRNA. Double-stranded RNA was isolated using a commercial RNA extraction kit and visualized in agarose gel electrophoresis. Isolates containing dsRNA were also investigated for their *in vitro* growth rate and ability to produce conidia. Three (50%) *G. abietina* and ten (56%) *D. pinea* isolates contained dsRNA that had an approximate molecular size of 1.6 kb.**

Key words: Scleroderris canker, calabrian pine, dsRNA.

INTRODUCTION

Diplodia pinea Dezmaz J. Kickx (*Sphaeropsis sapinea* (Fr.) Dyko & Sutton) and *Gremmeniella abietina* (Lagerb) Morelet are common pathogens causing shoot blight and dieback of coniferous tree species all around the world (Adams et al., 2002; Tuomivirta and Hantula, 2003a). *D. pinea* is one of the main fungal agents of shoot blight of Calabrian pines in the Mediterranean countries including Turkey. *G. abietina* was recently detected on *Pinus nigra* Arnold ssp. *pallasiana* (Lamb.) Holmboe most likely causing damage on saplings and seedlings at low temperatures under snow cover in Dedegül Mountain of Mediterranean Region of Turkey (Lehtijärvi et al., 2010).

*Corresponding author. E-mail: guldenaday@orman.sdu.edu.tr.

Mycoviruses with double stranded RNA genomes are widely spread in many major groups of plant pathogenic fungi. More than a hundred fungal species are known to be host for mycoviruses with most of these consisting of dsRNA (Buck, 1986). The main fungal mycovirus families are *Chrysoviridae*, *Hypoviridae*, *Partitiviridae* and *Totiviridae*. Double stranded RNA genomes can spread between mycelia via hyphal anastomosis, conidia, basidiospores, ascospores from yeasts and rarely through ascospores from filamentous ascomycetes (Buck, 1986). In general, mycoviruses do not change the host phenotype (Ghabrial, 1994), but there are several cases where virus infection results in marked structural or physiological changes. An accumulating number of cloned and sequenced viral genomes have enabled us to detect virus in increasing number of fungal species in recent years. *D. pinea* and *G. abietina* are known to con-

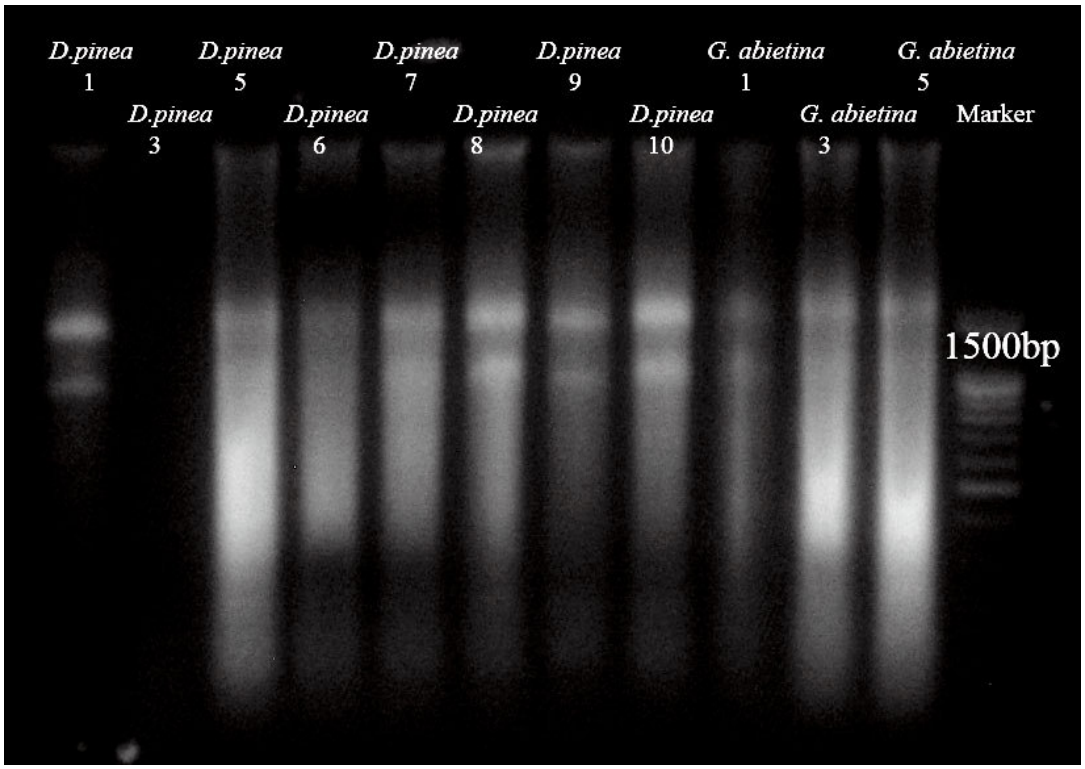

Figure 1. Banding pattern of dsRNA genomes in *D. pinea* and *G. abietina* isolates.

tain members of the virus families *Narnaviridae, Totiviridae* and *Partitiviridae* (Wu et al., 1989; Preisig et al., 1998; Steenkamp et al., 1998; De Wet et al., 2001; Adams et al., 2002; Tuomivirta and Hantula, 2003a,b), which can infect single fungal isolates. Some *Diplodia* and *G. abietina* isolates have different characteristics, such as reduced virulence and growth rate, lack of pigmentation, altered colony morphology, and reduction in conidial production due to presence of viral particles.

Several dsRNA genomes ranging from 600 to 7000 bp in size have been reported from single *Diplodia* isolates (Wu et al., 1989; Preisig et al., 1998; Steenkamp et al., 1998; De Wet et al., 2001; Adams et al., 2002). Two of these elements have been characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) (Preisig et al., 1998). Tuomivirta and Hantula (2003a,b) found two unrelated dsRNA patterns in *G. abietina* type A and they described *Totivirus, Partitivirus* and *Mitovirus* genera that are common in A type of *G. abietina* isolates. The aim of this study was to investigate Turkish *D. pinea* and *G. abietina* isolates for the presence of dsRNA.

MATERIALS AND METHODS

Fungal material

In this study, a total of 18 *D. pinea* and 6 *G. abietina* isolates were investigated for the presence of dsRNA. *D. pinea* isolates were obtained from *Pinus brutia.* Ten plantation site in Aşağı Gökdere in Isparta province which was as homogenous as possible regarding to tree size and shoot blight symptoms.

All *G. abietina* isolates were isolated from diseased shoots of *P. nigra* ssp. *pallasiana* in Dedegül Mountain in Yenişarbademli-Isparta province. *D. pinea* and *G. abietina* isolates were grown on potato dextrose agar (PDA) and modified orange serum agar (MOS–agar) respectively at 20°C.

RNA extraction

D. pinea and *G. abietina* isolates were grown on PDA and MOS–agar plates covered with cellophane membrane at 20°C for 10 days. The mycelium was then scraped off, placed into mortar, and ground with liquid nitrogen. Total RNA was isolated using RNA isolation kit following the instructions of the manufacturer and all isolates were screened in agarose gel electrophoresis. Isolates containing dsRNA were also checked for the growth rate and production of conidia.

RESULTS AND DISCUSSION

In visual inspection of the gels containing the total RNA of the fungal isolate fragments of approximate length of 1.6 kb were detected (Figure 1). Owing to the size of these fragments, they were regarded to be putative viral dsRNAs. Totally three (50%) *G. abietina* and ten (56%) *D. pinea* isolates contained these dsRNA fragments (Figure 1).

The size of the putative dsRNA in this study, present in

both *D. pinea* and *G. abietina*, were well within the 1 to 12 kbp range of known dsRNA genomes of mycoviruses (Ghabrial, 1994). In earlier studies, putative viruses of families; *Narnaviridae, Partitiviridae* and *Totiviridae* were detected in *G. abietina* (Tuomivirta and Hantula, 2003a, b) and *D. pinea* (De Wet et al., 2008). Therefore, it is likely that the putative dsRNA molecules found in this study also belong to these virus families. However, other possibilities cannot be excluded until we have determined and compared the sequences of the dsRNAs found in the present work with those in the viral genome database. The number of dsRNA genomes was not investigated in our study. It is common for mycelia to contain more than one dsRNA molecule (Ghabrial, 1998).

Some dsRNA virus molecules reduce the growth rate and spore production of a fungal pathogen like in *Cryphonectria parasitica* (Murr.) Barr (Ihrmark et al., 2001). In our study, the growth rate of *D. pinea* and *G. abietina* were 60 and 40 mm respectively and the dsRNA molecules were found to affect the spore production of only *D. pinea* isolates. These sizes were similar to those of the dsRNA molecules previously reported for viruses of *G. abietina* type A (Tuomivirta and Hantula, 2003a, b).

Conclusions

The results of this study showed that dsRNA fragments were common in Turkish *D. pinea* and *G. abietina* isolates. As the sample size was small, the result indicates that the frequency of the dsRNA in natural *D. pinea* and *G. abietina* populations is high. The dsRNAs from both fungi should be at least partially sequenced for sequence comparison with known viral sequences. In addition, the effect of the dsRNAs on the physiology of the fungal isolates should be investigated.

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Full Length Research Paper

Ash dieback caused by *Hymenoscyphus pseudoalbidus* in a seed plantation of *Fraxinus excelsior* in Austria

Thomas Kirisits* and Christian Freinschlag

Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.

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Ash dieback, an emerging fungal disease incited by *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*), causes immense damage to *Fraxinus excelsior* in many parts of Europe. There is hope that some individuals of this tree species display high levels of resistance to the disease. In 2009 and 2010, the intensity of ash dieback was investigated in an ash seed plantation in Upper Austria, consisting of 51 clones of local provenance. A specific rating system for visual inspections was developed to assess shoot, twig and branch dieback as well as leaf shedding. Considering all 187 evaluated trees, mean ash dieback intensity was 18.1% in July 2009 and 17.6% in July 2010. Disease intensity varied greatly between clones, ranging in both years from almost no dieback to more than 80% dieback in the most severely affected clone. Likewise, levels of leaf shedding in July and September differed considerably between clones. However, no clear relationship between leaf shedding and dieback intensity was observed. The results indicate that *F. excelsior* clones in the seed plantation may indeed differ substantially in their resistance to *H. pseudoalbidus*.

Key words: *Chalara fraxinea*, common ash, disease assessments, emerging fungal disease, resistance.

INTRODUCTION

Dieback of common ash (*Fraxinus excelsior*), narrow-leaved ash (*Fraxinus angustifolia*) and other *Fraxinus* species is caused by the recently described ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*; Kowalski, 2006; Bakys et al., 2009; Kowalski and Holdenrieder, 2009a, b; Kirisits et al., 2009, 2010a; Drenkhan and Hanso, 2010; Schumacher et al., 2010; Husson et al., 2011; Queloz et al., 2011). This emerging tree disease was first observed around 1992 in Poland and has by 2011 been recorded in at least 25 European countries (Timmermann et al., 2011; Kirisits, unpublished). Although its origin is still enigmatic, the high intensity and the gradual appearance of ash dieback in Europe, related to the successive spatial spread of the causative pathogen, may suggest that *H. pseudoalbidus* or an aggressive mutated strain of this species is behaving as an alien invasive organism (Husson et al., 2011; Queloz et al., 2011; Timmermann et al., 2011).

Ash dieback is characterized by a remarkably wide range of symptoms (Kirisits et al., 2009; Kowalski et al., 2010). The most conspicuous ones are necrotic lesions and cankers in the bark, combined with wood discoloration, leading to dieback of shoots, twigs, branches and smaller stems. Likewise, *H. pseudoalbidus* has been suggested to be associated with leaf symptoms on *F. excelsior* and other ash species (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Ogris et al., 2009; Drenkhan and Hanso, 2010; Kowalski et al., 2010; Kräutler and Kirisits, 2012). Leaf infections are also thought to play a key role in the disease cycle of ash dieback, as they have been postulated as a major path for the fungus to grow into shoots and twigs of their host trees (Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b; Schumacher, 2011; Kräutler and Kirisits, 2012).

*Corresponding author. E-mail: thomas.kirisits@boku.ac.at.

Ash dieback occurs on *F. excelsior* trees of all ages (Kowalski and Łukomska, 2005; Kirisits et al., 2009, 2011; Kowalski et al., 2010; Schumacher et al., 2010). It is often a lethal disease of seedlings and natural regeneration, but thicket- and pole-sized trees are also frequently killed. Older ash trees are severely damaged as well, but can endure the disease for a longer time and often succumb due to secondary damaging factors such as *Armillaria* spp. (Kowalski et al., 2010; Kirisits et al., 2009; Bakys et al., 2011). The disease is questioning the future of *F. excelsior* as economically valuable and for ecological reasons appreciated noble hardwood species (Kowalski et al., 2010; Kirisits et al., 2009, 2011).

Options for disease management are very limited (Kirisits et al., 2009; Kowalski et al., 2010). The observation of healthy and only slightly affected trees in severely diseased ash stands may, however, indicate that there are considerable differences in resistance between individuals of *F. excelsior* to the ash dieback pathogen (Kirisits et al., 2009, 2010b; McKinney et al., 2011). Investigations in seed plantations in Denmark revealed that damage levels of ash dieback vary greatly amongst 39 clones, indeed suggesting the presence of natural genetic resistance in *F. excelsior* to *H. pseudoalbidus* (McKinney et al., 2011). Moreover, the length of necrotic lesions in the bark following artificial inoculation of *H. pseudolabidus* differed amongst clones and showed a strong and significant correlation with phenotypic damage levels resulting from natural infections (McKinney et al., 2012). *F. excelsior* individuals with putative high resistance to ash dieback may form the basis for the maintenance of the species in the future. These genotypes are expected to be favoured by natural selection and *ex-situ* genetic conservation and breeding programs could be initiated, providing prospects that the overall resistance levels of populations of *F. excelsior* will increase over time (McKinney et al., 2011).

In Austria, there are three seed plantations of *F. excelsior*, one in the province of Upper Austria and two in the province of Styria. These plantations consist of clones of local provenance which have been selected based on traits desirable for timber production. The original aim of the plantations was to yield genetically diverse, site-adapted forest reproductive material of high quality. With the occurrence of ash dieback, seed orchards and clone collections have become a valuable resource for investigations on the resistance of *F. excelsior* to this emerging disease. Hence, from 2009 onwards, the intensity of ash dieback has been monitored in the three *F. excelsior* seed plantations in Austria. The objectives of these investigations were: (i) to document differences in the intensity of ash dieback between clones; (ii) to use the seed plantations as general monitoring plots to follow disease development seasonally within a year and between years; and (iii) to increase the knowledge on the symptomatology and the disease cycle of ash dieback. In this report, preliminary results from one of the seed plantations are presented.

MATERIAL AND METHODS

The investigations were carried out in a seed plantation of *F. excelsior* located in Feldkirchen an der Donau (14.02 E, 48.20 N, 264 m asl., flat terrain) in the Austrian province of Upper Austria. In this 1.36-hectare-large plantation, initiated in 1993 on former agricultural land, 51 different ash clones of local provenance (forest ecoregion 7.1, northern foothills of the Alps, western part, submontane altitudinal zone; Kilian et al., 1994) are represented. Originally four grafted ramets per clone and thus 204 ash trees were planted at a spacing of 7.50 m (distance between rows) and 8.66 m (distance of trees within rows). The trees were arranged in a randomized pattern in the plantation. Twelve trees died after planting. Further five trees were trimmed in order to maintain a telephone line crossing the plantation and they were therefore not considered in the assessments. The total number of investigated trees was thus 187; 35 clones were represented with four ramets, 15 clones with three ramets and one clone with two ramets.

Ash dieback was for the first time recognized in the plantation in 2008, but it is likely that it caused damage even earlier there, as the first unambiguous observations of the disease in Upper Austria were made in 2005 (Cech, 2006). On 30 April 2009 dead shoots and twigs or those with localized necrotic phloem lesions, representing early stages of ash dieback were collected from selected *F. excelsior* ramets. In the laboratory, about 4 to 6 cm long segments, containing the transition between necrotic and healthy phloem tissues and/or discoloured and healthy xylem were cut from the samples. After surface sterilization (1 min in 96% ethanol, 3 min in 4% NaOCl, 30 s in 96% ethanol), the outer bark was carefully peeled off and 3 to 10 mm wide discs containing wood and phloem tissues were cut under aseptic conditions and placed on malt extract agar (MEA; 20 g/L malt extract, 16 g/L agar, 100 mg/L streptomycin sulphate). Isolation plates were incubated at cool temperatures (between 4 to 10°C) in the dark. This was done in order to stimulate anamorph production of *H. pseudoalbidus* and to give it competitive advantage over other fungi, thereby increasing the likelihood to detect the ash dieback pathogen (Kirisits et al., 2009). *H. pseudoalbidus* was identified based on morphological characteristics of its *C. fraxinea* stage (colony morphology, phialophores and spores). Other fungi were not determined. Besides fungal isolation, leaf petioles and rachises on the ground were in both years examined for the occurrence of black pseudosclerotial layers and apothecia of *H. pseudoalbidus*. Observations were made at the dates of the ash dieback severity assessments (see below) and during visits in the plantation in mid-June 2009 and early June 2010.

For the assessments of the severity of ash dieback on ramets of the various clones, a specific rating system was developed (Figure 1). For the ratings, the crown of each individual tree was divided into thirds. Thereafter, each crown third was assigned to one out of seven ash dieback severity classes. Using class means (0, 2.5, 12.5, 35, 65, 90 and 100), the values of the three crown thirds were averaged, to obtain an ash dieback severity rating in percent for each ramet (Figure 1). The rating scheme of dieback of shoots, twigs and branches was tested on 30 April 2009, prior to the main surveys. For this, about 15 trees were evaluated independently by both authors and the results were compared. Subsequently, the second author did all assessments alone. Leaf shedding was evaluated in the same way as dieback of shoots, twigs and branches (Figure 1). Assessments were done at three dates in 2009 and 2010, at mid-May, at the end of July and in early to mid-September.

RESULTS

Fungal isolation and occurrence of apothecia of *H. pseudoalbidus*

H. pseudoalbidus was isolated from 17 out of 21 (81%)

Ash dieback rating system

Step 1: Divide crown into thirds.

Step 2: Rate each third separately. Each third is given a rating from 0 to 6, as described below.

0	No dieback	Class mean 0%
1	< 5%	Class mean 2.5%
2	> 5% to 20%	Class mean 12.5%
3	> 20% to 50%	Class mean 35%
4	> 50% to 80%	Class mean 65%
5	> 80 to 100%	Class mean 90%
6	100%	Class mean 100%

Step 3: Add ratings of crown thirds to obtain a total rating for a tree; the tree on the right will receive a rating of 4 + 5 + 5 = 14.

Step 4: Calculate an average percentage value of crown dieback for a tree, using class means (see above); the tree on the right will receive a value of (65 + 90 + 90) / 3 = 81.7 %.

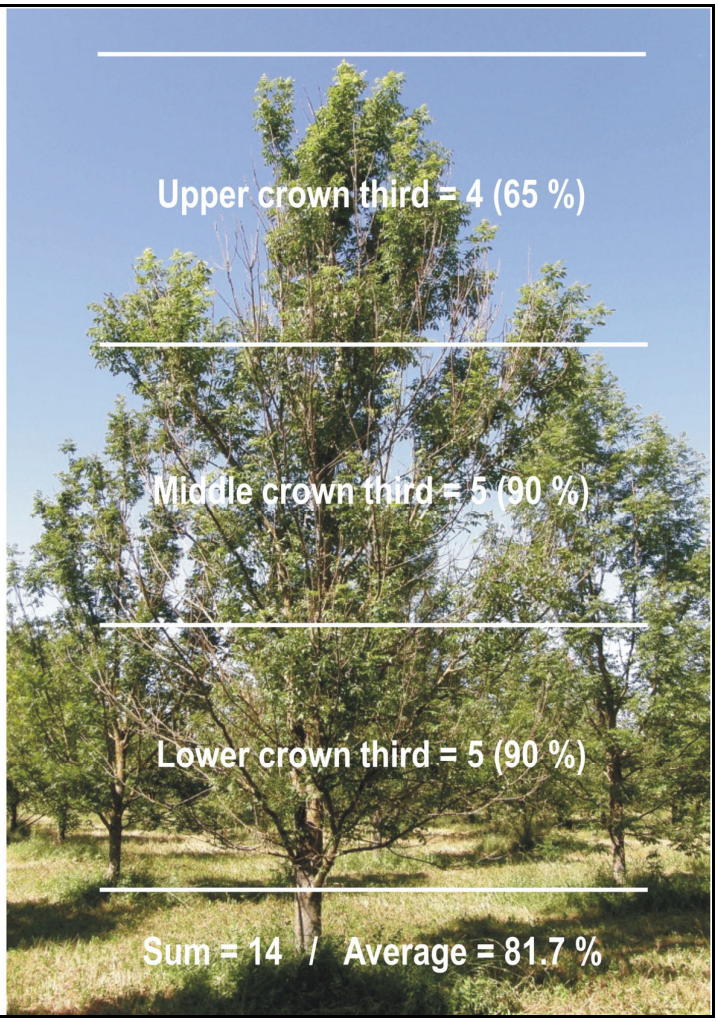

Figure 1. Rating system developed for the assessments of ash dieback severity in seed plantations in Austria.

symptomatic shoots and twigs collected from 20 ramets in the seed plantation. From 43% of the samples, it was obtained in pure culture, 38% of the isolations yielded *H. pseudoalbidus* in mixed culture with other fungi and from the remaining 19% of the samples other fungi, but not the ash dieback pathogen were isolated. Overall, *H. pseudoalbidus* was the most frequently isolated fungal species.

Leaf petioles and rachises with black pseudoclerotial plates were commonly observed in the litter during all visits in the seed plantation. Apothecia of *H. pseudoalbidus* occurred abundantly on leaf petioles and rachises at the end of July 2009 and 2010. Fruiting bodies were not seen at the inspections in May and were rare during the visits in September. In 2009, apothecia were observed on 17 June and in 2010 already on 9 June. Based on the irregular inspections in both years, the main period of ascocarp formation of *H. pseudoalbids* in the ash seed plantation likely extends from the middle or the end of June until late August or early September.

Dieback of shoots, twigs and branches

When testing the ash dieback rating system (Figure 1) in April 2009, the ratings of individual trees by the two authors were always identical. It was therefore decided to use this scheme for subsequent investigations. Considering all 187 evaluated trees, mean ash dieback intensity was 14.6% in May 2009, 18.1% in July 2009, 14.2% in May 2010 and 17.6% in July 2010. Due to early leaf shedding, evaluation of dieback severity proved to be difficult in September and the data of these assessments are therefore not presented.

Disease intensity varied considerably between clones, ranging in both years from almost no dieback to more than 80% dieback in the most severely affected clone (Figure 2). In May 2010 mean dieback intensity was below 10% in 28 out of the 51 clones (55%), between 10.1 and 20% in 13 clones (25%), between 20.1 and 30% in 5 clones (12%) and between 30.1 and 40% in one clone (2 %) (Figure 2). Only 4 clones (8%) had average

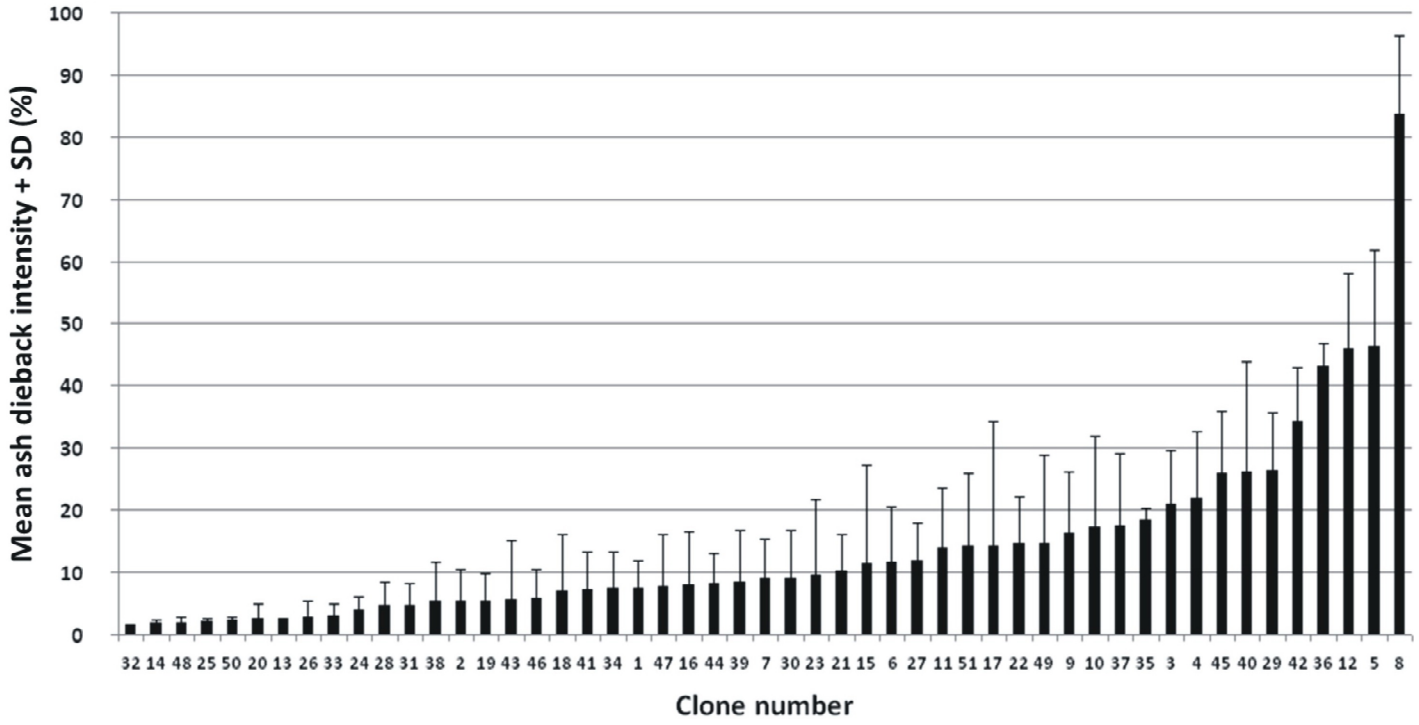

Figure 2. Mean ash dieback intensity (+ standard deviation, SD) in percent by *F. excelsior* clone on 13 May 2010 in the seed plantation in Feldkirchen an der Donau (Upper Austria). The overall sample size was 187 trees: 35 clones were represented with 4 ramets, 15 clones with 3 ramets and one clone with 2 ramets.

values higher than 40% (Figure 2). Ramets of two extreme clones (number 8, the most severely damaged clone and number 14, one of the least damaged clones) are shown in Figure 3. A comparison of the assessments in May 2009 and May 2010 showed that the mean ash dieback ratings remained unchanged for two clones, those of 28 clones had improved, while those of 22 clones got worse.

Leaf shedding

The overall patterns of leaf shedding were consistent in 2009 and 2010. In both years, the mean percentage of shed leaves was low at the end of July (8% in 2009, 1% in 2010), but high in late summer (67% in mid-September 2009, 46% in early September 2010). As it was observed for dieback, the intensity of leaf shedding varied considerably between clones, both in the July and September assessments (data not shown). While many clones showed high levels of defoliation by September, others were to a lesser degree affected by this symptom and a few remained densely foliated until late summer or even early autumn (for example clone number 14, Figure 3B). However, no obvious positive or negative relationships between the intensity of leaf shedding in September 2009 and dieback intensity in May 2010 were detected (data not shown). This is exemplified by clones numbers 8 and 14 (Figures 2 and 3), both of which are not so much affected by early leaf shedding, but differ substantially in the levels of dieback.

DISCUSSION

H. pseudoalbidus was isolated at high frequencies and often in pure culture from shoots and twigs displaying early symptom of ash dieback on selected ramets of *F. excelsior* clones in the seed plantation in Feldkirchen an der Donau. The massive occurrence of pseudosclerotial plates and apothecia on leaf petioles and rachises in the litter also confirmed the presence and abundant occurrence of the fungus. The results therefore clearly suggest that *H. pseudoalbidus* is associated with dieback of shoots, twigs and branches in the *F. excelsior* seed plantation.

The rating system developed in the present study is in our opinion convenient to monitor ash dieback over time and it may be useful for other studies evaluating the severity of this emerging disease. Assessments of disease severity based on visual inspections need to fulfil several criteria. They should be sufficiently accurate to reflect disease levels, easy to apply and assessments of the same tree by different observers should lead to the same result or at least be fairly comparable. Doing the assessments separately for crown thirds, which was

Figure 3. Differences in the intensity of ash dieback between *F. excelsior* clones in the seed plantation in Feldkirchen an der Donau (17 August 2011): A. Ramet of the most severely damaged clone (number 8), B. Ramet of one of the least damaged clones (number 14) with virtually no dieback symptoms. In both 2009 and 2010 ramets of this clone were also hardly affected by early leaf shedding and remained densely foliated until early autumn.

inspired by the 6-class dwarf mistletoe rating system (Hawksworth et al., 1996), and designation of the particular seven classes used for the ratings (Figure 2) contribute in our opinion to meet the three criteria mentioned previously (accuracy, easy application and comparability). All ratings in the seed plantation were done by the same person (C. Freinschlag), which should have a positive influence on data quality and guarantee comparability of assessments at various dates.

The *F. excelsior* ramets rated in the present study were relatively tall, justifying separate assessments for crown thirds. For smaller trees, dividing the crown into thirds is probably not necessary and it is preferable to evaluate the entire crown. In two other seed plantations in Austria, which consist of younger *F. excelsior* ramets, tall trees were rated separately for crown thirds, while for small trees a single value was assigned to the undivided crown. Using class means and averaging crown thirds, ash dieback severity can be calculated in percent, which can loosely be treated as a continuous variable in statistical analyses. These calculations also allow averaging disease severity data of trees which have been assessed separately for crown thirds and those for which ratings have been done for the entire crown.

Overall disease intensity changed very little during the entire observation period and in 2010 it was slightly lower than in 2009. This stagnation as well as the short-term changes in the performance of individual clones should, however, not be overestimated. Heavily diseased trees often respond intensively with the formation of auxiliary and epicormic shoots (Figure 3A), in order to compensate the loss of killed shoots and twigs. This often leads to a decrease in disease severity, but it is questionable, whether this reflects a long-term tendency. Likewise, killed twigs and branches become decayed, subsequently fall down and can therefore not be considered in future ratings. Moreover, it is possible that climatic factors contributed to the slight decrease in disease intensity from

2009 to 2010. In agreement with the assessments in the seed plantation, only a slight overall increase of ash dieback intensity was observed from 2008 to 2010 on monitoring plots in Lower Austria (Keßler et al., 2012) as well as from 2009 to 2010 in surveys in Norway (Solheim et al., 2011).

The assessments in the ash seed plantation provide added evidence that leaf symptoms are associated with ash dieback. In agreement with earlier reports (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Kowalski et al., 2010; Kräutler and Kirisits, 2012) necrotic lesions on leaf petioles and rachises as well as leaflet veins, followed by wilting and early leaf shedding were commonly observed on *F. excelsior* ramets in the plantation. Symptom observations, fungal isolations and inoculation studies suggest that *H. pseudoalbidus* is associated with these leaf symptoms and that leaf infections are indeed the cause of early leaf shedding (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Ogris et al., 2009; Kräutler and Kirisits, 2012). The phenology of early leaf shedding in the seed plantation, approximately starting in July and subsequently increasing in intensity, until many trees are to a large extent or even totally defoliated by September, corresponds well with the suggested period of ascocarp formation of *H. pseudoalbidus* from mid-/end of June to late August/early September. Given that ascospore production starts already in June and leaf symptoms first appear approximately by the middle of July, an incubation period (time from inoculation to the appearance of visible symptoms) of several weeks can be assumed for *H. pseudoalbidus*. The temporal pattern of leaf shedding also agrees well with the proposed disease cycle of ash dieback (Kirisits and Cech, 2009; Kirisits et al., 2009; Schumacher, 2011).

The intensity of shoot, twig and branch dieback and the levels of leaf shedding varied greatly amongst *F. excelsior* clones in the seed plantation. It is most likely that this variation reflects genetically determined differences in the resistance of ash genotypes to *H. pseudoalbidus*. No relationship between the intensity of leaf shedding and dieback intensity was detected. This is in contrast to the study in seed plantations in Denmark where a strong correlation between dieback intensity and late leaf shedding / leaf senescence was observed. Thus, clones retaining their leaves longer in autumn displayed higher levels of dieback (McKinney et al., 2011). Moreover, early leaf shedding was supposed as a means to partially escape disease, leading to lower dieback intensity (McKinney et al., 2011).

The results concerning leaf shedding in our study may not be directly comparable with those from Denmark by McKinney et al. (2011). Since the occurrence of ash dieback, the phenology of leaf shedding of *F. excelsior* has been substantially altered in many parts of Austria, due to direct leaf infections by ascospores of *H. pseudoalbidus* (Kirisits et al., 2009, 2010b; Kräutler and Kirisits, 2012). As observed in the course of this study, shedding of leaves often starts already in late summer and by mid-September a large portion of trees is substantially or totally defoliated (Kirisits et al., 2009, 2010b). McKinney et al. (2011) treated leaf senescence and shedding as a genetically determined phenological trait of *F. excelsior* which is not influenced by *H. pseudoalbidus* infections. We, however, argue that leaf shedding in our study, though varying greatly between clones, is mainly a consequence of leaf infections by the ash dieback pathogen. Because of leaf infections, we suppose that genetic differences in autumn leaf phenology of common ash can no longer be observed precisely in the seed plantation studied here. The inconsistent findings and interpretations in our study and that of McKinney et al. (2011) may be explained by differences in the phenology of the pathogen and the host in different environments, likely resulting in a different temporal development of the disease cycle of ash dieback. For example, formation of *H. pseudoalbidus* apothecia starts slightly later and the period of sporulation is shorter in Northern Europe than in Central Europe (Kirisits and Cech, 2009; Solheim et al., 2011; Timmermann et al., 2011).

Although induced by the pathogen, early leaf shedding could be a resistance mechanism to ash dieback in certain *F. excelsior* genotypes. This is because infected leaves may be shed before *H. pseudoalbidus* can enter bark and wood tissues adjacent to the leaf base (Kirisits et al., 2010b). The lack of a consistent association between dieback symptoms and leaf shedding suggests, however, that leaf shedding as a kind of 'defence reaction' does not operate successfully in all the clones which lose their leaves early. It is worth to note that some clones (for example number 14, Figure 3B) were only slightly affected by both dieback and leaf shedding. This may indicate that these clones possess resistance mechanisms in the leaves and possibly also in the bark and wood that limit infection by and/or spread of *H. pseudoalbidus*. In clones whose leaves are more intensively affected, but show only limited dieback, resistance traits in shoots may, however, be more important. Common ash clones that display limited damage of both leaves and ligneous tissues may in our opinion be most interesting for genetic conservation and breeding programs. Putative traits of leaves and ligneous tissues associated with resistance to ash dieback and the relative importance of resistance mechanisms operating in these two organs will require attention and study in the future, as discussed by McKinney et al. (2011, 2012).

Although this report is clearly preliminary, the assessments in the seed plantation in Upper Austria indicate that *F. excelsior* clones indeed differ substantially in their resistance to *H. pseudoalbidus*, with some clones displaying high putative resistance levels. The results agree well with the findings obtained in the *F. excelsior* seed plantations in Denmark (McKinney et al., 2011, 2012). For the Austrian seed plantations, thorough statistical data analyses are intended in the future,

to support the preliminary trends reported here. Likewise, it is planned to continue the assessments, in order to monitor the future development of damage levels and to check particularly, whether some clones will remain to be affected only to a limited degree by the disease. Moreover, artificial inoculation of *H. pseudoalbidus* onto leaves and branches of various clones displaying contrasting damage levels are desirable, to definitely determine that resistance to this emerging tree pathogen varies in *F. excelsior*.

The results presented in this report and by McKinney et al. (2011, 2012) are of considerable practical importance. They provide substantial evidence that at least a low portion of *F. excelsior* individuals displays high resistance levels to ash dieback. The recommendation to practitioners to maintain and promote disease-free and slightly diseased common ash trees of all ages (for example Kirisits et al., 2009, 2010b, 2011) thus appears to be a reasonable measure for disease management. Clearing ash trees irrespective of their health status bears the risk to eliminate individuals with high resistance levels from the populations.

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Full Length Research Paper

Reestablishing the health of secondary forests "*Satoyama*" endangered by Japanese oak wilt: A preliminary report

Kuroda K.[1]*, Osumi K.[2] and Oku H.[2]

[1]Graduate School of Agricultural Science, Laboratory of Forest Resources, Kobe University, Nada-ku, Kobe 657-8501, Japan.
[2]Forestry and Forest Products Research Institute, Kansai Research Center, Momoyama, Fushimi, Kyoto 612-0855, Japan.

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Japanese oak wilt caused by the fungus *Raffaelea quercivora* is increasing in secondary forests known as "Satoyama" that are surrounding rural communities. Oak wilt is occurring in stands that are 40 to 70 years old that have been used for fuel wood and charcoal production and then left unmanaged because those wood fuels were replaced with gas and kerosene since 1950s. An ambrosia beetle, *Platypus quercivorus*, which vectors the pathogen, can propagate effectively in thicker trunks. Due to the extensive population growth of this beetle in aged "Satoyama" forests, the infested areas are increasing annually. A drastic change occurs in the vegetation after the mass mortality of oak trees. Deterioration of biodiversity and soil erosion are of concern. To reduce oak mortality, rejuvenation of trees will be effective because the vector beetle cannot propagate in thin trunks. We are conducting an experiment to reestablish the health of "Satoyama" forests that are slightly affected by this disease. In this experiment, aged forests were clear-cut to promote sprouting from the oak stumps. This is a coordinated effort among the local governments and researchers. The management of the "Satoyama" combined with the utilization of biomass as fuel wood in the local area is essential for the success of this project.

Key words: Oak, wilt, *Raffaelea quercivora*, biomass, management.

INTRODUCTION

Secondary forests near residential areas, long used by residents as fuel resources, are called "Satoyama" in Japanese. Recently, the "Satoyama" is becoming popular internationally because of their high degree of biodiversity. In the Satoyama forests, mass mortality of trees by Japanese oak wilt has been increasing annually since the 1990s (Hijii et al., 1991; Ito et al., 1992; Kuroda, 2008; Nunokawa, 1993) (Figure 1). This wilt disease is caused by the fungus *Raffaelea quercivora* (Kuroda and Yamada, 1996; Kuroda, 2001; Kubono and Ito, 2002). Trees of fagaceaeous genera, except for *Fagus*, have been killed by this pathogen (Kuroda, 2008). This disease occurs primarily in the secondary forests that had been used for firewood and charcoal production and then left unmanaged since the 1950s, when the energy revolution, that is the replacement of fuels from wood to gas and kerosene started (Kuroda and Yamada, 1996). Damaged oak stands ranged from 40 to 70 years old because the traditional coppicing by periodical cutting at 15 to 30 year intervals had been fully discontinued in Japan by 1980. An ambrosia beetle, *Platypus quercivorus*, which vectors *R. quercivora* from dead to living oak trees, propagates effectively in trunks thicker than 10 cm (Kinuura and Kobayashi, 2006; Sone et al., 1998). Due to the extensive population growth of this beetle in abandoned and

*Corresponding author. E-mail: kurodak@garnet.kobe-u.ac.jp.

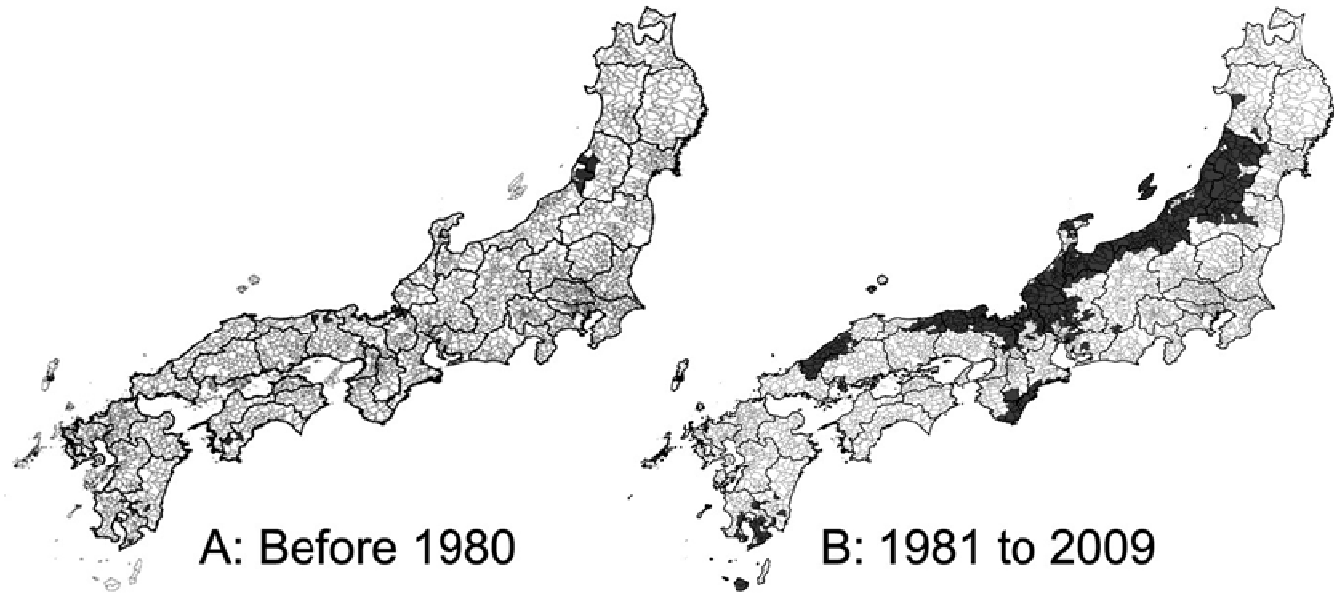

Figure 1. The Japan districts experiencing increased incidents of Japanese oak wilt after the 1980s. Infested areas (cities) are indicated in black."

aged forests, infested areas are expanding annually. Fundamental pathological aspects of this disease have been revealed in research over the last two decades (Kubono and Ito, 2002; Kuroda, 2001; Kuroda and Yamada, 1996; Murata et al., 2005). Although pesticides and fungicides for this disease have been developed (Kuroda, 2008) and an aggregation pheromone of the beetle became applicable for tree protection (Tokoro et al., 2007), the eradication of the disease is difficult, especially in severely damaged forests. In Korea, similar oak wilt as a result of fungus and a vector beetle of the same genera is increasing (Hong et al., 2006; Seo et al., 2010).

Some scientists believe that damaged oak stands will recover if left alone because of their natural resilience. However, a drastic change in vegetation is occurring following the mass mortality of thick trees. Itô et al. (2008) reported that only shrubs, small trees, and short-lived species are predominantly replacing the stands damaged by wilt. Tall deciduous species, including oak, did not grow under the shade of other trees. Deterioration of biodiversity and soil erosion are also of concern in these areas. As a strategy to reduce mass mortality by the Japanese oak wilt, rejuvenation of oak trees by the sprouting from stabs after the cutting of main stems will be effective to reduce the population growth of the vector beetle, *P. quercivorus*, because the beetle cannot propagate in young and thin oak trunks (Kinuura and Kobayashi, 2006). Clear-cutting of aged coppices will promote the regeneration of broadleaved trees, including oak species, by sprouting from stumps (Kuroda et al., 2009). We recommend this method to local governments and NPOs that have been trying to manage abandoned forests while failing to stop wilt damage. Simultaneously, we planned this experiment to reestablish the health of "Satoyama" forest that has not been affected by the disease or just slightly affected (Kuroda et al., 2010a, b).

In this experiment, "Satoyama" forests are being managed by the local district residents with the assistance of forestry researchers. Researchers in the field of forest ecology, forest pathology, forest economy, and landscape management cooperate and provide the residents with techniques suitable for reestablishing forest health. Educational programs on forest ecosystems were prepared for the understanding of the purpose of forest management and to obtain good results in experimental fields, targeting citizens who seldom have the opportunity to study science after finishing school. People's activities without fundamental knowledge on forestry sometimes induce unexpected bad effect for forests and therefore education is very important. Another important activity included in this plan is to restart the utilization of biomass as firewood in the districts. All activities related to the Satoyama management, including procedures and expected effects were monitored by researchers. After analyzing the factors behind the recent decline of oak trees, we discussed possible ways to reestablish the health of the "Satoyama" forests. In the present report, we show the process and preliminary results of our project in two experimental sites that is open to public.

MATERIALS AND METHODS

Analysis of the factors that promoting recent oak decline

The methods and results of the procedures used by local governments to control damage caused by this oak disease were surveyed

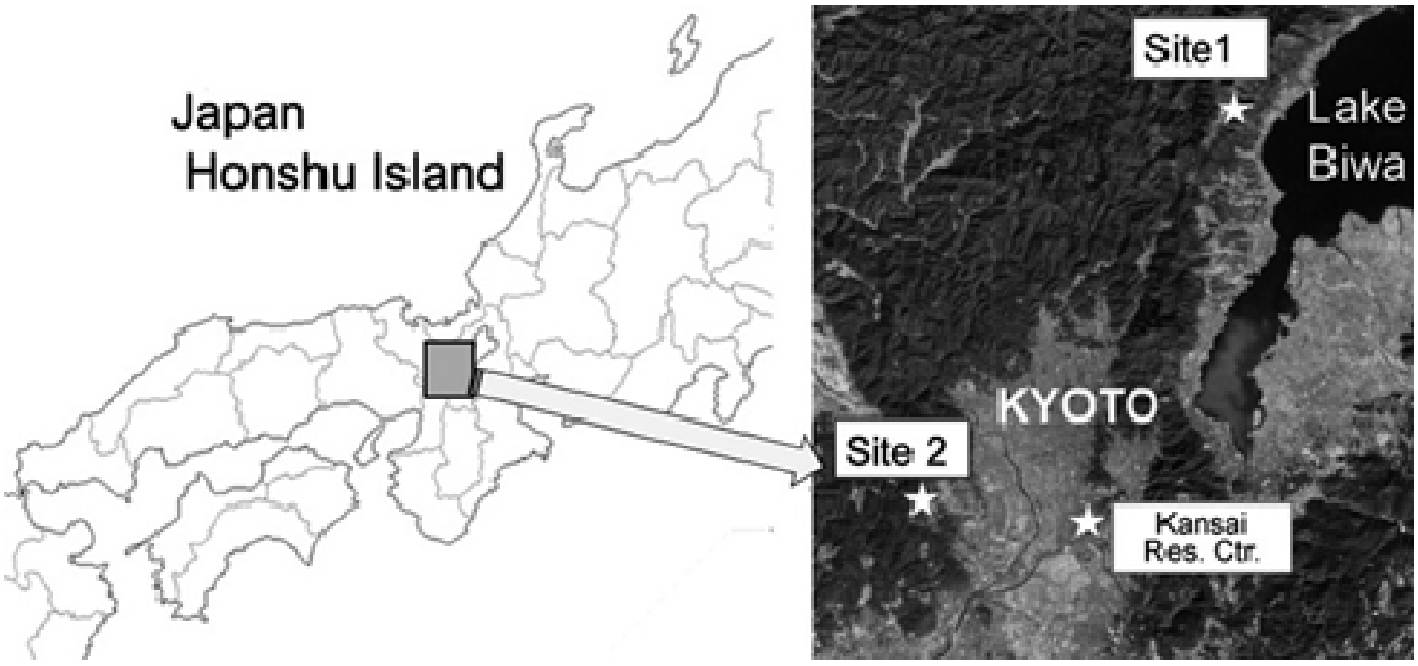

Figure 2. Two sites for the forestry experiments that started in 2008. Site 1: Shiga Prefecture, Otsu City, Site 2: Kyoto Prefecture, Nagaokakyo City.

in the Kansai District (Western Honshu Island, Japan). The survey focused on the effects of pesticide use in the area along with the delayed or nonexistent response of the local governments to the damage. We also examined the suitability of the methods used to control the forests. Based on the results, a useful and practical strategy against the mass mortality of oak trees was discussed.

Planning of a Social experiment to reestablish forest health

The purpose of this social experiment was to demonstrate the process of "Satoyama" regeneration to residents and local governments and its effectiveness to reduce the damage caused by Japanese oak wilt. Experiment sites in Shiga and Kyoto Prefectures were selected (Figure 2). This experiment was conducted as follows, and the outline of the experiment is shown in Figure 3.

1. Permission from the landowners (20 families at the Kyoto site and two families at the Shiga site) for clear-cutting was obtained through the local government (Kyoto) or from an influential person of the community (Shiga) (Figure 4).
2. The following activities were conducted by the district residents following our instructions. Ecological monitoring and survey of the biomass was made before cutting. Then, clear-cuttings were made during the winter, and firewood was obtained. Firewood was removed from the forests to prevent the vector beetle of wilt from being attracted to neighboring oak forests and was given to local residents along with wood stoves.
3. Regeneration from stumps was assessed the following summer at the experimental sites by counting and measuring sprouts and seedlings.
4. Wood stoves were furnished to families living near the experimental sites (Kyoto and Shiga) and public spaces including an elementary school (Kyoto). Fuel wood consumption, changes in lifestyle, and economy were monitored.

The results were analyzed to construct a more practical method.

Education and feedback on the "Satoyama" ecosystem and management

To educate individuals who are interested in nature and forests, including the participants of the social experiment, symposia and seminars on the ecosystem, Japanese oak wilt, forest health, and use of forest resources were hosted by local governments and the research group of Kansai Research Center, FFPRI. From the questions of the participants, the education programs were refined. To support the activities of citizens, a manual reporting the detailed process of "Satoyama" management was written on the basis of the present experiment.

RESULTS AND DISCUSSION

Factors preventing damage control

Difficulties of damage control

Eradication techniques with insecticides and fungicides have developed during the two decades. Infection can be reduced in oak stands by the extensive control of the vector beetles in the initial stage of transmission. However, landowners of private forests in "Satoyama" did not always follow those techniques and failed to remove dead trees from their forests, although they have a legal obligation to eradicate pests from their land. Because logs in "Satoyama" do not currently have economic value, landowners are indifferent to the condition of their forests. Despite pesticide application in the selected area, damage was not reduced due to the population growth of the vector beetle in the many dead trees left in wide areas

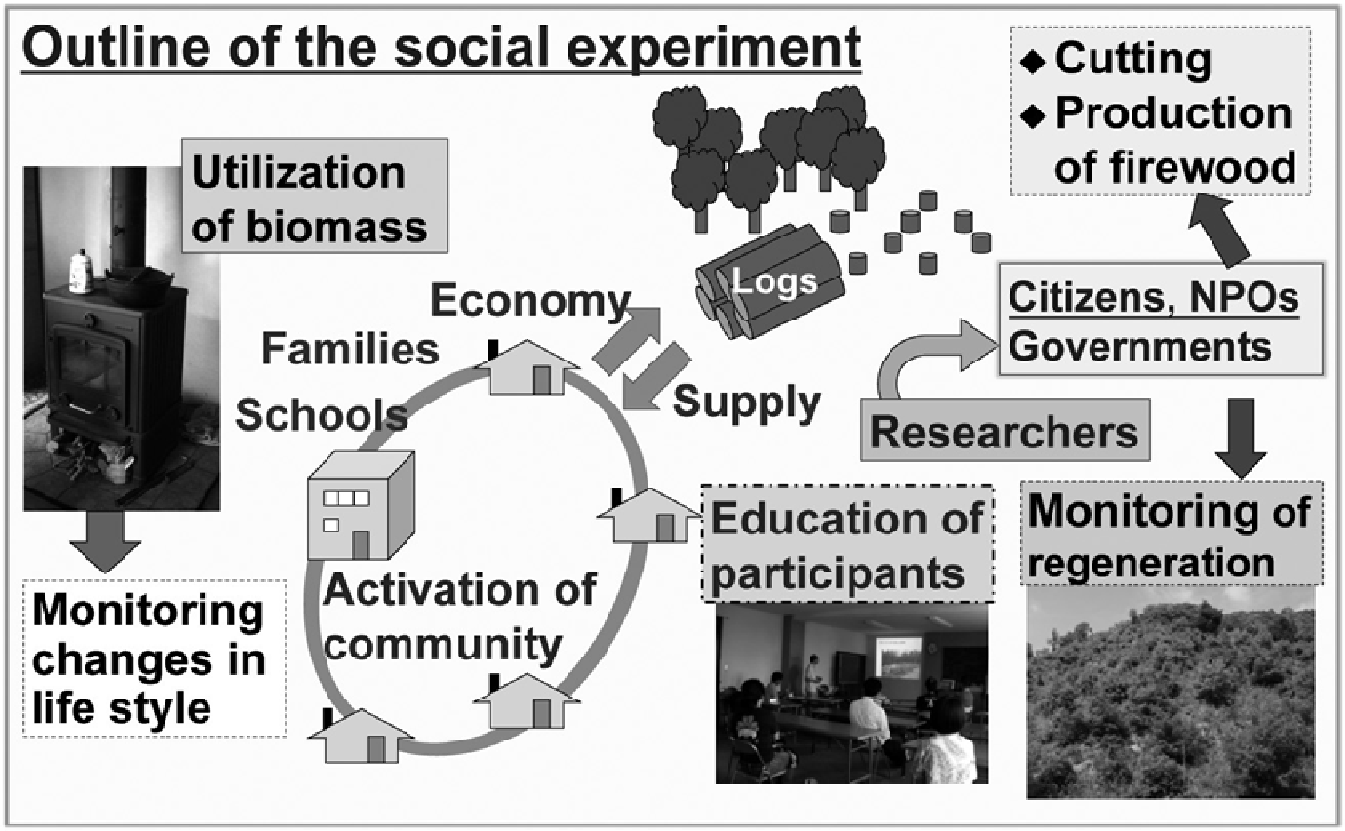

Figure 3. Illustration of the social experiment to reestablish healthy forests.

Figure 4. Clear-cutting at the experiment site in Kyoto (Figure 2, Site 1).

of private forests.

Local governments in Japan (prefectures, cities, towns, and villages) lack the funds to control infectious tree diseases, let alone another epidemic forest disease, pine wilt (Zhao et al., 2008), which is causing serious damage annually in Japan. Some local governments are seriously trying to stop wilt damage. However, cutting down dead trees and applying pesticides on the logs have been conducted only in limited areas even after the disease was widespread. Such methods are mostly ineffective to reduce future damage. In some parks or temple gardens, the trunks of valuable oak trees were wrapped with plastic from the ground level to a given height (at least 4 m) to prevent the beetle's invasion. This method seems to be effective in some cases. On the other hand, some local governments do not survey the damage in the forests or have a task force for damage control. These present conditions suggest that a strategy with expectant treatments with chemicals or plastic firm alone is not effective to reduce infection and damage in the forests. In severely damaged forests, the falling of big branches and trees that occurs due to the rapid decay after wilt is of concern. As to the reason that damage control had never been implemented in some prefectures or cities, it seems that governments did not realize the seriousness of the tree diseases and lacked sufficient funds in their budgets to combat them.

Problems related to the activities of citizens

Recently, citizens activities in the "Satoyama" are becoming popular. Residents usually rely on the combination of two methods, the removal of the undergrowth and the thinning of tall trees. The purpose is to make a beautiful stands suitable for walking or light trekking in the forest park. Although citizens contented that the forests look beautiful just after the activity, this method is not recommended for the following reason. Thinning of broadleaved forests prevents the regeneration of deciduous tall trees due to the shortage of light, and, therefore, shrubby evergreen species increase (Kuroda et al., 2009; Nishinaka et al., 2010). Due to the use of this garden type of forest management, the old oak trees scattered in the forest are liable to infect with the wilt disease because such low density stands attract the vector beetle.

Some citizen groups and NPOs that have joined Satoyama management are actively engaging in the cutting of living trees (thinning of undamaged forests). Mostly, cut logs are unused and abandoned in the stands. Fresh oak logs attract the vector beetle, *P. quercivorus*, and induce the infection and death of healthy oak trees surrounding those cut logs. In fact, this has been occurring in recently managed Satoyama. For suitable Satoyama management, we published a booklet of scientific guidelines" (Kuroda et al., 2009). However, this garden type method is still used in many sites. It is unfortunate that the activities of "Satoyama" management sometimes promote infection and decline due to lack of knowledge in forest health.

Social experiment to recover forest health

Forest management by citizens

An NPO (Shiga site) and two groups (Kyoto site) that are conducting activities for nature conservation and forest maintenance in "Satoyama" were selected as partners in our experiment. No landowner from the experimental sites was included. Surveying, cutting, and monitoring were conducted by volunteers from the groups identified above, mostly retired individuals over 60 years old and researchers of the Forestry and Forest Products Research Institute, Kansai Research Center. The natural conservation corps at the Kyoto site belongs to a committee established and managed by the Nagaokakyo City government. Preliminary surveys of vegetation before cutting, clear-cutting, and wood chopping were conducted in 2008, 2009, and 2010. Trees thicker than 20 cm were cut by professionals to avoid accidents. Nonetheless, cutting of thinner broadleaved trees is risky for untrained volunteers. Thus, a training program for tree cutting with a chainsaw and related logging skills must be prepared. A booklet of instructions with detailed procedures for "Satoyama" regeneration was prepared for the volunteers (Figure 5). A trial version will be improved on the basis of the feedback from this year's users.

Utilization of cut logs as fuel in homes and school

We are monitoring various aspects related to the use of the wood stoves that were furnished to two families, the library room of an elementary school, and a public space in the city park: the amount of used firewood, room temperature and humidity, hours of use for the wood stove, and energy usage relative to the cost of gas and electricity. Volunteers delivered firewood to the elementary school. After one winter using the wood stove, the participating families expressed satisfaction with the results. They valued highly that family members including children were apt to gather around the stove and spent longer hours with family. When enough data is accumulated, it will be analyzed and published by researchers on forest resources. The result of monitoring will be used to promote the utilization of biomass obtained from Satoyama in the future.

Education and feedback

Citizens believed that cutting trees was bad for the ecosystem, and that forests should not be cut because

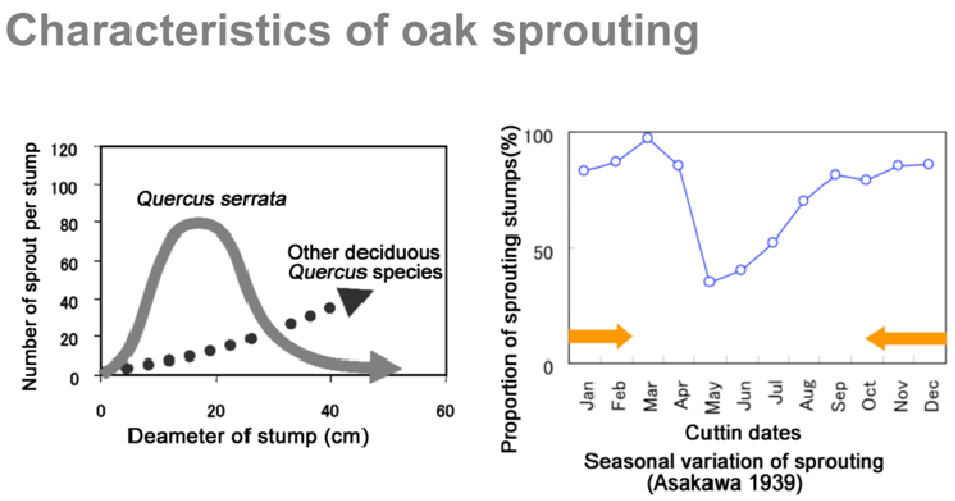

Figure 5. Page from the booklet for Satoyama management. Explanation of the sprouting of oak trees and monitoring the regeneration (Prepared by Osumi).

they will sustain themselves without management. At first, members of the cooperative found it difficult to agree on decisions about clear cutting, even in small areas smaller than 0.1 ha. Two or three times of explanations were necessary for them to understand that clear cutting is necessary for the regeneration of healthy forests dominated by deciduous broadleaved trees.

Two common perceptions expressed at symposia and seminars were that the eradication techniques needed to be improved and that chemicals should not be used in the forests. The opinions can be attributed to a lack of scientific knowledge regarding the chemicals. On the other hand, some individuals insisted on leaving green cut logs in the forests to decompose without regard for the admonitions that such logs attract vector beetles of the disease. For those people, "Satoyama management" is a hobby or just an amusement. A more forceful response from local governments may be necessary to prevent these harmful practices.

In areas where severe damage is evident, the need for rejuvenation of the forests is more readily understood. Our attempts to restore the forests are currently receiving media attention. These reports help convey the positive aspects of this work to the public. Education at all levels

is essential for the success of these efforts, and that includes the retired volunteers as well as school children. Programs for the younger generations, who will be in charge of the periodical cutting of trees (at 20 to 30 year intervals) that is necessary for the maintenance of healthy “Satoyama” forests, must be developed.

Conclusion

“Satoyama” areas include ca. 30% of the forests in Japan. Governments cannot continue to maintain such broad areas with the current level of budget. Two strategies are necessary to recover the health of “Satoyama”. From the standpoint of natural science, rejuvenation of secondary forests are effective for reducing damage due to wilt. In addition, public education is required. Encouraging local communities to take an active interest in the management of the forests is also important. However, we cannot return to the inconvenient lifestyle of a half century ago when we relied on forest biomass as energy source. Our proposal is to establish a new life style in which we utilize biomass as a part of our energy source and maintain the forest health. Forest management combined with the utilization of biomass and the contribution of the people of the district will be important (Kuroda et al., 2009). For that purpose, local government should play an active role by providing basic knowledge on sustainable forest management.

The framework of our proposal on “current style Satoyama management system” is as follows. Our primary approach requires the rejuvenation of forests by clear cutting and the maintenance of healthy secondary forests. Small-scale clear cutting is preferable as the traditional coppicing. Arboreal and deciduous species will grow dominantly after clear cutting. In addition, biodiversity will be kept high for the mosaic environment. Local participation of the citizenry, both urban and rural, is essential to the success of forest management. After that if there is some economical profit for selling coppice, it will attract the landowners to this activity. The utilization of forest biomass is key to the continued management by the local community. If woodstoves are used by a part of local residents, it will help to reduce CO_2 emission substituting petroleum. Biomass from the forests as a renewable energy may be an attractive aspect to the younger generation.

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Full Length Research Paper

Pathogenicity of some fungi isolated from cankers on *Cupressus sempervirens* var. *horizontalis* in Turkey

Lehtijärvi A.[1]*, Oskay F.[1], Doğmuş-Lehtijärvi H. T[1]. and Aday A. G.[2]

[1]Faculty of Forestry, Süleyman Demirel University, 32260 Isparta, Turkey.
[2]Yenişar Bademli Vocational School, Süleyman Demirel University, Isparta, Turkey.

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Natural stands of *Cupressus sempervirens* in Turkey are among the largest forests of this species in the world and are regarded as relicts of the centre of origin of var. *horizontalis*. In this study, we tested the pathogenicity of some of the most common fungal isolates originating from cankers on *C. sempervirens* by inoculating the isolates into the inner bark of *C. sempervirens* seedlings. The internal transcribed spacer (ITS) region of rDNA of the isolates was sequenced and compared with those in the GenBank. Among the isolates, eight ITS taxa were found. The isolates were inoculated into the inner bark of 2 year old *C. sempervirens* seedlings, on average 90 cm tall and 6 to 12 mm thick at the base. The seedlings were incubated seven weeks in a growth chamber at 70% mean relative humidity and 22.5°C mean temperature. The coaxial length of the lesion around the inoculation point on each seedling was measured. Among the eight ITS taxa *Pestalotiopsis funerea*, two other species of *Pestalotiopsis*, and two unidentified species belonging to the class Dothideomycetes caused lesions that were significantly larger than those in the controls while *Fusarium* sp., *Cytospora* sp. and an unidentified species belonging to Amphisphaeriaceae did not. In contrast to the *Pestalotiopsis* species, the two members of Dothideomycetes grew also into the sapwood of the seedlings.

Key words: *Cupressus sempervirens* var. *horizontalis*, canker, fungi.

INTRODUCTION

The natural stands of *Cupressus sempervirens* var. *horizontalis* (Mill.) Gordon in Turkey are considered among the most significant and largest natural Mediterranean cypress communities (Neyişçi, 1989; Özçelik, 2005), and regarded as relicts of the original source of *C. sempervirens* var. *horizontalis* due to the high diversity observed among the populations (Korol et al., 1997; Raddi and Sümer, 1999; Pichot et al., 1999). However, they constitute only 1392.5 ha of forests within Turkey, where more than 75% of the total is degraded (Anonymous, 2006).

In contrast to the many reports on phytopathological problems of Mediterranean cypress in areas where it has been introduced, information of the relict stands of *C. sempervirens* is available only for Greece and Cyprus (Xenopoulos and Diamandis, 1985; Tsopelas et al., 2007, 2008). The only exception is the study by Sümer (1987) reporting two pathogens in the Aegean coast of Turkey, *Seridium cardinale* (Wag.) Sutton and Gibson and *Pestalotiopsis funerea* (Desm.) Steyaert.

The aim of this study was to i) identify some of the most common fungal isolates originating from cankers on *C. sempervirens* var. *horizontalis* with the aid of the internal transcribed spacer (ITS) region sequences of their rDNA ii) to test the pathogenicity of these isolates by inoculating them into the inner bark of *C. sempervirens* seedlings.

MATERIALS AND METHODS

Fungal isolates

In a previous study, a total of 497 fungal isolates were obtained from cankers on *C. sempervirens* var. *horizontalis*. The trees were sampled during surveys in 2008 in two natural cypress stands locat-

*Corresponding author. E-mail: asko@orman.sdu.edu.tr.

Table 1. Identification and origin of the isolates used in the inoculations.

Isolate group	Isolate code	Origin of the isolate	Identification (Id)
PF	Pf-K1	Köprülü Kanyon, Antalya	*Pestalotiopsis funerea*
	Pf-K2	Köprülü Kanyon, Antalya	*Pestalotiopsis funerea*
	Pf-K3	Köprülü Kanyon, Antalya	*Pestalotiopsis* sp. 1
	Pf-K4	Köprülü Kanyon, Antalya	*Pestalotiopsis funerea*
	Pf-K5	Köprülü Kanyon, Antalya	*Pestalotiopsis* sp. 2
	Pf-K6	Köprülü Kanyon, Antalya	*Pestalotiopsis funerea*
SE	SE-K1	Köprülü Kanyon, Antalya	Mitosporic Amphisphaeriaceae
PO	Po-K1	Köprülü Kanyon, Antalya	Unidentified Dothideomycetes sp. 1
	Po-K2	Köprülü Kanyon, Antalya	Unidentified Dothideomycetes sp. 1
	Po-K6	Köprülü Kanyon, Antalya	Unidentified Dothideomycetes sp. 1
	Po-M1	Aydıncık, Mersin	Unidentified Dothideomycetes sp. 1
	Po-M2	Aydıncık, Mersin	Unidentified Dothideomycetes sp. 1
	Po-M3	Aydıncık, Mersin	Unidentified Dothideomycetes sp. 1
	Po-M4	Aydıncık, Mersin	Unidentified Dothideomycetes sp. 1
	Po-M5	Aydıncık, Mersin	Unidentified Dothideomycetes sp. 1
A	A-K1	Köprülü Kanyon, Antalya	Unidentified Dothideomycetes sp. 2
	A-M1	Aydıncık, Mersin	Unidentified Dothideomycetes sp. 2
CY	Cy-K1	Köprülü Kanyon, Antalya	*Cytospora* sp.
	Cy-M1	Aydıncık, Mersin	*Cytospora* sp.
FS	Fs-M1	Aydıncık, Mersin	*Fusarium* sp.

ed in the Köprülü Kanyon National Park, Antalya and in Aydıncık, Mersin, in the Mediterranean region of Turkey (Lehtijärvi et al., 2009). For the present study, 27 isolates, mainly representing the most common fungi isolated from the canker tissues were selected (Table 1).

DNA extraction

The isolates were cultured on cellophane membranes placed on either potato dextrose agar (PDA) with additional agar (20 g/l PDA, 20 g/l agar; Merck, Germany) or ground cypress needle amended PDA (CN-PDA; 20 g/l PDA, 20 g/l ground cypress needles). Cypress needles were used in order to stimulate the mycelial growth of some relatively slowly growing isolates (morphotypes PO and A). The cultures were incubated at 25°C until the mycelia had covered the cellophane membranes. The mycelia were harvested from the membranes and ground with mortar and pestle in liquid nitrogen. Immediately after grinding, the genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Amplification of the ITS region and DNA sequence analyses

Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region (ITS1, 5·8S and ITS2) of the rRNA genes of the isolates and the sequencing of the PCR products in both directions was performed by a commercial laboratory (IonTek, Istanbul, Turkey) using the primer set ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (White et al., 1990). The sequences were determined using an ABI PRISM automated sequencer.

The sequences were compared with those in GenBank database using the BLAST algorithm and the putative taxa of the isolates determined. The sequences showing a similarity above 95% with the query sequence were considered. Additionally, another online BLAST program by UNITE analysis (http://unite.ut.ee/analysis.php; Kõljalg et al., 2005) was used for comparisons. This molecular database for identification of fungi, provides only sequences from the ITS region and allows to search in the International Nucleotide Sequence Database (INSD), which contains all GenBank, EMBL and DDJB data.

Inoculation experiments

Cypress (*C. sempervirens* var. *horizontalis*) seedlings, 1+1 years old, were obtained from the state forest nursery in Denizli, Turkey. The plants were raised from seeds collected from urban amenity trees within the Denizli province. The height of the seedlings ranged from 56 to 136 cm with a mean of 90.6 cm (±0.7 SD). The average ground level and inoculation point diameters were 8.7 (±0.7 SD; range from 6-12 mm) and 6.9 mm (±0.1 SD; 4-11 mm), respectively. The seedlings were placed into a growth chamber and kept under controlled conditions prior to inoculation and during incubation. The mean daily temperature and relative humidity during incubation were 22.5°C and 70.7%, respectively. The seedlings were irrigated with two days intervals.

Twenty-seven day old isolates grown on CN-PDA were used for inoculations (Table 1). The seedlings were inoculated 10 cm above root collar. A cork borer (4 mm diameter) was used to remove the bark and expose the cambium for inoculation and to obtain mycelial plugs from growing cultures. A mycelial plug was placed into each wound with the mycelium surface facing the xylem. After inoculation, the wound was covered with Parafilm® M (Alcan inc.), to prevent contamination and desiccation of the wound and the inoculum. Each isolate was inoculated onto ten seedlings. In addition, ten seedlings were inoculated with sterile CN-PDA to serve as controls.

After seven weeks, the seedlings were harvested, the outer bark around the inoculation point was removed with a sterile scalpel, and the lesion lengths on the seedlings were measured. Reisolations were made from three randomly selected replicate seedlings per isolate. Three small pieces of tissues containing both necrotic and healthy tissues were taken from the lesion edges and placed onto PDA petri plates.

The SPSS GLM procedure (SPSS Inc., Chicago, IL, USA) was used to analyse the data. Duncan's multiple range test was used to determine the differences among mean lesion lenghts. Correlations between lesion lenghts and seedling size was calculated using Pearson's product moment correlation coefficient test.

RESULTS AND DISCUSSION

Molecular identification

When compared with sequences in GenBank, the ITS sequences of the isolates belonging to the PF group, with two exceptions, most closely matched those of *P. funerea*. The ITS sequence similarity of PF-K1, PF-K2, PF-K4 and PF-K6 isolates with *P. funerea* was greater than 95%, and therefore determined to belong to that species. The isolate PF-K5, in contrast, had a similarity percentage lower than 95% with *P. funerea*, and therefore identified only to be a member of the genus *Pestalotiopsis*. The isolate PF-K3, in turn, had higher similarity with *P. yunnanensis* J.G. Wei and T. Xu than with *P. funerea*.

The SE-K1 isolate, with cultural characteristics similar to those of the PF isolates, matched also with Pestalotioid fungi. However, the range of matching genera was larger than for the PF isolates, including *Pestalotiopsis*, *Sarcostroma* and *Truncatella*. Therefore, the isolate could only be identified to the family level as a member of Amphisphaeriaceae.

A comparison of ITS sequences of the isolates in the PO group with sequence data in GenBank resulted in only matches with low similarity, such as *Spencermartinsia* sp., *Diplodia medicaginis* Golovin, *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips, and *Botryosphaeria parva* Pennycook and Samuels, with 86 to 89% similarity. The best match, 93% sequence similarity with *Septoria pinithunbergii* S. Kaneko, was obtained with a BLAST search in UNITE database. As none of the matching ITS sequences showed similarity high enough, the identity of the PO group isolates could not be determined. Nevertheless, the PO isolates may represent a fungal taxon within the Pezizomycotina, class Dothideomycetes.

The A group isolates had 98% similarity with *Dothideomycetes* sp. However, the fungi in GenBank under the name Dothideomycetes sp. represent the fungal class Dothideomycetes, not a genus. The identity of the CY group isolates could be determined only to genus level as *Cytospora* sp., although they had high sequence similarity with *C. cedri* Syd., P. Syd. and E.J. Butler. Similarly, the single FS isolate was determined to be *Fusarium* sp. showing the highest sequence similarity with *F. equiseti* (Corda) Sacc. and *F. chlamydosporum* Wollenw. & Reinking.

In summary, the fungal isolates subjected to molecular identification were grouped into eight different taxa based on their ITS sequence, while grouping based only on the colony morphology resulted in six taxa. The PF group isolates were found to contain three *Pestalotiopsis* species: *P. funerea* and two unidentified ones.

Pathogenicity tests

All isolates used in the inoculation trial induced lesions on the *C. sempervirens* seedlings (Table 2). The mean lesion lengths ranged from 6.0 to 37.0 mm. Short lesions were formed also on the mock-inoculated control seedlings. The lesions in the inoculated seedlings resembled the cankers observed in the field. Furthermore, all fungal inoculations resulted in more or less necrotic needles around the inoculation point. Contrary to our field observations, no resin exudation was observed on the seedlings inoculated with any of the isolates tested.

Cankers resulting from inoculations with the PO (*Dothideomycetes* sp. 1) and A (*Dothideomycetes* sp. 2) isolates tended to have more distinct margins than those resulting from inoculations with the PF (*Pestalotiopsis* spp.), CY (*Cytospora* sp.), FS (*Fusarium* sp.) and SE-K1 isolates. Moreover, the PO isolates caused a remarkable discoloration in the sapwood in contrast to the PF isolates indicating a different ability to grow in sapwood.

The differences between the isolate groups in their ability to induce lesions on cypress seedlings were statistically significant ($P<0.01$). In general, the isolates in PO and the PF groups were virulent with mean lesion lengths of 17.2 (SE ± 0.5) and 17.1 (SE ± 0.8) mm, respectively. However, the isolates PF-K1, PF-K2 and PO-K3 from Köprülü Kanyon and PF-M1 from Mersin induced shorter lesions which did not differ statistically from those of the controls ($P<0.01$). Isolates belonging to all other groups (SE, FS, CY, A) were regarded as avirulent as they did not differ significantly in mean lesion length from the controls, with the exception of the A-M2 isolate.

Among the *Pestalotiopsis* species, the PF-K5 and PF-K6 isolates induced significantly longer lesions than the PF-K1, PF-K2, PF-K4, and PF-M1 isolates ($P<0.01$). The PF-K5 isolate, which was identified to be a species distinct from *P. funerea* within the genus *Pestalotiopsis*

Table 2. Mean lesion lengths produced by isolates.

Isolate group	ITS taxon	isolate	n	Mean lesion length (mm) ± SE	range (mm)
PF	*Pestalotiopsis* sp. 2	PF-K5	10	24.4 ± 2.4^{a}	13.0 – 37.0
	Pestalotiopsis funerea	PF-K6	10	21.8 ± 2.2^{abc}	10.0 – 36.0
	Pestalotiopsis sp. 1	PF-K3	10	18.6 ± 2.2^{abcd}	8.0 – 31.0
	Pestalotiopsis funerea	PF-K4	10	14.9 ± 1.1^{cdefg}	11.0 – 21.0
	Pestalotiopsis funerea	PF-K1	10	13.6 ± 1.5^{defgh}	8.0 – 23.0
	Pestalotiopsis funerea	PF-K2	10	13.2 ± 0.8^{defgh}	8.0 – 16.0
	Pestalotiopsis funerea	PF-M1	10	13.1 ± 2.0^{defgh}	9.0 – 28.0
SE	Mitosporic Amphisphaeriaceae	SE-K1	10	8.0 ± 0.4^{gh}	6.0 – 11.0
FS	*Fusarium* sp.	FS-M1	10	12.5 ± 1.0^{defgh}	8.0 – 18.0
CY	*Cytospora* sp.	CY-M1	10	9.5 ± 0.4^{efgh}	7.0 – 11.0
	Cytospora sp.	CY-K1	10	8.7 ± 0.2^{gh}	8.0 – 10.0
A	Unidentified Dothideomycetes sp. 2	A-M2	10	14.3 ± 1.7^{defg}	9.0 – 25.0
	Unidentified Dothideomycetes sp. 2	A-K3	10	10.5 ± 1.8^{efgh}	8.0 – 26.0
	Unidentified Dothideomycetes sp. 2	A-M1	10	10.0 ± 1.8^{efgh}	7.0 – 25.0
	Unidentified Dothideomycetes sp. 2	A-K2	10	9.0 ± 0.8^{fgh}	7.0 – 15.0
	Unidentified Dothideomycetes sp. 2	A-M3	10	8.6 ± 0.4^{gh}	7.0 – 11.0
	Unidentified Dothideomycetes sp. 2	A-K1	10	8.1 ± 0.4^{gh}	7.0 – 11.0
PO	Unidentified Dothideomycetes sp. 1	PO-M3	10	22.8 ± 1.2^{ab}	19.0 – 30.0
	Unidentified Dothideomycetes sp. 1	PO-K6	10	19.6 ± 1.4^{abcd}	13.0 – 25.0
	Unidentified Dothideomycetes sp. 1	PO-K4	10	19.3 ± 1.3^{abcd}	13.0 – 26.0
	Unidentified Dothideomycetes sp. 1	PO-M5	10	18.7 ± 1.3^{abcd}	11.0 – 24.0
	Unidentified Dothideomycetes sp. 1	PO-M4	10	18.3 ± 1.4^{abcd}	13.0 – 25.0
	Unidentified Dothideomycetes sp. 1	PO-M2	10	16.5 ± 2.0^{bcde}	6.0 – 28.0
	Unidentified Dothideomycetes sp. 1	PO-M1	10	16.0 ± 0.8^{bcdef}	13.0 – 22.0
	Unidentified Dothideomycetes sp. 1	PO-K1	10	15.2 ± 1.2^{cdefg}	11.0 – 25.0
	Unidentified Dothideomycetes sp. 1	PO-K2	10	14.1 ± 1.1^{defg}	10.0 – 21.0
	Unidentified Dothideomycetes sp. 1	PO-K3	10	11.0 ± 1.0^{efgh}	8.0 – 17.0
CONTROL			10	6.7 ± 0.3^{h}	5.0 – 8.0

Means are averages of N measurements and those followed by the same letter are not significantly different from each other at P < 0.01 significance level according to Duncan's multiple range test. ITS, Internal transcribed spacer.

based on its ITS sequence was found to be the most aggressive isolate among all isolates (24.4±2.4 mm). The PF-M1 isolate which was not subjected to molecular identification but accounted to be *P. funerea* based on its cultural and conidial similarities especially to those of PF-K6 produced the smallest lesions (13.1±2.0 mm).

Within each isolate group, the differences in mean lesion length between isolates originating from Mersin and Köprülü Kanyon were statistically insignificant. There was no correlation between the seedling size and lesion length. However, there was a negative correlation between the inoculation point diameter and lesion length produced by PF isolates ($r=0.302$, $p<0.05$).

Fusarium sp. could not be reisolated from the inoculated seedlings. However, the reisolation frequencies of the PO and A isolates were also lower than those of the PF isolates. Interestingly, *P. funerea* was isolated from nearly all seedlings, regardless of which fungal isolate they were inoculated. In addition, some fungal isolates resembling the A group isolates were isolated from seedlings which were inoculated with fungi other than the A isolates. This indicates that *P. funerea* could be an endophytic species occurring frequently in *C. sempervirens* (Panconesi et al., 1999; Santini and Di Lonardo, 2000). This fungal species is considered a weak pathogen of a wide range of conifer hosts including *Cupressus*, *Pinus*, *Juniperus* and *Thuja* spp (Madar et al., 1991; Sinclair et al., 1993; Santamaria et al., 2007). This species is endemic in Europe and also present in the native areas of cypress, and therefore considered to have co-evolved with *C. sempervirens.* Common moulds, such as *Alternaria* spp., *Cladosporium* spp., and *Penicillium* spp., were also isolated from the inoculated seedlings.

Conclusions

The most virulent isolates were found among the PF and PO groups. The PF group consists of three different *Pestalotiopsis* species. *Pestalotiopsis funerea* may belong to normal endophytic flora in the bark of *C. sempervirens* in natural stands without causing any signi-

ficant damage unless the trees are weakened (Panconesi et al., 1999; Santini and Di Lonardo, 2000). Drought stress could increase the susceptibility of the trees (Madar et al., 1991), but that was not tested in the present study. The PO group consists of isolates of unidentified Dothideomycetes sp. 1, which may be an opportunistic wound parasite.

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Full Length Research Paper

Nursery and field experiments to test conifers susceptibility to Pitch Canker disease

P. Martínez-Álvarez[1]*, J. Blanco[2], M. de Vallejo[2], F. M. Alves-Santos[1] and J. J. Diez[1]

[1]Sustainable Forest Management Research Institute, University of Valladolid – INIA, Avenida Madrid, 57. 34004 Palencia, Spain.
[2]Sección de Producción y Mejora Forestal, Servicio de Montes, Dirección General de Biodiversidad. Gobierno de Cantabria. Calle Rodríguez, nº 5, 1º. 39.071 Santander, Spain.

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Fusarium circinatum **is the causal agent of the Pitch Canker disease, which was first detected in Spain in 2004. Not only** ***Pinus*** **species seedlings in nurseries were affected but also** ***Pinus radiata*** **plantations in the forest. Thus, the pathogen has spread out over several pine forests of northern Spain producing substantial economical losses. Consequently, a resistant and viable pine species is required as alternative. The susceptibility of fifteen conifer species was tested in laboratory conditions measuring the germination of seeds and mortality of the emerged seedlings. In addition, a complementary field experiment was also established to evaluate natural infection of different conifers.** ***Chamaecyparis lawsoniana*** **and** ***Sequoiadendron giganteum*** **obtained the best results in the assay developed in the laboratory. On the contrary, the whole of** ***P. radiata*** **seedlings died, confirming that it is the most susceptible species, despite this species (together with** ***Pinus pinaster***) **showed the highest growth in the control stand of the field assay. Natural infection did not occur during the first two years after plantation.**

Key words: Susceptibility, conifer, *Fusarium circinatum*, pitch canker, pathogenicity.

INTRODUCTION

Pitch canker is a disease of pine species caused by the fungus *Fusarium circinatum* Niremberg & O'Donnell. The pathogen causes shoot die-back in adult trees (Correll et al., 1991) and is able to infect seedlings, thus causing damping off, shoot and tip die-back and the death (Viljoen et al., 1994). However, the most common symptom of the disease is a bleeding, resinous canker on the trunk, terminals or large branches (Hepting and Roth, 1946). The canker is usually sunken and the bark is retained, whereas the wood beneath the canker is deeply pitch-soaked (Dwinell et al., 1985). Since it was first detected in Spain during the winter of 2003-2004 (Landeras et al., 2005), the pathogen has spread along the north of the country affecting mainly *Pinus radiata* in nurseries and plantations. This pine species is the most commonly used exotic conifer for reforestation in northern Spain, covering an area of approximately 200,000 ha and producing 25% of the conifer timber in Spain (Hermoso et al., 2007). The presence of *F. circinatum* is a threat to the productivity in nurseries, pine plantations and native pine forests in Spain, and to the industries relying on them (Pérez-Sierra et al., 2007). Due to the economical and yield losses, an alternative species to radiata pine in the affected area is necessary. The aim of this study was to detect species resistant to Pitch Canker disease with high productivity in the area where *F. circinatum* is present.

*Corresponding author. E-mail: pmtnez@pvs.uva.es.

MATERIALS AND METHODS

Two different assays were performed to evaluate the susceptibility of the conifers. The first one was developed in the laboratory. A total of 15 different species (Table 1) were tested in this assay. Sixty four seeds of each one were washed repeatedly with sterile distilled water, left immersed in the water for twelve hours, and then

Table 1. Species employed in the lab and field assays and their respective provenance.

Species	Lab assay	Field assay
Pinus sylvestris	Sierra de Guadarrama	ES10 Sierra de Guadarrama
Sequoiadendron giganteum	USA	Unidentified
Larix decidua	Germany 83702	-
Pinus pinaster	ES11 Rodenales de Molina	ES08 Meseta castellana
Pseudotsuga menziesii	USA 430 Washington	430 Washington, Randle
Picea abies	East Europe	East Europe
Libocedrus decurrens	Segovia	-
Pinus uncinata	Pirineo Central	ESC Sierra de Gúdar
Juniperus thurifera	ES26 Serranía de Cuenca	-
Sequoia sempervirens	Navarra	-
Pinus strobus	USA	-
Pinus nigra corsicana	Corsica	902 Sud-ouest (France)
Abies alba	ES02 Pirineo Central	ES02 Pirineo Central
Pinus radiata	03 Litoral astur-cantabro-Galicia	Unidentified
Chamaecyparis lawsoniana	Navarra	Unidentified
Cedrus atlantica	-	Unidentified
Cupressocyparis leylandii	-	Unidentified
Thuja plicata lobbii	-	Unidentified

maintained for 30 min in hydrogen peroxide (3%). Finally they were washed twice with sterile distilled water to remove the remaining hydrogen peroxide and sown in nursery trays with a sterile mixture of peat and vermiculite (v:v). Half of the seeds were inoculated pouring one milliliter (1 ml) with one million spores of FcCa6 isolate of *F. circinatum*. To the other half of the seeds, 1 ml of sterile distilled water was poured and used as the control treatment. Seeds were watered once a week with 20 ml of sterile distilled water. Seed emergence was measured once a week and the number of dead seedlings were counted twelve weeks after the sowing. At the end of the experiment *F. circinatum* was re-isolated from the seedlings (10% were checked) to verify that it was the cause of the lesions.

The second experiment was performed in the field. Four different sites were selected in the province of Cantabria (Figure 1). In three of them the presence of the pathogen was confirmed and the other one was located in a *F. circinatum*-free area (control). Thirteen different conifer species were tested in this case (Table 1). Seedlings were planted in June of 2009. Every two months, sites were visited to look for symptoms and to measure collar diameter, height and diameter of the crown of the seedlings.

RESULTS AND DISCUSSION

In general, emergence rate of the species tested in the lab assay were slightly affected by *F. circinatum*. The lowest number of emerged seedlings after *F. circinatum* inoculation appeared on *Abies alba*, *Pinus pinaster* and *Pinus uncinata*. On the contrary, species like *Sequoiadendron giganteum*, *Picea abies* and *Chamaecyparis lawsoniana* got same emergence rate when *F. circinatum* was added to the substrate than in the control treatment. The species *Juniperus thurifera*, *Libocedrus decurrens*, *Pseudotsuga menziesii* and *Sequoia sempervirens* could not be used in the assay because they did not get enough number of emerged seedlings even in the control treatment.

In relation with the survival of the emerged seedlings, the most resistant species to the Pitch Canker disease were *C. lawsoniana* and *S. giganteum*. In both cases the number of healthy seedlings was the same in the treatment in which the pathogen was inoculated than in the control one. On the other hand *P. radiata* was severely affected by *F. circinatum* killing all emerged seedlings. The rest of *Pinus* spp. tested were also very susceptible to the disease, and less than 20% of the seedling survived in all cases. Despite *Picea abies* did not show any effect in the emergence, almost 60% of the seedlings died twelve weeks after the sowing.

The results of this assay confirmed *P. radiata* as the most susceptible species to the Pitch Canker disease. Other studies like the one performed by Viljoen et al. (1995) and Gordon et al. (1998) had already proven this statement.

In the assay developed in the field, natural infection did not happen eighteen months after the plantation. Until then *P. radiata* and *P. pinaster* species showed highest growths. On the contrary *A. alba* and *P. abies* were the species with the slowest growth. More time is needed to see the effect of the pathogen over the health and growth of the different conifers planted.

To our knowledge, except for *P. radiata*, *P. pinaster*, *P. menziesii* and *S. giganteum* (Mccain et al., 1987; Gordon et al., 1998, 2006; Landeras et al., 2005), this is the first time susceptibility to Pitch Canker disease of these species is checked.

Figure 1. One of the sites in which the different conifers were planted in order to check their susceptibility to the Pitch Canker disease.

Conclusions

Regarding the performed assays, *C. lawsoniana* and *S. giganteum* seem to be the best alternative to Monterey pine among the studied species, but it is necessary to wait for the results of the field assay in order to detect some other productive species alternative to the culture of *P. radiata*.

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Full Length Research Paper

Soil temperatures during prescribed burning and the occurrence of *Rhizina undulata* Fr.

Antti Uotila* and Janne Levula

University of Helsinki, Hyytiälä Forestry Field Station, Hyytiäläntie 124, 35500 Korkeakoski, Finland.

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Rhizina undulata **is a postfire fungus. The ascospores germinate after heating over 35°C. The prescribed burning of one forest compartment was done in the purpose to investigate the effects of forest fire to atmosphere at Hyytiälä Forestry Field Station in Southern Finland. The soil temperature measurements were one part of that research. One year after burning, the ascocarps of *R. undulata* appeared offering the possibility to use temperature data for studying the ecology of the species. The soil temperature was measured with 21 iButton sensors. Before and during the burning, all sensors were at 7 cm depth in the burned area. After burning, 10 sensors were moved to the unburned control area. The ascocarps were inventoried from 40 systematically located 10 m^2 plots. On average 6 ascocarps were found in one plot. Ascocarps were present on 75% of plots. Most ascocarps were found on spots for seeding with bare mineral soil visible. The fungus has not killed germlings of pine but 43% of planted seedlings were dead. The temperature during the burning reached 35°C in 10 points. In 4 points, the temperature was over 60°C, which could be too high temperature for spores to survive. It seems that the ascospores could exist in the soil in 2 to 10 cm depth if they can germinate. The temperatures after burning did not reach 35°C in 7 cm depth. More measurements are needed to show if the temperature in the surface of burned area reached critical point after burning.**

Key words: Postfire fungus, forest fire, ascocarps.

INTRODUCTION

Rhizina undulata is a postfire root rot pathogen occurring circumpolarily in Eurasian Taiga forest, Central Europe, Japan, Northern America and South Africa (CMI descriptions 489, 1993; Cha et al., 2009). The ascospores require a temperature over 35°C for germination (Jalaluddin, 1967a). The ascospores can survive years in the soil waiting for forest fire and high enough temperatures for germination (Jalaluddin, 1967). On the other hand, too high temperatures can kill the spores. The fungus grows in coniferous roots in acidic soil and it can kill living big trees near the fire place (Jalaluddin, 1967; Cha et al., 2009).

R. undulata has been shown to be homothallic (Vasiliauskas and Stenlid, 2001). It has at least 14 vegetative compatibility groups (Lygis et al., 2005).

*Corresponding author. E-mail: antti.uotila@helsinki.fi.

In Finland, *R. undulata* damages seemed to increase in 1960's (Laine, 1968). After that the areas of prescribed burning collapsed and nowadays it is not anymore an important pathogen, but it is ecologically interesting. It could have important role to kill trees after biodiversity burnings.

Fire protection is nowadays efficient and although the annual number of forest fires has varied between 100 and 3000 per year, the total burned area has been only 100 to 1600 ha/year (Finnish Statistical Yearbook of Forestry, 2010). Due to continental climate and far distances the big fires are still common in Russia. In history, prescribed burning has had different aims. First the forest was burned to cultivate rye, turnip and oats. This forest destroying way came to an end in the beginning of 1900's. After that prescribed burning was used in clear cuts for preparing soil easier to seed new forest. In the period, 1953 to 1966; the burned clear cut area annually exceeded 10000 ha in Finland (Yearbook

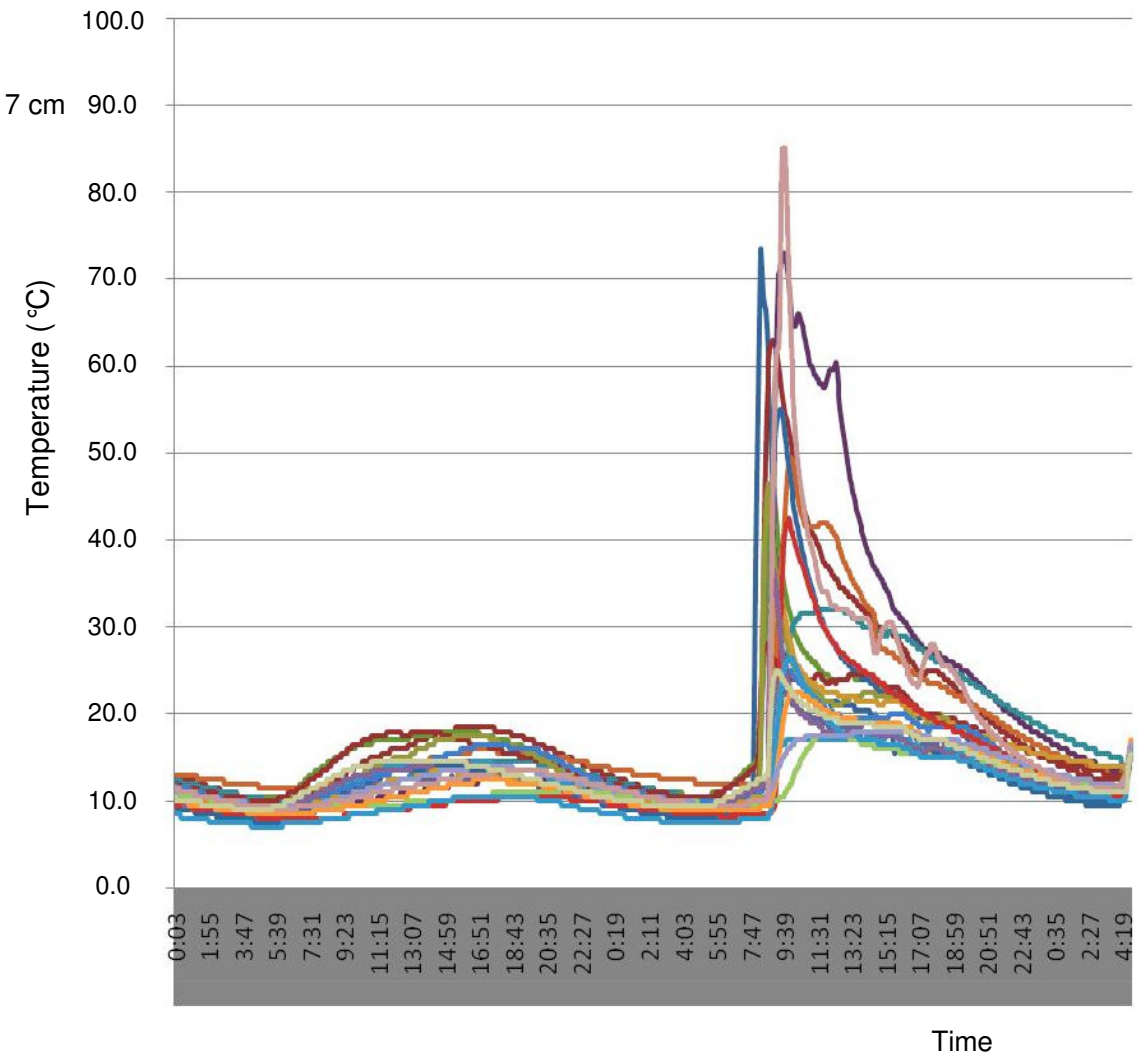

Figure 1. The soil temperatures of 21 points in 7 cm depth during the prescribed burning.

of Forest Statistics, 1988). After that machines took the role of burning in soil management; in recent years, burnings have also been done for increasing biodiversity.

The prescribed burning experiment was planned to measure the air pollutants of forest fire and carbon balance in burned area. *R. undulata* appeared one year after burning and then we decided to study the connection between occurrence of the fungus and soil temperatures during and after burning.

MATERIALS AND METHODS

The experiment area is located near the Hyytiälä Forest Station (61°51´N, 24°17'E). The 100 year old Myrtillus type spruce-pine forest was clearcut in February 2009 for the experiment. The soil of the site is medium coarse tilled and the average thickness of the organic surface layer is 7 cm. Main part of the one hectare clearcut was burned in 26th June, 2009. The slash and surface vegetation were fully burned and the thickness of the organic soil layer diminished 17%. In the beginning of June 2010 the burned area was seeded manually. The humus layer was removed with planting hoe from 30 × 30 cm spot. Pine seeds were sown manually on these spots. In addition, in the beginning of July one-year-old pot seedlings of *Pinus sylvestris* were planted on 20 × 30 m experimental plot. Occurrence of *R. undulata* was inventoried in the end of August 2010. The number of ascocarps were counted from 40 systematically located 10 m^2 plots. The ascocarps were counted separately on spots where the humus layer was removed. The ascocarps were photographed and microscoped in laboratory. Also the number of dead seedlings was observed.

The soil temperatures were measured with 21 iButton sensors at 7 cm depth systematically located in the area. The temperature measurements started before burning and half of the sensors were moved to the unburned control area after burning. During the burning the measurement interval was 4 min. After burning the temperature was measured at 1 h interval. The measurements continued over the year.

RESULTS AND DISCUSSION

The first ascocarp was observed in 19th July, 2010 one

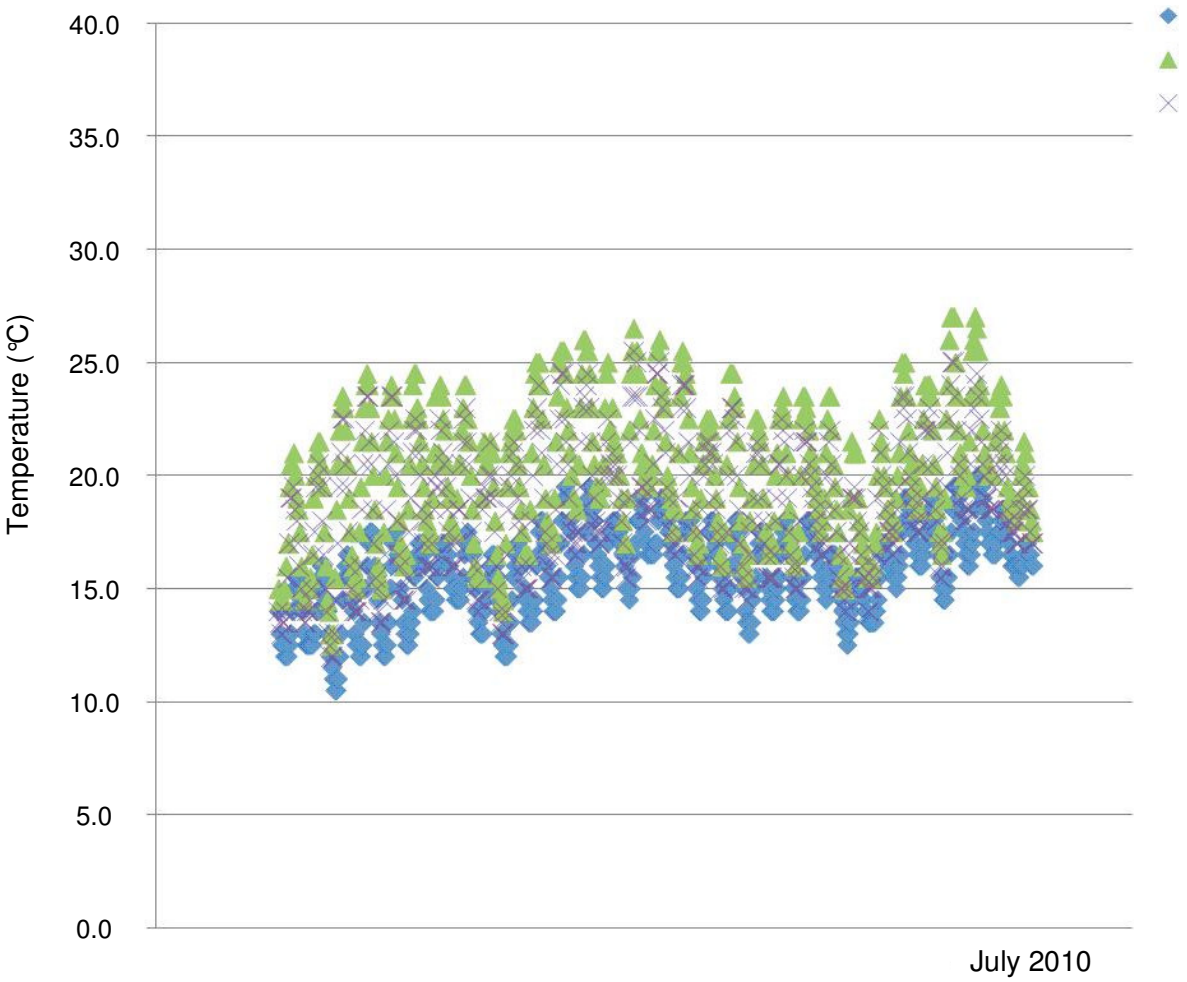

Figure 2. Soil temperatures in 7 cm depth one year after burning. B 16 and B 18 represent a burned area and Control represent an unburned area in the same clearcut.

year after burning. In the end of August the ascocarps were abundant on the whole burned area. Only one ascocarp was found outside the burned area. This one was growing on fire corridor 50 cm distance from burning edge. The ascocarps were observed on 75% of 10 m^2 plots. The mean ascocarp density was 0.6/m^2. In September the asci contents ripen ascospores ready for spreading. It seems that the ascocarps suffered from first night frosts. The ascocarps were much more common on seeding spots where the humus layer was removed and the mineral soil surface was visible. A total of 45% ascocarps were found on these spots.

Though the seeded pines have not yet suffered *R. undulata* infections, 43% of planted seedlings died due to infections or drought.

The temperatures in 7 cm depth from the surface of humus layer did not reach 35°C before and after burning. During the burning, soil temperatures rose over 35°C in 10 of 21 measuring points (Figure 1). The last of over 35°C temperature varied from 0 to 6 h. The critical 35°C is probably reached in depth 0 to 7 cm in the whole burned area. In four measuring points the temperature exceeded 60°C which could be lethal to ascospores.

The highest soil temperatures one year after burning in 7 cm depth reached 26.5°C (Figure 2). Air temperatures were at the same time over 30°C. The soil was clearly warmer in burned area, but the critical point for germination (35°C) was not reached in 7 cm depth.

The data shows that the suitable temperature for germination is present in varying depths in the same burning area. To be able to germinate, the living ascospores should be just there. The layer of suitable temperature for germination is only some centimetres thick. Earlier it has been shown that the fungus can spread 3 m outside bonfire places (Jalaluddin, 1967). The fire corridor was made so that all humus and in addition surface layer of mineral soil with roots were removed. This seems to stop spreading *R. undulata* outside the burned area. These also show us that living or dying roots are important for the *R. undulata* mycelium growth in the soil (Jalaluddin, 1967).

Further research is needed to find out if the high

temperature of black soil surface after the burning can start the germination process.

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Full Length Research Paper

Presence of Viral dsRNA molecules in the Spanish population of *Gremmeniella abietina*

Leticia BOTELLA[1]*, Tero T. TUOMIVIRTA[2], Jarkko HANTULA[2] and Julio J. DIEZ[1]

[1]Sustainable Forest Management Research Institute, University of Valladolid – INIA- 34004, Palencia, Spain.
[2]Finnish Forest Research Institute, Vantaa Research Unit, Jokiniemenkuja, 1, FI-01301 Vantaa, Finland.

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***Gremmeniella abietina* var. *abietina* has commonly been pointed out as a species complex, which includes different races and biotypes. Among them, the Spanish population seems to be a unique population derived from type A. Furthermore, *G. abietina* is known to harbour infections and co-infections of putative mycoviruses belonging to different families. In particular, *G. abietina* type A harbours putative members of families *Totiviridae* and *Partitiviridae* but also *Narnaviridae*, with members of genus *Mitovirus.* In case of *G. abietina* type B, a novel putative virus with endornavirus affinities has also been identified. Different types of *G. abietina* seem to host a divergent virus community. So, ninety-one isolates of the Spanish *G. abietina* were analyzed to check out the presence of viral dsRNA molecules. Thus, the 89% of Spanish isolates presented at least one dsRNA molecule, which is a significant frequency. Overall, eight dsRNA banding patterns were detected, suggesting the occurrence of putative members of different virus genera as *Partitivirus*, *Mitovirus* and *Totivirus*. This is the first approach to the study of fungal mycoviruses in the Spanish population of *G. abietina*.**

Key words: *Gremmeniella*, mycovirus, host, dsRNA, taxonomy.

INTRODUCTION

Mycoviruses are obligate parasites of fungi that generally produce cryptic infections because they are transmitted intracellularly by spores (sexual and asexual) and through anastomosis (Buck, 1986; Ghabrial, 1998). They are usually isometric particles of 25 to 50 nm in diameter and their genome is composed of single-stranded (ss) or double-stranded (ds) DNA or RNA (Van Regenmortel et al., 2000). Furthermore, retrovirus-like elements made of (+) ssRNA have also been found incorporating their genome into the host as dsRNA (Peterson-Burch and Voytas, 2002). In general, dsRNA genome, which codes for a RNA dependent RNA polymerase (RDRP), is the one most commonly associated with viral parasitism in fungi.

Classification of fungal viruses is based on virion morphology, genome organization, method of replication and the number and size of structural and non-structural viral proteins (Van Regenmortel et al., 2000; Bamford et al., 2002). According to the present taxonomical classification, eight families and one genus that is not related to any specific family, are recognized to infect fungi (Van Regenmortel et al., 2000; Ghabrial, 2009; Mayo, 2002). Thus, within dsRNA viruses, there are four families: The family, *Totiviridae,* which contains a single linear uncapped dsRNA molecule with a size of 4.6 to 7.0 kb. Family, *Partitiviridae*, whose genome contains two linear dsRNA segments of 1.4 to 3.0 kb. Family, *Chrysoviridae*, which members carry three to four linear dsRNA molecules with a length of 2.8 to 3.6 kb (Caston et al., 2003). Finally, the family, *Hypoviridae* with a linear genome that codifies a dsRNA molecule of 9 to 13 kb. To date, two families have been characterized within (+) ssRNA viruses, the families *Narnaviridae* and *Barnaviridae*. Members of *Narnaviridae* lack of true virions, and particularly, the genus *Mitovirus* has a linear genome of approximately 2.5 kb (Hong et al., 1999). Thus, the family *Barnaviridae* has only one member that infects to *Agaricus bisporus* and its genome has a length

*Corresponding author. E-mail: lbotella@pvs.uva.es.

of 4.0 kb (Revill et al., 1994). Within Retrovirus-like elements are two families: Family *Metaviridae* with a genome of (+) ssRNA (4 to 10 kb) as well as the family *Pseudoviridae* (Peterson-Burch and Voytas, 2002). Finally, dsDNA viruses are represented by genus *Rhizidiovirus* whose only member contains one linear dsDNA molecule of 25.5 kb in size (Hausner et al., 2000).

Consequently, as in Tuomivirta et al. (2002), the existence of different kinds of viruses in *G. abietina* isolates is widely explained, the aims of this study were: (i) to investigate if dsRNA particles were commonly isolated in the Spanish population of *G. abietina* and (ii) to try to identify which type of viruses they could correspond to.

MATERIALS AND METHODS

Isolates

Ninety-one Spanish isolates of *G. abietina* from *Pinus halepensis* stands located in Valle de Cerrato, Hontoria, Villalba de los Alcores and Astudillo (Botella et al., 2010).

DsRNA isolations

DsRNAs molecules were extracted following a modification of the protocol of Morris and Dodds (1979), which is based on the capability of modified CF-11 fibrose cellulose to bind specifically to dsRNA molecules.

All the samples were cultivated on MOS plates complemented with cellophane membrane to avoid negative interferences of the agar on the dsRNA isolation. Approximately, 1 to 1.5 g of mycelia was put into falcon tubes of 50 ml to be disrupted with 2.5 ml of lysis buffer (50 mM Tris-HCl pH 8.0 (ICN Biomedicals, Ohio, USA; J.T. Baker, Deventer, Holland), 50 mM EDTA (Riedel-de Häen, Seeize, Germany), 3% sodium dodecyl sulphate (SDS) (Acros Organics, Geel, Belgium), and 1% β-mercaptoethanol (YA-Kemia Oy, Helsinki, Finland)) and with a ULTRA-TURRAX® TP-18/10 (Janke & Kunkel GmbH & co KG IKA-Werk, Staufen, Germany) homogenizer. Then, 3 ml of phenol: Chlorophorm-isoamylalcohol (25: 24: 1) (Amresco, Ohio, USA; Tamro Medlab Oy, Vantaa, Finland; YA-Kemia Oy, Helsinki, Finland) were added to develop the extraction of total nucleic acids. Afterwards, 30 min of centrifugation (3000 rpm) was carried out, followed by a last extraction with chlorophorm:isoamylalcohol (24:1). Then, the samples treated with 30 µl NaCl and ethanol (16.5%) were kept during, at least, 20 min at -20°C. After this period, 50 mg of CF-11 was added into the tube and mixed during 10 min on ice. The following steps consisted of washing the samples with Tris/Sucrose/EDTA (TSE) buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) supplemented with 15% ethanol through handmade columns of 5 ml pipette tips and a cotton plug. Thus, the samples were transferred to the columns and washed 14 times with 1.2 ml of TSE buffer each time. Then, dsRNA molecules bound to CF-11 column were eluted with 400 µl TSE buffer without ethanol followed by 30 µl 5M NaCl and 1100 µl absolute ethanol for precipitation of dsRNA. Finally, after an incubation of 20 min at -20°C and 20 min centrifugation, the precipitate was dried up under vacuum and re-suspended in 10 µl Tris/EDTA (TE) (6 mM Tris-HCl pH 8.0, 1 mM EDTA). Albeit CF-11 cellulose is considered to bind specially dsRNA, isolations were repeated twice for positive isolates in order to discard the possibility of being artefacts or DNA.

DsRNA gel electrophoresis

DsRNA banding patterns were checked by electrophoresis in order to identify the possible virus molecules hosted in the Spanish isolates of *G. abietina*. Thereby, 6 µl of sample were pipetted into 2 µl of tracking dye supplemented with sucrose to be loaded in a 1% agarose gel containing 1 x TAE buffer with 50 µl of ethidium bromide during 60 min at 120 V. The size of the dsRNA was determined comparing the size with a Gene Ruler™ 1 kb DNA Ladder Plus (Fermentas). After running, the gel was observed under UV light and photographed.

RESULTS AND DISCUSSION

In this study, dsRNA molecules were found in the 89% of the Spanish isolates studied. This result not only confirms the presence of mycoviruses in *G. abietina* in Spain but also supports the common high dsRNA occurrence found in previous studies (Tuomivirta et al., 2002). Furthermore, 8 different dsRNA banding patterns were observed (Figure 1). According to the molecular size of the bands members of genus *Mitovirus*, *Partitivirus*, *Totivirus* and *Hypovirus* could be hosted in Spanish *G. abietina* isolates.

The existence of dsRNA molecules in the genome of fungi is very common in nature, as it has been demonstrated over the last years (Pearson et al. 2009). Basidiospores of *Heterobasidion annosum* are also often infected (Ihrmark et al., 2001, 2002), *Cryphonectria parasitica* strains normally infected by genus *Hypovirus*, *Mitovirus* in *Ophiostoma novo-ulmi* (Hong et al., 1999), *Totivirus* in *Sphaeropsis sapinea* (Preisig et al., 1998), Finnish *G. abietina* var. *abietina* type A (Tuomivirta et al., 2002) etc. In particular, *G. abietina* biotype A was shown to harbour dsRNA in 44% of all tested isolates (Tuomivirta et al., 2002) and even if samples originating from ascospores were excluded, the percentage increased until 55% of isolates. Thus, such high infection rate in the Spanish isolates of *G. abietina* is in accordance with *Discula destructive*, which hosts dsRNA molecules in 79% of its isolates (Rong et al., 2001) but it contrasts with the frequency of isolation of other species as *S. sapinea* with a rate of 32% (Steenkamp et al., 1998).

Although sequencing is the most reliable method to identify viruses, the molecular weight of the bands and the banding pattern can propose the type of virus. In this case, each banding pattern corresponded to an isolate and as it is observed in the Figure 1, more than one virus could be hosted per isolate.

Thus, according to Figure 1, the first banding pattern, which consisted of one band of about 3 kb, could refer to genus *Chrysovirus*, however, as it consist of only one band, it is more possible to belong to *Mitovirus* or *Narnavirus*. This band appeared in the banding patterns numbers 5, 6 and 7 as well. Then, the pattern number 8 could be the genus *Totivirus* (~6 kb) because it consists of a single molecule of about 4 to 7 kb as it is described in the literature. This band also appeared in 2 and 5.

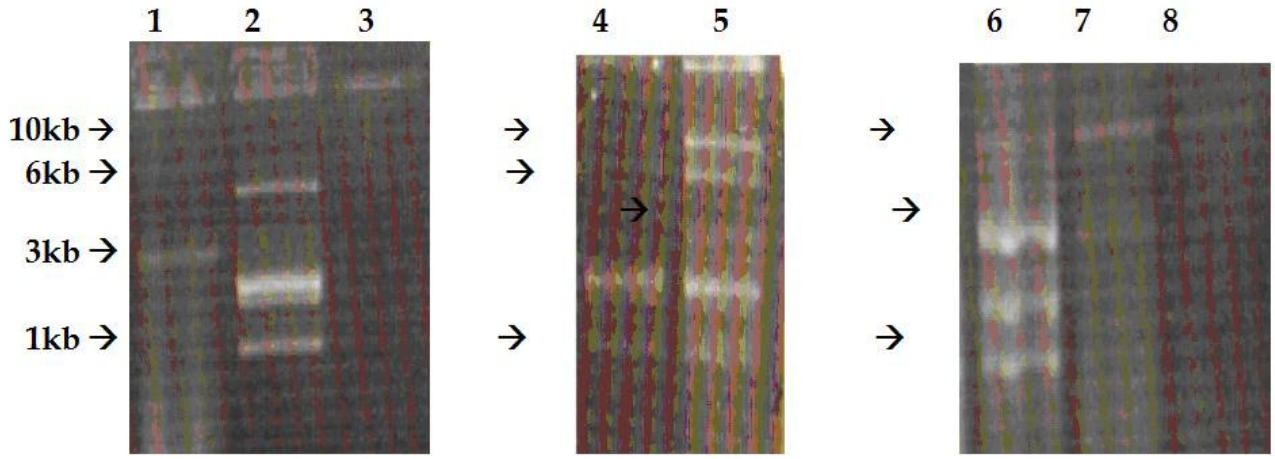

Figure 1. Common banding patterns found in the Spanish population of *G. abietina*.

Pattern number 4 has two similar linear molecules of 1.4 to 3.0 kb and one of about 1 kb, so it could correspond to *Partitiviridae*, which can also be observed in numbers 2, 5 and 6. In addition, banding pattern number 5 has a band over 10 kb that could be a member of the family *Hypoviridae*. However, as this family has not previously been confirmed in *G. abietina*, sequencing would be completely necessary. Finally, number 3 does not harbour any dsRNA molecule.

Conclusions

This study confirms that the Spanish population of *G. abietina* hosts dsRNA viral molecules at a significant high rate. Therefore, these results are interesting because they establish the bases for further phylogenic studies of the history of *G. abietina* and they open the possibility of finding hypovirulent strains of *G. abietina* that could behave as biological control agents.

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Full Length Research Paper

Using genomics to gain insights into the evolution and biology of *Pseudomonas syringae* pv. *aesculi* on European horse chestnut

Sarah Green[1]*, Bridget E. Laue[1], Heather Steele[1] and Paul M. Sharp[2]

[1]Forest Research, Northern Research Station, Roslin, Midlothian, EH25 9SY, UK.
[2]Institute of Evolutionary Biology, Kings Buildings, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JT, UK.

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***Pseudomonas syringae* pv. *aesculi* (*Pae*) causes a devastating bleeding canker disease of European horse chestnut (*Aesculus hippocastanum*) in northwest Europe. The pathogen can enter woody branches directly via lenticels, leaf scars and nodes, causing lesions in the cortex and phloem. Cankers can expand rapidly, causing bleeding symptoms on the stem and branches, and the trees suffer progressive crown dieback often leading to mortality. To gain insights into the evolutionary and biological adaptations of *Pae*, the draft genome sequences were generated for four strains of *Pae* including three strains from Britain and the type strain from India that causes leaf spot on Indian horse chestnut (*Aesculus indica*). The genomic data suggest that the British and Indian *Pae* strains share a recent common ancestor and that the three British *Pae* strains descend from a single, very recent introduction of the bacterium into Britain. A phylogenetic analysis based on a set of conserved genes showed that *Pae* belongs to a distinct clade of *P. syringae* pathovars adapted to woody hosts. Genomic comparisons with other *P. syringae* pathovars showed that *Pae* has acquired genes that may enable it to infect and live within the woody parts of the tree. These include genes involved in the degradation of plant-derived aromatic compounds, and others which likely have a role in disabling the tree's defense responses. These genes have not yet been found in other pathovars of *P. syringae* that infect herbaceous plants but may be conserved in other tree-infecting bacteria and thus, may be important to our understanding of the infection processes of bacterial tree diseases.**

Key words: Bleeding canker, *Aesculus hippocastanum*.

INTRODUCTION

There has been an unprecedented recent increase in the numbers of hitherto unrecognised diseases attacking trees throughout the world. Many of the causal organisms have been inadvertently introduced into new ecosystems through the increase in global commerce, via pathways such as trade in live plants, including soils (Brasier, 2008). Bleeding canker of European horse chestnut (*Aesculus hippocastanum*) is a destructive new disease affecting hundreds of thousands of European horse chestnut trees across several countries in northwest Europe, resulting in severe damage to rural and urban amenity landscapes (Webber et al., 2008; Green et al., 2009). Disease symptoms include bleeding cankers located on the stem and branches, foliar discoloration, and crown dieback often leading to tree death (Green et al., 2009). The causal agent was identified as the gram-negative fluorescent bacterium, *Pseudomonas syringae* pathovar *aesculi* (*Pae*) based on a partial sequence for its gyrase B gene, which was identical to that of the *Pae* type strain isolated from leaf lesions on Indian horse chestnut (*A. indica*) in India (Durgapal, 1971; Durgapal and Singh, 1980). Prior to the European epidemic, this was the only location where *Pae* had been reported. This

*Corresponding author. E-mail: sarah.green@forestry.gsi.gov.uk. Tel: +44 (0)131 445 6942.

suggests that *Pae* may have originated from India and been recently introduced into Europe.

Pae is highly virulent on European horse chestnut and apparently very mobile, since the pathogen can spread rapidly between, and within, infected trees causing dieback and mortality when it kills a large proportion of the phloem in the branches and stem. Observations of naturally infected trees have provided strong evidence that *Pae* initiates infection of European horse chestnut via lenticels, leaf scars and other natural openings in branches of various ages (Steele et al., 2010). *Pae* then colonises the cortex, phloem and cambium and has the potential to form extensive, continuous cankers within a single growing season. Thus, part of the success of *Pae* as a tree pathogen and the causal agent of a large-scale epidemic is due to an apparently highly effective capacity for direct aerial infection and colonisation of the woody parts of its host (Steele et al., 2010). Very little is known about the virulence traits of *Pae*. Due to its aggressiveness and rapidity of spread, *Pae* presents an excellent model system for gaining a greater understanding of bacterial tree diseases (Green et al., 2010).

Good quality draft genome sequences were generated for a strain of *Pae* recently isolated from a diseased European horse chestnut in Britain (strain 2250) as well as the Indian type strain of *Pae* (Green et al., 2010). Whole-genome re-sequencing data were also generated for two additional *Pae* strains (P6617 and P6623) from different geographical locations in Britain (Green et al., 2010). The aim of this study was to gain insights into the biology and evolution of *Pae* strains causing the current disease epidemic on European horse chestnut by comparing the *Pae* genome with sequences from other *P. syringae* pathovars and by determining the genomic variation among all four *Pae* strains.

MATERIALS AND METHODS

Pae strain 2250 was isolated in 2008 from necrotic phloem in the stem of a diseased horse chestnut near Pitlochry, Perthshire, Scotland. *Pae* strains P6617 and P6623 were isolated in 2006 from diseased horse chestnuts in Glasgow, Scotland and Farnham, England, respectively. Prior to sequencing, the pathogenicity of strain 2250 was confirmed by inoculating a cell suspension on to wounded horse chestnut shoots and observing subsequent development of lesions. The Indian strain of *Pae* (NCPPB3681) was isolated in 1969 from a leaf lesion on Indian horse chestnut in Northern India (Durgapal, 1971). DNA preparation and sequencing, genome assembly and alignment, bioinfomatic analyses and phylogenetic analyses were carried out as described in Green et al. (2010).

RESULTS AND DISCUSSION

Genome-wide Illumina sequence data were generated for the three British *Pae* strains and the Indian type strain of *Pae.* Since *Pae* is a recent disease of unknown origin, it is important to confirm the taxonomic placement of the British *Pae* strains and determine the evolutionary relationships between *Pae* and other *P. syringae* pathovars. On the basis of seven house-keeping genes (Sarkar and Guttman, 2004) the British *Pae* strains were identical to the Indian *Pae* type-strain. The close phylogenetic relationship of the British and Indian *Pae* strains is consistent with their classification within the same pathovar of *P. syringae* (Green et al., 2010). The nucleotide sequences of these seven marker genes were also used in phylogenetic analyses which showed that *Pae* belongs to a distinct clade of *P. syringae* pathovars adapted to woody hosts. These niche changes are likely to have required host-specific genetic adaptations (Green et al., 2010).

Several economically important tree diseases are caused by *P. syringae*, including pvs. *syringae* and *morsprunorum* on stone fruit, *savastanoi* on olive and *avellanae* on hazelnut, but their virulence traits remain unknown (Kennelly et al., 2007). In this study, comparative genomic analyses revealed genomic regions in *Pae* which are absent from other *P. syringae* pathovars that infect herbaceous hosts and which represent candidate genetic adaptations to infection of the woody parts of the tree. Of particular significance are the pathways for the degradation of plant-derived aromatic compounds such as lignin derivatives and other phenolics (Green et al., 2010). It is possible that these pathways enable *Pae* to utilize as carbon sources aromatic substrates specifically derived from the tissues of woody plants. Another mechanism in *Pae* that might be important to survival during host infection is the presence of two genes that have a predicted function in nitric oxide metabolism. Both enzymes encoded by these genes have a role in the protection of bacteria from NO which is an antimicrobial toxin shown to play a key role in plant disease resistance (Delledonne et al., 1998). The *Pae*-specific pathways identified here are potentially highly important for the understanding of bacterial diseases of woody plants (Green et al., 2010) and further studies are required to elucidate their function.

Rates of single nucleotide polymorphisms in the four *Pae* genomes indicated that the three British *Pae* strains diverged from each other much more recently than they diverged from the Indian strain of *Pae*. The lack of genetic diversity among the three geographically distinct *Pae* strains from Britain (only one or two nucleotide differences across 3 M bp) is consistent with a single introduction of the pathogen within the last few years (Green et al., 2010). This serves to highlight the environmental risks posed by the spread of exotic plant pathogens into new geographical locations.

Conclusions

Pae strains on European horse chestnut share a common ancestor with a *Pae* type strain isolated from Indian horse chestnut in India. The data also indicate that the

three British strains descend from a single, very recent introduction of the bacterium into Britain. Genomic comparisons with other *P. syringae* pathovars show that *Pae* has acquired genes that may enable it to infect and live within woody tree tissues. These genes have not yet been found in other pathovars of *P. syringae* that infect herbaceous plants but may be conserved in other tree-infecting bacteria and thus, may be important to our understanding of the infection processes of bacterial tree diseases.

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Extended Abstract

Himalayan dwarf mistletoe (*Arceuthobium minutissimum*) and the leafy mistletoe *Taxillus kaempferi* on blue pine (*Pinus wallichiana*) in Bhutan

Sangay Dorji[1], Edwin Donaubauer[1], Michael J. Wingfield[2], Dal Bahadur Chhetri[3] and Thomas Kirisits[1]*

[1]Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.
[2]Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.
[3]Renewable Natural Resources (RNR) Research and Development Sub-centre, Darla, Department of Forest and Park Services, Ministry of Agriculture and Forests, Royal Government of Bhutan, Darla, Chhukha, Bhutan.

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Blue pine, *Pinus wallichiana*, is an important tree species in temperate conifer forests in Bhutan. Disease surveys have shown that Himalayan dwarf mistletoe, *Arceuthobium minutissimum*, and the leafy mistletoe, *Taxillus kaempferi*, are important damaging factors on *P. wallichiana* in this Himalayan country. The knowledge on these two parasitic flowering plants in Bhutan is reviewed. A dwarf and leafy mistletoe survey in a study area in Western Bhutan documented high levels of mistletoe infection on *P. wallichiana*, especially by *A. minutissimum*. Recommendations for disease management, consisting mainly of sanitation, are given.

Key words: Parasitic flowering plants, Himalayas, temperate conifer forests, disease surveys, disease incidence.

INTRODUCTION

Blue pine or Himalayan blue pine, *Pinus wallichiana*, is an ecologically and socio-economically important tree species in many parts of the Himalayas and adjacent mountain ranges in Southern Asia. In Bhutan, it occurs in temperate conifer forests at elevations between 2100 and 3100 m asl. (Grierson and Long, 1983; Rosset, 1999). Blue pine is the preferred and most valuable softwood in this Himalayan country, being used for an array of purposes (Rosset, 1999). Forest tree disease surveys have shown that in some parts of Bhutan *P. wallichiana* is commonly and often severely infested with two parasitic flowering plants, Himalayan dwarf mistletoe, *Arceuthobium minutissimum*, and the leafy mistletoe, *Taxillus kaempferi* (Donaubauer, 1986; Chhetri, 1990, 1995; Tshering and Chhetri, 2000; Kirisits et al., 2002, 2007; Dorji, 2007). In this report, knowledge on these two parasitic flowering plants in Bhutan, emerging from collaborative studies since the 1980s (Kirisits et al., 2007), is briefly summarized.

GENERAL INFORMATION ON *A. MINUTISSIMUM* AND *T. KAEMPFERI* IN BHUTAN

A. minutissimum (Figures 1 and 2; Hawksworth et al., 1996) is widespread and very damaging in dry blue pine forests in the districts Paro, Ha and Thimphu in Western Bhutan (Donaubauer, 1986; Chhetri, 1990, 1995; Tshering and Chhetri, 2000; Kirisits et al., 2002; Dorji, 2007). As is true for all dwarf mistletoes *A. minutissimum* is a holoparasite. Infections are therefore a severe nutrient sink to *P. wallichiana* trees. Pathogenic effects of this parasitic flowering plant on the host include deformations (Figure 1A), stunted growth (Figure 2B), dwarfing

*Corresponding author. E-mail: thomas.kirisits@boku.ac.at.

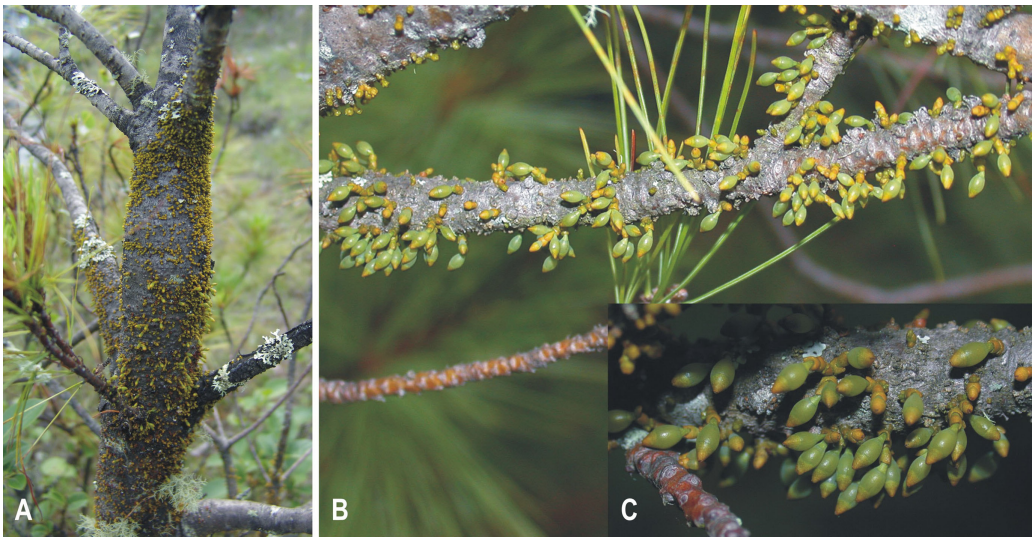

Figure 1. Himalayan dwarf mistletoe, *A. minutissimum* on *P. wallichiana*: (A) Stem swelling as symptom of dwarf mistletoe infection and shoots of *A. minutissimum*, (B) Pistillate (Female) plants of *A. minutissimum*, (C) Female *A. minutissimum* plants at higher magnification than in B. Shoots of this dwarf mistletoe species are on an average only 5 mm high.

Figure 2. Blue pine trees infected by *A. minutissimum*: (A) Mature tree with systemic witches' brooms, (B) Pole-sized tree showing stunted growth, (C) Sapling showing dwarfed growth and a 'bonsai-like' habit.

Figure 3. The leafy mistletoe *T. kaempferi* on *P. wallichiana*: (A) *T. kaempferi* plant with conspicuous red flowers, (B) Mistletoe bush in the crown of a Blue pine tree, (C) Infection of a blue pine stem by *T. kaempferi.*

(Figure 2C), systemic witches' brooms (Figure 2A), strong reduction of diameter and height growth, impaired wood quality, reduced cone production and mortality (Hawksworth et al., 1996; Dorji, 2007). Because of its severe impact on the host tree *A. minutissimum* is the most important pathogen of *P. wallichiana* in Bhutan. Even where insect pests and microbial pathogens are considered, it is most probably still the most important biotic damaging factor on this conifer species.

T. kaempferi (Figure 3) occurs most frequently on blue pine, but it also infests Himalayan hemlock, *Tsuga dumosa*, and Eastern Himalayan spruce, *Picea spinulosa* (Grierson and Long, 1983; Donaubauer, 1986; Chhetri, 1990; Kirisits et al., 2002; Dorji, 2007). It has a larger distribution range than *A. minutisimum*, occurring in Western and Central Bhutan, in the districts Thimphu, Wangdi Phodrang, Trongsa, Bumthang and Mongar (Grierson and Long, 1983; Donaubauer, 1986; Dorji, 2007). As a hemiparasite, the pathogenic effects of *T. kaempferi* on infected host trees are less serious than those caused by *A. minutissimum*. However, *T. kaempferi* infections weaken trees and can contribute to tree mortality, especially on dry sites, where prevalence of this parasitic plant is highest.

DWARF AND LEAFY MISTLETOE SURVEY IN A STUDY AREA IN WESTERN BHUTAN

In August 2004, a forest inventory incorporating a dwarf and leafy mistletoe survey was conducted in a 156-hectare-large area of blue pine forests on xeric sites at elevations between 2604 and 3024 m asl. in the district of Thimphu in Western Bhutan (Dorji, 2007). The inventory was based on 7-m-diameter, fixed-sized, systematic sample plots. Horizontal distance between plots was 100 m and the total number of plots was 98. At each sample plot all trees with a height exceeding 1.3 m above the ground were recorded. Various biometric characteristics, particularly diameter at breast height, were measured for each sample tree and subsequently processed for forest inventory calculations. Blue pine trees were also inspected for infections by *A. minutissimum* and *T. kaempferi*. In addition, various site characteristics were recorded.

The study area was occupied by open, degraded, cattle-grazed, low-stocked forests dominated by blue pine (Dorji, 2007). Admixed species included *Rhododendron arboreum*, *Populus* sp., oak species (mainly *Quercus semecarpifolia*), *Salix* sp. and *Picea spinulosa*. The mean number of trees per hectare was 2286, among which 66%

were *P. wallichiana*. Mean basal area per hectare was 12.6 m^2, approximately 79% of which being blue pine. Mean standing timber volume of blue pine in the study area was about 90.5 m^3 per hectare.

High levels of mistletoe infection on *P. wallichiana*, especially by *A. minutissimum* were recorded in the survey (Dorji, 2007). *A. minutissimum* occurred on 58%, *T. kaempferi* on 52% and both mistletoes on 30% of the 97 sample plots containing blue pine. Of the 2282 blue pine trees evaluated, 29.4% were infested with *A. minutissimum* and 4.9% with *T. kaempferi*, with both species occurring on 1.5% of the trees. Incidence of *A. minutissimum* increased slightly with tree diameter, however, it was also prevalent on small trees, exemplified by the smallest diameter class (0.1 to 5.0 cm diameter at breast height), in which 25.3% of the trees were infected. Incidence of *T. kaempferi* also increased with tree diameter, and highest infection levels were recorded on pine trees in larger diameter classes. Incidence of *A. minutissimum* was higher on blue pine trees growing on ridges (37%) and lower slopes (43%) than on trees occurring on sites at other topographic positions (valley bottom - 21%, middle slope - 19%, upper slope - 24%). There were no clear relationships between dwarf mistletoe infection levels and other site characteristics.

CONCLUSIONS

Mistletoes, especially Himalayan dwarf mistletoe, are serious forest pathogens in Bhutan. The high levels of incidence and infection severity caused by *A. minutissimum* and *T. kaempferi* on blue pine in parts of Western Bhutan suggest that past and present forest management has favoured infestation of *P. wallichiana* with these parasitic flowering plants. This is because it is common practice to preferentially cut uninfected trees with good wood quality and to leave infested residual trees. We recommend incorporating principles of disease management, particularly sanitation in a silvicultural system to treat blue pine forests heavily affected by these parasitic plants in Bhutan (Donaubauer, 1986; Chhetri, 1990; Tshering and Chhetri, 2000; Dorji, 2007).

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Extended Abstract

Quantification of *Dothistroma septosporum* spores by real-time polymerase chain reaction (PCR)

Josef Janoušek[1,2]*, Rebecca McDougal[2], Martin Mullett[3], Libor Jankovský[1], Anna Brown[3] and Rosie E. Bradshaw[2]

[1]Department of Forest Protection and Wildlife Management, Faculty of Forestry and Wood Technology, Mendel University in Brno, Zemědělská 3, 613 00 Brno, Czech Republic.
[2]Institute of Molecular BioSciences, Massey University, Palmerston North 4442, New Zealand.
[3]Forest Research, Alice Holt Lodge, Farnham, Surrey GU10 4LH, United Kingdom.

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Many fungal plant pathogens can be spread over long distances by airborne or rain splash spore dispersal. These spores can infect susceptible hosts causing disease and in some cases mortality and for this reason spore monitoring followed by control is needed. Air sampling can be combined with modern sensitive molecular methods such as quantitative polymerase chain reaction (qPCR), real-time PCR (West et al., 2008, 2009). A method for absolute quantification of *Dothistroma septosporum* (teleomorph *Mycosphaerella pini*) spores by real-time PCR is being developed. We report here the optimisation of plasmid standards and standard curves that are required for quantification using qPCR. Preparation of a DNA standard is necessary to make a robust and reliable estimation of the number of spores. The most consistent results have been obtained by using plasmid DNA with insert containing target sequence (Dhanasekaran et al., 2010). The standard can be used to determine the number of DNA copies in a spore trap sample, and thus the number of spores. Two plasmids, with different lengths of the target gene inserted (single copy gene), were prepared and both linear and circular forms of these were used for standard curve generation. Linear plasmid amplified in average of 2 to 3.5 cycles (C_q - quantification cycle) earlier than circular plasmid when using a SYBR Green detection system. However, no differences in C_q value were recorded when using a hydrolysis detection system. Addition of carrier tRNA to plasmid solution caused inhibition of PCR at high plasmid concentration. Plasmid with the short insert, the same length as the amplified product, generated standard curves with efficiencies closer to 2.0 than plasmid with long insert. The final standard curve was prepared with circular plasmid containing short insert without adding the tRNA. DNA extraction from *D. septosporum* spores will be optimised and then tested with spore trap tape samples. Standard curves developed from DNA extracted from spores will be correlated with those issued from plasmid as a DNA template. The method for quantification of *D. septosporum* spores will increase accuracy of epidemiological studies. This type of spore quantification method will be suitable to form part of the complex method for management of disease caused by *Dothistroma septosporum*.

Key words: *Dothistroma septosporum*, quantification, real-time PCR, spores.

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*Corresponding author. E-mail: janousek.jose@gmail.com.

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Extended Abstract

Dieback of ash (*Fraxinus excelsior* and *Fraxinus angustifolia*) in Eastern Austria: Disease development on monitoring plots from 2007 to 2010

Marion Keßler[1], Thomas L. Cech[1], Martin Brandstetter[1] and Thomas Kirisits[2]*

[1]Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Protection, Seckendorff-Gudent-Weg 8, A-1131 Vienna, Austria.
[2]Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.

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Dieback of *Fraxinus excelsior* and *Fraxinus angustifolia*, caused by *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*), is presently the most important damaging factor of hardwood trees in Austria. Results from permanent monitoring plots in Lower Austria show that disease development on mature ash trees was slow from 2008 to 2010. In 2008, mean dieback intensity ranged from 1 to 34% on the 14 plots (mean 11%). In 2010, mean dieback intensity per plot varied between 2 and 38% (mean 16%). Only on three of the 14 monitoring plots one out of the 20 sample trees had died during the observation period. Disease intensity was higher on most plots in the western parts of Lower Austria than on most plots in the eastern parts of the province. Relations between disease intensity and site and stand factors are discussed.

Key words: *Hymenoscyphus pseudoalbidus*, *Chalara fraxinea*, ash dieback, emerging forest disease, disease monitoring.

INTRODUCTION

Ash dieback (Figure 1) caused by *Hymenoscyphus pseudoalbidus* (Figure 2; anamorph *Chalara fraxinea*) was first recorded in Austria in 2005 and has since then become the most important damaging factor of hardwood trees in this Central European country (Cech, 2006, 2010; Kirisits et al., 2009, 2011). Both *Fraxinus excelsior* and *Fraxinus angustifolia* are affected by the disease (Cech, 2008, 2010; Halmschlager and Kirisits, 2008; Kirisits et al., 2009, 2010). In 2007, shortly after the massive occurrence of ash dieback, a monitoring project was initiated in the province of Lower Austria, aiming at surveying the extent and intensity of damage and studying the etiology of this emerging and at that time poorly understood phenomenon (Cech, 2008). Monitoring of ash dieback was subsequently continued on permanent plots from 2008 to 2010 (Kirisits et al., 2011). The main results of this research are briefly summarized in this presentation.

ASH DIEBACK MONITORING 2007

In 2007, 50 monitoring plots in mature ash stands (48 composed of *F. excelsior* and two of *F. angustifolia*) were established in various parts of Lower Austria (Cech, 2008). On each plot 20 mature ash trees were selected. For each sample tree the percentage of crown volume affected by dieback was visually estimated in 5% classes. Likewise, various other biotic and abiotic damaging factors were recorded. Assessments were done from July to August.

In this year, ash dieback was significantly less intensive in the plain and drier eastern parts of Lower Austria than in the mountainous and more humid western parts. In addition, suppressed individuals showed higher mean

*Corresponding author. E-mail: thomas.kirisits@boku.ac.at.

Figure 1. Ash dieback on *F. excelsior*: (A) Heavily damaged, declining pole-sized tree (photo: Christian Freinschlag), (B) Intensive dieback of shoots, twigs and branches in a pole-sized stand, (C) Severely damaged mature ash tree (photo: Josef Wampl).

dieback intensity than dominant ash trees, and female and hermaphroditic trees were more severely affected than male ones. In almost every monitoring plot trees without symptoms were observed. For more detailed results of the survey in 2007 see Cech (2008).

ASH DIEBACK MONITORING FROM 2008 TO 2010

From 2008 onwards, annual monitoring was continued on 14 selected permanent plots (Kirisits et al., 2011), representing the major forest ecoregions in Lower Austria, where ash is a common tree species. Thirteen plots contained *F. excelsior* and one, near Hohenau/March along the river Morava, *F. angustifolia*. Visual damage assessments as described above were done on 20 permanently marked sample trees per plot. The monitoring was conducted from late July to early August each year.

In 2008, mean dieback intensity ranged from 1 to 34% on the 14 plots, the mean value was 11%. In 2010, mean dieback intensity per plot varied between 2 and 38% and the overall mean value for all plots had slightly risen to 16%. On some plots disease intensity had changed very little and was still at low levels in 2010. Mean dieback intensity was below 5% on 5 plots (36%), between 5.1 and 10% on 2 plots (14%), between 10.1 to 20% on 3 plots (21%), and between 30.1 and 40% on 4 plots (29%). Mortality was still very low in 2010. Only on three of the 14 plots one out of the 20 sample trees (5%) had died.

In agreement with the assessments in 2007 most plots in the western parts of Lower Austria were still more severely affected in 2010 than most plots in the eastern parts of the province. In contrast to 2007, however, differences in disease intensity in relation to social position and gender were not observed any more. On 13 out of the 14 plots trees without dieback symptoms were still found in 2010; their proportion ranged from 5% (1 tree) to 80% (16 trees) per plot. Amongst secondary damaging factors, ash bark beetles (mainly *Leperisinus varius*) were detected in about one third of the plots already in 2007. Until 2010, however, no attacks of bark

Figure 2. Apothecia of the ash dieback pathogen *H. pseudoalbidus* on ash leaf petioles and rachises from the previous year in the forest litter. The sizes of the disc flats of the apothecia shown on the photos range from about 1.5 to about 6 mm.

beetles on living trees were observed.

DISCUSSION AND CONCLUSIONS

Although ash dieback has been present in Lower Austria at least since 2005, disease intensity on mature ash trees remained relatively low during the entire observation period, reaching the highest level in a pure ash stand in riparian forests along the Danube near Korneuburg. Though the disease generally increased slightly in intensity, the changes from 2008 to 2010 show a wide variation and on some sites even a decrease in disease intensity was recorded. The considerable variation in dieback intensity between plots may be related to factors influencing sporulation of *H. pseudoalbidus* and the infection biology of the ash dieback pathogen. These include stand characteristics, the amount of ash trees in the vicinity and site-dependent humidity during the infection period. In the eastern parts of Lower Austria incidence of ash is lower than in the western parts and the sites are usually drier, which may explain the lower levels of ash dieback intensity on most of the plots in the eastern part of the province. The occurrence of ash trees without dieback symptoms on many plots may indicate that some individuals display high levels of resistance to *H. pseudoalbidus*.

Although ash bark beetles were recorded from the beginning of the monitoring, up to 2010 there was no evidence of an outbreak of these secondary pests on the survey plots. In 2011, however, attacks of bark beetles on living trees were observed in the province of Styria. This indicates that these insects should be carefully observed in the future and sanitation measures, if required, should be considered.

In summary, the monitoring results presented here

indicate that ash dieback damages mature ash trees gradually or even slowly over time and that damage levels vary greatly between sites. Field observations in many parts of Austria suggest, however, that damage and mortality levels are much higher on nursery seedlings, in afforestations, on natural regeneration as well as in thicket-sized and pole-sized stands (Figure 1A and B; Kirisits et al., 2011). Thus, ash dieback causes immense problems for establishing and tending young stands, while old trees appear to be capable to endure the disease for a relatively long time.

It is envisaged to continue the ash dieback monitoring in Lower Austria in the next years, in order to document the disease development on mature trees over time and to strengthen the knowledge regarding the role of site, climatic and stand factors for the epidemiology of this emerging forest disease. The detection of ash trees with putative high levels of resistance to *H. pseudoalbidus* could also be a valuable result of this survey. Ultimately, this research aims at leading to science-based recommendations for disease management to practitioners.

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Extended Abstract

Alien pathogens of forest trees in Austria

Thomas Kirisits[1]* and Thomas L. Cech[2]

[1]Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.
[2]Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Protection, Seckendorff-Gudent-Weg 8, A-1131 Vienna, Austria.

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Based on information in the literature and unpublished records a brief synthesis on alien pathogens of forest trees in Austria is presented. A total of 29 pathogens were recorded, consisting of one bacterium, nine *Phytophthora* species, 14 ascomycetes, three anamorphic fungi and two basidiomycetes. The majority of alien pathogens (23 out of 29) occur on hardwood species. Six pathogens have had a high impact on their host trees and have considerably impaired their use in forestry and/or their role in ecosystems. Four of the six pathogens with high impact affect hardwood trees. Due to climate change the importance of some alien forest pathogens already present in Austria may increase in the future.

Key words: Invasive forest pathogens, introduced pathogens, emerging forest diseases, biological invasion, forest pathology.

INTRODUCTION

Alien pathogens are an important threat to the world's forests and the various ecosystem services they provide to humans. In recent years, there have been various initiatives to synthesize information on alien organisms in general and forest pathogens specifically. Based on information in the literature and own, hitherto unpublished records, assembled as part of the EU-funded projects FORTHREATS and ISEFOR (see acknowledgements), we present a synthesis of alien pathogens occurring on forest trees in Austria (see also Kirisits, 2010). Pathogens were classified either as definitely or ambiguously alien, the latter including those species for which it is not precisely known, whether they are non-native or not. In the synthesis, only host trees recorded by the Austrian Forest Inventory (2007 to 2009, www.waldinventur.at) were considered, but not species mainly occurring as shade trees.

LIST OF ALIEN FOREST PATHOGENS IN AUSTRIA

A total of 29 pathogens of forest trees were recorded for Austria, 15 of which are definitely alien and 14 whose status is ambiguous. As the Dutch elm disease fungus *Ophiostoma ulmi* has already become extinct, the number of species presently known to occur is 28. The Austrian list of alien and possibly alien forest pathogens includes one bacterium (*Erwinia amylovora*), nine *Phytophthora* species and 19 true fungi. Amongst fungi, ascomycetes dominate with 14 species, three are anamorphic fungi and two are basidiomycetes, both being rust fungi. For 12 out of the 15 definitely alien pathogens, the continent of origin is known. Seven are native to North America and five to Asia. Special cases are *Phytophthora alni* which originated from hybridization of two or three *Phytophthora* species, including at least one alien taxon, and *Cronartium ribicola* which is native to the Alps, but the epidemic on *Pinus strobus* was likely caused by strains of the fungus originating from Asia.

AFFECTED HOST TREES

Amongst conifers native to Austria only pine species (*Pinus* spp.) and Norway spruce (*Picea abies*) are affected by a total of three definitely or ambiguously alien pathogens. Three pathogens occur exclusively or mainly on non-native conifer species (*P. strobus* and *Pseudotsuga menziesii*) and two exclusively on non-

*Corresponding author. E-mail: thomas.kirisits@boku.ac.at.

Figure 1. Examples of diseases caused by alien or ambiguously alien forest pathogens: (A) Dutch elm disease caused by *Ophiostoma novo-ulmi* on *Ulmus glabra*, (B) Diplodia shoot blight caused by *Diplodia pinea* on *Pinus nigra*, (C) Ash dieback caused by *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) on *Fraxinus excelsior*, (D) Dothistroma needle blight caused by *Mycosphaerella pini* (anamorph *Dothistroma septosporum*) on *Pinus cembra.*

native hardwood trees. One fungal pathogen occurs on both native and non-native trees (*Populus nigra* and its hybrids with North American *Populus* spp.). The remaining 20 pathogens use various native deciduous tree species as hosts.

IMPACT OF ALIEN PATHOGENS

While the ecological and socio-economic impacts of most pathogens have been or are presently considered as low, six species which cause serious diseases and can lead to

tree killing have been particularly problematic. These include *Ophiostoma novo-ulmi* (Figure 1A, on *Ulmus glabra*, *Ulmus laevis* and *Ulmus minor*, first observed around 1955), *Cryphonectria parasitica* (on *Castanea sativa*, first observed in 1970), *C. ribicola* (on *P. strobus*, first observed in the 1890s), *P. alni* (on *Alnus glutinosa* and *Alnus incana*, first recorded in 1996), *Diplodia pinea* (Figure 1B, mainly on *Pinus nigra* in Eastern Austria, observed since around 1990 as frequent and important pathogen) and *Hymenoscyphus pseudoalbidus* (Figure 1C, anamorph *Chalara fraxinea*, on *Fraxinus excelsior*, *Fraxinus angustifolia* and *Fraxinus pennsylvanica*, first observed in 2005). Five of these particularly damaging forest pathogens have emerged during the last 60 years. At an average, this corresponds to one serious disease caused by a definitely or ambiguously alien pathogen every 12 years. Due to climate change the importance of a number of alien forest pathogens is suspected to increase in the future. Examples include *Mycosphaerella pini* (Figure 1D, anamorph *Dothistroma septosporum*, on various *Pinus* spp.) and *Mycosphaerella dearnessii* (anamorph *Lecanosticta acicola*, on *Pinus mugo* and *Pinus sylvestris*).

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Extended Abstract

Ash dieback associated with *Hymenoscyphus pseudoalbidus* in forest nurseries in Austria

Thomas Kirisits*, Peter Kritsch, Katharina Kräutler, Michaela Matlakova and Erhard Halmschlager

Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.

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Dieback of *Fraxinus excelsior, Fraxinus angustifolia* and other *Fraxinus* species is an emerging infectious disease caused by the ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*). Investigations in five forest nurseries in Austria from 2008 to 2011 showed that ash dieback is a common and important disease of nursery seedlings. *H. pseudoalbidus* was consistently isolated at high frequencies from symptomatic *F. excelsior* and *F. angustifolia* seedlings, confirming that this fungus is associated with ash dieback. Symptom observations on potted seedlings revealed progression of the disease outside the vegetation period and thus a long incubation period in the disease cycle of ash dieback. Apothecia of *H. pseudoalbidus* were occasionally observed on dead ligneous parts of common ash, including stems of nursery seedlings. This suggests that a low portion of diseased seedlings can initiate new infections, which may be important for moving the pathogen to new areas. Based on our observations and studies in forest nurseries and the present knowledge on the disease cycle of ash dieback, recommendations for disease management in tree nurseries and concerning artificial regeneration are presented.

Key words: *Chalara fraxinea*, *Fraxinus excelsior*, *Fraxinus angustifolia*, emerging forest disease, disease management.

INTRODUCTION

During the last two decades, ash dieback caused by the ascomycete fungus *Hymenoscyphus pseudoalbidus* (Figure 1; anamorph *Chalara fraxinea*; Kowalski, 2006; Kowalski and Holdenrieder, 2009a, b; Queloz et al., 2011) has successively emerged in many European countries (Husson et al., 2011; Timmermann et al., 2011). Everywhere the disease appears it causes immense damage to common ash (*Fraxinus excelsior*) and in some parts of Europe also to narrow-leaved ash (*Fraxinus angustifolia*) (Kowalski and Łukomska, 2005; Kirisits et al., 2009, 2010; Schumacher et al., 2010; Schumacher, 2011). In addition, ash dieback has also been reported on a few ash species not native to Europe (Drenkhan and Hanso, 2010).

Its gradual spread and the high disease intensity may indicate that *H. pseudoalbidus* is an invasive alien organism (Husson et al., 2011; Queloz et al., 2011; Timmermann et al., 2011). Trees of all ages, in the forest, the landscape and in urban environments, both naturally regenerated and planted ones, are affected (Kowalski and Łukomska, 2005; Kirisits et al., 2009; Schumacher, 2011). Moreover, the disease is an economically important problem in shade tree and forest nurseries (Kowalski and Łukomska, 2005; Schumacher et al., 2010; Schumacher, 2011). Here, we report on observations and investigations on ash dieback in forest nurseries in Austria.

ASSOCIATION OF *H. PSEUDOALBIDUS* WITH ASH DIEBACK IN NURSERIES

Investigations on ash dieback were conducted from 2008 to 2011 in five forest nurseries. Seedlings were inspected for disease symptoms on the stem and on side twigs. From seedlings showing early symptoms of disease (rela-

*Corresponding author. E-mail: thomas.kirisits@boku.ac.at.

Figure 1. Apothecia of the ash dieback pathogen *H. pseudoalbidus* on ash leaf petioles and rachises from the previous year in the forest litter. Note black pseudosclerotial plates (see also inset) on petioles and rachises, from which apothecia emerge. Ash trees are infected by airborne ascospores produced in the apothecia.

tively small necrotic lesions in the bark), fungal isolation was attempted. After surface sterilization (1 min in 96% ethanol, 3 min in 4% NaOCl, 30 s in 96% ethanol) of stem segments, the outer bark was carefully peeled off and small discs containing wood and phloem were cut near the transition zone between necrotic and healthy phloem and placed on malt extract agar (MEA; 20 g/L malt extract, 16 g/L agar, 100 mg/L streptomycin sulphate). The primary isolation plates were at first incubated at room temperature in diffuse daylight (nursery 1, in 2008), but later (nurseries 1 to 5, in 2009 and 2011) at cool temperatures (between 4 to 10°C) in the dark. The latter was done in order to stimulate anamorph production of *H. pseudoalbidus* and to give it competitive advantage over other fungi, thereby increasing the likelihood to detect the ash dieback pathogen (Kirisits et al., 2009). *H. pseudoalbidus* was identified based on morphological characteristics of its *Chalara fraxinea* stage (colony morphology, phialophores and spores). Other fungi were not determined.

Typical symptoms of ash dieback, including necrotic lesions in the bark and phloem, wood discoloration as well as dieback of shoots and entire seedlings were frequently observed in all five nurseries (Figure 2), on one-year-old to three-year-old ash seedlings. *H. pseudoalbidus* was consistently isolated at high frequencies and often in pure culture from symptomatic *F. excelsior* seedlings in all five nurseries and in one nursery also from *F. angustifolia* seedlings. Overall, the ash dieback pathogen was obtained from 174 of the 192 (91%) investigated *F. excelsior* seedlings and from 14 of the 15 (93%) examined *F. angustifolia* plants. In agreement with a number of recent studies (for example Kowalski, 2006; Bakys et al., 2009; Kirisits et al., 2009, 2010; Kowalski and Holdenrieder, 2009a; Drenkhan and Hanso, 2010; Schumacher et al., 2010; Husson et al., 2011), the results clearly suggest that *H. pseudoalbidus* is associated with ash dieback and the cause of this disease.

DISEASE PROGRESSION OUTSIDE THE VEGETATION PERIOD

In mid-November 2010, three-year-old *F. excelsior* seedlings, rated as 'disease-free' based on external inspections were obtained from forest nursery 1. They were potted and placed in the garden of the institute. In spring 2011, the plants were again examined for the occurrence of ash dieback and isolations were done from diseased seedlings as described above. Upon inspection on 19 April 2011, 196 out of the 464 seedlings (42%) showed symptoms of ash dieback. The ash dieback pathogen was confirmed from all 41 plants (38 times in pure culture), from which isolations were made. As ascocarps of *H. pseudoalbidus* do not occur from October to May (Kirisits and Cech, 2009; Timmermann et al., 2011) and spores of *C. fraxinea* are most likely not infectious (Kirisits et al., 2009), the seedlings must have

Figure 2. Symptoms of ash dieback on seedlings of *F. excelsior*: (A) Necrotic bark lesion adjacent to a leaf scar, (B) Necrotic bark lesion with a small dead twig in the centre, (C and D) Extensive bark necrosis, (E) Package of ash seedlings prepared in autumn to be sold next year, containing diseased plants in spring.

been already infected by November, but were asymptomatic at that time and symptom progression took place outside the vegetation period. This is consistent with reports by forest nursery managers that considerable portions of apparently healthy ash seedlings, selected in autumn to be sold next year, were diseased when inspected again the following spring (Figure 2E). Disease progression during the cold period of the year is also consistent with the proposed disease cycle of ash dieback (Kirisits and Cech, 2009; Kirisits et al., 2009; Schumacher, 2011).

SPORULATION OF *H. PSEUDOALBIDUS* ON LIGNEOUS PARTS OF COMMON ASH

Structures resembling pseudosclerotial plates of *H. pseudoalbidus* were seen on stems, shoots and twigs of a few dead ash seedlings, in spring 2009 in nurseries 3 and 4 and in spring 2011 in nurseries 1 and 5, indicating that the fungus can probably form its apothecia on these seedlings later in the year. In summer 2011 stem segments of selected seedlings (from nursery 1) and natural regeneration as well as dead ash shoots and twigs with pseudosclerotial layers, collected from the forest litter at various localities, were placed in boxes filled with potting soil and incubated under moist conditions. Apothecia of *H. pseudoalbidus* subsequently developed, sometimes in high numbers (Figure 3), on a large portion of the incubated stems of nursery seedlings and naturally regenerated saplings as well as the ash shoots and twigs. This is consistent with previous reports on the occasional occurrence of apothecia on stems and shoots of one- to three-year-old dead nursery seedlings (Kowalski and Holdenrieder, 2009b) as well as on dead ash shoots and twigs in the forest litter (Kirisits and Cech, 2009). In contrast, teleomorph formation on ligneous tissues of ash is not known for *Hymenoscyphus albidus*, the long-known and apparently native sibling species of the ash dieback pathogen (Kowalski and Holdenrieder, 2009b; Queloz et al., 2011).

H. pseudoalbidus is effectively disseminating via its airborne ascospores that are primarily produced in apothecia on leaf petioles and rachises from the previous year on the ground (Figure 1; Kirisits and Cech, 2009; Kowalski and Holdenrieder, 2009b; Timmermann et al., 2011). This mode of dispersal likely explains the rapid recent spread of the fungus in Europe (Kowalski and Holdenrieder, 2009b; Timmermann et al., 2011). However, trade with infected nursery seedlings could have

Figure 3. Apothecia of *H. pseudoalbidus* on *F. excelsior* twigs. Note black pseudosclerotial layers that had formed underneath the epidermis on each of the twigs. The size of the largest apothecial disc flats shown on each of the photos is about 5 mm.

been and still may be an important pathway to introduce the pathogen into new areas and to accelerate its spread (Kirisits et al., 2010; Timmermann et al., 2011). The occasional observation of *H. pseudoalbidus* apothecia on ligneous tissues of common ash indicates that nursery seedlings can indeed be infectious. It is likely that the movement of apparently and latently diseased seedlings, on which inoculum subsequently develops, can lead to the initiation of new disease centres, even far away from natural infection sources. Likewise, infected ash leaf petioles and rachises, including those with pseudosclerotial plates from the litter, may be moved together with bare-root and container-grown plants (Timmermann et al., 2011).

RECOMMENDATIONS FOR DISEASE MANAGEMENT

Although the origin of *H. pseudoalbidus* remains enigmatic, the pathogen behaves as an invasive tree pathogen and is a serious threat to *F. excelsior* and other

Fraxinus species (Kowalski, 2006; Bakys et al., 2009; Kirisits et al., 2009, 2010; Drenkhan and Hanso, 2010; Schumacher et al., 2010; Husson et al., 2011; Queloz et al., 2011; Timmermann et al., 2011). The disease cycle of ash dieback is now understood sufficiently well (Kirisits and Cech, 2009; Kirisits et al., 2009; Kowalski and Holdenrieder, 2009b; Schumacher, 2011; Timmermann et al., 2011) that recommendations for disease management concerning seedlings and artificial regeneration can be given. Measures are grouped according to the strategies presented by Tainter and Baker (1996). As knowledge on ash dieback is still limited amongst many practitioners and stakeholders, especially in countries and areas, where the disease presently does not occur, raising awareness on the disease forms the basis for implementing disease management measures.

Exclusion

i. Plant quarantine measures for nursery seedlings (import bans, imports from confirmed disease-free areas only, plant inspections, plant passports and certifications) may be effective to avoid or delay the movement of the ash dieback pathogen to geographically isolated parts of Europe such as the British Isles and to other continents. However, the long inclubation period in the disease cycle of ash dieback makes inspection of ash plants extremely difficult.
ii. Import bans for nursery seedlings and possibly other commodities (for example ash logs and timber) and thus closing potentially dangerous pathways would be the most effective measures to avoid the movement of *H. pseudoalbidus*, but are difficult to enforce politically.

Avoidance

i. Due to the risks posed by the disease, wide afforestation of susceptible ash species (especially *F. excelsior* and *F. angustifolia*) is no longer recommended. Site adapted alternative species should be given preference. If ash is used to some degree, it should be planted in mixture with other species.
ii. Observations in a number of countries (for example Schumacher, 2011) suggest that disease intensity is lower on drier sites within the ecological amplitude of *F. excelsior*. Still using ash on such sites may thus be justified, as well as in situations where wood production is not the main management goal, for example in shelterbelts, landscape and urban plantings.
iii. Flowering ash (*Fraxinus ornus*) that has so far not been affected by ash dieback may in some situations be an alternative.
iv. Little is known on the susceptibility of non-native *Fraxinus* species to the disease, but Drenkhan and Hanso (2010) reported ash dieback and confirmed the occurrence of *H. pseudoalbidus* on *F. americana*, *F. nigra* and *F. pennsylvanica* from North America and *F. mandshurica* from Asia. Exotic ash species should thus be used only with caution as alternative to native *Fraxinus* spp.
v. As sporulation of and infection by *H. pseudoalbidus* is favoured by high soil and air humidity, ash seedling beds should be established at sites with fairly dry microclimate, offering the prospect of lower disease levels.
vi. In order to decrease infections, seedling beds should be located large distances away from natural inoculum sources in the surroundings.
vii. Both producers and buyers should carefully inspect nursery plants for the occurrence of ash dieback. It is recommended that users continue the inspections after trees have been planted. If plants have been unambiguously infected in the nursery, buyers can claim for compensation.
viii. Many seedlings appear to be disease-free in autumn, but develop externally visible symptoms during winter and early spring. Purchasing trees in spring therefore increases the chance to obtain mostly healthy plants.

Inoculum reduction

i. The removal and destruction of shed ash leaves may in some situations be economically feasible to reduce inoculum and thus to decrease infections in nurseries. Leaves should be burnt, buried, ploughed into nursery beds or covered with soil.
ii. Routine removal of leaves in cities, in addition to dry climatic conditions in urban environments, not very conducive for inoculum production of and infection by *H. pseudoalbidus*, may explain why ash dieback is presently often of relatively minor importance on shade, avenue and park trees.

Protection

Fungicide treatments may be an option in nurseries, however, as the infectious period of *H. pseudoalbidus* is long (mid-June to early September in Central Europe) many applications are likely necessary. Overall, fungicide treatments will be of limited value to manage ash dieback, as seedlings remain susceptible when planted in the field.

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Extended Abstract

Chalara fraxinea incidence in Hungarian ash (*Fraxinus excelsior*) forests

A. Koltay[1]*, I. Szabó[2] and G. Janik[1]

[1]Forest Research Institute, Hungary, Mátrafüred, Hungary.
[2]University of West Hungary, Sopron, Hungary.

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Chalara fraxinea **was identified for the first time in Hungary in the first half of 2008, in western Hungary near Kapuvár and Sárvár, in 4 to 6 years old mixed (seed and coppice shoot) origin European ash (*Fraxinus excelsior*) stands (Szabó, 2008). In the same time in Budapest, under an older Turkey Oak – Sessile Oak – European ash stand we also detected the symptoms and the pathogen on the saplings of the natural regrowth. The local foresters first thought, that the wilting was caused by the frost, but in that period of time there were no frosty days. With the typical symptoms and the examinations of the collected samples, we were able to definitely identify the *Chalara fraxinea* as pathogen (Szabó, 2009). This pathogenous fungus was also identified on Narrow-leafed ash (*Fraxinus angustifolia*), from the samples of the western part of Hungary (Kirisits et al., 2009). In 2008-2009, we thoroughly researched the distribution of the pathogen in Hungary, and the volume of the caused damages. As a result, we confirmed that the pathogen spread to the whole area of Hungary (Figure 1). It appears both in young and older stands, but it causes damages more frequently in 2 to 10 years old forestations. Because of the characteristics of the symptoms and the measures of the dieback, we concluded that the pathogen appeared in Hungary 2 to 3 years before. The degree of the infections in the examined forest stands is significantly diverse. The most severe infestation was observed in Eastern-Hungary, near Debrecen, in the summer of 2009. This European ash stand was 10 years old, with 0.5 ha of area, and was planted with 2 years old saplings. Every single tree showed the symptoms of *C. fraxinea* infection (Figure 2). In the examined part of the forest-stand, the mortality reached 37%. Among the still living trees, the rate of the infected and died stem parts varied between 20 to 90%. From the symptoms of the dead trees we diagnosed, that the first infections in this area also occurred a few years ago. We do not know much about the environmental conditions assisting the infestation. The examinations of the infested forest-stands of Western-Hungary show that the infestation is more frequent on sites with frost-hollow, deep soil and plenty of water. In the same time we also noticed that the symptoms are also frequent on forest sites drier than average and exposed to extreme cold (Szabó et al., 2009). According to the surveys, the fungus is more common in younger stands, but this can be affected by the fact, that we have lesser amount of samples from older and bigger trees, for collecting samples and identifying them from large crowns is more difficult. After the survey in Bükk-mountains, North-eastern-Hungary, we found that the extent of the infection is at least the same on older or middle aged trees, than on the youngest ones. Contrarily, in the western part of the country in mixed species forest stands we experienced mass and severe infections of the natural ash regrowth, while older trees showed only small degree of typical symptoms in their crowns. The complete death of older trees takes more time, so major mortality occurs on young ones, 2 to 10 years old trees. In August, 2009 we surveyed the degree of *C. fraxinea* infestations in some forestry's of the Bakony-mountains, in different aged and in different tree-species composition forests. Based on this survey we pointed out that in the significant majority of the surveyed stands the rate of infected ash trees is under 5%, and in only 2 forest-parts are there 5 to 10% infestations (Table 1).**

Key words: *Chalara fraxinea*, ash dieback, *Fraxinus excelsior*, *Hymenoscyphus pseudoalbidus*.

*Corresponding author. E-mail: koltaya@erti.hu.

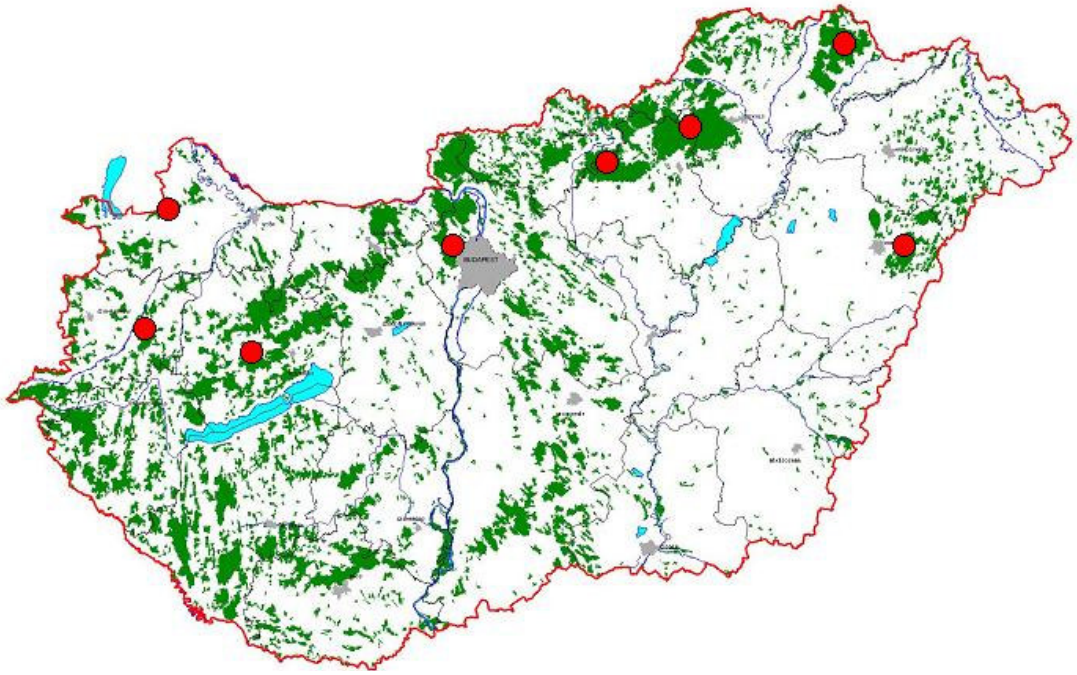

Figure 1. Proven occurrence of *C. fraxinea* in Hungary 2008 to 2009.

Table 1. The measure of *C. fraxinea* infection in Bakony mountain August, 2009.

Forest subcompartment	Measure of shoot dying			Area (ha)	Age	Tree species (%)
	1	2	3			
Tés 9H	X			6.3	76	*Fagus 80; Carpinus 15; Fraxinus 5*
Tés 9I	X			4.2	76	*Fagus 100; Fraxinus natural regrowth in spots*
Tés 10B	X			18.8	112/7	*Fagus 100; Fraxinus natural regrowth in spots*
Várpalota 55G		X		2.7	9	*Q. cerris 40; F. excelsior 30; F. ornus 30*
Várpalota 55F	X			4.7	110/9	*Q cerris 40; Fagus 30; F. excelsior 20; Carpinus 10*
Várpalota 56D	X			5.2	8	*Q. petrea 50; Fagus 10; F. excelsior 15; Q. cerris 15; Carpinus 10*
Várpalota 57A	X			11.2	127/6	*Q. cerris 100; Fraxinus natural regrowth in spots*
Iszimér 63H		X		2.5	9	*Q. cerris 60; F. excelsior 30; F. ornus 10*
Nagyvázsony 92B	X			19.0	20	*Q. cerris 80; F. excelsior 5; F. ornus 10; Carpinus 5*
Ajka 8G	X			0.6	19	*F. excelsior 100*
Lókút 11A	X			2.6	11	*Q. petrea 40; Q robur 15; F. excelsior 35; Fagus 10*
Eplény 47B	X			17.7	16	*Q. cerris 40; Q. petrea 10; Fagus 15; Carpinus 30; F. excelsior 5*
Porva 16C	X			2.5	30	*F. excelsior 50; Fagus 10; Carpinus 40*
Zirc 41A	X			10.3	36	*F. excelsior 35; Fagus 15; Q. cerris 15; Carpinus 35*
Zirc 41C	X			8.0	44	*F. excelsior 35; Fagus 5; Q. cerris 20; Carpinus 20; Q. petrea 10; Acer 10*

Measure of shoot dying - **1**: *Chalara fraxinea* infection under 5% on all of *Fraxinus excelsior* trees, **2**: *Chalara fraxinea* infection 5-20% on all of *Fraxinus excelsior* trees, and **3**: *Chalara fraxinea* higher than 20% on all of *Fraxinus excelsior* trees.

CONCLUSIONS

To summarize our researches so far, it seems that in Hungary the European and Narrow-leafed ash forests are seriously endangered by *C. fraxinea*, especially the young stands. The results of the extended life-cycle examinations of this pathogen are indicating that we are defenceless against the pathogens infestation; we cannot effectively control the pathogen or decrease the severity of infestations. In future presumably natural selection will

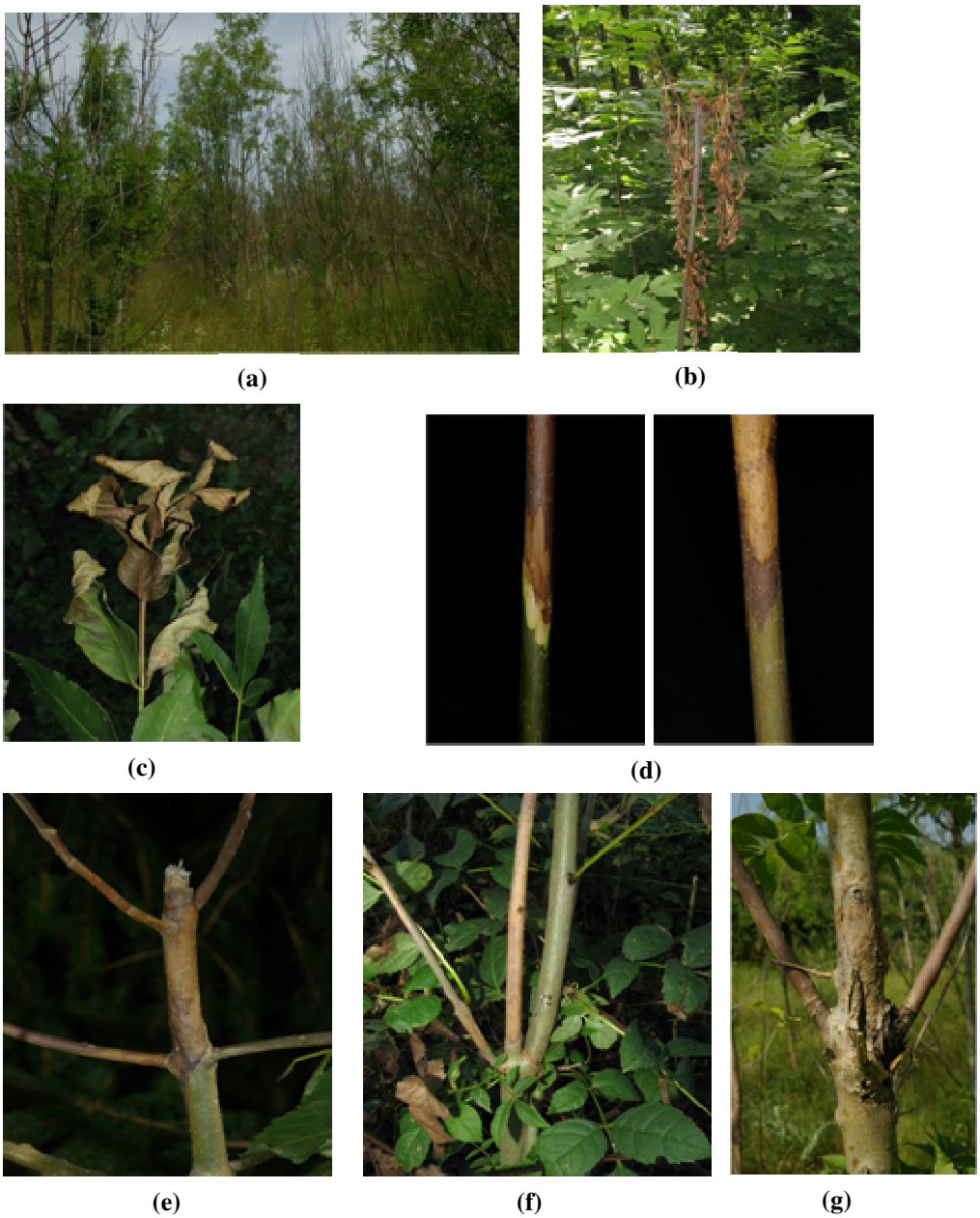

Figure 2. Symptoms of *C. fraxinea* infection (a) Heavy *C. fraxinea* infection in a young ash stand, (b) New infection on top of a young tree in spring time, (c) New infection on the leaves, (d) The infected tissue dies, (e) Infected tips of the branches are decaying, (f) Usually a new shot grows next to the infected branch and (g) Old *C. fraxinea* necrosis on the bark.

work among ashes which will seriously affect us with mass mortality of trees. In the same time it is our task to assist these processes with the selection of more resistant tree individuals, and with the mas propagation of these samples using them in forestry practice.

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Extended Abstract

Antagonistic effect and reduction of *Ulmus minor* symptoms to *Ophiostoma novo-ulmi* by elm endophytes

Juan Antonio Martín[1]*, Johanna Witzell, J.[2], Kathrin Blumenstein[2] and Luis Gil[1]

[1]Universidad Politécnica de Madrid, Departamento de Silvopascicultura. Ciudad Universitaria s/n, 28040 Madrid, Spain.
[2]Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, P.O. Box 49, SE-230 53 Alnarp, Sweden.

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The interactions between 31 elm endophytic fungi and *Ophiostoma novo-ulmi* were *in vitro* tested, and 18 endophytes inhibited the pathogen growth. Four selected endophytes were also *in vivo* tested against *O. novo-ulmi*, and three of them significantly reduced the tree wilting symptoms caused by the pathogen.

Key words: Dutch elm disease, endophytes, biocontrol, *Ophiostoma novo-ulmi.*

INTRODUCTION

Ophiostoma novo-ulmi is the causal agent of Dutch elm disease (DED), which has developed into one of the most devastating forest diseases (Martín et al., 2010). Although often ignored, endophytic symbionts may play an important role in plant fitness, protecting the plant against biotic and abiotic stressors (Arnold et al., 2003). The objectives of the present study were: (i) to evaluate the ability of elm endophytes to suppress *O. novo-ulmi* using *in vitro* tests; and (ii) to evaluate *in vivo* the potential use of elm endophytes as biocontrol agents of DED.

In vitro experiment

We have isolated endophytic fungi from bark and xylem tissues of healthy *Ulmus minor* trees from Spain. Several elm populations in Majorca Island and a clone collection in Madrid, including elm genotypes with high resistance level to DED were sampled. The interactions between 31 endophytic fungi and two *O. novo-ulmi* strains were *in vitro* tested by means of dual plate assays in malt extract agar (MEA). Three types of interactions were evaluated: i) antibiosis: growth-inhibition determined by the presence of an inhibition zone; ii) competition for substrate: overgrowth of one organism by another; and iii) myco-parasitism: direct parasitism on the hyphae of the pathogen. In each case, we determined which fungus "won" or "lost" the interaction and by which type of activity. Endophytes won the interaction against *O. novo-ulmi* at 58% of the dual tests, mostly by competition for substrate. Four endophytic strains were selected for the *in vivo* experiment on the basis of their strong antagonistic effect against *O. novo-ulmi*, the level of resistance to DED of their hosts, and the tree organ from where they were isolated (isolates labelled C31-1, X23-1, C9-2 and X32-1).

In vivo experiment

The selected four endophyte isolates were inoculated in 4- to 8-year-old *U. minor* plants 10 days before challenge with *O. novo-ulmi*. Four independent experiments, one per each endophyte strain, were done. Isolates C31-1, X23-1 and C9-2 were inoculated in field-grown trees located in experimental plots. Isolate X32-1 was inoculated in potted trees under controlled conditions. Four treatments were applied in each experiment (N = 12): i) endophyte inoculation, ii) endophyte inoculation

*Corresponding author. E-mail: juan.martin.garcia@upm.es.

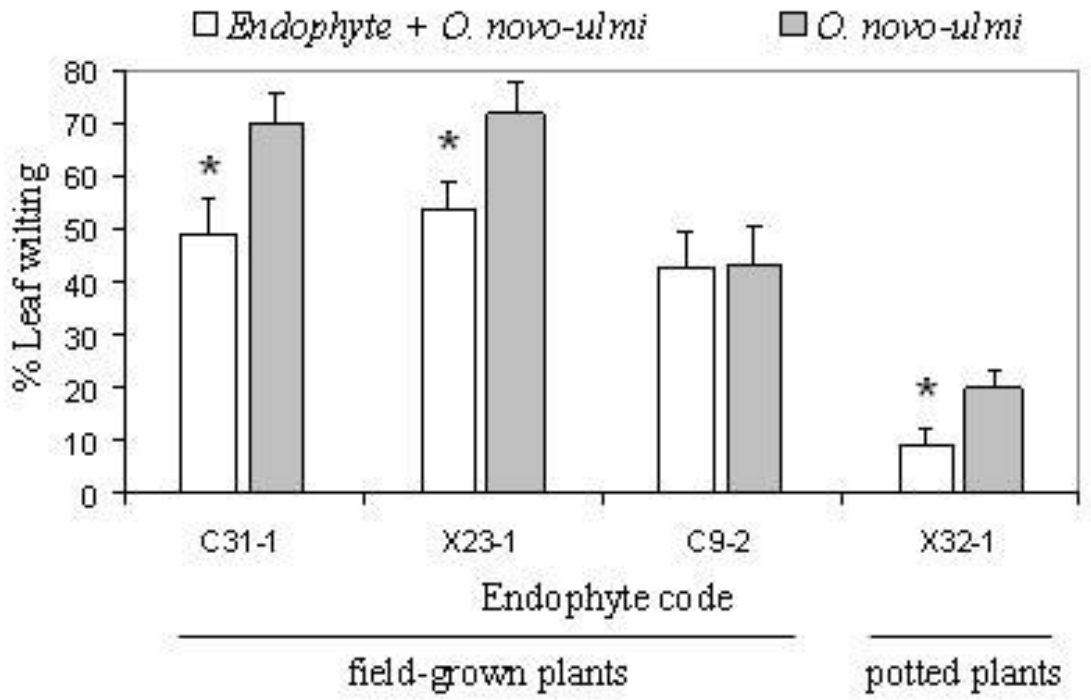

Figure 1. Wilting symptoms (%) shown by elm trees inoculated with different endophyte isolates before challenge with *O. novo-ulmi* (white bars) and with *O. novo-ulmi* alone (grey bars). For each endophyte isolate (C31-1, X23-1, C9-2 and X32-1), asterisks indicate significant differences between endophyte-treated and non-treated trees (LSD, $P < 0.05$).

plus *O. novo-ulmi* inoculation, iii) *O. novo-ulmi* inoculation, and iv) control treatment.

For inoculation of endophytes, a bark piece (0.5 × 1 cm) was removed from the base of the trunk of each tree. Then, a small piece of active mycelium growing in MEA (or sterile MEA as control) was inserted into the wounds on the surface of the sapwood. The wounds were sealed with parafilm and plastic sheets to protect the inoculations from desiccation and contamination.

O. novo-ulmi was inoculated in the opposite side and 3 cm below the endophyte inoculation point. Fungal spores were grown in Tchernoff's liquid medium (Tchernoff, 1965). A spore suspension in sterilized distilled water (0.1 mL, 10^6 conidia/mL) was introduced into the sap stream through a knife wound. Control trees received water. Wilting symptoms were evaluated 40 days after inoculation with the pathogen.

Three of the four endophyte isolates inoculated before challenge with the pathogen significantly reduced DED symptoms as compared to trees inoculated only with the pathogen (Figure 1). Trees inoculated only with the endophytes and control trees showed no wilting symptoms.

The low wilting symptoms shown by potted plants in comparison with field-grown plants (Figure 1) was probably due to the limited root and xylem development of the potted plants. Although more evidence is needed to fully evaluate the potential of endophytes in biocontrol of DED, these results open new prospects to elucidate the functional roles of endophytes in forest ecosystems and to develop future strategies for sustainable disease management.

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Authors are very grateful to the personnel working in Puerta de Hierro Forest Breeding Centre (Madrid) for their technical assistance. This work was supported by the research projects AGL2009-09289 and FORMAS 2008-1090 and by an agreement established between DGMN (Ministerio de Medio Ambiente y Medio Rural y Marino) and ETSI de Montes (UPM) in Madrid.

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Extended Abstract

REsource INFrastructure for monitoring and adapting European Atlantic FORests under Changing climatE (REINFFORCE): Establishing a network of arboretums and demonstration sites to assess damages caused by biotic and abiotic factors

Cristina Prieto-Recio*, Felipe Bravo and Julio J. Diez

Sustainable Forest Management Research Institute, University of Valladolid- INIA, Avda. Madrid 44, 34004, Palencia, Spain.

*Corresponding author. E-mail: cristina.prieto@pvs.uva.es.

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The reality of global warming is recognized worldwide, and most of the climatic models in the optimistic scenarios of IPCC forecast a 4°C temperature rise over the next 50 years. Nevertheless, the regional consequences are still fuzzy, especially in the case of ocean areas because there are many unknown factors like the climatic, economic and environmental conditions at regional level. However, some specific threats are appearing such as, disturbances in the life cycle of tree species, the introduction of new pathogens, or the mis-adaptation of tree species to new climatic conditions. So the potential climate change impacts on tree diseases could affect many aspects like population and community structure, micro-evolutionary processes and plants dynamics (Chakraborty et al., 2000). European Union Project (INTERREG IVB) REINFFORCE offers the opportunity to install a network of arboretums and demonstration sites unique in the world, located between latitudes 37° and 58° for monitoring the adaptation of European Atlantic forests to climate change through the study of the tree growth, its phenology and the forest health (Figure 1). The participants belong to eleven institutions from United Kingdom, France, Spain and Portugal. This is a key issue for sustainability of Atlantic forest resources, as the trees that are now being planted, will be harvested in 50 years facing new climatic conditions.

The goals of the REINFFORCE project were: i) to establish protocols for the installation of infrastructures and data collection; ii) to perform the technical and administrative evaluation of the work; iii) to create a network of 37 arboretums to anticipate the effects of climate change; iv) to implement a network of 32 demonstration sites to compare usual silviculture with other adaptative measures; and v) to develop databases to share online.

To achieve these objectives, The University of Valladolid is responsible to create, manage and explore; two arboretums and two demonstration sites located in Cantabria and three arboretums and two demonstration sites located in Castilla y León (Figure 2). Thirty one tree species with 3 to 9 provenances of each species are going to be tested in these arboretums (Table 1). One block of 12 seedlings per provenance will be planted in homogeneous plots. Each arboretum will be divided into conifers (species of the genus *Pinus* and other conifers) and broadleaves (*Quercus* species and other hardwoods). In each section, the seedlings will be distributed randomly in framework 3 m × 3 m, according to the parameters of growth and tolerance.

One important point of the study is the susceptibility to biotic and abiotic hazards. This issue depends on individual tree attributes which in turn depends on stand composition, site preparation and silvicultural treatments (Jactel et al., 2009). Hence, these arboretums will be planted following a homogeneous site preparation and concrete distribution of the species. Also, the common protocols will be developed to measure growth parameters, to study plant phenology, and to assess biotic and abiotic damages at different levels. Particularly, the health common protocol will be developed at tree level and at leaf or shoot level (Table 2).

On the other hand, the demonstration sites are mixed plantations based on Nelder Wheels (Nelder, 1962) following the type “a” scheme (Figure 3) designed to evaluate the influence of the density on the mortality, the size, the pathogenic damages and the biomass allocation of each plant. The data analysis will be performed using logistic regression

and spatial analysis. In each demonstration site two tree species or clones associated with the different diseases will be tested (Table 3) varying the degree of composition of both species and using systematic designs in concentric circles with different grid of plant positions.

Nowadays, these infrastructures and protocols are in process of creation and development. In the near future these arboretums and demonstration sites will offer a large data collection for monitor and adapt the European Atlantic forests to climate change.

Key words: Climate change, arboretums, demonstration sites, pathogens, silviculture.

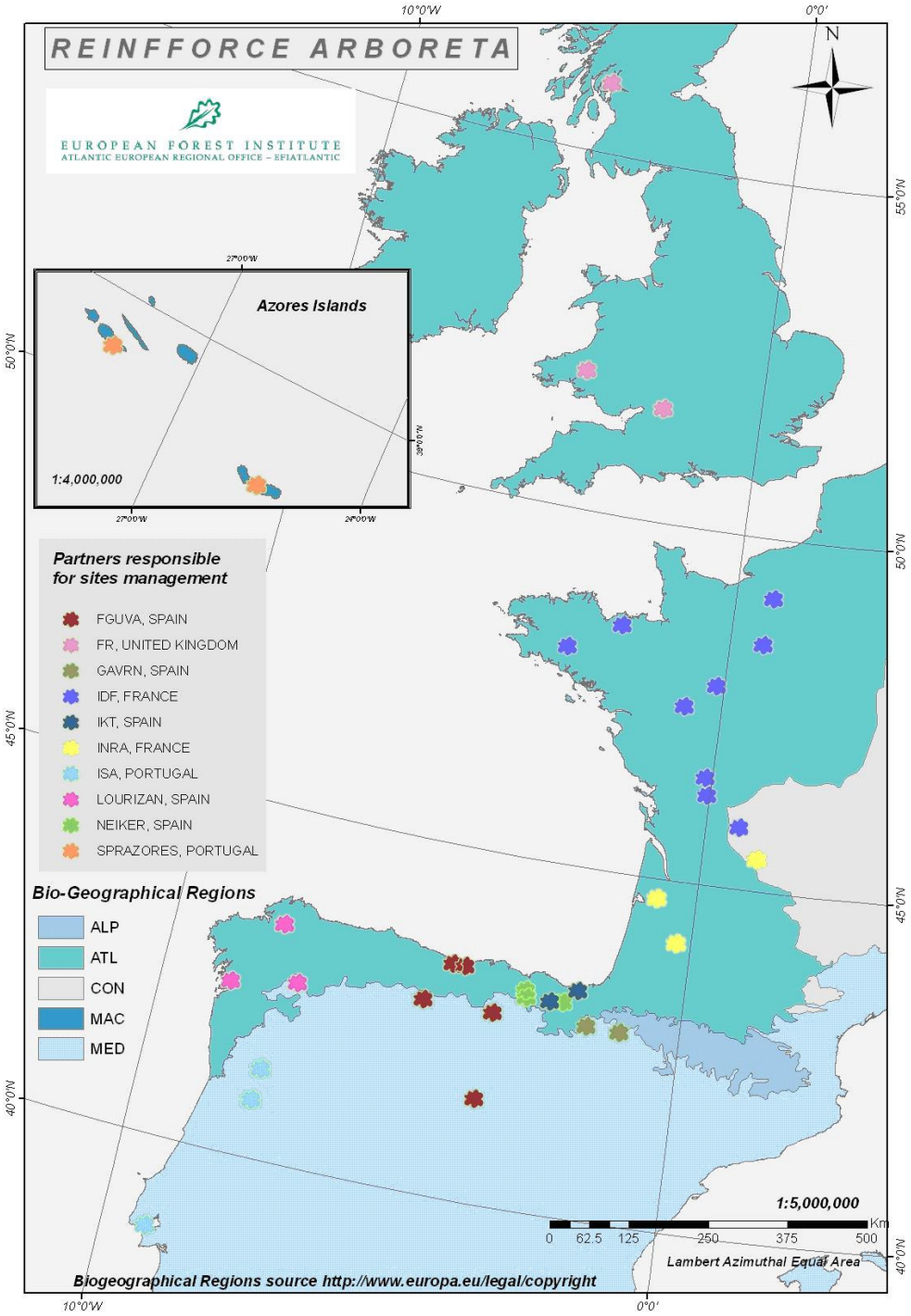

Figure 1. Distribution Map of the Arboretums (Biogeographical Regions of Europe).

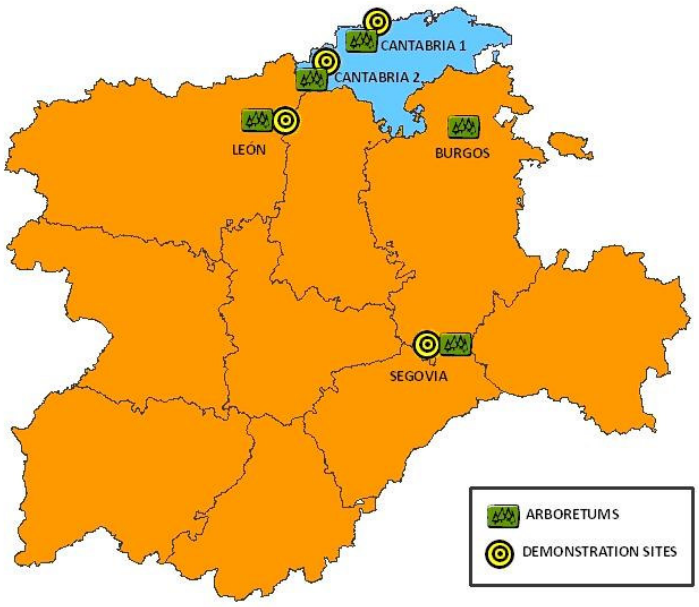

Figure 2. Distribution Map of the Arboretums and Demonstration Sites in Cantabria and Castilla y León.

Table 1. List of common species of the Arboretums.

S/N	Specie of Arboretums
1	*Acer pseudoplatanus*
2	*Betula pendula*
3	*Calocedrus decurrens*
4	*Castanea sativa*
5	*Cedrus atlantica*
6	*Cedrus libani*
7	*Ceratonia siliqua*
8	*Cunninghamia lanceolata*
9	*Cupressus sempervirens*
10	*Eucalyptus sp.*
11	*Fagus orientalis*
12	*Larix decidua*
13	*Liquidambar styraciflua*
14	*Pinus brutia*
15	*Pinus elliottii*
16	*Pinus nigra*
17	*Pinus peuce*
18	*Pinus pinaster*
19	*Pinus pinea*
20	*Pinus ponderosa*
21	*Pinus sylvestris*
22	*Pinus taeda*
23	*Pseudotsuga menziesii*
24	*Quercus rotundifolia*
25	*Quercus petraea*
26	*Quercus robur*
27	*Quercus rubra and Quercus phellos*
28	*Quercus suber*
29	*Robinia pseudoacacia*
30	*Sequoia sempervirens*
31	*Thuja plicata*

Table 2. Forest health protocol.

Biotic and abiotic damage assessment		
Tree level	Tree branch mortality	Record tree mortality and send dead trees to lab for diagnosis
	Crown conditions	Defoliation 0 (no) / 1-10% (low) / 11-50% (moderate) / > 50% (high) Discoloration (yellow, red, brown) 0 / 1-10% / 11-50% / > 50%, 100%
Leaf / shoot level	Damage types	Forest hervivore (Chewers / Gall makers / Leaf-miners / Skeletonisers / Leaf-rollers and tiers / Sap feeders / Shoot deformation / Stem / bark borers / Mammal grazer) Forest disese (Rust / Mildew / Leaf Necrosis / Red Bands / Canker / Stem - shoot Necrosis) Abiotic (Drought / Frost / Wind)

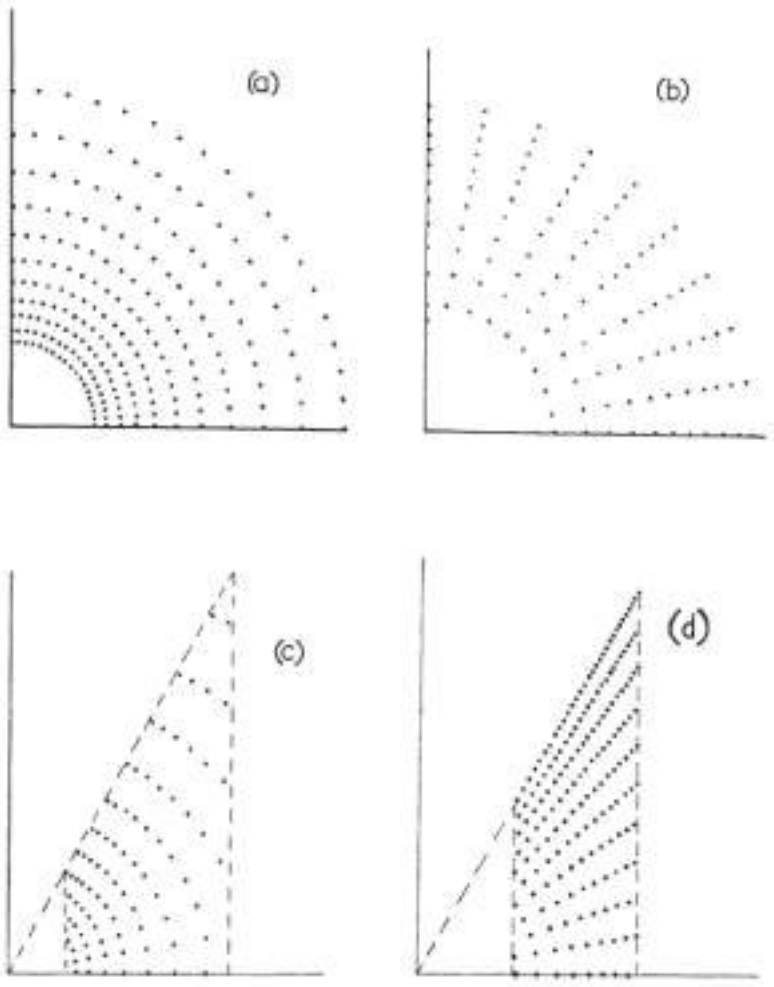

Figure 3. The Nelder Systematic Designs (Nelder, 1962).

Table 3. List of species of the demonstration sites.

Site	**Specie 1**	**Specie 2**	**Disease**
CANTABRIA 1	*Eucalyptus nitens I*	*E. nitens II (Resistant code to Mycosphaerella* sp.*)*	Area affected by *Mycosphaerella* sp.
CANTABRIA 2	*Pinus radiata*	*Pinus sylvestris*	Area affected by *Fusarium circinatum*
LEÓN	*Pinus sylvestris*	*Quercus pyrenaica*	Study of possible pathogens asociated
SEGOVIA	*Pinus pinaster*	*Quercus ilex*	Area affected by *Pinus pinaster* decline

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Extended Abstract

Foliage diseases on true fir (*Abies* spp.) in Norway

Venche Talgø* and Arne Stensvand

Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Plant Health and Plant Protection Division, 1432 Ås, Norway.

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Fir plantations in Norway are mainly for Christmas trees, a production situated to a large extent in western Norway, where the mild, humid climate is also ideal for fungal diseases. Thus, a number of airborne fungi causing foliage diseases on true fir (*Abies* spp.) have been found in nursery, Christmas tree, bough, and/or landscape plantings in Norway, with the most serious ones being *Botryotinia fuckeliana*, *Delphinella abietis*, *Herpotrichia parasitica*, *Melampsora abieti-capraearum*, *Phaeocryptopus nudus*, *Pucciniastrum epilobii*, *Rhizosphaera kalkhoffii* and *Sydowia polyspora* (Talgø, 2009).

Key words: *Botryotinia fuckeliana, Delphinella abietis, Herpotrichia parasitica, Melampsora abieti-capraearum, Phaeocryptopus nudus, Pucciniastrum epilobii, Rhizosphaera kalkhoffii, Sydowia polyspora.*

Botryotinia fuckeliana (grey mould, imperfect stage is *Botrytis cinerea*)

This is mainly a problem in nurseries, but damaged shoots have also been observed in Christmas tree fields with wet conditions during shoot elongation. We have found damage by *B. fuckeliana* on subalpine fir (*A. lasiocarpa*) (Figure 1), nordmann fir (*A. nordmanniana*), Korean fir (*A. koreana*), noble fir (*A. procera*), and white fir (*A. concolor*).

Delphinella abietis

This destroys current year needles, and in severe cases entire shoots. The needles curl downwards along the edges and are usually covered by numerous, black pseudothecia. We have found the disease on subalpine fir (Figure 2), Turkish fir (*A. bornmuelleriana*), Siberian fir (*A. sibirica*), nordmann fir, and noble fir in western Norway.

Herpotrichia parasitica (herpotrichia needle browning)

This kills both old and young needles. The stomatal areas of the needles get covered by brown hypha. The needles turn greyish and hang straight down from the twigs (Figure 3), only attached by mycelium. We have seen severe damage in south western Norway on silver fir (*A. alba*) in a forest stand, and on Turkish fir and nordmann fir in Christmas tree fields.

Melampsora abieti-capraearum

This is a rust fungus on true fir needles. Goat willow (*Salix caprea*) is the alternating host. We found the fungus on nordmann fir (Figure 4) in a Christmas tree field in south western Norway.

Phaeocryptopus nudus (interior needle blight)

This has been found on *A. lasiocarpa* (corkbark fir and subalpine fir) in southern Norway. It is problematic in the subalpine fir Christmas tree production. The symptoms appear approximately one year after infection, and thus the current year shoots appear healthy, while the older needles turn brown. In severe cases shoots die (Figure 5).

Pucciniastrum epilobii

This is a rust fungus that we so far have found on nordmann fir (Figure 6) and subalpine fir Christmas trees, and

*Corresponding author. E-mail: venche.talgo@bioforsk.no.

Figure 1. *Botrytis cinerea,* the anamorph stage of *Botryotinia fuckeliana,* on subalpine fir (*Abies lasiocarpa*). Photo: Venche Talgø.

Figure 2. *Delphinella abietis* on a current year shoot of subalpine fir (*Abies lasiocarpa*), dead needles are covered with black pseudothecia. Photo: Venche Talgø

Figure 3. *Herpotrichia parasitica* on Turkish fir (*Abies bornmuelleriana*) in a Christmas tree plantation. Photo: Venche Talgø.

Figure 4. Chlorotic foliage on nordmann fir (*Abies nordmanniana*) caused by *Melampsora abieti-caprearum*. The underside of the needles expose whitish peridia (wall) around the aeciospores. Photo: Venche Talgø.

Figure 5. Interior needle blight caused by *Phaeocryptopus nudus* on subalpine fir (*Abies lasiocarpa*). Photo: Venche Talgø.

on noble fir in bough plantations. Damage has been observed in years with high precipitation during shoot elongation. Willow herbs (*Epilobium* spp.) are alternating hosts.

Figure 6. Nordmann fir (*Abies nordmanniana*) with aecidia and yellow aecidiospore mass from *Pucciniastrum epilobii.* The peridia are longer than those formed by *Melampsora abieti-caprearum* (Figure 4). Photo: Venche Talgø.

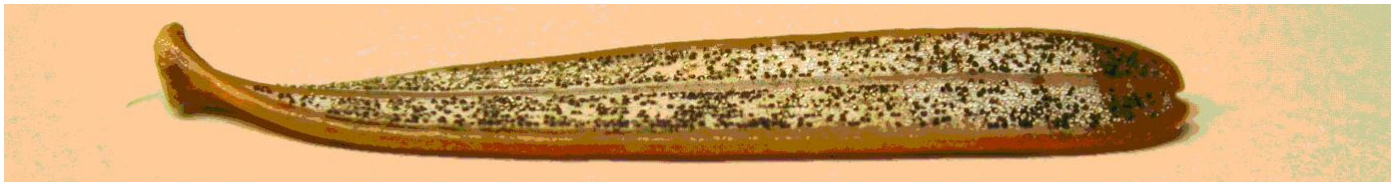

Figure 7. *Rhizosphaera kalkhoffii* on Korean fir (*Abies koreana*). The black spots in the stomatal area are pycnidia. Photo: Venche Talgø.

Rhizosphaera kalkhoffii

This causes needle cast on true fir. We have found severe damage on nordmann fir, subalpine fir, and Korean fir in Christmas tree fields. Small, black, globose pycnidia cover the stomatal bands (Figure 7).

Sydowia polyspora

This is involved in two serious diseases on fir Christmas trees in Norway and elsewhere; "*Sclerophoma* shoot dieback" (conidial stage often referred to as *Sclerophoma pithyophila*) and "current season needle necrosis" (CSNN) (conidial stage referred to as *Hormonema dematioides*). We have proven the two conidial stages to be identical. The former may kill the entire shoot (Figure 8), while CSNN gives necrotic spots and bands on new needles, often followed by severe needle cast. We have isolated the fungus from noble fir, nordmann fir, grand fir (*A. grandis*), and subalpine fir.

Figure 8. *Sclerophoma* shoot dieback on nordmann fir (*A. nordmanniana*). The black spots are the pycnidia. Photo: Venche Talgø.

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Extended Abstract

Neonectria-canker on trees in Norway

Venche Talgø*, May Bente Brurberg and Arne Stensvand

Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Plant Health and Plant Protection Division, 1432 Ås, Norway.

*Corresponding author. E-mail: venche.talgo@bioforsk.no.

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Neonectria spp. have been found on a number of tree species in Norway. *Neonectria ditissima* is commonly detected from diseased trees in apple orchards and a *N. ditissima*–like species has caused an epidemic on white fir (*Abies concolor*).

In Norway, *Neonectria* spp. attack numerous plant species including true fir (*Abies* spp.), sycamore maple (*Acer pseudoplatanus*), alder (*Alnus glutinosa*) (Figure 1), dogwood (*Cornus* sp.), ash (*Fraxinus excelsior*), holly (*Ilex aquifolium*) (Figure 2), apple (*Malus* × *domestica*), spruce (*Picea abies*), poplar (*Populus* sp.), bird cherry (*Prunus padus*), pear (*Pyrus* sp.), and rowan (*Sorbus aucuparia*). Attacks by *Neonectria* spp. may result in canker wounds and dieback. Red fruiting bodies (perithecia) containing asci and ascospores are often present. Usually they form clusters that are clearly visible to the naked eye. In culture, *Neonectria* spp. produce microconidia (*Cephalosporium* spp.) and macroconidia (*Cylindrocarpon cylindrioides*).

Neonectria-canker is a serious problem in Norwegian apple orchards (Figure 3), and in 2010 we investigated if host plants other than apple trees were potential inoculum sources. We sequenced the internal transcribed spacer (ITS)-regions from *Neonectria* cultures isolated from apple trees and two other host plants in the rose family (*Rosaceae*); rowan (*S. aucuparia*) (Figure 4) and bird cherry (*P. padus*) (Figure 5). Cultures from all 3 hosts produced floccose, white mycelium. The cultures had identical sequences and they were identical to the sequences of *N. ditissima* (syn. *Neonectria galligena*) deposited in GenBank. Inoculation tests have not yet taken place, thus, we have no indications that cross infections take place in nature. Further research is needed to find out if infected rowan or bird cherry in the vicinity of apple orchards may increase the disease pressure.

Interestingly, cultures isolated from dying conifers in Norway in 2008 differed from *N. ditissima* by five out of the 550 base pairs included in the ITS-sequence. Previously, *Neonectria fuckeliana* has been reported on spruce species in Norway, and on spruce and fir species in other countries, but *N. fuckeliana* differs by more than 20 base pairs from the *N. ditissima*-like isolates we obtained from conifers in 2008. The *N. ditissima*-like fungus might be a new species related to *N. ditissima*, possibly imported to Norway. *N. ditissima* has to our knowledge never been described as a pathogen on conifers, but the *N. ditissima*-like fungus we isolated from conifers in 2008 was clearly pathogenic. We first discovered a serious disease outbreak on white fir (*Abies concolor*) in southern Norway (Figure 6), and the *N. ditissima*-like fungus was isolated from dying trees in two counties in south western and four counties in south eastern Norway. Both old and young trees were dead or dying. The *N. ditissima*-like fungus was later isolated from Siberian fir (*A. sibirica*), subalpine fir (*A. lasiocarpa*), and Norway spruce (*Picea abies*) in south eastern Norway. Perithecia in canker wounds from the conifer samples were dark around the ostiole. This morphological characteristic is known from *N. ditissima,* but not from *N. fuckeliana*. Sequencing showed that all the *Neonectria*-isolates from different conifer hosts in 2008 were identical in their ITS-region. The cultures were white. Cultures from *N. fuckeliana* are brownish. *N. fuckeliana* is common on Norway spruce in our country (Roll-Hansen and Roll-Hansen, 1995), and commonly associated with dieback on white fir in Europe and western North America (Callan, 1997). In Canada, *N. fuckeliana* caused dieback on subalpine fir (*A. lasiocarpa*) (Funk, 1981).

Inoculation tests with *N. ditissima*-like isolates were carried out in 2009 on subalpine fir, white fir, and Norway spruce. Map pins (16 mm SHF top grip map pins, Pålsboda, Sweden) were used to inoculate and easily trace the inoculation points. The pins were autoclaved and placed on potato dextrose agar (PDA) together with an agar plug (0.5 mm in diameter) from the *Neonectria*–culture to be tested. After approximately one week at room temperature the pins were covered with mycelium, and the needle tips were inserted into the bark or dormant buds (3 pins or more were inserted

per plant – depending on the size and shape of the plant). As a control, unwounded trees or trees wounded by autoclaved map pins from sterile PDA were used. The fungus was pathogenic on all three fir species tested. Figure 7 shows symptoms on inoculated subalpine fir. Control trees showed no symptoms.

Key words: *Neonectria fuckeliana, Neonectria ditissima, Abies, Picea, Malus, Sorbus, Prunus*.

Figure 1. *Neonectria* sp. on alder (*Alnus glutinosa*) (left); discoloration below infected bark (middle), and red perithecia (right). Photos: Venche Talgø.

Figure 2. In natural stands of holly (*Ilex aquifolium*) on the west coast of Norway (left) damage by *Neonectria* sp. has occasionally been found (middle and right). Photos: Venche Talgø.

Figure 3. *Neonectria ditissima* on apple (*M* × *domestica*); red fruiting bodies (perithecia) on apple tree branches (left and middle) and dead tissue in a canker wound (right). Photos: Venche Talgø.

Figure 4. *Neonectria ditissima* on rowan (*Sorbus aucuparia*); malformed branches and twigs (left and middle) and red perithecia in a canker wound (right). Photos: Venche Talgø.

Figure 5. *Neonectria ditissima* on bird cherry (*Prunus padus*); swellings and canker wounds (left), red perithecia (middle), and a whitish culture with light brown patches (right). Photos: Venche Talgø.

Figure 6. White fir (*Abies concolor*) attacked by a *Neonectria ditissima*-like fungus; dead and dying trees (left), slightly sunken canker wound (middle), and resin flow (right). Photos: Venche Talgø.

Figure 7. Subalpine fir (*Abies lasiocarpa*) inoculated with a *Neonectria ditissima*-like fungus isolated from subalpine fir; dieback symptoms on inoculated plants compared to control plants (left), resin flow and sunken, discoloured bark (middle), and dead shoot (right). Photos: Venche Talgø.

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Extended Abstract

Fungal and bacterial diseases on horse chestnut in Norway

Venche Talgø*, Juliana Irina Spies Perminow, Arild Sletten, May Bente Brurberg, Maria Luz Herrero, Gunn Mari Strømeng and Arne Stensvand

Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Plant Health and Plant Protection Division, 1432 Ås, Norway.

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Horse chestnut (*Aesculus hippocastanum*) is mainly grown in gardens, public parks and alleys in Norway. It has been known as a tree with no disease problems, but since 2006, a number of new diseases have been detected (leaf blotch, powdery mildew, anthracnose, and bleeding canker). These new diseases have most likely entered the country by imported horse chestnut transplants. The diseases mentioned here may reduce the aesthetical value of horse chestnut to such an extent that we no longer recommend it for planting in Norway. Fortunately, the insect horse chestnut leaf miner (*Cameraria ohridella*) has not yet been found in Norway.

Key words: *Aesculus hippocastanum, Guignardia aesculi, Erysiphe flexuosa, Colletotrichum* spp.*, Pseudomonas syringae* pv. *Aesculi*.

Leaf blotch (*Guignardia aesculi*)

This was found in south eastern Norway in August 2006. Diseased leaves had brown, irregular blotches with a yellow halo (Figure 1), and heavily infected leaves were wrinkled. In the beginning of the last century, *G. aesculi* was the most important disease on horse chestnut in North America, and later it also became widespread in Europe (Pawsey, 1962). Probably the North Sea, which acts as a barrier for natural spread of diseases into most areas of southern Norway, stopped the pathogen from entering the country.

Powdery mildew (*Erysiphe flexuosa* syn. *Uncinula flexuosa*)

This was discovered in August 2006, while searching for trees with leaf blotch symptoms. Infected leaves had a grey appearance, and chasmothecia were present (Figure 2), but the disease was not widespread at the time. By 2010, horse chestnut in several locations in south eastern Norway had severe powdery mildew attacks. The pathogen originates from North America and has become widespread in Europe during the last decade (Kiss et al., 2004).

Anthracnose (*Colletotrichum acutatum* and *C. gloeosporioides*)

This was found in 2006 on horse chestnut leaves. After incubation (room temperature, 100% RH) sporulation became visible along the midrib and veins. *C. acutatum* was most commonly found, but in one case *C. gloeosporioides* was detected on horse chestnut leaves from western Norway (Figure 3, left). Internal transcribed spacer (ITS) sequencing was used to distinguish the two *Colletotrichum* species. Cultures from *C. acutatum* had a pinkish appearance (Figure 3, right), while *C. gloeosporioides* was greyish. *Colletotrichum* spp. are commonly causing anthracnose on woody plants worldwide included on horse chestnut (Sinclair and Lyon, 2005).

Bleeding canker (*Pseudomonas syringae* pv. *aesculi*)

This is the latest and most serious of the recently discovered pathogens on horse chestnut in Norway. It

*Corresponding author. E-mail: venche.talgo@bioforsk.no.

Figure 1. Leaf blotch (*Guignardia aesculi*) symptoms on horse chestnut (*Aesculus hippocastanum*) in south eastern Norway. Photo: Venche Talgø.

Figure 2. Powdery mildew (*Erysiphe flexuosa*) makes the leaves appear greyish on horse chestnut (*Aesculus hippocastanum*) (left). Chasmothecia (right) are often present on infected leaves; asci with ascospores emerging from the chasmothecia. Photo: Venche Talgø.

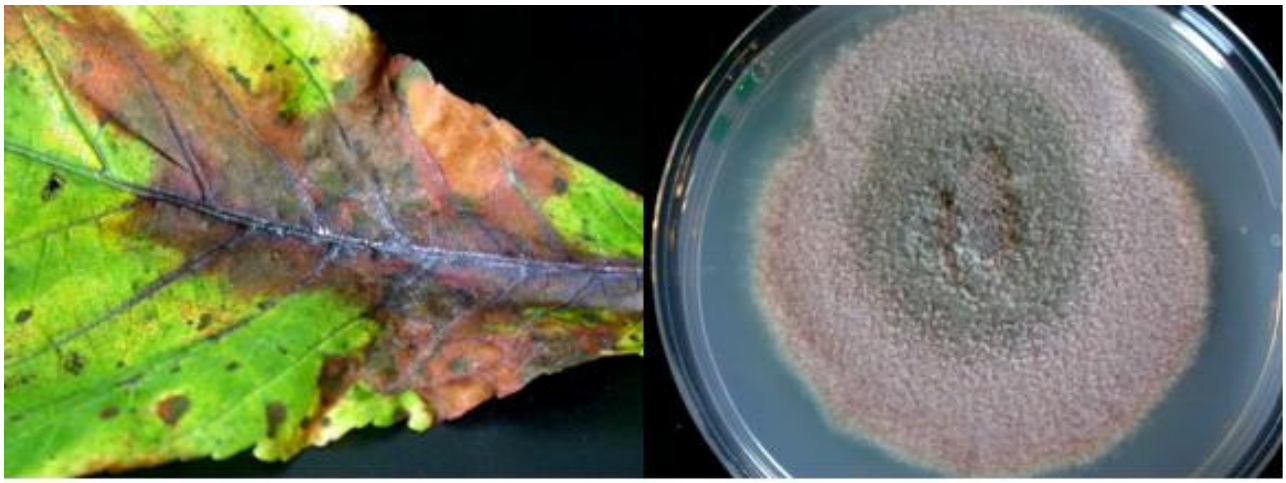

Figure 3. Sporulation of *Colletotrichum gloeosporioides* along the midrib and veins on a horse chestnut (*Aesculus hippocastanum*) leaf (left). Culture of *C. acutatum* isolated from horse chestnut (right). Photo: Venche Talgø.

Figure 4. Bleeding canker (*Pseudomonas syringae* pv. *aesculi*) on horse chestnut (*Aesculus hippocastanum*). From left to right; dieback symptoms in the crown, bacterial exudates, bleeding canker wounds, and discoloured tissue under the bark. Photo: Venche Talgø.

was detected in south western Norway in June 2010. Diseased trees had dieback symptoms in the crown, bacterial exudates on infected stems, bleeding canker wounds in the bark, and discoloured tissue below infected bark (Figure 4). In Europe, the bacterium was first detected in the Netherlands in 2002 (Dijkshoorn-Dekker, 2005), and has since been found in several European countries.

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Extended Abstract

Evaluating methyl jasmonate for induction of resistance against *Fusarium oxysporum*, *Fusarium circinatum* and *Ophiostoma novo-ulmi*

María Vivas[1], Juan Antonio Martín[2] and Alejandro Solla[1]*

[1]Ingeniería Forestal y del Medio Natural. Universidad de Extremadura. Avenida Virgen del Puerto 2, 10600 Plasencia, Spain.
[2]Departamento de Silvopascicultura, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

*Corresponding author. E-mail: asolla@unex.es.

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Worldwide, damping off is probably the most severe disease affecting seedlings in forests nurseries. In south-western Europe, the pitch canker and the Dutch elm disease cause relevant economic looses in forests, mostly in adult trees. Plants protect themselves against a diversity of attackers through constitutive and inducible defense strategies. Exogenous applications of Methyl Jasmonate (MeJA) have been successfully used to artificially induce chemical defense responses and resistance of forest trees against several insects and pathogens (Huber et al., 2005; Zeneli et al., 2006; Moreira et al., 2009). MeJA is usually mixed with the surfactant Tween 20 (0.1% v/v) and applied by spraying or by brushing the plant stem. Conscious that induced resistance occurs in pines and elms (Huber et al., 2005; Martín et al., 2010); we conducted three different experiments to evaluate if MeJA induces resistance in *Pinus pinaster* against *Fusarium oxysporum* and *Fusarium circinatum*, and in *Ulmus minor* against *Ophiostoma novo-ulmi*.

First, different concentrations of an aqueous solution of MeJA were applied to *P. pinaster* seeds and seedlings. Seeds were treated by (i) spray with 5 mM of MeJA solution, (ii) immersion 10-min in 5 mM of MeJA solution, or (iii) spray with water. During sowing, seeds were inoculated with *F. oxysporum* (5 mL; 10^5 and 10^7 spores mL^{-1}) on the ground. In non-inoculated seeds, MeJA treatments did not significantly affect seed germination but originated 44% of seedling mortality 1 month after treatments. In inoculated seeds, MeJA treatments caused a significantly higher mortality by *F. oxysporum* in relation to the control treatment (P=0.008). Seedlings were treated with MeJA solutions (0, 0.1, 0.5, 1, 5 and 10 mM) by spraying the stems. Time-periods between treatments and challenge inoculations (10^5 and 10^7 spores mL^{-1}) were 1 or 7 days. Seedlings treated at doses above 1 mM MeJA showed symptoms of toxicity. Seedlings treated at doses below 1 mM showed higher mortality rates than untreated seedlings. Final mortality of seedlings depended on the concentration of MeJA and the dose of *F. oxysporum* used, but the challenging treatments did not significantly protect the seedlings against the pathogen.

In the second experiment, 6-months-old *P. pinaster* seedlings were sprayed with 0 and 25 mM of MeJA. One month after treatments, half of the seedlings were inoculated by contact mycelium of *F. circinatum* with a wound onto the stem. The exogenous application of MeJA in non-inoculated seedlings significantly reduced above and belowground plant growth (P<0.05), increased the average number of resin ducts per transverse section (P<0.01) and marginally increased the relative conductive area of resin ducts (P=0.056) in relation to the control plants. Mortality of seedlings inoculated with *F. circinatum* was 58%, and mortality of seedlings treated with MeJA and subsequently inoculated with *F. circinatum* was 60%, thus the challenging treatment did not show any positive effect against the pathogen tested.

Finally, 4-year-old *U. minor* trees were sprayed with 0, 50 and 100 mM of MeJA. Half month after treatments, half of trees were inoculated with *O. novo-ulmi*. 100 mM of MeJA was slightly toxic to the trees, causing leave spots and some wilting. However, time to bud burst and tree growth was not altered by MeJA treatments. Dieback symptoms, evaluated 120 days and one year after inoculations, revealed that MeJA did not protect the plants against *O. novo-ulmi*. In fact, one year after inoculation the trees treated with MeJA showed higher dieback symptoms than the control trees (P=0.03).

MeJA did not protect *P. pinaster* seeds and seedlings against *F. oxysporum*, probably because plants were too young for the physiological mechanisms responsible for resistance to be induced. Based on the morphological changes observed in the treated 6-months-old *P. pinaster* seedlings (reduction of growth, increased resin duct density), there is evidence that MeJA could have activated the mechanisms of resistance. However, 25 mM MeJA did not reduce plant

mortality, probably because the spread of the virulent *F. circinatum* strain within the tree tissues was faster than the formation of effective defense responses. Based on the lack of phenological changes observed in the treated elms, there is no evidence that MeJA would cause induction of resistance. This is the first work reporting the effect of MeJA on *U. minor* and *P. pinaster* seeds, and the first approach to test MeJA against three pathogens previously not used for this purpose. Based on our results, the use of MeJA to prevent *F. oxysporum* and *F. circinatum* in *P. pinaster* seedlings in nurseries and *O. novo-ulmi* in *U. minor* trees should be discarded.

Key words: *Pinus pinaster, Ulmus minor*, Induced resistance.

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Extended Abstract

The ash dieback pathogen *Hymenoscyphus pseudoalbidus* is associated with leaf symptoms on ash species (*Fraxinus* spp.)

Katharina Kräutler* and Thomas Kirisits

Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.

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Ash dieback caused by the ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) is characterized by a wide range of symptoms. Leaf symptoms have previously been related to this emerging infectious disease. In fungal isolations from necrotic lesions on leaf petioles and rachises as well as leaflet veins of *Fraxinus excelsior*, *H. pseudoalbidus* was consistently obtained at high frequencies. When inoculated onto leaf rachises of *F. excelsior*, the ash dieback pathogen induced symptoms identical to those seen after natural infections (necrotic lesions, wilting and leaf dropping). The fungus also caused symptoms on leaves of *Fraxinus angustifolia* and *Fraxinus ornus* in artificial inoculations and was re-isolated at considerably high frequencies from all three *Fraxinus* spp. Kochs postulates were thus fulfilled to conclude that *H. pseudoalbidus* is associated with leaf symptoms on *F. excelsior*. On *F. angustifolia* leaf damage resulting from natural infections still remains to be detected. In contrast, *F. ornus* may not be a natural host of *H. pseudoalbidus*, although it proved to be somewhat susceptible in the leaf inoculation experiments.

Key words: *Chalara fraxinea*, *Fraxinus excelsior*, *Fraxinus angustifolia*, *Fraxinus ornus*, emerging forest disease.

INTRODUCTION

Ash dieback, an emerging infectious disease of common ash (*Fraxinus excelsior*) and other *Fraxinus* species in Europe, is caused by the ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*; Kowalski, 2006; Bakys et al., 2009; Kowalski and Holdenrieder, 2009; Kirisits et al., 2009, 2010a; Drenkhan and Hanso, 2010; Queloz et al., 2011). The disease is characterized by a wide range of symptoms (Bakys et al., 2009; Kirisits et al., 2009; Kirisits and Cech, 2010). Besides necrotic lesions in the bark, cambium and phloem as well wood discoloration leading to dieback of shoots, leaf symptoms have been related to ash dieback (Figure 1). These include necrotic lesions on leaf petioles and rachises (Figure 1A and B) as well as leaflet veins (Figure 1C), often followed by wilting (Figure 1A) and early leaf shedding (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Kirisits and Cech, 2010).

Besides damaging ash trees in their own right, leaf infections incited by the pathogen's ascospores have been suggested to be the primary path enabling *H. pseudoalbidus* to grow into shoots and twigs of its host trees (Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b; Timmermann et al., 2011). This view is based on the temporal sequence of appearance of symptoms and on the observation that small, localized necrotic lesions, representing early stages of disease, frequently occur around leaf scars (Figure 2; Kirisits et al., 2009, 2010b; Timmermann et al., 2011). So far it has, however, not been definitely established that *H. pseudoalbidus* is associated with leaf symptoms on *Fraxinus* spp. and the role of leaf infections in the disease cycle of ash dieback remains to be proven.

*Corresponding author. E-mail: katharina.kraeutler@boku.ac.at.

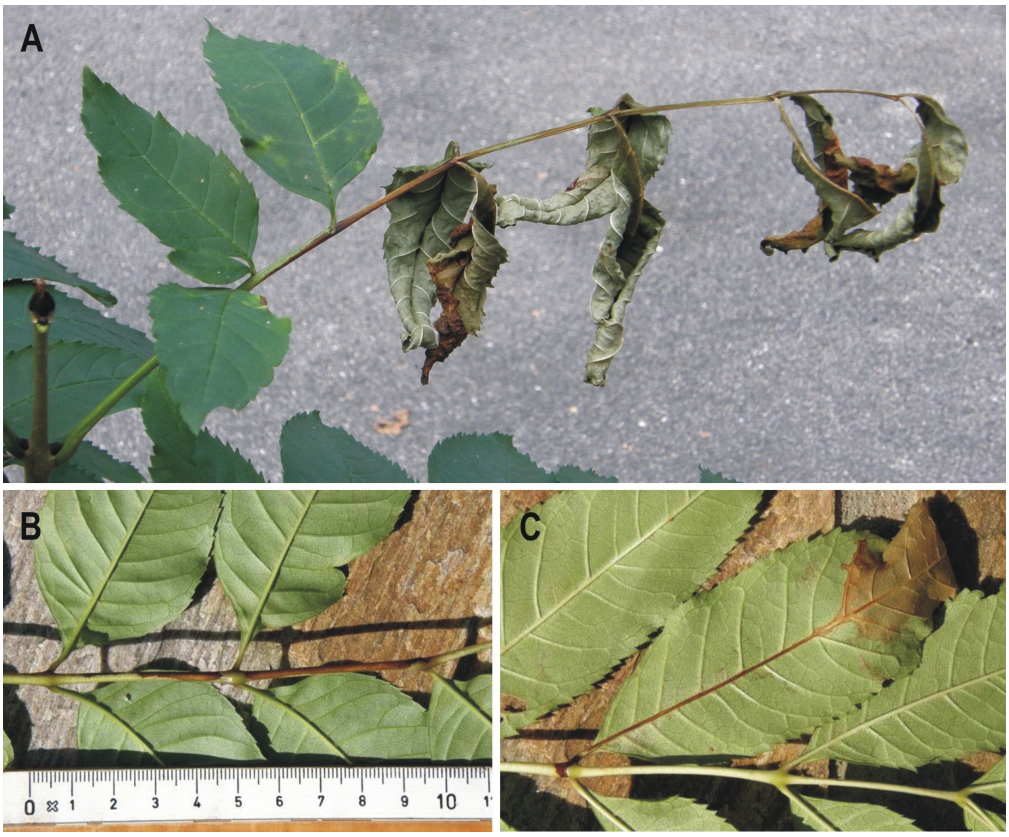

Figure 1. Leaf symptoms on *F. excelsior* commonly seen from late summer onwards in connection with ash dieback: (A) Necrotic lesion on a leaf rachis leading to wilting in the part of the leaf distal to the lesion, (B) Detailed view of a necrotic lesion on a leaf rachis, (C) Necrotic lesion on a leaflet vein extending to the leaf rachis.

FUNGAL ISOLATION FROM SYMPTOMATIC *F. EXCELSIOR* LEAVES

In early September 2008 and early October 2009 symptomatic leaves (126 in total for both years) were collected from young *F. excelsior* trees at one site in Vienna (Schafberg) and fungi were isolated from necrotic lesions on leaf petioles and rachises (Figure 1A and B; subsequently referred to as 'leaf rachises' only). After surface sterilization (1 min in 96% ethanol, 1 to 3 min in 4% NaOCl, 30 s in 96% ethanol), the epidermis was carefully peeled off and small fragments of necrotic tissue were cut and placed on malt extract agar (MEA; 20 g/L malt extract, 16 g/L agar, 100 mg/L streptomycin sulphate). Isolation plates were incubated at cool temperatures (between 4 to 10°C) in the dark. This was done in order to stimulate anamorph production of the ash dieback pathogen and to give it competitive advantage over other fungi, thereby increasing the likelihood to detect the fungus (Kirisits et al., 2009). *H. pseudoalbidus* was identified based on morphological characteristics of its *Chalara fraxinea* stage (colony morphology, phialophores and spores).

In both years *H. pseudoalbidus* was isolated at high frequencies from necrotic leaf rachises and it was the most frequently isolated fungus. The overall isolation frequency of the ash dieback pathogen was 89.7%. From 52.4% of the rachises the fungus was obtained in pure culture. Various other fungi were also isolated, especially in October 2009, but they were not determined. None of the other fungal species reached the high frequency of *H. pseudoalbidus*. In other isolation series from necrotic leaf rachises, using leaves collected in late summer and early autumn 2008 at five localities in Lower Austria and in August 2011 at one site in Upper Austria, the ash dieback pathogen was also recovered abundantly, with isolation frequencies ranging from 80 to 100% at the various sites. Moreover, at the site Vienna-Schafberg *H. pseudoalbidus* was obtained from 26 out of 35 (74.3%) necrotic leaflet veins (Figure 1C), from which isolation had been attempted.

The high isolation frequencies of the ash dieback pathogen from symptomatic leaves in the present study agree well with the investigations by Bakys et al. (2009). Using fungal isolation on various media and molecular detection methods directly from plant tissues, these authors detected *H. pseudoalbidus* commonly in ash leaf rachises and leaf blades. Ogris et al. (2009) also obtain-

Figure 2. Small, localized necrotic lesions on *F. excelsior* shoots, representing early stages of ash dieback (Vienna, Penzing, March 2010). The position of the lesions adjacent to leaf scars may indicate that shoot infections occurred via leaves.

ed the fungus from necrotic leaf petioles of *F. excelsior.* Moreover, Drenkhan and Hanso (2010) isolated *H. pseudoalbidus* from petioles of four *Fraxinus* species not native to Europe (*F. americana*, *F. mandshurica*, *F. nigra* and *F. pennsylvanica*).

LEAF INOCULATION EXPERIMENT

At the end of June 2010 leaf rachises of potted *F. excelsior*, *F. angustifolia* and *F. ornus* seedlings were wound-inoculated with five *H. pseudoalbidus* isolates. Inoculum consisted of autoclaved wood fragments (approximately 5 mm long and 2 mm in diameter) obtained from *F. excelsior* shoots that had been placed for 24 days on the various *H. pseudoalbidus* cultures on MEA. Sterile wood fragments of similar size were used as control treatment. Inoculation was done by initiating an approximately 1 cm long superficial wound on the upper surface of a leaf rachis, placing inoculum on the wound and fixing it with parafilm. For *F. excelsior* and *F. angustifolia* 20 plants per treatment were inoculated, while for *F. ornus* 10 seedlings per treatment were used. After inoculation the seedlings were regularly inspected for leaf symptoms until early November, when the remaining inoculated rachises were harvested. Upon dropping of an inoculated leaf from a seedling or at the termination of the experiment, re-isolation of *H. pseudoalbidus* from the leaf rachis onto MEA was attempted as described above. The positions of the inoculated leaves on the shoots were marked and during autumn, winter and spring 2010/2011 shoots were examined at irregular intervals for the occurrence of necrotic lesions.

Leaf symptoms (necrotic lesions on the rachises, wilting and leaf dropping) appeared on all three ash species tested. Some inoculated leaves showed typical symptoms

already two weeks after inoculation. The temporal patters of leaf shedding varied considerably between the three ash species. For example, five weeks after inoculation only one fungus-inoculated *F. ornus* leaf had dropped, whereas the proportions of shed leaves were more than 50% in *F. excelsior* and 11% in *F. angustifolia*. Necrotic lesions on the leaf rachis developed on 91% of the *F. excelsior* seedlings, 56% of the *F. angustifolia* seedlings and 53% of the *F. ornus* seedlings inoculated with *H. pseudoalbidus*. The values for the occurrence of leaf wilting were 74% (*F. excelsior*), 29% (*F. angustifolia*) and 30% (*F. ornus*). Besides a few natural infections, no symptoms occurred on the leaves that had received the control treatment.

H. pseudoalbidus was re-isolated from all three *Fraxinus* spp., often in pure culture, but results varied between species. Re-isolation rates from fungus-inoculated seedlings were 64% for *F. excelsior*, 53% for *F. angustifolia* and 27% for *F. ornus*. Until the end of May 2011 necrotic lesions in the bark of shoots and/or the main stem had developed on a small portion of the test seedlings. All the lesions resulted from natural infections and none of them could unambiguously be related to the leaves that had been inoculated with *H. pseudoalbidus* in the previous summer.

DISCUSSION AND CONCLUSIONS

Kochs postulates were fulfilled to conclude that *H. pseudoalbidus* is indeed associated with leaf symptoms on *F. excelsior*. Likewise, in the inoculation experiment the ash dieback pathogen caused leaf damage on *F. angustifolia* and also on *F. ornus*. On these two ash species, leaf symptoms resulting from natural infections have so far not been reported in connection with ash dieback. It is, however, most likely that they occur also on *F. angustifolia* which is clearly affected by the disease (Kirisits et al., 2009, 2010a). In contrast, ash dieback has till now not been confirmed to occur on *F. ornus*. This ash species may therefore be immune or highly resistant to *H. pseudoalbidus*, although it proved to be somewhat susceptible in stem (Kirisits et al., 2009) and leaf inoculation experiments (this study). In the leaf inoculation trial reported here, it appeared to be the least susceptible species amongst the three *Fraxinus* spp. tested.

The ash dieback pathogen predominantly forms its apothecia on previous year's ash leaf petioles and rachises in the forest litter (Kowalski and Holdenrieder, 2009; Kirisits and Cech, 2009). This emphasises the great importance of leaves as primary habitat of *H. pseudoalbidus* and for the entire life cycle of the fungus. Observations and circumstantial evidence suggest that green leaves are infected by ascospores and colonized by the fungus during the vegetation period, while further development of *H. pseudoalbidus* on leaf petioles and rachises as well as leaflet veins, particularly formation of black pseudosclerotial layers, takes place during the following autumn, winter and spring (Kowalski and Holdenrieder, 2009; Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b; Timmermann et al., 2011). Depending on latitude, altitude and varying climatic conditions apothecia first appear at the end of May, June or early July in the year following infection (Kowalski and Holdenrieder, 2009; Kirisits and Cech, 2009; Kirisits et al., 2009; Queloz et al., 2011; Timmermann et al., 2011).

Although we were not able to induce shoot infections in the leaf inoculation experiment, we still suppose that *H. pseudoalbidus* can grow from infected leaves into phloem and xylem tissues of ash to cause necrotic phloem lesions and wood discoloration (Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b). Field observations suggest that only a relatively low portion of leaf infections may lead to shoot infections, because in many cases leaves are shed before the ash dieback pathogen has reached phloem and/or xylem tissues (Kirisits et al., 2010b). This phenomenon likely also occurred in our leaf inoculation experiment. Thus, due to methodological problems it may be difficult to fully mimic natural infections in inoculation trials with the ash dieback pathogen. Further studies are required to fully elucidate the infection biology of *H. pseudoalbidus* and particularly to definitely determine the organs and tissues of the hosts which are subjected to infections. Such studies include especially also experiments using ascospores as source of inoculum.

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Extended Abstract

Ophiostomatoid fungi associated with the Eastern Himalayan spruce bark beetle (*Ips schmutzenhoferi*) in Bhutan: Species assemblage and phytopathogenicity

Thomas Kirisits[1]*, Heino Konrad[1,2], Michael J. Wingfield[3] and Dal Bahadur Chhetri[4]

[1]Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.
[2]Present address: Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Genetics, Unit of Ecological Genetics and Biodiversity, Hauptstraße 7, A-1140 Vienna, Austria.
[3]Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.
[4]Renewable Natural Resources (RNR) Research and Development Sub-centre, Darla, Department of Forest and Park Services, Ministry of Agriculture and Forests, Royal Government of Bhutan, Darla, Chhukha, Bhutan.

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A brief synthesis of recent studies on the ophiostomatoid fungi associated with the Eastern Himalayan spruce bark beetle, *Ips schmutzenhoferi* and on the pathogenicity of selected fungal associates of this insect to *Picea spinulosa* and *Pinus wallichiana* is presented. *I. schmutzenhoferi* is intimately associated with ophistomatoid fungi and eleven fungal associates belonging to the genera *Ceratocystis*, *Ceratocystiopsis*, *Grosmannia*, *Ophiostoma*, *Leptographium* and *Pesotum* were documented in a survey in Western Bhutan in 2001. In inoculation experiments with four ophiostomatoid fungi, conducted in 2005, *Leptographium* sp. 1, the most common fungal associate of *I. schmutzenhoferi*, displayed high levels of virulence to *P. spinulosa*. In contrast, *P. wallichiana* was highly resistant to inoculation with all four fungal species. The pathogenicity trials indicate that fungal associates of *I. schmutzenhoferi* and especially *Leptographium* sp. 1 prefer *P. spinulosa* over *P. wallichiana* as host, as is true of the insect itself.

Key words: *Ceratocystis bhutanensis*, *Ophiostoma sensu lato*, blue-stain fungi, fungal associates, insect-fungus symbiosis.

INTRODUCTION

The Eastern Himalayan spruce bark beetle, *Ips schmutzenhoferi* is a socio-economically important pest in temperate conifer forests at altitudes between about 2500 and 3400 m asl. in the Bhutan Himalayas (Kirisits et al., 2002, 2012). This bark beetle species preferentially infests Eastern Himalayan spruce, *Picea spinulosa*, but also Blue pine, *Pinus wallichiana* and occasionally logs of Eastern Himalayan larch, *Larix griffithiana*. *I. schmutzenhoferi* is an aggressive scolytine species that caused a destructive outbreak in Western and Central Bhutan in the 1980's.

Most conifer bark beetles live in association with ophiostomatoid fungi belonging to the ascomycete genera *Ophiostoma*, *Grosmannia*, *Ceratocystiopsis* and *Ceratocystis* and related anamorph genera such as *Leptographium* and *Pesotum*. They cause discoloration in the sapwood of conifers and are, therefore, commonly referred to as blue-stain fungi. Some fungal associates of conifer bark beetles are phytopathogenic and are able to kill their host trees.

Here, we briefly summarize recent studies on the ophiostomatoid fungi associated with *I. schmutzenhoferi* and on the pathogenicity of selected fungal associates of this insect to *P. spinulosa* and *P. wallichiana* (Kirisits et al., 2002, 2012; van Wyk et al., 2004; Konrad, 2006).

*Corresponding author. E-mail: thomas.kirisits@boku.ac.at.

Table 1. Ophiostomatoid fungi associated with *I. schmutzenhoferi* in Western Bhutan and their frequencies in relation to sources of isolation and isolation methods (directly from beetles collected from a *P. spinulosa* log, from the sapwood of a beetle-infested *P. spinulosa* tree and from insect galleries on *P. spinulosa* and *P. wallichiana*).

Fungal species	Sources of isolation / isolation methods		
	Beetles	**Sapwood**	**Beetle galleries**
Ceratocystiopsis cf. *minuta*	Not isolated	Not isolated	Common
Ceratocystis bhutanensis	Common	Not isolated	Not recorded
Grosmannia cf. *cucullata*	Common	Not isolated	Common
Leptographium sp. 1 (*Grosmannia* sp.)	Uncommon	Common	Dominant
Leptographium sp. 2	Rare	Rare	Rare
Ophiostoma cf. *ainoae*	Common	Common	Common
Ophiostoma floccosum (*Pesotum aureum*)	Rare	Not isolated	Not recorded
Ophiostoma quercus	Rare	Not isolated	Not recorded
Pesotum cf. *quercus*	Rare	Not isolated	Not recorded
Ophiostoma cf. *piceae*	Rare	Rare	Common
Ophiostoma setosum (*Pesotum cupulatum*)	Not isolated	Rare	Common

OPHIOSTOMATOID FUNGI ASSOCIATED WITH *I. SCHMUTZENHOFERI*

In July 2001, we conducted a survey of ophiostomatoid fungi associated with *I. schmutzenhoferi* in Western Bhutan (Kirisits et al., 2002, 2012; van Wyk et al., 2004; Konrad, 2006). Specimens of *I. schmutzenhoferi*, excavated from their galleries or collected from pheromone traps, stem discs and stem sections from *I. schmutzenhoferi*-infested *P. spinulosa* and *P. wallichiana* trees and logs as well as bark and wood samples containing bark beetle breeding galleries were collected. These collections were made in forests and at wood depots at various localities in the districts of Thimphu and Wangdue Phodrang. Fungi were isolated on malt extract agar and cycloheximide-malt extract agar. Isolations were made directly from the insects, from the sapwood of a beetle-infested *P. spinulosa* tree and from ascospores and conidia taken from perithecia and asexual fungal structures in and around breeding galleries of the insects.

Eleven fungal species were found to be associated with *I. schmutzenhoferi* (Table 1). These included *Ceratocystiopsis* cf. *minuta*, *Ceratocystis bhutanensis* (van Wyk et al., 2004), *Grosmannia* cf. *cucullata*, *Leptographium* sp. 1 (associated with a *Grosmannia* teleomorph), *Leptographium* sp. 2, *Ophiostoma* cf. *ainoae* and five taxa in the *Ophiostoma piceae* species complex (Kirisits et al., 2002, 2012; Konrad, 2006). The latter comprised *Ophiostoma floccosum* (*Pesotum aureum*), *Ophiostoma quercus*, *Ophiostoma* cf. *piceae*, *Ophiostoma setosum* (*Pesotum cupulatum*) and *Pesotum* cf. *quercus* (Konrad, 2006; Kirisits et al., 2012). The assemblages and frequencies of ophiostomatoid fungi varied greatly in the niches that were examined and depended on the isolation methods used (Table 1). Overall, *Leptographium* sp. 1 appeared to be the dominant fungal associate of *I. schmutzenhoferi* and *O.* cf. *ainoae* was also common.

PATHOGENICITY OF FUNGAL ASSOCIATES TO *P. SPINULOSA* AND *P. WALLICHIANA*

In spring 2005, two isolates each of *C. bhutanensis*, *Leptographium* sp. 1 (*Grosmannia* sp.), *O.* cf. *ainoae* and *O.* cf. *piceae* were included in a pathogenicity trial on *P. spinulosa* and *P. wallichiana* in the district of Bumthang in Central Bhutan (Kirisits et al., 2012). Each of 15 pole-sized *P. spinulosa* and *P. wallichiana* trees (diameter at breast height between 20 and 27 cm) were wound-inoculated twice with each of the isolates. Sterile malt extract agar was used as control treatment. About seven weeks after inoculation, the outer bark was removed around the inoculation sites and the lengths of the necrotic lesions in the phloem were measured. All four fungi caused only small necrotic lesions in the phloem of *P. wallichiana* trees, with average lesion lengths ranging from 17.2 to 28.2 mm. *Ceratocystis bhutanensis*, *O.* cf. *ainoae* and O. cf. *piceae* also caused relatively small lesions on *P. spinulosa* (range of average lesions lengths: 29.6 to 34.4 mm). In contrast, *Lepographium* sp. 1 incited very long phloem lesions on *P. spinulosa* (Figure 1), averaging 223.2 and 296.3 mm for the two isolates. In a mass inoculation experiment on small trees (diameter at breast height between 10 and 12 cm), also conducted in 2005, *C. bhutanensis* displayed a very low level of virulence to *P. wallichiana* and a moderate level of virulence to *P. spinulosa* (Kirisits et al., 2012). Thus, this fungus appears to have a very limited or no ability to kill its host trees, even when inoculated at high dosages.

CONCLUSIONS

Our studies in Bhutan (Kirisits et al., 2002, 2012; van Wyk et al., 2004; Konrad, 2006) have shown that *I. schmutzenhoferi* is intimately associated with ophio-

Figure 1. A. Necrotic lesion in the phloem (right) and on the corresponding sapwood surface (left) of a *P. spinulosa* tree caused by *Leptographium* sp. 1. in the pathogenicity trial, B. The longest phloem lesion incited by *Leptographium* sp. 1 on *P. spinulosa*, measuring 69.5 cm.

stomatoid fungi. The most common fungal associate of this bark beetle species, *Leptographium* sp. 1 displayed high levels of virulence to *P. spinulosa*. In contrast, *P. wallichiana* appears to be highly resistant to the fungal associates of *I. schmutzenhoferi*. Moreover, the pathogenicity trials suggest that fungal associates of *I. schmutzenhoferi* and especially *Leptographium* sp. 1 prefer *P. spinulosa* over *P. wallichiana* as host, as is true of the insect itself. Our studies on *I. schmutzenhoferi* (Kirisits et al., 2002, 2012; van Wyk et al., 2004; Konrad 2006) are the first to consider the association of ophiostomatoid fungi with a conifer bark beetle in the Himalayan region. They also contribute to knowledge regarding the occurrence, taxonomy, ecology and pathogenicity of these organisms in a largely unexplored part of Asia.

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Journal of Agricultural Extension and Rural Development (JAERD)

Global change and tree diseases: New threats and new strategies

M. J. Wingfield

Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.
Email: Mike.Wingfield@fabi.up.ac.za

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Robert Hartig's classic first forest pathology text book "Lehrbuch der Baumkrankheiten" was published in 1882, about 130 years ago. It is interesting that this work, and indeed forest pathology as a discipline, emerged at more or less the same time as some of the most notable examples (white pine blister rust, chestnut blight, Dutch elm disease) of epidemic tree diseases caused by accidentally introduced pathogens. Research in subsequent years has led to a broad understanding of the biology, ecology and management of tree pathogens. It is well established that tree pathogens are regularly being moved to new environments and the field of invasion biology has emerged as an important component of forest pathology. New tools, particularly those linked to DNA-based technologies have led to fascinating new discoveries relating to tree pathogens. Exciting and important developments include the ability to recognise cryptic pathogen species that were previously not detectable. This has also made it possible to detect important and unexpected tree pathogen host shifts. Furthermore, tree endophytes have emerged as important in tree health, both as pathogens and possibly relating to the protection of trees against the onslaughts of pests and diseases. The growing numbers of pathogen and tree genomes available for study, new generation DNA sequencing and metagenomics will influence the future of forest pathology dramatically. Against the backdrop of these powerful new tools to study and better understand tree diseases, there will be many threats to tree health in years to come. Examples of negative impacts of global climate change on tree health are emerging and this is a trend that will most likely continue. The movement of trees to new environments will lead to increasing numbers of "new encounter" diseases. Likewise, growing global trade and tourism will make it increasingly difficult to avoid the movement of tree pathogens to new environments, where they come into contact with susceptible hosts.

Key words: Introduced pathogens, climate change, tree health, global trade.

Hot water treatment to reduce *Fusarium circinatum* contamination on *Pinus radiata* seeds

C. Agustí-Brisach, M. Berbegal, A. Pérez-Sierra, J. Armengol* and J. García-Jiménez

Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022, Valencia.

*Corresponding author. E-mail: jarmengo@eaf.upv.es

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Fusarium circinatum is the causal agent of pitch canker on *Pinus* spp. and *Pseudotsuga menziessi*. The use of pathogen-free seeds is the most important means to prevent infections in nurseries and long-distance spread. In this work, different temperature-time combinations of hot water treatment (HWT) were evaluated to reduce *F. circinatum*

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contamination of *Pinus radiata* seeds without compromising seed quality. Specific objectives of the research were: (i) to evaluate the sensitivity of *F. circinatum* isolates to HWT *in vitro*, (ii) to evaluate *P. radiata* seed germination after HWT, and (iii) to assess the effect of HWT on naturally infected seeds of *P. radiata*. Four *F. circinatum* isolates (two of each mating type) were used to evaluate mycelial growth and conidial germination after treatments at 47, 48, 49, 50, 51, 52, 53 and 54°C for 30 or 45 min. Results showed a significant reduction of mycelial growth and conidial germination associated with an increase of treatment temperature and duration time. Mycelial growth was not observed at temperatures above 50 and 51°C for isolates in *MAT*-2 and *MAT*-1, respectively. Differences were observed on conidial germination among the isolates depending on the mating type: At 49°C no conidial germination was observed for *MAT*-2 isolates; however, *MAT*-1 isolates tolerated temperatures up to 52 to 53°C although percentages of germination were very low. *P. radiata s*eed germination was evaluated after treatments at 50, 51, 52, 53 and 54°C for 30 or 45 min. Percentage of germination in non-treated seeds was 90% and, in treated seeds, percentage of germination decreased significantly with increasing temperature and time. Percentage of germination values ranged from 80% at 50°C for 30 or 45 min to 55 to 40% at 54°C for 30 and 45 min, respectively. Reduction of *F. circinatum* contamination was tested on naturally infected *P. radiata* seeds after treatments at 50, 51, 52, 53 and 54°C for 30 or 45 min. HWT effects were evaluated by assessing the percentage of infected seeds plated onto Komada medium. Percentage of infection of non-treated seeds in Komada was above 70% while only 1% of infected seeds were detected after 50°C-30 min, 50°C-45 min and 52°C-45 min treatments. Results obtained indicate that HWT reduce *F. circinatum* contamination of *P. radiata* seeds.

Key words: *Fusarium circinatum,* hot water treatment, *Pinus radiata* seeds.

Phytophthora diseases of chestnut trees in black sea region of Turkey

S. Akıllı[1], Ç. Ulubas Serçe[2], Y. Z. Katırcıoğlu[3] and S. Maden[3]*

[1]Department of Biology, Faculty of Sciences, Çankırı Karatekin University, Çankırı, Turkey.
[2]Mustafa Kemal University, Faculty of Agriculture, Department of Plant Protection, Hatay, Turkey.
[3]Department of Plant Protection, Agricultural Faculty, Ankara University, 06110, Dışkapı, Ankara, Turkey.

*Corresponding author. E-mail: salihmaden@hotmail.cm.

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Phytophthora disease of chestnut trees in Black Sea region of Turkey was investigated by using 85 soil and root samples collected from the trees showing dieback symptoms. Samples were mainly collected from chestnut trees, showing more intensive dieback symptoms. In addition, presence of *Phytophthora* diseases was investigated in important forest nurseries growing chestnut trees. *Phytophthora* spp. in the chestnut forests and nurseries was determined by using baiting technique with the younger leaves of the chestnut saplings and direct planting of diseased roots on selective media respectively. *Phytophthora* spp. were identified by using morphological and cultural aspects of the isolates and by analysing their gene sequences of their ITS regions. In the chestnut forests of this region, the following *Phytophthora* spp. were recovered; *P. cambivora, P. cinnamomi,* and *P. plurivora. P. cinnamomi* was also found in 3 nurseries. The most widespread species on chestnut was *P. cinnamomi*. All the *Phytophthora* species were found pathogenic on its host. The most aggressive species on chestnut were *P. cambivora*, *P. citrophthora* and *P. cinnamomi*.

Key words: Chestnut, *Phytophthora*, Black Sea region, Turkey.

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Journal of Agricultural Extension and Rural Development (JAERD)

Semiochemicals for monitoring and control of the pine wood nematode vector *Monochamus galloprovincialis* (Coleoptera: Cerambycidae)

G. Álvarez Baz[1], I. Etxebeste[1], G. Pérez[2], A. Martín[2], E. Sánchez-Husillos[1], D. Hall[3] and J. A. Pajares[1]*

[1] Sustainable Forest Management Research Institut, University of Valladolid, Palencia, Spain.
[2] Calabazanos Forest Health Center. Junta de Castilla y León, Palencia, Spain.
[3] Natural Resources Institute, University of Greenwich, United Kingdom.

*Corresponding author. E-mail: jpajares@pvs.uva.es.

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Monitoring and control of *Monochamus galloprovincialis*, the sole known cerambycid vector of the pine wood nematode *Bursaphelechus xylophilus* in Europe, are among the most promising strategies against this disease. Recent research has unravelled the very complex, chemically mediated, reproductive behaviour in *M. galloprovincialis.* Males and females locate suitable hosts for feeding and oviposition by responding to host and bark beetle semiochemicals. Then, a male emitted pheromone increases the chance for both sexes to meet on the host tree and contact chemical recognition of females is performed before mating. Such findings led to the development of a very efficient attractant lure, consisting in a blend of the aggregation pheromone plus the kairomones, which is already available to researchers and managers. There exist many potentialities of this tool in pine wilt disease integrated management programs along Europe. Determination of dispersal range and mass trapping of the sawyer are among the most relevant. Two dispersal experiments based on the recapture of marked-released adult insects were carried out in natural and reforested pine stands. Recapture in the pheromone-kairomone baited traps was highly successful, accounting for 33.9 and 29.5% of the 174 and 350 released beetles, respectively. Both experiments showed that most insects have low/moderate dispersal, as some 75% of the beetles were recaptured less than 100 or 141 m away, but 6.7% of them were caught at 500 and 6.86% more than 760 m away. One beetle was caught 1.5 km from the release point in the first experiment. Time elapsed between the release and recapture was extended to 91 and 84 days respectively, showing a high life span for *M. galloprovincialis* adults. Results on one mass trapping experiment to test the rate of population extraction and on an operational mass trapping program aimed to vector eradication in the 2008 focus at Sierra de Dios Padre (Extremadura, Spain) are also commented.

Key words: Pine wilt disease, *Monochamus galloprovincialis*, semiochemicals, aggregation pheromone, insect dispersal.

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Effects of leaf spotting caused by mycosphaerella leaf disease and eucalyptus rust on *Eucalyptus globulus* in Uruguay

G. Balmelli[1]*, S. Simeto[1], N. Altier[1], V. Marroni[2] and J. J. Diez[3]

[1]Programa Nacional Forestal, Instituto Nacional de Investigación Agropecuaria (INIA). Ruta 5, Km 386. Tacuarembó, Uruguay.
[2]Plant and Food Research, Private Bag 4704. Christchurch, New Zealand.
[3]Departamento de Producción Vegetal y Recursos Forestales. Instituto de Gestión Forestal Sostenible. Universidad de Valladolid. Palencia, Spain.

*Corresponding author. E-mail: gbalmelli@tb.inia.org.uy.

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Mycosphaerella leaf disease (*Mycosphaerella* spp. and *Teratosphaeria* spp.) and Eucalyptus rust (*Puccinia psidii*) are important diseases of eucalypt plantations in Uruguay. *Eucalyptus globulus* is highly susceptible to both diseases; however production losses caused by them have not been properly quantified in this country. In this study, the severity of foliar damage caused by Mycosphaerella leaf disease and Eucalyptus rust and the long term effects on growth and survival were assessed in a progeny test of *E. globulus* located in Rocha, Uruguay. The severity of leaf spots was quantified eight months after planting and tree growth and mortality were evaluated two, four and six years later. The trial presented a high incidence of spotting (88.2% of trees showed leaf spots), with a mean severity of 28.7%. The greatest impact of foliar damage, both on growth rate and mortality, occurred in the first two years after damage was assessed. During this period, spot severity less than 40% did not affect growth rate, while survival was affected by spot severity of 70% or higher. When spot severity reached 80% or more, a loss of up to 25% in diameter and an accumulated mortality of 71.7% were registered by the sixth year. It is concluded that, under the intensive Uruguayan productive conditions, *E. globulus* trees tolerate a relatively high degree of leaf spotting. However, severe foliar damage in the first months can cause considerable production losses, compromising the success of the plantation.

Key words: Mycosphaerella leaf disease, Eucalyptus rust, leaf damage, *Eucalyptus globulus*.

Effects of pruning on pitch canker disease in *Pinus radiata* plantations

D. Bezos*, J. M. Lomba, P. Martínez-Álvarez, M. Fernández and J. J. Diez

Sustainable Forest Management Research Institute, University of Valladolid – INIA. Avenida Madrid, 57. 34004 Palencia, Spain.

*Corresponding author. E-mail: dbezosg@funge.uva.es.

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Fusarium circinatum (Niremberg and O´Donell, 1998) is the causal agent of pitch canker disease (PCD) in *Pinus*

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species, causing necrosis and deformation in trunk as well as dieback. The disease appeared recently in northern Spain associated with *Pinus* spp. seedlings at forest nurseries, and *Pinus radiata* plantations in the forest. The aim of this study was to evaluate the effect of pruning on PCD in *P. radiata* plantations in Cantabria, so the study was carried out on 50 *P. radiata* plots (pruned and unpruned) distributed along this region. Symptoms of PCD were evaluated in 25 trees in each plot following the ICP forest methodology and were related with dendrometric factors including pruning. A significant relationship was found between pruning and the number of cankers per tree concluding that management affects PCD severity.

Key words: Pruning, *Fusarium circinatum*, Cantabria, Monterey Pine, Spain.

Can global warming affect the survival and impact of *P. alni* subsp. *alni*?

Karel Černý[1,2]*, Veronika Strnadová[1] and Nela Filipová[1,2]

[1]Silva Tarouca Research Institute for Landscape and Ornamental Gardening, Publ. Res. Inst., Průhonice 25243, Czech Republic.
[2]Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Prague 16521, Czech Republic.

*Corresponding author. E-mail: cerny@vukoz.cz.

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The winter survival of the invasive pathogen *Phytophthora alni* subsp. *alni* in black alder stems was studied after two different winter seasons: cold one in 2008/2009 with an average temperature of –1.96°C and extremely mild one in 2006/2007 with an average temperature of 2.54°C. The difference resembles the expected potential climate change in Central Europe in this century. After the cold winter of 2008/2009, the pathogen survived in only 13.91% samples with an average survival rate of 2.70%. After the mild winter of 2006/2007, the pathogen survived much better and was successfully isolated from 86.09% samples with an average survival rate of 25.52%. Moreover, the total thickness of the covering tissues and exposure to the most heated south western quadrant of stem girth positively affected the pathogen survival. In laboratory experiment with incubation of ten isolates in different temperature and frost duration conditions; the influence of temperature, frost duration and their interaction on *P. a. alni* viability was confirmed ($p < 0.01$). The temperature –0.1 and –2.5°C did not have significant effect on the viability after 4 week incubation. The pathogen viability significantly decreased ($p < 0.01$) when the temperature –5.0°C persisted at least one week. At –10.0°C; no isolate survived till 3 days. The pathogen was very sensitive to hard frost – its median survival time (t_{50}) was only 1 day at –10°C. The survival of *P. a. alni* importantly depended on the temperature. Likely, the pathogen will react to the climate change by having a higher survival rate in aerial tissues and surface roots and a higher disease impact in its existing locations. Likely, the pathogen escapes the influence of hard frost in continental Europe by surviving in roots in non-frozen water and soil.

Key words: *Phytophthora alni*, winter survival, temperature, frost, phytophthora alder disease.

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Assessment of the early effects of climate change on forest health

C. Colinas[1]* and V. J Monleon[2]

[1]Department of Crop and Forest Sciences, University of Lleida, Lleida, Spain.
[2]Pacific Northwest Research Station, U.S. Forest Service, 3200 SW Jefferson Way, Corvallis, Oregon, USA.

*Corresponding author. E-mail: carlos.colinas@pvcf.udl.es.

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The current warming trend is expected to result in species migration northwards in latitude and upwards in elevation. Colonization of higher elevations and latitudes has been documented elsewhere, but a warmer climate may also increase mortality at the lowest latitude and elevation of species ranges. While local conditions may mediate some of the effects of global climate change, the trend should be detectable at large geographical scales. Given tree size and longevity, mass mortality in the warmer boundary of species distributions may not be apparent at the early stages of warming. However, pathogen damage may increase, because trees are less capable of resisting pathogens when growing under limiting conditions. So, under a global warming hypothesis, trees at the receding edge of the species habitat must be the first to suffer disease outbreaks. In order to test this hypothesis, we used data from the forest inventory of Washington, Oregon and California (Forest Inventory and Analysis Program, Forest Service, Department of Agriculture, USA) to study whether there was a pattern of higher disease damage associated with southern latitude and lower elevation of a species range, and with SW aspects. The area under study covers 35 million ha. of forestland across a wide range of environmental conditions. A total of 14,000 plots, 45 pathogens and 37 tree species were included. The results confirm the presence of damage at the hypothesized sites at regional scale, but also indicate a more complex pattern. The damage is pathosystem dependent, and there seem to be some fundamental differences between diseases and insect attacks. These results would be useful to modelers in order to refine the projected effects of climate change on future tree distribution.

Key words: Global warming, pathogen damage, disease outbreaks.

Fungi in shoots and foliage of *Fraxinus excelsior* and *Fraxinus angustifolia* in Eastern Ukraine

K. Davydenko*, S. Bengtsson, J. Stenlid and R. Vasaitis

Swedish University of Agricultural Sciences, Ukraine.

*Corresponding author. E-mail: kateryna.davydenko@slu.se.

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During the last years, the massive dieback of ash (*Fraxinus* spp.) caused by *Hymenosyphus pseudoalbidus* is observed over large areas of Europe. Until 2010, there was little concern regarding ash health condition in Ukraine, as no dieback symptoms were reported. During the last year, however, some morphological symptoms of ash decline have been observed in the eastern part of the country: Uneven flushing, occasional shoot necroses, discoloration of wood and

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premature leaf-shedding. The main aim of our work was to investigate fungal communities in necrotic and healthy-looking shoots, and in petioles of leaves that were shed during previous vegetation season. The detection of fungi has been accomplished using molecular methods: direct extraction of DNA from plant tissue, its amplification using polymerase chain reaction (PCR) with internal transcribed spacer (ITS) primers, subsequent sequencing, and comparison of the sequences with the sequences of fungi originating from disease-devastated areas. The 176 samples symptomatic and healthy (that is, having necrotic lesions) of shoots and leaves were collected for molecular identification of fungal community and isolation of fungal cultures. In addition, health condition of different ash provenances was visually assessed on seven monitoring plots (24 test trees in every plot) at two locations in eastern Ukraine. The plots represented ash stands from different ages (from 10 to 80) with different crown condition.

Key words: *Hymenosyphus pseudoalbidus*, ash, dieback, ITS primers, crown condition.

Susceptibility of *Pinus nigra* and *Cedrus libani* to Turkish *Gremmeniella abietina* isolates

Doğmuş–Lehtijärvi H. T.*, Oskay F., Karadeniz M. and Lehtijärvi A.

Faculty of Forestry, Süleyman Demirel University, 32260 Isparta, Turkey.

*Corresponding author. E-mail: tugbadogmus@sdu.edu.tr.

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Virulence of Turkish *Gremmeniella abietina* isolates was investigated in a field experiment. Five isolates obtained from dead branches of *Pinus nigra* subsp. *pallasiana* and *Pinus sylvestris* in high altitude mountainous areas in the Black Sea Region and the Lakes District were used. The lower branches of 15 to 20 year-old *P. nigra* and *Cedrus libani* in a plantation site at 1050 m a.s.l. in Isparta, were inoculated at 1 to 2 month intervals during September to January. Each isolate was inoculated into one branch per tree and repeated ten times on both tree species at each inoculation date; a total of eighty trees and four hundred-eighty branches were inoculated. The branches were harvested at the end of February, after 166, 112, 64 and 33 days of incubation and lesion length in the inner bark was measured (inoculation wound excluded). The mean lesion length on *P. nigra* and *C libani* were 10.6 ± 0.8 and 3.8 ± 0.2 mm, respectively. In general, differences in the mean lesion length between the isolates were small. Nevertheless, there were significant differences between the isolates on *P. nigra* in November and January inoculations, and on *C. libani* at all four inoculation times. The mean lesion length for all isolates was the highest ($p < 0.05$) in December inoculations for both *P. nigra* (22.0 ± 1.9) and *C. libani* (5.6 ± 0.7). There was no difference between the September and January inoculations on *P. nigra,* despite the almost six-fold difference in incubation period. On *C. libani,* in contrast, the shortest necroses were found in January inoculations ($p < 0.01$). During the December inoculations, the trees were most likely in winter dormancy, which would explain the large lesions.

Key words: *Gremmeniella abietina, Pinus nigra* subsp. *pallasiana, Cedrus libani*.

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Powdery mildew fungi on some deciduous tree species in Turkey

Dogmus-Lehtijarvi H. T.[1]*, Aday A. G.[2] and Lehtijarvi A.[1]

[1]Faculty of Forestry, Suleyman Demirel University, 32260 Isparta - Turkey.
[2] Süleyman Demirel Üniversity, Yenişar Bademli Vocational School, Isparta- Turkey.

*Corresponding author. E-mail: tugba@orman.sdu.edu.tr.

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Powdery mildew is quite a common disease that appears as a white powdery substance occurring on the leaf surface, stem and fruits of many deciduous tree species in European forests. As a result of heavy infection, leaves and affected parts of the tree frequently are distorted and in worst cases infected trees may exhibit symptoms of defoliation or decline. In this study, some deciduous tree species such as *Platanus orientalis, Quercus vulcanica, Quercus robur, Castenea sativa* were inspected for the occurrence of powdery mildew. Additionally, infection rates and distribution were studied from the visible symptoms of the disease observed on *P. orientalis* saplings from seed orchards of Çınarcık, Yalova. *Q. vulcanica* which is an endemic oak species of Turkey shows disease symptoms every year in Kasnak Oak National Park in Isparta Province together with *Q. robur*. *C. sativa* has also powdery mildew infections observed abundantly in one of the recreation areas in Isparta. As a result of macroscopic and microscopic studies, several different fungal species causing powdery mildew on these hosts were found. Mainly characteristics of cleistothecia were used for the identification of the powdery mildew fungi. While *Microsphaera alphitoides* was common fungus on the upper surface of *Q. vulcanica* and *Q. robur*, only *Phyllactinia roboris* which is known as a rare species was detected on the lower surface of the leaves of *Q. vulcanica*. Sweet chestnut and plane trees were infected by *Phyllactinia guttata* and *Microsphaera platani*, respectively. The infection rate and the distribution of *M. platani* on 100 *P. orientalis* saplings were investigated. The saplings were approximately 2.5 cm in diameter and 160 cm in height. In each tree, three topmost lateral shoots and the terminal shoot were checked. All leaves of each shoot were counted and investigated for the presence of the fungus. While all *Platanus* seedlings were found to be infected with *M. platani*, the disease was more common on terminal shoots (85.8%) than on the lateral ones (52.4%).

Key words: Forest disease, *Microsphaera alphitoides*, *Phyllactinia roboris, Phyllactinia guttata, Microsphaera platani*.

Dothistroma septosporum: Incidence of spore production and weather condition

M. Dvořák* and L. Jankovský

Department of Forest Protection and Wildlife Management, Faculty of Forestry and Wood Technology, Mendel University in Brno, Czech Republic.

*Corresponding author. E-mail: Milon.Dvorak@seznam.cz.

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Dothistroma septosporum (Dorog.) Morelet. and *Dothistroma pini* Barnes are fungal pathogens responsible for a very

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serious needle disease Dothistroma needle blight (DNB), also known as Red band needle blight. DNB was previously considered as a disease supported by global climate change. The influence of locally enhanced precipitations to the spreading of the disease in temperate zone was emphasized. Although a number of papers were published about phenology of DNB, no attempt was made to describe the weather conditions, which are necessary for production of conidia and general pattern of conidia releasing during the year. The aim of this study was to examine the temporal spore dispersal patterns of *D. septosporum*. Spore trapping was done for a period of two seasons from March to December 2009 and 2010 in a 13 year old (2009) plantation of austrian pine (*Pinus nigra*) in the forest district "Soutok", near Lanžhot, South Moravia, Czech Republic. This plantation was strongly infected by *D. septosporum* for a few years. For trapping the Dothistroma spores the automatic volumetric spore trap of usual Hirst, Burkard or Lanzoni VPPS construction was used. Our spore trap (AMET Velké Bílovice, Czech Republic) was placed inside the plantation with the orifice 0.3 m above soil level. Close to the spore trap, a SIGNALIZATOR automatic climatic station (AMET Velké Bílovice, Czech Republic) was installed 2 m above the ground. From the evaluation of sticky tapes from the trap was noticed, that the production of spores occurred solely during days with average day temperature above 10°C and only in the part of season without frost days. Such conditions started in the third decade of April and finished at the beginning of October. During the spore-active season some periods of interruptions were apparent. The longest period without any spore was the first and the second decade of June. It is characterized by average daily temperature above 18°C and average daily relative humidity under 75%. Upon such dry conditions the sporulation ceases within two days. On the other hand, the optimal weather conditions are average daily temperatures 15 to 20°C and average daily relative humidity above 90%. This optimal period was taken from the end of July to the half of September. The highest amount of spores in one cubic meter of air was 3.89. The lowest detectable amount was 0.07 spores/m^3 of air.

Key words: Spore trap, conidia releasing, Dothistroma needle blight, weather.

Phytosanitary conditions of *Quercus cerris* in tuscany evaluated by monitoring, geographic information system (GIS) applications and molecular techniques

M. Feducci[1], N. Luchi[1,2], V. Mancini[1] and P. Capretti[1]*

[1]Università degli Studi di Firenze, Dipartimento Biotecnologie Agrarie, Sezione di Protezione delle Piante, Piazzale delle Cascine 28, 50144 Firenze, Italy.
[2] Consiglio Nazionale delle Ricerche, Istituto di Protezione delle Piante, Via Madonna del Piano 10, 50019, Sesto Fiorentino, Firenze, Italy.

*Corresponding author. E-mail: paolo.capretti@unifi.it.

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Data from the phytosanitary monitoring program in Tuscany (META) collected during the last four years showed an increasing number of *Quercus cerris* stands affected by *Biscogniauxia mediterranea* (from 12 to 78%) followed by a two-years period of decreasing values. Since the occurrence of this fungus is considered as a good indicator of oak stress in central Italy, a study was performed to verify whether environmental parameters are involved in oak decline. The study was based on the use of geographic information system (GIS) technology and was validated as fungal presence from symptomless oak twig samples. Fungal detection was assessed by using isolation and real-time polymerase chain reaction (PCR) already optimized from this research group in a *B. mediterranea*/*Quercus* pathosystem. During summer

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2009 geographical and monitoring data were obtained from twenty Oak areas in Tuscany. Stands dendrological information and crown conditions evaluated according to international co-operative programme (ICP)-Forest monitoring method were registered; damages and severity index for crown damages were also calculated. Finally, all data were stored and managed by a GIS system by ArcMap 9.3 software. Using climatic data obtained from official local meteorological networks, the mean temperatures and total rainfall during the vegetative seasons were calculated and then data points were interpolated by using Ordinary Kriging technique to obtain prediction climatic maps at local scale. Vector maps of chemical, physical and biological properties of soil according to United Sates Department of Agriculture (USDA) classification, digital elevation model, viability map and *Q. cerris* regional distribution map were lastly stored into GIS system. Results of monitoring showed that the most common damages observed were diebacks and necrosis of branches. Kruskal-Wallis test allowed grading the areas on the base of severity of crown damages level. Results of laboratory analysis confirmed that samples collected from damaged areas hosted a significantly higher amount of *B. mediterranea* DNA in oak asymptomatic tissue compared to areas with lower damages. All geographical and climatic data stored in GIS system were used as predictors. Temperature and soil characteristics showed inverse relationship with crown damages in multiple regression model, while rainfall, elevation, and quantitative of clay and sand were positively linked to crown damages.

Key words: *Biscogniauxia mediterranea*, oak decline, real time PCR, ICP-forest, severity, crown damages.

Use of *Chondrostereum purpureum* in controlling hardwood sprouting

J. Hantula[1]*, L. Hamberg[1], H. Vartiamäki[1], K. Korhonen[1] and A. Uotila[2]

[1]Finnish Forest Research Institute, PO Box 18, 01301 Vantaa, Finland.
[2]University of Helsinki, Hyytiälä Forestry Field Station, Hyytiäläntie 124, 35500 Korkeakoski, Finland.

*Corresponding author. E-mail: jarkko.hantula@metla.fi.

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Hardwood sprouting is a considerable problem in forest regeneration, road sides and also under electric lines. As chemical control is not used any more due to environmental problems and public opinion, biocontrol has become an interesting option. There is a biocontrol product available in Canada, but importing North American pathogen to Europe may have risks, and probably would not even satisfy the public opinion. Therefore, we have studied the possibilities to use Finnish *Chondrostereum purpureum* isolates for this purpose. Our results on birch (*Betula pendula* and *Betula pubescens*) showed that sprout control efficiencies of more than 80% may be reached using natural strains. Already this figure might be satisfactory for practical application, but we intended to test, if it could be increased by breeding. This project is still under its way, but preliminary first generation results do not show considerable improvements. In Nordic countries also *Phlebia gigantea* is used for biocontrol in forestry. In this project, we tested, whether both biocontrol agents (*C. purpureum* and *P. gigantea*) could be used with the same equipment, or if small residuals of one agents hampers the other. The results show that the viability of both fungi decreased when small amounts of the other one was added. However, in efficacy testing the control of both fungi surprisingly increased against both hardwood sprouting or *Heterobasidion parviporum*, respectively.

Key words: *Chondrostereum purpureum*, biological control, hardwood sprouting, forest regeneration, Heterobasidion root, butt rot.

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Susceptibility assessment of common alder seedlings to *Phytophthora alni* and other *Phytophthora* species

M. M. Haque* and J. J. Diez

Sustainable Forest Management Research Institute, University of Valladolid – INIA. Palencia, Avenida Madrid, 57. 34004 Palencia, Spain.

*Corresponding author. E-mail: mhaque@pvs.uva.es.

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Common alder (*Alnus glutinosa*) (L.) Gaertn seedlings were tested *in vitro* for their susceptibility towards alder pathogen *Phytophthora alni* and other *Phytophthora* species. Isolates of *P. alni* and other *Phytophthora* species (*Phytophthora cinnamomi*, *Phytophthora citrophthora*, *Phytophthora nicotianae* and *Phytophthora palmivora*) were used in the assay. Seedlings were inoculated with uniform mycelial blocks of agar. Susceptibility was assessed in terms of seedling mortality percent after 67 days of inoculation. Seedlings were found highly susceptible to *P. alni* and also to other *Phytophthora* spp. which varied from higher to lesser extent. Results implied that common alder seedlings are at risk to be infected by *Phytophthora* spp. and showed relative host-nonspecificity of this genus.

Key words: *Alnus glutinosa*, inoculation, susceptibility, *Phytophthora alni*, *in vitro*.

Sequencing and assembly of a fungal genome

T. Hsiang

School of Environmental Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1, Canada. E-mail: thsiang@uoguelph.ca.

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With the next generation sequencing technologies, it is now feasible, within a small research program, to attempt *de novo* sequencing and assembly of small eukaryotic genomes (<100 Mb in size). In late 2009, this study decided to attempt sequencing of the genome of the fungus *Colletotrichum cereale*. Since this species had been split from *Colletotrichum graminicola* in 2006, and the rough genome assembly of *C. graminicola* became available in 2008, it is thought that these two genomes would be similar enough for *C. graminicola* to act as the reference genome for sequencing of *C. cereale*. This study prepared genomic DNA of an isolate of *C. cereale* using standard methods, and sent in 10 µg as requested. The sequencing center then took two weeks for library construction, and another two weeks to run out on a Illumina GAIIx sequencer. They generated ~25-fold coverage in 35 bp paired-end reads of this 60 Mb genome (1.5 Gb worth of sequence data plus 4 Tb worth of image files and other data associated with sequencing). They attempted assembly of the 35 bp reads, based first on the *C. graminicola* genome and then other fungal genomes, and then provided the results. Attempt of this data was assembled with a variety of programs and a multitude of settings. With genomes of species that have already been sequenced, the new sequencing technologies may be feasible to obtain the genome of an isolate of the same species; but for species lacking a reference genome, the technology in terms of software and perhaps equipment may have limitations. The sequencing technology and the results of this attempt at fungal genome sequencing and assembly are discussed.

Key words: Illumina Solexa, fungi, genomic, reads.

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Enhancing systemic resistance of maple against tar spot disease

T. Hsiang*, A. Darbyson, P. Goodwin, A. Cortes-Barco and B. Nash

School of Environmental Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1, Canada.

*Corresponding author. E-mail: thsiang@uoguelph.ca.

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Plants are known to possess natural defense mechanisms against stresses including diseases. In cultivated systems or under high disease pressure, these natural mechanisms may be insufficient to guard the plants against disease outbreaks. There are chemicals that have been observed to stimulate the natural resistance pathways in plants. We have been investigating the mechanism of action of certain new compounds in their role of defense activation against diseases in plants, including tar spot of maple caused by *Rhytisma* species. These compounds generally do not have strong direct anti-fungal effects, but activate signaling pathways within the plant to either cause direct expression of defense-related genes prior to pathogen attack (induction) or allow expression of defense-related genes more quickly in response to pathogen attack (priming). Using tests in the lab and in the field, we found that applications of such chemicals either alone or in combination can reduce plant diseases significantly. This presentation will explore the use of such chemicals for plant disease control, and discuss their advantages and possible disadvantages.

Key words: Induced systemic resistance, systemic acquired resistance, *Acer*, tar spot.

The potential of soil bacteria and their biosurfactants to suppress *Phytophthora* diseases of forest trees

M. Hultberg[1], K. Blumenstein[2] and J. Witzell[2]*

[1]Department of Horticulture, Faculty of Landscape Planning, Horticulture and Agricultural Sciences, Swedish University of Agricultural Sciences, Box 103, Sundsvägen 14, 230 53 Alnarp, Sweden.
[2] Faculty of Forest Sciences, Swedish University of Agricultural Sciences, Southern Swedish Forest Research Centre, Box 49, Rörsjövägen 1, 230 53 Alnarp, Sweden.

*Corresponding author. E-mail: johanna.witzell@slu.se.

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Diseases caused by the fungi-like organisms, *Phytophthora* spp. (Oomycetes), are a major problem in deciduous forests. Alarmingly, problems with *Phytophthora*-diseases are expected to be further magnified and intensified in the future, since the conditions under the predicted climate change are likely to favor the growth and spread of these pathogens also in the Northern Europe. New control strategies are thus urgently needed. The use of microorganisms as biocontrol agents is an environmentally sound alternative to chemical pesticides and could be an integrated part of sustainable management of *Phytophthora* pathogens of forest trees. A group of microorganisms with high potential in biocontrol are the fluorescent pseudomonads (Pseudomonas spp.). They are indigenous in the environment and may excrete metabolites that are inhibitory to pathogenic microbes. An important group of metabolites produced by fluore-

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scent pseudomonads are biosurfactants. Due to their zoosporicidal activity, these chemicals have attracted increased attention as a potential tool in biocontrol of pathogens belonging to Oomycetes. We initiated studies to evaluate the potential of biosurfactant-producing pseudomonads to protect deciduous forest trees against *Phytophthora* spp. Firstly we used in vitro tests to validate that biosurfactants produced by a fluorescent pseudomonad, *P. koreensis* 2.74, isolated from a horticultural system would effectively lyse the zoospores of oak pathogenic *P. quericina.* Secondly, we conducted a greenhouse experiment with young oak seedlings to study whether the mortality of oak seedlings due to *P. quercina* infections could be reduced by treating the plants with biosurfactant-producing pseudomonads. Thirdly, we investigated whether the treatment with bacteria could also induce alterations in the potentially defensive phenolic metabolites in oaks. The implications of the results for control strategies in nurseries and natural habitats were discussed.

Key words: Biocontrol, sustainable management, *Pseudomonas spp.*, phenolic metabolites.

New alternate hosts for *Cronartium* spp. in Finland

J. Kaitera[1]* and R. Hiltunen[2]

[1]Finnish Forest Research Institute, Oulu, P.O. Box 413, FI-90014 Oulu, Finland.
[2]Botanical Gardens, University of Oulu, P.O. Box 3000, FI-90014 Oulu, Finland.

*Corresponding author. E-mail: juha.kaitera@metla.fi.

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Cronartium flaccidum causes serious rust epidemics on *Pinus sylvestris* in northern Fennoscandia. The rust spreads via *Melampyrum* spp. in diseased stands. The main alternate host for the rust in genus *Melampyrum* is *Melampyrum sylvaticum* in northern Finland. The rust occurs also commonly on *Vincetoxicum hirundinaria* in southern Finland, but it has also been found on *Pedicularis* spp. and *Paeonia* spp. in natural forests and garden plants in Finland. In artificial inoculations, the rust has been shown to infect several other species in other genera elsewhere in Europe and Asia. *Cronartium ribicola* is most common on five-needle pines in arboretums and botanical gardens in southern Finland. The rust spreads via a high number of cultivars of *Ribes* spp. The rust is known to be very host-specific in Europe. A number of inoculation experiments were conducted in the laboratory and greenhouse to test the susceptibility of alternate hosts in potential plant genera to *C. flaccidum* and *C. ribicola* in 2008-2010. Both *C. flaccidum* and *C. ribicola* formed uredinia and telia on *Pedicularis palustris* ssp. *palustris*. Either uredinia or telia of *C. flaccidum* and *C. ribicola* developed also on several earlier unreported species in previously unreported families. The high number of new alternate hosts capable of spreading the rust showed the low host specificity of *C. flaccidum*. The results suggest that the virulence of the European *C. ribicola* is much wider than earlier reported. Sampling of natural samples of the new alternate hosts is needed to clarify their role in spreading rust epidemics in practice.

Key words: Alternate hosts, *Cronartium flaccidum, Cronartium ribicola, Pinus sylvestris,* scots pine blister rust, white-pine blister rust.

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Journal of Agricultural Extension and Rural Development (JAERD)

Mycosphaerella dearnessii M. E. Barr (Brown-Spot Needle Blight of Pine) in Austria

M. Kessler[1]*, T. L. Cech[1], C. Tomiczek[1] and E. Halmschlager[2]

[1]Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Protection, Seckendorff-Gudent-Weg 8, A-1131 Vienna, Austria.
[2]Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Hasenauerstraße 38, A-1190 Vienna, Austria.

*Corresponding author. E-mail: marion.kessler@bfw.gv.at.

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Mycosphaerella dearnessii M. E. Barr (syn. *Scirrhia acicola*; anamorph: *Lecanosticta acicola* Thüm, syn. *Septoria acicola*) is an ascomycetous pine needle pathogen and the causal agent of brown spot needle blight. Potential hosts comprise various pine species and even *Picea glauca* can be infected when exposed to heavy spore loads. The disease is known from North, Central and South America, Asia, South Africa and Europe. Since it is widespread in North and Central America, it is assumed to be of Central American origin. The global spread of the fungus is attributed to the expanded pine trade in the last decades. In Europe *M. dearnessii* is mostly limited to local sites and often occurs on Mountain pine (*Pinus mugo*) and Scots pine (*Pinus sylvestris*) in urban habitats (parks, gardens) as well as in arboreta. In forests, *M. dearnessii* is known to occur on *Pinus uncinata* in swamps and more rarely in *Pinus sylvestris* / *Pinus radiata* stands. In Austria, brown spot needle blight was identified originally from Mountain pine (Pinus mugo) in 1996 in the town Hollenstein/Ybbs (Lower Austria). Annual surveys by the Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW) from 1996 to 2007 revealed a slowly increasing number of infested trees (*P. mugo*, *P. uncinata* and *P. sylvestris*) but infestation was still limited to urban sites in that town. In August, 2008, however, the species was found for the first time in mixed forest stands on Scots pines (*P. sylvestris*) adjacent to the town of Hollenstein. In autumn 2009 newly infested trees were found at the border of the municipal area of Hollenstein close to further mixed pine forest stands. In autumn 2010 *M. dearnessii* was detected for the first time on urban trees in five other towns up to 40 km distant to Hollenstein. Austrian black pine (*Pinus nigra austriaca*) which is common in the town of Hollenstein and known to be susceptible was never infected by *M. dearnessii*. This point might be due to a probable competition effect between *Dothistroma septosporum* (Dorog.) M. Morelet, which is extremely common on this pine species in Austria, and *M. dearnessii*. Swiss stone pine (*Pinus cembra*) which was planted as an ornamental tree in Hollenstein was also never infected. Whether the disease spread naturally from Hollenstein to the other towns, or infections were the result of multiple introductions via infested plant material is subject of discussion. Pathogen spread can occur over short distances by rain splash and wind, however, it is also likely that spores can be transported by clothes, shoes, or vehicles. Heavily infested trees, suffering from intense needle losses for many years, show branch dieback extending upwards in the crown. It is very likely, that such trees become attacked by secondary invaders. Currently, a doctoral thesis is conducted at the University of Natural Resources and Life Sciences (BOKU), Vienna and the BFW. One goal of this thesis was to investigate population diversity and potential patterns of spread of *M. dearnessii* in Austria as well as genetic diversity of *M. dearnessii* in Europe and other continents.

Key words: Brown spot needle blight, Austria, spread of disease, host range, *Mycosphaerella dearnessii, Lecanosticta acicola, Dothistroma septosporum*.

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Phytophthora-infection in a sweet chestnut (*Castanea sativa*) Orchard in Transdanubia, Hungary

J. Kovács*, I. Szabó and F. Lakatos

University of West-Hungary, Institute of Silviculture and Forest Protection, H-9400 Sopron, 5 Ady E. str., Hungary.

*Corresponding author. E-mail: csendes.judit@gmail.com.

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In the first decades of the 20th Century, a big amount of sweet chestnut (*Castanea sativa*) which stands in the South-Europe died. The cause was the ink disease. Since then, the ink disease is one of the most serious diseases of the sweet chestnut in the whole Europe. There are several *Phytophthora* species in contact with the appearance of the ink disease. The most frequent species are *Phytophthora cambivora* and *Phytophthora cinnamomi*. The appearance of ink disease is sparse in Hungary. In September 2010, we found dying trees in a sweet chestnut orchard of 14 year old in South-Transdanubia, Hungary. The symptoms were specific: small, yellowish leaves, sparse, drying foliage, necrotic bark lesions at the stem basis and main roots. The fine roots were rot. Some saplings also died in the same orchard. We isolated *Phytophthora* species on selective agar media from soil samples taken from the rhizosphaere of the dying trees and saplings by baiting with *Rhododendron* leaves. The species identification was carried out by morphologic examination and the internal transcribed spacer (ITS1) and ITS2 sequences of the ribosomal DNA. We identified *P. cambivora* from the soil of dying trees and of saplings, too. Now, the appearance of ink disease in the orchard is confined to a small clump. It is an urgent task to find the adequate methods to confine the disease.

Key words: Ink disease, internal transcribed spacer, ribosomal DNA, *Phytophthora cambivora*.

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White pine needle diseases in Eastern Canada

G. Laflamme[1]*, C. Côté[1] and L. Innes[2]

[1]Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, P.O. Box 10380, Québec, QC, Canada G1V 4C7, Canada.
[2]Ministère des Ressources Naturelles et de la Faune, Direction de l'environnement et de la protection des forêts, 2700 rue Einstein, Québec, QC, G1P 3W8.

*Corresponding author. E-mail: Gaston.Laflamme@RNCan-NRCan.gc.ca.

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In 2009, yellowing of white pine (*Pinus strobus*) needles was reported from several regions in three Canadian provinces: New Brunswick, Québec and Ontario. A similar problem was seen also in eastern United States. Several causal agents

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were presented as hypotheses: Drought, pollution as well as several needle diseases. In 2010, samples of white pine needles were collected in areas where symptoms were seen in previous year. Samplings were done by the three provincial agencies. In addition, one white pine was sampled every month in Québec City and some endophytic fungi were isolated from diseased needles collected in June. At least 6 fungal species were more common on these needles but some were secondary fungi like *Hendersonnia pinicola*. The most common pathogen found was *Canavirgella banfieldii* which seems to be a synonym of *Lophophacidium dooksii.* The yellowing of the current year needles is visible mainly in August. The discoloration affects only a distal portion of the needles. Some white pine seems to be resistant to this disease. The teleomorph is visible mainly on previous year needles in early summer. A second pathogen, *Mycosphaerella dearnessii*, appears in June on previous year needles: the whole needle becomes yellow and red bands are visible near the infection point. Both pathogens were collected on the same tree on few occasions. All these fungi are being sequenced and this should clarify the synonymy at some fungal species level and their classification at the family level.

Key words: *Pinus strobus, Lophophacidium dooksii, Mycosphaerella dearnessii*, needles diseases.

Seasonal variation in the infection level of *Cedrus libani* needles by *Ploioderma cedri*

A. Lehtijärvi* and H. T. Doğmuş-Lehtijärvi

Faculty of Forestry, Süleyman Demirel University, 32260 Isparta, Turkey.

*Corresponding author. E-mail: asko@orman.sdu.edu.tr.

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Cedrus libani forests are presently found mainly in the Taurus Mountains of Turkey while only small populations of the once extensive and magnificent cedar forests remain in Lebanon and Syria. Since 2004, we have observed browning of needles in spring in the lower part of the canopy of the trees in some *C. libani* stands in the lakes district of Turkey. The disease occurred both on saplings growing as understory in mixed forest as well as on approximately 10 m tall trees in an even aged stand. The frequent fruiting of *Ploioderma cedri* on dead parts of otherwise green needles indicates that the fungus was the causal agent of the disease. There has been considerable variation in the level of infection from one year to another. In the present study, we estimated the effect of the disease on needle biomass.

Key words: Cedar forest, Turkey, *Ploioderma cedri*, disease.

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Neonectria sp., a new pathogen causing cankers on Norway spruce?

Arja Lilja[1]*, Anna Rytkönen[1], Marja-Leena Napola[2], Venche Talgø[3], Marja Poteri[4] and Jarkko Hantula[1]

[1]Finnish Forest Research Institute, Vantaa, Finland.
[2]Finnish Forest Research Institute, Läyliäinen, Finland.
[3]Norwegian Institute for Agricultural and Environmental Research, Plant Health and Plant Protection Division, 1432 Ås, Norway.
[4]Finnish Forest Research Institute, Suonenjoki, Finland.

*Corresponding author. E-mail: arja.lilja@metla.fi.

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In 2001, dieback and cankers with resin flow were found in a Norway spruce stand in Saarijärvi. A trial with one progeny from Poland was established by breeders in 1985. In a second evaluation in 2001, breeders noticed that several trees had their tops dying and in addition they had strange, black wounds on their trunks. The trial was thinned and dying trees were removed in 2003. In 2008, the trial was assessed again and 13% of the trees in Finnish families had wounds while 37% of the trees were damaged in the Polish provenance. In 2005, similarly damaged trees were found in other progeny trials and plantations. In all trials, trees representing southern origin had more damages than Finnish spruces. More recently, 5 to 30 year-old stands suffering from the same kind of cankers and necrotic lesions were found in private forests in different parts of Finland. In most cases, the origin of Norway spruce seedlings used for planting has not been possible to trace. The same disease has been reported from Norway on white fir, Siberian fir, subalpine fir and also on Norway spruce, especially in south eastern Norway. Fungal isolations done in Finland and Norway resulted in several fungi, but the only common fungal genus was *Neonectria*. The sequencing of the internal transcribed spacer (ITS) of ribosomal DNA showed that most isolates from Finland were identical to *Neonectria fuckeliana,* while the Norweigian isolates were most similar to *N. ditissima* (*N. galligena)*. In inoculation tests both species were pathogenic.

Key words: Progeny, necrotic lesions, resin flow, Finland, Norway.

Transmission of *Diplodia pinea* via the new invasive insect *Leptoglossus occidentalis*

N. Luchi[1,2], V. Mancini[1], M. Feducci[1] A. Santini[2] and P. Capretti[1]*

[1]Università degli Studi di Firenze, Dipartimento Biotecnologie Agrarie, Sezione di Protezione delle Piante, Piazzale delle Cascine 28, 50144 Firenze, Italy.
[2]Consiglio Nazionale delle Ricerche, Istituto di Protezione delle Piante, Via Madonna del Piano 10, 50019, Sesto Fiorentino, Firenze, Italy.

*Corresponding author. E-mail: paolo.capretti@unifi.it.

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During the last years global changing showed its impact on forest ecosystems. In this context, the presence of new invasive species introduced from different geographical areas, may have stronger effects on native species leading to changes on tripartite interactions among plant-pathogen-insect associations. Since 1999 in Italy a new insect species, *Leptoglossus occidentalis*, originated from North America has been introduced. This insect which is able to cause several damages on cones of conifer trees has spread on *Pinus pinea* (Italian stone pine) plantations. This pine species is also one of the hosts of a native fungus (*Diplodia pinea*) which is becoming an increasing threat because of stresses due to global warming. Since both insect and fungus have been found living in the cones of the same host, a possible interaction between these two organisms has been hypothesized and a molecular method developed by using real-time polymerase chain reaction (PCR) (TaqMan™ chemistry) was used to detect and quantify the fungal presence on the insect body. The aim of this study, supported by the project PINITALY (MiPAAF DM 256/7303/2007), was to ascertain an association between *L. occidentalis* and *D. pinea* on *P. pinea* cones. For this purpose groups of individuals insects collected in the forest but also laboratory-grown were analyzed. In the lab, insects were processed after: i) artificial contamination with a conidial suspension of *D. pinea*; ii) walking on pine cones infected with *D. pinea.* Samples not treated were used as negative control. The conidial contamination by soaking insect body reproduced a possible source of conidia after rainfall, while the walking on infected cones simulated another possible type of insect contamination. Molecular analysis after real-time PCR showed the presence of *D. pinea* DNA on insects, either in the case of those collected from forest and also from laboratory, showing significant differences among the different samples. The use of rapid and sensitive molecular tools leads to detect a fungal pathogen in a DNA extracted from insect body, revealing the association between the native fungal pathogen, *D. pinea*, and the invasive insect species, *L. occidentalis*.

Key words: Global warming, invasive insect species, real time PCR, fungal pathogen.

Red band needle blight – Molecular screening of the Czech Republic

T. Májek* and V. Tomešová

Department of Forest Protection and Wildlife Management, Faculty of Forestry and Wood Technology, Mendel University in Brno, Czech Republic.

*Corresponding author. E-mail: t.majek@seznam.cz.

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Red band needle blight is considered to be a serious disease of pine trees and some other conifers. It is caused by two cryptic deuteromycete species, *Dothistroma septosporum* (teleomorph *Mycosphaerella pini* Rostr.) or *Dothistroma pini* (teleomorph unknown) (Ioos, 2010). Originally considered as one species, *D. seprosporum* and *D. pini* were distinguished in 1993 by Barnes using morphological a molecular markers. Identification just by morphological characteristics is not easy and in most cases even impossible. Using specific primers, screening and differentiation of these two species is feasible. The entire collection of *Dothistroma* strains of the Department of Forest Protection and Wild Life Management was screened. DNA of *Dothistroma* specimens was isolated using commercially available kits, the specific recognition regions were analyzed using polymerase chain reaction (PCR) and visualized on agarose gels.

Key words: *Dothistroma septosporum*, molecular markers, polymerase chain reaction.

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Endophyte communities associated with northern Spain forests: Influence of environmental variables

P. Martínez-Álvarez*, J. Martín-García and J. J. Diez

Sustainable Forest Management Research Institute, University of Valladolid – INIA Palencia. Spain, Avenida Madrid, 57. 34004 Palencia, Spain.

*Corresponding author. E-mail: pmtnez@pvs.uva.es.

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Over 60's a lot of pine plantations were established in the north of Spain, coexisting with native forests of *Quercus pyrenaica* Willd. Nowadays, they need to be conserved because of their ecological relevance. For this purpose, it is necessary to know thoroughly the existing relationships among members of forest systems. Fungal endophytes, which colonize living plant tissues without causing any immediate overt negative effects are still poorly known, especially in the Mediterranean region. Furthermore, it is also important to study the effect of different environmental and silvicultural variables on fungi. Branches and needles/leaves of *Pinus sylvestris*, *Pinus nigra*, *Pinus pinaster* and *Q. pyrenaica* from 36 trees (in twelve sites, three per species) of the province of Palencia were collected. Thus, environmental, dendrometric and dasometric features of the host were measured in order to find a relation with respect to the fungal endophytic community. A total of 46 fungal species were isolated and some relations with variables studied were found.

Key words: Endophytic fungi; environmental variables; soil properties; crown condition; dendrometric variables.

Differences in twig endophyte assemblages between native black poplar (*Populus nigra*) and a cultivated hybrid poplar (*Populus x euramericana*)

J. Martín-García[1,2]*, M. M. Müller[3] and J. J. Diez[1]

[1]Sustainable Forest Management Research Institute, University of Valladolid – INIA. Palencia. Spain. Avenida Madrid, 57. 34004 Palencia, Spain.
[2]Forestry Engineering, University of Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain.
[3]The Finnish Forest Research Institute, P. O. Box 18, FIN-01301 Vantaa, Finland.

*Corresponding author. E-mail: jorgemg@pvs.uva.es.

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The European black poplar (*Populus nigra*) is considered as one of the most seriously endangered indigenous tree species in Europe. The question remains whether a possible extinction of *P. nigra* through replacement by *Populus x euramericana* or a possible drastic decrease in genetic variation through intensive introgression would imply the extinction of those organisms that are obligatory associates of *P. nigra*. To achieve this, we compared the endophytic mycota in twigs of native poplar (*P. nigra*) and hybrid poplar plantations (*P. x euramericana* clone I-214). Twig endophytes were isolated from native and hybrid poplar stands in Palencia (N. Spain), and identified according to

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sequences of the internal transcribed spacer (ITS) region of their rDNA. The results reveal that the endophyte community on poplar forests may be affected by an extinction of *P. nigra* or a drastic decrease in genetic variation, because it differed considerably between native and hybrid poplars of this study.

Key words: Native, hybrid, poplar, endophyte, twig.

Changes of the shrub structure in a Turkey-oak forest after a tree decline in North-Hungary

Tamás Misik[1]*, Béla Tóthmérész[2] and Imre Kárász[1]

[1]Department of Environmental Science, Eszterhazy Karoly College, Eger, H-3300 Hungary.
[2]Department of Ecology, University of Debrecen, Debrecen, H-4032 Hungary.

*Corresponding author. E-mail: misikt@ektf.hu.

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Similar to other European countries there was an oak decline at the end of the 70's in Hungary; 68% of the *Quercus petraea* specimens of the forest's trees died at the site. This decline resulted in an opening of the canopy; the canopy cover decreased from 80% (1972) to 36% (2007). The aim of our study was to analyse the structural changes in the forest interior after oak decline. We focused on those specimens which were higher than 8 m (so-called secondary trees). Height and diameter of these specimens were registered; their location and cover percentage were mapped from 1972. We focused on the following questions: (1) What are the most important structural changes in the forest interior after the oak decline? (2) Which woody species are the most successful shade-tolerant species and what are the possible ecological reasons of this process? In 2007, there were 130 specimens of *Acer campestre*, 22 specimens of *Cornus mas* and 4 specimens of *Acer tataricum* between 8 - 13 m height and created a secondary tree layer below the primary tree layer of oaks. The mean cover of these species increased remarkably after the tree decline. The canopy cover was 32% of high shrub layer cover in this newly formed layer. Our study reveals that the forest responded to the oak decline by structural changes in the shrub layer and three woody species of secondary tree layer compensated the remarkably loss of tree canopy cover.

Key words: Oak decline, *Acer campestre*, *Quercus petraea*, density, canopy cover, secondary tree layer.

Does long distance gene flow occur between subpopulations of *Lophodermium piceae*, the most common needle endophyte of Norway spruce?

M. M. Müller[1]* and T. Sieber[2]

[1]Finnish Forest Research Institute, Vantaa Research Unit, P. O. Box 18, FI-01301 Vantaa, Finland.
[2]ETH Zürich, Institute of Integrative Biology, Forest Pathology and Dendrology, Switzerland.

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*Corresponding author. E-mail: Michael.Mueller@metla.fi.

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Lophodermium piceae is a ubiquitous endophytic inhabitant of Norway spruce needles (*Picea abies)*. It can generally be found in the majority of older (> 2 yr) needles of single trees and it may be one of the most numerous fungi in spruce forests. The fungus is transmitted by aerial ascospores, which are formed on dead needles still attached to twigs in the tree crown or on fallen senescent needles. Locally, *L. piceae* is a highly diverse fungus and it is difficult to find identical (characterized by DNA markers) isolates even within a single needle. The aim of this study was to examine the degree of differentiation within and among Eurasian subpopulations separated by various distances and geographical barriers. For this purpose, samples of seven subpopulations (including 14 to 46 isolates/subpopulation) were collected along a north-south transect stretching from the northern timberline in Finnish Lapland to the southern border of the distribution area of Norway spruce in northern Italy. Additionally, isolates obtained from areas nearby Irkuts, Siberia, were included. The investigation included in total of 227 isolates. Differentiation between *L. piceae* subpopulations was determined from DNA sequences of three genetic markers. One of the markers was the internal transcribed spacer (ITS) of the ribosomal DNA and the other two (LP1 and LP2) were sequence characterized amplified regions (SCAR) found in *L. piceae*. Results including sequences of Finnish, Belarusian, Swiss, Italian and Siberian isolates showed low differentiation among populations. According to analysis of molecular variance, among the subpopulation variation was 1, 2 and 3% in ITS, LP1 and LP2 markers, respectively. This low variation among subpopulations indicates high gene flow between them.

Key words: Genetic differentiation, SCAR, DNA markers, fungi, ascomycete.

Monitoring damage from foliage, shoot and stem diseases in New England and New York.

I. A. Munck* and R. L. Lilja

USDA Forest Service, Northeastern Area State and Private Forestry, Forest Health Protection, 271 Mast Road, Durham, NH 03824, USA.

*Corresponding author. E-mail: imunck@fs.fed.us.

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Forests are an important resource in New York and the six states that comprise New England. Sixty-six percent (206,646 km^2) of the land area is forested, 83% of which is privately owned. The region's forests are ecologically diverse. The most common forest types are maple (*Acer* spp.), spruce/fir (*Picea*/*Abies* spp.), other hardwoods (*Fagus*, *Prunus*, *Fraxinus*, *Betula*, *Carya*, *Quercus* spp.), hemlock (*Tsuga canadensis*) and pine (*Pinus* spp.). Foliage, shoot, and stem diseases are common in the region due to favorable climate and the wide variety of available host tree species. To protect forest resources, State Forestry Agencies in cooperation with the US Forest Service monitor forest health conditions annually. States participate in national aerial detection surveys and visit resource-specific and pest-specific permanent plots. Data from these surveys are analyzed to assess impact of forest diseases and insect pests. For instance, data from aerial and ground surveys are used to map the spread of beech bark disease, which is endemic in New England and New York but continues to expand throughout the range of American beech. Beech bark disease is a disease complex involving the beech scale (*Cryptococcus fagisuga*) and canker fungi in the genus *Neonectria*. Aerial and ground surveys are also used to identify forest disease trends. For example, foliar diseases and shoot blights including anthracnoses, conifer needle casts, and *Sirococcus* shoot blights proliferated since at least 2006 due to

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unusually wet spring weather. Results from forest health monitoring efforts are made publicly available via web-based products such as the Forest Pest Portal (http://www.foresthealth.info/) and Forest Health Highlights (http://fhm.fs.fed.us/fhh/fhmusamap.shtml).

Key words: Forest health, detection, invasive, exotic, alien pests

Impact of Beech bark disease on the sustainability of American Beech in New York

I. A Munck[1]* and P. D. Manion[2]

[1]USDA Forest Service, Northeastern Area State and Private Forestry, Forest Health Protection, 271 Mast Road, Durham, NH 03824, USA.
[2]State University of NY-College of Environmental Science and Forestry (SUNY-ESF), 1 Forestry Drive, Syracuse, NY 13210.

*Corresponding author. E-mail: imunck@fs.fed.us.

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Beech bark disease (BBD) is a disease complex involving the beech scale (*Cryptococcus fagisuga*) and canker fungi in the genus *Neonectria*. Beech bark disease has affected American beech (*Fagus grandifolia)* in eastern North America since its introduction in the 1890s. Northeastern forests comprised diverse tree species. Factors associated with tree species composition could affect the distribution of the BBD causal agents, which in turn could impact the sustainability of beech. A sustainable forest ecosystem was defined as one that offsets current growth with current mortality, thus maintaining a stable size-structure relationship. Data from 539 plots with beech, containing 2,495 beech trees, were analyzed. Eleven forest types with beech were identified. For American beech populations within each forest type, the baseline mortality required to maintain a stable size-structure relationship was compared to the observed mortality. Consistently greater than predicted mortality in the mid to large diameter (dbh = 31 - 46 cm) classes indicate sustainability problems in these dbh classes for beech populations in the sugar maple (*Acer saccharum*)-beech, red maple (*Acer rubrum*)-beech, and eastern hemlock (*Tsuga canadensis*) forest types. Cutting had contributed to the mortality in dbh classes >26 cm in the sugar maple-beech forest type, which had the greatest proportion of beech (43% of all trees). Beech regeneration is abundant and BBD-free trees were present even in the large DBH classes, suggesting the future build up of a resistant population.

Key words: Nectria, forest structure, landscape-scale analyses, invasive, exotic, alien pests.

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Epidemic and pathogenicity of *Chalara fraxinea* causing ash dieback in Hungary

L. Nagy[1,2]* and I. Szabó[2]

[1]Szombathely Forestry Corporation, Sárvár Forestry Management, H-9700 Szombathely, Sáághy István u. 15.
[2]University of West Hungary, H-9400 Sopron, Bajcsy Zs. u. 4, Hungary.

*Corresponding author. E-mail: nagyl@sarvar.szherdeszet.hu.

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In Hungary the ash dieback caused by *Chalara fraxinea* was first observed in spring 2008 in the plots of the Sárvár and South-Hanság Forestry Management units. The disease affected both artificially planted saplings and natural regrowths. Symptoms included leaf and shoot wilting, brown discolouration in the bark as well as greyish-brownish discolouration of the wood. Since then the disease has been proved to be widespread and to endanger the health state of ash trees of different age seriously. The susceptibility of ash species was examined by artificial infection of one-year-old saplings in a nursery. More hundred saplings were wound-inoculated with the mycelium of the pathogen. Common ash and narrow-leafed ash were found susceptible in the experiment: 24 and 21% of the inoculated trees showed wilting after 2 to 3 weeks. The green ash and the flowering ash did not alter. Between 2008 and 2010 investigations were carried out in an artificial common ash regeneration. The frequency of the symptoms and their dynamics were observed in three survey plots of 0.1 ha each. In 2008, the frequency of the symptoms of the disease was low, only 0.8 to 1.2%. In 2009, the disease spread significantly: 8.2 to 20.9% of the trees showed fresh symptoms. In spring 2010, the number of infected trees decreased again: 2.7 to 9.3% of the shoots wilted. This change is connected with the lower amount of rainfall in summer and autumn of the previous year. The precipitation in the infectious period (August-September) should determine ash dieback in spring next year.

Key words: *Chalara fraxinea*, *Fraxinus*, susceptibility, inoculation.

Red pine logging debris as a potential source of inoculum of *Diplodia* shoot blight pathogens

B. W. Oblinger, D. R. Smith and G. R. Stanosz*

Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, USA.

*Corresponding author. E-mail: grs@plantpath.wisc.edu.

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Following the observation of a high incidence of *Diplodia* shoot blight on recently planted red pine (*Pinus resinosa*) seedlings growing where mature red pine stands previously had been clearcut, the potential of logging debris as a source of inoculum of *Diplodia* pathogens was investigated. Cones, bark, needles, stems from shoots bearing needles, and stems from shoots not bearing needles (both suspended above the soil and in soil contact) were collected from debris left at sites that were previously clearcut. Conidia were extracted in water, quantified, tested for germinability, and *Diplodia* species were identified from samples using a polymerase chain reaction (PCR) assay. Conidia of *Diplodia*

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species were detected from all study sites. Repeated sampling of the same sites at 6, 12, and 18 months postharvest revealed an initial increase in the numbers of conidia. Although fewer conidia were obtained from debris collected from additional sites at greater intervals since harvest (and fewer of these germinated), all substrates yielded many germinable conidia even 5 years postharvest. The type of host substrate from which conidia were extracted had an effect on the number of conidia quantified and the percentage of conidia that germinated. Also, more conidia were obtained and a greater percentage germinated from debris that was suspended above soil at the time of collection than from debris in soil contact. Because red pine seedlings are commonly planted in close proximity to logging debris on clearcut sites and germinable conidia were abundant, debris could be a potential persistent source of inoculum for *Diplodia* shoot blight pathogens to planted seedlings. The results of this study should prompt further consideration by land managers of the potential forest health risks, in addition to benefits, that may be associated with logging debris.

Key words: Conidia, debris, *Diplodia, inoculum*, pine, *Pinus resinosa*, *Sphaeropsis*.

Cedrus libani - A new host for *Herpotrichia juniperi*

F. Oskay[1], E. Halmschlager[2]*, A. Lehtijarvi[1] and H. T. Doğmuş- Lehtijarvi[1]

[1]Faculty of Forestry, Süleyman Demirel Üniversity, 32260 Isparta-Turkey.
[2]Institute of Forest Entomology, Forest Pathology and Forest Protection, Hasenauerstraße 38 A-1190 Wien-Austria.

*Corresponding author. E-mail: erhard.halmschlager@boku.ac.at.

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Lebanon cedar (*Cedrus libani*) is an ecologically, economically and historically important tree species that has been exposed to severe anthropogenic impacts through intensive cutting, burning and goat grazing over the past 5000 years resulting in an excessive reduction in population size. Today, excluding small and degraded populations in Lebanon and Syria, the primary natural distribution of Lebanon cedar is in the Taurus Mountains of Asia Minor. *C. libani* is one of the most important tree species occurring at high altitudes up to 2100 m in Turkey thereby contributing to the highly diverse protective functions of high mountain forests. Efforts to protect existing forests and natural regeneration of this endangered tree species were undertaken in recent years; however, there is a lack of special techniques to develop and protect high mountainous forest ecosystems in Turkey. Recently, brown felt blight caused by *Herpotrichia juniperi* was observed for the first time on Lebanon cedar in Turkey. *H. juniperi* is known to have the potential to destroy a whole natural regeneration after a winter with a long lasting, thick snow cover, often combined with snow melt late in spring. This pathogen seems to be an endemic species coevolved with its host species. However, it may play an important role in distribution and existence of *C. libani* and other host species at high altitude forest as a disturbance agent that affects the survival and growth of its hosts. The current situation, possible threats and the impact of climate change on the occurrence and the magnitude of damage of *H. juniperi* is discussed.

Key words: *Cedrus libani*, brown felt blight, *Herpotrichia juniperi*, Turkey, high elevation forests.

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Evaluation of genetic resistance to *Fusarium circinatum* in *Pinus* species

E. Quintana[1], Y. Serrano[2], N. Mesanza[1], M. Elvira-Recuenco[2], R. Raposo[2] and E. Iturritxa[1]*

[1]NEIKER, Granja Modelo – Arkaute, Apdo. 46, 01080 Vitoria-Gasteiz, Spain.
[2]CIFOR-INIA, Ctra. Coruña Km 7.5, 28040 Madrid, Spain.

*Corresponding author. E-mail: eiturritxa@neiker.net.

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Pitch canker is *a* disease caused by the fungal species *Fusarium circinatum.* This disease is the main cause of damages in nurseries and plantations of *Pinus* in the northern area of Spain. Its presence involves applying eradication measures producing serious economical, ecological and social impacts in the Cantabrian coast. Susceptibility to these pathogens could be due to a variety of factors such as drought, physical damage or other environmental stresses, and host species. Currently, control, prevention and eradication of *Fusarium circinatum* are hard to achieve and disease management becomes difficult and highly expensive. The objective of this study was to evaluate the response of the main conifer species grown in Spain, *Pinus sylvestris* L., *Pinus nigra* Arnold, *Pinus pinaster* Aiton, *Pinus radiata* D. Don, *Pinus halepensis* Mill, *Pinus pinea* L. and *Pinus uncinata* Mill. Ex Mirb. to the inoculation of 5 isolates of *F. circinatum* (Mat 1 and Mat 2). Artificial inoculations are considered to be a convenient and relatively effective way of evaluating the inter-specific resistance of pines to *F. circinatum*. Accordingly, two-year-old shoots were inoculated with a drop of spore suspension placed in a wound previously done. Lesion length was measured three weeks after inoculation. The experiment design was a completely randomized factorial. Analysis of variance and multiple comparison procedure of Bonferroni were performed on the lesion length. Preliminary results show that *P. radiata* was the most susceptible species to *F. circinatum*, whereas the most resistant were *P. pinea*, *P. halepensis*, *P. nigra* and *P. pinaster*. Mat 2 isolates were more virulent than Mat 1 isolates.

Key words: Pine, Spain, inoculation, susceptibility.

Effects of temperature, pH and osmotic potential on *in vitro* mycelial growth of *Gremmeniella abietina* isolates infected by mitoviruses

Carmen Romeralo[1]*, Leticia Botella[1], Oscar Santamaría[2] and Julio Díez[1]

[1]Sustainable Forest Management Research Institute, University of Valladolid-INIA, Avda. Madrid 44, Building E, 34004 Palencia, Spain.
[2]Dpto. Ingeniería del Medio Agronómico y Forestal. Escuela de Ingenierías Agrarias (Universidad de Extremadura). Ctra. de Cáceres, s/n. 06007 Badajoz, Spain.

*Corresponding author. E-mail: carmen.romeralo@pvs.uva.es.

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Mitoviruses have been found in several forest pathogens (example *Gremmeniella abietina*) and because they may reduce virulence of the host fungi their use is being studied for biocontrol purposes. A preliminary study was carried out to test the effect of temperature (5, 15, 25 and 35°C), pH (4, 5, 7 and 9) and osmotic potential ([KCl] of 250, 500, 750 and 1000 mM) on mycelial growth of seven *G. abietina* isolates under laboratory conditions. Four of the isolates hosted mitoviruses and three of them did not. During the experiment, mycelial growth was measured every week for a period of 8 weeks. The highest colony sizes were observed in the Petri dishes with pH 4 and 5, temperature of 15°C and 1000 mM of KCl, and lowest sizes in the ones placed at 35°C. No differences were observed among isolates in experiments developed at 5 and 25°C and pH of 9. However, Petri dishes placed at 15°C presented differences on mycelial growth if isolates were grouped in mitovirus and not mitovirus presence. Colony areas measured in pH of 4, 5, 7 and 750 mM KCl treatments presented differences among isolates when analyzed altogether. Mycelial growths of isolates with mitovirus were higher than the ones without mitoviruses at 15°C (p=0.0188) and 1000 mM KCl (p=0.0001). On the contrary, they were lower in dishes with 250 mM (p=0.0086) and 750 mM (p<0.0001) KCl.

Key words: Mitoviruses, Scleroderris canker, *in vitro*, *Gremmeniella abietina*.

Investigations on *Phytophthora plurivora and Phytophthora pini* in Finland

A. Rytkönen[1]*, A. Lilja[1], M. Soukainen[2], P. Parikka[3], S. Werres[4], M. Poteri[5] and J. Hantula[1]

[1]Finnish Forest Research Institute, P. O. Box 18, FI-01301 Vantaa, Finland.
[2]Finnish Food Safety Authority Evira, Mustialankatu 3, FI-00790 Helsinki, Finland.
[3]MTT Agrifood Research Finland, FI-31600 Jokioinen, Finland.
[4]Julius Kühn Institute (JKI) - Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Horticulture and Forests, Messeweg 11/12, D-38104 Braunschweig, Germany.
[5]Finnish Forest Research Institute, Juntintie 154, 77600 Suonenjoki, Finland.

*Corresponding author. E-mail: anna.rytkonen@metla.fi.

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The ornamental plant trade has unwittingly trafficked alien Oomycetes, such as pathogens in the genus *Phytophthora*, around the globe. So far, four *Phytophthora* species have been identified on seedlings in Finland: *Phytophthora cactorum, Phytophthora plurivora*, *Phytophthora pini* and *Phytophthora ramorum.* The recently described *P. plurivora* is found to be abundant in semi-natural ecosystems and nurseries across Europe, causing bark necroses, fine root losses and dieback on numerous host tree species. We isolated *P. plurivora* [previously identified as *P. inflata* (Lilja et al., 2007)] for the first time in 2004 from rhododendron cultivars, and later from *Syringa vulgaris* in two nurseries in Southern Finland. Since the original finding, it has been found almost every year, despite attempted eradication procedures. Also *P. pini* was isolated once in 2007. *P. pini* has been found widespread in the eastern USA, causing damage and mortality to introduced species such as *Fagus sylvatica*. In Europe, it has only been found in nurseries, which indicates a recent introduction to the continent. We tested the susceptibility of Finnish tree species in addition to other forest plants to infection by *P. plurivora* and *P. pini*. In our pathogenicity trials, both species were able to infect most host plants including *Fragaria* x *ananassa*, *Rhododendron* sp., *Betula pendula, Alnus incana, Alnus glutinosa, Picea abies, Vaccinium uliginosum, Vaccinium myrtillus, Vaccinium vitis-idaea* and *S. vulgaris.* The only resistant woody species in our trials was *Pinus sylvestris*. Both hyphae and oogonia were seen in cortical cells of Norway spruce seedlings inoculated with *P. plurivora*. In preliminary trials for control measures, none of the tested chemicals (Aliette, Restart, Cumin oil) was effective with the concentrations and application schedules used. None of the *Phytophthora* species

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present in Finnish nurseries has been found in natural ecosystems. As *P. plurivora* has been very recently encountered on many tree species in semi-natural ecosystems in other Scandinavian countries, further screening for this tree pathogen in addition to other *Phytophthora* spp. will be conducted in Southern Finland in 2011.

Key words: Oomycetes, nursery, plant trade, oogonia.

REFERENCE

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Research on Oak decline disease in Spain

G. Sanchez[1]*, J. J. Tuset[2], C. Hinarejos[2], J. L. Mira[2] and M. Prieto[1]

[1]Ministerio de Medio Ambiente y Medio Rural y Marino, Spain.
[2]Instituto Valenciano de Investigaciones Agrarias, IVIA, Spain.

*Corresponding author. E-mail: gsanchez@mma.es.

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Holm oak and cork oak are the two *Quercus* species affected by oak decline (named “seca” in Spanish) in Spain. *Phytophthora cinnamomi* is the soil fungus associated with this disease. Since 1991 this fungus has been isolated from feeder roots of both oak species and after analyzing more than 700 soil and root samples, all from oak areas with a considerable number of damaged trees, it is considered the main cause of oak decline. Disease symptoms have been obtained by inoculating mycelium and zoospores of *P. cinnamomi* both in adult trees in the field and in seedlings (two years old) in the greenhouse. Studies on disease dynamics (zoospore production, influence of cations and anions in fungal development, influence of soil moisture, host-pathogen interaction, and zoospore infection) have been carried out over the last fifteen years both in the laboratory and the greenhouse. In addition, an experimental plot containing the Spanish *Quercus* species has been planted in a high soil contaminated area by *Phythopthora*, and monitored during the last years. A summary of all this study lines will be presented, and the results of sampling campaigns done in the affected areas during the last 20 years.

Key words: Holm oak, cork oak, *Phytophthora cinnamomi*, soil moisture.

Interaction between *Gremmeniella abietina* and several fungal endophytes

O. Santamaría[1]*, C. Romeralo[2], L. Tejerina[3] and J. J. Diez[2]

[1]Dpto. Ingeniería del Medio Agronómico y Forestal. Escuela de Ingenierías Agrarias (U. de Extremadura). Ctra. de Cáceres, s/n. 06007 Badajoz, Spain.
[2]Sustainable Forest Management Research Institute, University of Valladolid – INIA. Avenida Madrid, 57. 34004 Palencia, Spain.
[3]Laboratorio de Cultivo In Vitro. Vivero Forestal Central. Junta de Castilla y León. Cañada Real, 222 C.P. 47008 Valladolid. Spain.

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*Corresponding author. E-mail: osantama@unex.es

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Gremmeniella abietina is the causal agent of Scleroderris canker and Brunchorstia dieback in many conifer species. It has caused severe losses in nurseries and plantations worldwide and several outbreaks have occurred in North America and Central-North Europe in recent decades. In Spain, it was first isolated from symptomatic *Pinus halepensis* trees in 1999. Since then, several studies have been conducted to either determine or to evaluate its morphological, physiological and genetic variability, pathogenicity, and potential control strategies to be used if necessary. Among these studies, the evaluation of several fungal endophytes to be used as potential biocontrol agents against *G. abietina* has been carried out. In the present communication, the results of co-inoculations of both *G. abietina* and the fungal endophyte in *P. halepensis* seedling under greenhouse conditions are presented. Two isolates of *G. abietina* and two endophyte species, which previously exhibited an antagonistic behaviour against *G. abietina in vitro*, were used in the experiments. Inoculations were made on 1-year-old *P. halepensis* seedlings by wounding their bark at 8 and 4 cm below the shoot apex. Mycelia of *G. abietina* and the endophyte were placed in the lower and upper wound of *P. halepensis*, respectively. Fifteen seedlings per treatment were inoculated. In addition, single inoculations with each fungal specimen were also made to evaluate their pathogenicity in *P. halepensis*. Ten weeks after inoculation, the degree of disease caused by *G. abietina* was evaluated by the extent (length) of necroses caused by the pathogen. Statistical analyses show that none of the endophytes has a significant influence on *G. abietina* isolates as the length of necroses caused by *G. abietina* when it was inoculated alone was the same as when it was inoculated with the endophyte. It was also ascertained that the isolates of *G. abietina* showed different aggressiveness and that endophytes were not pathogens as they did not cause a necrotic length greater than the ones detected in the control. Further studies, including different timing and inoculation techniques, would be required in order to evaluate in more detail the antagonistic effect *in vivo* of such endophytes, previously stated to show a great antagonism on *G. abietina in vitro*.

Key words: *Gremmeniella abietina*, endophytes, antagonism, *in vivo*, pathogenicity.

Analysis of factors influencing canopy loss in pine stands infected by *Gremmeniella abietina* in Northern Spain

Antonio V. Sanz-Ros[1,2]*, F. Valladares[3,4] and J. J. Diez[1]

[1]Sustainable Forest Management Research Institute, University of Valladolid – INIA, Avenida Madrid, 57. 34004. Palencia, Spain.
[2]Centro de Sanidad Forestal de Calabazanos (Junta de Castilla y León). Polígono Industrial de Villamuriel. 34190, Villamuriel de Cerrato, s/n. (Palencia).
[3]Institute of Natural Resources, CCMA, CSIC, Serrano 115, E-28006 Madrid, Spain.
[4]Department of Biology and Geology, Escuela Superior de Ciencias Experimentales y Tecnológicas, University Rey Juan Carlos, c/Tulipán s/n, 28933 Móstoles, Spain.

*Corresponding author. E-mail: tonisanz@pvs.uva.es.

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In this study, the influence of environmental and silvicultural factors on canopy condition was evaluated in *Pinus halepensis* stands from Northern Spain where *Gremmeniella abietina* infections have been reported. Canopy condition was evaluated estimating defoliation and leaf area index (LAI). Hemispherical photography analysis was used as an

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indirect method to estimate LAI, and International Co-operative Programme for the Intensive Monitoring of Forest Ecosystems (ICP-Forest) methodology was used for defoliation estimation. Several environmental and stand parameters were measured in order to test their influence on canopy condition. Multivariate and multiple regression analyses were carried out to identify the main factors influencing defoliation and LAI. *G. abietina* has been found to produce severe damages on *P. halepensis* in this area, and its activity is influenced by several factors such as canopy depth, age, basal area and elevation. On the other hand, LAI is determined by mean diameter, tree density and canopy openness. The relationship between defoliation and LAI was very weak and factors affecting both parameters were different. The way in which *G. abietina* produces dieback and defoliation, being the latter heterogeneously distributed through the canopy (affecting mainly the upper part of the canopy) decrease the correlation between them. The contrary was found for other defoliating agents, such as after some insects outbreaks, in which this relationship was stronger and clearly proved. This suggests that symptoms produced by pathogens causing dieback will be not detected just estimating LAI, being necessary a visual evaluation for this purpose.

Key words: *Gremmeniella abietina*, defoliation, dieback, leaf area index, LAI, hemispherical photography, *Pinus halepensis*.

Effect of temperature on survival of *Fusarium circinatum*

Y. Serrano[1], E. Quintana[2], M. Elvira-Recuenco[1], E. Iturritxa[2] and R. Raposo[1]*

[1]CIFOR-INIA, Ctra. Coruña Km 7.5, 28040 Madrid, Spain.
[2]NEIKER, Granja Modelo – Arkaute, Apdo. 46, 01080 Vitoria-Gasteiz, Spain.

*Corresponding author. E-mail: raposo@inia.es.

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Needles and wood pieces of *Pinus radiata* were inoculated with an isolate of *Fusarium circinatum* and placed on Fusarium-free soil in plastic containers stored at 5, 20 and 30°C. Three replicates for each temperature and type of tree part were done. Needles and wood pieces were sampled periodically to estimate survival of *Fusarium circinatum*. This was assessed as the percentage of tree parts cultured on Selective Fusarium Agar (SFA) from which the fungus was recovered. Positive isolation was microscopically confirmed by the presence of circina growing on Spezieller Nährstoffarmer Agar (SNA) medium. *F. circinatum* was recovered from 100% of the inoculated wood pieces during the first seven and nine months at 20 and 5°C respectively, while it decreased very slowly from the beginning at 30°C. After 380 days, *F. circinatum* was recovered in more than 70% of the inoculated wood pieces for all temperatures tested. In needles, *F. circinatum* survived in almost 100% of the samples after nine months at 5 and 20°C. Survival on needles at 30°C began to decrease after three months, and it was the lowest (89%) after 380 days.

Key words: Needles, wood, inoculation, *Pinus radiata*.

Distribution of *Mycosphaerella* leaf disease on Eucalyptus in Portugal

M. C. Silva[1]*, H. N. Machado[1], L. Neves[2], C. Valente[3] and A. J. L. Phillips[4]

[1]Instituto Nacional dos Recursos Biológicos, Edifício da ex-Estação Florestal Nacional, Quinta do Marquês, 2780-159 Oeiras, Portugal.

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[2]Altri Florestal, S.A., Quinta do Furadouro 2510 – Amoreira OBD.
[3]Instituto RAIZ, Quinta de S. Francisco Ap. 15 – 3801 – 501 Eixo – Aveiro.
[4]Centro de Recursos Microbiológicos, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal.

*Corresponding author. E-mail: marcia.silva@inrb.pt.

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Eucalypt plantations represent the main source of wood for the pulp and paper industry and are affected by an important foliage disease worldwide - the complex of *Mycosphaerella* and *Teratosphaeria* species (*Mycosphaerella* leaf disease). These genera affect mainly young trees with juvenile-phase foliage, causing premature defoliation, decreased growth and wood production. Species of *Mycosphaerella senso lato* reported on eucalypts in Portugal are *Mycosphaerella communis, Mycosphaerella heimii, Mycosphaerella lateralis, Mycosphaerella madeirae, Mycosphaerella marksii, Mycosphaerella walkeri, Teratosphaeria africana, Teratosphaeria molleriana, Teratosphaeria nubilosa and Teratosphaeria parva.* Since 2004, in order to complete the survey, symptomatic leaves were collected from *E. globulus* plantations. Morphological and molecular characterization was used to give a clear indication of the population composition and the main species.

Key words: *Mycosphaerella*, *Teratosphaeria*, leaf disease, MLD, *Eucalyptus*.

Pathogenicity trials with *Gremmeniella* fungi collected on conifers in Canada

E. Smerlis and G. Laflamme*

Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, Québec, QC, Canada G1V 4C7.

*Corresponding author. E-mail: Gaston.Laflamme@RNCan-NRCan.gc.ca.

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Gremmeniella abietina var. *balsamea* isolated from balsam fir (*Abies balsamea*) and spruces (*Picea* spp.) was tested for pathogenicity on different conifer hosts including *A. balsamea* and *Picea* spp. Pathogenicity of the fungus was positive on balsam fir only. This pathogen could not colonize other conifers, not even spruces which are hosts included in the taxonomic entity *G. abietina* var. *balsamea*. Also, inoculation trials with isolates from spruces and pines on several conifer species are specific to their respective hosts. These results raise questions on the taxonomic status of the two pathogens classified as var. *balsamea*. We believe that both pathogens on spruce and balsam fir should be promoted to the species level for two reasons: 1) isolates from balsam fir, spruces and pine are specific to their hosts, and 2) they have a colour in pure culture that is characteristic of each three groups of isolates. The species *G. laricina* is morphologically very different from all other known species of *Gremmeniella*. All *Gremmeniella* native to North America cause damage only on shoots in the snow.

Key words: Scleroderris canker, *Gremmeniella* spp., *Abies balsamea, Picea* spp.

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Storage conditions influence cultural detection of the shoot blight pathogen *Diplodia pinea* on or in asymptomatic red pine nursery seedlings

D. R. Smith[1], J. Albers[2] and G. R. Stanosz[1]*

[1]Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, USA.
[2]Division of Forestry, Minnesota Department of Natural Resources, Grand Rapids, MN 55744, USA.

*Corresponding author. E-mail: grs@plantpath.wisc.edu.

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The pine shoot blight and canker pathogen *Diplodia pinea* has been shown to persist on or in asymptomatic red pine nursery seedlings, with later potential to rapidly proliferate after outplanting to cause disease, including seedling mortality. After lifting from nursery beds, seedlings are routinely kept in cold storage at nurseries, but during and after shipment to customers may be maintained without refrigeration for several days prior to planting. The potential for both the duration and temperature of storage to influence the frequency of cultural detection of *D. pinea* from asymptomatic red pine seedlings was investigated. In the first two experiments, surface-disinfested stem segments from seedlings were culturally assayed for *D. pinea*: shortly after lifting in spring; after 3 weeks of cold storage (approximately 4°C in experiment 1) or 4 weeks of cold storage (approximately 8°C in experiment 2); or after 3 weeks of cold storage followed by 1 week of storage at approximately 24°C in both experiments). Probably due to implementation of a program of scrupulous sanitation and application of preventative fungicidal sprays at the nursery, *D. pinea* was infrequently detected, and no effects of storage were apparent. In two additional experiments, seedlings were inoculated with a suspension of *D. pinea* conidia and then similarly assayed: after 3 weeks of cold storage (approximately 4°C in experiment 3) or 4 weeks cold storage (approximately 8°C in experiment 4); or after 3 weeks of cold storage followed by 1 week of storage at approximately 24°C in both experiments. In experiments 3 and 4, in which the pathogen was initially present due to inoculation, frequency of detection of the pathogen was greater after longer storage and after storage at a warmer temperature. This indicates that the association of the pathogen with seedlings may be affected by storage conditions. Thus, when inoculum is present, minimization of the duration of storage and maintenance of cold temperatures during storage may inhibit persistence of *D. pinea* on or in seedlings, and help to reduce later seedling mortality.

Key words: *Diplodia pinea*, pine, *Pinus resinosa*, seedling, *Sphaeropsis*, storage.

Cultural detection of *Diplodia* shoot blight pathogens from red pine and Jack pine seeds

D. R. Smith, J. Albers and G. R. Stanosz*

[1]Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, USA.
[2]Division of Forestry, Minnesota Department of Natural Resources, Grand Rapids, MN 55744, USA.

*Corresponding author. E-mail: grs@plantpath.wisc.edu.

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The pine shoot blight and canker pathogens *Diplodia pinea* and *Diplodia scrobiculata* are among the many fungi associated with seed cones of conifers. Each of these species has been found to commonly and abundantly sporulate on cones of red pine (*Pinus resinosa*) and jack pine (*Pinus banksiana*) collected in Wisconsin and Minnesota forests. Cultural methods were used to investigate the incidence of these fungi on or in seeds obtained from government nurseries in Minnesota (three red pine seedlots) and Wisconsin (five red pine seedlots and five jack pine seedlots). Seeds were extracted, cleaned, and stored using standard methods at each nursery. In each of the three replicate trials, seeds of each lot were assigned to four treatments: 1) not surface disinfested, 100 seeds; 2) surface disinfested, 50 seeds; 3) surface disinfested and then inoculated with *D. pinea* conidia, 50 seeds; or 4) not surface disinfested but then inoculated with *D. pinea* conidia, 50 seeds. Each seed was placed in a slant containing tannic acid agar and autoclaved pine needles, and incubated for up to 6 weeks. Development of pycnidia with conidia consistent with those of *Diplodia* species indicated the presence of either pathogen. For red pine seeds, the mean percentage positive was 2.7% for treatment 1 and 1.3% for treatment 2. Jack pine seeds were less frequently positive for both treatments. Using species-specific polymerase chain reaction (PCR) primers, the *Diplodia* species cultured was identified as *D. pinea* in almost every case, with identification of *D. scrobiculata* only rarely. *D. pinea* was much less frequently detected from seeds that were not surface disinfested but then inoculated (treatment 4) compared to seeds that were inoculated with *D. pinea* after surface disinfestation (treatment 3). This indicated that the presence of seed-surface microflora led to underestimation of the actual presence of the pathogen in treatment 1. Results confirm the potential for dissemination of *D. pinea* on red pine and jack pine seeds. Although the frequency of positive seeds was low, the large numbers of seeds planted in nurseries suggest that seeds may be a potentially important route of entry of *D. pinea* into nursery beds.

Key words: *Diplodia pinea, Pinus banksiana, Pinus resinosa*, pine, seed, *Sphaeropsis*.

Expansion in the known geographic distribution and host range of the shoot blight pathogen *Sirococcus tsugae*

G. R. Stanosz[1]*, D. R. Smith[1], J. P. Sullivan[2], A. M. Mech[3], K. J. K. Gandhi[3], M. J. Dalusky[4], A. E. Mayfield[5] and S. W. Fraedrich

[1]Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, USA.
[2]Georgia Forestry Commission, Gainesville, GA 30507, USA.
[3]Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602, USA.
[4]Department of Entomology, University of Georgia, Athens 30602, USA.
[5]USDA Forest Service, Asheville, NC 28804, USA.
[6]USDA Forest Service, Athens, GA, 30602, USA.

*Corresponding author. E-mail: grs@plantpath.wisc.edu.

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Eastern hemlock (*Tsuga canadensis*) is an ecologically and economically important conifer from the north-central United States to the east coast of North America to the southern Appalachian Mountains. In early spring 2010, blighted shoot tips of eastern hemlock were observed at widely separated locations in the Chattahoochee National Forest in north Georgia. Damage did not appear to be directly related to hemlock woolly adelgid (*Adelges tsugae*) activity, which was sporadic or absent in some areas where symptoms were observed. A preliminary survey in March 2010 revealed that incidence of blighted shoots on individual trees varied, but was as high as 70%. Stems of shoots produced the previous

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year were frequently necrotic, had lost needles, and bore pycnidia with hyaline, two-celled conidia consistent with those of *Sirococcus tsugae*. Later in the spring and summer, shoots of the current year's growth became blighted, with sporulation of *S. tsugae* also on dead and dying needles. While *S. tsugae* previously has been reported on *T. heterophylla*, *T. mertensiana*, *Cedrus atlantica*, and *C. deodara* in western North America, it has only recently been reported on eastern hemlock, and its ability to induce shoot blight had not been proven. Pure cultures were obtained on streptomycin-amended potato dextrose agar (PDA) and their identity was confirmed by species-specific polymerase chain reaction (PCR) primers. Nuclear rDNA internal transcribed spacer sequence also was obtained and was identical to sequences for *S. tsugae* previously deposited in GenBank. Two isolates were used to inoculate potted 2-year-old eastern hemlock seedlings in a growth chamber at 20°C with a 16-h photoperiod. Conidia were collected by flooding 1-month-old colonies on PDA with sterile water. Expanding shoots on one branch of each seedling were wounded using scissors to cut the tips off needles and stems, while another branch remained unwounded. Ten seedlings per isolate were inoculated by spraying to runoff with a conidial suspension sterile water, and five similarly treated control seedlings were sprayed with sterile water. Seedlings were covered with plastic bags to maintain high humidity for 4 days. Symptoms were evaluated and reisolation was attempted on streptomycin-amended PDA 2 months after inoculation. Symptoms of seedlings inoculated with either isolate included chlorotic and necrotic needle spots, browning of cut edges of needles, browning and death of needle tips and entire needles, death of stem tips with retention of dead needles, and needle loss. Symptoms of control seedlings were limited to slight browning of cut edges of needles. The fungus was reisolated from wounded shoots of 17 of 20 inoculated seedlings and nonwounded shoots of 5 of 20 inoculated seedlings and was not cultured from control seedlings. To our knowledge, this is the first report of *S. tsugae* in Georgia and also the first demonstration of its ability to produce symptoms that have been attributed to it on any tree species.

Key words: Hemlock, shoot blight, *Sirococcus tsugae*, *Tsuga canadensis*.

Gremmeniella epidemic in Sweden in 1999 and 2001 - Recovering of the forest

E. Stenström[1]*, J. Oliva[1], L. G. Wichmann[1], K. Wahlström[1], M. Jonsson[1], I. Drobyshev[2] and J. Stenlid[1]

[1]Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, P. O. Box 7026, SE 750 07 Uppsala, Sweden.
[2]Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, P. O. Box 49, SE 230 53 Alnarp, Sweden.

*Corresponding author. E-mail: Elna.Stenstrom@slu.se.

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During 1999 and 2001, the most severe *Gremmeniella abietina* epidemics ever appeared in Sweden. More than 500,000 ha forest were severely attacked and the forest industry lost milliards of Euros. In order to follow the development of the forest after one or two *Gremmeniella* infections, we studied seven experimental sites established in the most affected areas in middle of Sweden. In total, we followed the defoliation and growth of 360 trees exposed to two epidemics and of 250 trees exposed to one epidemic. When the experiment started in 2000 and 2001, trees were chosen according to different defoliation: healthy (<20% defoliation), medium (60-70%) or severely defoliated (80-90%). The number of epidemics affected the survival of trees in the medium defoliation class, but not in the severely and healthy classes. When subjected to two epidemics, survival after 10 years of medium defoliated trees was almost similar to that of severely defoliated trees (40% vs. 25%, respectively). When only attacked once, both medium and severely defoliated trees showed a higher survival (65% vs. 35%). After one epidemic, surviving trees presented a lower defoliation (25%)

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than when subjected to two epidemics (50%) that is, less defoliated trees survived. We also observed different patterns amongst surviving trees subjected to one or two epidemics. Severely defoliated trees subjected to one epidemic recovered the growth at a similar rate than trees with medium defoliation. Severely infected trees subjected to two epidemics recovered the growth at a lower rate than trees with medium defoliation. Recurrent epidemics severely diminish the capacity of survival and recovery from *G. abietina* attacks. Knowledge on preceding attacks may be used to optimize tree removal after the epidemics. Trees shall be removed based upon different defoliation thresholds depending on the previous history of the stand.

Key words: *Gremmeniella abietina*, defoliation, growth, survival.

Climate change and forest diseases: Using today's knowledge to address future challenges

Rona N. Sturrock

Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, 506 West Burnside Road, Victoria, British Columbia, Canada V8Z 1M5. E-mail: Rona.Sturrock@nrcan.gc.ca.

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The health of the earth's forests and urban green spaces is increasingly challenged by the outcomes of human activities, including global climate change. As climate changes, the role and impact of diseases on trees in both forest ecosystems and in urban settings will also change. Knowledge of relationships between climate variables and diseases affecting forest and urban trees is reviewed, with specific emphasis on those affecting foliage, shoots, and stems. Evidence that forest diseases are already responding to the earth's changing climate is examined (example Dothistroma needle blight in northern British Columbia) as are predicted scenarios for future changes in impact on forests by other tree diseases. Outbreaks of tree diseases caused by native and alien pathogens are predicted to become more frequent and intense – this and other general predictions about the effects of climate change on forest and tree diseases are discussed. Despite the uncertainty that accompanies such predictions it is imperative that researchers, forest and urban tree managers, and policy makers work together to develop and implement management strategies that enhance the resilience of the worlds' forests and urbanized trees. Strategies discussed include monitoring, forecasting, planning, and mitigation.

Key words: Tree diseases, forest pathogens, forest health, urban forests, plant disease management.

Dothistroma septosporum and *Lecanosticta acicola* in Czech Republic: Current situation and inoculation tests

V. Tomešová*, J. Janoušek, L. Jankovský, D. Palovčíková, M. Dvořák and T. Májek

Department of Forest Protection and Game Management, Faculty of Forestry and Wood Technology, Mendel University in Brno, Zemědělská 3, 613 00 Brno, Czech Republic.

*Corresponding author. E-mail: tomesovav@seznam.cz.

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Severe spreading of Needle blights has been noticed within the past 15 years. Ascomycetes *Dothistroma septosporum* (Dorog.) Morelet (teleomorph: *Mycosphaerella pini* Rostr.) and *Dothistroma pini* Hulbary (teleomorph: unknown) causing Dothistroma Needle Blight (Red Band Needle Blight) and *Lecanosticta acicola* (Thüm.) Syd. (teleomorph: *Mycosphaerella dearnessii* M. E. Barr), causing Brown Spot Needle Blight, are considered as the causal agents. The occurrence of these pathogens from new sites as well as from new host tree species has been reported. Scandinavia can be an example of such crucial spreading of the disease. The Red Band Needle Blight has been occurring in northern Europe since the year 2008 in a big scale mainly on *Pinus sylvestris*. Woods et al. (2005) consider Dothistroma needle blight (DNB) as a disease supported by global climate change. He emphasizes the influence of locally enhanced precipitations to the spreading of the disease in temperate zone. The increasing amount of new infested sites has been noticed also in Czech Republic. The fast disease spreading plays the key role in this current situation. The observation of the disease development has been carried out in 132 sites with host tree species so far. DNB has been confirmed on 79 sites and from 23 of those pure cultures have been successfully cultivated. The occurrence of Brown Spot Needle Blight is not too alarming in the CZ as DNB from the point of the amount of infested sites. Nevertheless Brown Spot Needle Blight in Southern Bohemia occurs in a big scale hence its spreading to new areas is not out of the question. The current occurrence of Needle Blights in a huge scope and its relatively easy and fast spreading has became the main reason of the inoculation spraying test. The purpose of the inoculation test is to verify the symptomology of Needle Blights. The inoculation test will be done simultaneously with the Real-Time polymerase chain reaction (PCR), whereby only a little DNA concentration of the organism in a sample can be detected. By this it is possible to detect an occurrence of a disease in its early stage, which is a problem for diagnostics. The identification of the pathogen in the early stage could help the use of protective spray for prevention of the occurrence and spreading of the disease. Another purpose of the inoculation test is the question of susceptibility of various conifer species on the *Dothistroma* and *Lecanosticta* infection. Inoculation tests have been carried out since spring 2010 and a quarantine greenhouse in Praha-Ruzyne and former Hacker's nursery have been chosen for the purpose of this experiment. Pure cultures of *D. septosporum* and *L. acicola* were used as a basic inoculation suspension, whereas their conidias were washed by double distilled water. Inoculation suspension was applied on seedlings with atomizer. Selected seedlings were consequently covered with unwoven fabric for encouraging favourable microclimatic conditions. Following seedling species have been placed in the greenhouse or/and planted *in natura* in former nursery: *Pinus nigra, Pinus mugo, Pinus sylvestris, Pinus uncinata, Picea pungens, Picea abies, Picea sitchensis and Pseudotsuga menziesii*. With respect to actual epidemic situation in many countries, it is necessary to discuss the role of climatic factors in Europe and trade with plant material as the main risk factor for the spreading of both diseases.

Key words: *Dothistroma septosporum* and *Lecanosticta acicola*, Czech Republic, Dothistroma needle blight (DNB).

Occurrence of *Dothistroma septosporum* in different types of forests in Finland

M. Vuorinen

Finnish Forest Research Institute (Metla), Eastern Finland Regional Unit, Suonenjoki Unit, Juntintie 154, FI-77600 Suonenjoki, Finland. E-mail: martti.vuorinen@metla.fi.

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Red band needlecast has been found in Finland during the last years. It has been distributed almost all over Finland, but seems to be most common in the southern and central part of the country. The aim of this study was to find out the frequency of red band needle blight distribution in different type of pine stands. Normally the pine stands in dry forest

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sites are healthy without any needlecast. Although the reasons for disease distribution are unclear, the increased length and increased humidity of growing seasons may favour the dispersal and infection of spores. Earlier epidemics caused by *Lophodermium seditiosum* could outbreak especially in southern Finland, but during the last years red band needle blight has also caused needlecast in pine stands, too. The amount of red band needlecast varies a lot between sites, depending on the density of trees vegetation and the type of soil etc.

Key words: *Lophodermium seditiosum*, Finland, needlecast.

Control of chestnut canker with hypovirulent strains of *Cryphonectria parasitica* in Castilla y León (Spain)

P. Zamora[1,3]*, A. B. Martín[1], R. San Martín[2] and J. J. Diez[3]

[1]Centro de Sanidad Forestal de Calabazanos, Consejería de Medio Ambiente, JCyL, Polígono de Villamuriel, 34190 Villamuriel de Cerrato, Palencia, España.
[2]Departamento de Estadística e Investigación Operativa, ETSIIAA Palencia, U. De Valladolid, Avda. Madrid 57. 34004 Palencia, España.
[3]Departamento de Producción Vegetal y Recursos Forestales, ETSIIAA Palencia, U. De Valladolid, Avda. Madrid 57. 34004 Palencia, España.

*Corresponding author. E-mail: zambrapa@jcyl.es.

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Hypovirulence within a virus reduce the virulence of the fungus, and is a biological control for *Cryphonectria parasticia* used with effectiveness in different European countries since its first detection in Italy. In this study, the objective was to determine if the hypovirulent isolates found in the field can be used for controlling the expansion of chestnut canker in Castilla y León. During 2007 and 2008, four inoculation assays were conducted in chestnut stands in Castilla y León. Three of the inoculations were done in León where the hypovirulence has been found naturally distributed in different orchards. One assay was done in Zamora where no hypovirulence has been found so far. The first inoculation was conducted in autumn 2007 with hypovirulent isolates of the vegetative compatibility group EU11. In 2008 the inoculations were conducted in spring and autumn with isolates from EU11 and EU1. At the inoculation time the cankers were measured and the area was calculated with the ellipse formula. The effectiveness of the inoculation was measured calculating the relative increment of growth after 6, 12 or 18 months since inoculation. In autumn, the inoculations had good results reducing the growth of the cankers with all the treatments assayed after 12 or 18 months. With both vcg tested, EU1 and EU11 reduced the canker growth. The inoculation conducted in spring had no differences between the inoculated and the control cankers in exception of one treatment in Zamora. All the isolates used were efficient controlling canker growth and the best moment to conduct the inoculation was autumn. The current vcg distribution and the number of hypovirulent strains isolated represent a good opportunity for an effective biological control of chestnut canker in Castilla y León.

Key words: Hypovirulence, *Cryphonectria parasticia*, chestnut canker, Castilla y León.

UPCOMING CONFERENCES

52nd Conference on the German Society of Economic and Social Sciences in Agriculture (GEWISOLA), Stuttgart, Germany , 26 Sep 2012

28th International Conference on Agricultural Economists (ICAE), Foz Do Iguacu, Brazil, 18 Aug 2012

Conferences and Advert

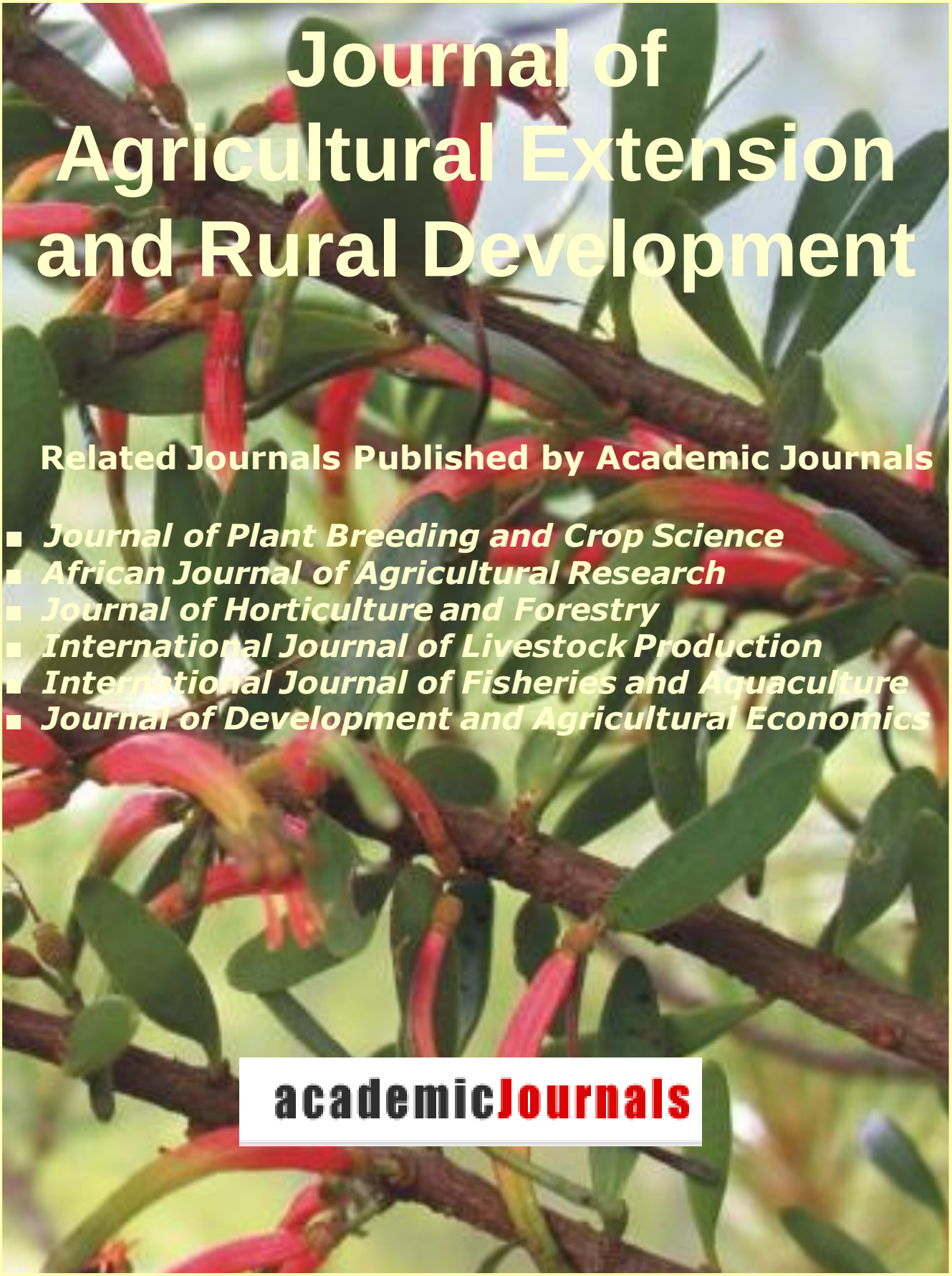
September 2012

II Asia Pacific Symposium on Postharvest Research Education and Extension (APS2012), Bogor, Indonesia, 18 Sep 2012

International Conference on Agricultural Science, Biotechnology, Food and Animal Science (ABIFA '12), 20 Sep 2012

November 2012

International Conference on Agricultural and Food Engineering, Serdang, Malaysia, 26 Nov 2012



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