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H. Tuğba DOĞMUŞ-LEHTIJÄRVI

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Preface

The meeting of IUFRO Working Party (WP) 7.02.02 "Foliage, Shoot and Stem Diseases of Forest Trees" was held in Eğirdir, Isparta, Turkey. Local organizers were Dr. H. Tuğba Doğmuş-Lehtijärvi and her colleagues from the Faculty of Forestry, Süleyman Demirel University. In the opening ceremony, vice rectors of the university, Dr. Vecihi Kurdemir and Dr. Mehmet Ali Koyuncu and also dean of the Forestry Faculty, Dr. Musa Genç and the Coordinator of the IUFRO Forest Health Division, Dr. Gaston Laflamme welcomed the participants of the WP. We thank the attendees who presented oral and poster presentations, local organisers for their great effort, Süleyman Demirel University for their kind support and The Scientific and Technological Research Council of Turkey (TUBİTAK) for financial support.

Forest pathology has developed a lot during the past 40 years. The most remarkable progress is the possibility to use DNA methods in studying genetics of pathogens and host plants. DNA methods are helping us also when we try to find out the origin of new diseases, alien or original? What is the next step? Also the development of information technology has affected a lot of our daily working compared to time before computers and internet. The first step in forest pathology is to describe the pathogen and disease symptoms. The pathogenicity should be tested according to Koch's postulates. Then the ecology of the disease should be examined experimentally including the interactions between the pathogen, host, other microbes and environment. DNA methods can be used in studying the variation of pathogens, taxonomy, endophytes and pathogenesis.

The first meeting of the IUFRO Working party 7.02.02 was arranged in 1973 in Minneapolis, USA. Now Turkey was the 10th country to host the meeting so far. In the first meetings one of the main subjects was Scleroderris canker which was an important disease in Europe, Northern America and Japan. Surprisingly, the subjects of the presentations in this meeting were scattered, including some other important biotic and abiotic diseases of forest trees.

When new disease appears we should start from the beginning. Thanks to new methods or tools we can get the same knowledge much faster than in the 1900´s. It seems that new diseases appear continuously, f. ex. Phytophthora alni and Chalara fraxinea in Europe. The human interest to grow exotic tree species and even the commercial international seedling trade keeps the forest pathologists in work in future too.

The climate change means also challenges to forest pathologists. The warming climate is a fact based on the laws of physics. The concentration of carbon dioxide and other greenhouse gases are rising fast in atmosphere. Near the arid areas the warming means the drought problems and in the north it means the new pathogens from milder climates together with faster forest growth. The climate change can disturb the evolutionary balance between the plants and the pathogens. But the climate change highlights also the importance of forests. At the same time, forests produce renewable materials and energy and bind the carbon dioxide from the air. A healthy forest is important in controlling the warming. This is a fact which is very important to get to a common knowledge of people and this is our task.

Nowadays we can exchange information quickly by internet. Why to fly to another side of the earth to meet colleagues? I think the human being needs human contacts and conversation. This is an excellent opportunity to discuss and to start co-operation. The internet has not yet changed human genes. We had once more a very successful meeting in Eğirdir with very sincerely and relaxed atmosphere and found the opportunity to share the experiences and create the new ideas coming from basically all age groups, from very young to seniors, and we had close to 50 participants from 15 countries with 29 oral presentations and 15 posters. This proceeding includes full papers as well as extended and short abstracts and we would like to mention that the responsibility for the published papers lies with the authors.

Finally, we would like to see you in the next meeting to be held in Spain, 2011 organised by new deputy Julio Javier Diez Casero!

Antti Uotila
Coordinator
IUFRO WP 7.02.02

H. Tuğba Doğmuş-Lehtijärvi
Local organizer & Deputy

Full Papers

Foliage Diseases of Conifers

Needle Diseases

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DOTHISTROMA AND LECANOSTICTA NEEDLE BLIGHT IN THE CR

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ABSTRACT

Dothistroma needle blight is widespread in the Czech Republic now, although the first finding was noted in 2000. To date, it has been identified on 21 species of Pines, 4 species of Spruces and also on Douglas fir in the CR. Records on Scots pine were exceptionally rare in the CR and also in Europe up to spring 2008. Brown spot needle blight caused by *Lecanosticta acicola* was for the first time reported in the Czech Republic on June 2007, actually is known from 2 localities on *Pinus rotundata*. *Lecanosticta acicola* coincides in observed localities with Dothistroma needle blight on Scots pine *Pinus sylvestris*, bog pine *Pinus rotundata* and their hybrid *P. digenea,* however no finding of both diseases on the same tree was observed.

Key words: *Dothistroma septosporum*, *Lecanosticta acicola*, needlecast, alien species

1. INTRODUCTION

Dothistroma needle blight *Mycosphaerella pini* E. Rostrup, resp. its anamorph *Dothistroma septosporum* is known from most of European countries, eg. France, Italy, Portugal, Spain, Georgia (Ivory, 1994), UK (Murray and Batko, 1962), Croatia (Novak-Agbaba et al., 1997; EPPO, 2005), Montenegro and Serbia (Karadzic, 1989, 2004), Romania (Gremmen, 1968) etc. From Central Europe it was reported from Austria (Petrak, 1961), Slovenia (Macek, 1975), Germany (Butin, 1983; Richter, 1983) and Poland (Kowalski and Jankowiak, 1998) where it

was found in May 1990, Slovakia (Kunca and Foffová, 2000), Hungary (Koltay, 1997) and Czech Republic (Jankovský et al., 2000, 2004). Recent findings are from Netherlands (EPPO, 2007) and Belgium (EPPO, 2008a). Up to 2008 there was no report on Dothistroma from Scandinavia, actually there are reports from Estonia (Hanso and Drenkhan 2008), Finland (EPPO, 2008b), Sweden (Stenlid, oral communication; DNA isolated from needle, no symptoms); it is also reported from Lithuania (Fig 1). European strains belong mostly to *Dothistroma septosporum*, however Barnes et al. (2004, 2007) recorded also *D. pini* from samples from Ukraine.

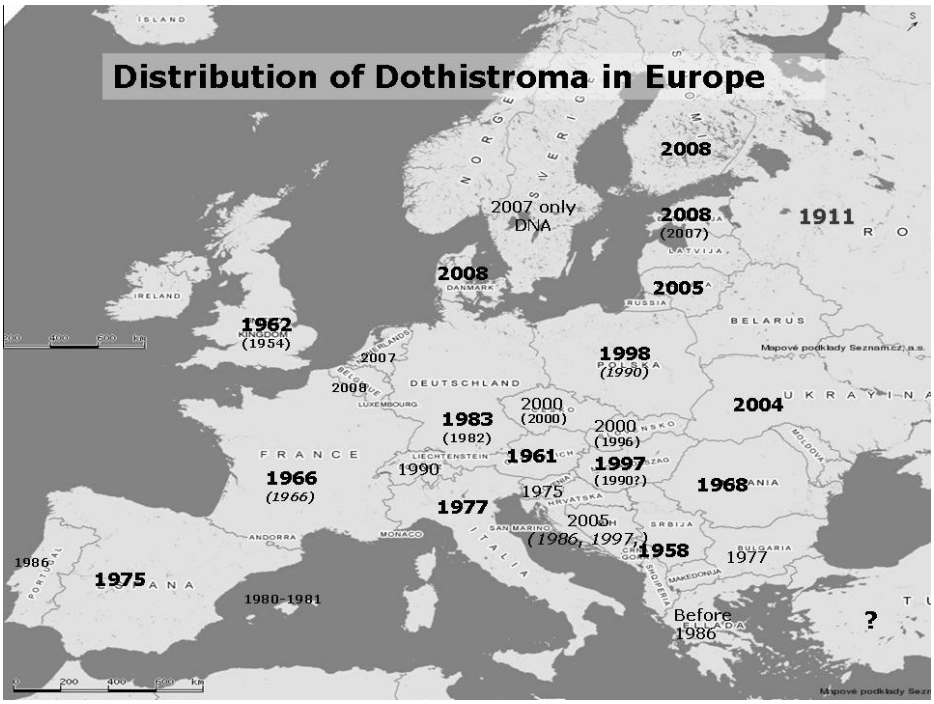

Fig. 1 Distribution of Dothistroma needle blight in Europe. Years mean first published report, in parentheses are years of findings, if differ from year of publishing.

Brown spot needle blight *Mycosphaerella dearnessii* M. E. Barr, resp. anamorphic stage *Lecanosticta acicola* (Thüm.) Syd. is in Europe reported (Fig. 2) from Austria (Petrak, 1961; Brandstetter and Cech, 1999, 2003; Kirisits and Cech, 2006), France (Chandalier et al., 1993), Italy (Porta and Capretti, 2000), Germany (Butin and Richter, 1983; Pehl, 1995), Switzerland (Holdenrieder and Sieber, 1995), Bulgaria and formerly Yugoslavia (Holdenrieder and Sieber, 1995), Serbia (Milanovic and Karadzic, oral communication), in 1979 it was reported from Croatia (Novak-Agbaba and Halambek, 1997; EPPO, 2007). Some new records origin from Estonia (Cech, 2008, oral communication) and Slovenia (Jurc, 2008).

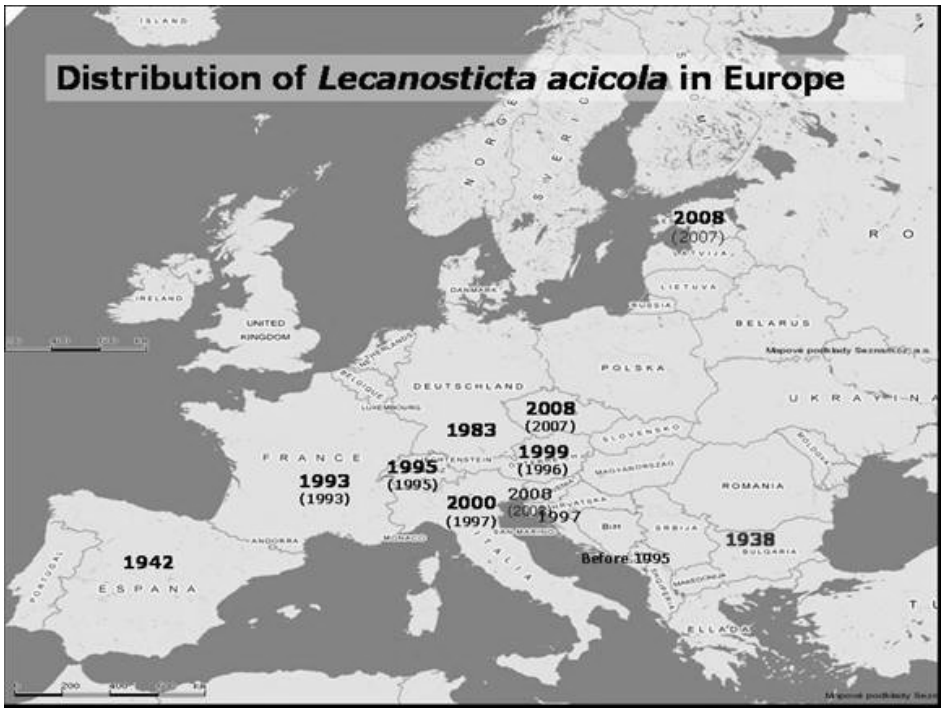

Fig. 2. Distribution of *Lecanosticta acicola* in Europe. Years mean first report, in parentheses are years of findings, if differ from year of publishing.

2. MATERIAL AND METHODS

Records from the Czech Republic are based on monitoring carried out in 2000 – 2008. Pine and also Spruce and Douglas fir needle samples were examined, mainly from regions of Southern and Central Moravia, Silesia, Eastern and Central Bohemia.

The presence of the pathogen was always investigated according to characteristic symptoms such as red bands, dying tips of needles or the occurrence of subepidermal sporocarps, acervuli. Exact identification was proved on the basis of microscopic analyses of conidia.

Isolation of culture was made on 3% MEA containing malt extract 30 g/l, pepton 5 g/l, agar 15 g/l, without addition of any antibiotics. Pieces of needles with acervuli 3 - 5 mm long were on surface sterilized by sodium hypochlorite 7%, subsequently by ethanol 96% and washed by sterilized water and put on malt extract agar. After 3 weeks of incubation, when new conidia occurred on fruiting bodies, conidia were inoculated into new medium.

Herbarium specimens are deposited at Herbarium of Faculty of Forestry and Wood Technology (BRNL).

3. RESULTS AND DISCUSSION

Dothistroma needle blight is widespread across the CR now, although the first finding was noted in 2000 (eg. Jankovský et al., 2000, 2004; Bednářová et al., 2007). More than 80 host species of Dothistroma needle blight are mentioned from all continents (Bednářová et al., 2006). To date, it has been identified on 21 species of pine, 4 species of spruce and also on Douglas fir in the CR: *Pinus aristata* Engelm., *P. attenuata* Lemon, *Pinus banksiana* Lamb., *Pinus cembra* L. var. *sibirica* (Du Tour) G. Don, *Pinus contorta* Douglas ex Loudon, Pinus x digenea Beck (=*P. rotundata x P. sylvestris*), *Pinus heldreichii* H. Christ, *Pinus heldreichii* H. Christ var. *leucodermis* (Antoine) Markgraf ex Fitschen, syn. *Pinus leucodermis* Ant., *Pinus jeffreyi* Grev. et Balf, *Pinus mugo* Turra, *Pinus nigra* Arnold, *Pinus ponderosa* Douglas ex Lawson, *Pinus pungens* Lambert, *Pinus rigida* Miller, *Pinus rotundata* Link = *Pinus mugo* nothosubsp. *rotundata* (Link) Janchen & Neumayer, *Pinus strobus* L. var. *sibirica*, *Pinus sylvestris* L., *Pinus tabuliformis* Hort. ex Carrière, *Pinus taeda* L., *Pinus thunbergii* Parlatore, syn. *Pinus thunbergiana* Franco, *Pinus wallichiana* A. B. Jackson, *Picea abies* L. Karst., *Picea pungens* Engelm., *Picea omorika* (Pančić) Purkyně, *Picea schrenkiana* Fisch. & C. A. Mey, *Pseudotsuga menziesii.* Austrian pine *Pinus nigra* Arnold*,* mountain pine *Pinus mugo* Turra, *Pinus ponderosa* Douglas ex Lawson, *Pinus jeffreyi* Grev. are the most frequent and most susceptible hosts. As for species of other genera *Picea pungens* Engelm., *Picea abies* L. Karst., *Picea omorika* Purkyně and *Picea schrenkiana* Fisch. & C. A. Mey were noted as hosts. *Dothistroma septosporum* was also isolated from needles of *Pseudotsuga menziesii*. Symptoms on needles of Douglas fir were not so clear, acervuli were observed exceptionally.

Records on Scots pine were exceptionally rare in the CR and also in Europe up to spring 2008. Risk of Dothistroma needle blight for Scots pine in Europe is noted eg. by Lang and Karadzic (1987). According to Gadgil (1984), *Pinus sylvestris* is highly susceptible. Contrary, according to data from Great Britain, Peterson (1982), mentions that the attack occurs very rarely. However several hectares of Scots pine plantations, infested by *Dothistroma septosporum*, about 10 years old, were registered in Southern Bohemia, in Forest district Nové Hrady, Třeboň area, in March, 2008. In 2009 progress of infection in the same plot contrary precedent year. Large infestations were registered also on Scots pine in peat bog nature reserve Soběslavská blata in Southern Bohemia. Infected trees were origin from natural regeneration. Surrounding commercial forest was not affected. *Dothistroma septosporum* outbreak on Scots pines was observed simultaneously in large areas across the Central Finland in spring 2008 (EPPO, 2008). Dothistroma seems to be threat for Scots pine, including natural stands in Europe, although it was mostly reported from plantations of introduced species eg. *Pinus nigra*, *P. ponderosa*, *P. contorta* etc.

Brown spot needle blight caused by *Lecanosticta acicola* was for the first time reported in the Czech Republic on June 10, 2007 (Jankovský et al. 2008). The first record is from the peat bog National Nature Reserve Červená Blata in South

Bohemia, close to town Třeboň; coord. N 48°51'37.06", E 14°48'44.09". The disease was observed on 10 – 40 years old trees of *Pinus rotundata*, (Jankovský et al., 2008). The new record in the CR is from the same host species, 10-60 years old in National Nature Reserve Borkovická blata, near town Soběslav (coord. N 49°14'16.3" E 14°37'54.2") on August 7, 2008 (Jankovský et al., in press). Both places are very strictly protected areas. Typical symptoms (according to EPPO, 2005) were observed in current year needles declined from tips in middle of July and in August. Brown spots with apparent yellow separation were present on green needles as well. Visible yellow belts were present between dead tissues of killed tips and green tissues. Studied conidia were subhyalinne, even dark olive green, surface of conidia echinulate to verrucose or tuberculate, straight to curved, with one to five septae, fusiform to cylindrical, size 3 - 5 µm × 21 - 44 µm. On 3% MEA medium, the fungus produced grayish green olive to olive black stromatic colonies, producing slime with conidias

Lecanosticta acicola coincides in localities with causal agent of Dothistroma needle blight *Dothistroma septosporum* on Scots pine *Pinus sylvestris*, bog pine *Pinus rotundata* and their hybrid *P.×digenea.* Nevertheless, the threat of the disease spreading to Scots pines frequently planted in the region remains unclear yet. While bog pines inside the nature reserves display remarkable needle defoliation, Scots pines in surrounding managed stands are without visible symptoms of infection by *Lecanosticta acicola*.

4. CONCLUSIONS

With respect to actual epidemic situation in some countries, it is necessary to discuss the role of climatic factors in Europe and trade with plant material as main risk factors for spreading of both diseases. Dothistroma needle blight and brown spot needle blight are relatively quickly spreading needle casts in Central and also in Northern Europe. Within past 15 years, Dothistroma was reported from many new areas. Spreading of these diseases should be considered as result of climatic extremes and also one of exhibitions of climatic changes (eg. Woods et al., 2005). However reasons of spreading are still not sure. We cannot exclude also some other factors as trade with plant material. Reasons of occurrence of disease of strictly protected areas without any human interventions for many decades are not sure. Control of these needle casts is problematic due distribution in large infested areas. Dothistroma needle blight is established in most areas across Europe now and seems to be problem in Central and North Europe now due to very quick spreading and adaptation for climatic and natural conditions in new areas.

5. ACKNOWLEDGEMENTS

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TWO NEW SPECIES OF *Lophodermium* COLONISE SCOTS PINES NEEDLES IN SCOTLAND

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ABSTRACT

Previous work has indicated that Scots pine needles are colonized by three species of *Lophodermium,* of which two are the endophytes *L. pinastri* and *L. conigenum* and the third is the pathogen *L. seditiosum*. Recent work on a DNA based *Lophodermium* phylogeny found huge variation among *L. pinastri* isolates which was interpreted as the presence of two subspecies within this taxon. In this study we use a combination of sequence data, molecular markers and culture morphology to demonstrate the existence of three distinct taxa within the entity that was previously classified as *L. pinastri*. These three taxa co-occur within the native pine woods of Scotland

Keywords: Ascomycete, *Lophodermium pinastri*, *Pinus sylvestris,* Phylogeny

1. INTRODUCTION

There is increasing evidence that the diverse endophytic communities within the leaves and needles of trees confer protection against pathogens (Petrini, 1991; Arnold et al., 2003). A situation in which protection by endophytes could be commercially important is found in the ascomycete genus *Lophodermium* Chev which is ubiquitous in pines and is distributed worldwide. Many *Lophodermium* species live asymptomatically as endophytes inside the needles of pines for at least part of their life cycle (Minter, 1981a; Diwani & Millar, 1987; Wilson, 1995) and could potentially help to protect against needle cast diseases (Minter, 1981b). However in order to determine the importance of endophytic *Lophodermium* species in protecting pines from needle diseases, it is essential to clarify their taxonomy so that they can be readily identified and their ecology and behaviour can be studied.

Lophodermium Chev. includes 145 species, mostly from pine hosts, and there is some degree of host specificity (Kirk et al., 2008; Ortiz-Garcia et al., 2003). Morphological characteristics of this genus include a single longitudinal slit opening of the apothecia, and the fusiform shape of the ascospores (Darker 1967). As it is known today, the *Lophodermium* species complex on *P. sylvestris* in Scotland includes two endophytes and one pathogen. The two endophytes differ in

their ecology. *L. pinastri* ascocarps are found on naturally shed needles, while *L. conigenum* fruits on prematurely killed needles (Minter and Millar, 1980). The pathogen *L. seditiosum* causes needlecast disease which is particularly a problem on young *P. sylvestris* (Diwani and Millar, 1987).

Preliminary studies of *Lophodermium* isolates made from naturally shed needles in Scottish native pinewoods showed striking variation among cultures grown on malt agar. PCR-RFLP and sequence analysis of the ITS region in these isolates also showed greater variability than had hitherto been reported for *L. pinastri* (Johnston et al., 2003). In this manuscript we explore the hypothesis that the current taxon *L. pinastri* includes more than one species. The hypothesis is tested using data from a DNA based phylogeny and analysis of DNA markers and cultural morphology.

2. MATERIAL AND METHODS

2.1. Fungal Isolates and DNA extraction

L. pinastri was isolated onto malt agar from naturally shed, surface sterilized needles collected in the Scottish native pine woods at Glen Affric (NH278278) and Amat (phylogenetic and population genetic analysis) and in Glen Affric, Abernethy (NJ015155) and Loch Maree (NG995654) for the analysis of culture morphology (Table 1). DNA was extracted from pure cultures grown in liquid Malt Extract medium using the Plant DNAeasy Qiagen kit.

Table 1: Numbers of isolates of each clade and population included in the culture morphology test

Population	Clade Ia	Clade Ib	CladeII
Loch Maree	10	20	3
Glen Affric	15	20	18
Abernethy	20	20	2
Total	*45*	*60*	*23*

2.2. Sequencing and Sequencing Analysis

ITS regions of the Ribosomal DNA and partial ACTIN was amplified using primers ITS1 / ITS4 (White et al., 1990) and ACT-512F / ACT-783R (Carbone and Kohn 1999) respectively. Sequencing of both strands was conducted using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Foster city, USA). Sequences were aligned with Clustal X2 (Thompson et al., 1997; Larkin et al., 2007).

2.3. Phylogenetic Analysis

Phylogenetic analysis was conducted using parsimony in PAUP* 4.0 (Swofford, 2003) and Baysian inference in MrBayes-3.1.2 (Huelsenbeck and Ronquist, 2001;

Ronquist and Huelsenbeck, 2003). Additional ITS sequences derived from Genbank were included in a broader ITS phylogeny to look at the relationship between *L. pinastr*i isolates from Scotland and from other parts of the globe.

2.4. Amplified Fragment Length Polymorphism and Corresponding Analysis

AFLP (Amplified Fragment Length Polymorphism) markers were obtained using the protocol of Vos et al. (1995). Eight primer combinations were chosen which gave 549 markers across all *L. pinastri* isolates (Table 2). Bands were scored as present or absent, a distance matrix was calculated using the Jaccard coefficient, and this was used to conduct a principal coordinate analysis in PAST.

Table 2: Numbers of AFLP markers found in each putative Lophodermium species

Primer Combination	Total	Clade Ia	Clade Ib	Clade II
AAC/CAA	74	27	23	24
AAC/CG	86	31	33	33
AAC/CT	98	24	39	48
AAC/CC	91	26	33	46
AAC/CAGA	53	26	7	28
AAC/CTA	58	18	10	34
AAC/CCG	60	20	13	32
AAC/CAG	29	12	5	12

2.5. Culture Morphology

Isolates collected as described above from Glen Affric, Loch Maree and Abernethy (Table 1) were identified to clade on the basis of their ITS sequence. Isolates were then chosen at random within clades and populations. Inoculum measuring 5mm diameter was cut from cultures and transferred onto a 2% malt agar Petri dish. Radial growth was measured once a week. The experiment followed a randomised block design with four blocks and included two replicates of each isolate. Results were analysed using R 2.8.0 (R Development Core Team, 2008)

3. RESULTS

3.1. Phylogenetic and Genetic Marker Analysis

Phylogentic analysis showed a total of five monophyletic clades with strong bootstrap or prior probability support within the *Lophodermium* complex on *Pinus sylvestris*. Isolates derived from shed needles in Scottish pinewoods, and previously classified as *L. pinastri*, fell into three of these clades named Ia, Ib and II. These clades are consistent when using both Parsimony and Baysian inference of phylogeny on combined data from the ITS and Actin loci (Fig. 1 and 2). Isolates from these three clades also form three distinct groupings in the Principal Coordinate analysis based on genetic distances obtained by scoring AFLP markers.

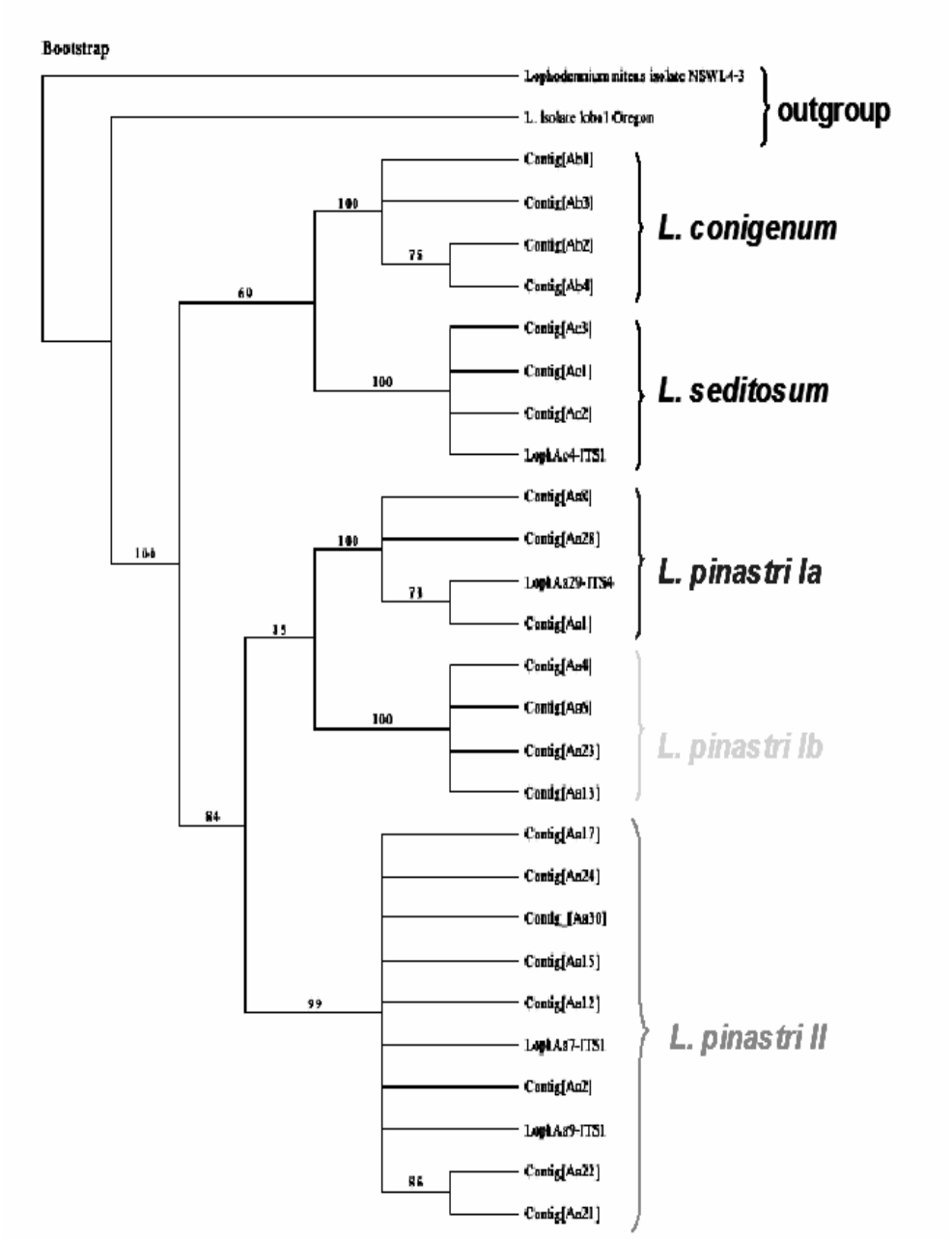

Figure 1: Phylogeny of *Lophodermium* species isolated from *Pinus sylvestris* needles from Scotland by Parsimony criterion phylogeny of the ITS. Bootstrap values are annotated on the branch located before the corresponding node

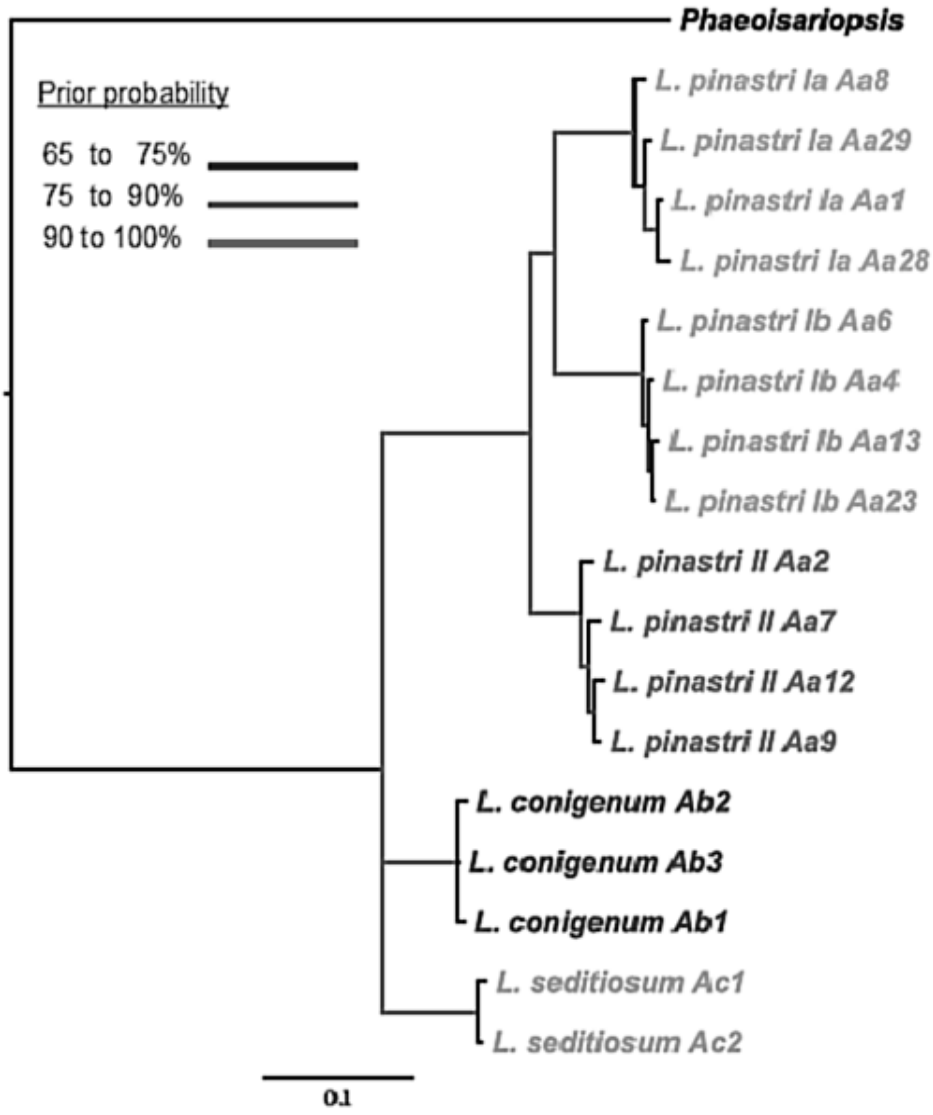

Figure 2: Phylogeny of *Lophodermium* species isolated from *Pinus sylvestris* needles from Scotland by Baysian inference of phylogeny of combined ITS and Actin

3.2. Culture Morphology

Growth rate differs significantly among the three clades identified in the phylogenetic and genetic marker analyses (P< 0.001 ANOVA, Fig. 3). Clade Ib shows the slowest and clade Ia the fastest growth.

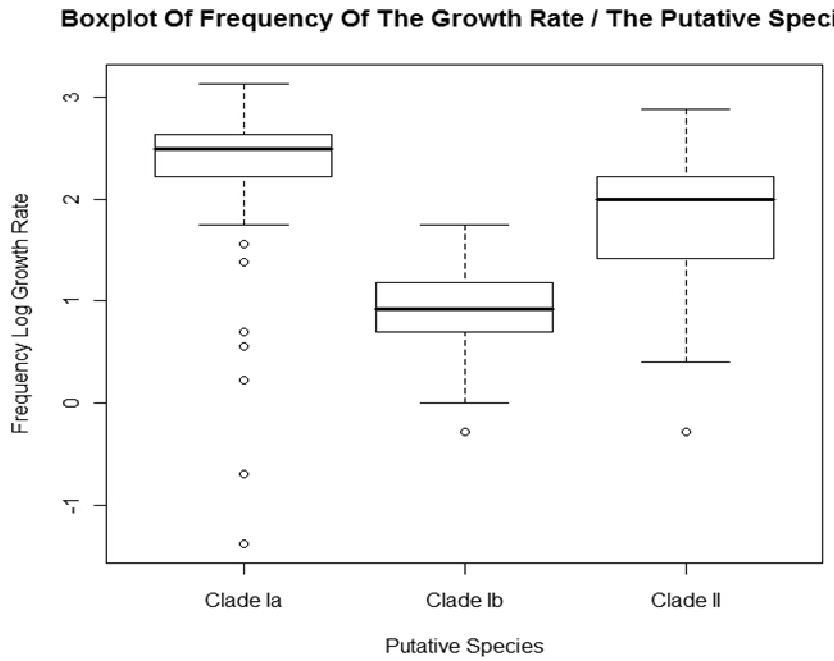

Figure 3: Box plot representing growth rate difference between each phylogenetic clades

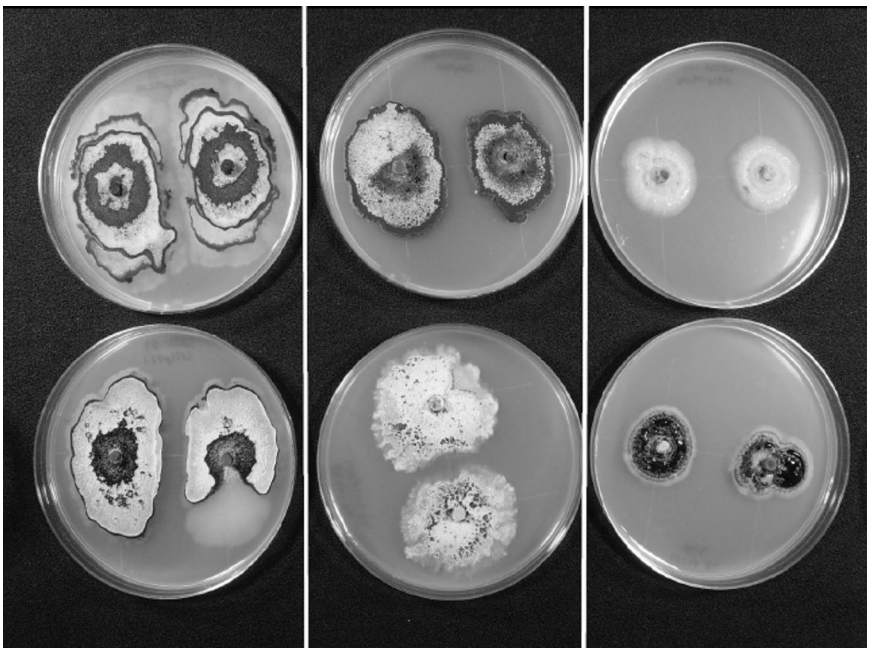

Figure 4: Eight weeks old colonies of two individuals per clade. From left to right: Clade Ia, Clade II and clade Ib.

3.3. Relationships among *Lophodermium* Species

Clade Ia includes Genbank sequences of *L. pinastri* from Europe on *Pinus sylvestris* and from Canada on *P. strobus* (Fig. 5). There are no Genbank sequences of clade Ib except for those derived from the native pinewood at Glen Affric (Scotland). Clade II includes Genbank sequences of *L. pinastri* from *P. ponderosa* in North America and from *P. pinaster* in New-Zealand. It also includes a different species of *Lophodermium*, *L. kumaunicum*, described from the Himalayas.

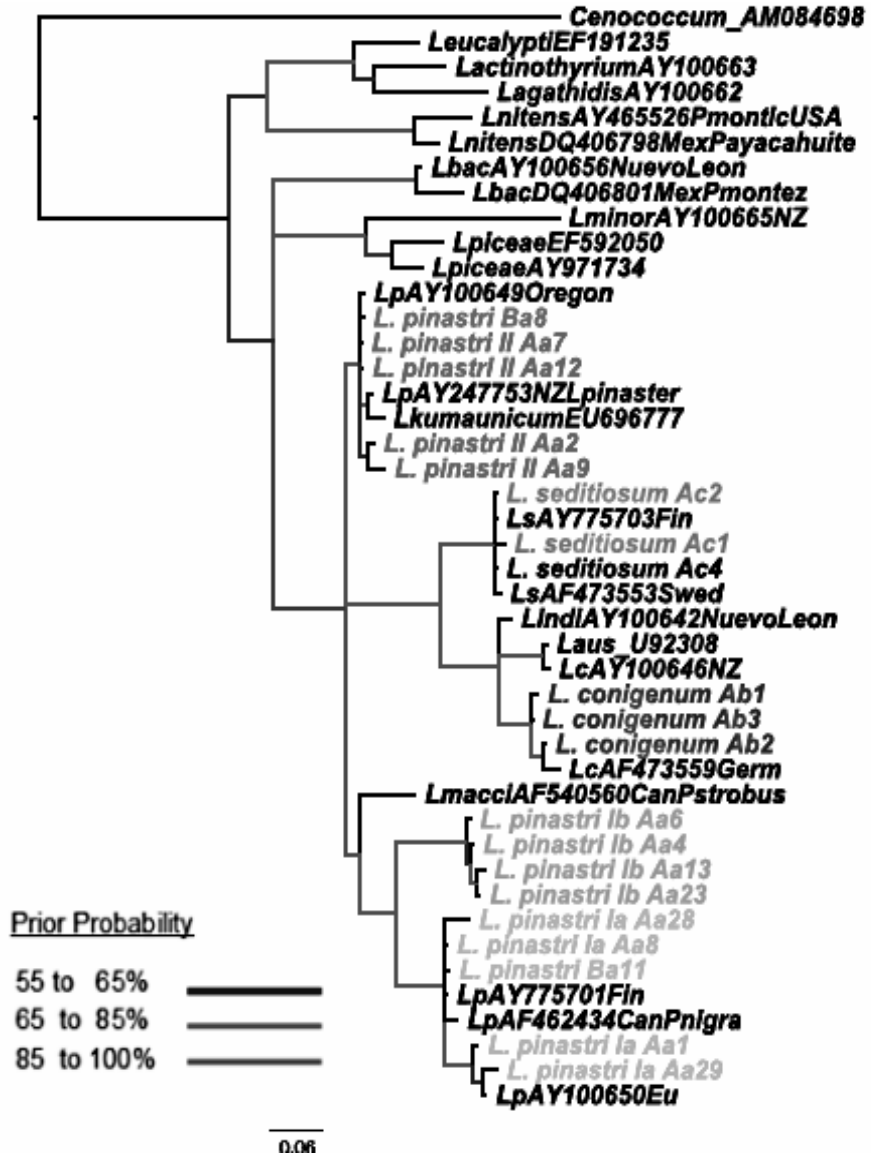

Figure 5: Phylogenetic relationship between species of Lophdermium Chev.

4. DISCUSSION

Multigene genealogy and genetic marker analysis coupled with culture morphology data demonstrate that *Pinus sylvestris* in Scotland is colonized by five species of *Lophodermium,* four of which are endophytes. Isolates previously classified in a single taxon, *L. pinastri*, fall into three distinct species. At least one of these species, corresponding to clade II, is distributed worldwide. This clade includes Genebank entries given the name *L. pinastri* and more recently *L. kumaunicum*. Clade II of *L. pinastri* was previously classified as a subspecies in the phylogeny of *Lophodermium* published by Ortiz-Garcia et al. (2003).

The present study has uncovered a greater diversity of endophytes than was previously known. Clarification of their taxonomy, and the ability to recognize the *Lophodermium* taxa on Scots pine in Scotland will allow us to compare the genetic diversity, gene flow and mating system of each species. Ultimately this will help us to understand the differences that exist and the interactions that occur between closely related pathogens and endophytes within *Lophodermium*.

5. ACKNOWLEDGMENT

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RED BAND NEEDLE BLIGHT IN FINLAND, SYMPTOMS AND DISTRIBUTION

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ABSTRACT

Red band needle blight is cused by ***Mycosphaerella pini*** Rostrup **(1957)** also known as ***Scirrhia pini*** A. Funk & A.K. Parker and conidial state is ***Dothistroma septosporum* (Dorog.) M Morelet.** First time it has described as *Cytosporina septospora* Dorog. 1911 and later the asexual state has called *Septoria septospora* (Dorog.) Arx, *Dothistroma pini* Hulbary 1941, *Dothistroma pini var. keniense* M.H. Ivory 1967, *Dothistroma pini var. lineare* Thyr & C.G. Shaw 1964, *Dothistroma septospora*, *Dothistroma septosporum* (G. Doroguine) M. Morelet, *Dothistroma septosporum var. keniense* (M.H. Ivory) B. Sutton 1980, *Dothistroma septosporum var. lineare* (Thyr & D.E. Shaw) B. Sutton 1980, *Dothistroma septosporum var. septosporum* (Dorog.) M. Morelet 1968, *Eruptio pini* (Rostr.) M.E.Barr 1996. The disease is called red band needle cast or dothistroma needle cast or dothistroma needle blight or pine needle blight. In Finnish it is called punavyökariste. Reproduction happens mostly asexual through conidia during the growing season in moist conditions, in Finland from May to October. Needles of all ages are infected by *D. septospora*. If infection is strong, needles can fall down during the same growing season, but more often they can stay on branches to the next season.

First symptoms of red band needle cast are yellow spots on needles which turn later on brown. Red-brown coloration, commonly associated to the disease is caused by dothistromin which is a potent and broad–spectrum toxin and is responsible for the characteristic necrotic lesions and red bands on needles. *Mycosphaerella pini* is believed to be native to the cloud forest of Central America, but the first description has made in Russia (Dorogin, 1911). Red band needle blight has now a days world wide distribution and is most serious disease in *Pinus radiata* plantations in Southern Hemisphere, East Africa, New Zealand and Chile and *Pinus contorta* in Northern Hemisphere in British Columbia, in Canada is particularly susceptible.

There are altogether 80 host species: 60 *Pinus* species and t.e. *Picea* and *Pseudotsuga*.

1. INTRODUCTION

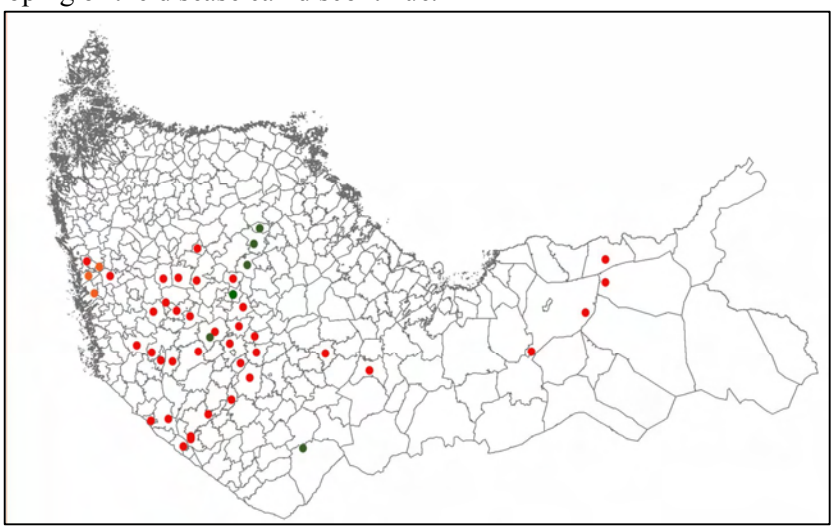
Occurrence in Finland

Red band needle blight is most common in dense 5-15 years stands and infects needles in branches mostly in 0.5m-2 m high. It can also find in the same young pine stands, where is or has been Pine needle cast epidemic caused by *Lophodermium seditiosum*. Jankowsky (2008) has also observed infections in 60-80 years stands. In *Pinus sylvestri*s records has made in Hungary, Poland, Zhech Republic and Estonia, but records were rare until spring 2008 (Jankowsky, 2008) when has happened rapid outbreak throughout Europe.

Limiting factors to occurrence?

In Finland red band needle blight is recorded first time in autumn 2007 and in spring 2008 pycnidias and germinated conidia was found and first tree isolates have done from Hartola, Kangasniemi and Suonenjoki and ITS sequenced. The sequences are identical with each others to a number of *M. pini* sequences of the GenBank database.

Cold tolerance test has been made in test chambers to the needles, where was able to find fresh pycnidias: one day interval +5 ºC/-5 ºC, continuous -10 ºC, one day interval +5 ºC /-20 ºC, continuous -70 ºC in one week testing time and control samples were stored in cold room in +4 ºC. No clear differences could be observed in the germination of conidia between temperatures, which mean that winter temperature is not a limiting factor. Humid climate favors the outbreak of red band needle cast, but dry periods in growing season are a stress to pathogen and the developing of the disease can discontinue.



Observations of the distribution of Red band needle blight in Finland 2008 by occasionally selected areas. Observations were not made systematically in whole country, e.g. south western Finland was outside observation area.

These observations show areas where red band needle cast was noticed in dense pine stand. Most of the observations in 2008 have made in southern and central Finland, but some observations have made in northern Finland, too. That means, that red band needle cast has spread out to some extend in whole country.

CONCLUSIONS OF THE DISEASE OUTBREAK ACCORDING TO OBSERVATIONS 2008

Red band needle blight has been prevalent since 1960s in pines planted as exotics in plantation forests, particularly in Southern hemisphere, but caused normally not serious damage to native pine stands. During the last few years the incidence of red band needle blight has increased dramatically in the Northern Hemisphere and the epidemic in British Columbia, Canada, has caused extensive mortality of pines in their native ranges and the disease has been correlated with climate change (Woods et. al., 2005). Climate change could have a positive impact to the occurrence of *Dothistroma pini* if weather is humid and rainy during growing season. Drought during growing season is a stress to host and to pathogen too and the outbreak can stop.

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Scleroderris Canker

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THE OCCURRENCE OF MICROCONIDIA ON *Gremmeniella abietina* (LAGERB.) MORELET

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ABSTRACT

The *Gremmeniella abietina* microconidia were observed during 1981-2002 in numerous finnish specimen and pure cultures of the fungus. Microconidia existed in type A and B on *Gremmeniella abietina*. Microconidia were found in pycnidia and apothecia. Grey microconidial mass was produced also in pure cultures. Size of microconidium was 3-6 µm X 1 µm. The germination was not observed. The attempts to produce apothecia with the help of microconidia in lab failed.

1. INTRODUCTION

Finn and Helka Roll-Hansen first reported microconidia in 1973. They found microconidia in pycnidia in Norwegian samples of *G. abietina*. The colour of microconidial mass was grey instead of pink as conidial mass. Bergdahl and Tsajkowski (1982) reported microconidia in North America. According to them microconidia rarely germinate. Microconidia are also described in taxonomical work of Petrini et al., 1989, when microconidia were found on different races or types of *G. abietina*. My purpose was to check the occurrence of microconidia in two types of *G. abietina* existing in Finland. In addition apothecia were tried to produce by transferring microconidia.

2. MATERIAL AND METHODS

These results based on notes which I have done on different experiments and cultures of numerous *G. abietina* isolates during 1981-2002. Hundreds of microscope slides were examined. They were done from natural pycnidia or apothecia and also from pure cultures in laboratory. The types of *G. abietina* were determined by conidial septa and disease symptoms. Later the types were confirmed with fatty acid (Müller & Uotila, 1997) or RAMS method (Hantula &Müller, 1997; Uotila et al., 2000). After determining mating alleles of G. abietina (Uotila, 1992) the pairing experiment with microconidia was done. *G. abietina* has two mating alleles, mat 1 and mat 2. The microconidia from the monospore culture were transferred to culture having different mating allele. The cultures were grown in erlenmeyer bottles on barley groats +pine needles medium.

3. RESULTS AND DISCUSSION

The size of microconidia is 3-6 µm X 1 µm (Fig. 1 and 2). Microconidium contain one nucleus. Microconidia were found regularly in types A and B of Gremmeniella. Microconidia are produced also in pure cultures of monoascospore isolates. The color of microconidial mass in pure culture or in pycnidia is grey instead of pink color of macroconidial mass. I have not seen germinating microconidia. The microconidia were not germinating in monospore cultures.

Figure 1. Microconidia formation in pure culture of *Gremmeniella abietina*. Stained with anilin blue.

The microconidia are formed from mycelia (Fig. 1 and 3.), conidia (Fig. 2.) or ascospores. When the contents of pycnidium with microconidia and macroconidia is spread on the agar the microconidia adhered close to macroconidia (Fig. 2.). The change of nuclei is possible in these conditions. The role of microconidia is propably to transfer the nuclei before meiosis. This sounds reasonable, but why there are microconidia also in apothecia? I tried to produce apothecia in lab with help of microconidia. *G. abietina* is heterothallic with two mating alleles. So the microconidia were added in cultures with different mating allele, but apothecia did not appear in these cultures. In nature we can produce apothecia by inoculating compatible mycelia close to each other in the same seedling.

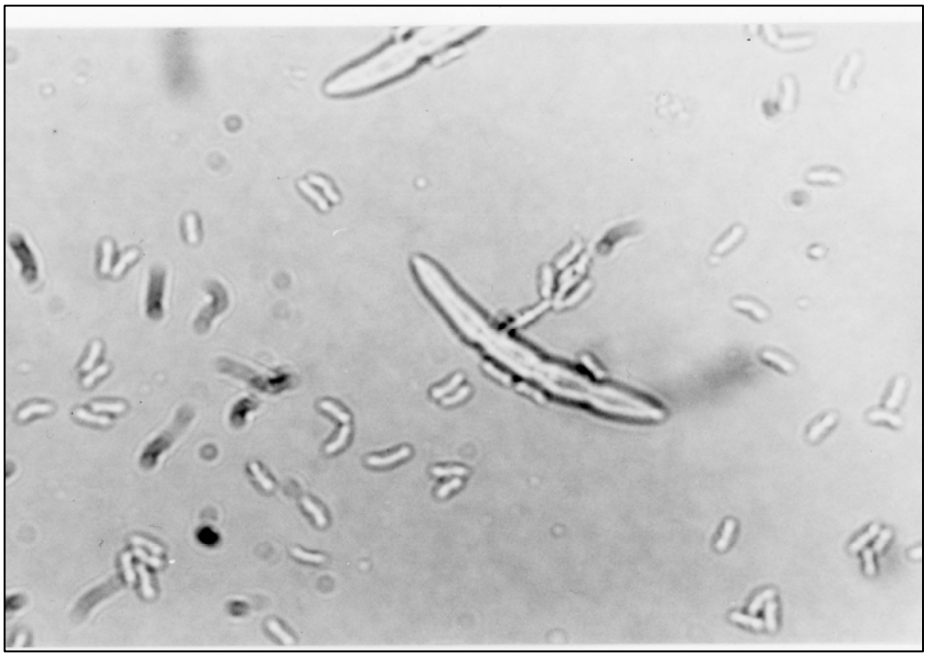

Figure 2. The macroconidium seems to produce microconidia.

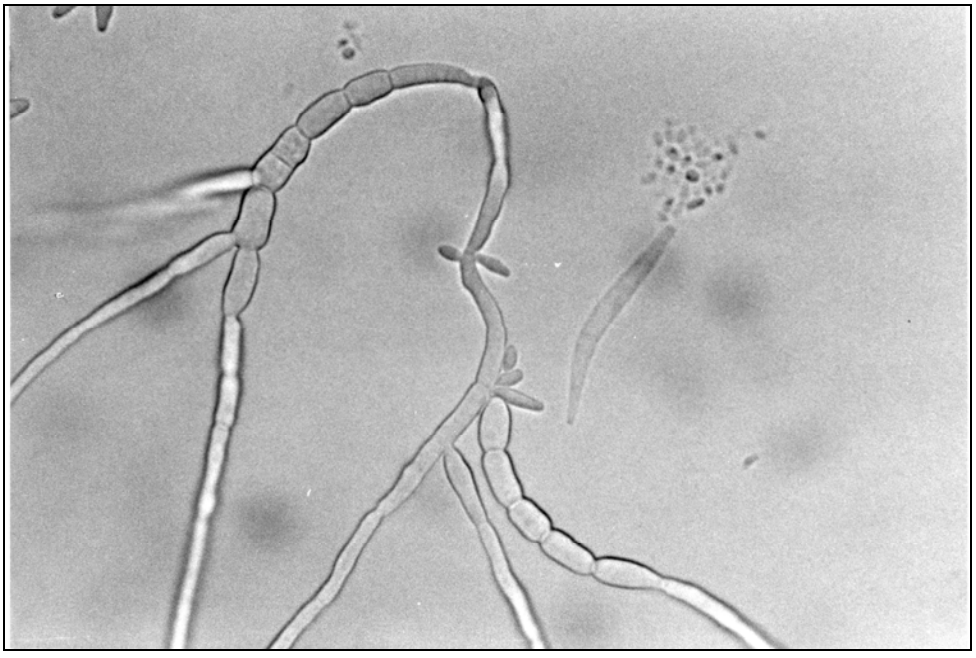

Figure 3. Germinated macroconidia and the mycelia forming microconidia.

It is still needed more research to describe the exact process of pairing and so to understand the role of microconidia for the fungus. This knowledge do not offer direct possibilities to control the disease, but it is important to understand the enemy.

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CENTRAL NEWFOUNDLAND: ESCAPE from QUARANTINE

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ABSTRACT

Scleroderris canker, European race, was first detected on Austrian pine in St. John's, Newfoundland in 1979. To prevent spread of this exotic disease, a quarantine zone was established in 1980 to all areas north of the Witless Bay Line. Later, red pine mortality near Torbay (1981), Upper Island Cove and along Salmonier Line (1996) resulted in extending the quarantine zone in 1998 to all areas east of Route #202 at the isthmus of the Avalon Peninsula. Infection on these pines was tracked back to planting stock produced at the Back River Nursery on Salmonier Line. These seedlings were planted on the Avalon and Bonavista Peninsulas from1937 to1952. Until 2007, the slow rate of spread and natural quarantine boundary limited this disease for over 60 years to the Avalon Peninsula. In 2007, the European race of Scleroderris canker was detected in an isolated red pine plantation in central Newfoundland at Berry Hill Pond, 400km outside of the quarantine zone. Field observations showed that conducive conditions for the pathogen were always present in the area, explaining rapid development of the epidemic compared to slow progression in plantations on the Avalon Peninsula. Failure to publicize and enforce the quarantine and apply preventative control measures has now resulted in threats to native red pine stands and plantations established throughout central Nfld. Pruning red pines in that region will prevent any new outbreak. We cannot rely on quarantine measures alone to prevent spread of this disease.

Keywords: Scleroderris canker, *Gremmeniella abietina*, *Pinus resinosa*, outbreak, quarantine.

1. INTRODUCTION

Scleroderris canker cause by *Gremmeniella abietina* (Lagerb.) Morelet is a serious disease of hard pines, causing shoot blight, branch dieback, stem cankers and tree mortality. Two races of the disease affect pines in North America, the native North American (NA) race and the introduced European (EU) race (Dorworth et al., 1977)). The NA race causes infection on lower branches in the snow, inducing dieback and mortality in pine seedlings or pines less than 2 m in height. The NA race has never been found in Newfoundland. The EU race, introduced in North America, is a very serious disease; it is not restricted at the snow level; the whole crown of large trees can be affected. Red pine (*Pinus resinosa* Ait.) is very susceptible to this disease (Skilling, 1975); Scots (*Pinus sylvestris* L.) and Austrian pines (*Pinus nigra* Arnold) are moderately affected (Bernhold et al., 2009) while jack pine (*P. banksiana* Lamb.) is the most resistant (Laflamme and Blais, 2000).

2. FIRST REPORTS OF THE DISEASE IN NEWFOUNDLAND

Scleroderris canker, EU race, was first detected on Austrian pine in St. John's, Newfoundland, in 1979 (Singh et al., 1980). To prevent spread of this exotic disease, a quarantine zone was established in 1980 to all areas north of the Witless Bay Line (Figure 1). No movement of conifer stock from the area north of Witless Bay Line was allowed out of this zone. An information pamphlet was produced and distributed. It showed photos of symptoms to help identification of the disease as well as a map of the hazard and quarantine zone for Scleroderris canker. Within this quarantine zone, severe infection followed by tree mortality of red pine was detected in 1981; it was found in the Torbay plantation located 15 km north of St. John's (Figure 1).

3. BACK RIVER NURSERY

In mid 1980's, Scots pine dieback and mortality caused by Scleroderris canker EU race was observed along Salmonier Line and at a site called the old "Back River Nursery". This tree nursery has an important role in understanding the introduction of the disease on the Island, as we will see later. Back River Nursery was outside the quarantine zone (Figure 2).

In early 1990's Scleroderris canker infection and limited mortality was observed in a number of mixed pine plantations along the southern shore of Conception Bay, again outside the quarantine zone. Planting stock for all affected plantations came from the Back River Nursery.

In 1996, Scleroderris canker was observed in a red pine plantation at Upper Island Cove and the whole plantation was completely destroyed by the disease.

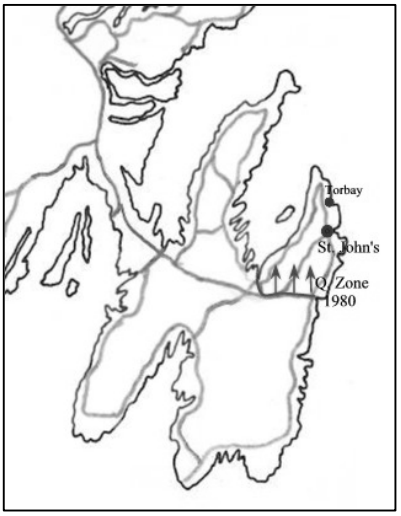

Figure 1: Quarantine zone north of Witless Bay Line established in 1980 to prevent spread of Scleroderris canker outside this area.

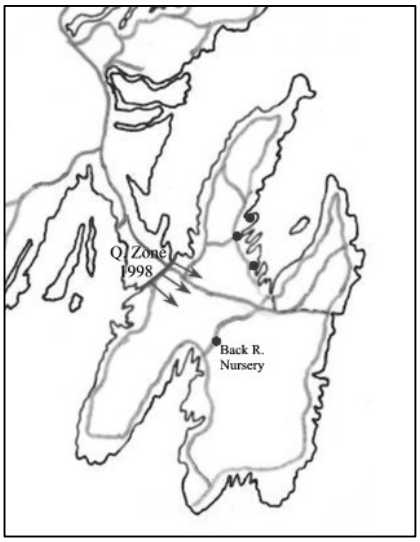

Figure 2: In1998, the quarantine zone for Scleroderris canker, European race, was extended to all areas east of Route #202, a natural barrier for the Avalon Peninsula.

Following this extension of the disease, the quarantine zone was extended in 1998 to all areas east of Route #202, a natural barrier for the Avalon Peninsula (Figure 2). Unfortunately, no public information or pamphlet was given or produced

After these new findings, the senior author did a search of historical data on the Back River Nursery, to have information on the provenance of seed or seedlings in this nursery and the location of plantations established with seedlings produced in this nursery. A report on Reforestation in Newfoundland, from 1937 to 1952, summarized the establishment and activities of the Back River Nursery (Doyle, 1967). It gives details of planting stocks produced, and identified 16 plantations established on the Avalon, Burin and Bonavista Peninsulas between 1938 and 1951. This report indicated also that all stocks at the Back River Nursery were produced from seeds: white spruce (*Picea glauca* (Moench) Voss), white pine (*Pinus strobus* L.) and balsam fir (*Abies balsamea* (L.) P.Mill.) were of local origin while Norway spruce (*Picea abies* (L.) Karst.), red pine, Scots pine and jack pine came from Ontario, Canada. It is important to note that Scleroderris canker cannot be transmitted by seed. Another report provided a detailed historical account of the Back River Nursery (Baker and Miller-Pitt, 1998). In 1939 the Newfoundland Forestry Division received a gift of 30,000 seedlings of red, white, jack and Scots pines from the Province of Ontario, Canada.

With this information on hand, a survey of localized plantations was done in 1998. The 16 plantations established with Back River Nursery stock, revealed that most plantations were in very poor condition, but Scleroderris canker EU race was present in a number of them. The majority of red pine had died but the disease was still present and viable in the surviving Scots and jack pines which show resistance to the disease

(Bernhold et al., 2009; Laflamme and Blais, 2000). Even if these pines are surviving with the disease for several years, the host persists as an endemic carrier of the pathogen. Infected plantations outside the Avalon Peninsula, at Sunnyside and on the Bonavista Peninsula, were sanitized with expectations of limiting the disease to the Avalon Peninsula, and keeping the 1998 quarantine zone in place. The quarantine and natural barrier at the isthmus of the Avalon Peninsula was successful in limiting the spread of Scleroderris canker EU race for over 60 years (Figure 2).

4. OUTBREAK IN SOUTH-CENTRAL NEWFOUNDLAND

In 2007, severe red pine mortality was reported in an 18-year-old red pine plantation at Berry Hill Pond, 90 km down the Bay D'Espoir highway from the Trans Canada Highway, approximately 400 km outside the quarantine zone. (Figure 3). *G. abietina* was isolated and molecular tests have proven the disease to be Scleroderris canker EU race. Damage assessment done in

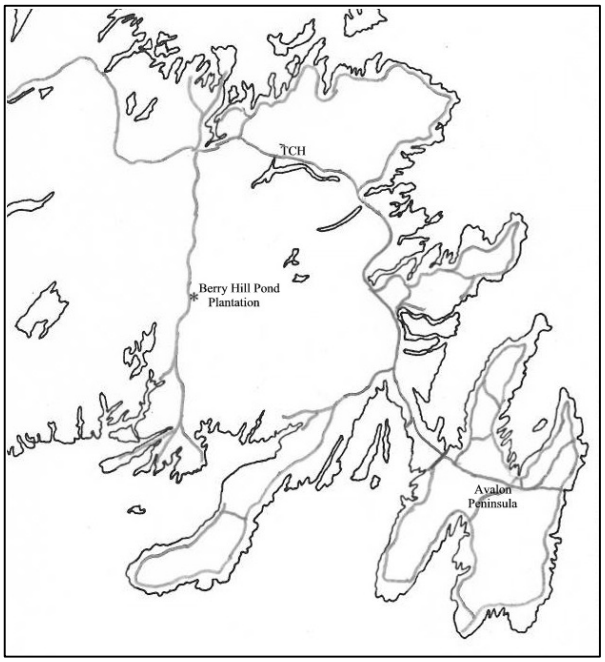

Figure 3: Outbreak in Central Newfoundland, 400 km from the quarantine zone for Scleroderris canker, European race on the Avalon Peninsula.

2007 and 2008 revealed the disease had been present for 7-8 yr, building up inoculum by infecting lower branches in a center of infection. Then, optimum conditions conducive for spore release, dispersal and infection occurred 2-3 years prior to 2007 resulting in killing pines in the center of infection by shoot infection of the whole crown. This high spore load in the top of crown exposed to wind, rain and snow helped the disease to spread on top of surrounded healthy pines. Spores from these tops

were rain splashed and disseminated on lower shoots, killing the residual trees the following year.

Planting stock for this plantation was produced in the Wooddale provincial tree nursery in central Newfoundland. This plantation and several others in the forest management district were established in 1989 using the same red pine planting stock. An inspection in the fall of 2007 of the other plantations showed no other signs of infection, ruling out infected planting stock as the source of the disease. Geographic isolation of this plantation, surrounded primarily by bog and scrub forest with no pine content, has all but ruled out natural spread of the disease from the Avalon Peninsula. Forestry personnel from the Bay D'Espoir office commented that locations along the road through the plantation were common camp sites for moose hunters from the Avalon Peninsula in the late 1980's early 1990's. Two tall communication towers along the road to the plantation were easy landmarks for hunters to locate the campsites. It is suspected that hunting groups from the Avalon Peninsula brought their own kindling and firewood with them, which would have been readily available from recently killed red pine in the infected Conception Bay plantations.

5. DISCUSSION

After 15 years of observations on the development of a Scleroderris canker epidemic, EU race, in 50 red pine plantations located in Quebec, we can sum up the results into three steps:

1- There is a build up of inoculum in a centre of infection.

2- Followed by a spread of infection on lower branches in the snow over a large area.

3- Finally, under conducive climatic conditions, conidia of *G. abietina* produced on lower branches will spread the disease higher in the crown of trees.

In south-central Newfoundland, we observed a different pattern. The epidemic started in a centre of infection and killed the trees in that centre relatively rapidly. The large number of infected shoots in the crown of dead and dying trees produced a large amount of inoculum in the upper part of trees. The strong wind prevailing in that region, with rain and snow had spread the disease to the top of surrounding trees, causing their death in less than 2 to 3 years. The disease became so widespread that nothing could be done to save the plantation. Because of the climatic conditions favourable to the disease in that region, the other red pine plantations should be pruned to prevent any build up of inoculum in the eventuality of an introduction of *G. abietina*.

From the historical reports, seedlings of pine were imported into the Island of Newfoundland from Ontario, Canada. It is difficult to conclude that the introduction of the disease came with this nursery stock: the European race was not

found in Ontario until 1985 and the disease had been present in Back River Nursery several years before. So the origin of the introduction of the disease on the island is still unknown. Based on preliminary molecular study, the Newfoundland introduction would be different than the one on continental North America; moreover, the pathogen population in Nfld. shows more relationship with the pathogen population from Europe (Hamelin et al., 1998). The introduction could have come from Austrian pine seedlings imported from Europe; this pine species has been planted in St. John's area for many years. This tree species was never imported for reforestation; it was imported as ornamental probably not too long after the establishment of the Back River Nursery, but this hypothesis remains to be proven.

The breach of the quarantine zone now places the natural red pine stands and numerous plantations in central Newfoundland region at a serious risk of extinction. A communication plan to the public should be undertaken through information, pamphlets, display notices, roadside signs and kiosk.. It is necessary to maintain the Scleroderris canker quarantine on the Avalon Peninsula. The isthmus of the Avalon Peninsula is a natural barrier with limited alternate access, where quarantine notices, signage and material deposition depots can be setup.

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Shoot Blights

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CONE DAMAGES BY *Diplodia pinea* AND SEED BORING INSECTS ON *Pinus pinea* L. (ITALIAN STONE PINE) IN CENTRAL ITALY

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ABSTRACT

In Italy *Pinus pinea* L. (Italian Stone Pine) is cultivated for several purposes but mainly for dune protection, landscape conservation, tourism, and seeds production. This economic activity is annually negatively affected by the occurrence of fungi, insects, and abiotic agents but also by a new alien pest (*Leptoglossus occidentalis* Heidemann) recently introduced from North America. In order to clarify the main reasons of seed losses, monitoring investigation have been organized in different pinewood located along the Tyrrhenian cost in Tuscany. During the period 2006 – 2008 cones, having different ages (from 1 to 3 years old) were analyzed. The main agents responsible of biotic damage were identified and their incidence on losses was ranked.

The study showed that: - about 64% of 1-yr old and 36% of 2-yrs old cones were affected by *D. pinea*; - more than 80% of old cones collected on the ground in pinewoods were colonized by the fungus; - up to 57% of old infected cones produced available inoculum, able to germinate in 6h at 25°C. The most consistent damages on the harvested mature cones were caused by *D. pinea*, followed by small mammals and insects including *L. occidentalis*, although differences among forest occurred. In infected cones by the fungus seed losses were up to 64%.

Keywords: *Pinus*, edible seeds, insects, diseases, fungi

1. INTRODUCTION

Pinus pinea L., the Italian Stone Pine with its umbrella-shape crown is one of the most characteristic and attractive Mediterranean trees. It is usually grown in pure pinewood plantation along the coasts, or mixed with other Mediterranean species in temperate forests. Often it is also cultivated as an ornamental tree, in gardens and public parks areas of Southern Europe (Bernetti, 1995).

P. pinea is also well known for its edible seeds (pinoli) about two cm long that quite often constitute the main income for pinewoods owners (Bernetti, 1995; Ducci et al., 2001; Saporito, 2002). Seeds are produced by pines after a long process of maturation. The cones ripen after three years expanding to oval-shaped, 300 gr. weigh. During that long period they are exposed to several biotic threats

due primarily to insects *Ernobius impressithorax,* (*Coleoptera,Anobiidae)* (*Dioryctria mendacella* Stgr. (*Lepidoptera, Pyralidae*), *Pissodes validirostris* Gyll. (*Coleoptera, curculionide*), small mammals (squirrels and dormouse) (CanakcioglU, 1969; Roques, 1983; El Hassani and Messaoudi, 1986; Innocenti and Tiberi, 2002) and fungi, mainly *Diplodia pinea* (Petri, 1917; Petri and Adani, 1916; Verona, 1950; Maresi et al., 2002). More recently a new alien insect, *Leptoglossus occidentalis* Heidemann (*Heteroptera Coreidae*), was accidentally introduced in Italy (Bernardinelli and Zandigiacomo, 2001) and contribute to enhance the amount of seed damages.

Since the 2006-2007 the amount of seed production dropped down causing serious economic concern for the market and several survey activities were organized in order to evaluate the main causes of seed losses.

2. MATERIALS AND METHODS

The studies on the reduction of seed production from *P. pinea* trees were carried out in Tuscany, Central Italy during the period 2006 – 2008 in different pine forests. Two areas were located in the North, close to Pisa, in the Regional Park "Parco Migliarino S. Rossore Massaciuccoli" MSRM-1 (Migliarino) and MSRM-2 (Tirrenia), while the third was situated in the South of region, close to Grosseto, in the "Parco Naturale della Maremma" PNM (Alberese). All the mentioned areas are particularly large pinewood forests from where every year cones are usually harvested and seeds are send to the market.

The study was organized following different aspects:

2.1. - Surveying on the Occurrence of *D. pinea* on cones of different age. Pinewood **a**reas MSRM-1 and MSRM-2.

a) Occurrence of *D. pinea* on immature 1- 2 yr-old cones. During the winter 2006 five trees were felled and 150 green cones (n.100 1yr-old and n.50 2yr old samples) were randomly collected from the crown. Cones were then put in moist chambers up to 15 days and regularly checked to detect the occurrence of *D. pinea* (Feducci, 2007).

b) Occurrence of *D. pinea* on mature and old cones lying on the ground. Pinewood **a**rea MSRM-1. About 600 cones were collected under the pine crown in order to detect the occurrence of the fungus. Cones were ranked according to the age (1, 2 >2 yrs) and percentage of scales cover by pycnidia (< 15% ; 15 – 50 ; > 50%) (Pepori, 2006).

2.2. Detecting the inoculum availability from mature cones on the ground. Pinewood area MSRM-1. A sample of 135 cones was collected from 5 different particles under the crown of 27 pines. Cones were processed according to Munck and Stanosz (2009). The method was modified and adjusted to the size of cones (350 ml of water was used in rinsing the cones). A conidia suspension was

obtained for each cone and used for germinability test on 2% Malt-agar in Petri dishes at 25°C for 6 hours (Cambi, 2008).

2.3. Assessing the sanitary conditions of harvested mature cones (3 yrs-old). Pinewood areas MSRM-1, MSRM-2, and PNM. In order to evaluate the occurrence of damage on cones ready to the extraction process, about 3000 cone samples were collected (1000 from each previously mentioned area). Symptoms of damages, if present, were visually detected and classified according their main causes: insects (*Dioryctria sp.*, *Ernobius impressithorax, Pissodes validirostris), L. occidentalis,* small mammals (Dormouse, *Glis glis;* Squirrel, *Sciurus vulgaris*), *Diplodia pinea* and abiotics. Total observed: 3045 cones (Migliarino 1045, Tirrenia 1000, Alberese 1000).

2.4. Test seed viability. Pinewood **a**reas MSRM-1, MSRM-2, and PNM. From a sample of 100 cones, 80 affected by *D. pinea* and 20 apparently healthy as control (see 2.3) all seeds were extracted and than checked for their viability conditions, first visually and later by using the Tetrazolium Test (International Seed Testing Association., 2003; 2007; Feducci, 2007).

3. RESULTS

3.1. Occurrence of *D. pinea* on cones of different age.

a) Immature 1-2 yr-old cones. *D. pinea* is a fungal parasite quite common in young pine cones. After evaluation about 64% of 1-yr old and 36% of 2-yrs old cones were affected by the fungus. Percentages of apparently healthy cones increase according to their age (Table 1).

Table 1. Occurrence of *Diplodia pinea* on *Pinus pinea* cones of different age.

Cone conditions	1 year-old cones		2 years- old cones	
	Apparently healthy	Affected by *D. pinea*	Apparently Healthy	Affected by *D. pinea*
data in %	36.6±13.9	63.4±13.9	66.7±4.3	33.3±4.5

b) Mature and old cones lying on the ground. Observations on cones on the ground under the crown of pines show that the number of old ones was quite relevant. The percentage of cones having more than 50% of scales covered by *D. pinea* was also consistent (Table 2).

Table 2. Occurrence of pycnidia of *Diplodia pinea* on *Pinus pinea* cones.

Cone conditions	Age of cones			Occurrence of *D. pinea* on cones*			
	1 yr	2 yrs	>2 yrs	0	1	2	3
Data in % ±st	6,7±0,8	12,1±5,6	81,2±4,0	5,9±2,1	7,4±1,1	15,0±0,2	71,3±3,2

* Occurrence of *Diplodia pinea*. 0= none or few pycnidia; 1 = pycnidia detected on < 15% of cone scales ; 2 = 15 – 50 % ; 3 = > 50%.

3.2. Detecting the inoculum availability from mature cones on the ground. A large amount of cones colonized by *D. pinea*, and showing pycnidia (Table 3), were still actively releasing conidia. Among them 55.6% of 1-yr old cones and 63.9% of >3-yrs cones were still producing inoculum. Percentage of conidia germination after 6 h at 25°C was 47.4%.

Table 3. Inoculum availability of *Diplodia pinea* (pycnidia) on *Pinus pinea* cones.

Ages of cones	Percentage of cone scales showing *D. pinea* pycnidia		
	<15	15-50	>50
1-yr	15.6±0,8	66.7±5,6	17.8±4,0
2-yrs	0.0	42.2±5,6	57.8±4,0
>3-yrs	35.6±0,8	53.3±5,6	11.1±4,0

3.3. Sanitary conditions of harvested mature cones (3 yrs-old).

Data obtained from cones ready for seed extraction showed that damages ranged from 28-55% according to the different forest areas considered. The most consistent damages were caused by *D. pinea* (average 16%) followed by small mammals and insects (about 6%) and *L. occidentalis* (4%) (Table 4). Differences values among forests were quite pronounced particularly for the fungal parasite (Figure 1).

Table 4. Main damages observed on 3-yrs old *Pinus pinea* cones.

Sampling area	Cone conditions		Causes of damage				
	Healthy (n.1900)	Damaged (n.1145)	Insects*	*L. occid.*	Small mam.**	*D. pinea*	Other causes
MSRM-1	71.1	28.9	6.3	2.5	2.5	14.5	3.1
MSRM-2	44.8	55.2	4.5	8.0	1.3	32.0	9.4
PNM	70.9	29.1	7.5	3.2	15.6	2.4	0.4
Average±sd	62.4±15.1	37.6±15.1	6.1±1.5	4.5±3.0	6.4±7.9	16.3±14.9	4.3±4.6

**Insects: Dioryctria sp.*, *Ernobius impressithorax, Pissodes validirostris.*

** Small mammals: Dormouse (*Glis glis*), Squirrel (*Sciurus vulgaris*).

Total cones: 3045 (Migliarino 1045, Tirrenia 1000, Alberese 1000)

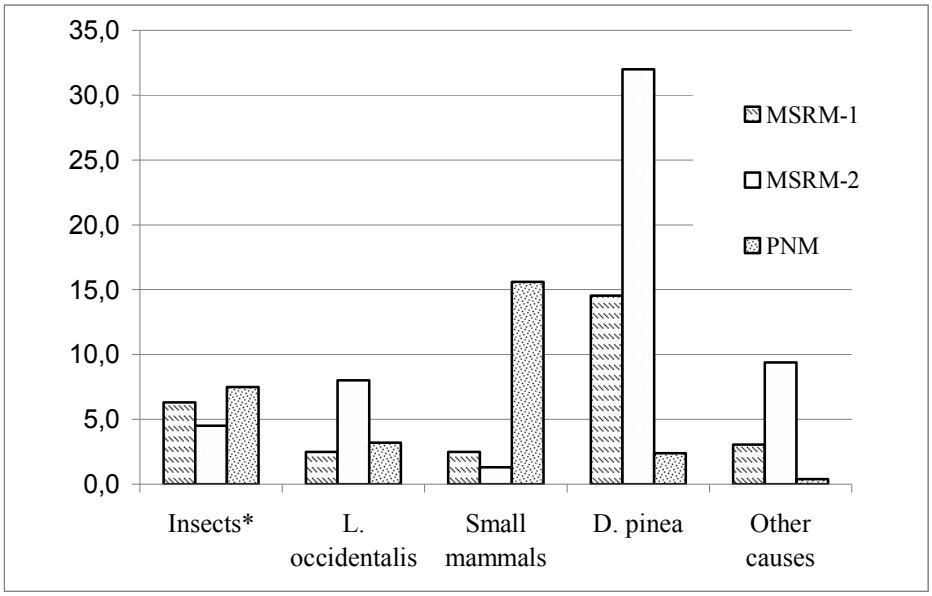

Figure 1. Main damages observed on 3-yrs old *Pinus pinea* cones according different pine forests. (MSRM-1 "Parco Migliarino S. Rossore Massaciuccoli" MSRM-2, Tirrenia; PNM "Parco Naturale della Maremma"

3.4 Test seed viability. Quality of seeds resulted quite variable after visual assessment and TZ test. Seeds discarded were about 35% from apparently healthy cones and raised up to 55% and 64% from partially injured and injured cones respectively (Table 6).

Table 6. Percentage of seed losses in healthy and injured cones by *D. pinea*.

Percentage of seed losses after/	Seeds from healthy cones (n.503)	Seeds from partially Injured cones (n.1056)	Seeds from Injured cones (n.1221)	TOT (n.2780)
Visual assessment	34,4	50,5	60,9	52,1
TZ test	0,9	4,6	3,8	3,4
T. losses	35,3	55,1	64,6	55,5

4. DISCUSSION

Due to the losses of seed production several survey activities have been promoted by Italian local regional governments. In this study, conduced during the period 2006-2008, mainly supported by the Tuscany region, the surveys showed that seed losses can be attributed to different causes but mainly to *D. pinea*, the fungal parasite which is able to colonize a large amount of cones since the first

year after their development (up to 64% of 1-yr losses). Later on the fungus can survive for long time on old cones on the ground under the canopy (up to 80% of old cones sampled). Large part of these cones, confirming Munck and Stanosz (2009), produced available inoculum, able to germinate in 6 h at 25°C and able to infect the new cones generation.

Ranking the most common types of damage on the harvested mature cones, we found that large part of losses can be attributed to *D. pinea*, followed by small mammals and insects, including *L. occidentalis*. However the occurrence of the fungus was clearly related with site conditions and was more pronounced in humid areas whilst was lower in dry forests where insects and small mammals prevailed.

On the basis of this data seed losses related to *L. occidentalis* were not so evident. Probably the role of the insect is more dangerous during the early stages of cone growth and also it remain difficult to be detected on mature cones.

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SUSCEPTIBILITY OF DIFFERENT CONIFEROUS SEEDLINGS INOCULATED WITH *Diplodia pinea*

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ABSTRACT

In this study, virulence of 5 *Diplodia pinea* isolates on *Pinus nigra*, *Pinus brutia*, *Pinus sylvestris*, *Cedrus libani*, *Abies nordmanniana* ssp. *bornmülleriana* and *Juniperus excelsa* seedlings was investigated. Needle fascicles on terminal and three lateral shoots of each seedling were removed to create small wounds. Agar plugs with *D. pinea* mycelia were placed in the wounds and secured in position with laboratory film. Five seedlings were used for each species-isolate combination and a randomized complete block design was used in the trial. Nine weeks later dead shoots, lesion length and fungal growth were recorded. Dead shoots occurred in almost all isolate–host combinations: the only exceptions were two isolates on *J. excelsa*. Within host variation in dead shoot rates among the isolates was low. However, there was high variation in the mean dead shoot rate among the hosts, with the highest rate (98.0%) on *C. libani,* and the lowest (4.0%) on *J. excelsa.* On *C. libani* all isolates caused remarkable lesion, while on the other host species some of the isolates caused lesion. The average lengths of lesion were 15.72 mm on *C. libani,* 8.12 mm on *P. nigra*, 2.4 on *P. sylvestris*, 1.2 on *P. brutia* and 0.08 mm on *A. nordmanniana* ssp. *bornmülleriana* and *J. excelsa*. Similarly, average linear extension of *D. pinea* in the shoot tissues was high on *C. libani* and *Pinus* spp. and low on *J. excelsa*. The results indicate high virulence of *D. pinea* on *C. libani* and *P. nigra.*

Keywords: Pathogenicity, *Pinus nigra*, *Pinus brutia*, *Pinus sylvestris*, *Cedrus libani*, *Abies nordmanniana* ssp. *bornmülleriana*, *Juniperus excelsa*

1- INTRODUCTION

Diplodia pinea (Desm.) Kickx. (=*Sphaeropsis sapinea* (Fr.) Dyko & Sutton) has a worldwide distribution and causes the disease known by the common name of Diplodia tip blight of pine (Stanosz et al., 1996). The fungus can affect the trees from early to older ages causing shoot blight, twig blight, dead top, sap stain, root

disease and cankers on stems and branches (Brookhouser and Peterson, 1971; Peterson, 1977) and can have devastating effects on various conifers when it is associated with stress-inducing factors (Sinclair et al., 1987; Swart et al., 1987; Chou and Mackenzie, 1988; Nicholls and Ostry 1990; Stanosz et al., 2001).

Although diseases caused by the fungus are usually on trees under stress, healthy trees, especially nursery seedlings can also be infected (Palmer and Nichols, 1985). *Pinus* species are the most common hosts of the fungus, but *Abies* Mill., *Chamaecyparis* Spach., *Cupressus* L., *Larix* Mill., *Picea* A. Dietr., *Pseudotsuga* Carriere and *Thuja* L. species are among hosts of the pathogen (Punithalingam and Waterston, 1970; Swart and Wingfield, 1991). Previous studies reported significant interactions among host species, environmental factors and the virulence of the isolates (Swart and Wingfield, 1991; Blodgett and Stanosz, 1997; Adams et al., 2002; Blodgett and Bonello, 2003).

Two different *S. sapinea* morphotypes have been described and are referred to as A and B (Palmer et al., 1987; Smith and Stanosz, 1995; de Wet et al., 2000; Burguess and Wingfield, 2001). While *D. pinea* is accepted name for morphotype A, morphotype B has been recognized as *Diplodia scrobiculata* J. de Wet, B. Slippers & M. J. Wingfield (de Wet et al., 2003). It is reported that they differ in morphology, cultural characteristics and aggressiveness against host plants (Smith and Stanosz, 1995; Blodgett and Stanosz, 1997; Blodgett and Bonello, 2003).

Eventhough *D. pinea* was recorded sixteen years ago for the first time on *Pinus pinea* and *Pinus pinaster* (Unligil and Ertas, 1993), very little is known about the distribution and the damage of the disease by far in Turkey (Doğmuş- Lehtijärvi, et al. 2007). Studies carried out in the western part of Taurus Mountains located in the Mediterranean part of Turkey, in Isparta province showed that *D. pinea* was the main agent of the shoot blight of Calabrian pines (*Pinus brutia* Ten.) (Doğmuş-Lehtijärvi et al., 2007). The aim of this study was to determine the pathogenicity of *D. pinea* isolates obtained from *P. brutia* showing shoot blight symptoms, on 6 conifer species, under field conditions.

2- MATERIALS AND METHODS

Five-year-old potted *Pinus nigra* Arnold, *Pinus brutia* Ten, *Pinus sylvestris* L., *Cedrus libani* A. Rich., *Abies nordmanniana* ssp. *bornmülleriana* Mattfelt seedlings obtained from Eskişehir and Balıkesir forest nurseries and 3- year-old *Juniperus excelsa* Bieb seedlings obtained from Eğirdir forest nursery were used as the plant materials. Fungal materials were the five *D. pinea* isolates obtained from the Calabrian pine (*P. brutia*) shoots showing blight symptoms in Aşağı-Gökdere location of Isparta province, Turkey. The isolates were confirmed as *D. pinea* by Glen Stanosz, (Department of Plant Pathology, University of Wisconsin-Madison, personal communication) using species-specific primers (Smith and Stanosz 2006).

Pathogenicity trial was carried out in the campus area of Süleyman Demirel University, during October-November, 2007. The *D. pinea* isolates were grown on PDA (Merck) at 24ºC in the dark, for one week. Four wounds of 2 x 2 mm were made approximately 2 cm below the shoot apex, on one terminal and three lateral shoots of each seedling, removing a needle fascicle by a scalpel and agar plugs with *D. pinea* mycelia cut from the actively growing culture were placed mycelia-side-down on the wounds (Blodget and Stanosz, 1997). Wounds were then wrapped with parafilm. Non-colonized agar plugs were placed on control seedlings. Five seedlings were used for each species-isolate combination and a randomized complete block design was used in the trial. Seedlings were incubated under field conditions for 9 weeks. Average temperature in October was 14.4°C (maximum up to 28.2 °C, minimum 0.8 °C) and in November 7.4 °C (maximum up to 22.9 °C, minimum -10°C). Average relative humidity was 58 % in October and 76 % in November. The seedlings were regularly irrigated and controlled for characteristic disease symptoms. Dead shoots were recorded.

At the end of the inoculation period, inoculated terminal and lateral shoots were cut 15 cm below the inoculation point and brought to the laboratory. Based on the color changes on the shoots and needles, lesion sizes were measured. Then the needles on the shoots were removed and the shoots were cut into 1 cm long segments, from the inoculation point to the cut end of the shoots. The segments were surface sterilized by keeping them 10 seconds in 96 % ethyl alcohol and 4 minutes in 1 % NaOCl and dried between sterile paper towels. They were then placed on petri plates with 2.5 % bacto agar and 0.05 % tannic acid, 5 segments per plate, in a clockwise serial order.

The plates were incubated at room temperature for 4 weeks and examined under stereomicroscope for the presence of *D. pinea* colonies growing from the segments. Dead shoot rate, lesion length and fungal growth data obtained for each tree species-isolate combination were statistically analyzed by using SPSS program.

3- RESULTS

Susceptibility of the tree species to *D. pinea* and the virulence of the *D. pinea* isolates among those host species were different from each other. Dead shoots were observed in all isolate-host combinations, except for P047 and P097 isolates on *J. excelsa*. Considering the dead shoot rates, within host variation of the isolates was low, while host species had high variation. The highest rate was on *C. libani* (98.0%), followed by *P. nigra* (51.0%) and *P. brutia* (31.9%). Dead shoot rates were low on *Abies* (16.0%), *P. sylvestris* (12.8%) and *J. excelsa* (4.0%) (Figure 1).

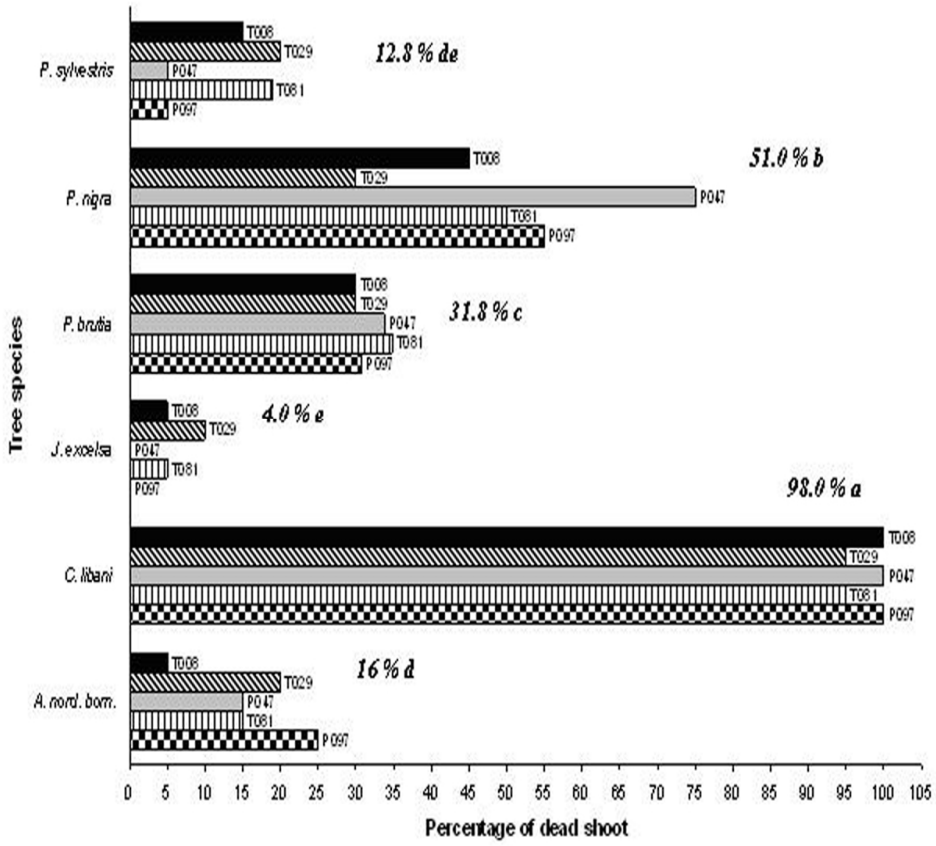

Figure 1. Dead shoot rates of five tree species inoculated with five D. pinea isolates.

On *C. libani* all isolates caused remarkable lesion, while on the other host species only 1-3 of the isolates caused lesion. The length of lesion ranged from 12.4 to 21.4 mm on *C. libani* and from 9.0 to 18.6 mm on *P. nigra*, while on the other hosts the length did not exceed 6.0 mm (Figure 2).

There was a significant correlation ($r= 0.53$, $p< 0.01$) between length of lesion and linear fungal extension on all host-isolate combinations. Average length of the lesion was greater than average fungal extension in all host species including all isolates of the fungus (Table 2, Figure 3). The average fungal extension was found 27.6 mm on *P. nigra*, 24.0mm on *P. brutia,* 18.4mm on *C. libani*, 16.4 mm on *P. sylvestris* and 8.4 mm on *A. nordmanniana bornmülleriana.* Similar to length of lesion , average fungal extension was the lowest on *J. excelsa* (1.6 mm). Control seedlings did not show any symptoms of disease.

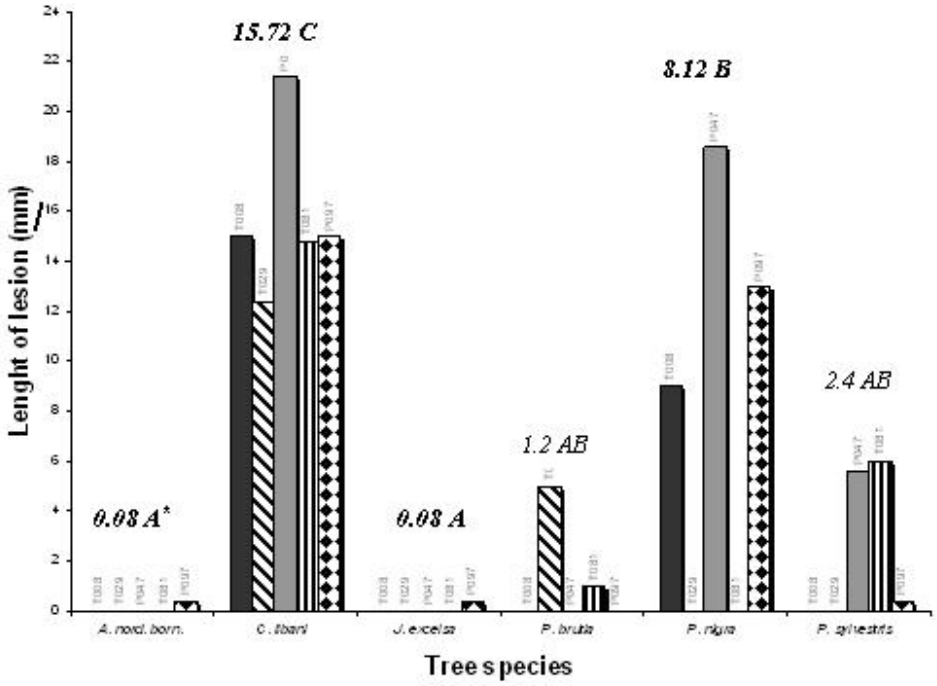

Figure 2. Length of lesions of five tree species inoculated with five *D. pinea* isolates.

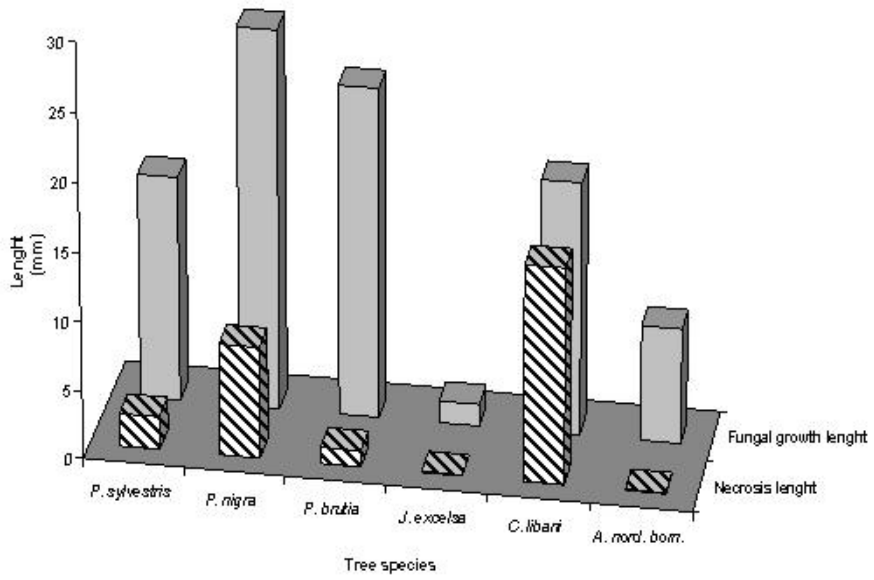

Figure 3. Average fungal extension and length of lesion caused by *D. pinea*.

Table 1. Linear extension of *D. pinea* in the shoots of five tree species inoculated with different isolates (mm).

Tree species	Isolates						
	T008	T029	P047	T081	P097	Control	Averages
A.n.bor.	6.0 B ab	8.0 B a	10.0 BC a	10.0ABC a	8.0 B a	0.0 Ab	8.4AB
C. libani	12.0 AB b	34.0 A a	14.0 B b	20.0AB ab	12.0 B b	0.0 Ac	18.4 BC
J. excel.	2.0 B a	2.0 B a	2.0 C a	2.0 C a	0.0 C a	0.0 Aa	1.6 A
P. brutia	20.0 A ab	38.0 A a	20.0 B ab	24.0 A b	18.0AB ab	0.0 Ac	24.0 BC
P. nigra	22.0 A abc	22.0ABabc	54.0 A a	6.0 BC bc	34.0 A ab	0.0 Ac	27.6 C
P.syl.	12.0AB ab	8.0 B bc	28.0 AB a	24.0 A ab	10.0 B abc	0.0 Ac	16.4 ABC

Means in the same column followed by the same uppercase letter and means in the same row shown by the same lowercase letter were not significantly different from each other according to Duncan's Multiple range test (P=0.05)

4- DISCUSSION

Inoculations with the five *D. pinea* isolates resulted in necrotic needles and dead shoot tips on the tested coniferous tree species, showing high virulence on *C. libani* and *P. nigra* and low on *A. nordmanniana* ssp. *bornmülleriana* and *J. excelsa*. Even though most of the coniferous tree species have been tested for their sensitivity to *S. sapinea* sensu lato (s.l.) (Chou, 1976; Brookhouser, 1971; Blodgett and Stanosz, 1997; Flowers, 2001; Blodgett and Bonello, 2003; Luchi et al., 2007; Munoz et al., 2007) this is the first study mentioning the high susceptibility of *C. libani* to *D. pinea* isolates.

The time of the inoculation significantly affects the relative aggressiveness of the fungus. (Literatür). The results of the experiment indicate that *D. pinea* were pathogenic on tested conifer tree species, especially on *C. libani* and *Pinus* spp. However, inoculations with most of the fungal isolates resulted in little or no measurable symptoms on shoots of *J. excelsa* and *A. nordmanniana* in October and November when the experiment was carried out. To confirm these results it may be necessary to repeat the experiment during spring when the conditions are much more favorable for the natural spread of the fungus.

Blodgett and Stanosz (1997) indicated that the wound inoculation technique provides a reproducible method for comparing the aggressiveness of the isolates belonging to different species of the fungus but, they did not found this method reliable for comparing the aggressiveness of the isolates within the species. In the present experiment, the same technique was used to find out the susceptibility of the host species to the disease, and similarly, there were no significant differences between the five isolates on any of the tree species. Blodgett and Stanosz (1997) conducted the inoculation experiments also with conidial suspensions to be able to

understand the penetration capacity of the fungus to the healthy tissue. Also in the present study more realistic results of the virulence of the isolates could have been obtained by spraying the seedlings with conidial suspensions.

Virulence of *S. sapinea* s.l. isolates on pine varies depending on several factors, such as ecological and climatic conditions, season, species of the fungus and where the experiment takes place (Swart and Wingfield, 1991; Blodgett and Stanosz, 1997). It is also known that *S. sapinea* s.l. is able to live without typical symptoms in its latent phase (Stanosz et al., 1997; Flowers et al., 2001; 2003; Stanosz et al., 2005; Maresi et al., 2007). In that sense, it is important to have knowledge about the latent infections in nurseries and forest stands. *D. scrobiculata* was not found in the area where the isolates used in the present experiment were collected. As the isolates of *D. pinea* were directly obtained from pycnidia located on diseased shoots, instead of isolating from host tissues, only the frequently fruiting species could be found. Since the asymptomatic persistence of the fungal species in the host tissues is important for their wide distribution, a study focusing in latent infections is needed to shed light on the Diplodia-species composition on the local tree species and their infection potential.

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SITE AND STAND CHARACTERISTICS OF A *Pinus brutia* STAND INFECTED WITH *Diplodia pinea* IN TURKEY

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ABSTRACT

Shoot blight can be a very costly disease for coniferous forests as it results in defoliation and reduced growth. In this study, some of the site and stand characteristics of a *Pinus brutia* Ten. stand infected with *Diplodia pinea* (Desm.) Kickx has been investigated. Blight occurrence was investigated for two different years, and also some soil and foliar nutrients were determined. Our results showed that at the first sapling time in 2006, 18 out of 90 shoot samples had infection, while in 2008 this number fell down to 5 out of 90 samples. Results for soil analysis generally indicated somewhat poor site conditions. On average, soil had sandy clay loam texture, reaction was 7.72 and organic matter content was moderate with 3.84 percent. However, soil had a coarse fraction up to 62 % indicating relatively poor nutrition. Similarly, foliar nutrient levels also showed poor site quality. Overall, needle N concentration was lower than 1 %, indicating N deficiency on these sites. These poor site conditions and extreme droughts may have played a role in development of the shoot blight in these stands.

Keywords: *Diplodia pinea*, *Pinus brutia*, drought, nutrients

1. INTRODUCTION

Pinus brutia Ten. is the most commonly distributed coniferous forest tree species in Turkey, covering approximately 5.4 million hectares. It can be found at elevations between sea level and 1500 m. *Pinus brutia* grows best at elevations of 600-800m, below which very high temperature and demand for evapotranspiration, and above which cold weather restrict its distribution and growth (Boydak, 2000)

Diplodia pinea (Desm.) Kickx is one of the most common disease agents in coniferous forests. It causes necrosis on needles, and therefore defoliation and death of shoots. In turn, it results in reduced growth and substantial economical losses (Stanosz et al., 2004).

Several studies indicate higher risk of disease if plants are stressed. Especially, water stress seems to increase the occurrence of this disease. Paoletti et al. (2001) showed that water stressed *Pinus halepensis* Mill. seedlings had longer cankers of *Sphaeropsis sapinea* sensu lato 5 months after inoculation. Blodgett et al. (1997a) have also shown that 3-year-old red pine (*Pinus resinosa* Sol. ex Aiton) seedlings

had higher *S. sapinea* infection when the seedlings were subjected to water stress. Similarly, in a 9–year–old red pine plantation in Wisconsin, trees with lower predawn water potentials had more severe development of disease (Blodgett et al., 1997b). Also, nutritional imbalances and alterations of nutrients can affect development of disease (Stanosz et al., 2004). Blodgett et al. (2005) showed that fertilized red pine trees had higher foliar N and greater lesion size.

In 2004 and 2005, *Diplodia* had devastating effect on *P. brutia* stands on lower ranges of its distribution area (Lehtijärvi et al., 2007). It caused defoliation of the pines and reduced growth. In this study, the objectives were to determine the occurrence of disease on pines and also to determine the site and stand characteristics of this infected site.

2. MATERIAL AND METHOD

The study site is located in Asagi Gokdere district of Isparta, along the Isparta-Antalya highway (37^{o} 33" N, 30^{o} 45" E). The altitude is 350 m above sea level and the distance to the Mediterranean coast is 75 km. The site was planted in mid 1980's, and it became somewhat dense over time with natural seeding and lack of tending operations. Number of trees per hectare was 2100 on average and it reached as high as 2775 on one site. Mean annual precipitation is 1052 mm in Antalya weather station, and there is almost no rainfall between June and September during the growing season. Monthly average temperatures range from 0 ^{o}C to 23.4 ^{o}C, but maximum daily temperatures can be as high as 41 ^{o}C during the summer, indicating very high demand for evapotranspiration.

For the study, four different treatments with three replications were applied to the stands to control the disease. These treatments included control, pruning, thinning and pruning + thinning. Each plot was 20 x 20 m in size. Remains of pruning and thinning operations were removed from the plots afterwards.

We conducted some measurements to determine the disease rate and site parameters. These measurements included fungal survey, soil analysis, foliar analysis and water potential.

2.1. Fungal survey

10 trees were samples from each plot for fungal survey in two different years (2006 and 2008). One dead branch from each tree was cut and the shoots were investigated under the stereomicroscope for the presence of fungal structures. Samples were also isolated to identify the fungal species based on conidial and pycnidial morphology. Number of shoot samples with *D. pinea* was recorded.

2.2. Soil sampling

Soil samples were taken from 5 points in each plot at 0-20 cm depth. Coarse material larger than approximately 3 cm in diameter was not included in soil

samples. Samples were mixed for each plot, sieved to pass 2 mm after air drying, and physical and chemical properties were determined in laboratory.

2.3. Needle samples

Prior to sampling, every tree was labelled and their health condition was recorded. Five healthy and 5 unhealthy trees were chosen for foliar sampling in each plot (Figure 1). 40 fascicles were taken from each tree with a shotgun. Samples were dried at 70°C for 24 hours. The dry weights were measured and the needles ground, then chemical analysis was performed for nutrients.

2.4. Plant water potential

We assumed that water potential of the trees may have played a role in development of this fungal disease. Five trees were chosen in each plot for water potential measurements. From each tree, representative needle samples were taken from the bottom 1/3 of the canopy, and needle water potentials were measured with a pressure bomb. However, the data was not conclusive, so the initial results were not included here.

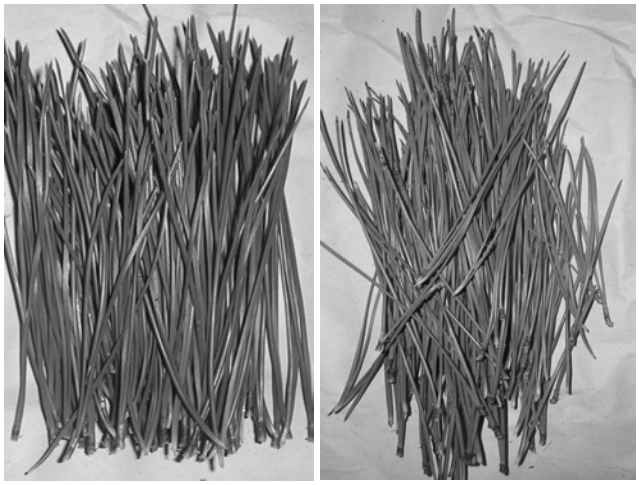

Figure 1. Healthy (a) and unhealthy (b) pine needles.

3. RESULTS

3.1. Fungal survey

Since start of the fungal disease in 2004, the rate of the disease has decreased over time. In 2006, the ratio of samples with *Diplodia* was 20 %, while in 2008 the ratio fell down to 5.5 %. As of today, the stand looks quite healthy.

3.2. Soil samples

On average, soil texture was sandy clay loam (Table 1). Soil reaction was relatively neutral. Organic matter and nutrient concentrations were moderate. However, soil

organic matter and nutrient contents were low considering that the coarse fraction (up to 3 cm) was as high as 62 %, and fraction larger than 3 cm in size estimated to be more than 50 % in some plots.

3.3. Needle samples

Similarly, foliar nutrient levels also showed poor site quality (Table 2). Overall, needle N concentration was lower than 1 per cent. Usually, less than 1.2 % N indicates some deficiency and N concentration is usually between 1.4-1.6 % in first quality sites. There was no significant difference between healthy and unhealthy needles in terms of nutrient concentrations (Figure 2), however fascicle weight of health needles were significantly greater (Figure 3).

Table 1. Soil characteristics of the infected stands.

Parameters	Mean	Std. Error
Sand (%)	50.11	1.958
Silt (%)	27.32	1.297
Clay (%)	22.57	1.570
Coarse faction (%)	34.63	5.664
EC	188.53	8.909
pH	7.72	0.044
Lime (%)	16.08	1.899
Organic matter (%)	3.84	0.116
N (ppm)	1360.33	71.736
P (ppm)	16.38	1.425
K (ppm)	256.17	14.053
Ca (ppm)	8130.92	361.213
Mg (ppm)	248.08	13.118
Na (ppm)	16.47	0.925

Table 2. Foliar characteristics of the infected stands.

Parameters	Mean	Std. Error
N (%)	0.97	0.024
P (%)	0.13	0.003
K (%)	0.33	0.010
Ca (%)	0.83	0.030
Mg (%)	0.21	0.007
Fe (ppm)	37.24	1.069
Cu (ppm)	3.13	0.197
Mn (ppm)	32.60	3.640
Zn (ppm)	15.00	0.414
B (ppm)	19.07	1.418
Fascicle weight (g)	6.53	0.204

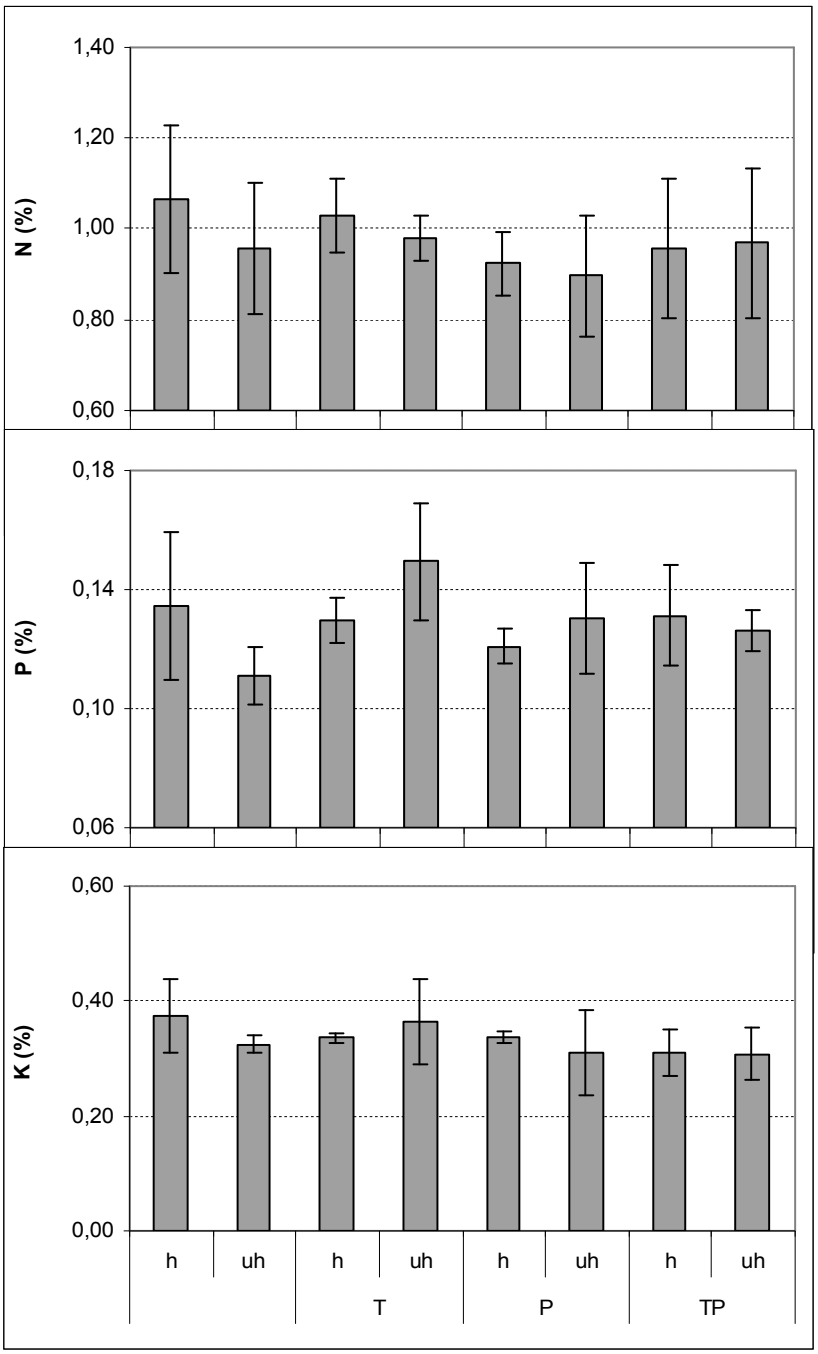

Figure 2. Nitrogen, phosphorus and potassium concentrations of healthy (h) and unhealthy (uh) needles in control (C), thinning (T), pruning (P) and thinning plus pruning (TP) treatments.

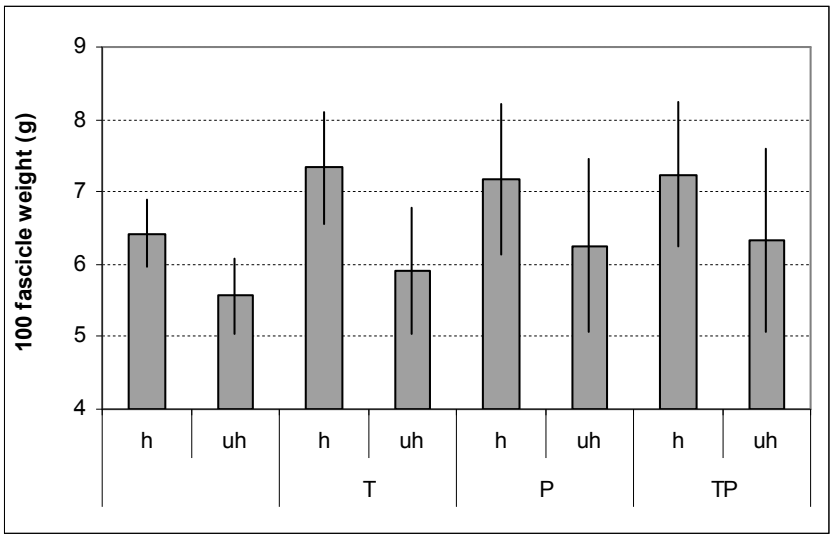

Figure 3. Fascicle weights of healthy (h) and unhealthy (uh) pine needles in control (C), thinning (T), pruning (P) and thinning plus pruning (TP) treatments.

4. CONCLUSION

Diplodia had some very serious effects on pine stands of Asagi Gokdere region. The symptoms diminished over the past four years since its first occurrence in this site. The site characteristics indicated poor site with low nutrients and rocky soil. Nutrient level in the needles also showed very low levels of N, indicating nitrogen deficiency. In fact, N levels in needles were almost one half of the one in a healthy needle. In addition, these stands have received no silvicultural treatments in recent years and the number of stems in these stands was almost twice as much as it should be.

Although the site is located in a region which receives approximately 1000 mm rainfall, most of it is received in winter, and summer droughts are very common. Temperatures reaching above 40°C during the summer months intensify the effects of drought, creating a very high demand for evapotranspiration.

These poor site conditions and extreme droughts may have played a role in development of the shoot blight in these stands. Apparently, the site was both nutrient and water limited, especially during the growing season. This limitation was most apparent when the canopy of the stand was investigated. In most areas of the site, the individual trees had very thin and sparse canopy due to lack of growing space and site resources to develop healthy crown (Figure 4). Timely and appropriate silvicultural treatments may reduce the effects of poor soil conditions and droughts, leading to stronger and more resistant trees beforehand, and better recovery after an infection.

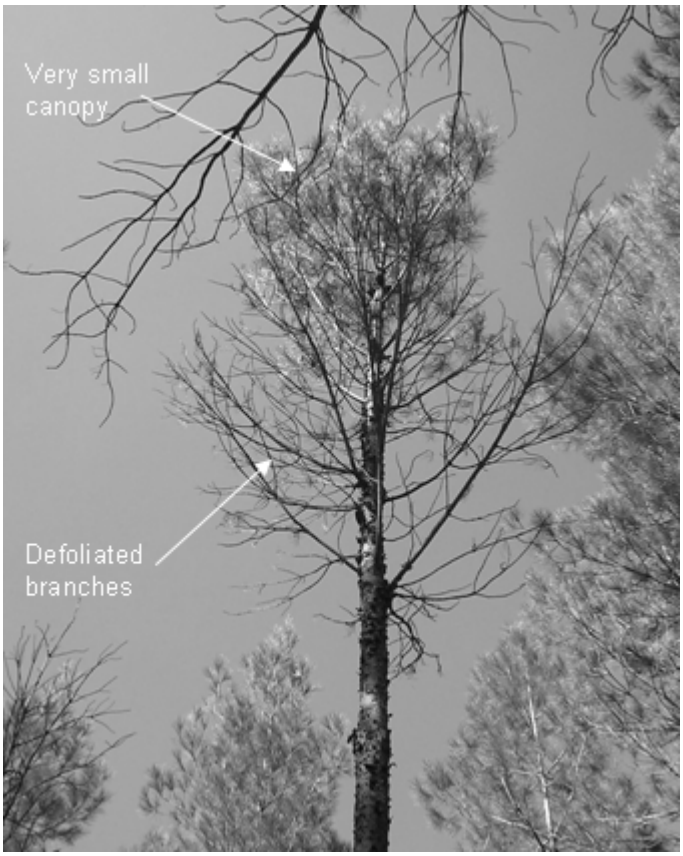

Figure 4. Defoliated branches and remaining thin canopy of *Pinus brutia* infected by *Diplodia pine.*

5. ACKNOWLEDGEMENTS

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THE EFFECTS OF *SIROCOCCUS* SHOOT BLIGHT AND VITALITY FERTILIZATION ON GROWTH OF MATURE NORWAY SPRUCE

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ABSTRACT

The impact of *Sirococcus* shoot blight and vitality fertilization on the growth of mature Norway spruce (*Picea abies* (L.) Karst.) was studied in a single tree fertilization experiment, established in autumn 2000. A total of 144 sample trees were selected among the dominant and co-dominant trees of the 90-year-old Norway spruce stand. Half of the trees exhibited severe symptoms of *Sirococcus* shoot blight whereas the other half were apparently healthy and vigorous. A randomised block design with the factors "slope section" (lower slope versus upper slope) and "*Sirococcus* shoot blight" (severely affected versus healthy trees) was used. Within these blocks sample trees were randomly assigned to one of the three treatments (dolomitic liming, application of gypsum and kieserite, unfertilized control). Due to tree mortality caused by bark beetle infestation the experimental design became unbalanced and therefore final analyses were performed with the volume growth data of 125 sample trees only. The effects of *Sirococcus* shoot blight and fertilization treatments on current annual volume increment were investigated by analysis of covariance, using the average volume increment of the period 1977–1980 as a covariate attribute (assuming that tree growth was not yet affected by *Sirococcus* shoot blight during this period). Indeed results indicated that *Sirococcus* shoot blight started in 1981 in the experimental stand and trees with shoot blight symptoms had a significantly lower increment over the whole period 1981–2006. *Sirococcus* induced increment reduction of the nonfertilized trees continuously increased from 7,5 ± 2,9% in 1981 to 37 ± 3,8% by the year 2000. A significant positive effect of vitality fertilization was only achieved with the gypsum and kieserite variant from 2002 to 2006. The highest surplus increment was found in 2004 with 31,6 ± 15,2%, calculated as average over the diseased and healthy group. However, a mitigation of increment loss caused by *Sirococcus* shoot blight was statistically significant only for the year 2003.

Keywords: *Sirococcus conigenus*, Norway spruce, increment reduction, vitality fertilization

1. INTRODUCTION

Since the early 1980s shoot blight by *Sirococcus conigenus* (DC.) P. Cannon & Minter has caused severe damage on Norway spruce (*Picea abies* [L.] Karst.) in some parts of Austria (Fig. 1). Previous studies revealed insufficient Mg and Ca supply, enhanced N/Mg and N/Ca-ratios in the needles of severely diseased trees (Anglberger et al., 2003) and demonstrated that application of appropriate fertilizers mitigated disease severity and promoted tree recovery (Halmschlager et al., 2007). The present study was carried out in the same individual-tree-fertilization experiment and aimed to investigate the effect of *Sirococcus* shoot blight on growth of mature Norway spruce (for further details see: Huber et al., 2009).

Figure 1. Severely affected mature Norway spruce.

2. MATERIAL & METHODS

The experiment was established in a mature Norway spruce stand in Upper Austria. Two fertilizer treatments and an unfertilized control variant were applied on a total of 144 dominant or co-dominant trees in a randomized block design (Fig. 2) in April 2001. Half of the trees exhibited symptoms of *Sirococcus* shoot blight ("diseased"), whereas the other trees were "healthy". The average basal area increment of the two groups diverged after the year 1980, therefore analysis of covariance was applied for testing the effects of fertilization and Sirococcus symptoms on volume increment, using the average of the current annual increment in the period 1977 to 1980 as the covariate.

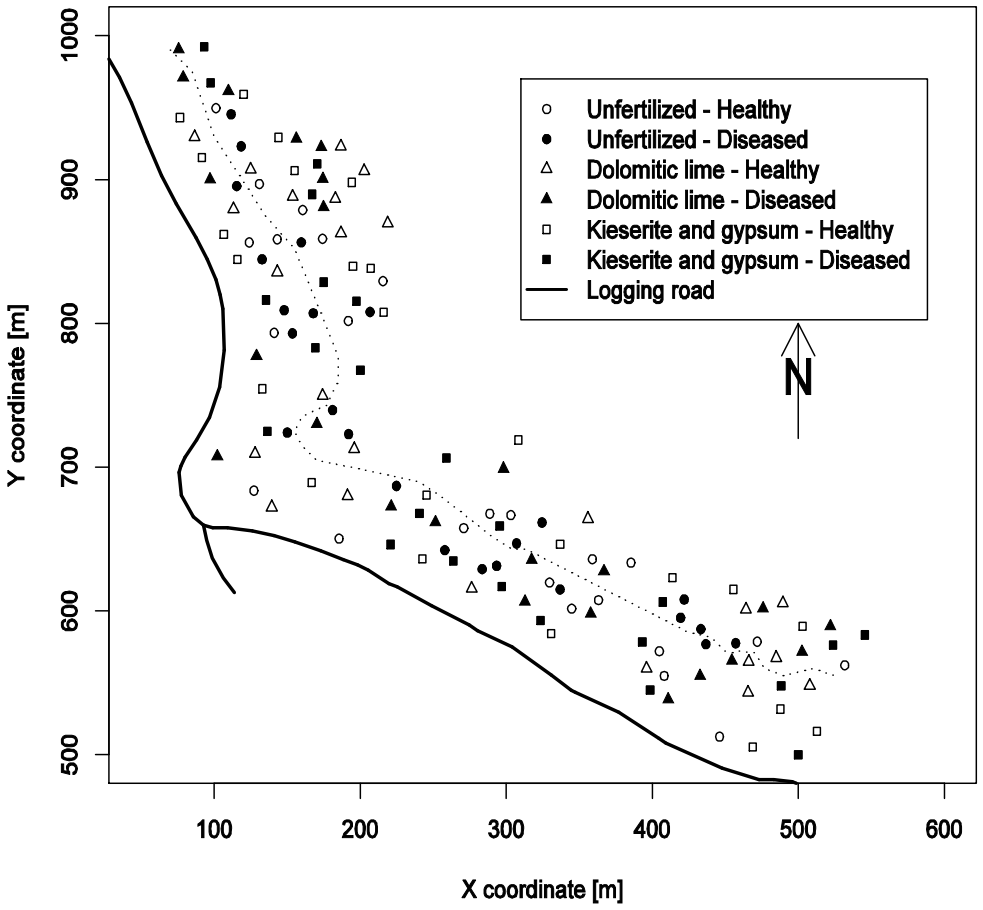

Figure 2. Position of the sample trees within the experimental stand. The tree position is marked with different symbols for the variants. Trees from the slope position "upper slope" are found northern to the dotted line. (Figure reprinted from Huber et al. 2009 with permission from Elsevier.)

3. RESULTS & DISCUSSION

Tree ring analyses indicated that *Sirococcus* shoot blight started in 1981 in the experimental stand and trees with shoot blight symptoms had a significantly lower increment over the whole period 1981–2006 (data not shown). *Sirococcus* induced increment reduction of the nonfertilized trees continuously increased from 7,5 ± 2,9% in 1981 to 37 ± 3,8% by the year 2000 (Fig. 3). Results therefore clearly revealed a volume-growth decreasing effect of *Sirococcus* shoot blight in mature Norway spruce, which has been been observed only on young Norway spruce trees yet (Halmschlager et al., 2000).

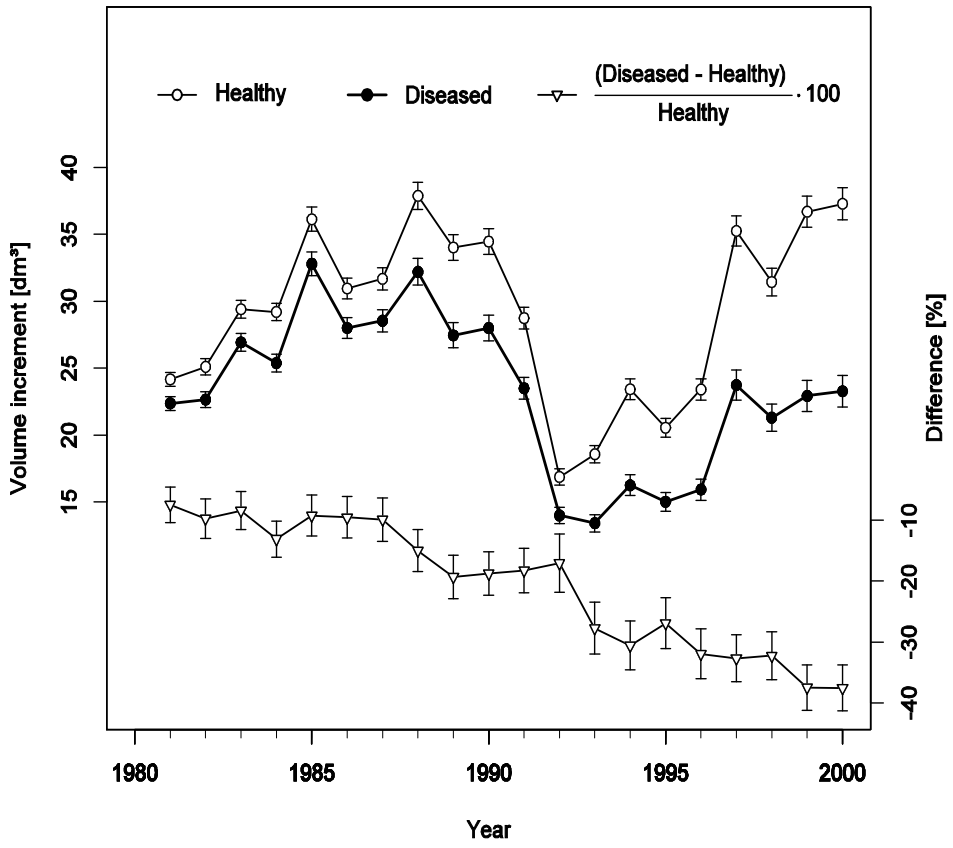

Figure 3. Current annual increment of the unfertilized "diseased" and "healthy" sample trees, adjusted for the same average current annual increment of 21.0 dm^3 in the period 1977 to 1980, and the difference between the current annual increment of "diseased" and "healthy" trees in percent of the current annual increment of "healthy" trees. The error bars indicate the standard error of the adjusted means. (Figure reprinted from Huber et al. 2009 with permission from Elsevier.)

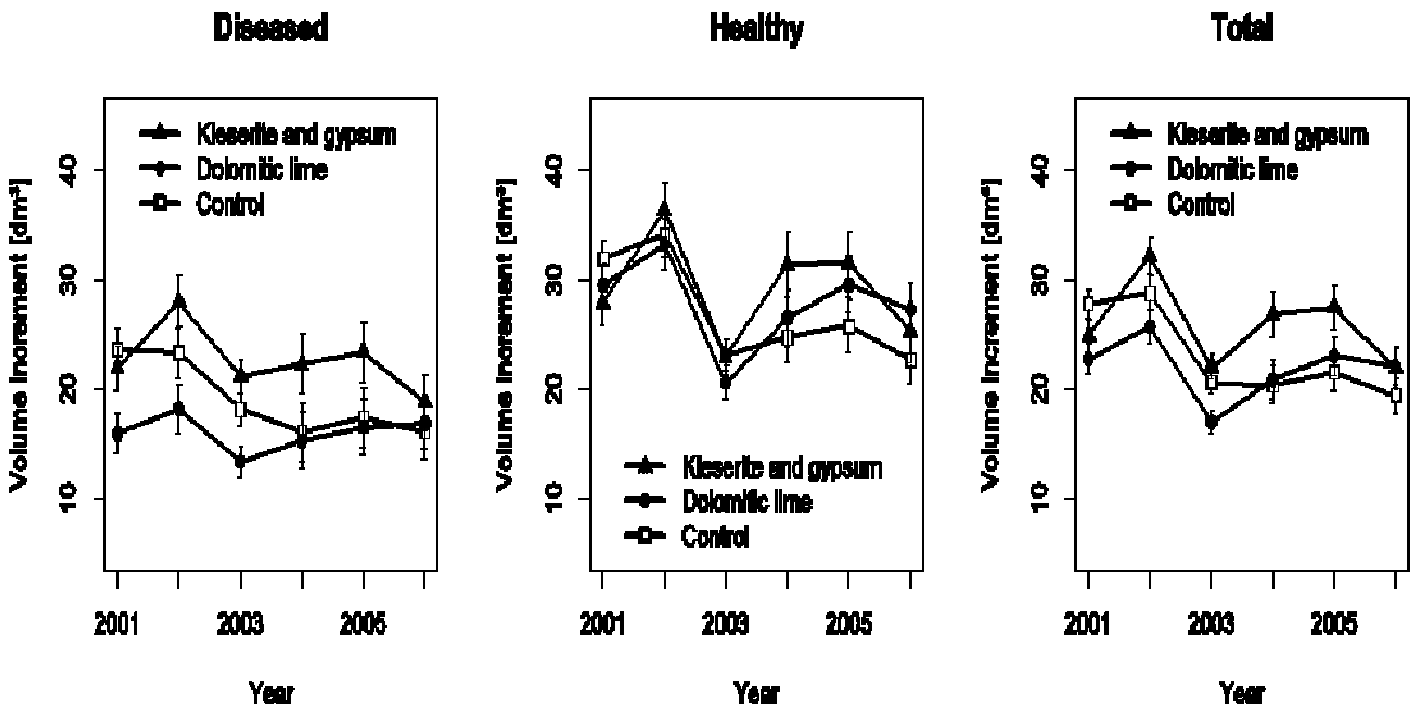

Figure 4. The development of the adjusted average increment per tree by fertilization treatment for the "diseased" and "healthy" trees. The error bars indicate the standard errors of the means. The means in the subfigure on the right were calculated as average over the "diseased" and "healthy" group. (Figure reprinted from Huber et al. 2009 with permission from Elsevier.)

The effect of fertilization on tree volume growth was negative in the first year after fertilization in both treatments. A positive effect of vitality fertilization was achieved for the first time 2 years after fertilization with the gypsum and kieserite variant *(Fig. 4)*. This significant surplus in increment was found from 2002 to 2005 and culminated in 2004 with 32 ± 15%, calculated as average over the diseased and healthy group. The rapid response of the fertilized trees in this treatment can be explained by the quick plant availability of Ca and Mg from these water soluble fertilizers. In the dolomitic lime variant a positive effect occurred at first in 2004 on the healthy trees and in 2006 also on the diseased trees.

A significant interaction between *Sirococcus* symptoms and fertilization with kieserite + gypsum was found in 2003, where the increment increasing effect by fertilization was higher for the "diseased" trees. Therefore a possible mitigation of *Sirococcus*-induced increment losses by fertilization can be suggested.

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INTERACTION BETWEEN *Diplodia pinea*, *D. scrobiculata* AND SEVERAL FUNGAL ENDOPHYTES IN RED AND JACK PINE SEEDLINGS

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ABSTRACT

Sphaeropsis sapinea sensu lato is a conifer fungal pathogen that causes mainly shoot blight and stem cankers. Recently, the former *S. sapinea* has been divided in two new species, *D. pinea* and *D. scrobiculata*. The aim of the study was to evaluate the interaction between those two fungal pathogens and among them and several fungal endophytes isolated from healthy shoots of *Pinus resinosa* and *P. banksiana* adult trees. Interaction was evaluated by means of co-inoculations in Red and Jack pine seedling under greenhouse conditions. Symptom severity (distance below the inoculation site at which necrotic needles were observed) was recorded after four weeks of incubation and used as response variable. The results showed *D. pinea* to be much more aggressive on both hosts than *D. scrobiculata*. When both pathogens where inoculated in a single plant, the symptom development was mainly due to *D. pinea*. Furthermore, *D. scrobiculata* showed antagonism with *D. pinea*, since when both pathogens co-occurred in a single seedling, symptom severity caused by *D. pinea* was lower than that caused when *D. pinea* acted alone. The results also suggested that two of the endophytes, *Trichoderma atroviride* and *Rosellinia subiculata,* were able to inhibit the pathogen spreading and therefore they could be considered biocontrol agents against *D. pinea*. Further studies would be needed to confirm biocontrol.

Keywords: *Sphaeropsis sapinea*, biocontrol, *Pinus* sp, inoculation.

1. INTRODUCTION

Sphaeropsis sapinea sensu lato is a conifer fungal pathogen of worldwide distribution which has caused significant economic damage in nurseries, plantations, and natural stands (Gibson, 1979; Nicholls and Ostry, 1990; Davison et al., 1991). The parasite can infect most parts of host plants, causing a broad range of disease symptoms: shoot blight, stem cankers, branch dieback, dead tops, death, and blue staining of cut wood (Nicholls and Ostry, 1990; Stanosz and Cummings-Carlson, 1996).

Two groups, A and B, were distinguished within *S. sapinea sensu lato* (Palmer et al., 1987) based on several characteristics such as morphology, growth, virulence and molecular markers (Palmer et al., 1987; Smith and Stanosz, 1995; de Wet et al., 2000; Burguess and Wingfield, 2001). Recently, the B group has been recognized as a distinct species and assigned the name *Diplodia scrobiculata* J. de Wet, B. Slippers & M. J. Wingfield (de Wet et al., 2003). At the same time, the A group was named as *Diplodia pinea* (Desmaz.) J. Kickx fil. (syn. *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton in Sutton) (de Wet et al., 2003). In terms of pathogenicity, *D. pinea* has been shown to be more aggressive than *D. scrobiculata* (Blodgett and Stanosz, 1997; Blodgett and Bonello, 2003).

The reported geographic and host ranges of *D. pinea* and *D. scrobiculata* are broad and overlapping. Not only do distributions and hosts overlap, but *D. pinea* and *D. scrobiculata* also are known to occur together. In this sense, both species have been isolated from individual red pine (*Pinus resinosa* Aiton.) plantations (Palmer, 1991; Stanosz et al., 2005) or even from a single tree (Morelet and Chandelier, 1993) or a single sample (Smith and Stanosz, 2006). Those experiments stated the potential for intimate association between *D. pinea* and *D. scrobiculata* within host tissues, but relatively little is known about their local co-occurrence and the implications of this fact for the disease development, and subsequent survival.

Complete eradication of the pathogen is difficult due to latent infection of symptomless tissues of apparently healthy trees (Flowers et al., 2001; Stanosz et al., 2005; Maresi et al., 2007; Stanosz et al., 2007); however, proper control measures could reduce the spread and virulence of the disease. Among those measures, the use of biological control is of increasing interest since it provides an effective and environmentally safer alternative to chemical application. Several microorganisms, mainly fungi, have been observed to cause 'systemic induced resistance' in the host after their inoculation into the plant, which may prompt a lower host susceptibility to later infections with *D. pinea* (Luchi et al., 2005; Blodgett et al., 2007; Muñoz et al., 2008). On the other hand, several endophytes, which have been shown to produce secondary metabolites, some of them with antifungal properties (Tan and Zou, 2001; Schulz et al., 2002), have shown antagonism with several pathogens and they have been assessed as biological control agents (Mehrotra et al., 1988; Holdenrieder and Greig, 1998; Roy et al., 2001). However in the literature, few studies dealing with the use of endophytes as biological control agents against *D. pinea* can be found.

Therefore, the main aim of the study was to analyse the effect of *D. scrobiculata* and several fungal endophytes, isolated from healthy shoots of *Pinus resinosa* and *P. banksiana* Lamb. (jack pine) adult trees, on the symptom severity

caused by *D. pinea* by means of co-inoculations on *P. resinosa* and *P. banksiana* seedlings, with the goal of evaluating their potential role as biological control agents of *Diplodia pinea*.

2. MATERIALS AND METHODS

2.1. Plant material

Dormant, 1-year-old jack pine and 2-year-old red pine nursery seedlings were lifted at the beginning of winter and transplanted into Deepot cones (conical tubes, 6.4 cm wide x 25.4 cm deep; Stuewe & Sons Inc., Corvallis, OR) in a soil mix (1:1 vol/vol) of Plainfield sand (containing 89% sand and 7% silt) from a 24-year-old red pine plantation in central Wisconsin and Fafard growing mix no. 2 (Conrad Fafard Inc., Inkerman, New Brunswick, Canada). Red pine seedlings had a mean stem height of 16.9 cm ± 0.3 standard error (SE) and jack pine had a mean stem height of 26.8 cm ± 0.6 SE at the time transplanted. Seedlings were placed in a greenhouse supplemented with artificial light (maximum recorded ambient greenhouse photon flux density was 1,560 $\mu mol \cdot s^{-1} \cdot m^{-2}$; supplemental photon flux density averaged 132 $\mu mol \cdot s^{-1} \cdot m^{-2}$) to provide a 16-h photoperiod. The seedlings were watered to field capacity every 2 to 3 days. The climatic conditions during the experiments in the greenhouse were: average temperature (T) 30.6º C ± 0.7 SE, average relative humidity (RH) 30.0% ± 1.5 SE.

2.2. Fungal material

Diplodia mycelia inoculum was produced for two monoconidial isolates of each species, *D. pinea* and *D. scrobiculata*, collected from various pine species and locations in the north central United States (Table 1). Thirty four endophytes were isolated from vigorous external twigs located at 1.5 m above ground in the canopy of 20–30 year old dominant red and jack pine trees. For isolations, needle-free shoots were surface sterilized by immersion for 30 s in 95% ethanol, and then two immersions for 2 min each in 1.05% NaClO plus two drops Tween-80 per litre. This procedure is similar to those routinely used for detection of endophytic fungi (Bills, 1996). Then, for each shoot, four 5-mm long plant segments including bark were cut and plated into a Petri dish containing PDA (Potato Dextrose Agar, 39 $g \cdot l^{-1}$, Difco Laboratories, Detroit, MI) medium and incubated at room temperature for 1 week before examination. Outgrowing colonies were transferred to PDA and incubated at 24º C. Four endophytes (Table 1) among the total were selected to perform the experiments based on a preliminary study (data not shown) where the antagonism between all of them and the

Table 1: Origin of *Sphaeropsis sapinea* sensu lato and endophyte isolates used in the experiments.

Isolate	Isolate no.[a]	Species	Pine host	Geographic origin
P1	411	*D. pinea*[b]	*Pinus resinosa*	Clearwater Co., MN
P2	04-126	*D. pinea*[b]	*Pinus banksiana*	Wood Co., WI
S1	124	*D. scrobiculata*[b]	*Pinus banksiana*	Jackson Co., WI
S2	462	*D. scrobiculta*[b]	*Pinus resinosa*	Clearwater Co., MN
E1	08-08	Not determined	*Pinus banksiana*	Jackson Co., WI
E2	08-09	Not determined	*Pinus resinosa*	Jackson Co., WI
E3	08-10	*Trichoderma atroviride* Karst.[c]	*Pinus resinosa*	Jackson Co., WI
E4	08-13	*Rosellinia subiculata* (Schwein.) Sacc.[c]	*Pinus banksiana*	Jackson Co., WI

[a] Culture collection numbers of M. A. PALMER (3-digit number) or G. R. STANOSZ (04-xxx).

[b] The isolates were previously characterized according to the specific primers given by SMITH and STANOSZ (2006).

[c] The most homologous species given in the GeneBank after introducing the ITS sequence.

Diplodia isolates was evaluated *in vitro*. Identifications of these four endophytes were carried out by obtaining the ITS region sequence and later comparison with the GeneBank. The fungal DNA was extracted directly from mycelia previously grown in PDB (Potato Dextrose Broth, 39 $g \cdot l^{-1}$, Difco Laboratories, Detroit, MI) by using the slightly modified method of Gilbertson et al. (1991) outlined in Smith and Stanosz (1995). The extracted DNA was amplified using ITS1 and ITS4 primers, and the entire region containing both internal transcribed spacers (ITS) was sequenced by the University of Wisconsin Biotechnology Center on an Applied Biosystems 3730XL DNA sequencer.

2.3. Interaction between *D. pinea* and *D. scrobiculta* experiment

The elongating, asymptomatic terminal shoot of each seedling was inoculated in the greenhouse approximately 11 weeks after transplanting in the case of red pine, and 6 weeks in jack pine. On each shoot, two wounds (in the opposite sides of the shoot) were made by removing a needle fascicle per side (by a sterile scalpel cut flush to the stem) approximately 2 cm below the shoot apex. Inoculations were made by placing fungus-side-down a 4-mm-diameter plug, cut from the margin of an actively growing culture on PDA (Potato Dextrose Agar, 39 $g \cdot l^{-1}$, Difco Laboratories, Detroit, MI), on each of the two wounds. In the controls, seedlings were inoculated with sterile PDA in both wounds. Another five nonwounded seedlings for each host were incorporated in the experiments as additional controls but those were not included in the statistical analyses. Parafilm (American National Can Co., Chicago, IL) was wrapped around the shoots for 7 days. Five seedlings per treatment combination (isolate and fungal species; Table 1) were used in each of two trials separated by two weeks. Thus, for each tree species were inoculated

five seedlings per each one of the following treatments: 1) P1+S1, 2) P1+S2, 3) P2+S1, 4) P2+S2, 5) P1+P1, 6) P2+P2, 7) S1+S1, 8) S2+S2, and 9) Agar A+A. Then, a total of 45 seedlings per trial and tree species were inoculated. All treatments were assigned randomly. Four weeks after inoculation, the distances below the inoculation site at which necrotic needles and cankers were present were measured (symptom severity). Most of the seedlings were carried to the lab and, by means of a molecular analysis with specific primers (Smith and Stanosz, 2006), the presence of *D. pinea* or/and *D. scrobiculata* was verified in order to assure they were the causal agents of the necroses.

2.4. Interaction between *D. pinea* and several endophytes experiment

This experiment was carried out on 1-year-old jack pine seedlings. Shoots were inoculated as explained above, although *D. pinea* was inoculated 2 cm below the shoot apex and the endophyte 5 cm below the shoot apex. The endophytes were inoculated one week before the *Diplodia* inoculation. Two types of controls were used: seedlings inoculated with sterile PDA instead of the endophyte into the lowest wound (D+PDA) and seedlings nonwounded in the lower part (D). Other nonwounded seedlings were incorporated in the experiments as additional controls but those were not included in the statistical analyses. Five seedlings per treatment combination (*D. pinea* isolate and endophyte species) were used in each of two trials separated by two weeks. Thus, a total of 60 seedlings were double-inoculated per trial and considered in the analysis. All treatments were assigned randomly.

Two and four weeks after inoculation, the distances below the site inoculated with *D. pinea* isolates at which necrotic needles and cankers were present (symptom severity) were measured. At the end of the experiment, 40% of the seedlings were cut and carried to the lab where, after needles were removed, cross sections, 1 cm long, centered at 0, 1.5, and 3 cm from the site inoculated with *Diplodia*, were aseptically cut and surface disinfected as described above. Each of the three cross sections was transferred to a PDA plate and incubated for 1 week at 25ºC and light. The presence of *Diplodia* isolates and endophyte species in the plates was recorded.

2.5. Statistical analysis

Symptom severity was analyzed by two-factor analysis of variance with interaction. Factors used as main effects were treatment and trial. Fisher´s least significant difference (LSD) test was used for multiple comparison among treatments by means of the General Linear Model Procedure of SAS (Statistical Analysis Software v. 9.1.3, SAS Institute Inc., Cary, NC) when significant differences were found in the ANOVA table. The ln (x+1) transformation of the response variable was used to stabilize the residual variance, although back-transformed values are presented in tables and figures for clarification. Assumptions of normality and homoscedasticity were assured by Kolmogorov-Smirnov and Levene´s tests respectively. In the endophyte experiment, since symptom severity was recorded at two and four weeks after inoculation, a repeated measurements analysis was also applied by means of Repeated Procedure of SAS, to test the effect of time on the symptom severity.

3. RESULTS

3.1. Interaction between *D. pinea* and *D. scrobiculta* experiment

Inoculated seedlings of both tree species produced symptoms similar to those reported for seedlings in field and nursery studies, including necrotic needles, stem cankers, and crooked and dead shoot tips. Symptoms were observed in 100% of the seedlings inoculated with *D. pinea*, in 60% of the seedlings inoculated with *D. scrobiculata* and in 95% of the seedlings inoculated with both pathogens. No symptoms developed on wounded or nonwounded controls of any of the two hosts. In jack pine, the two-factor analyses of variance of the symptom severity (distance below the inoculation site at which necrotic needles were present) indicated a significant effect of the treatment ($F = 50.75$, $p < 0.01$), but not of the trial ($F = 1.92$, $p = 0.170$). Thus, the multiple comparison of the treatments was performed with the pooled data of the two trials.

In jack pine, symptom severity was greater on seedlings inoculated with isolates of *D. pinea* than on seedlings inoculated with isolates of *D. scrobiculata* (Figure 1). Symptom severity on seedlings inoculated with the isolate S2 of *D. scrobiculata* was not significantly different from that on control seedlings. It was observed that symptom severity also differed between the two isolates of *D. pinea*. On seedlings inoculated with the most aggressive isolate (P1), there were not statistical differences in the symptom severity between seedlings inoculated only with P1 and those inoculated with P1 in combination with either isolate of *D. scrobiculata* (Figure 1). However, on seedlings inoculated with the less aggressive isolate of *D. pinea* (P2), the co-occurrence of both fungal species in the seedling significantly reduced symptom severity in comparison to that caused by P2 when it was inoculated alone (Figure 1).

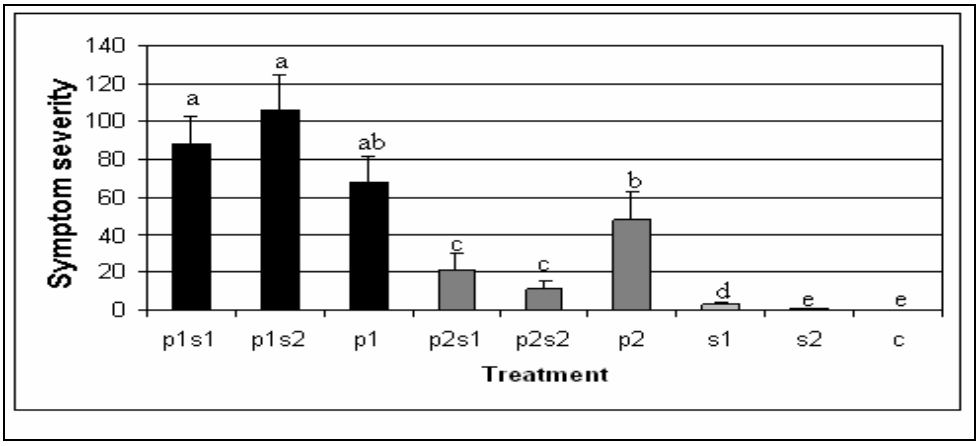

Figure 1: Mean symptom severity (distance below the inoculation site at which necrotic needles were present) for each treatment on jack pine seedlings. Averages with the same letter are not significantly different according to Fisher´s least significant difference (LSD) test at a significant level of 0.05. Vertical bars indicate Standard Error.

In the case of red pine seedlings, two-way ANOVA of the symptom severity showed a significant effect of the treatment ($F = 15.89$, $p < 0.01$), the trial ($F = 16.29$, $p < 0.01$), but not of their interaction ($F = 0.34$, $p = 0.88$). Therefore, multiple comparison was performed separately for each trial (Figure 2). As in the case of jack pine, isolates ranked in the same order in relation to their aggressiveness, although in general terms, symptom severity was lower in red pine than in jack pine. In red pine, it was also observed that the presence of either isolate of *D. scrobiculata* in the seedling reduced the symptom severity caused by either isolate of *D. pinea* (except in trial 1 for P2 isolate), although in red pine, the differences between treatments were not so evident (Figure 2). Molecular analyses indicated that symptom severity was caused mainly by *D. pinea*. *D. scrobiculata* was only detected in the proximities of the inoculation site.

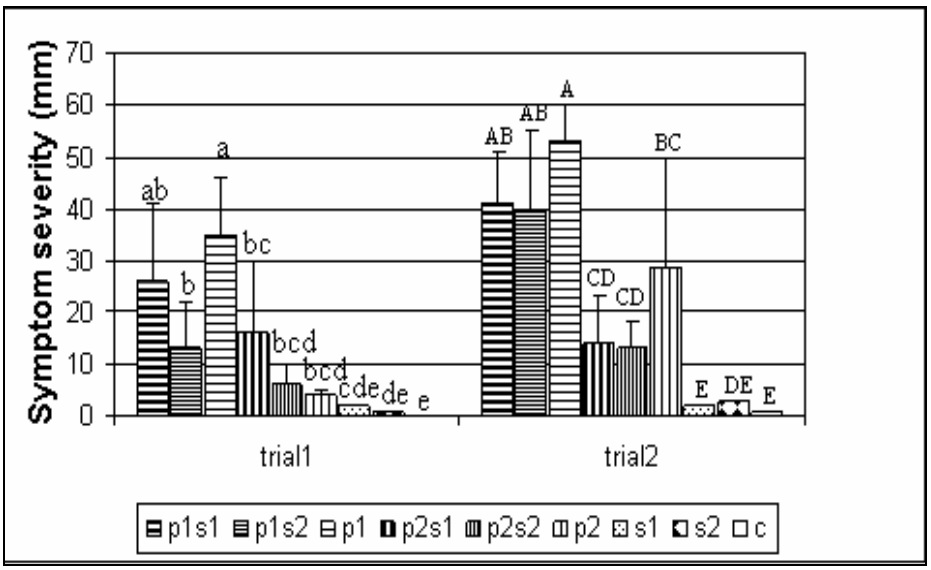

Figure 2: Mean symptom severity (distance below the inoculation each trial on red pine seedlings. Averages with the same letter are not significantly different according to Fisher´s least significant difference (LSD) test at a significant level of 0.05. Vertical bars indicate Standard Error.

3.2. Interaction between *D. pinea* and several endophytes experiment

Jack pine seedlings inoculated with *D. pinea* isolates also produced symptoms similar to those reported above. No symptoms developed on nonwounded controls. After 2 weeks, the two-factor analyses of variance of the 'necrosis length' (distance below the inoculation site at which necrotic needles were present) indicated a significant effect of the 'treatment' ($F = 4.24$, $p < 0.01$), the trial ($F = 6.53$, $p = 0.012$), but not of the interaction ($F = 1.41$, $p = 0.18$). The two-factor ANOVA of the 'necrosis length' caused by *Diplodia* isolates after 4 weeks showed that only the 'treatment' effect was significant ($F = 2.91$, $p < 0.01$). Only the 'Time' effect

influenced the 'treatment' effect (interaction time*treatment: $F = 3.94$, $p < 0.01$). LSD test showed the inoculation with endophytes 08-10 and 08-13 to produce a significantly lower symptom severity caused by the second isolate (p2) of *D. pinea* after 2- and 4-weeks of incubation but only when compared with the control seedlings non-wounded in the lower part (Fig. 3). However, those fungi did not cause reduced symptom severity when compared with those caused by *Diplodia* on the controls wounded in the lower part and inoculated with PDA instead of endophyte mycelia (Controls PDA). For the most aggressive isolate of *D. pinea* (the p1 isolate), no endophyte was able to reduce significantly the symptom severity caused by *Diplodia* (Fig. 3). Re-isolation percentages of each fungus can be observed in Table 2.

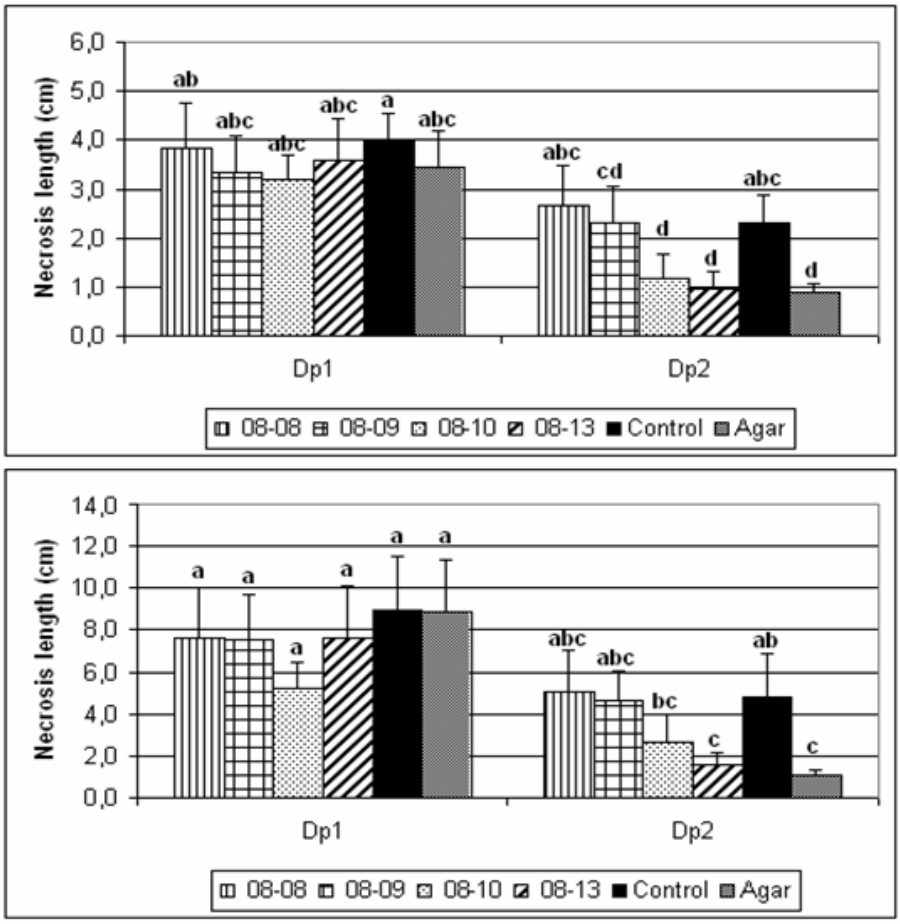

Figure 3: Mean necrosis length caused by the *D. pinea* for each endophyte treatment after 2 (figure a) and 4 weeks (figure b) of incubation. Averages with the same letter are not significantly different according to Fisher´s least significant difference (LSD) test at a significant level of 0.05. Vertical bars indicate Standard Error. 'Control' was not wounded 5 cm below the apex; and 'Agar' was a control wounded and inoculated with agar 5 cm below the shoot apex.

Table 2: Re-isolation percentage of each fungal species from the seedlings where they had been inoculated.

Isolate	Cross section[a]		
	Initial	Mid	Final
P1	100%	85%	55%
P2	100%	60%	40%
08-08	0%	0%	0%
08-09	0%	0%	0%
08-10	0%	25%	81.3%
08-13	0%	0%	6.3%

[a].- Initial: 1-cm cross section including the *Diplodia* inoculation site; Mid: 1-cm cross section between both inoculation sites; Final: 1-cm cross section including the endophyte inoculation site.

4. DISCUSSION

Inoculations with *D. pinea* isolates resulted in greater severity of symptoms than inoculations with *D. scrobiculata* isolates on seedlings of red and jack pine. This result agrees well with those stated in previous studies (Blodgett and Stanosz, 1997; 1999) which used a similar inoculation technique on the same or different host seedlings. However, in Blodgett and Stanosz (1997) *D. scrobiculata* was more aggressive in jack pine and *D. pinea* in red pine. In our study, although results are not statistically comparable since they were obtained in separated experiments, jack pine appeared to be the most susceptible host for both fungal species.

The molecular analysis confirmed that, when both pathogens were inoculated in the same seedling, *D. pinea* was mainly responsible for the necrosis length observed in the seedlings. This fact was consistent with the greater aggressiveness shown by *D. pinea*. In general terms, when both species were inoculated in the same seedling, symptom severity observed was lower than that recorded when *D. pinea* was inoculated alone. This fact suggests a potential antagonism between both pathogens when they co-occurred in the same plant tissue. This may have very important implications, because it is already known *D. pinea* and *D. scrobiculata* occur together in nature. In this sense, both species have been isolated from individual red pine plantations (Palmer, 1991; Stanosz et al., 2005) or even from a single tree (Morelet and Chandelier, 1993) or a single sample (Smith and Stanosz, 2006). Therefore it would be very interesting to develop further studies to investigate whether where both pathogens co-occur in those plantations, the disease incidence caused by *Diplodia* is lower than in plantations where just one pathogen is present.

However, this result should be taken cautiously because the reduction of the symptom severity caused by *D. pinea* when *D. scrobiculata* was also present, was not always statistically significant. One possible explanation for this lack of significance could be related to the great variability recorded between the repetitions. This great variation could be a consequence of the natural presence of both pathogens, in a latent state, in the plant material used in the experiments. Latency has already been described in previous studies as a very common state for *Sphaeropsis sapinea sensu lato* (Stanosz et al., 1997; Flowers et al., 2001; 2003; Stanosz et al., 2005; Maresi et al., 2007), which was isolated from asymptomatic plant tissues in a percentage range of 20-85%. Even in the present study, *D. pinea* and *D. scrobiculata* occurred asymptomatically in several of the non inoculated seedlings (data not shown). Despite this natural presence of the pathogen in the seedlings of our experiments, control seedlings did not become diseased and did not show any symptom of the disease. This result was predictable since it has been proposed by several authors that activation from this latency to a pathogenic state may take place under different conditions of stress (Stanosz et al., 1997; Smith et al., 2002); and seedlings used in the present study were grown in optimal conditions. However in the case of inoculated seedlings, the fungal spreading and colonization may cause stress to the plant that could activate the latent infections of the pathogen, if present. In such cases, the symptom severity recorded could be overestimated.

In general terms, when two of the endophytes, 08-10 and 08-13, were also inoculated in the seedling, symptom severity casused by the *D. pinea* isolates was lower than that recorded when *D. pinea* was inoculated alone. This fact suggests a potential antagonism between those two endophytes and *D. pinea* when they co-occurred in the same plant tissue. However, that reduction of the symptom severity caused by *D. pinea* was only statistically significant on the less aggressive isolate of *D. pinea* (p2). Endophytes have been demonstrated to be suitable candidates as biological control agents, as they seem to be part of the defence system of trees (Barklund and Unestam, 1988; Ranta et al., 1995) and have already been shown to be antagonistic to many fungal pathogens, including D*iplodia* spp. (Holdenrieder and Greig, 1998; Roy et al., 2001; Campanile et al., 2007).

Among the endophytes used in the present study, isolate 08-10 was identified as *Trichoderma atroviride* Karst. Since the potential of this genus as a biocontrol agent of plant pathogens was first recognized in the early 1930s (Weindling, 1932), continuous studies have demonstrated the effectiveness of this species in the biological control against a number of plant pathogenic fungi, including *Diplodia* spp., on several forest hosts (Knudsen et al., 1991; Mousseaux et al., 1998; Campanile et al., 2007; Schubert et al. 2008). *T. atroviride* is a mycoparasite which, once it recognizes and attacks the fungal host, uses it's nutrients killing the host before or just after invasion (Chet et al., 1998). The endophyte 08-13, which was also able to reduce the symptom severity caused by *Diplodia* on pine seedlings, was identified as *Rosellinia subiculata* (Schwein.) Sacc. This fungal endophyte has been shown to produce sordarin, which is an antibiotic with antifungal properties against a number of plant pathogenic fungi (Bills et al., 2002).

This fact is in agreement with a preliminary assay carried out in the present study for the endophyte selection, where *R. subiculata* was observed to produce a reddish compound on PDA culture media that inhibited strongly *Diplodia* growth on contact with mycelium.

Among the three types of interaction between antagonistic organisms proposed by Adams (1990), competition, antibiosis and hyperparasitism, the former could be the most important mechanism acting for *D. scrobiculata*, although antibiosis could be also acting since *S. sapinea sensu* lato has been shown to produce metabolites with antifungal activity (Cabras et al., 2006). For *T. atroviride* hyperparasitism may be the main process involved in the reduction of symptom severity caused by *D. pinea* in most of the cases. This is corroborated with the fact that in the 92.8% of the seedlings where *T. atroviride* was re-isolated, *D. pinea* isolates were not able to cause necroses farther than the site inoculated with the endophyte. In the case of *R. subiculata* it seems clear that the type of interaction involved in the antagonism would be antibiosis as expressed above.

Systemic induced resistance (SIR) is a host defense mechanism which has also been proposed by several authors (Blodgett et al., 2007; Muñoz et al., 2008) to explain why inoculations with *D. scrobiculata* and other fungal endophytes reduced the symptom severity on seedlings inoculated later with *D. pinea*. In such studies, those fungi were inoculated several weeks before inoculating with *D. pinea*, because plants need some time to produce the defensive action. Therefore, the reduction of symptom severity caused by *D. pinea* when *D. scrobiculata* is also inoculated into the same seedling at the same time, as it is our case, could not be explained by this mechanism of SIR. In the case of the endophytes, however, those were inoculated one week before the *D. pinea* inoculation. Therefore, SIR could be implicated in the reduction of the symptom severity caused by *D. pinea* when endophytes had been previously inoculated into the plant. This is consistent with the fact that in the present study the reduction in the symptom severity caused by *D. pinea* when endophytes were also inoculated into the seedlings, was only significant when compared with the controls non-wounded in the lower part, but it was not with those wounded and inoculated with PDA plugs. This fact may indicate that wounding could activate that defensive mechanism. This mechanism has been already shown to occur after wounding without any later fungal inoculation in several important components in pine resistance such as resin flow (Luchi et al., 2005). Nevertheless, this is in disagreement with the results obtained by other authors who indicated that wounding alone does not induce SIR in the case of *Pinus nigra* Arn. (Blodgett et al., 2007) or in *P. radiata* D. Don. (Bonello et al., 2001).

In conclusion, the results presented here suggested that, when both *D. pinea* and *D. scrobiculata* co-occurred in a single plant, the symptom development was mainly due to *D. pinea*. Furthermore, *D. scrobiculata* and two of the endophytes tested (the isolate 08-10 of *Trichoderma atroviride* and the isolate 08-13 of *Rosellinia subiculata*) showed some antagonism to *D. pinea*, since, when both

fungi co-occurred in a single seedling, symptom severity caused by *D. pinea* was lower than that caused when *D. pinea* was acting alone (at least this happened with the less aggressive isolates of *D. pinea*).

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ADELGID GALLS ON SPRUCE AS A RESERVOIR INOCULUM SOURCE FOR THE SHOOT BLIGHT PATHOGEN *Diplodia pinea*

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ABSTRACT

Diplodia pinea is a shoot blight and canker pathogen of many conifers and sporulates on killed needles, stems, and mature, opened seed cones. Although Colorado blue spruce (*Picea pungens*) is an atypical host, pycnidia of this fungus were observed on galls induced by the Cooley spruce gall adelgid (*Adelges cooleyi*). The elongate, cone-like galls that form on ends of shoots as a result of feeding by nymphs of this insect normally do not seriously impact tree health, but they can be considered unsightly. A survey was conducted to determine the incidence and abundance of inoculum of *D. pinea* that could be obtained from these galls on an otherwise healthy, ornamental Colorado blue spruce in Madison, WI, USA. Ten arbitrarily selected galls were collected from one branch at each of four directions in the top, middle, and bottom thirds of the tree crown (120 galls total). A washing and filtration technique was used to determine presence and estimate the numbers of conidia extracted from these galls, and species-specific PCR primers were used to confirm the identity of the pathogen. Conidia were obtained from each gall, but the number of spores varied greatly from gall to gall. Some galls yielded few spores, a result that suggests these conidia may have been produced elsewhere. In contrast, many thousands of spores were obtained from galls on which pycnidia were abundant. Thus, in the absence of usual host trees, insect-altered organs of an atypical host can be an alternative substrate for this pathogen and a reservoir inoculum source of *D. pinea*.

Keywords: spruce, *Picea*, gall, *Adelges*, *Diplodia*, inoculum

1. INTRODUCTION

Elongate, cone-like galls form on ends of shoots of several *Picea* A. Dietr. species as a result of feeding by nymphs of the Cooley spruce gall adelgid (*Adelges cooleyi* Gillette) (Cumming, 1959; USDA Forest Service, 1985). This insect is native to North America and transcontinental in distribution. Primary hosts include Colorado blue spruce (*P. pungens Engelm.*), Englemann spruce (*P. englemannii* Parry ex Englem.), Sitka spruce (*P. sitchensis* (Bong.) Carrière), and white spruce (*P. glauca* (Moench) Voss. Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is an alternate host. Similar galls are induced by the eastern spruce gall adelgid (*Adelges abietis* L.), which was introduced from Europe and is now found in

eastern North America on native spruce species and on Norway spruce *(Picea abies* (L.) H. Karst). Although sometimes numerous, these galls normally do not seriously impact spruce health. They are, however, considered unsightly on trees grown as ornamentals or as Christmas trees.

Although a single host species can support multiple generations of the Cooley spruce gall adelgid, its complex life cycle normally involves alternation between spruce and Douglas-fir hosts. The life history of this insect has been described in detail by Cumming (1959) and summarized by the USDA Forest Service (1985). Immature females overwinter as nymphs on the most recent year's twigs of spruce. After emergence and maturation in spring, the "stem-mother" deposits up to 350 eggs covered by a mass of white, waxy secretion. Eggs hatch in 1 to 2 weeks, and nymphs feed at bases of new needles on the elongating shoots. Young, green to purple, fleshy galls develop rapidly, elongating (up to 75 mm) and expanding in girth (up to 18 mm) to enclose the feeding nymphs. By late summer galls open to allow nymphs to move to needles where they molt into winged adults that fly to Douglas-fir. On Douglas-fir the adelgid is able to reproduce parthenogenically and sexually, overwinter as nymphs, and develop a winged stage that returns to spruce. Although feeding occurs on Douglas-fir, galls similar to those on spruce are not produced on this host.

Diplodia pinea (Desmaz.) J. Kickx fil. (syn. *Sphaeropsis sapinea*) is a widely distributed, asexual fungal pathogen of conifers in native forests and where planted as exotics (Punithalingam and Waterston, 1970). Reports of severe damage caused by *D. pinea* most frequently involve pines (*Pinus* species), especially two- and three-needled pines of the subgenus *Diploxylon*. However, the fungus occasionally has been reported from spruce hosts or substrates (Farr et al., 1989; Punithalingham and Waterston, 1970). Rain-splashed conidia of *D. pinea* can be dispersed throughout the growing season (Palmer et al., 1988) and germinate quickly followed by penetration directly (Brookhouser and Peterson, 1971; Chou, 1978) or through wounds. Disease may develop rapidly, or *D. pinea* may persist on or in asymptomatic hosts (Stanosz et al., 2005) with subsequent proliferation to cause disease under conditions that induce host stress (Stanosz et al., 2001). Damage includes seed rot and seedling collar rot, shoot blight, branch and bole cankers, crown wilt, and blue stain of sapwood (Chou, 1976; Chou, 1987; Palmer, 1991; Rees and Webber, 1988; Stanosz and Cummings Carlson, 1996). *Diplodia pinea* is frequently found sporulating on needles and stems it has killed, and also on mature, opened seed cones (Waterman, 1943; Peterson, 1977).

Close examination of galls of the Cooley spruce gall adelgid from an ornamental Colorado blue spruce revealed presence of pycnidia on the galls (and attached needles) (Figure 1) that yielded *D. pinea* conidia. The objective of this study was to examine the frequency of occurrence and amount of potential inoculum of this pathogen from galls induced by the Cooley spruce gall adelgid. Procedures used were modified from those developed by Munck and Stanosz (2009) for water extraction of conidia from seed cones of pines.

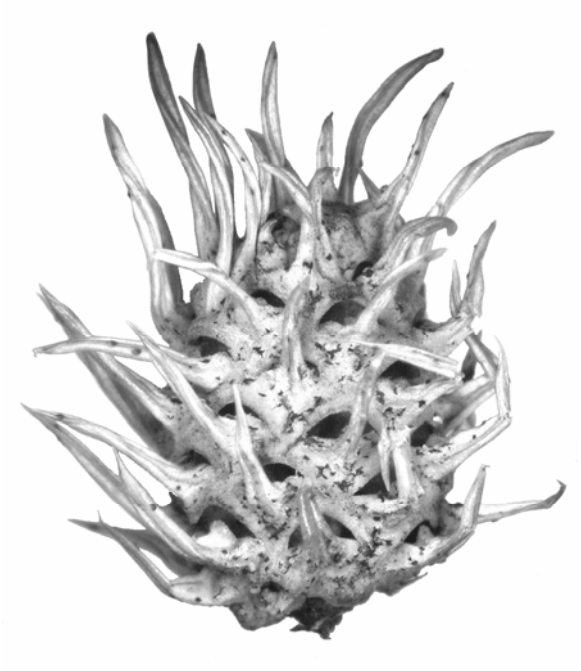

Figure 1: Abundant pycnidia of *Diplodia pinea* on a gall induced by the Cooley spruce gall adelgid.

2. MATERIAL AND METHODS

Galls were collected in summer from a single mature Colorado blue spruce tree (approximately 12 m tall) growing as an ornamental on the University of Wisconsin-Madison campus (43.07 N, 89.40 W). Shoots on this tree did not exhibit symptoms attributable to *D. pinea*. Two ornamental Austrian pines (*Pinus nigra* Arnold) that were damaged by shoot blight and bore *D. pinea* pycnidia on shoots and cones were located within approximately 20 m. The galls that were collected had matured to release nymphs the previous year and were dead. Ten arbitrarily selected galls were collected from one branch at each of the four cardinal directions in the top, middle, and bottom thirds of the tree crown (120 galls total). Galls were bagged separately and frozen until extraction of conidia.

Galls were processed individually using procedures similar to those described for pine cones by Munck and Stanosz (2009). Each gall was placed in a 100 ml plastic beaker containing 50 ml of distilled water with 2 drops of Tween 80 l^{-1} (Fisher Scientific Company, Fair Lawn, NJ) and washed for 3 h on a rotary shaker at 110 rpm. The resulting suspension was then filtered through 0.8 µm pore size filters printed with a grid to delineate 3 mm x 3 mm squares (Cat. No.: AAWG047SP, Millipore Corporation, Billerica, MA). The cup with the gall then was rinsed with an additional 50 ml of distilled water with Tween and this liquid

also was filtered. The filter for each gall was examined with the aid of a microscope for presence or absence of conidia recognized as those of *D. pinea* based on morphological characteristics. For three randomly selected galls from each branch, conidia were counted in five compound microscope fields randomly located within the filtered area at magnifications of 40 to 200x. Lower magnification was used for filters with relatively few conidia and higher magnification (i.e., smaller fields) was used for filters with many conidia. The number of conidia in five fields was multiplied by respective factors to adjust for total filtered area. Galls were then oven dried and weighed to also allow expression of conidial numbers on the basis of oven dry weight (odw).

To confirm pathogen identity, 12 filters (corresponding to four galls from each third of the tree crown) on which numerous conidia had been deposited were selected. A piece of the filter approximately 5 mm x 5 mm was excised and placed in a microcentrifuge tube. This was ground and then DNA was directly extracted using methods described by Smith and Stanosz (1995). DNA was amplified using species-specific primers developed by Smith and Stanosz (2006) that allow differentiation of *D. pinea* from the similar conifer pathogen *D. scrobiculata*.

3. RESULTS AND DISCUSSION

Every gall yielded conidia morphologically consistent with those of *D. pinea*, although the estimated numbers of conidia obtained varied widely. The range per gall was from 176 to 1,099,695 (mean = 249,599, standard error = 57,756). The range per gram odw was from 169 to 3,447,320 (mean = 462,691, standard error = 120,975). The numbers of galls categorized according to the estimated numbers of conidia extracted from each (for the 36 for which conidia were quantified) are displayed in Figure 2. For the majority of galls, this number was $>10^4$ conidia on both per gall and per gram odw bases.

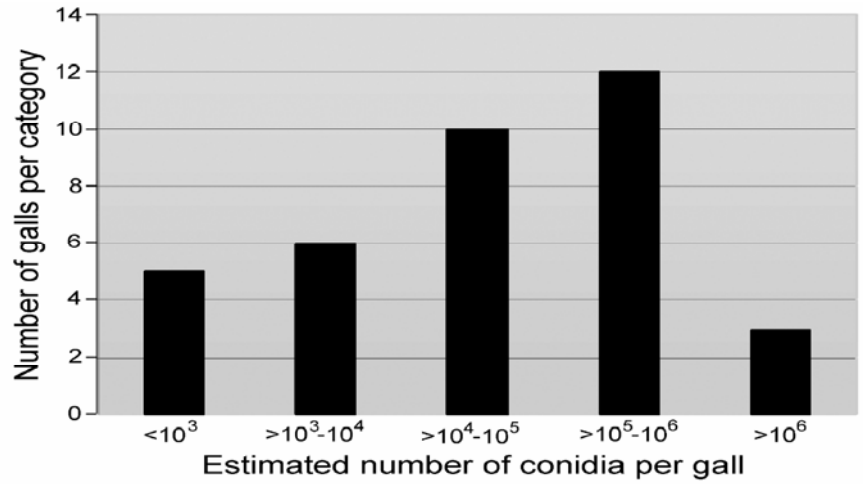

Figure 2: Numbers of Cooley spruce gall adelgid galls categorized by the estimated number of conidia extracted per gall.

The identity of the pathogen was confirmed as *D. pinea*. Eleven of the 12 filters that were tested were positive for this fungus. The test of one filter produced no result. No filters tested positive for *D. scrobiculata*.

The estimated numbers of conidia extracted from galls (when expressed on the per gram odw basis) often were within the range or even greater than numbers reported by Munck and Stanosz (2009) for pine cones. In that study, cones from which *D. pinea* (or less frequently *D. scrobiculata*) were cultured were collected from the crowns of red pines (*P. resinosa* Aiton) and jack pines (*P. banksiana* Lamb.) in which typical shoot blight symptoms were not apparent. The greatest mean number of conidia extracted per gram odw for any of these locations was 23,181, for cones from red pine crowns. The large numbers of conidia obtained from Cooley spruce gall adelgid galls indicate that, at least in this case, inoculum production by *D. pinea* is not limited by its exploitation of an atypical host as substrate.

Previous researchers have noted a diversity of relationships between *D. pinea* and insects or host material altered by insects. Epidemics characterized by severe damage to Scots pine (*P. sylvestris* L.) in Ontario was associated with injuries resulting from feeding of the pine spittle-bug (*Aphrophora parallela* Say) (Haddow and Newman, 1942). In contrast, Feci et al. (2003) found *D. pinea* only infrequently on red pine shoots damaged by insects, primarily the red pine shoot moth *Dioryctria resinosella* Mutuura. Other studies provide evidence that the cone bug *Gastrodes grossipes* De Geer has a role in movement of this fungus to cones of Austrian pine (Feci et al., 2002) and support the conclusion that the bark beetle *Ips pini* (Say) may vector *D. pinea* (Whitehill et al., 2007).

Researchers also have noted a previous relationship between an apparent disease and adelgid galls on spruce. Audley and Skelly (1994) noted the occurrence of necrotic twigs of red spruce (*Picea rubens* Sargent) that bore galls of the eastern spruce gall adelgid. A *Phomopis* Harter species was isolated from 14 of 33 dying, adelgid-galled twigs. This fungus was used to inoculate seedlings, and produced cankers in 29% of the attempts.

Documentaton of the sporulation of *D. pinea* on insect altered, necrotic spruce tissues does not clarify the potential confusion about the ability of this fungus to infect and kill normal spruce shoots under natural conditions. Although spruces are included on lists of trees on which *D. pinea* has been reported (Farr et al., 1989; Punithalingam and Waterston, 1970) such sources do not provide details necessary to know if the fungus was causing disease or merely was present saprophytically. Interpretation of reports of *D. pinea* from spruces is further complicated by current knowledge that past references to *D. pinea* sensu lato may have referred to either of two species (i.e., *D. pinea* or the similar fungus *D. scrobiculata* that was described by deWet et al., 2003). For example, Myren (1991) attributed killing of stems and branches of stressed black spruce (*P. mariana* (Mill.) B. S. P.) in an Ontario seed orchard to *D. pinea* (as *S. sapinea*). However, isolates later collected from black spruces at that seed orchard and each of four other Ontario seed orchards all were

proven to be *D. scrobiculata* (Hausner et al., 1999). Blodgett and Stanosz (1999) did demonstrate the ability of well-characterized isolates of *D. pinea* and *D. scrobiculata* to kill shoots of small, potted Colorado blue spruce seedlings following wounding and inoculation in a greenhouse experiment. But the relative lack of reports of damage to spruces, even when grown in the same locations where more susceptible species are attacked (e.g., red pine in the Great Lakes region of the USA) suggests that spruce species are infrequent hosts, or perhaps not often severely damaged by *D. pinea*. Unambiguous identification of the *Diplodia* species associated with disease symptoms of spruces should allow a better understanding of the relationships between these fungi and *Picea* species.

Regardless of possible ambiguity in the status of *D. pinea* as a potential pathogen of spruce, galls of the Cooley spruce gall adelgid serve as a substrate. The production of a very large number of conidia on any individual gall is magnified by the potential for a single spruce crown to bear many hundreds of galls. This utilization of galls by *D. pinea* to produce very large numbers of conidia confirms that, in the absence of normal disease development, altered tissues on an atypical host tree species can be a potentially significant reservoir inoculum source for this pathogen.

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Dieback And Canker Diseases

Dieback Diseases

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THE CURRENT SITUATION OF ASH DIEBACK CAUSED BY *Chalara fraxinea* IN AUSTRIA

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ABSTRACT

In many parts of Europe common ash, *Fraxinus excelsior*, is presently affected by a serious dieback of shoots, twigs and branches, causing decline and mortality of trees of all age classes. Initially thought to be primarily incited by abiotic damaging factors, accumulating evidence suggests that ash dieback is a new infectious disease caused by the hyphomycete *Chalara fraxinea* and its teleomorphic state, *Hymenoscyphus albidus*. In Austria, ash dieback was first observed in 2005 and in 2008 it occurred in all Austrian provinces. In heavily affected forests mortality is common amongst saplings and young trees. Moreover, in some areas dying of mature trees has started to occur. *Chalara fraxinea* was for the first time recorded in Austria in June 2007. Subsequent surveys have shown that the pathogen is widespread in the country. Until June 2009 it was isolated from symptomatic ash trees at 82 localities in eigth out of the nine Austrian provinces. Apart from *F. excelsior*, *C. fraxinea* was isolated from narrow-leaved ash, *F. angustifolia* subsp. *danubialis*and from weeping ash, *F. excelsior* 'Pendula'. *Chalara fraxinea* was consistently isolated at high frequencies from ash shoots, twigs and stems showing early symptoms of disease. In inoculation experiments using potted *F excelsior* and *F. angustifolia* seedlings, Kochs postulates were fulfilled for *C. fraxinea*, clearly suggesting that this fungus is the primary causal agent of ash dieback. It also displayed pathogenicity to *Fraxinus ornus* seedlings. Besides reviewing the situation of ash dieback in Austria and summarizing the results of some of our research since 2007, we generally review the knowledge on this emerging disease in Europe, describe its symptoms, present a hypothetic disease cycle for ash dieback and discuss options for disease management.

Keywords: *Hymenoscyphus albidus, Fraxinus excelsior*, *Fraxinus angustifolia* subsp. *danubialis*, emerging disease, fungal disease, new forest health problem

1. INTRODUCTION

Since the early 1990s common ash, *Fraxinus excelsior*, has been affected by an unprecedented, serious dieback of shoots, twigs and branches, causing decline and mortality of trees of all age classes. Ash dieback was first noticed around 1992 in Poland (Kowalski and Holdenrieder, 2008), where it has been causing serious damage (Przybył, 2002; Kowalski, 2006; Kowalski and Holdenrieder, 2008). It was subsequently recorded in many other European countries and threatens common ash in large parts of its distribution range. By 2009 ash dieback has been reported to occur in Lithuania (Lygis et al., 2005), Latvia (T. Gaitnieks, personal communication), Estonia (M. Hanso and R. Drenkhan, personal communication), area Kaliningrad (Russia, R. Vasaitis, personal communication), Danmark (Thomsen et al., 2007; Skovsgaard et al., 2009), Sweden (Bakys et al., 2009a; 2009b), Norway (Talgø et al., 2009), Finland (J. Hantula, personal communication), Germany (Schumacher et al., 2007), Slovakia (A. Kunca, personal communication), Czech Republic (Jankovský et al., 2008), Austria (Cech, 2006b; Halmschlager and Kirisits, 2008; Kirisits et al., 2008a; 2008b; 2009), Hungary (Szabó, 2008), Slovenia (Ogris et al., 2009), Rumania (D. Chira, personal communication), Switzerland (Engesser et al., 2009) and eastern France (Chandelier et al., 2009; Ioos et al., 2009) It can be expected that this emerging forest health problem will in the future also occur in other parts of Europe, where it has thus-far not been found.

Initially, ash dieback was suspected to be primarily incited by abiotic damaging factors (frost, drought and abrupt changes of periods with warm and cold weather conditions), with secondary, weakly virulent fungal pathogens and endophytes contributing to the syndrome (Przybył, 2002; Pukacki and Przybyl, 2005; Cech, 2006b; Cech et al., 2007). This view changed with the discovery and description of the anamorphic fungus *Chalara fraxinea* that was in Poland frequently isolated from shoots in the early phase of the pathological process (Kowalski, 2006). In the meanwhile, *C. fraxinea* has been detected in many of the above mentioned countries and accumulating evidence suggests that it is the cause of ash dieback (Schumacher et al., 2007; Halmschlager and Kirisits, 2008; Szabó, 2008; Kowalski and Holdenrieder, 2009a; Talgø et al., 2009; Bakys et al., 2009a; 2009b; Engesser et al., 2009; Ogris et al., 2009; Kirisits et al. 2009; Chandelier et al., 2009; Ioos et al., 2009).

Because of the sudden appearance, the rapid spread and the high intensity of ash dieback, *C. fraxinea* was thought by some forest pathologists to be an alien invasive organism (Halmschlager and Kirisits, 2008; Kirisits and Halmschlager, 2008; Kowalski and Holdenrieder, 2008; Ogris et al., 2009). However, *Hymenoscyphus albidus*, a discomycete native to Europe has recently been identified as the teleomorph of *C. fraxinea* (Kowalski and Holdenrieder, 2009b). This ascomycete fungus has been known since 1850 as harmless decomposer of leaf rachises (referred to as leaf petioles by Kowalski and Holdenrieder [2009b] but we think that 'leaf rachis' is the more appropriate botanical term) of common ash

and it is unknown, why this fungus suddenly causes a serious, emerging disease on *F. excelsior* (Kowalski and Holdenrieder, 2009b). Kowalski and Holdenrieder (2009b) proposed that the fungus they assigned to the morphospecies *H. albidus* may have undergone genetic change by mutation or hybridization with an unknown introduced species. Another possibility is that the teleomorph of *C. fraxinea* is not the 'original' *H. albidus*, but an exotic invasive species, that is morphologically virtually identical to *H. albidus*. Finally, the fungus may show unprecedented aggressiveness towards ash because of environmental factors or/and weather extremes, that could either have predisposed the host trees to fungal attack or provided ideal conditions for fungal infections (Kowalski and Holdenrieder, 2009b). These three theories are the conceptual basis for future research regarding the question what triggered the epidemic of *H. albidus* and its anamorphic state *C. fraxinea*.

Ash dieback is presently amongst the most important forest health problems in Austria. Here we review the situation of this emerging disease in this Central European country. We also summarize some research that has been conducted since 2007, present a hypotheticAL disease cycle for ash dieback and discuss options for disease management.

2. ASH SPECIES IN AUSTRIA

Three ash species are native in Austria including common ash, *Fraxinus excelsior*, also known as European ash, narrow-leaved ash, *F. angustifolia* subsp. *danubialis* and flowering ash, *F. ornus* (Adler et al., 1994). While European ash is widespread on appropriate sites in many parts of the country (Schadauer, 1994), the two other species are at the edge of their distribution ranges in Austria and are thus rare (Adler et al., 1994; Zukrigl, 1997).

With a share of 2.5% (based on the number of trees) and 1.8% (based on the growing stock) ash species are the third most frequent group of hardwood species in managed forests in Austria (Table 1). All three species are included in the relative proportions reported in Table 1, but the vast majority of the share of *Fraxinus* spp. refers to *F. excelsior*. Based on the growing stock only European beech (*Fagus sylvatica*) and oak species (mainly *Quercus petraea* and *Quercus robur*) occur more frequently than *Fraxinus* spp. and based on the number of trees *F. sylvatica* dominates amongst hardwood species, while European hornbeam (*Carpinus betulus*) is slightly more frequent than ash. The share of ash in the nine Austrian provinces is shown in Table 1. Ash is particularly abundant in the provinces Upper Austria, Lower Austria, Vorarlberg and Vienna and occurs least frequently in the province Tyrol that is to a large extent located in areas of the Alps that are inappropriate for the growth of *F. excelsior*.

Table 1: Share (%) of ash, based on number of trees and based on growing stock in managed forests in the nine Austrian provinces and in entire Austria. The data include all three ash species native in Austria, but the vast majority is *F. excelsior,* while the proportions of *F. angustifolia* and *F. ornus* are negligible. Source: Austrian Forest Inventory 2000-2002, Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Inventory (http://bfw.ac.at/rz/bfwcms.web?dok=35).

Austrian province	Based on number of trees	Based on growing stock
Burgenland	2.1	1.2
Carinthia	1.7	1.0
Lower Austria	3.4	2.9
Salzburg	2.0	1.1
Styria	1.6	1.2
Tyrol	0.4	0.1
Upper Austria	5.2	3.7
Vienna	9.4	7.5
Vorarlberg	4.6	2.2
Austria total	**2.5**	**1.8**

Common ash has its optimum on moist, nutrient-rich sites, but in areas with limestone as geological bedrock it can also occur on drier sites (Mayer, 1984). It is mainly found from the lowlands up to elevations of 900 m asl. (Schadauer, 1994; Nationalpark Kalkalpen, 2007) In the Alps it rarely occurs at elevations higher than 1200 m asl. (Mayer, 1984; Schadauer, 1994; Nationalpark Kalkalpen, 2007). *Fraxinus excelsior* occurs in a large number of forest types. It is a characteristic component of floodplain forests along big rivers, forests along streams and in glens, but also occurs in other hardwood-dominated forests on moist and sometimes drier sites (Jelem, 1974; Mayer, 1974; 1984; Nationalpark Kalkalpen, 2007). For forest owners managing riparian forests, along the Danube for example, common ash is usually the most economically important timber species (Jelem, 1974). In many areas, especially on sites at lower elevations it has received much attention for the production of valuable timber and as an alternative to Norway spruce (*Picea abies*) (Wolf and Jasser, 2003). It is also important for various ecosystem services, for example stabilization of riverbanks and slopes (Mayer, 1984). Moreover, *F. excelsior* is an attractive and appreciated landscape tree in the countryside and shade tree in urban areas. Due to its nutrient-rich foliage it was the most preferred tree species for lopping in the Alps, providing fresh or dry fodder for cattle, sheep and other domestic animals.

Narrow-leaved ash, *Fraxinus angustifolia* subsp. *danubialis* (hereafter referred to just as '*F. angustifolia*'), is mainly distributed in lowland areas of south-eastern Europe. In eastern Austria it reaches one of its distribution limits and is therefore rare (Jelem, 1974; 1975; Adler et al., 1994; Zukrigl, 1997). It forms part of riparian forest ecosystems along the lower reaches of the rivers March, Danube, Fischa

(province Lower Austria) and Leitha (provinces Lower Austria and Burgenland) (Jelem, 1974; 1975; Adler et al., 1994; Zukrigl, 1997). Hybrids between *F. angustifolia* and *F. excelsior* have been reported to occur in areas in Austria, where the distribution ranges of the two species overlap (Jelem, 1974; 1975). While it is mainly a botanical curiosity and of interest for nature conservation, this ash species is an economical important timber species in floodplain forests along the March (Jelem 1975; Damm, 1997).

Flowering ash mainly occurs in southern and south-eastern Europe, but its natural distribution range just reaches southern Austria (Mayer, 1974; Adler et al., 1994; Zukrigl, 1997). It is mainly found in southern and eastern Carinthia (Adler et al., 1994), where it usually occurs on steep, rocky, warm and dry sites on limestone (Zukrigl, 1997). Together with European hop-hornbeam, *Ostrya carpinifolia*, *F ornus* often forms dense bush forests on such sites (Mayer et al., 1974; Zukrigl, 1997). Apart from Carinthia, flowering ash also occurs in other Austrian provinces (Eastern and Northern Tyrol, Styria, Lower Austria and Burgenland), where it is generally rare (Adler et al., 1994; Zukrigl, 1997). Most of the occurrences in these provinces are likely not native, but *F. ornus* has become naturalized in some areas (Zukrigl, 1997). This ash species has no importance for forestry in Austria, but is of interest for nature conservation, as it forms part of rare and ecologically valuable forest types. It is occasionally used as shade and ornamental tree and planted in shelterbelts (Zukrigl, 1974).

Green ash (*F. pennsylvanica*) and white ash (*F. americana*), two introduced species from North America are occasionally planted as ornamentals (Adler et al., 1994). Some decades ago they also received some interest as plantation trees in floodplain forests, but as their growth potential and timber quality did not fulfill the expectations of foresters they are presently no longer planted. *Fraxinus pennsylvanica* has become naturalized in some riparian areas, especially in those along the lower reach of the Danube and the March (Essl and Rabitsch, 2002). It is therefore considered as alien invasive species in Austria (Essl and Rabitsch, 2002; Essl et al., 2006).

3. SYMPTOMS OF ASH DIEBACK

The symptoms of ash dieback share characteristics of a bark disease, a vascular wilt disease and a leaf disease (Figure 1 and 2; Thomsen et al., 2007; Halmschlager and Kirisits, 2008; Kirisits et al., 2008a; 2008b; Kowalski and Holdenrieder, 2008; Szabó, 2008; Bakys et al., 2009b; Engesser et al, 2009; Kirisits and Cech, 2009; Kirisits et al., 2009; Ogris et al., 2009). Typical symptoms occur in the bark, phloem and wood of shoots, twigs, branches and stems as well as on leaves of ash trees. Necrotic lesions and wood discoloration can also extend into the roots, from where the fungus infects coppice sprouts, but the disease clearly starts at above-ground parts of the tree (Kowalski and Holdenrieder, 2008).

Figure 1. A stand of common ash severely affected by shoot, twig and branch dieback (Laussa, Upper Austria, July 2007).

The most obvious symptom is dieback of shoots, twigs and branches (Fig. 1). Shoot dieback is caused by localized necrotic lesions that initially are small, but as they expand they girdle the phloem and sapwood occlusion occurs, too. When phloem girdling and sapwood occlusion take place in winter time, shoots do not flush in spring, however, when they happen during the vegetation period, simultaneous wilting of leaves above the lesions occurs (Fig. 2A). Leaves then dry, turn brown to black and remain attached to the shoots for a long time. Elongated, often elliptical necrotic lesions and cankers in the bark are characteristic symptoms of ash dieback (Fig. 2B and 2C). These lesions either form around a dead side twig (Fig. 2B) or occur around a leaf scar (Fig. 2C). On larger shoots, twigs, branches as well as on younger stems, the tree often defends itself against the pathogen attack, at least for some time, leading to perennial cankers.

Necrotic lesions and cankers are usually accompanied by brownish to grayish discoloration of the wood (Fig. 2D) that frequently extends longitudinally beyond necrotic areas in the bark. Diseased trees react with prolific formation of epicormic shoots and the silhouettes of heavily affected trees look tousled and distorted (Fig. 1). *Chalara fraxinea* also causes symptoms on leaves, resulting from direct leaf infections (Kirisits et al., 2008b; Ogris et al., 2009; Bakys et al., 2009b). These symptoms include brown to blackish necrotic lesions on leaf rachises and leaflet veins, followed by wilting of parts of the leaves distal to the necrotic lesions. Early leaf shedding is often a consequence of these leaf symptoms.

4. ASH DIEBACK IN AUSTRIA

In Austria, first unambiguous observations of ash dieback were made in 2005, mainly on young trees (Cech, 2006a; 2006b). From 2006 to 2007 damage levels increased dramatically, particularly in the provinces Lower and Upper Austria as well as Styria (Cech and Hoyer-Tomiczek, 2007; Hagen, 2007; Fachabteilung Forstwesen-Forstdirektion, 2009). In 2008 the phenomenon was widespread and symptoms were observed in all Austrian provinces (Kirisits et al., 2008a; Kirisits and Cech, 2009).

Prior to the widespread occurrence of dieback of shoots, twigs and branches, early leaf shedding on ash trees, occurring already in late August and early September, was observed in parts of the provinces Lower and Upper Austria in 2005 and Styria in 2006 (Cech, 2005; Hagen, 2005; Fachabteilung Forstwesen-Forstdirektion, 2009). In the following year, thus in 2006 in Lower and Upper Austria and in 2007 in Styria, ash dieback was for the first time recorded at high intensity (Cech, 2006b; Hagen, 2007; Fachabteilung Forstwesen-Forstdirektion, 2009). Originally, this early shedding of *F. excelsior* leaves was thought to be caused by powdery mildews (*Phyllactinia fraxini*) and other microfungi (Cech, 2005). However, as *C. faxinea* is now known to cause also symptoms on ash leaves (Thomsen et al., 2007; Bakys et al., 2009b; Kirisits and Cech, 2009; Ogris et al., 2009), it is likely that the episodes of early leaf shedding in 2005 and 2006 were the first obvious indications of ash dieback. In 2008 leaf symptoms and early leaf shedding occurred again, for example in parts of Styria (Fachabteilung Forstwesen-Forstdirektion, 2009).

Incidence and severity of ash dieback varies considerably in different parts of the country. It appears to be most serious in the Northern Limestone Alps in the provinces Lower and Upper Austria, Styria and Salzburg. Likely, there are still areas, where the disease does not occur, especially in parts of the Alps, where common ash is present at a low density (Kirisits and Cech, 2009). Apart from *F. excelsior*, dieback also occurs on narrow-leaved ash and weeping ash (*Fraxinus excelsior* 'Pendula'), an ornamental variety of common ash (Kirisits et al., 2008a; 2009; Kirisits and Cech, 2009). No symptoms have thus-far been observed on

F. ornus as well as the exotic *F. pennsylvanica* and *F. americana* (Kirisits, 2008; Kirisits et al., 2008a; Kirisits and Cech, 2009).

The disease occurs on ash trees of all ages, both on natural regeneration and planted trees and on the entire spectrum of sites and forest types, where ash is found (Cech, 2008). Ash dieback is damaging on both forest and shade trees and it also causes serious problems in nurseries, where rearing of healthy ash seedlings has become difficult, if not impossible. In heavily affected forests mortality is common amongst saplings and young trees. Moreover, in some areas dying of mature trees has started to occur. It is expected that the future use of *F. excelsior* as economically and ecologically valuable noble hardwood species will be substantially impaired by this emerging disease.

Figure 2. Symptoms of ash dieback: (A) Wilting of leaves due to girdling of the phloem and sapwood occlusion, (B) A necrotic lesion in the bark with a small dead twig in the centre, (C) A necrotic lesionr in the bark with a leaf scar (arrow) in the centre, (D) Discoloration of the wood.

A number of surveys are presently underway to obtain more precise information on the geographical distribution, incidence, severity and temporal development of ash dieback in Austria. In 2007 and 2008 monitoring plots have been established in Lower Austria (Cech, 2008) and additional plots in other provinces will be installed in 2009. On these plots, disease intensity will be monitored on permanently marked ash trees over the next years. From 2009 onwards the disease is also included in the 'Documentation of forest damage factors' (German: 'Dokumentation der Waldschädigungsfaktoren – DWF'), a monitoring system that is based on expert opinions and appraisals of staff of the district forest authorities (Steyrer et al., 2008). Finally, assessments on the geographical distribution and incidence of ash dieback will be carried out as part of the field work of the Austrian Forest Inventory.

5. *Chalara fraxinea* ASSOCIATED WITH ASH DIEBACK IN AUSTRIA

Starting in June 2007, we aimed to examine the role of *C. fraxinea* in ash dieback in Austria. Shoots, twigs, branches, stems and leaves of young *F. excelsior* trees showing symptoms of the disease were collected in many different parts of the country. From January 2008 onwards special attention was given to collect only samples from trees showing early symptoms of ash dieback, particularly shoots with small, localized necrotic phloem lesions. About 4 to 6 cm long segments, containing the transition between necrotic and healthy phloem tissues and/or discolored and healthy xylem were cut from symptomatic ash organs. These segments were surface sterilized as described by Kowalski (2006). Thereafter, the outer bark was carefully peeled off and 3 to 10 mm small discs containing wood and phloem tissues were cut under aseptic conditions and put onto malt extract agar (MEA; 20 g malt extract, 16g agar, 1000 ml tap water supplemented after autoclaving with 100 mg streptomycin sulphate). In the earlier isolation series until January 2008, the outer bark was not peeled off and not discs, but small pieces of phloem or wood were removed and placed on MEA plates.

Initially the isolation plates were incubated at room temperature (23-25°C), but from August 2008 onwards they were immediately stored at low temperatures (approximately +4°C) in refrigerators. At cool temperatures, the growth of many fungi competing with *C. fraxinea* is more inhibited than that of *C. fraxinea* itself. In addition, phialophore formation of *C. fraxinea* is greatly enhanced by low temperatures (Halmschlager and Kirisits, 2008; Kirisits et al., 2008a). Both factors increase the likelihood to detect the fungus. *Chalara fraxinea* was identified based on morphological characteristics (colony morphology, phialophores and conidia; Kowalski, 2006; Halmschlager and Kirisits, 2008; Kowalski and Holdenrieder, 2008; Kirisits et al., 2008a).

Chalara fraxinea was for the first time isolated in Austria in June 2007, at one locality in Upper Austria (Edt bei Lambach, 48°06'51" N, 13°53'29" E) and another one in Styria (Altaussee, 47°38'34" N, 13°45'36" E) (Halmschlager and Kirisits,

2008; Kirisits and Halmschlager, 2008). Subsequent surveys have shown that the pathogen is widespread in the country and apparently occurs everywhere, where ash dieback is present. Until June 2009 the fungus was obtained from symptomatic ash trees at 82 localities in eight out of the nine Austrian provinces (Table 1). The differences in the number of records of *C. fraxinea* in the various provinces (Table 1) do not allow inferring about the intensity of ash dieback in various parts of Austria, but just reflect differences in the intensity of sampling. In the future it is planned to examine more samples and sites in those provinces, where extensive collections have thus-far not been conducted, especially in Tyrol, Vorarlberg, Burgenland and Carinthia

In the early isolation series carried out in 2007 *C. fraxinea* was rarely isolated, because samples were mainly collected from ash trees showing relatively late symptoms of disease. We suppose that on such plant material the slow growing *C. fraxinea* is in most cases already outcompeted by fast-growing endophytic and saprotrophic fungi (Kowalski and Holdenrieder, 2008). However, when isolations were made from shoots, twigs and stems showing early symptoms of disease, *C. fraxinea* was the most consistently and most frequently isolated fungus and in most cases the only one that was recovered. For example, isolation frequencies of *C. fraxinea* at ten localities in six Austrian provinces ranged from 81% to 100% of the examined shoots and necrotic lesions (Table 2). Overall, the fungus was obtained from 94% of the samples, from which isolations were made. Isolation of *C. fraxinea* was successful throughout the year, given that samples were collected from ash trees showing early disease symptoms (Table 2).

Apart from *F. excelsior*, *C. fraxinea* was isolated from young, planted *F. angustifolia* trees in floodplain areas along the river Morava near Hohenau/March and from symptomatic seedlings of this species in a nursery in Lower Austria (Kirisits et al., 2009; Table 2). In addition, it was obtained at a few localities from *F. excelsior* 'Pendula' (Table 2). To our knowledge, these are the first and thus-far only European records of the fungus from hosts other than *F. excelsior*. The fungal isolations from *Fraxinus* ssp. have shown that *C. fraxinea* is associated with early symptoms of ash dieback, as it is typical for the primary causal agent of a plant disease. These results agree well with other studies in Europe, in which this fungus was consistently isolated or detected with molecular markers from diseased ash trees (Kowalski, 2006; Bakys et al., 2009b; Ioos et al., 2009; Chandelier et al., 2009).

In May 2008 potted, one-year-old common ash seedlings were wound-inoculated with five *C. fraxinea* isolates and in June 2008 with five other strains. Similarly, potted, two-year-old narrow-leaved ash seedlings were inoculated with one *C. fraxinea* isolate in May 2008 (Kirisits et al., 2009) and with two other isolates in June 2008. Inoculum consisted of small pieces of autoclaved *F. excelsior* phloem (approximately 10 x 4 x 2-3 mm) that had been placed for 15 to 30 days on the various *C. fraxinea* cultures on MEA.

Table 2: Overview of the records of *Chalara fraxinea* in eight Austrian provinces from June 2007 to June 2009. Only in Tyrol the fungus has thus-far not been reported, although its presence as causal agent of ash dieback in this province is also very likely.

Austrian province	**First record (month, year, locality)**	**Ash species and varieties**	**Number of localities, where *C. fraxinea* has been recorded**
Burgenland	October 2008, BOKU Forest Demonstration Centre 'Rosalia'	*F. excelsior*	1
Carinthia	August 2008 Saberda, Sattnitz	*F. excelsior*	3
Lower Austria	August 2007 Langau	*F. excelsior* *F. angustifolia* subsp. *danubialis*	30
Salzburg	October 2008 Fuschl	*F. excelsior* *F. excelsior* 'Pendula'	14
Styria	June 2007 Altaussee	*F. excelsior* *F. excelsior* 'Pendula'	10
Upper Austria	June 2007 Edt bei Lambach	*F. excelsior*	11
Vienna	January 2008 Neuwaldegg, 17th district	*F. excelsior* *F. excelsior* 'Pendula'	12
Vorarlberg	May 2009 Götzis	*F. excelsior*	1
Total	-	-	**82**

Table 3: Isolation frequencies of *Chalara fraxinea* from young common ash (*Fraxinus excelsior*) trees showing early symtoms of ash dieback (recently died shoots as well as shoots, twigs and stems with localized necrotic lesions and wood discoloration) at ten localities in six Austrian provinces. Isolations were done from August 2008 to May 2009 and localities are arranged in chronological order of the date of isolation.

			Necrotic shoots or necrotic lesions[1]	
Locality	**Austrian province**	**Date**	**Number of samples**	**Isolation frequency of *Chalara fraxinea* (%)[2]**
Saberda, Sattnitz	Carinthia	August 2008	17	100
Wien, Hermannskogel	Vienna	November 2008	14	93
Muggendorf, Unterberg	Lower Austria	December 2008	14	93
Wien, Steinhofgründe	Vienna	January 2009	17	100
Lofer	Salzburg	April 2008	11	100
Ungerdorf, Hinterleiten	Styria	April 2009	8	88
Bad Gleichenberg[3]	Styria	April 2009	11	91
Feldkirchen an der Donau[3]	Upper Austria	May 2009	21	81
Kaumberg-Araburg	Lower Austria	May 2009	8	100
Leutschach	Styria	May 2009	9	100
All ten localities	-	-	**130**	**94**

[1] Usually one shoot or necrotic lesion per tree was used for fungal isolation, but occasionally more than one sample was collected from a single tree. [2] In most cases *C. fraxinea* was obtained in pure culture and only occasionally in mixed culture with other fungal species (data not shown). [3] Seed orchards affected by ash dieback.

Inoculation of seedling stems was done by cutting an approximately 2 cm-long slit into the bark, to the level of the cambium, pulling the bark slightly away, inserting inoculum and wrapping parafilm around the wound to seal the bark flap back to the stem, to minimize contamination and to prevent rapid drying of the phloem and wood. Each isolate was inoculated onto 20 seedlings and twenty other seedlings of each ash species were inoculated with sterile pieces of ash phloem to serve as control. In each experiment, seedlings were observed for external symptoms during a period of approximately three months. Thereafter, they were dissected and lengths of necrotic lesions and longitudinal extension of wood discoloration were recorded.

On both ash species all isolates tested caused symptoms virtually identical to those occurring on naturally infected trees: wilting of leaves and dieback (Fig. 3A) due to girdling of the phloem and sapwood occlusion around the inoculation site, necrotic lesions in bark (Fig. 3B), phloem (Fig. 3C) and cambium as well as brown-greyish discoloration in the wood (Fig. 3D) (Kirisits et al. 2008a; 2009). On the control seedlings the inoculation wounds were partly or entirely closed and no necrotic lesions or wood discoloration occurred. The three isolates that were tested on both ash species caused more intensive symptoms (longer necrotic lesions, more plants displaying wilt and dieback) on *F. angustifolia* than on *F. excelsior*, which may indicate that the former species is more susceptible to *C. fraxinea* than the latter species. *Chalara fraxinea* was consistently re-isolated from the inoculated seedlings, while it was not recovered from any of the control plants. On *F. excelsior* the re-isolation rates of the various *C. fraxinea* isolates ranged from 25 to 73 %. On *F. excelsior* and *F. angustifolia* Kochs postulates were thus fulfilled for *C. fraxinea*, clearly suggesting that this fungus is the primary causal agent of ash dieback. This is in agreement with studies in Poland (Kowalski, 2006; Kowalski and Holdenrieder, 2008; 2009a), Sweden (Bakys et al., 2009b), Hungary (Szabó, 2008), Slovenia (Ogris et al., 2009) and Norway (Talgø et al., 2009).

Flowering ash was also included in the inoculation experiments. Two *C. fraxinea* isolates, one in May 2008 and the other one in June 2008, were wound-inoculated onto one-year-old seedlings of this ash species as described above. In both experiments *C. fraxinea* displayed pathogenicity to *F. ornus*. While in the first experiment some plants showed wilting of leaves and dieback, and necrotic lesions of similar size as those on *F. excelsior* developed, no dieback occurred in the second experiment and necrotic lesions were rather small. Flowering ash may be less susceptible to *C. fraxinea* than the other two European ash species which is supported by the fact that natural infections have thus-far not been observed. Further disease surveys and inoculation trials are, however, required to definitely appraise the susceptibility of *F. ornus* to the ash dieback pathogen.

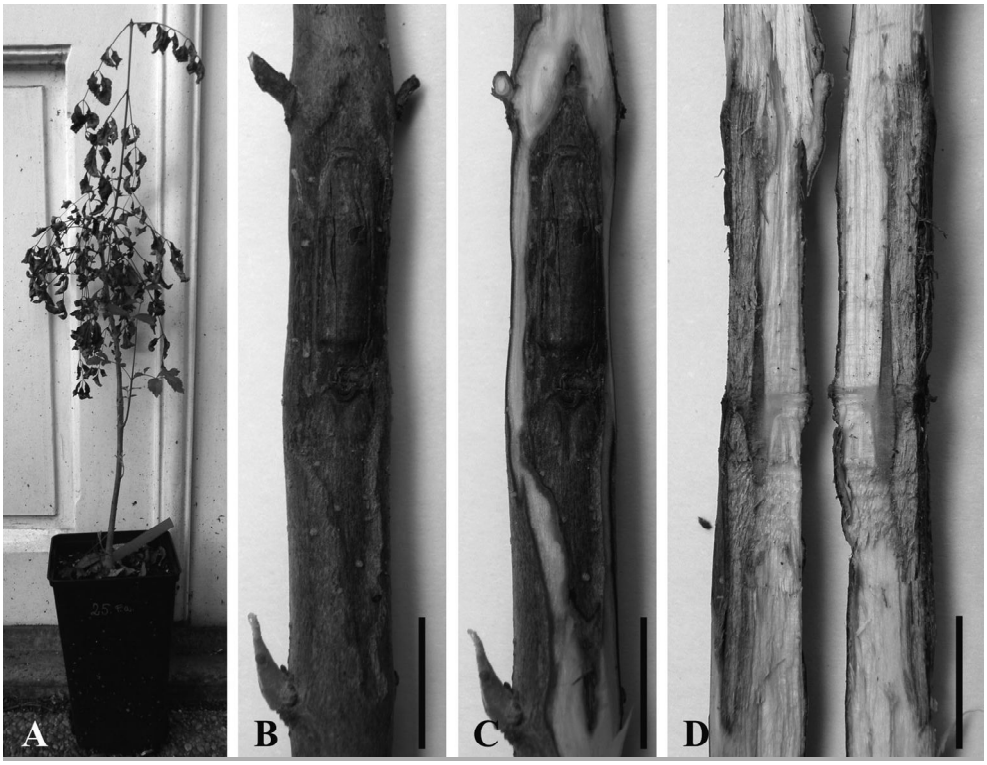

Figure 3. Symptoms on potted *Fraxinus angustifolia* seedlings following wound-inoculation with *Chalara fraxinea*: (A) Wilting of leaves, (B) Superficially visible necrotic lesion in the bark, (C) Necrotic lesion in the phloem, (D) Discoloration of the wood (Bar for B, C and D [each showing the same inoculation point] = 1 cm). See Kirisits et al. (2009) for colored versions of the photographs.

6. INFECTION BIOLOGY OF *Chalara fraxinea* AND HYPOTHETIC AL DISEASE CYCLE OF ASH DIEBACK

Until recently, the infection biology of *C. fraxinea* was totally enigmatic. There were no published reports of the fungus sporulating on dead shoots, necrotic lesions or cankers and its mode of dispersal was unknown. The conidia of *C. fraxinea* are sticky, accumulate in droplets on the top of phialophores and do not appear to be adapted to wind-dispersal (Kowalski, 2006; Kowalski and Holdenrieder, 2008). It was therefore speculated that the fungus is transmitted by animal vectors such as the ash bark beetle *Leperesinus varius* (Kowalski and Holdenrieder, 2008). No clear evidence was found, however, that vectors are involved and circumstantial evidence, for example the occurrence of the disease on trees of all age classes and the low degree or lack of association between insect infestations and ash dieback, made vector-dispersal of the fungus unlikely.

Figure 4. Apothecia of *Hymenoscyphus albidus* on leaf rachises of *Fraxinus excelsior* from the previous year (Neuwaldegg-Dornbach, Vienna, 14 and 16 June 2009).

The enigma, how the ash dieback pathogen is transmitted was solved, at least partly, by Kowalski and Holdenrieder (2009b) who discovered the teleophorph of *C. fraxinea* and linked it to *Hymenoscyphus albidus*. Similar as in other ascomycetes, the ascospores of *H. albidus* are likely to be wind-dispersed (Kowalski and Holdenrieder, 2009) and appear to play the key role in inciting infections of ash trees. Ascospore dispersal by wind would also explain the rapid spread of the ash dieback pathogen in Europe, if it had changed genetically or were an invasive alien organism (Kowalski and Holdenrieder, 2009b). In contrast, we suppose that the spores of *C. fraxinea* are unable to cause infections and they may play a different, if any role in the biology of *H. albidus*. In May 2008 we inoculated ash shoots and leaves with suspensions of *C. fraxinea* spores, but no symptoms developed on any of the test seedlings. In addition, we repeatedly aimed to test the germination of spores of *C. faxinea*, but without any success. They did not germinate on MEA, V8 agar or an agar medium containing an extract from ash leaves that stimulated mycelial growth of *C. fraxinea*, but not conidial germination. The spores also did not germinate after *in vitro* inoculation of detached ash leaflets. We thus speculate that the spores of *C. fraxinea* are not conidia, but probably

spermatia that play a role in exchanging nuclei and in fertilization of the fungus, if they are of any biological significance.

Independent from the discovery of *H. albidus* by Kowalski and Holdenrieder (2009b), from September 2008 onwards we started to comprehend the importance of leaf rachises for the infection biology and epidemiology of the ash dieback pathogen. Fungal isolation from necrotic lesions on leaf rachises in September and October 2008 has shown that *C. fraxinea* is clearly associated with these leaf symptoms, as it was the most frequently isolated fungus and was often obtained in pure culture. Repeated isolations from shed leaf rachises collected from the forest floor in autumn, winter and spring confirmed that *C. fraxinea* persists and overwinters in these parts of ash trees. Given that isolation of *C. fraxinea* from dead shoots and necrotic lesions can be difficult (e. g. Bakys et al., 2009a), it was surprising to isolate it as the most frequent fungus from decaying leaf remnants collected on the ground. Occasionally, also phialophores of *C. fraxinea* were found, sometimes abundantly. Furthermore, from late November 2008 onwards, most leaf rachises were covered by black, pseudosclerotial plates, resembling the structures often occurring in cultures of *C. fraxinea*, and now, with hindsight, known to be associated with *H. albidus* (Kowalski, 2006; Kowalski and Holdenrieder, 2008; 2009b; Halmschlager and Kirisits, 2008; Kirisits et al., 2008a).

While the discovery of *H. albidus* as teleomorph of *C. fraxinea* (Kowalski and Holdenrieder, 2009b) came surprising for us, we were not so surprised that the apothecia of this fungus are predominantly formed on leaf rachises from the previous year. In spring 2009 we repeatedly inspected leaf rachises for the occurrence of *H. albidus*. At one site in Vienna, developing apothecia with unripe ascospores were first seen at the end of May, while in mid-June the first fully developed fruiting bodies with ripe, germinating ascospores occurred abundantly (Fig. 4). Since then, apothecia of *H. albidus* have been recorded at several sites in various parts of Austria, indicating that they occur widespread and in high numbers. Intriguingly, apothecia were observed much earlier in the year (June) than previously reported in the literature (Kowalsi and Holdenrieder, 2009a and references therein).

Based on the discovery of *H. albidus* as the teleomorph of *C. fraxinea*, published information on the disease as well as own observations and studies, we propose a hypothetical disease cycle for ash dieback (Fig. 5). This scheme (Fig. 5) shall also emphasize knowledge gaps and form the conceptual basis for future investigations on the infection biology and epidemiology of *H. albidus*/*C. fraxinea*. Infection of ash trees is thought to occur by wind-dispersed ascospores of *H. albius* which form mainly on leaf rachises from the previous year, lying on the forest floor (Kowalski and Holdenrieder, 2009b; Fig. 5). Occasionally they also occur on dead shoots (Kowalski and Holdenrieder, 2009b). Ascospores are likely released from June to early October (Fig. 5). The length of the infectious period will depend on the local climate and will likely vary from year to year according to the weather conditions.

How infection by ascospores exactly takes place is unknown, but careful observations of symptoms suggest that leaves are an important target for infections (Fig. 5). In 2008 we observed leaf symptoms occasionally already in June and July, but they were most conspicuous from August onwards. In our opinion they can lead to early leaf shedding in late August and in September, as it has been repeatedly observed in Austria since 2005 (Cech, 2005; Hagen, 2005; Fachabteilung Forstwesen-Forstdirektion, 2009). We suppose that *H. albidus* is able to grow from the leaves into the shoots of ash trees (Fig. 5), where it causes necrotic lesions and wood discoloration. When examining numerous young ash trees showing early stages of disease, necrotic lesions occurred either around leaf scars (Fig. 2B) or around dead side twigs (Fig. 2C), but lesions were never seen in other positions. Dead side twigs are for sure entrance points of the pathogen into main shoots, bigger branches and stems of ash trees and the location of lesions around leaf scars may support the suspicion that the pathogen can enter the phloem and xylem via leaves. Direct infections of shoots possibly also occur (Fig. 5). Whatever organ is concerned, we assume that wounding is not required for infection. Environmental factors, particularly high amounts of precipitation and high levels of air humidity are likely conducive for ascospore release and for infections to be successful.

In 2008 small, localized necrotic lesions on shoots, often located around leaf scars (Fig. 5), where leaves had already been shed, were first observed in early August, but more commonly in September and October. These symptoms must have originated from current-years infections. Observations from 2007 to 2009 suggest, however, that many infections may remain latent for a while and that most of the host colonization and symptom progression takes place outside the vegetation period (Fig. 5). This view is supported by wound-inoculation of *F. angustifolia* with *C. fraxinea* in early December, resulting in dieback of many test seedlings in early spring. Because symptom development occurs to a large extent in autumn and winter, high damage levels become obvious in spring, when shoots do not flush and die, wilting of leaves occurs and trees show extensive dieback (Fig. 5). On bark tissues killed a while ago, saprotrophic or endophytic fungi sporulate (Fig. 5) and outcompete *C. fraxinea*, making isolation of the primary pathogen difficult or impossible. With the occurrence of *H. albidus* apothecia on leaf rachises on the forest floor (Fig. 5), the disease cycle starts again.

7. RECOMMENDATIONS FOR DISEASE MANAGEMENT

Ash dieback is another example of a serious disease damaging a valuable hardwood tree species in Europe, thereby causing problems for forestry, nature conservation and shade tree management. Common ash is not amongst the main timber species in Austria, but on many sites and forest types it is an economically and ecologically important species. On some sites there are hardly any or no attractive alternatives to ash for the selection of tree species. If ash dieback in Austria develops similar as in other countries, so that also old trees are seriously

affected, considerable losses will occur and ash may lose much of its importance in silviculture.

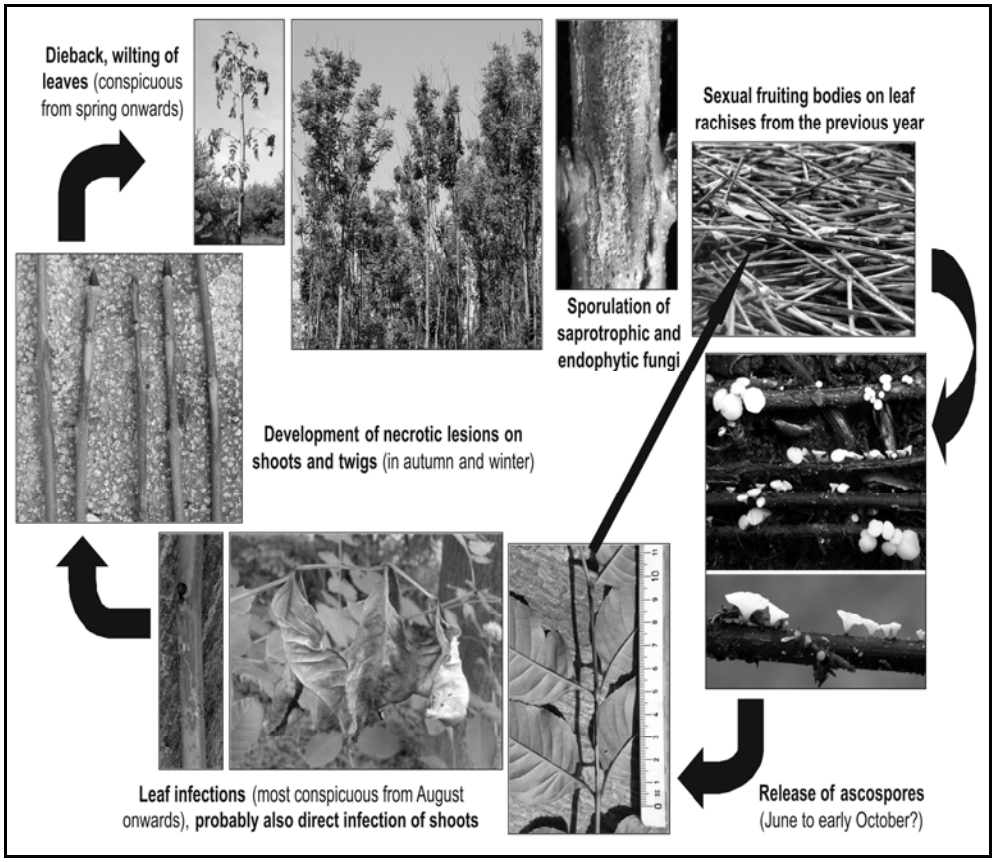

Figure 5. Hypothetical disease cycle of ash dieback caused by *Hymenoscyphus albidus*/ *Chalara fraxinea*. See text for explanations

Although many aspects are still unknown, much progress has recently been made to better understand ash dieback and, based on this knowledge, to recommend measures for disease management. However, this new phenomenon reminds us, how little can in most cases be done against emerging forest health problems. As a consequence of ash dieback the silvicultural characteristics of ash need to be re-appraised. While it used to be a 'stable' tree species that was little affected by diseases, insect pests and abiotic damaging factors, it is presently threatened by this new phenomenon. It is therefore recommended to plant common ash less extensively as before and mix it with other site-adapted tree species. Plants for planting should be carefully inspected for the occurrence of symptoms by nursery managers, forest owners and foresters. Likewise, it should be avoided to bring diseased seedlings into areas, where ash dieback has thus-far not been recorded. Wherever it is possible, leaves shed in autumn and leaf rachises on the

ground prior to the occurrence of apothecia of *H. albidus* should be removed, ploughed into the soil or covered with soil. Such sanitation measures are probably possible and economic feasible in nurseries and urban areas. However, the dispersal distances of ascospores of *H. albidus* are presently unknown and it will depend on these distances, whether infections can be effectively prevented by local removal of leafs and leaf rachises. In nurseries fungicide application to protect plants from infections by ascospores may be another measure, but thus-far there is no experience regarding the fungicides to be effectively used as well as the precise timing of the applications. It is likely that infections can occur over a long period of time, from June to early October (Fig. 5), which would make many applications necessary. While fungicide treatments may be a useful method to raise healthy ash seedlings, their general importance for disease management will be limited, as seedlings will become infected after having been planted in the field.

Thus-far there are no reliable recommendations for the silvicultural treatment of stands affected by ash dieback. It is, however, recommended not to abandon ash too early, and, if there are no other reasons, to harvest only dead and severely damaged trees. It has been observed that *C. fraxinea* can grow from epicormic shoots into the wood of ash stems and causes discoloration there (Kowalski and Holdenrieder, 2008; Thomsen et al., 2009), thereby lowering the timber quality and value. To avoid such losses as well as damage caused by wood-decay fungi, timely felling of severely diseased trees is recommended. Ash dieback will likely weaken the populations of ash trees and secondary pathogens such as *Armillaria* spp. and ash bark beetles may become more important and need to be considered (Kowalski and Holdenrieder, 2008; Thomsen et al., 2009). When intensive care is possible, individual trees can be rescued by cutting infected shoots, twigs and branches. Likewise, young trees can be cut to rescue the stump and root system, from where suckers will develop. However, in both cases, new infections of *H. albidus*/*C. fraxinea* are likely to occur and thus, trees need to be inspected and treated repeatedly.

The most promising potential option for disease management may be the existence of considerable levels of resistance or tolerance within the populations of common ash. In heavily affected areas it is not rare to see severely diseased ash trees growing aside of still healthy or little affected trees. Likewise, investigations in seed plantations in Denmark strongly suggest that there are considerable differences in the resistance levels of ash clones towards ash dieback, with some clones hardly being affected (Olrik et al., 2007; Kjær et al., 2009). Preliminary assessments in clonal seed orchards in Austria support this view (C. Freinschlag, C. Jasser, A. Gaisbauer and T. Kirisits, unpublished data). It is therefore recommended that forest owners and foresters record, mark and promote healthy and slightly diseased ash trees growing in severely affected stands and promote natural regeneration of these potentially resistant or tolerant trees. Thereby they may facilitate that resistance levels in the ash populations are maintained, get

stabilized or even increase. Breeding for resistance may be another, more intensive option for disease management in the future.

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DIEBACK ON *Fraxinus ornus* IN KONYA REGION

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ABSTRACT

In many European countries, intensive dieback of ash has been observed in all age classes, independent of forest type and geographic position during the last 10 years. There are 3 species of *Fraxinus* in Turkey: *Fraxinus excelsior* L., *F. angustifolia* Vahl.. and *F. ornus* L.. They are fast growing trees and have valuable wood which is widely used in furniture industry. In addition, the trees are used in landscape architecture.

In this study, existence and causal agents of dieback was investigated in *F. ornus* plantations located in Dutlukır (7.2 ha) and Altınapa Dam (6.4 ha) in Konya. Condition of the shoots, characteristics of the cankers and lesions, height and diameter (at root collar) of the trees, and signs of insect attacks were recorded. Almost all, 98.2%, of the sampled trees were bearing cankers, 4.1% had signs of insect attacks, and 24.7% of the trees had dry shoots.

Keywords: Dieback, *Fraxinus excelsior, Fraxinus angustifolia, Fraxinus ornus*

INTRODUCTION

Dieback of ash has been one of the most important diseases on ash in European countries during the last 10 years. Recently the casual agent of the dieback was reported to be *Chalara fraxinea* T. Kowalski (Kowalski, 2006; Cech and Hoyer-Tomiczek, 2007; Kirisits et al., 2008; Halmschlager and Kirisits, 2008*)*. Symptoms are necrosis of leaf rachises and leaflet veins, shoot, twig and branch dieback as well as necrotic lesions and cankers in the bark. Bark necrosis is often accompanied with brownish discolouration of the wood. Wilting of leaves can sometimes be seen on recently girdled shoots and twigs (Bakys et al., 2008; Kowalski, 2006; Kowalski and Holdenrieder, 2008; Halmschlager and Kirisits, 2008; Krisits *et al., 2008)*.

There are three species and seven subspecies of *Fraxinus* distributed in Marmara, Black sea, Aegian and Mediterranean Region of Turkey (Yaltırık, 1978). *Fraxinus ornus* (Manna ash) is a native species to southern Europe and south western Asia. Manna ash is an important tree species widely used in furniture industry and music instrument manufacturing. In addition, it is used in landscape architecture.

The aim of the present study was to investigate the frequency of dieback in Manna ash plantations located in Dutlukır and near Altınapa Dam in Konya province.

MATERIALS AND METHODS

Manna ash plantations located in Dutlukır and near Atınapa Dam in Konya province were investigated in April 2009. The origin of the trees in the study areas is not known.

The plantation site near Altınapa Dam, approximately 16.5 km west of the city of Konya, covers 6.4 ha on a minor slope facing the dam at an altitude of 1280 m a.s.l. The 7.2–hectare plantation in Dutlukır is located on flat terrain, 1075 m a.s.l., approximately 9 km southwest of Konya. In Konya region, winters are cold and snowy and summers are hot and dry. In the study area, annual precipitation ranges from 300 to 400 mm (Fig. 1). The average number of days with precipitation per month is 9-11 from December to May, and 2-7 from June to November. Between July and September there are only 2-3 rainy days per month. During these three summer months the total precipitation is below 30 mm. The average annual temperature is +11.4˚C (Anonymous, 2009).

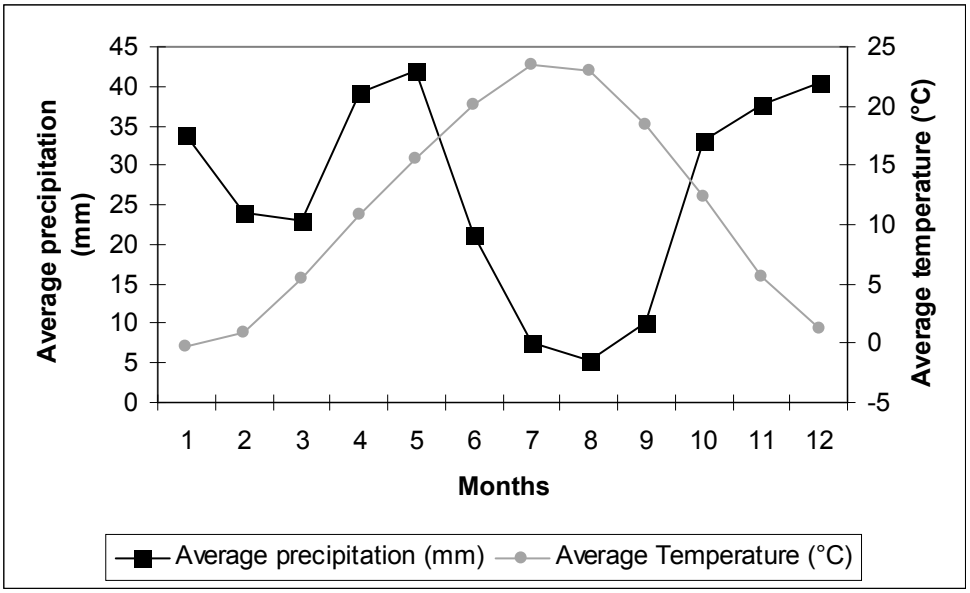

Figure 1: Monthly means for precipitation and temperature in Konya 1975-2007.

The plantations were investigated using a systematic sampling strategy. Condition of the shoots, characteristics of the cankers and lesions, tree height and stem diameter at root collar, and signs of insect attacks were recorded for every 4th tree in Dutlukır and for every 6th tree in Altınapa plantation. Shoot and bark

samples were collected from trees bearing cankers and lesions; totally 340 samples were collected for fungal isolation. The samples were kept in paper bags at +4˚C until isolation.

The isolations were made from cankers and lesions on the shoots and the stem (Fig. 2).

Figure 2: Cankers and lesions on the stem and the shoot of *F. ornus*.

After surface sterilization with 70% ethanol and removing the surface bark, pieces of shoots were removed and placed in petri dishes containing 2% malt extract agar. The cultures were incubated at room temperature in dark conditions.

RESULTS & DISCUSSION

The average diameter of the sampled trees was 3.2 cm in Dutlukır and 2.6 cm in Altınapa and the average height of the sampled trees was 235 cm in Dutlukır and 233 cm in Altınapa.

Almost all, 98.2% of the sampled trees were bearing cankers, 4.1% had signs of insect attacks and 24.7% of the shoots had dried. In addition, wood discolouration and necrosis on the shoots were observed. The study is still going on and identification of the fungi is under progress.

The high proportion of the trees bearing cankers and dry shoots indicates that the trees are either growing at an unsuitable site or that the trees are frequently attacked by a biotic agent. As the origin of the trees is not known, the possibility that they are poorly adapted to the local climate in the study area can not be excluded**.** The fact that the plantation sites are located outside of the natural distribution of *F. ornus* in Turkey (cf. Yaltırık, 1978) supports a hypothesis of weather related damage.

Periods of low rainfall can be one of the abiotic factors correlated with the initiation of the disease (Hibben and Silverborg, 1978). However, there has not been any unusually dry periods, that could explain the high frequency of dead shoots, in Konya during the last 3 years. On the other hand, insufficient adaptation to the local climate could result in e.g. delayed winter hardening of the current–year shoots increasing susceptibility to frost damage or winter drought. The shoots had dried after bud formation indicating that the shoots had died between late summer and early spring. The frequency of dry shoots was similar in both sites. Therefore differences in microclimate between the sites, caused by the dam in Altınapa, seemed not to have had any effect on the occurrence of the damage.

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ASH DIEBACK IN THE CZECH REPUBLIC

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ABSTRACT

Ash dieback was observed in Baltic states since middle of 90`s, however the progress of disease was observed within last years. The new fungus *Chalara fraxinea* was described as a causal agent of ash dieback in 2006. The ash dieback was observed in some local areas in the Czech republic since middle of 90`s and it was connected mostly with extreme climatic conditions. Progress of disease was observed since 2003. Ash decline was observed practically in all regions of the CR, *C. fraxinea* were confirmed in the CR in September 2007. Occurrence of *Chalara fraxinea* was confirmed in *Fraxinus excelsior* and *F. angustifolia.* Perfect stage of *C. fraxinea Hymenscyphus albidus* is known as common saprophytes on leaf-stalks. In areas with esh dieback was observed aphid *Prociphilus bumeliae*, however the role of this insect is actually discus.

Key words: ash decline, *Chalara fraxinea,* ash, ash dieback

1. INTRODUCTION

Common ash (*Fraxinus excelsior*) is threatened in large parts of Europe by ash dieback (eg. Cech, 2006; Lygis et al., 2005). *Chalara fraxinea* has been recently determined to be the causal agent of this disease (Kowalski, 2006; Kowalski & Holdenrieder, 2009a; Bakys et al., 2009).

Chalara fraxinea was reported in Poland (Kowalski, 2006), Denmark (Thomsen et al., 2007), Germany (Schumacher et al., 2007), Austria (Halmschlager and Kirisits, 2008), Hungary (Szabo, 2008a; 2008b), Finland (EPPO, 2008a), Lithuania (R. Vasaitis, personal communication), Norway (EPPO, 2008b; H. Solheim, personal communication), Sweden (Bakys et al., 2009), Switzerland (Engesser and Holdenrieder, unpublished) and France (Ioos, personal communication). Symptoms of ash dieback were also reported from Slovakia in 2008 (Kunca, personal communication); the disease was noted also in Slovenia (Ogris et al., 2009) and Croatia (own observation) in 2008. From the Czech republic is pathogen reported from 2007 (Jankovský and Holdenrieder, 2009). The ascomycete *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips was identified as the teleomorph of *C. fraxinea* by Kowalski and Holdenrieder (2009b).

2. MATERIAL AND METHODS

Ash twigs (4-8 mm diameter) with dieback (comprising a necrotic distal portion and a proximal living portion) were collected and transferred to the laboratory. The surface were disinfected by spraying with concentrated ethanol and superficially dried on a clean bench. The tissue samples (diameter 2-5 mm, about 2-3 mm long), were dissected from the sapwood below necrotic lesions, after bark removal. Samples were then surface sterilized by immersion in sodium hypochlorite (7 - 10 %) for 60 - 90 sec, then immersed in 96% ethanol for 60 - 90 sec, washed in sterilized water and placed on the medium. Tissue samples were aseptically transferred on malt extract agar (MEA 3; 30 g/L, peptone 5 g/L, agar 15 g/L) and, according to Kowalski (2006), streptomycin (100 mg/L) added after autoclaving. Identification was made on the bases of colony morphology and microscopic features.

3. RESULTS AND DISCUSSION

Ash dieback associated with bark necroses and withering of young shoots was recorded in several areas in the CR during 2004 – 2008. The locations affected include mostly all area of the CR, eg. Beskydy Mts., Jeseniky Mts., Giant Mts., Bayerischer Wald Mts., Central Bohemia, Prague, Eastern Bohemia, Czech Moravian Highland, the area at the junction of the Thaya and Morava Rivers, and along the Czech, Austrian and Slovak borders. Ash dieback has extended across the entire country since 2004. The symptoms were also noted in nurseries, especially on saplings in South Moravia. However wooly aphids from genus *Prociphilus* spp. were observed in this areas abundantly as a important harmful factor in early spring.

The first record of *Chalara fraxinea* in the CR originated from samples collected at Drahany Highland, Arboretum Krtiny, from *Fraxinus excelsior* and in some other locations (Jankovský and Holdenrieder 2009). Ash diaback were observed on *Fraxinus excelsior* and its cultivars, especially sensible is *F. excelsior* cv. „Pendula“, and *F. angustifolia*.

On the area of South Moravia samples has been taken especially from the district of Židlochovice forest enterprise, close to Austrian and Slovak borders. Health condition of young forest stands is serious on some locations and there is a danger of large damages of these young plantations. The culture of *C. fraxinea* origin from flood-plain forest by the village Tvrdonice in this area. It is located near of the boarder with Slovakia. The other samples were collected in the Ivaň area (adjacent to the Nove mlyny reservoir) and Soutok game preserve (National nature reserve Cahnov).

The occurrence of ash decline is continually monitored, especially in the area of south Moravia.

According to the experience with symptoms of ash wilting we can assume that *C. fraxinea* is spread through the whole area of the CR.

Table 1. Occurrence of Ash dieback on some monitored plots.

	LOCALITY	DATE OF COLLECT.	CONCLUSION	NOTICE	POSITION
1	Arboretum of Křtiny	26.09.2007	Pozitive	*Fraxinus excelsior "Pendula"*	49°19'7"N/ 16°44'35"E
2	Ochoz u Brna	29.09.2008	Pozitive		49°15'24"N, 16°43'56"E
3	Hradčany u Brna	06.10.2008	Pozitive		49°19'36"N, 16°25'58"E
4	Brno Lesná - Soběšice	08.10.2008	Symptom of disease		49°14'37.5"N, 16°37'5.728"E
5	Lomnice u Tišnova (Sýkoř hill)	12.10.2008	Pozitive	Shoots along the way	49°24'34.51"N, 16°24'46.765"E
6	LZ Židlochovice - NPR Cahnov	13.10.2008	Symptom of disease	Regeneration under the cover of older stand	48°39'17.223"N, 16°56'32.378"E
7	District Lednice-Valtice area - Rendez vous	30.10.2008	Symptom of disease		48°44'54.48"N, 16°47'39.299"E
8	LZ Židlochovice - Ivaň	04.02.2009	Symptom of disease		48°55'18.304"N, 16°34'56.446"E
9	Kroměříž - zámeček	07.02.2009	Symptom of disease		49°16'55.536"N, 17°27'50.746"E
10	LZ Židlochovice – forest district Tvrdonice	18.02.2009	Pozitive	Compartment 909	48°47'29.587"N, 17°4'21.828"E
11	LZ Židlochovice – forest district Tvrdonice	18.02.2009	Symptom of disease	Compartment 904	48°47'23.761"N, 17°4'21.588"E
12	Forest nursery Hadovna - region Kroměříž	30.05.2009	Symptom of disease		49°18'24.285"N, 17°39'4.909"E
13	Arboeko co. - Smržice	30.04.2009	Symptom of disease		49°23'56.145"N, 17°11'26.528"E

Dry lesions show circular shape at first, they gradually change into elliptical oblong, depressed necrosis, which gradually extends on the surface; it was observed in most places. Below the bark, the dieback of the cambium is evident. The necrosis extends both into transpiration flow and assimilation flow. The infection gets from necrosis also into the wood part, which is discolored by grey brown. One year shoots die above the necrosis especially in autumn months (from end of August till October). Necrosis on 2 years old and perennial shoots can be occluded and there can be superficial cankers with callus on the edge created. Dark brown necrosis is formed on petioles and trees shed premature, from end of August till October, their still green leaves. One year old, rarely also older shoots die on older trees. Typical of this is a creation of compressed crown and disturbance of parallel trunk.

Hymenoscyphus albidus is noted by Kowalski and Holdenrieder (2009) as the teleomorph of *Chalara fraxinea.* The species is widespread in Europe. According to the literature, *H. albidus* occurs exclusively on ash petioles from ash litter (Breitenbach and Kranzlin, 1984; Ellis and Ellis, 1985). In the CR, it is well known as a saprophyte on leaf-stalks in the litter, it is considered to be a common species in mycofloristic research.

Transfer of infection nor vector has not been clarified till now. One can assume that infection occurs via ascospores of *H. albidus* which are formed on leaf fall of ash during summer months. It corresponds to time period of development of newly attacking infection. Rapid and especially sudden dissemination of this disease throughout Europe is not typical for tree disease, is it more typical for non biotic diseases or for insect outbreaks. Especially an area, which is being occupied during relatively short period of time, is enormous. For pathogens, it is difficult to penetrate structural suberin barrier which is represented by unharmed bark. The influence of sucking insect can be discussed. Remarkable is the fact that lesions occur practically at the same place like the colony of woolly aphids *Prociphilus spp.* Aphid`s colonies are located on shoots and petioles of leaves. In nurseries, where chemical control against sucking insect was applied, symptoms of ash dieback disappeared. Woolly aphids are not reported to occur in all areas where ash decline was observed. Sucking insect can play a role of agent which creates a defect in the bark. In sucking locations, tissues suffer from necrosis and fungi like *C. fraxinea* penetrate through the tissues. There is an apparent separation of reason – sucking of aphids during spring time and formation of necrosis during summer time when the connection to sucking insect needs not to be evident.

4. CONCLUSION

The bionomy of the fungus, infection cycle, pathology, resp. pathogenicity of the fungus and genetic structure are main topics of contemporary research. Although pathogenicity of the *C. fraxinea* has been proved, the mechanism of penetration of fungus into host is not known yet. The role of sucking insect, mainly woolly aphids, should be crucial due to production of lesion around sucking points at the position of previous aphid colonies. The relationship of sucking insects and *C.fraxinea* in ash dieback pathology requires further investigations.

5. ACKNOWLEDGEMENTS

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Canker Diseases

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HORSE CHESTNUT BLEEDING CANKER – BAGGING THE BUG !

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ABSTRACT

Bleeding canker of horse chestnut, caused by the bacterial pathogen, *Pseudomonas syringae* pv. *aesculi*, has rapidly established and become widespread throughout parts of northern Europe, including Great Britain, over the last five years. Despite the fact that this disease is having a devastating effect on an important amenity tree species, very little is known about the infection processes of this pathogen, or the genetic and physiological factors which cause it to be so highly damaging. One difficulty associated with studying this pathogen is the lengthy procedure required to confirm its presence on the host. Real-time PCR primers were developed based on gyrase B gene sequence data for the specific detection of *P. syringae* pv. *aesculi* in infected horse chestnut. This quantitative real-time PCR assay provides the facility to study several important aspects of the biology of *P. syringae* pv. *aesculi* on horse chestnut including its potential for epiphytic survival on healthy trees, as well as its dissemination in different environmental substrates, such as soil, water and infected tree debris. As part of ongoing work, the complete genome of *P. syringae* pv. *aesculi* is currently being sequenced to determine its complement of virulence and fitness genes. Molecular tools are also being used to determine the origin of *P. syringae* pv. *aesculi,* the location/s and time of its introduction and geographical routes of spread within Great Britain and Europe.

Keywords: *Pseudomonas syringae* pv. *aesculi*, bleeding canker, *Aesculus hippocastanum*, real-time PCR.

1. INTRODUCTION

In the last 10-15 years, there has been an unprecedented 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), poorly treated timber products and international trade in bonsai (Brasier, 2008). European horse chestnut (*Aesculus hippocastanum*), an important amenity tree species throughout much of Great Britain and northern Europe, is suffering from two major problems of recent introduction into the continent: bacterial bleeding canker and the horse chestnut leaf miner. Native to northern Greece and Albania (Phillips, 1978), horse chestnut was introduced into the UK in the late 16th Century and was planted widely in parks and gardens in both urban and rural areas, often in avenues bordering roads. Horse chestnut is highly regarded for its qualities as a shade tree, for its showy white flowers in spring and the production of its fruits or ‘conkers’.

Since 2003, an epidemic of a bleeding canker disease of horse chestnut has become widespread across Great Britain as well as a number of other European countries, including the Netherlands, Belgium, France and Germany. The disease has been attracting a great deal of media attention in Great Britain over the last couple of years due to the severity of damage on affected trees. Symptoms of the disease include bleeding cankers located on the stem and branches, with rust-coloured liquid oozing from cracks in the bark, necrotic phloem, foliar discolouration, and crown dieback often leading to tree death. A Great Britain-wide survey of 2629 horse chestnut trees conducted in 2007 found that over 70% of trees surveyed in parts of England exhibited symptoms typical of bleeding canker, with 36% and 42% of surveyed trees showing these symptoms in Wales and Scotland, respectively (Forestry Commission, 2008). The reason that bleeding canker disease of horse chestnut is currently of such high profile is due to its dramatic impact on an important amenity tree species. The disease is now a key tree health issue in Great Britain, particularly in the context of 'urban greening' and with climate change in mind there is increasing recognition of the need to maintain healthy populations of shade trees within urban areas.

The causal agent responsible for this new epidemic of bleeding canker disease of horse chestnut has only very recently been identified as a gram-negative fluorescent *Pseudomonas syringae* bacterium, which has an identical partial gyrase B gene sequence to a strain isolated from leaf lesions on Indian horse chestnut (*Aesculus indica*) in India in 1980 (Schmidt et al., 2008; Webber et al., 2008). There are at least 50 closely related pathovars of the species *Pseudomonas syringae* which can be distinguished by host range, and which infect a wide range of herbaceous and woody plants. It is thought that *P. syringae* pv. *aesculi* may have originated from India, being native on Indian horse chestnut, and has been recently introduced to Great Britain and Europe, possibly via imported, infected nursery stock (Brasier, 2008). If it is a new introduction to Europe, *P. syringae* pv. *aesculi* has found a new host, European horse chestnut, on which it is considerably more aggressive than its native host on which it only causes leaf lesions. Britain's forests are under continuous risk from new, exotic diseases as a result of the increased movement of plant stock between countries over ever greater distances. Bacterial diseases of trees present a particular risk to Britain's forests and woodlands since their activity in northern Europe may be favoured by the milder, wetter winters predicted under future climate change scenarios and because so little is currently known about the routes of invasion, spread and survival of these pathogens on woody hosts.

One of the obstacles to studying *P. syringae* pv. *aesculi* is the difficulty of confirming its presence in host tissues. To date, this bacterium is detected on symptomatic trees by isolation and culturing, PCR amplification and sequencing of the gyrase B gene using universal primers, and comparing sequence homology with a type strain of *P. syringae* pv. *aesculi* published in the US National Center for Biotechnology Information (NCBI) GenBank sequence database. However, this is

time consuming, through the need to obtain pure bacterial cultures from diseased horse chestnut tissues which may be colonised by a range of bacterial genera (Green et al., 2009). Recently, real-time PCR has proven to be a very useful tool for the detection of plant pathogenic fungi and bacteria, being highly sensitive, specific and rapid, with the added capacity for quantification of the pathogen in host tissues (Schaad and Frederick, 2002; Vandroemme et al., 2008). The first aim of this study was to develop and test a robust, reliable, quantitative real-time PCR assay which is specific to *P. syringae* pv. *aesculi*, and to demonstrate its use for detecting the bacterium in inoculated and naturally infected horse chestnut trees (Green et al., 2009). Also, briefly discussed are ongoing projects aimed at i) characterising the key genetic and physiological factors determining virulence and epiphytic fitness of *P. syringae* pv. *aesculi* on European horse chestnut and ii) tracing the epidemiological origins of this devastating bacterium.

2. MATERIALS AND METHODS

For the development of the real-time PCR assay, a total of 65 bacterial isolates were collected from various parts of diseased or healthy horse chestnut in Britain, the DNA extracted and the partial gyrase B gene region amplified using universal, degenerate primers. The amplified fragments were sequenced and aligned with other bacterial gyrase B gene sequences available in GenBank to design real-time PCR primers specific to *P. syringae* pv. *aesculi*. The specificity and sensitivity of the real-time PCR primers was tested using nine strains of *P. syringae* pv. aesculi, 17 other strains of *P. syringae*, 11 other non-pathogenic *Pseudomonas* spp. and 14 other species of bacteria isolated from horse chestnut trees. The ability of the real-time primers to amplify and quantify DNA of *P. syringae* pv. *aesculi* in diseased horse chestnut tissues was also demonstrated.

3. RESULTS

The real-time primer pair and reaction conditions developed to detect *P. syringae* pv. *aesculi* amplified all isolates of *P. syringae* pv. *aesculi*, but did not amplify the DNA extracted from the included reference bacteria or horse chestnut when 1 ng of DNA was used as the template. The real-time primers reliably amplified *P. syringae* pv. *aesculi* down to 1 pg of extracted DNA, with and without the presence of host DNA, and also amplified unextracted DNA in whole cells of the bacterium down to at least 160 colony forming units. The real-time PCR assay detected and quantified DNA of *P. syringae* pv. *aesculi* in sixteen out of seventeen tissue samples collected from naturally infected or artificially inoculated horse chestnut, with generally good consistency between the two PCR runs in terms of Ct values and quantity of pathogen DNA detected.

4. DISCUSSION

A novel, quantitative real-time PCR assay has now been developed to detect the pathogenic bacterium, *P. syringae* pv. *aesculi*, which is currently causing a severe bleeding canker disease of horse chestnut trees in several European countries (Green et al., 2009). The real-time PCR primers were shown to be both highly specific, giving exponential amplification only with the target pathogen, and highly sensitive, allowing detection of *P. syringae* pv. *aesculi* down to 1 pg DNA in diseased horse chestnut tissues with the optimised reaction conditions used (Green et al., 2009). This advance in methodology now provides a tool for the accurate and sensitive quantitative detection of *P. syringae* pv. *aesculi* in host tissues, as well as in different environmental substrates. This assay will be used to examine the ability of *P. syringae* pv. *aesculi* to survive epiphytically in host material, and its potential to contaminate rainwater and soil. The aim of this is to determine the routes for transmission of the pathogen, and to explain the reason for its high mobility.

Research into *P. syringae* plant pathogens is currently of high profile internationally due to their detrimental impact in the horticultural, agricultural and forestry sectors (Kennelly et al., 2007; Perez-Martinez et al., 2008). Ongoing research into the dentification of the key virulence and fitness genes of *P. syringae* pv. *aesculi* through genome sequencing, combined with *in vivo* studies on pathogenicity, will provide novel insights into the factors determining the pathogenicity and fitness of an important and newly damaging *P. syringae* pathovar on a woody host; this being an area for which information is currently scarce.

The effectiveness of import and quarantine regulations and disease management strategies designed to protect the agricultural, horticultural and forest industries against exotic pathogens relies on a thorough understanding, based on sound scientific data, of the routes of introduction and spread of exotic pathogens in new locations. This is reflected in the current high priority given to these types of studies within the European Union (EU), particularly given the recent, rapid expansion of the international plant trade and an appreciation of the threat that this presents. Current research on *P. syringae* pv. *aesculi* will identify the most suitable genetic markers, based on genome sequence data, for studying the recent evolutionary history of *P. syringae* pv. *aesculi*. These markers will then be used for phylogenetic analyses to elucidate whether this pathogen is a new introduction to Europe, and if so, when and from where it was introduced and routes of subsequent spread within regions. The research briefly described here will be essential for the development of more effective phytosanitary measures and disease management strategies to guard against future threats posed by exotic bacterial pathogens. The project will ultimately inform and guide management and mitigation strategies for an important bacterial disease of an amenity tree species through increased understanding of the causal agent.

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AN OVERVIEW OF POTENTIAL INFECTION COURTS FOR *Neonectria fuckeliana*, THE CAUSAL AGENT OF NECTRIA FLUTE CANKER IN *Pinus radiata* IN NEW ZEALAND

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ABSTRACT

Nectria flute canker is a disease of *Pinus radiata* stems in the southern parts of New Zealand caused by the pathogen *Neonectria fuckeliana*. Although tree crowns generally remain healthy, stem cankers and associated defect reduce the commercial value of the timber. In Northern Hemisphere countries where *N. fuckeliana* is endemic, open wounds, dead attached branches and branch stubs have been identified as the primary infection courts for *N. fuckeliana*. In New Zealand the development of the Nectria flute canker disease is primarily associated with pruned branch stubs however recent studies suggest that this is not the only possible infection court as the fungus has been found in trees prior to pruning. Three separate field trials were established to examine potential infection courts for *N. fuckeliana* in *P. radiata* in New Zealand. These infection courts included stem wounds, pruned stubs, branch crotches and branch collars. Stem depressions, the usual precursor to flute cankers, were created following inoculation of deep and shallow stem wounds and of some branch collars. Inoculation directly into pruned stubs resulted in only a few, small stem depressions and the fungus was largely contained within the branch trace. Infection through branch crotches was not successful. Both inoculation types tested (ascospores and conidia) resulted in similar canker development and fungal spread within the tree. The trials described in this paper are ongoing.

Keywords: *Neonectria fuckeliana*, stem cankers, *Pinus radiata*.

1. INTRODUCTION

Pinus radiata D. Don is the most important plantation tree species grown in New Zealand, comprising more than 89% of the plantation estate (NZFOA, 2009). Many of the plantations are managed to produce clear, knot-free wood by pruning from one to three times during the rotation (NZFOA, 2009). Stem cankers, often associated with pruned stubs, have become increasingly noticeable in some *Pinus radiata* plantations in the lower South Island of New Zealand over the last 15 years (Dick and Crane, 2009; Gadgil et al., 2003). The long, narrow cankers, commonly referred to as "flute cankers" for their elongated appearance, can extend for several metres above and for a shorter distance below a pruned branch stub. Formation of cankers associated with natural injuries on the stem internodes has rarely been observed (Dick and Crane, 2009). Although tree crowns generally remain healthy, affected trees are susceptible to decay, to wind breakage at infected whorls, and wood quality can be affected.

Neonectria fuckeliana (C. Booth) Castl. & Rossman (*Nectria fuckeliana* C. Booth) (Ascomycota: Nectriaceae) is the fungus most commonly found in association with the flute cankers (Dick and Crane, 2009). This pathogen is thought to be endemic to Northern Europe, Scandinavia and North America where it has been recorded principally as a common wound invader or weak pathogen of species of *Picea* and *Abies* (e.g. Roll-Hansen and Roll-Hansen, 1979; Schultz and Parmeter, 1990; Vasiliauskas and Stenlid, 1998). Pathogenicity of the fungus has been reported infrequently in *Pinus* spp., and this has been primarily as the result of artifical inoculations (Smerlis, 1969). The pathogen has three spore states. In addition its teleomorph, in which ascospores are produced in perithecia (the *Neonectria* phase), two anamorphs are formed under certain conditions: an *Acremonium* state with unicellular spores and a *Cylindrocarpon* state (*Cylindrocarpon cylindroides* var. *tenue* Wollenweber) with multicellular spores. Vasiliauskas and Stenlid (1997) demonstrated that, in Europe, the *N. fuckeliana* ascospores are probably the major dispersal propagules. The importance of the anamorphs in the pathogen life cycle and in disease development is not fully understood.

In the Northern Hemisphere, open wounds, dead attached branches and branch stubs have been identified as the primary infection courts for *N. fuckeliana* (Roll-Hansen and Roll-Hansen, 1979). In New Zealand, since the development of the Nectria flute canker disease is primarily associated with pruned branch stubs, it was assumed that these branch stubs were the primary infection court (e.g. Bulman, 2007). Recent studies by Power and Ramsfield (2006, 2007) however, suggest that this is not the only possible infection court for *N. fuckeliana*. In a study of 90 pruned and 90 unpruned trees, the pathogen *N. fuckeliana* was found in approximately 22% of trees and no significant difference in frequency of the pathogen was found between pruned and unpruned trees. None of the trees examined showed symptoms of Nectria flute canker. This suggests that *N. fuckeliana* is able to enter trees prior to pruning using some other infection court/s. This paper outlines a number of trials currently being undertaken in southern New Zealand to identify possible alternative infection courts for *N. fuckeliana*.

2. INFECTION THROUGH STEM WOUNDS

A field trial was undertaken to examine the importance of different wound types and inoculum sources for disease development and fungal infection. Specifically the trial aimed to determine whether pruned branch stubs were an effective infection court for *N. fuckeliana* and, following on from Vasiliauskas and Stenlid (1997), whether ascospores were the most effective inoculum source. Forty-five 6-year-old *Pinus radiata* were subjected to one of three wound types (shallow stem wound, deep stem wound or pruned branch stub) and one of three inoculation types (ascospore inoculation, conidial inoculation or a water control). Trees were assessed for the formation of stem depressions (the typical precursor to flute cankers) after 6, 12 and 18 months, after which time they were harvested and the

spread of *N. fuckeliana* was examined within the tree using isolations. Wound type was found to be very important for flute canker development, with trees with deep wounds showing largest stem depressions and most spread of *N. fuckeliana* within the tree. Shallow stem wounds also resulted in some stem depressions however these were usually small and there was only a little vertical movement of the fungus through the stem. Inoculation of branch stubs resulted in few or small stem depressions and the fungus was largely contained within the branch trace. Both inoculation types (ascospores and conidia) resulted in similar levels of stem depressions and fungal spread within the tree.

3. INFECTION THROUGH BRANCH CROTCHES

Pinus radiata grown in the southern regions of New Zealand is frequently subjected to snow events during the winter months. Due to the acute branching angle of *P. radiata* this often results in severe branch bending and can lead to branch breakage. This severe branch bending can lead to openings in bark of the branch crotch which may provide an infection court for fungal spores. Forty eight-year-old *Pinus radiata* trees were used to examine this theory. On each tree, six branches of similar size were selected on the same or adjacent whorls. Each branch was then subjected to one of two bending treatments (bent with string to simulate the weight of snow on the branch, or unbent) and one of three inoculum sources (control, *Acremonium* conidia on a colonised twig, or a piece of bark containing *N. fuckeliana* perithecia with ascospores). The inoculum source was glued or stapled directly above the branch crotch. Trees were checked after 6 and 12 months for any canker development and no change to the trees was observed. After 18 months, 20 of the trees were felled and dissected through the branch traces. Isolations were undertaken to determine whether *N. fuckeliana* was now present within the stem tissue. No *N. fuckeliana* was isolated. The remaining 20 trees will be monitored for a further 6-12 months and may be felled if any external symptoms of Nectria flute canker develop.

4. INFECTION THROUGH BRANCH COLLARS

Although inoculations directly into pruned stubs were not always successful at initiating *N. fuckeliana* spread throughout the stem (see section 2), the symptoms of Nectria flute canker are almost exclusively associated with pruned branches. Incidence of Nectria flute canker in a stand can be much higher than 22% (the proportion of colonisation recorded in unpruned trees) and so some infection may be occurring at the time of pruning. If the branch collar was damaged during pruning, it is possible that this branch collar may act as an infection court for *N. fuckeliana*. To investigate this, 41 eight-year-old *P. radiata* trees were pruned in the lower third of the stem and three branch collars on each tree were inoculated with an *Acremonium* spore suspension. Great care was taken to prevent the spore suspension spreading onto the rest of the pruned branch surface. The remaining

branch stubs on each whorl were treated as controls and were not inoculated. After 6 months, eight of the trial trees were showing very slight canker development from inoculated sites. Following 12 months stem depressions were recorded above 17 of the 123 inoculated branch collars**.** This trial is ongoing and isolations will be made from trees felled after 18-24 months to determine whether *N. fuckeliana* had infected the stem and how far it has spread.

5. DISCUSSION AND FUTHER RESEARCH

The inoculation experiments described in this paper have given some insight into the potential infection mechanisms for *N. fuckeliana* in *P. radiata* in New Zealand. In the first trial, infection and symptom development was clearly demonstrated using both ascospores and conidia from the *Acremonium* stage. This indicates that spores from both these lifestages could potentially play a role in infection of this pathogen. In New Zealand however, although the *Acremonium* stage is produced in culture, it is rarely observed in the field. In contrast, perithecia producing ascospores of the teleomorph are frequently observed in the field and, due to their abundance, are much more likely to play a role in dispersal and infection of the pathogen.

While both deep and shallow stem wounds in the first trial resulted in successful infection of *N. fuckeliana* and development of some stem depressions, it is unlikely that these infection mechanisms play an important role in infection in the field. Few wounds are found on *P. radiata* in plantations. Thus wounding is unlikely to play a role as a primary infection court for *N. fuckeliana*.

Although no *N. fuckeliana* was isolated from inside the stems in the branch crotch trial this does not rule out branch crotches as an infection court for this pathogen. During the experiment, it was very difficult to simulate the effect of snow weight on branches, particularly any repetitive opening and closing of the branch crotch associated with branch movement. As a result, the experimental conditions may not have been sufficient to allow penetration of spores into the stem. Further trials of this nature are planned.

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PRELIMINARY RESULTS OF MYCOFLORA ASSOCIATED WITH CANKERS ON *Cupressus sempervirens* var. *horizontalis* (Mill.) GORDON IN TURKEY

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ABSTRACT

Natural stands of *C. 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*. However, phytopathological status of these stands was not investigated so far. In this study, canker formations were investigated in trees, saplings and seedlings of *C. sempervirens* var. *horizontalis* within natural stands located in Köprülü Kanyon National Park, Antalya and Aydıncık, Mersin. Incidence of the cankers and some other disease associated symptoms and signs of insect attacks on sampled trees were recorded. Isolations were made from cankers, and the obtained fungal cultures identified morphologically.

Cankers were present on the trunk or branches of 34.4% of the totally 1023 trees sampled. Canker incidence was greater in Aydıncık than in Köprülü Kanyon, 38.0 % vs. 29.8%, respectively. Totally 497 fungal isolates, representing 30 genera, were obtained. The most common species isolated from both Aydıncık and Köprülü Kanyon was: *Phomopsis* cf. *occulta* (44 and 22 %), unidentified coelomycete (22 and 6%), *Alternaria* spp. (5 and 13%), *Cladosporium* spp. (5 and 5%), *Cytospora* sp. (4 and 2%) and *Pestalotiopsis funerea* (1 and 37%, respectively). While *P.* cf. *occulta* was the most common species in Aydıncık, *P. funerea* - which was rare in Aydıncık - was the most frequently isolated species in Köprülü Kanyon. To our knowledge, with the exception of *P. funerea,* these species are new records on cypress in Turkey. Moreover, both *P. occulta* and *P. funerea* are reported to be pathogenic on cypress, especially under stress conditions.

Keywords: *Cupressus sempervirens* var. *horizontalis,* Canker, Fungi

1. INTRODUCTION

Cupressus sempervirens L.*,* the Mediterranean cypress or common cypress, is a long established cultivated forest tree species exterior to its natural geographic range. However its natural geographic distribution had been restricted to disjoint and often relict populations within Iran, Syria, Jordan, Lebanon, Libya, the Aegean Islands, Crete, Turkey, and Cyprus, which are thought to be being remnants of an extensive *C. sempervirens* forest, (Raddi and Sümer, 1999; Ducrey et al., 1999). 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 the relicts of the original source of *C. sempervirens* var. *horizontalis* due to the high diversity observed among the populations (Koral et al., 1997; Raddi and Sümer, 1999; Ducrey 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). The largest pure and mixed stands (mixed with *Pinus brutia* Ten.) of the species are located in Köprülü Kanyon National Park, Antalya and in Aydıncık, Mersin. Other cypress forests in Turkey are located mostly in the south-western part of Turkey as small stands mainly mixed with *Pinus brutia* Ten. (Sabuncu, 2004). As everywhere else within its distribution area, the Mediterranean cypress forests in Turkey have been frequently endangered by human activities, such as deforestation and wild fires, as well as by overgrazing.

Several species of fungi have been associated with diseases of natural and cultivated varieties of *C. sempervirens*. Among these fungi, *Seiridium cardinale* (Wag.) Sutton and Gibson, the causal agent of the canker disease which had caused heavy damage in forests, nurseries and ornamental plantations, especially in the Mediterranean countries, has taken the most attention (Graniti, 1986, 1998). On the other hand, many pathogens, such as *Diplodia pinea* f.sp. *cupressi* Solel, Madar, Kimchi & Golan, *Phomopsis occulta* (Sacc.) Trav., *Pestalotiopsis funerea* (Desm.) Steyaert, *Fusarium* sp. Link, *Cytospora* sp. and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., (syn: *Botryodiplodia theobromae* (Pat.)) have also been found to be the causal agents of the cankers on cypress (Solel et al., 1987; Bruck et al., 1990; Madar et al., 1991, 1996; Linde et al., 1997; Ducrey et al., 1999; Gonthier and Nicolotti, 2002; Bajo et al., 2008).

Although there are many reports on the phytopathological problems – especially associated with *Seiridium cardinale* – of Mediterranean cypress 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 paper by Sümer (1987) reporting two pathogens in the Aegean coast of Turkey, *S. cardinale* (in Muğla) and *P. funerea*. No further studies have been performed since then.

In this study, the presence of canker formations, top and crown diebacks, foliage symptoms, resin exudation from the trees and insect associations were investigated within natural cypress stands in two locations. In addition, isolation of fungi from cankered tissues was tried.

2. MATERIALS and METHODS

2.1. Survey locations and sampling

The surveys and samplings were conducted in two natural stands of *C. sempervirens* var. *horizontalis* located within Köprülü Kanyon National Park in Antalya province and in Aydıncık Forestry Enterprise in Mersin, during September and December 2008, respectively. The location, altitude, and some topographic and stand characteristics of the sampling plots are given in Table 1.

The Köprülü Kanyon National Park (37º01′N and 31º14′E) is located in the western part of the Taurus Mountains in Antalya province in southern Turkey. The annual average rainfall is 1140.5 mm and the annual average temperature is 18.3 ºC. The cypress forest in the national park is composed of pure (195.6 ha) and mixed stands with *P. brutia* (255.6 ha) at elevations ranging from 650 to 950 m (Anonymous, 2008).

Aydıncık (36º11′N and 33º27′E) is situated along hillsides of the central part of the Taurus Mountains facing Mediterranean Sea coast in Mersin province, 325 km east of Antalya. The annual average rainfall is 936.2 mm and the annual average temperature is 19.1ºC. While degraded (292.5 ha) and normal (88.5 ha) stands comprised a total of 381 ha of cypress forest in Aydıncık, within the same basin the total cypress forests made up 1247.5 ha, at elevations from 20 to 120 m, representing the largest distribution area of this variety (Özçelik, 2005). The cypress forest within Aydıncık, composed of pure and mixed stands (*P. brutia*) was mostly spread along the valley of the Sipahili (Babadili) stream.

Table 1: Characteristics of the sampling plots

Sampling plots	Location	Coordinates		Altitude (m a.s.l.)	Exposure	Description
		Latitude (N)	Longitude (E)			
KK1	Köprülü Kanyon	37 º12′	31 º09′	710	S	Groups of seedlings and saplings along road side
KK2	Köprülü Kanyon	37 º13′	31 º08′	844	S	Groups of seedlings and saplings along road side
KK3	Köprülü Kanyon	37 º13′	31 º08′	736	SE	Groups of seedlings and saplings along road side
KK4	Köprülü Kanyon	37 º13′	31 º08′	151	S	Groups of seedlings and saplings along road side
KK5	Köprülü Kanyon	37 º12′	31 º09′	740	SE	Groups of seedlings and saplings along road side
KK6	Köprülü Kanyon	37 º11′	31 º11′	737	S	Groups of seedlings and saplings along road side
M1	Aydıncık	36° 11'	33° 27'	49	W	Degrade, Mixed with *P. brutia*
M2	Büyükeceli	36 º11'	33° 27'	6	W	Degrade, Mixed with *P. brutia*
M3	Aydıncık, Babadili	36°12'	33° 27'	24	SE	Degrade, Mixed with *P. brutia*, Gene protection forest
M4	Aydıncık, Babadili	36°12'	33° 27'	29	E	Degrade, Mixed with *P. brutia*, Gene protection forest
M5	Aydıncık, Babadili	36°12'	33° 27'	172	E	Pure, Gene protection forest
M6	Aydıncık, Karaseki	36°10'	33°23'	61	NE	Degrade, Pure, along stream
M7	Aydıncık, Babadili	36° 12 '	33° 27'	157	E	Pure, Gene Protection Forest
M8	Aydıncık, Babadili	36° 11'	33 °27'	114	NE	Pure
M9	Aydıncık, Babadili	36° 11'	33 °27'	72	E	Degrade, Pure
M10	Aydıncık, Duruhan	36°13'	33°17'	482	N	Degrade, Mixed with *P. brutia*, along stream and agricultural fields

In Köprülü Kanyon, seedlings and saplings of *C. sempervirens* along the road sides within 6 sampling plots, and in Aydıncık cypress trees of different age classes

within 10 sampling plots were investigated for the canker formations on branches and trunks. In Aydıncık, sampling plots were consist of four circular sub-sampling plots with a 10–m–radius, located 25 m to the north, east, south and west from the plot centre. While in Köprülü Kanyon the length of the cankers and the height of the cankers above ground, as well as tree features were recorded, no such measurements were done in Aydıncık. Contrary to the survey conducted in Köprülü Kanyon, in Aydıncık trees were also assessed for top and crown dieback, resin exudation, foliage symptoms, and insect damage.

2.2. Fungal isolation and identification

Branch and trunk samples with cankers were collected from a number of trees within the sampling plots and transferred to the laboratory. The samples were surface sterilized with 70% ethanol, the outer tissues near the canker margins were removed with a sterile scalpel, and small pieces of the affected bark transferred onto potato dextrose agar plates (PDA; Merck, Darmstadt, Germany). Some of the samples were incubated in moist-chamber in order to induce formation of fruiting bodies on plant tissues. The cultures incubated at 25 ºC were identified according to their morphological characteristics.

3. RESULTS AND DISCUSSION

During the surveys, in Köprülü Kanyon and in Aydıncık, 449 and 574 trees, respectively, were examined (totally 1023 trees). Bark cracks and cankers, as well as resin drops and moderate to heavy resin flows were present on the trunk, branches and twigs at all sites. Of the 1023 trees examined, 34.4% (352) were bearing at least one canker. Canker incidence was greater in Aydıncık than in Köprülü Kanyon, 38.0 and 29.8%, respectively. Branch cankers were more frequent than trunk cankers in Aydıncık (31.7% and 17.9%, respectively), while in Köprülü Kanyon trunk cankers were significantly more common. Bark cracks exuding resin, especially on branches and twigs, were observed often in Aydıncık, where small perennial resin soaked cankers on declining lower branches were also found.

While in Köprülü Kanyon the length of the cankers and the height of the cankers above ground, as well as the tree height and diameter were recorded (Table 2), no such measurements were done in Aydıncık. In Köprülü Kanyon, among the seedlings and saplings investigated, the average height and diameter at breast height of 134 canker bearing individuals were 360.5 and 5.6 cm, respectively. In this site, majority of the cankers observed were on the road–facing parts of the saplings, mostly under breast height (mean 81.1cm). On the other hand, grazing damage was also remarkably more prevalent in Aydıncık, especially on lower branches where canker formations were also common. Therefore it is likely that wounds resulting from grazing have worked as suitable entrance points for canker–causing pathogens.

Table 2: Results of Köprülü Kanyon sampling area

Sampling plot	No of trees	No of trees with cankers	Proportion of trees with cankers (%)	(trees with cankers) average values for; (cm)		Average height of cankers above ground (cm)
				Height	$D_{1,3}$	
KKK1	30	5	16.7	282.6	3.4	66.3
KKK2	162	62	38.3	385.6	7.3	91.3
KKK3	127	17	13.4	262.9	6.1	133.0
KKK4	56	32	57.1	490.0	6.0	119.1
KKK5	36	15	41.7	250.0	3.3	23.0
KKK6	38	3	7.9	491.7	7.5	53.6
Total	449	134	-	-	-	-
Avarage	74.83	22.33	29.2	360.5	5.6	81.1

In Aydıncık, the number of trees per sampling plot varied from 28 to 86 with a mean value of 57.4 (±21SD). The average tree height and diameter in the whole Aydıncık sampling area were 586.7 (±368.8 SD) and 14.9 cm (±12.6 SD), respectively. There were significant differences in average diameter and height of trees between sampling plots (Table 3). While the highest averages were in the sampling plot M7, the lowest ones were in the M8. On the other hand, correlations of symptom incidences with tree size (Table 4) were not significant, with the exception of the negative correlation between tree diameter and foliage symptoms.

The highest canker incidence was in the M5 plot (52.3%), followed by the M8, M4, M9, M3 plots (range approximately 40-50%). The canker incidence (7.7%) as well as the other assessed symptoms had the lowest values in the M6 plot. The highest incidence of top dieback, crown dieback, resin exudation, and foliage symptoms was in the M9 (23.5%), M4 (18.1%), M8 (66.2%) and M10 plots (51.0%), respectively. Insect damage was the most frequent in the M8 plot (53.8%) and occasional in the M1 plot (3.6%).

Table 3: Number and size of trees and incidence of symptoms in the sampling plots in Aydıncık.

Sampling plot	No of trees	Tree sizes		Number of individuals exhibiting symptoms of;					
		Diameter cm	Height cm	Canker	Top dieback	Crown dieback	Resin exudation	Foliage symptoms	Insect Damage
M1	28	17.6±11.9b	555.5±319.8b	6	1	1	4	1	1
M2	53	15.0±10.2bc	572.8±374.7ab	16	3	1	10	5	1
M3	83	15.4±11.9bc	609.3±380.3ab	33	8	11	47	5	9
M4	83	15.2±12.7bc	634.8±415.8ab	36	9	15	50	17	20
M5	86	13.0±10.0bc	583.3±307.9ab	45	5	7	50	17	22
M6	39	17.3±11.8b	606.8±264.7ab	3	0	0	3	3	3
M7	37	24.8±20.0a	734.4±480.1a	12	4	3	20	6	18
M8	65	10.5±6.0c	497.4±353.1b	32	6	8	43	18	35
M9	51	12.7±10.7bc	497.4±353.1b	21	12	9	24	23	15
M10	49	14.6±16.8bc	576.8±481.4ab	14	4	2	27	25	18
Σ	574	-	-	218	52	57	278	120	142
Avr. ± SD	57.4±20.9	14.9±12.6	586.7±368.8	21.8±14.0	5.2±3.7	5.7±5.1	27.8±18.7	12.0±8.9	14.2±10.8

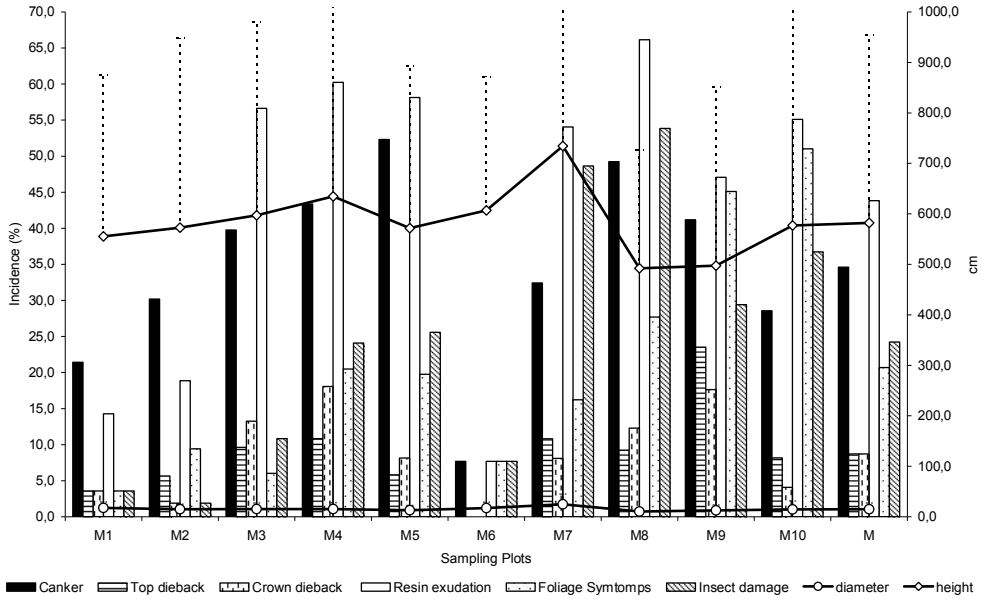

Figure 1: The mean incidences of canker, top and crown dieback, resin exudation, foliage symptoms and insect damage within sampling plots (M1-M10) and in the whole sampling area (M) in Aydıncık, as well as the mean diameter and height of the trees (the bars on height and diameter lines indicate the standard deviations).

Top dieback, crown dieback and resin exudation were significantly correlated with canker formations ($p<0.001$). Top dieback co-occurred with crown dieback ($P<0.001$) and resin exudation ($p<0.005$). Insect damage, in turn, was associated with the resin exudation and foliage symptoms ($p<0.005$) (Table 4).

Table 4: Correlations between tree sizes and symptoms and insect damage

	Tree features		Symptom incidences					
	Diameter	Height	Canker	Top Dieback	Crown Dieback	Resin Exudation	Foliage symptoms	Insect Damage
Diameter	1,000							
Height	0,697*	1,000						
Canker	-0,564	-0,164	1,000					
Top Dieback	-0,529	-0,164	0,766**	1,000				
Crown Dieback	-0,359	-0,006	0,802**	0,957**	1,000			
Resin Exudation	-0,474	-0,012	0,936**	0,756*	0,829**	1,000		
Foliage symptoms	-0,744*	-0,256	0,433	0,624	0,480	0,544	1,000	
Insect Damage	-0,530	-0,079	0,616	0,544	0,569	0,752*	0,702*	1,000

*Significant $p < 0.05$; **significant $p < 0.01$.

Totally 146 cankers were sampled from 83 trees for fungal isolation. As a result of this isolations, 497 fungal isolates, representing 30 genera, were obtained from the 1390 sections of canker tissues placed on PDA plates. The most common

species isolated from both Köprülü Kanyon and Aydıncık were; *P.* cf. *occulta* (22 and 44 %), unidentified coelomycete (6 and 22%), *Alternaria* spp. (13 and 5 %), *Cladosporium* spp. (5 and 5%), *Cytospora* sp. (2 and 4%) and *P. funerea* (37 and 1%), respectively. While *P.* cf. *occulta* was the most common species in Aydıncık, *P. funerea* - which was rare in Aydıncık - was the most frequently isolated species in Köprülü Kanyon. Fungi isolated from both sampling area agrees slightly with those stated in previous studies (Munoz and Ruperez, 1980; Ducrey et al., 1999; Madar et al., 1991; Gonthier and Nicolotti, 2002; Bajo et al., 2008). Moreover, *P.* cf. *occulta*, *P. funerea* and *Cytospora* sp. are reported to be pathogenic on cypress, especially under stress conditions. However, neither *S. cardinale* nor other cypress canker related *Seiridium* species was recovered in this study. Nor were there well–known canker causing fungal pathogens of cypress, such as *B. theobromae* and *D. pinea* f.sp. *cupressi*, among the isolates. This indicates that either the pathogens were missing from the studied stands or replaced by other fungi in the sampled cankers. On the other hand, as the study areas were large, a sampling strategy focusing in sampling only the fresh cankers could have given different results. Nevertheless, the absence of e.g. *S. cardinale* in the study areas may not indicate that the native populations would be highly resistant against the pathogen, since the previously tested Turkish provenances (including Köprülü Kanyon –Zerk–, but not Aydıncık) tended to have only intermediate resistance against *S. cardinale* (Santini and Di Lonardo, 2000). The absence may be due to other factors including geographical barriers (Santini and Di Lonardo, 2000).

In Köprülü Kanyon, *P. funerea* was isolated nearly from all cankers sampled. This species is endemic in Europe and is also present in the native areas of cypress, and therefore considered to have co-evolved with *C. sempervirens* (Santini and Di Lonardo, 2000). It is considered a weak pathogen of a wide range of conifer hosts including species in the following genera: *Cupressus*, *Pinus*, *Juniperus* and *Thuja* (Madar et al., 1991; Sinclair et al., 1993; Santamaria et al., 2007). Moreover, it is also thought to be capable of replacing other pathogens such as *S. cardinale* (Sanches and Gibbs, 1995). During the survey in Köprülü Kanyon fruiting structures on plant tissues were not noticed, however in Aydıncık, there were unripe fruiting structures on the plants. Ecology of *Cytospora* sp., *P. funerea* and *P.* cf. *occulta* could differ between the two study areas, however, the differences could have been caused by the different sampling times or climate as well.

In conclusion, canker incidence in natural *C. sempervirens* var. *horizontalis* stands in southern Turkey was relatively high. To our knowledge, all obtained fungal species, except *P. funerea,* are new records on cypress in Turkey. None of the fungi is reported to be an aggressive pathogen, but could be harmful under stress conditions.

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SOME MORPHOLOGICAL ASPECTS OF EUTYPELLA CANKER OF MAPLE (*Eutypella parasitica*)

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ABSTRACT

Eutypella canker of maple originates from North America and was recently reported from Slovenia, Austria and Croatia. Disease distribution and frequency in the surveyed forest stands in Slovenia, Eutypella canker shape and its extent on the trunk, fungi present in discolored wood, perithecia density, and ascospore discharge were explored. Diseased maples were usually grouped into infection centers and the disease occurred on 3–5% of all maple trees at surveyed forest stands. However, incidence up to 30% was recorded. The canker was usually oval shaped and the average area of the canker was 48% of the affected part of the trunk. Canker width measured about a half (0.44) of canker length. 54.8% of all isolates (2,276) from discolored wood were identified as *Eutypella parasitica.* Perithecia covered an average of 32% of the total cankered area. A good correlation existed between the area with perithecia and the whole cankered area. In average, 647,000 perithecia per canker were found. The average discharge was 506,000 ascospores cm^{-2} h^{-1}. One Eutypella canker discharged from 65 million to 3.3 billion ascospores h^{-1}, with an average of 1.0 billion ascospores $hour^{-1}$ under favorable environmental conditions. The inoculation potential of the fungus is enormous but its rapid colonization of European forests is prevented by ineffective mode of transmission and slow development of the disease.

Keywords: shape, area, perithecia density, ascospore discharge, frequency, *Acer*

1. INTRODUCTION

Eutypella canker of maple caused by the fungus *Eutypella parasitica* R. W. Davidson & R. C. Lorenz is a well-known disease in North America in the area around the Great Lakes where it was first found and described (Davidson and Lorenz, 1938). In Slovenia and Europe, the disease was not reported until 2005 although it seems to have been present for some time prior to this (Jurc et al., 2006). The disease was also reported from Austria (Cech, 2007) and Croatia (Ogris et al., 2008). The means of the disease introduction into Slovenia is not known. The hosts of the disease are maples (*Acer* spp.). When a disease is introduced to new location, new hosts can emerge. Similar scenario was observed with Eutypella canker, when field maple (*A. campestre* L.) has been found to be a new host of *E. parasitica* (Ogris et al., 2005). About 35% of known cankered trees in Slovenia are field maples. The most susceptible maple in Slovenia is sycamore maple (*A.*

pseudoplatanus L., 54% of cankered trees), while only 3.5% trees with Eutypella canker are Norway maple (*A. platanoides* L.). In North America, the disease is a common perennial canker on sugar maple (*Acer saccharum* Marsh.), red maple (*A. rubrum* L.), infrequently occuring on boxelder (*A. negundo* L.), Norway maple, silver maple (*A. saccharinum* L.), black maple (*A. nigrum* Mich.), sycamore maple, striped maple (*A. pennsylvanicum* L.), and bigleaf maple (*A. macrophyllum* Prush.) (Davidson and Lorenz, 1938; French, 1969; Kliejunas and Kuntz, 1972; 1974).

One of the reasons for performing additional research on Eutypella canker was the outcome of a spread risk assessment of the disease for Europe (Ogris et al., 2006). 13% (1,404,000 km^2) of Europe's land area was found to be at very high risk for *E. parasitica* due to favorable host range and suitable climatic conditions. Regions with very high spread risk extends in the Balkans (Slovenia, Bosnia and Herzegovina, Serbia and Montenegro, Croatia), Southern Europe (some parts of the Apennines, the central part of the Pyrenees), Central Europe (all parts of Austria except the eastern part, the whole Czech Republic, northern and southern parts of Slovakia, central and southern part of Germany, almost all of Poland except the northeastern part), Western Europe (northern half of Switzerland, eastern part of France), and Eastern Europe (some parts of Moldova, eastern region of Ukraine, Caucasus). The sycamore maple, field maple, and Norway maple are predicted to be the most endangered.

The aim of this work was to explore the morphological characteristics of the Eutypella canker that may explain the as yet unrevealed capacity of the disease to spread. This work represents a supplement to the current knowledge of the disease. Parts of the study are tests of validity that have been stated in different papers earlier but not yet tested; some hypotheses are checked again. This work supplies information about Eutypella canker shape, area, fungi present in discolored wood, perithecia density, ascospore discharge, the disease distribution tendencies and frequency in the stand.

2. MATERIALS AND METHODS

2.1. Description of the sites

Samples were taken from six locations. (1) Rožnik hill is an urban forest just 1 km from the center of Ljubljana, the capital city of Slovenia. Rožnik hill rises about 130 m above Ljubljana (429 m above sea level) and covers over 380 hectares. For analysis, 16 diseased trees (13 sycamore maples, 3 field maples) were taken from three infection centers. (2) A single specimen on sycamore maple was collected at Topol at Medvode (650 m a.s.l.); this is one of the westernmost locations in Slovenia where Eutypella canker has been found and is 9 km far from Ljubljana. The other four sites are located in the eastern part of Slovenia. (3) Jelški hill (310 m a.s.l.) is 60 km east from Site 1 and is located near Sevnica rising over the Sava River. Two specimens on sycamore maple were found at this location and both trees were cut down and included in the analysis. (4) The fourth site is 7 km

from Site 3 and is near to Sevnica. It is located on Kremenc hill (350 m a.s.l.) where two specimens of Eutypella canker on sycamore maple were found and collected on shady side of the hill. (5) A single diseased sycamore maple was found at Bohor hill (580 m a.s.l.), near the town of Kozje, which is located 22 km east of site 4. (6) Site 6 is located at Rogaška Slatina (230 m a.s.l.) where 55 Eutypella cankers were found – all on field maple. This site is the most eastern place where Eutypella canker of maple was found in Slovenia. It is located about 1 km from the border with Croatia. At Site 6 one field maple was gathered. Distance from Site 1 is 90 km. Altogether, of the 23 diseased trees collected, 19 trees were sycamore maple and 4 trees were field maple. Sites 1–5 had rich, rather moist soils in common which suits the sycamore maple's demands for water, while Site 6 parent material was sandstone, soils were quite warm and dry, and location was on sunny side of the hill.

Site 1 and 6 were chosen to determine the frequency of Eutypella canker of maple in forest stands.

2.2. Common measurable characteristics

Some basics measurements were made for all specimens collected. (1) The position of canker on trunk was measured from the ground to the center of the canker which is usually represented by a dead branch stub (Davidson and Lorenz, 1938). When the centre of the canker was within arm's reach the position was determined by tape measure, otherwise a Haglöf Sweden Vertex III height measurer was used. (2) The diameter of trees at breast height was measured by log calliper. (3) Canker circumference was determined by tape measure at the centre of the canker. (4) Canker length was determined by tape measure.

2.3. Area and shape of the canker on the trunk

The canker area was measured and calculated for all 23 specimens collected. Area measurement was performed by fastening a transparent plastic foil to canker by pins and drawing the outline of the canker using permanent marker. Three different densities of perithecia, bark without perithecia, and areas where bark had already fallen off were also marked on the foil. The foil was then put down on a flat surface with white paper for background and the contours were digitalized. Each digital photo was taken with a 10 cm long scale bar to allow for the correct calibration of the photos. The areas were calculated using Soft Imaging System analySIS® Pro function for measuring arbitrary areas. The areas of different densities of perithecia were used later for calculating ascospore discharge at the level of the whole canker.

2.4. Isolates

Dissectional study enabled us to determine the places where the fungus actually lives and is active. It also enabled us to search for other species of fungi present in the canker. Trunk cross sections were taken at 10 cm intervals. The isolations were made as described below. Samples were taken within a period of 24 hours after

dissecting the trunk into discs. Samples of discolored or decayed wood were taken from each second disc from eight cankered trunks. One trunk was analyzed in detail and samples were taken from each disc. At least three samples of healthy wood (showing no discoloration) from each trunk were taken as a control. When taking samples, attention was given to the margin of decayed wood, the various colors of decayed wood, the different phases of decay, and, in some cases, to the dried zone of wood observed around the margin of the decayed wood. The samples were taken with a boring machine with a wood borer of 12 mm diameter. Between each boring the borer was sterilized by flaming with 96% (v/v) ethanol and was then cooled down by dipping it in cool distilled water for few seconds. First, a 1–3 mm deep hole was made to eliminate possible contaminants and after flame sterilization of the borer the sample for fungal isolation was taken. The boring chips were collected in sterile Petri dishes. Up to five samples were taken per disc. From each sample four isolations on 2% (w/v) malt extract agar (MEA) were made with two repetitions. The sample was represented by a boring chip 2–3 mm × 2–3 mm in size. Altogether, 2276 isolations were made. Pure cultures were made of every fungus growing from the sample. Cultures were incubated at 24°C for 4 weeks.

2.5. Density of perithecia and ascospore discharge

Three different densities of perithecia were distinguished when the area of the Eutypella canker was measured. This enabled an assessment of ascospore production of the canker in a more accurate way. Three different densities of perithecia were then sampled. Each sample was checked for the maturity of its perithecia before it was used in the test. The maturity test was performed on the bark area just next to the area of perithecia that was taken into the test of ascospore discharge. Mature perithecia are full of dark brown ascospores (Davidson and Lorenz, 1938), young perithecia are white inside, and old perithecia are empty when moistened. After the maturity test, the samples were cut to approximately 1 cm^2. The exact area of the sample was determined after the test using analySIS software. Because the samples had been air-dried they had to be immersed in water for at least 30 min (Johnson and Kuntz, 1979). We immersed the samples in distilled water for 1 h, and then the bark samples with perithecia were put on moist filter paper for 3 hours, which is necessary for ascospore discharge to begin (Lachance, 1971; Johnson and Kuntz, 1979). The sample with underlaying moist filter paper was fixed to a rubber stopper with thin needle. Ascospores were discharged into test tubes with a 2 cm diameter to which 2 ml 0.01% (v/v) detergent Tween® 80 solution had been added. Ascospores were allowed to discharge for 3 hours at room temperature about 22°C. Ascospores were counted using a Bürker-Türk counting chamber. The calculation of the number of ascospores discharged per cm^{-2} h^{-1} was corrected using the exact area of the sample. The samples were also histologically examined and the number and maturity of the perithecia were determined.

3. RESULTS

3.1. Incidence of the disease in forest stands

Site 1 had the greatest number of diseased trees, altogether 70 trees. Eutypella cankers usually form infection centers, while single occurrences are rarely found (Kliejunas and Kuntz, 1974; Martinez, 2003) since ascospores are disseminated only over short distances (Johnson and Kuntz, 1979). There were four infection centers and six single trees distributed around the hill. A full inventory of one infection centre at Site 1 was performed. All trees were identified and measured for their diameter at breast height (DBH) within an area of 2.7 hectares. In the searched area, 20 tree species were determined. The most common species was the sycamore maple (41.7% of all trees), followed by the big leaf linden (*Tilia platyphyllos* Scop.) with 15.3%, the common hornbeam (*Carpinus betulus* L.) with 10.6%, and the northern red oak (*Quercus rubra* L.) with 9.7%. Among 606 sycamore maples in the 2.7 hectare area, 3.3% were diseased by *E. parasitica*. This is within the range of the usual incidence of Eutypella canker (Gross, 1984b).

At Site 6 on the area of 7.2 ha, frequency of Eutypella canker was determined. 28.6% of 192 field maples had canker.

3.2. Common measurable characteristics

Table 1 shows a summary of the statistics for four variables. The sample size was different for each variable measured. Eutypella cankers were found over the entire DBH range from thin to large, as previously reported by French (1969). The average trunk diameter at breast height was 25 cm. The relative frequency of cankered trees among 7 classes showed that 52% of diseased trees had a DBH between 11 and 22 cm.

65% of all trees with Eutypella canker occurred on first 220 cm of trunk above the ground line, and 92% of all cankers occurred on the first 435 cm of trunk. These results are comparable to French (1969), who noted that 60% of all cankers occurred on the first 244 cm of trunk and 81.5% on the first 488 cm of trunk.

33 cankers were measured for canker length. 85% of cankers did not exceed a length of 120 cm. Gross (1984a) measured 27 trees and the range of canker length was from 10 cm to 170 cm with an average of 67 cm. Wide range of canker length was directly related to the age of the canker and could be explained as such.

Table 1: Summary statistics for common measurable characteristics of Eutypella canker

	Trunk DBH (cm)	Vertical distribution (cm)	Length of canker (cm)	Width of canker (cm)
Sample size	38	37	33	31
Minimum	11	35	30	23
Maximum	61	1150	233	115
Average	25	216	102	59
95% confidence interval (±)	3.6	73.9	18.6	9.5

Canker width was correlated to canker length, which was due to canker shape, since the majority of cankers were oval shaped. Regression analysis with a linear model (canker width = 14.6 + 0.44 × canker length) gave correlation coefficient of 0.87. Coefficient 0.44 tells that canker width measured usually about a half of canker length.

Possible entry points for *E. parasitica* are branch stubs and bark wounds (French, 1969). Great majority of cankers were traced to branch stubs as the avenue of entry. Only 3 out of 89 cankers were associated with bark wounds and all three wounds resulted from logging operations.

3.3. Area and shape of canker on the trunk

The largest Eutypella canker measured 1.7 m^2, although the average was much lower, i.e. 0.5 m^2 (Table 2). The canker area varied a lot because it depended upon the canker age. The cankered area ranged from 4% to 90% of the trunk area with the canker, with an average of 48%.

The area without bark also depended upon the canker age and thus the whole canker area. In order to determine if in fact there was a relationship between the canker area without bark and whole canker area, regression analysis using a linear model was carried out. The linear model described a relationship between canker area without bark and whole canker area. The equation of the model was: area without bark = –2.32 + 0.25 × whole canker area. The high correlation coefficient (0.83) showed a good linear relationship between the canker area without bark and whole canker area. The coefficient of the equation tells that the area without bark represented usually a quarter of the whole canker area when the canker was old enough to bark fall off. The bark begins to fall off a trunk due to high degree of decay or/and high activity of bark beetles.

Perithecia do not usually develop nearer than the fourth or fifth annual callus ring from the margin of the cankers (Davidson and Lorenz, 1938). Therefore, Davidson and Lorenz (1938) stated that perithecia are confined to only a small central portion of large cankers. During this study, their hypothesis about the area size covered with perithecia was tested. The part of their hypothesis was rejected. It was confirmed that perithecia are usually located in the central portion of the canker but this area is not small. The total area of bark covered with perithecia could exceed 68% of the whole canker area. An average of 32% (±7% at 95% confidence level) of the canker area was covered with perithecia. Further analysis was performed and it was found that the correlation between the total area of perithecia and the area of the whole canker was very good. Simple regression analysis produced a correlation coefficient of 0.94. A simple regression was made using a linear model: total area of perithecia = –3.93 + 0.42 × whole canker area. Three different densities of perithecia were delineated. The high densities of perithecia were usually located nearer to the canker centre while low densities were located nearer to canker margin. It should be noted that a middle density of perithecia could hardly be distinguished from high density. Therefore, some

portion of the middle density of perithecia was included in the high density. It was suggested that about one-third of the total area covered with perithecia belonged to low density class, one third to middle density, and one third to high density class.

20 out of 23 cankers were oval shaped, two cankers were triangular, and only one was shaped irregularly. All triangular shaped cankers had the centre of the canker close to the ground line. The irregular shape of the canker area has been already observed and explained (Davidson and Lorenz, 1938). The annual extension of the cankered areas is usually uniform and therefore the shape of canker area is usually oval. However, this extension is occasionally stopped suddenly by some unknown cause, around either the entire margin or only a portion of it. Such sudden cessation of the extension gives the canker an irregular shape. The extension of the canker is faster in the longitudinal direction than in the transverse direction. This leads to the elongated oval shape of canker area. Cankers could be from 1.32 to 2.62 longer than they are wide while the average length/width proportion was 1.74 (± 0.18 at 95% confidence level).

Within the canker shape analysis it was observed that the trunk gradually grows thicker up to canker centre and then the trunk thins down. The thickness of the canker was measured for 14 specimens at 10 cm intervals. It was determined that the thickness of the Eutypella canker was represented by a parabola very well. Polynomial regression using a second order polynomial model was performed for each of the 14 specimens. The R^2 was up to 97.8% and the average R^2 was 76.7%. This shows again that the canker shape is a regular oval.

Table 2: Summary statistics for different kinds of areas of Eutypella cankers

Canker area (dm^2)	Whole	Without bark	Perithecia (averages)			
			low density	middle density	high density	Total
Minimum	12.3	0	0	0	0	0
Maximum	168.0	43.7	14.9	16.6	45.8	64.7
Mean	53.3	11.3	6.5	3.9*	10.9	20.5
95% confidence interval (±)	20.6	6.3	2.2	1.8	7.3	9.2

*Middle density of perithecia could hardly be distinguished from high density. Therefore, some portion of the middle density of perithecia was included in the high density.

3.4. Isolates

Davidson and Lorenz (1938) stated that a single species of *Eutypella* has been consistently isolated from discolored wood from under the centre and from near the margins of the discoloration. Our study strongly supports this statement. From 9 dissected trees, 97 discs and 237 boring holes, 1896 samples were cultivated on

MEA for possible fungal presence. The most frequent fungus isolated was *E. parasitica* which represented 54.8% of all isolates. The second most frequent organisms isolated from discolored wood were bacteria (27.6% of all isolates). 17.6% of all isolates were represented by 25 macroscopically different species of fungi among which were *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Mucor circinelloides* Tiegh., *Umbelopsis vinacea* (Dixon-Stew.) Arx, *Armillaria* spp., *Nectria* spp., *Pestalotia* spp., *Fusarium* spp., and *Pythium* spp.

The controls were made by cultivating 74 boring chips that were taken from healthy wood. The result of this test was that 65 of the isolations were sterile and only 9 Petri dishes were populated by bacteria and three different genera of fungi. No *E. parasitica* was present in controls.

Dried healthy wood was present quite often at the center of the canker or near the margin of canker. 64 isolations were made for testing for the presence of *E. parasitica* in this area of the wood. After a month of incubation almost all isolations were negative and only seven had bacteria.

As part of testing for the presence of *E. parasitica* in different parts of the wood, 420 isolates were made from the canker margin in the wood. *E. parasitica* was also the dominant fungus here (58.6%). The second most frequently found were bacteria. 23.6% of isolates contained eight macroscopically different fungi. 17.9% of samples were sterile.

3.5. Growth in pure culture

Average radial growth in pure culture at room temperature of about 25°C is reported to be about 20 mm in seven days (Davidson and Lorenz, 1938). We measured the growth rate of 45 pure cultures on 1.5% (w/v) MEA at 24°C; 1712 measurements were made. The average radial growth per day was 2.4 mm (± 0.1 mm at 95% confidence level). The slowest measured average radial growth was 0.9 mm per day and the fastest was 5.6 mm per day.

3.6. Density of perithecia and ascospore discharge

When measuring the area of the canker, three different densities of perithecia were determined (Table 2). The low density perithecia ranged from 181 to 210 perithecia cm^{-2} with an average of 196 perithecia cm^{-2}. The middle density ranged from 234 to 269 perithecia cm^{-2} with an average of 255 perithecia cm^{-2}. The high density ranged from 301 to 378 perithecia cm^{-2} with an average of 348 perithecia cm^{-2}. These average densities of perithecia were used for calculating the number of perithecia at the whole canker level and the total ascospore production per canker. The total number of perithecia per whole canker could be from 25,000 for young cankers to over 2,102,000 perithecia for old cankers. The average number of peritecia per canker was 647,000 for the 23 Eutypella cankers analyzed. It should be noted that not all of these perithecia discharged ascospores because some of them were still developing, while others were too old and empty. The maturity test was performed on those samples that were used in the study of ascospore

discharge. Maturity of perithecia varied substantially. There were some samples that were 100% old and empty, then there were some samples with only young perithecia incapable of discharging the ascospores, and there were whole range of samples that had a mixture of young, mature and old perithecia. Generally, the maturity tests showed that areas of canker with a lower density of perithecia have more young perithecia, while those with higher density have more old perithecia.

Because the maturity test showed that there was no strict rule of maturity of perithecia among different densities, the results of ascospore discharge could not be shown by low, middle, and high class densities of perithecia but only as average value. The range of ascospore discharge was from 360,000 to 718,000 ascospores cm^{-2} h^{-1} while the average discharge was 506,000 ascospores cm^{-2} h^{-1}. These results are comparable to the results of Lachance (1971) and Johnson and Kuntz (1979) who reported 450,000 ascospores cm^{-2} h^{-1}.

On average, a perithecium discharged 2520 ascospores per hour i.e. 315 octads. The average ascospore discharge (506,000 ascospores cm^{-2} h^{-1}) was used for calculating the total ascospore discharge of cankers which represents the potential of disease spread. One Eutypella canker could discharge from 65 million to 3.3 billion ascospores per hour with an average 1.0 billion ascospores per hour under favorable environmental conditions. This kind of ascospore production represents enormous inoculation potential.

4. DISCUSSION

This research pointed out some of morphological aspects of Eutypella canker of maple that had not been analyzed before or were hypothesized and not checked, while others are checked again. A total of 23 Eutypella cankers were analyzed in detail. This is the required minimum for reliable statistics and it is comparable to other dissectional studies (Gross, 1984a).

While ascospore discharge of Eutypella canker can be enormous under favorable environmental conditions, the question is raised as to why the disease is not more frequent. Specific demands of the fungus for successful colonization are the main reason for generally low occurrence of Eutypella canker. The fungus needs an exposed xylem for successful infection, i.e. branch stubs or bark wounds. Since the branch stubs represent a small area and ascospores are disseminated only over short distances (25 m), it is hard for infection to take place despite enormous ascospore production. There are some other environmental limitations. Ascospore discharge is greatest at temperatures between 24 and 28 °C (Lachance, 1971; Johnson and Kuntz, 1979). Laboratory tests show no ascospore discharge and dissemination at temperatures below 4 °C or higher than 36 °C. Free moisture (rainfall) induces mature perithecia to discharge the ascospores. At least 3 mm of rain has to penetrate the tree canopy to initiate discharge (Lachance, 1971; Johnson and Kuntz, 1979). Spore ejection begins about 2 hours after rain has started. High relative humidity alone is not sufficient to induce discharge of spores but it can influence the rate of drying of bark on cankers and prolongs discharge after periods of rainfall (Johnson and Kuntz, 1979).

The findings of the study show some morphological characteristics of Eutypella canker that help in understanding the disease spread. The very high average ascospore discharge of 506,000 ascospores cm^{-2} h^{-1}, or 1.0 billion ascospores h^{-1} for the whole canker area is important for the success of disease spread. The study of the isolates showed that *E. parasitica* is the dominant fungus in infected and decayed wood. The fungus has proven its pathogenic ability with progressive attack tactics where the maple rarely has a chance to protect itself with callus or wound-wood. The Eutypella canker grows faster in longitudinal direction that in transverse direction. Therefore, the canker shape is in most cases almost regular or elongated oval. When the maple succeeds in defending itself from the fungus by forming wound-wood, the fungus can reinfect the wound-wood and bark from the underlaying wood. When the host is killed or snapped due to canker progress, the fungus acts as a saprobe and continues to produce ascospores for some years. The disadvantages of the fungus are its heavy ascospore octads which are disseminated only over short distances and the fact that they can infect only exposed xylem that occurs up to 4 m from the ground level.

When a disease is introduced to a new distant location, e.g. another continent, it is hard to predict disease behavior and its spread speed. Environmental conditions could be more favorable for the disease and thus it could accelerate its spread rate. In a new environment new vectors of the disease could be present which could also add to the success of disease spread. A third factor that could help the disease to spread and establish are hosts. In a new environment new hosts are possible and host distribution can be over a wider area and contiguous. A new host, i.e. the field maple, was found and described in Slovenia (Ogris et al., 2005), but there are numerous other possible hosts (*Acer* spp.) present in Europe that have not yet been identified. There are at least 10 autochthonous maple species in Europe that could be the target of *E. parasitica* (Ogris et al., 2006). An inoculation study should be planned and carried out to test possible susceptibility. The results of that study would give needed information for more accurate assessment of the disease spread risk.

Eutypella canker has been recognized as a new disease of maple in Europe. Despite intensive searching by the Slovenian Forestry Service only 225 Eutypella cankers were found from 2005–2009. We suspect that there are many undetected cases of Eutypella canker. Since it is assumed that cankers are present for a longer time period and they are dispersed over wide area we do not believe that the eradication of the disease is possible (Jurc et al., 2005). The disease can also have an economical impact. Incidence of the canker in North America is usually less than 5% (Gross, 1984b). Stands with over 10% of maples cankered were fairly common, and instances of over 40% cankering have been observed; in Slovenia there was a case with almost 29 % incidence. The disease frequently kills trees less than 7.5 cm in diameter and larger trees are predisposed to wind snap at the canker (French, 1969; Kliejunas and Kuntz, 1974). Cankered trees lose up to 50% of their merchantable cubic volume because most of the cankers occur below a height of 3

m, which represents the most valuable portion of trunk (Gross, 1984b). Control measures are proposed within the framework of sanitation felling procedures. Part of the trunk with a canker at least 40 cm below and above the canker margin should be removed from the forest and destroyed by fire; when left in forest, the canker should be turned with face towards ground.

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OCCURRENCE OF *Pseudomonas syringae* ON POPLAR DAMAGED BY NECROSIS AND CANKER

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ABSTRACT

In poplar and aspen clones, established in a collection with more than 250 different genotypes, in nursery practice as well as in stock plant cultures necrosis and canker symptoms have appeared at 1 – 3 year-old shoots for some years. The injuries occur in winter season are characterized by ring necrosis at shoots, canker and cracking. The most frequent bacteria type that was isolated from damaged clones was identified as *Pseudomonas syringae* by means of 16S rDNA analysis. The pathogenicity of the isolates was evident at cuttings of eight several poplar clones tested under greenhouse conditions. The deleterious effect was visible at young shoots with symptoms of discoloration and leaf spots as well as blackening and wilting of buds and young shoots. The eight poplar genotypes differently responded to bacterial infection.

Keywords: *Populus*, stem necroses, canker, *Pseudomonas syringae*

1. INTRODUCTION

Populus species are widely distributed and abundant in many different environments. A broad range of species and hybrids can be found as nursery crops grown for biomass production in short rotation coppice plantations and for reforestation and restoration purposes. In nurseries, plant material of the most poplar species is normally propagated with hardwood cuttings whereas aspen may be propagated from seeds.

In poplar and aspen clones, established in a collection with more than 250 different genotypes, in nursery practice as well as in stock plant cultures necrosis and canker symptoms had appeared at 1 – 3 year-old shoots in North-Eastern Germany for some years. The injuries were characterized by ring-shaped necroses with constrictions of the shoot, canker formations and spontaneous cracking. The extend of disease symptoms like necroses formation and shoot cracking increased during the winter season and disrupted in early spring. This pattern was different from the known damages caused by the more common pathogens like *Aplanobacter populi* and *Cryptodiaporthe populea*.

The frequency of necroses formation was changing depending on from the poplar genotype. At some susceptible genotypes, single shoots showed up to 10 necroses. Some shoots with lesions that did not crack, dried above the damaged spots.

Above and below the smaller necroses and lesions a wound periderm had developed, varying in thickness with the poplar genotype (fig. 1-3). Inside the shoots, tissue showed a hypertrophic ring-shaped zone with dark discolorations.

2. METHODS AND RESULTS

2.1. Bacteria identification

During the search for the cause of losses, no indication for fungal pathogens or phytophagous insects could be found. Rather, investigations of damaged and broken shoots resulted in a range of bacterial strains, which were isolated directly from the margin of necrotic tissues and discoloured zones in winter season. The isolations followed a standard protocol using Yeast extract-Mannitol Agar (YMA) (Elkan and Bunn, 1992). The plates were incubated at 28 °C for three days. The most frequent type could be identified by means of 16S rDNA sequence analysis (primer pairs: fd1/ 926r and 799f/1492r, resp.) as *Pseudomonas syringae* pv. *syringae*. This species could be found only in the damaged annular areas with inner brown discolorations. The shoot segments between the lesions were free of *P. syringae*. Further species like *Rahnella* sp., *Pantoea agglomerans, Pseudoclavibacter helvolus*, *Cellulomonas* sp., and *Microbacterium oxydans* were found with lower frequency. Only one strain could be isolated from *Xanthomonas* sp. and *X. campestris*, resp., *Curtobacterium flaccumfaciens,* as well as some unspecified isolates.

Figure 1, 2: Ring-shaped necrosis with wound periderm (left) and cracking of shoot (right)

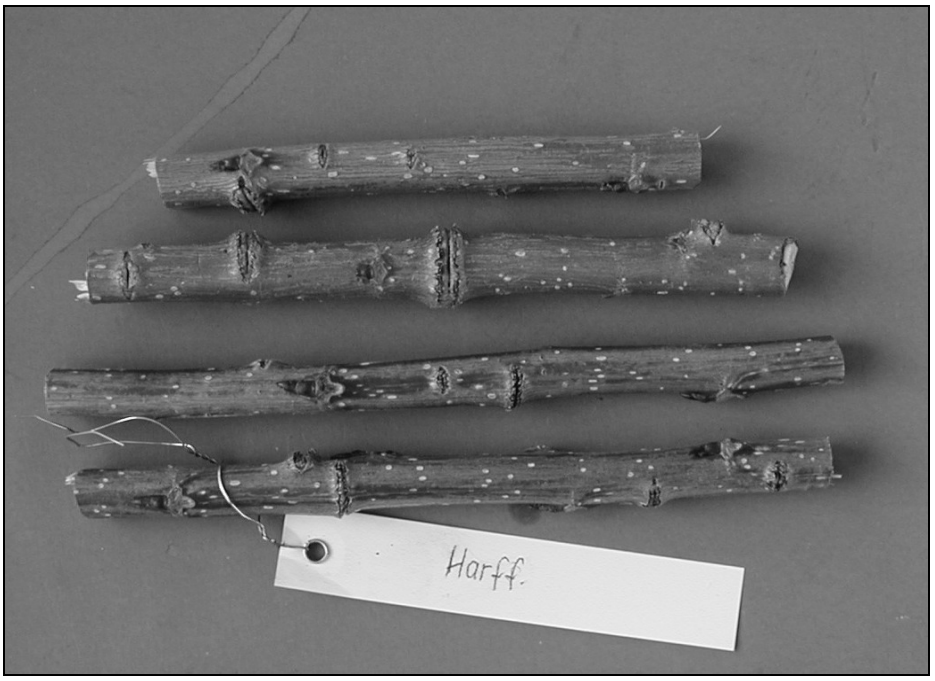

Figure 3: Cuttings with small necroses

2.2. Pathogenicity testing

The pathogenicity of two strains of *P. syringae* was analysed at cuttings of eight different poplar clones tested at 20-21°C under greenhouse conditions. The cuttings that were intact from outward appearance with a length for 12 – 15 cm were harvested after a frost period in winter, and were grown with illumination (35-60 µE $m^{-2}s^{-1}$) in beakers with water for 8 days. After bud break, the cuttings were inoculated with bacterial suspension (10^8 cfu/ml) for two days, plugged into moist sand, and cultivated for further five weeks at 20-22°C with additional light of 4 h (180-200 µE $m^{-2}s^{-1}$) in greenhouse. The deleterious effect caused by the bacteria was assessed by counting the cuttings with symptoms weekly. Symptoms that were visible on young leaves and shoots had started with discoloration and leaf spots after 3 weeks and resulted in blackening and wilting of buds and young shoots. In general, 37.8 % and 40.9 % of the cuttings (n=88 and 87, resp.), treated with two different *P. syringae* strains developed severe symptoms on young shoots (table 1). Most of the shoots of affected cuttings died off within the observation time of five weeks, even though the eight poplar genotypes responded to bacterial infection quite differently. The *P. maximowiczii* hybrids as well as the aspen clone were more susceptible than the other clones tested. Within the observation time, some single control cuttings of three poplar genotypes (4 % of total) died off by unknown causes.

Table 1: Pathogenicity of *Pseudomonas syringae* strains on poplar clone cuttings

Species / Hybrid	Clone	Cuttings with visible damages (%)		
		P. syringae strain 47	*P. syringae* strain 48	control
Populus trichocarpa	30	21 (14)[1]	0 (11)	0 (13)
Populus trichocarpa	872	33 (9)	25 (12)	0 (9)
Populus trichocarpa	936	27 (11)	45 (11)	0 (10)
Populus trichocarpa x *P. deltoides*	995	29 (12)	18 (11)	0 (10)
Populus tremula x *P. tremuloides*	L 211	56 (16)	79 (14)	8 (13)
Populus deltoides x *P. maximowiczii*	960	56 (9)	67 (9)	13 (8)
Populus maximowiczii x *P. berolinensis*	Geneva	37 (8)	33 (9)	0 (9)
Populus maximowiczii x *P. nigra*	Rochester	44 (9)	60 (10)	11 (9)

[1] in brackets: number of cuttings used for average

The limited number of cuttings as well as the fact that some cuttings did not root resulted in the different number of replications per clone and treatment. Only those cuttings were finally assessed regarding bacteria sensitivity, which showed a sufficient root system. The inadequate rooting ability of the poplar clones can could have induced water stress resulting in similar wilting symptoms like the disease symptoms.

3. DISCUSSION AND CONCLUSION

P. syringae is a known pathogen on woody plants, not only in poplars, but also in ash, willow, cherry, horse chestnut, and others. In recent time, to *P. syringae* more than 50 pathovars were assigned. On woody plants, the pathogen causes bark necroses with slime flux and canker. The newly-discovered pathovar of horse-chestnut, *P. syringae* pv. *aesculi* can act as a cambium destroyer with severe dieback (Stobbe et al., 2008; Kaminski et al., 2007).

In poplars, the pathogen played only a secondary role for a long time because poplar cultures were less important for timber industry in Germany. In other countries like New Zealand, Sweden, and Romania, observations of *P. syringae* were made on poplars for a long time (Spiers, 1990; Ramstedt et al., 1994; Mocanu & Poleac, 1965). The pattern of damages that was described were spotted leaves, wilting of leaves and shoots in the whole, as well as bark necroses. In current investigations in a Chinese poplar breeding program *P. syringae* pv. *syringae* was identified as the reason for injuries like bark necroses on cuttings of various hybrid clones (Xiang et al., 2001). Moreover, this species could be found in symptomless hybrid clones of different poplar sections (Ulrich et al., 2008).

The disease transmission of the pathogen is unclear until now. Possibly mechanical bark wounds induced by insects and other animal vectors are responsible, considerably earlier in summer. The invasion of bark tissue is also possible by bark micro-cracks following weather conditions like summer drought or autumn frost. The propagation of the pathogen takes place within a large temperature range and can still be found slightly above zero. Sequent changing of warm and cold periods with freeze stress could have promoted the infection by the pathogen and their distribution within the tissue. The formation of necroses in the cold season can be caused by pathovars belonging to ice nucleation inducing strains of *P. syringae*. Those pathotypes are able to induce the formation of ice crystals within plant cells downward from -1°C. As a result, the cell walls burst and the protoplasm can serve as nutrient resources for further bacteria growth. Moreover, some pathovars of the *P. syringae* group are known as phytotoxine producers. Analysed compounds are the lipodepsipeptides Syringomycin and Syringopeptin, possessing a cell wall destroying influence (Hutchinson and Gross, 1997).

In recent time, poplars became more important as a fast growing tree species for biomass production in short rotation coppice plantations, and a high demand for suited propagation material exists. A basic precondition of utilizing poplar for this purpose is the provision of proved propagation material with high quality and free of harmful organisms. The use of cuttings grown in infected stock plant cultures can lead to a fast distribution of the disease. It is difficult to appreciate a possible role of *P. syringae* as a serious pathogen for poplar cultures in Germany. Therefore, work on epidemiology, vectors, and host-specific association of various pathovars is recommended as well as research referring to resistance / susceptibility of clones and cultivars of economical importance.

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Rust Diseases

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SEASONAL FRUITING AND SPORULATION OF *THEKOPSORA* AND *CHRYSOMYXA* CONE RUSTS IN NORWAY SPRUCE CONES AND ALTERNATE HOSTS IN FINLAND

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ABSTRACT

Seasonal fruiting and sporulation of cone rusts were investigated in Norway spruce cones and alternate hosts in 2006-2008. Current-year and one-year-old cones and leaves of alternate hosts, *Pyrola* spp. and *Orthilia secunda*, were collected from case-stands in southern and northern Finland bi-monthly or monthly and checked for rust fruitbodies. Fruitbodies and different fruiting structures of the rusts were examined using light microscopy (LM), and field emission scanning electron microscope (FESEM).

Spermogonia of *Chrysomyxa pirolata* and aecia of *Thekopsora areolata* developed in current-year cones in June, while *C. pirolata* aecia developed and began to sporulate in July. *Thekopsora areolata* aecia sporulated mainly in previous year's cones in May-August. Uredinia, telia and basidia of *C. pirolata* developed in overwintered *Pyrola* spp. and *Orthilia secunda* leaves in May, and sporulated in May-June. Uredinia of *T. areolata* developed in current-year *P. padus* leaves in June and sporulated in June-August. Telia of *T. areolata* developed in late summer, but no basidia developed in overwintered *P. padus* leaves in March-May.

Keywords: Cone rusts, *Thekopsora areolata*, *Chrysomyxa pirolata*, sporulation

1. INTRODUCTION

Good quality seed crops of *Picea abies* (L.) Karst. are irregular due to insects and pathogens reducing both the amount and quality of seed crop in seed orchards and natural forests throughout Finland (Kangas, 1940; Rummukainen, 1960; Nikula and Jalkanen, 1990; Tillman-Sutela et al., 2004)). *Thekopsora areolata* (Fr.) Magnus, and *Chrysomyxa pirolata* Wint., cause severe damage on *Picea* spp. throughout the northern hemisphere (Savile, 1950; Gäumann, 1959; Roll-Hansen, 1965; Ziller, 1974). *Thekopsora areolata* infects *Prunus* spp. (Gäumann, 1959), while *C. pirolata* infects species in genera *Pyrola*, *Moneses* and *Orthilia* (Savile, 1950; Gäumann, 1959; Ziller, 1974). Uredinia and telia develop on alternate hosts after aeciospore infection.

In 2006, florescence and cone crop of Norway spruce were abundant, but due to fungal injuries the seed crop was severely reduced in some seed orchards. The aim of this study was to collect information of rust sporulation after a serious rust outbreak to improve disease control. For a thorough description of the study, see Kaitera et al. (2009).

2. MATERIAL AND METHODS

Norway spruce cones were collected in a seed orchard (Stand 1) in southern Finland, and in a naturally regenerated stand (Stand 2) in northern Finland. A random sample of previous year's (2006) and current-year (2007) cones, were collected in Stand 1 in 5 times in 2007. Similar sampling was performed in Stand 2 for the current-year cones in 5 times in 2007 and for the previous year's cones in 8 intervals in 2006-2007. The cones were cut from sample trees either using branch scissors (Stand 1) or a lifting cage (Stand 2).

Overwintered and current-year leaves of *Prunus padus*, *Pyrola* sp. and *Orthilia secunda* were collected along the cone sampling in May-October 2007 in Stand 1, and 7-19 times in 2007-8 in Stand 2. In a third group of seed orchards (Stand 3) in southern Finland, leaves of *P. padus* and *Pyrola* sp. were collected in 8 times in 2007. Overwintered *P. padus* leaves were collected in March-May and current-year leaves were collected between early May and early October.

The occurrence, incidence and distribution of rust fruitbodies (spermogonia, aecia), and proportion of sporulating fruitbodies were recorded in cones under stereo microscope. Leaves of alternate hosts were investigated for the occurrence, incidence and stage of sporulation of rust uredinia, telia and basidia per leaf. Rust fruiting stages were also selected and further studied using a JEOL JSM 6300F field emission scanning electron microscope (FESEM).

3. RESULTS

3.1. *Rust incidence and sporulation in current-year cones*

In Stand 1, cones collected in late May to mid-June bore no rust fruitbodies. In late June, about 2 % of the sample cones carried *C. pirolata* spermogonia and 5 % carried immature *T. areolata* aecia. None of the aecia were sporulating. The *T. areolata* aecia located on both sides of cone scales along the entire cone. No fungal structures resembling *T. areolata* spermogonia were observed. In the early August, 7 % of the sample cones bore *C. pirolata* aecia that located on outer (abaxial) side of scales, being currently sporulating or already had finished sporulating and ruptured. Two percent of the sample cones carried *T. areolata* aecia that occurred in all the scales of cones. None of the *T. areolata* aecia sporulated yet. In the early October, 2 % of the cones carried both *T. areolata* and *C. pirolata* aecia in cone scales. *Chrysomyxa pirolata* aecia were ruptured with only individual aeciospores within aecia. *Thekopsora areolata* aecia were immature and non-sporulating.

3.2. *Rust incidence and sporulation in previous years' cones*

3.2.1. Stand 1

Only *T. areolata* aecia were observed in previous year's cones. In late May, 35 % of the sample cones bore *T. areolata* aecia with 92 % of the cone scales being

infected. All observed aecia located on both sides of cone scales with 96 % of the aecia per cone being currently sporulating or had finished sporulating being ruptured. From the mid-June to early October, 22 % - 45 % of the sample cones carried *T. areolata* aecia. All infected cones carried aecia on both sides of cone scales with 93 % - 100 % of the cone scales per cone carrying aecia.

3.2.2. Stand 2

In cones collected in October 2006, 90 % carried *T. areolata* aecia and 10 % carried *C. pirolata* aecia. Aecia of *T. areolata* were non-sporulating and located in both sides of cone scales with 94 % of the scales per cone being infected. *Chrysomyxa pirolata* aecia had all finished sporulating, locating in 77 % of the infected cones on the outer side of cone scales.

In late March in 2007, 90 % of the scales carried *T. areolata* aecia on both sides of the scales in previous year's cones that were non-sporulating. About 2 % of these cones carried already sporulated and ruptured *C. pirolata* aecia on outer side of the scales with 70 % of the scales carrying aecia per infected cone. In the early May of 2007, 43 % of the sample cones carried *T. areolata* aecia and 7 % carried *C. pirolata* aecia .

From late May to late June, 95 % of the sample cones bore *T. areolata* aecia and 15 % bore *C. pirolata* aecia. Aecia of *T. areolata* occurred on both sides of cone scales in most of the cones with 86 % - 93 % of the scales per cone carrying fruitbodies. All *C. pirolata* aecia had finished sporulating.

In late July and early October, 92 % - 94 % of the sample cones carried *T. areolata* aecia, and 0 % - 19 % of them carried *C. pirolata* aecia. The average proportions of cone scales bearing *T. areolata* aecia were 89 % - 95 % per cone. On average, 18 % - 23 % of the scales per cone carried sporulating *T. areolata* aecia.

3.3. *Rust incidence and sporulation on alternate hosts*

3.3.1. Stand 1

In late May 2007, 1 % of the overwintered *O. secunda* leaves bore *C. pirolata* uredinia and undifferentiated fruitbodies. Undifferentiated fruitbodies were common on *O. secunda* in late June, but after that they became rare. Uredinia were common between mid-June and early August, after which they became rare. No sporulation was observed in these uredinia until early August in 2007, after which uredinia finished sporulating and ruptured. None of the current-year leaves of *P. padus* bore any *T. areolata* fruitbodies in late May, but practically all leaves carried uredinia from mid-June on and telia without basidia since late-June.

3.3.2. Stand 2

In late March 2007, none of the overwintered *O. secunda* and *Pyrola* sp. leaves carried *C. pirolata* fruitbodies. Undifferentiated fruitbodies occurred first in early

May, but they were most common between mid-May and late May in 2007-8. Uredinia appeared in mid-May, being most frequent in late May with low coverage per infected leaf (2 % - 10 %) in 2007. Uredinia were, however, most frequent fruitbodies in 2008 with their coverage ranging between 47 % - 83 %. Uredinia started to sporulate immediately after their formation and continued throughout the sample collection. Telia of *C. pirolata* were the most frequent fruitbodies in 2007, but they were less common than uredinia in 2008. Telia with basidiospores were observed throughout the collection, but they were most common between late May and mid-June in 2007 and between early and mid-June in 2008. In 2007 telia began to rupture from late July on.

Practically all overwintered *P. padus* leaves bore *T. areolata* telia without external basidia between late March and late May in 2007-8. Uredinia of *T. areolata* occurred on the lower leaf surface with less frequency than telia on overwintered *P. padus* leaves. Uredinia started to sporulate immediately after formation in early June, and they began to rupture in early July. Until mid-September, all uredinia had finished sporulating.

3.3.3. Stand 3

In late April in 2007, no fruitbodies of *C. pirolata* occurred on the overwintered leaves of *O. secunda* or *Pyrola* sp. Undifferentiated fruitbodies occurred on all such leaves with the highest coverage of 70 % per leaf in mid-May, after which their coverage decreased. First *C. pirolata* uredinia were observed with a low coverage per infected leaf in mid-May. Thereafter, uredinia occurred in increasing frequency until early October with a coverage of uredinia per leaf lower than 40 %. Sporulation of uredinia started in mid-May and continued until early October. Since early July the proportion of uredinia that had finished sporulating increased, and after late July uredinia ruptured.

Telia were the most common fruitbodies of *C. pirolata* in 2007. The first telia appeared in late May in almost all infected leaves with high (87 %) coverage per infected leaf. Telia with basidia were frequent until mid-June, when they began to rupture. Ruptured telia were common from early July to early October.

Almost all of the overwintered *P. padus* leaves carried *T. areolata* telia without basidia in late April. Sporulated and ruptured uredinia occurred on 38 % of the current-year sample leaves with a low (6 %) coverage per infected leaf. A few uredinia occurred in 17 % of the infected leaves, which all sporulated in late May. From mid-May until early October, almost all *P. padus* leaves carried sporulating *T. areolata* uredinia, and the proportion of uredinia that had finished sporulation increased after early July. Telia were observed in all infected leaves between early July and early October without external basidia.

4. DISCUSSION

In this study, *T. areolata* and *C. pirolata*, which are causes for severe rust epidemics in Finland, fruited and sporulated in young current-year cones the next year after a serious rust outbreak. The high incidence of *T. areolata* aecia in previous year's cones confirmed that it was the main cause for the serious rust outbreak in the study areas in 2006. In all stands, *T. areolata* uredinia and telia were frequent on both overwintered and current-year *P. padus* leaves indicating that alternate host infection took frequently place both in 2006-2007. The high disease incidence coincided with the incidence of aecia in previous year's cones, too. In this study, *T. areolata* aecia sporulated mainly in one-year-old cones as reported (Jørstad, 1925), but single sporulating aecia could be found already in late summer after infection. The observed aeciospore and urediniospore size and morphology was in accordance with the reports elsewhere (Gäumann, 1959; Saho and Takahashi, 1970). As practically all scales in infected cones bore *T. areolata* aecia, the rust was highly pathogenic and systemic in the cones and hindered efficiently seed formation.

The occurrence and morphology of spermogonia, spermatia, aecia, aeciospores, uredinia, urediniospores, telia and basidia of *C. pirolata* corresponded well to earlier reports (Gäumann, 1959; Sutherland et al., 1984; Crane and Hiratsuka, 2000). As most scales in infected cones were covered by *C. pirolata* aecia, the rust was highly pathogenic and systemic in cones. Therefore, the rust had a great impact on cone development causing seed deformation in diseased cones. The gelatinuous young fruiting structures, undifferentiated fruitbodies, corresponded to those described previously (Crane and Hiratsuka, 2000), and reported to develop either into uredinia or telia depending on the amount of moisture and free water.

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PRELIMINARY STUDIES ON GENETIC VARIATION IN *Gymnosporangium fuscum* in the LAKES DISTRICT OF TURKEY DETECTED WITH M13 MINISATELLITE MARKER

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ABSTRACT

Gymnosporangium fuscum infections on the trunk and branches of *Juniperus excelsa* are common in natural stands in the Lakes District of Turkey. In the present study, level of genetic variation among *G. fuscum* isolates was estimated. Telial horns were obtained from trunk lesions in Sütçüler, Bucak-Aziziye and Beşkonak sites. From each telium DNA was extracted by using plant mini kit. PCR amplification profiles were run using the M13 minisatellite core sequence. Preliminary results indicated low variation among the isolates.

Key words: European pear rust, Turkey, Minisatellite, M13, genetic variation

1. INTRODUCTION

Crimean juniper (*Juniperus excelsa* Bieb) contributes to 5.6 % of forest area in Turkey. It grows on dry rocky slopes of hills and mountains at elevations ranging from 150 to 2700 m above sea level, and often forms the tree line in the Taurus Mountains. Crimean juniper has been under certain level of protection since 1996 when all silvicultural treatments in the juniper forests were ceased due to the bad condition of the stands (Güner et al., 2000).

European pear rust is caused by the fungus *Gymnosporangium fuscum* DC. like rusts in general, it alternates between two hosts: *J. excelsa* and *Prunus* spp. The infections are perennial on the coniferous host, on which in spring it develops the characteristic telial horns.

European pear rust is widely distributed throughout Europe with observations (including) extending to Asia Minor (Lebanon, Syria and Turkey) and North Africa (Algeria and Morocco). The pathogen has also been introduced to North America (California, Washington, and British Columbia) probably through the importation of junipers from Europe (Laundon, 1977; Hollebone, 2006). In Turkey, perennial lesions caused by the rust are common on Crimean juniper in the Lakes district (Doğmuş-Lehtijärvi et al., 2008).

Minisatellite sequences are repeated over the genome and exist in most organisms providing an obtainable and highly variable probe and PCR amplification (Karlsson, 1993; Högberg et al., 1995). In most cases they have been used to study variation among populations.

In our study, we used M13 minisatellite core sequence as a primer in PCR based DNA fingerprinting technique to study level of genetic variation among *G. fuscum* populations.

2. MATERIAL AND METHODS

Telial horns were collected from trunk lesions in Sütçüler, Bucak-Aziziye and Beşkonak sites. In Sütçüler three stands 3-8 km apart were sampled. The stands in Beşkonak and Bucak-Aziziye were 40 and 70 km west from the Sütçüler stands, respectively.

In each stand telia horns were sampled from ten trees, placed into plastic bags, and stored in the laboratory at -20 ºC until DNA extraction.

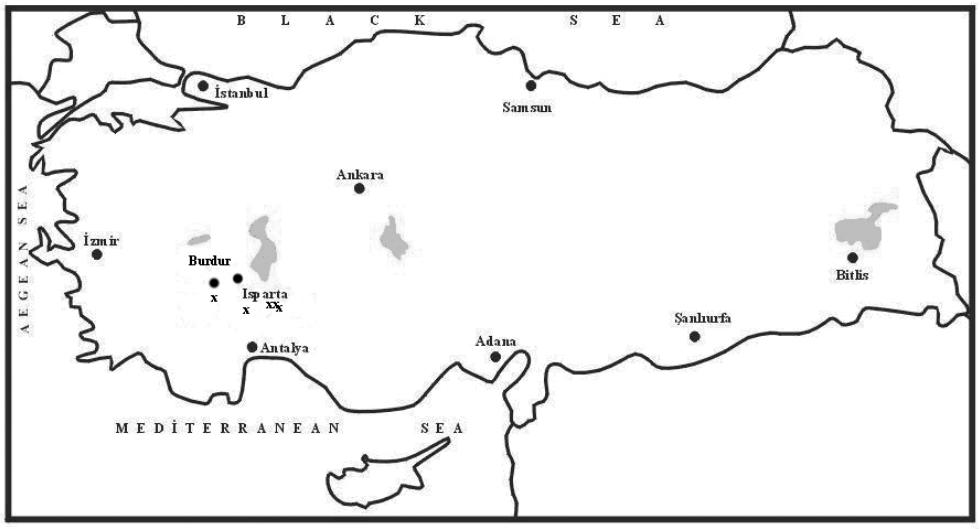

Figure 1. Locations of the sampled stands (crosses).

2.1. DNA extraction and PCR amplification

Telia were grounded using liquid nitrogen and DNA was extracted with Qiagen DNeasy Plant Mini Kit. PCR amplification profiles were obtained using M13 minisatellite core sequence as a primer. Reactions were performed in volumes of 50 µl containing Tris-HCl 10 mM, pH 8; dNTPs 0.2 mM; $MgSO_4$ 2 mM; Tsg polymerase 1,25 U; primer (M13) 2 µM; approximately 10 ng of template DNA. PCR was conducted using a hot start step at 95°C for 10 min, followed by 45 cycles at 95°C for 1 min, 48°C for 30 s, 72°C for 2 min and a final extension at 72°C for 10 min.

2.2. Analyzes of amplification products

Amplification products were separated by electrophoresis (90 V for 120 min) in 1.5 % agarose gels in TAE buffer and length of the products were determined by using 100 bp DNA Ladder. The presence or absence of amplification products was scored to analyze the variation among the populations.

3. RESULTS

The amplifications yielded a total of 18 markers ranging from 300 to 1200 bp in size. The amplification profiles were very similar for most of the *Gymnosporangium* telial horns; the M13 primer seemed not detect any significant level of variation within 50 samples.

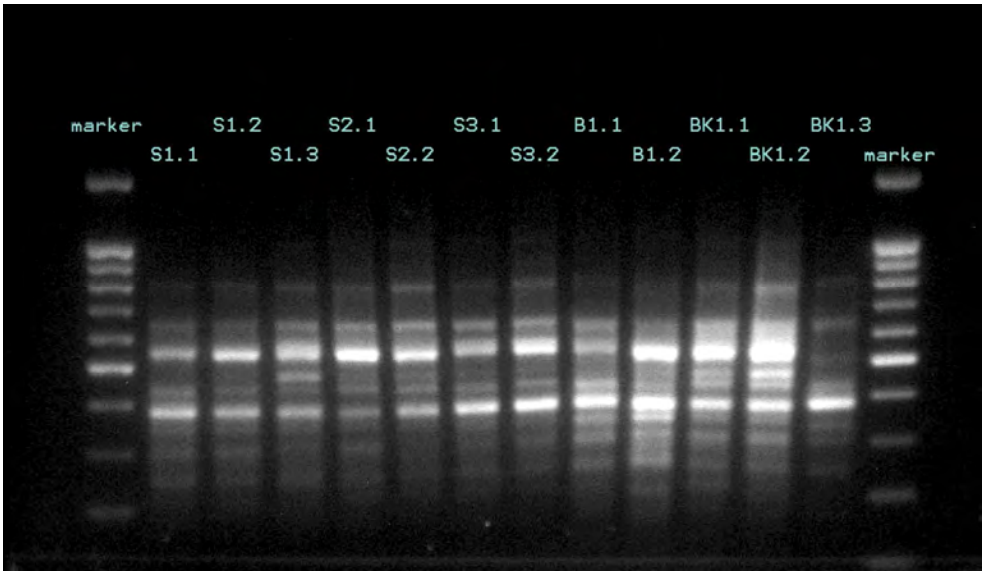

Figure 2: Amplification products of *G. fuscum* isolates from different sites.

4. DISCUSSION

The very low level of variation detected by M13 amplifications was surprising. Within its 2- year life cycle *G. fuscum* produces relatively limited number of genetically identical spores. Each aecidium on pear is initiated by a single basidiospore and therefore could be expected to be (at least in practise) genetically different from other aecidia. As the fungus lacks an uredinial stage on pear the number of infections on pear caused by single genets is not multiplied. As a result, genetic variation among aecidiospores infecting junipers should be high. As new infections on juniper occur annually, one could expect to find a high level of genetic variation among *G. fuscum* isolates on that host.

As the results are preliminary we can not exclude the possibility of non-optimal conditions in the PCR reactions. However, the amplicon profiles looked normal without signs of e.g. abnormally long fragments. Therefore other explanations are more likely.

Arbitrarily primed PCR using M 13 core sequence as a primer detects variation in several of species of fungi growing on forest trees including such as; basidiomycetes *Heterobasidion annosum* s.l. (Fr) Bref. (Hantula et al., 1996; Stenlid et al., 1993)and *Fomitopsis pinicola* (Schwarts: Fr) Karst. (Högberg et al., 1995), ascomycetes *Ophiostoma ulmi* (Buism), *Ceratocystis fimbriata* f.sp. *platani* (Santini et al., 2000), and *Nectria fuckeliana* C. Booth (Vasiliauskas and Stenlid, 1997) and *Sphaeropsis sapinea* Dyko& Sutton (Xiaoqin et al., 2007) among fungi imperfecti. Among biotrophs the primer has been used successfully for powdery mildews (ascomycete) but to our knowledge not for smuts and rusts (basidiomycetes). It is possible that annealing sites for the M13 primer within *G. fuscum* genome are few. Another reason for the low variation could be that the distances between the sampled stands were short.

Table 1. Presence and absence vector of amplification products.

Isolates	330bp	360bp	380bp	390bp	400bp	450bp	540bp	550bp	590bp	650bp	660bp	680bp	710bp	810bp	870bp	910bp	950bp	1200bp
S1.1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
S1.2	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
S1.3	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
St2.1	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	1	1	1
St2.2	1	1	0	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0
St2.3	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
St2.6	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
B3.1	0	1	0	0	0	0	1	1	0	1	1	1	0	0	1	0	0	1
B3.2	0	1	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	1
B3.3	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
B3.4	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
B4.4	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1
Be4.5	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1
Be4.6	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0	0
Be5.8	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
Be5.9	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
Be5.10	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0

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FACTORS FAVOURING BROOM RUST INFECTION IN ADVANCE PLANTINGS OF *Abies alba* IN SW-GERMANY

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ABSTRACT

For ecological reasons, even aged stands of Norway spruce (*Picea abies*) are going to be converted to mixed forests, including silver fir *(Abies alba)* and other native tree species. In order to avoid clear cuttings, the alternative tree species are often introduced into the spruce stands by advance plantings. However, after about two decades of planting, several stands of planted silver fir turned out to be severely infected by *Melampsorella caryophyllacearum*. If the stems are affected, wood quality is deteriorated by burl formation and even more by the risk of secondary infection by wood decay fungi like *Phellinus hartigii* which makes the stems break in timber age.

A study was performed in order to quantify the disease incidence in advance plantings of silver fir under Norway spruce by using circular sample plots. Brooms respective cankers formed after *Melampsorella*-infections in branches and in stems were counted separately. Amongst others the stand structure and the presence of caryophyllaceous host plants (mainly *Stellaria nemorum*) bearing the dicaryotic phase of the rust fungus in the neighbourhood of the plots were recorded. The presence of alternate host plants turned out to be the most crucial factor for disease incidence, whereas, there was no evidence that geology, altitude, or stand density could play a major role. Since *Stellaria* plants were mainly found along forest roads or in logging lines, it is recommended that silver fir not be planted right beside such places.

1. INTRODUCTION

More than 100.000 ha (7.6 %) of the forest area of the SW-German land Baden-Wuerttemberg is covered by silver fir *Abies alba* MILL. This is by far the biggest area of this tree species in Germany. In order to achieve a more nature-oriented forest, the state forest administration is going to raise the proportion of *A. alba* to 11% and to diminish monocultures of Norway spruce (*Picea abies)*, presently by far the most dominant forest tree species. In contrast to the latter species, silver fir is neither object of butt rot by *Heterobasidion* spp. nor endangered by severe bark beetle attacks.

In order to avoid clear cuttings, silver fir is often introduced into the spruce stands by advance plantings in small gaps. However, after about two decades of planting, several stands of planted silver fir turned out to be severely infected by *Melampsorella caryophyllacearum* SCHROET. Forest administration of Baden-

Wuerttemberg reports fir broom rust to be economically important on 575 ha (Schroeter et al., 2009).

The increment of the trees is not directly affected by the rust infection (Solla et al., 2006). However, if the stems are affected, wood quality is deteriorated by burl formation and even more by secondary infection by wood decay fungi, primarily *Phellinus hartigii,* which makes the stems break in timber age. Thus, besides the economical damage the rust fungus is driving higher biodiversity in silver fir stands (Holdenrieder, 1994).

We performed a study in order to quantify the disease incidence in advance plantings of silver fir under Norway spruce. In the past, broom rust was designated as a major problem in silver fir forest (Heck, 1894), so it should be elucidated whether this is true under the given circumstances today.

Figure 1. *M. caryophyllacearum*-stem canker (arrows) in two *Abies alba* plants.

Figure 2. Several cankers on the trunk and in branches closed to the trunk of a silver fir.

2. MATERIALS AND METHODS

Eleven forest stands with advance plantings and two stands with natural regeneration of silver fir were selected in the eastern slope of Black Forest in the SW-German districts of Breisgau-Hochschwarzwald, Schwarzwald-Baar, and Tuttlingen. Altitude and geology are listed in Table 1. Age class and density of regeneration and canopy were evaluated according to forest inventory methods (Kramer and Akca, 1995). Three to seven circular sample plots of 25 m² each were placed at random within these stands.

Presence of brooms respective cankers formed in consequence of *Melampsorella*-infections in were counted separately for branches and trunks (Figure 2). Branch cankers which are closer than 10 cm to the trunk are expected to fuse with the stem in a few years and hence were regarded like stem cankers. The presence of caryophyllaceous host plants (mainly *Stellaria nemorum*) bearing the

dicaryotic phase of the rust fungus in the plots and in their neighbourhood were recorded. Silver fir can only be infected by the rusts basidiospores, exclusively originating from the alternate host.

Table 1: List of monitoring plots(a.p.: advance planting; n.r. natural regeneration)

Stand Number	District	Geology	Forest Com-partment	Regenera-tion type	Altitude (m s l)	N plots
1	Schw.-Baar	Braunjura	XI_1	a.p.	808	4
2	Schw.-Baar	Keuper	IV_2	a.p.	739	4
3	Schw.-Baar	Keuper	IV_3	a.p.	737	4
4	Br.-Hschw.	Buntsandstein	III_2	a.p.	1036	5
5	Br.-Hschw.	Muschelkalk	XXV_6	a.p.	732	3
6	Br.-Hschw.	Muschelkalk	XXV_7	a.p.	873	3
7	Br.-Hschw.	Muschelkalk	XXV_9	a.p.	811	4
8	Br.-Hschw.	Muschelkalk	XXVII_1	a.p.	722	3
9	Schw.-Baar	Braunjura	X_6	n.r.	822	7
10	Tuttlingen	Braunjura	Gew.Teilenw.	n.r.	802	4
11	Br.-Hschw.	Granit/Gneis	Urishof	a.p.	1062	4
12	Schw.-Baar	Braunjura	XI_1	a.p.	806	4
13	Br.-Hschw.	Granit/Gneis	Urishof	a.p	1050	4
					Mean **846**	Total **53**

3. RESULTS

Incidence of broom rust in the stands (average of percentage infected firs per plot) stretched from 2.2 till 100 % (Table 2). In five stands, 100% were reached; the mean amount was 63.8%. The percentage of stem cankers was lower (in average by 35.5%) and decreased parallel with the general disease incidence. In seven stands, more than 50% of the trees bared stem cankers (mean 42.4 %).

As alternate host of the rust fungus nearly exclusively *S. nemorum* was recorded. In most cases, it was found numerously at roadsides and in logging lines, in the vicinity of the plots rather than in the plots themselves. This plant species was found much more frequently in stands with higher disease incidence.

Neither density of canopy and young stand nor geological factors nor age class of the plantings respective regenerations showed evidence to be related with the percentage of the diseased silver fir trees. Advance plantings seemed to be more infected than natural regenerations.

Table 2: Characteristics and disease incidence of monitoring plots. Stands are given in the order of descending disease incidence. (I: seedlings; II: saplings, III: young pole stage)

Stand Number	n *A. alba* counted	Density of young stand	Density of canopy	Age Class	Alternate host recorded	Disease Incidence %*	Stem cankers %*
7	45	+++	0	I	++	100	100
13	33	++	0	I	++	100	93
2	27	+++	0	II	+	100	76
11	25	+	0	II	++++	100	67
1	14	++	+	II	++	100	65
12	15	++	+	I - II	++	94	60
5	7	++	0	III	0	83	28
9 n.r.	127	+	++	I - II	++	70	52
3	46	+++	0	I - II	0	39	2
6	14	+++	0	II	+	22	8
8	14	++	0	II	0	14	0
4	16	++	0	II	+	5	0
10 n.r.	304	+++	+++	I	+	2	0
	Total **687**					Mean **63,8**	Mean **42,4**

* mean of plots

4. DISCUSSION

The results show the potential of the rust fungi to infect young plantations of silver fir up to 100 % and in rare cases up to 100 % incidence of stem canker. Thus, *M. caryophyllacearum* may under certain circumstances still be a serious threat for high quality timber growth of *A. alba* under modern silviculture. However, a disease incidence up to 50% of the trees is mostly restricted to harmless branch cankers. To provoke a certain amount of stem cankers, the infection pressure must be higher. This occurs both in natural regeneration as well as in advance plantings, if a close association with an alternate caryophyllaceous host is given.

Data shows that all age classes from seedling stage till pole stage can equally be infected. Roth (1955) found stem cankers between 1.5 and 15.2 m tree height (average 6.1 m) in Swiss fir forest. Heck (1894) found stem cankers between 0.5 and 18.5 m (average 5.0 m) in SW-Germany. Both give evidence that even late pole stage firs can also be infected at the leaders or twigs in the upper crown.

Nicolotti et al. (1994) and Solla and Camarero (2006) stated a positive correlation between disease incidence and shorter distance to rivers. This can be due to air humidity in sites or regions where humidity is a limiting factor for the infection process of the fungus. Furthermore, under German conditions, the main alternate host *S. nemorum* is rather linked with plant associations in sites close to ditches, rivers, or to other moist and nutrient rich sites (e.g. “Stellario-Alnetum”)

than to typical silver fir forest (Oberdorfer, 2001). Consequently, the distance to rivers may be identical with the source of spore dispersal from alternate host plants. Influencing the infection process by opening the canopy to achieve lower air moisture seems to be an option in Mediterranean countries rather than in Black Forest conditions where moisture is hardly a limiting factor.

The silvicultural consequences from this study are considered as follows:
a) Broom rust is a natural phenomenon in silver fir forests. Thus, in close to nature forestry some infections on branches must be taken into consideration.
b) Stem canker incidence up to about 60% in the regeneration can be eliminated during successive regular thinnings. Premature elimination of infected trees (sanitation treatment) is not necessary, since infection from tree to tree does not take place. Furthermore, exaggerative opening of the canopy respective regeneration may promote damage by the aphid *Dreyfusia nordmannianae* (Schröter et al., 2009).
c) Expensive plantings of silver fir should not take place close to road sides, logging lines, or in other disturbed sites where alternate hosts are abundant. These sites should be planted with non-host trees like European beech or Norway spruce.
d) Dense stands may prevent growth of herbaceous alternate hosts.
e) Selective pruning of infected branches may be meaningful, before cankers are going to merge with stems. Too intensive pruning should be avoided since silver fir tends to grow epicormic shoots, which may give entrance to new broom rust infections close to the trunk.

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Foliage Diseases of Hardwood

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NON-NATIVE HOSTS AND CONTROL OF *Rhytisma acerinum* CAUSING TAR SPOT OF MAPLE.

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ABSTRACT

Tar spot of maple is an increasingly common disease in eastern North America. *Rhytisma acerinum*, causing large tar spot, was apparently introduced from Europe, and causes the most extensive problems on introduced maple species such as *Acer platanoides* (Norway maple). However, this pathogen was recently confirmed via molecular methods on native North American species of maple, *A. saccharum* and *A. negundo*. This raises the possibility of pathogenic adaptation to native species that will result in more widespread epidemics. Ascospore production on Norway maple was observed to occur over a relatively short period annually (May 25 - June 22, 2006; May 25 - July 3, 2007; and May 21 - June 13, 2008). The duration of spore dispersal was dependent on the frequency of rainfall from the start of dispersal. Field studies on fungicidal control of tar spot on Norway maple were conducted in summer 2008, using nine chemicals and a water control. All nine chemicals were found to be effective if applied between late May and early June. A single application was sufficient to control disease in summer 2008.

Keywords: disease, fungi, *Acer*, fungicides

1. INTRODUCTION

Tar spot of maple is caused by species of the ascomycete genus *Rhytisma,* and has a worldwide distribution wherever maples are found. Tar spot has been increasing in abundance across Eastern North America in the last 15 years, with leaves of Norway maple (*Acer platanoides*) bearing multiple black spots. There has been relatively little research done on tar spot in North America. The only scientific reports have come from Connecticut (Waterman, 1941) and New York (Hudler et al., 1987; 1998). The most recent peer-reviewed research report is one from New York (Hudler et al., 1998), which found that the fungus *Rhytisma acerinum* is the cause of tar spot on Norway maple, both of which (host and pathogen) are immigrant species, while a native fungal species, *R. americanum*, occurs on the native red and silver maples (*A. rubrum and A. saccharinum*). This is probably the reason that a Norway maple may be heavily infected with tar spot while an adjacent red maple (*A. rubrum*) or silver maple (*A. saccharinum*) may have no spots. The work report here continues from the earlier report (Hsiang and Tian, 2008) which examined the spore dispersal and identity of the fungus on various maples in North America. The purpose of this work was to continue to examine the epidemiology of this disease, by gathering overwintered maple leaves from southern Ontario weekly from March through August 2007 and 2008, and

inspecting the asci for the presence of filiform ascospores, which initiate infections. Another objective of this research was to confirm the genetic identity of the organism causing tar spot on a range of maples in Ontario. And the final objective was to look at fungicidal control of tar spot.

2. METHODS

2.1 Sporulation

After snowmelt, overwintered leaves of Norway maple bearing tar spots caused by *Rhytisma acerinum* were collected from a copse of maples at the Guelph Turfgrass Institute, Guelph, Ontario every week from March through August in 2007 and 2008. Diseased Norway maple leaves were soaked in distilled water for 24 h to allow the apothecia to open, and many spots were examined, with several cross-sections per spot. The percent asci that were empty was estimated. Maple phenology and weather conditions were also recorded at each sampling.

2.2 Genetic Identity

Samples of tar spot from a variety of different maple species were collected from Ontario and Quebec, Canada in 2007 and 2008. We used the Qiagen DNAeasy kit (Qiagen Inc., Mississauga, Ontario, Canada), to extract DNA from these samples. This DNA was then amplified with conserved ITS primers which target the internal transcribed spacer region of ribosomal DNA spanning the 3' end of the 18S gene to the 5' end of the 28S gene. The primer pair, ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were from White et al. (1991). The 12 5 ul reaction mixture for PCR amplification contained the following : 10 ng DNA, 1 DNA polymerase buffer, 0 5 ìm of each primer, and 1 U Tsg DNA polymerase (Biobasic, Scarborough, Ontario, Canada). Amplifications were performed in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA), with an initial denaturation step of 94 for 2 min, followed by 35 cycles of 94 for 30 s, 55 for 1 min, and 72 for 2 min, and a final extension at 72 for 7 min. These PCR reactions were sent for sequencing at the Laboratory Services Division, University of Guelph with both forward and reverse primers. At least two tar spot sequences from each maple species were used for analyses.

2.3 Fungicidal Control

The fungicide trials were conducted at the Guelph Turfgrass Institute, Guelph, Ontario, Canada on 2 m tall plants. These plants had been obtained from a local nursery as bareroot saplings over 2 m tall, and were planted in the local Fox Sandy Loam soil in early May 2007. The trees were placed in four rows adjacent to a older stand of Norway maple trees. There was 1.5 m between the rows and 1.2 m between the trees. Each row was considered a block and consisted of 10 trees.

Treatments were applied to each tree at two-week intervals but to different branches or twigs on each of five dates in 2008: May 6, May 20, June 4, June 17,

June 30. On each date, the number of leaves per twig was assessed, and twigs were sprayed until runoff with each treatment (up to 5 mL per twig, but depending on the number of leaves and leaf sizes). A plastic board was held behind each twig while spraying to prevent overspray onto other twigs. There were nine chemical treatments and a water control (Table 1).

Table 1: Treatments applied to 2 m tall maple trees at the Guelph Turfgrass Institute, Guelph, Ontario, Canada, in Spring 2008 for control of tar spot.

Treatment	Common Name	Product/ L
Water control	water	0 ml
Banner MAXX	propiconazole (15.6%)	0.245 mL
Compass 50WG	trifloxystrobin (0.16%)	0.175 g
Daconil Ultrex	chlorothalonil (82.5%)	1.5 g
Dithane DG	mancozeb (75%)	3 g
Eagle WP (Nova)	myclobutanil (40%)	0.34 g
Heritage WG	azoxystrobin (50%)	0.3 g
Rovral Green GT	iprodione (25%)	10 mL
Senator WP	thiophanate-methyl (70%)	1 g
Sulfur	sulfur (92%)	10 g

The number of spots per leaf was assessed weekly from the start of the trial until the end of June, and then assessed monthly until the end of September. The morphology and phenological state of the maple leaves was also recorded during each assessment.

3. RESULTS AND DISCUSSION

As observed in previous years (Hsiang & Tian 2008), the first symptoms of tar spot on Norway maple in Southern Ontario appeared in late June as small, round, light green, chlorotic spots, 2 mm across. Spots enlarged to 15 mm by mid-August, and developed small black tar-like raised structures on the adaxial surface with a yellow margin. Conidia, which are considered non-infective and possibly spermatizing, appeared as a shiny layer on the black stroma at this time. By early September, the individual spots merged into a circular black spot up to 2 cm across.

Overwintered Norway maple leaves collected in March 2007 and 2008, had stroma, paraphyses and asci (56-80 µm × 8.5-10.6 µm), but no ascospores were visible. By the middle of April, the asci were still undifferentiated, but were found to contain globular vacuoles or bodies. The asci became swollen as spores developed, and filiform ascospores were first observed in early May, averaging 55 × 2.0 µm. By late May, after soaking in water, slits in the hysterothecia (modified

apothecia) on the leaf surface opened, and contained a grayish milky substance. At this time, Norway maples were abundantly producing and shedding pollen, and small samaras were formed, with leaf sizes averaging 10 cm × 15 cm. By late May in 2007 and 2008, a few partially filled or empty asci were observed, with ascospore release through the tips, and paraphyses becoming curled beside asci after spore release. In early June, Norway maple leaves reached their full size (20 cm × 24 cm), and 10% (2007) to 30% (2008) of the asci had fully discharged their spores. By the beginning of July 2008, nearly all the asci were empty and this sporulation period was longer than that observed in 2006 (Hsiang and Tian, 2008) because of much drier conditions. However in 2008, the sporulation period was shortened with full spore release by mid June because of much wetter conditions.

In 2007, we found a few tar spots on trees tentatively identified as sugar maple (*A. saccharum*). We confirmed the identity of these maple trees based on DNA sequencing of a chloroplast gene. The fungal DNA was also sequenced, and it turned out to match *R. acerinum*, the European species. This was very surprising since the European fungal species should not occur on a native North American maple species. The same trees were visited again in 2008, and samples were collected during the growing season. These also yielded DNA confirmed as belonging to *A. acerinum*. In 2008, we also collected specimens of *A. campestre* and *A. negundo* from Ontario, and other samples of *A. saccharum* from Quebec. All specimens were infected with tar spot caused by *R. acerinum* as identified by sequences of the ITS region. This result was not unexpected for *A. campestre* since both the host and pathogen are European species. However, this was another unexpected result for *A negundo*, and *A. saccharum*. The first species is considered a weed, but the second species is the major source of maple syrup. The occurrence of a European tar spot species on North American maple species raises the possibility of pathogenic adaptation to native species that could result in epidemics more widespread than currently seen on Norway maples.

In Hsiang & Tian (2008), we predicted that based on spore production periods of *R. acerinum*, "the practical implication is that fungicide protection against tar spot, if necessary, needs only to be applied during a very short period, which begins near the end of full leaf expansion in Norway maple". We tested this hypothesis in spring 2008 with application of fungicides at different times. We found that a single fungicide application in late May or early June was efficacious in reducing the number of spots per leaf assessed on September 2, 2008, from over 20 to none for many of the fungicide tested. All fungicides were found to significantly suppress tar spot compared to the water control when applied at either of these two times (May 20 or June 4). Applications of fungicides on May 6 or June 30 were not effective in reducing tar spot, while the application on June 17, which was just at the end of the spore production period, was effective for some fungicides but not others. The implication of these results is that if fungicides are used, only a single application between late May and early June is needed for control of tar spot of Norway maple in Southern Ontario, but this may depend on weather and growth, with another application possibly necessary if early June weather is dry.

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BIOLOGICAL CONTROL TRIALS OF BEECH BARK DISEASE UNDER LABORATORY CONDITIONS

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ABSTRACT

Beech bark disease (BBD) causes mortality of American beech (*Fagus grandifolia* Ehrh.). BBD involves an attack by the beech scale insect *Cryptococcus fagisuga* Lind. followed by the native fungal pathogen *Neonectria faginata* (Lohman et al.) Cast. & Rossman. *C. fagisuga* was introduced into Halifax, Nova Scotia, from Europe through seedlings around 1890. Damage to American beech was observed 20 years later. Our objective is to use entomogenous fungi to control the insect. *Lecanicillium muscarium* (Petch) Zare & W. Gams, common in European infested sites, was retained as well as *Beauveria bassiana* (Bals.-Criv.) Vuill. Our first trials were done on non-crawling nymphal stage on bark disks, 24 mm in diameter, kept individually in Solo cups ® at 20°C or 25°C. To expose the insects, the "wool-like" wax covering the colony was removed. The treatment consisted of an application of 125 µL of 10^6 spores/mL of water and oil. A second trial was conducted by spraying spore suspensions of *L. muscarium* (100 µL) on eggs kept at 25°C. Both biological control agents reduced the crawlers' population by 50% after 11 days. Eggs treated with *L. muscarium* showed low mortality, but their development was slowed down. Fungi seen on the surface of the eggs invaded the first instars. Field trials are underway.

Keywords: Beech bark disease, *Cryptococcus fagisuga, Neonectria faginata*, *Lecanicillium muscarium,* entomogenous fungi, *Beauveria bassiana*

1. INTRODUCTION

American beech (*Fagus grandifolia* Ehrh.) forests suffer significant mortality caused by beech bark disease (BBD); this complex disease has a permanent negative impact on the forest ecosystem in North America. A symposium on this important disease was held in New York State to summarize the status of our knowledge on BBD, and to identify the most important knowledge gaps of this phenomenon (Evans et al., 2005). Rapidly spreading across eastern North America, BBD involves a preliminary attack by the beech scale insect *Cryptococcus fagisuga* Lind. and fungal species of the genus *Nectria* (Ehrlich, 1934), now known under the genus *Neonectria* (Figure 1). The insect *C. fagisuga* was introduced into Canada from Europe, around 1890, through European beech (*F. sylvatica* L.) seedlings planted as ornamentals in Halifax, Nova Scotia, Canada.

Damage to our native American beech was first observed 20 years later. The pathogen was thought to be the exotic fungus *Neonectria coccinea* from Europe, but recent taxonomic studies showed that the new species *Neonectria faginata* (=*Nectria coccinea* var. *faginata*) is the pathogen of this BBD complex in North America (Castlebury et al., 2006). Also, *N. ditissima* (=*Nectria galligena*), a pathogen causing cankers on *Acer* spp. and *Betula* spp. in North America, is often involved in the BBD (Houston, 1994).

Figure 1: A- The beech scale insect *Cryptococcus fagisuga* colonizing the bark of the American beech (*Fagus grandifolia*). B- Multiple small cankers caused by *Neonectria faginata*.

The control of BBD is not an easy task. Different approaches have been considered. The selection and propagation of resistant beech is one of them (Koch and Carey, 2005; Loo et al., 2005). Using silviculture to improve the health status of beech populations in newly infested stands (Heyd, 2005) or aftermath forests (Ostrofsky, 2005) is a second approach. Biological control of the scale insect and fungal pathogens is a possibility that has been suggested in the past, but so far only preliminary trials have been reported (Lonsdale, 1983).

In our project, we are testing different entomogenous fungi and hyperparasite fungi to control the insects and the pathogens involved in the BBD complex. The objective of this study is to use entomogenous fungi to reduce the population of *C. fagisuga* under laboratory conditions.

2. MATERIALS AND METHODS

Since the entomogenous fungus *Lecanicillium muscarium* (= *Verticillium lecanii* Vegas) was observed to be common in heavily infested sites in England (Lonsdale, 1983), it was retained in our first experiment on the non-crawling nymphal stage. This instar is a crawling immature but has its stylet fixed in the bark and produces the typical “wool-like” wax. We are using the isolate *L.*

muscarium (Mycotal®). Another fungus, *Beauveria bassiana* (Bals.-Criv.) Vuill. currently being tested against other forest insect pests in our laboratory, has been added to part of this trial.

The first experiment was done on 24 mm diameter bark disks colonized by *C. fagisuga* collected on different trees in the fall and winter. To expose the insect nymphs to the fungus, the white "wool-like" wax was gently removed with fine tools to facilitate observations. The treatment consisted of an application of 125 µL of 10^6 spores/mL of water (added with oil (0.5%) and Tween 80 (0.0025%)). Bark disks were kept individually in Solo cups at 20°C (65% HR) and/or 25°C (85% HR) and photoperiod of 16D:8L. A control was also used with the same formulation but without the spores. Observations were done regularly over a period of two weeks. Table 1 presents the total number of live scale insects observed on the bark disks in each treatment on samples collected in fall and winter.

Table 1: Total number of scale insects used according to treatments and sampling seasons.

Treatment	Sampling season	Number of bark discs	Total number of live non-crawling nymphal stage on bark discs
Control-Oil	Fall	15	88
ycotal-Oil	Fall	11	119
Beauveria-Oil	Fall	7	84
Control-Oil	Winter	12	70
Mycotal-Oil	Winter	17	99

The second experiment was run using a spore suspension of *L. muscarium* (100 µL of 10^6 spores/mL) with the same formulation as the first experiment, sprayed on eggs (61 eggs for the control and 74 for the treatment) and kept at 25°C (85% HR and photoperiod of 16D:8L). Observations were done regularly over a period of three weeks.

3. RESULTS AND DISCUSSION

Both biological control agents reduced the non-crawling nymphal stage population by 50% after 10-11 days, but *L. muscarium* (Mycotal ®) showed slightly better results (Figure 2). Mortality of each female was based on apparent drying symptoms associated with cuticle darkening and hyphal growth on the insect body.

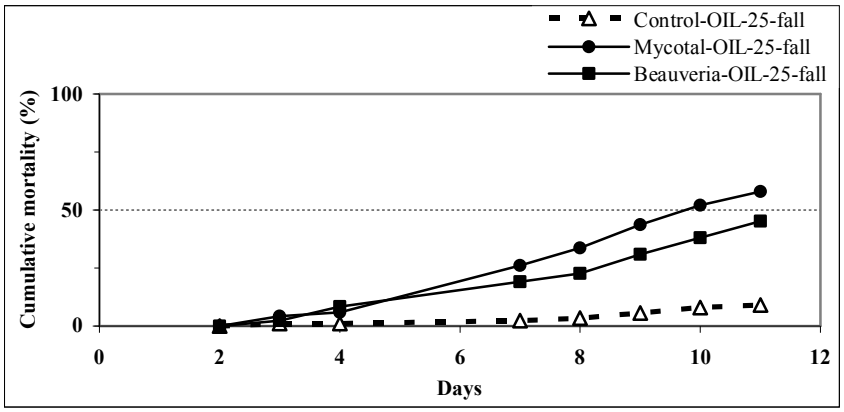

Figure 2: Cumulative mortality of *Cryptococcus fagisuga* exposed to *Lecanicillium muscarium* (Mycotal®), *Beauveria bassiana* and control over a period of 11 days.

A higher mortality level of scale insects treated with *L. muscarium* was observed on samples collected in winter, compared with the fall samples. The winter mortality may have been caused by very cold temperatures (-35°C) measured in February (Environment Canada). Also, the measured mortality rate with *L. muscarium* was increasing much faster, reaching 50% in only 7 days compared with 10 days in the previous fall tests (Figure 3). Our observations are in agreement with Crosby and Bjorkbom (1958), who consider that severe winter temperatures of -35°F (-37°C) or lower are lethal to beech scale insects.

Eggs treated with *L. muscarium* showed very low mortality, but the treatment slowed down their development (Figure 4). Fifty percent hatching was reached after 8 and 12 days respectively for the *L. muscarium* treatment and control. The fungus was observed to colonize only the external surface of the egg chorion. However, when they hatch, first instars become rapidly infected by the fungus already present on the egg chorion.

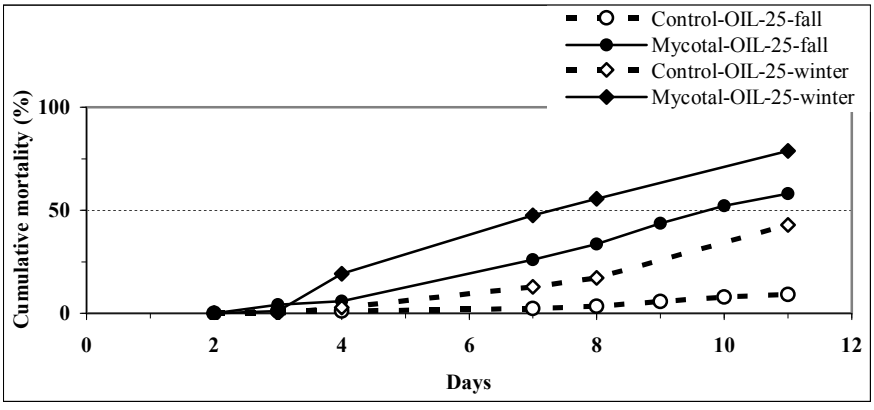

Figure 3: Mortality of scale insects over a period of 11 days from samples collected in the fall and winter, after treatment with *Lecanicillium muscarium* (Mycotal®) and control.

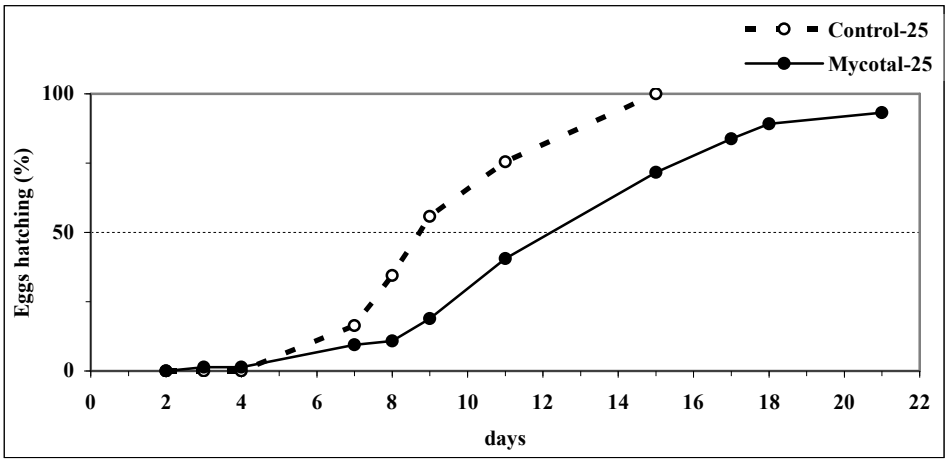

Figure 4: Percentage of *Cryptococcus fagisuga*'s eggs hatching after treatment with *Lecanicillium muscarium* (Mycotal®) over a 21-day period.

4. CONCLUSION

The scale insect *Cryptococcus fagisuga*, exposed to *Lecanicillium muscarium* (Mycotal®) and *Beauveria bassiana* under laboratory conditions, reduced the non-crawling nymphal stage population by 50%. This percentage could have been higher, but the experiment on bark disks cannot last longer than 10 to 12 days; because of the high rate of humidity, fungal hyphae invade the disks making further observations impossible. The fall population treated with entomogenous fungi had a mortality rate reaching 50% in 10 days. Scale insects collected in winter show a higher rate of mortality after biological treatment; the difference with fall is probably the mortality caused by the very cold temperatures during the winter. Finally, if eggs are not directly invaded by fungal hyphae, the young crawlers are rapidly colonized by the fungi soon after hatching (data not shown). Field trials with these biological control agents will be the next step of this study.

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PATHOGENICITY OF *Fusarium circinatum* NIREMBERG & O'DONNELL ON SEEDS AND SEEDLINGS OF RADIATA PINE

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ABSTRACT

Pathogenicity of seven *Fusarium circinatum* isolates from Northern Spain was evaluated on Monterey pine (*Pinus radiata*) seeds and seedlings. The objectives of our study were also to investigate emergence and post-emergence damping-off damage, and to observe differences in pathogenicity among the isolates.

The effect of *F. circinatum* on seed emergence was approximately 10 to 19% lower than the control treatment. However, all *F. circinatum* isolates severely affected pine seedlings, causing 63% to 90% mortality of plants 30-days post inoculation. Sixty days after inoculation, isolate FcCa7 killed all the seedlings, while the less aggressive FcCa2 affected 79% of the plants. We believe this homogeneity in aggressiveness among *Fusarium* isolates may possibly be attributed to the recent introduction of the pathogen in this region.

Key words. Pitch Canker, Biological control, *Pinus radiata*, Nurseries, Plant health care

1. INTRODUCTION

Fusarium circinatum is a pathogenic fungi with great virulence in species of the genus *Pinus*, causing a disease called pitch canker. It was first discovered as a pathogen in California during 1986 (Mccain et al., 1987). Since then, *F. circinatum* was also found in Mexico (Rodriguez, 1989), South Africa (Viljoen et al., 1994; Nirenberg and O'Donnell, 1998; Crous et al., 2000; Steenkamp et al., 2002; Coutinho et al., 2007), Japan (Aoki et al., 2001; Kobayashi, 2007), Chile

(Wingfield et al., 2002; Jacobs et al., 2007), Korea (Cho and Shin, 2004), Italy (Carlucci et al., 2007) and Spain (Landeras et al., 2005; Perez-Sierra et al., 2007).

One of the most important actions to control the disease is to have a better understanding about the populations of the fungus, and it behaviour over plants host. Thus, the aim of this work is to investigate the effect of *F. circinatum* over seeds and seedlings of *Pinus radiata*, and to observe the differences in pathogenicity between isolates.

2. MATERIAL AND METHODS

2.1. Fungal material

Seven different isolates of *Fusarium circinatum* obtained from *Pinus radiata* plantations of the Autonomous Community of Cantabria, in the northern Spain, were used for this study. Malt extract media (20 g/l) was prepared to achieve the spore dissolution of the fungus used in the inoculation (50 ml of autoclaved media in a Erlenmeyer flask). Once esterilized, four pieces of fungal mycelium grown in PDA-S (potato-dextrose-agar with 0.5 g/l of streptomycin sulfate) were placed inside the flasks. Production of spores was induced by using an orbital shaker. After that, the media was filtered in order to collect only spores in the dissolution. In order to obtain the fitted concentration (10^6 spores/ml), we used a Thoma counting chamber.

2.2. Plant material

A total of 672 seeds were sown to observe the effect of the fungus over the plant material. Before sowing them, seeds were washed with sterile distilled water repeatedly and kept there for twelve hours. After that, seeds were maintained in hydrogen peroxide (3%) for 30 minutes. Finally they were washed twice with sterile distilled water to remove the remaining hydrogen peroxide permeating the seeds.

2.3. Substrate. A mixture of peat and vermiculite at 50% were used for the experiment. Before filling the nursery trays, the substrates were autoclaved twice during one hour at 120 ºC.

2.4. Seeds sowing

Seeds were grown in nursery trays, placing four seeds in each hole. Twenty one holes were used for each isolate. After sowing, trays were covered with a transparent plastic paper to prevent the aerial contaminations. The assay was developed in controlled conditions of temperature (20 ºC) and photoperiod (16/8) inside a growth chamber (Figure 1). Seedlings were watered once a week, with twenty millilitres of sterile distilled water, and checked for the progress of the assay.

Figure 1: Growth chamber with the trays inside.

2.5. Data capture

Seed germination was measured once a week. Futhermore dead seedlings were counted ten weeks after the sowing. At the end of the experiment, attempts to reisolate the pathogen from the seedlings were made to verify it presence in the necrotic lesions.

2.6. Statistical analysis

A Kruskal-Wallis test was performed with Statgraphics Plus 5.1. to find differences between germination and mortality taxes of the different isolates.

3. RESULTS AND DISCUSION

3.1. Germination

Despite genus *Fusarium* is considered as one of the most important causes of pre-emergence and post-emergence damping-off (Machon et al., 2006; Pinto et al., 2006), in our present assay fungus did not cause great damages over seeds but, decreasing slightly germination rates. As it can be observed in the Figure 2, CONTROL was the treatment where seeds were more germinated. There were significant differences between this and the others treatments in which *F. circinatum* was present. On the other hand, no differences were observed among the seven isolates of the fungus used in the assay.

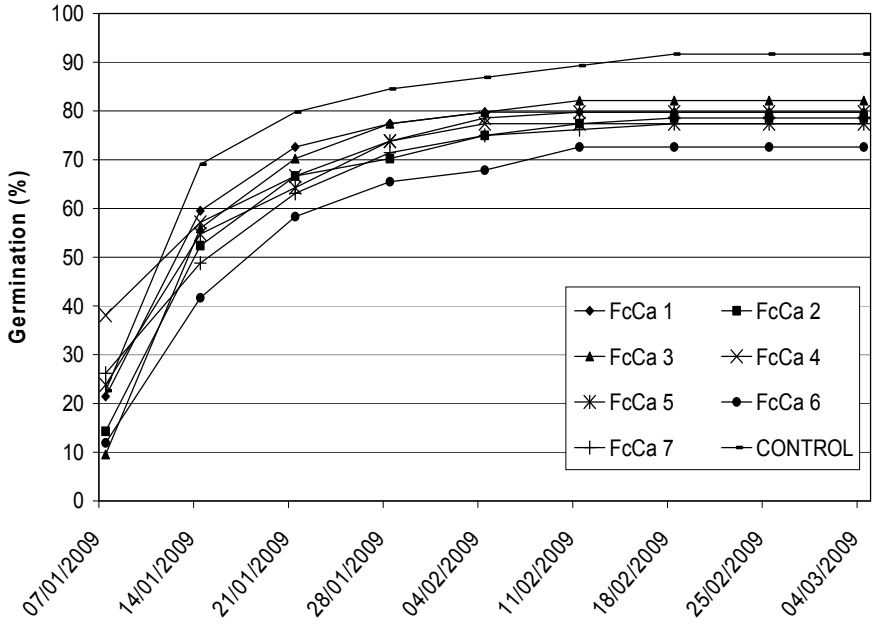

Figure 2: Rates of germination of the seeds depending on the isolate of *Fusarium circinatum* used

3.2. Virulence

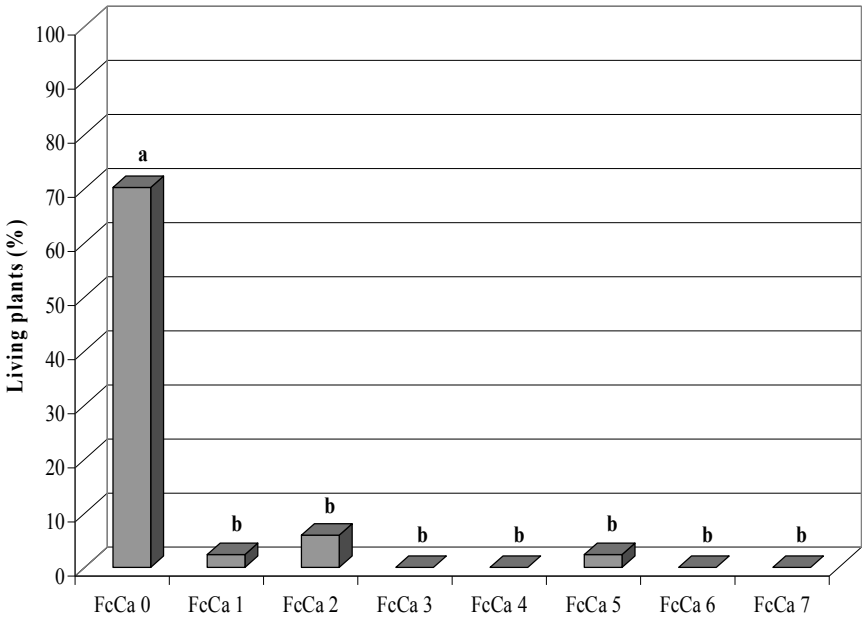

Figure 3: Percentage of living plants ten weeks after the inoculation according to the treatment used

All isolates of *F. circinatum* were highly virulent and significant differences among them were not observed. Aegerter and Gordon (2006) obtained the rates of seedling mortality ranging from 3.5 to 52%, however in our investigation most of the seedlings died after ten weeks of the inoculation. On the other hand, 70% of seedlings were still alive in case of the treatment where the fungus was not present (p-value < 0.001).

4. CONCLUSIONS

1. The effect of *Fusarium circinatum* over seed germination was small, reducing the rate of germinated seeds in between 10 and 20%. No differences were seen in germination among the seven isolates used.

2. Mortality of *Pinus radiata* seedlings inoculated with the *Fusarium circinatum* was high for the seven isolates. Four isolates killed all the *P. radiata* seedlings.

3. No big differences were found in virulence among the seven isolates.

5. ACKNOWLEDGEMENTS

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POWDERY MILDEW ON WOODY PLANTS IN THE CZECH REPUBLIC

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ABSTRACT

There were identified 30 different species of powdery mildew on woody plants in the Czech Republic. From this number of identified *Erysiphales* is *Phyllactinia roboris* reported as a missing species actually. Eleven species of reported powdery mildews can be termed alien for the Czech Republic, including oak powdery mildew *Erysiphe alphitoides* (syn. *Microsphaera alphitoides* Griff.) which is naturalized species throughout Europe now. Newly recorded species are *Erysiphe arguata, E. azaleae, E. elevata, E. flexuosa, Erysiphe palczewskii, Erysiphe syringae, Erysiphe vanbruntiana* var. *sambuci-racemosae. Erysiphe euonymi-japonici* was reported from herbarium specimen in 1941 only.

Keywords: Powdery mildews, *Erysiphales*, Alien species

1. INTRODUCTION

Several important alien diseases of woody plants were introduced in Europe within 20th century. The largest number of newly discovered alien diseases is belonging to the powdery mildew *Erysiphales.* Powdery mildew was observed by Theoprastis on roses 300 year B.C. already. The survey of taxonomy of this group is given eg. by Jaczewski (1927), Blumer (1967), Braun et al. (1978, 1981, 1987, 2006, 2007), Zheng (1985), Gelyuta (1989), Takamatsu et al. (2007) etc. The main area of powdery mildew distribution is temperate zone of Northern Hemisphere. Occurrence in tropics and subtropics is rare, in conidial stage mostly. Some genera of powdery mildews occur also in boreal and arctic areas, eg. in Scandinavia, Northern America, polar areas of Russia, Island and Greenland (Braun, 1995).

Preliminary list of alien disease in the Czech Republic (CR) include more than 30 most important species, including 4 quarantine pests, most of them belonging to *Erysiphales.* The aim of this paper is to evaluate of spectrum of powdery mildews in the CR focused to alien species.

2. MATERIAL AND METHODS

Distribution, identification and pathology of individual species of powdery mildew have been studied on set of 240 examined samples collected on amenity trees and woody plants in parks in the CR within 2004 - 2008. Leaves infected with powdery mildew were collected in North and South Moravia and East Bohemia. Identification was provided according Braun (1987, 1995) and Paulech C. (1995), taxonomy was corrected by Takamasu (2007). Identification was made according to morphological features; critical findings were confirmed by H.D. Shin, new species as *Erysiphe elevata* and *E. azaleae* were confirmed on the bases of DNA. Herbarium specimens are deposited at Herbarium of Department of Forest Protection and Wildlife Management, Faculty of Forestry and Wood Technology.

3. RESULTS, DISCUSSION

There were identified 30 different species of powdery mildew on woody plants belonging to following genera: *Erysiphe, Microsphaera, Phyllactinia, Podosphaera, Sawadaea, Sphaerotheca, Uncinula, Uncinuliella* (Tab. 1). From this number of identified *Erysiphales* is *Phyllactinia roboris* reported as a missing species actually and *Erysiphe euonymi-japonici* (Vienn.-Bourg.) U. Braun & S. Takamatsu is known only from herbarium specimen collected in 1941; recent records are missing. Nearly 11 species of reported powdery mildews can be termed alien, although origin of several species is not clear. Newly recorded species are *Erysiphe arguata, E. azaleae, E. elevata, E. flexuosa, Erysiphe palczewskii, Erysiphe syringae, Erysiphe vanbruntiana* var. *sambuci-racemosae.*

Table 1. List of powdery mildews observed in the CR

Powdery Mildew	Hosts	Origin/ distribution	First record from Europe	References	First record in the CR	References
***Erysiphe adunca* (Wallr.) Fr.** **(syn. *Uncinula adunca* (Wallr.) Lév.)**	*Salix appendiculata, S. caprea, S. renii*	- / Europe, Asia, N. America		Braun 1987 Paulech 1995	2006	Palovčíková et al 2007
***Erysiphe adunca* var. *adunca* (Wallr.) Fr.** **(syn. *Uncinula adunca* var. *adunca* (Wallr.) Lév.)**	*Populus nigra*	- / all Europe, all Asia, N. America		Braun 1987 Paulech 1995	2005	Palovčíková et al 2007
***Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam. (syn. *Microsphaera alphitoides* var.**	*Quercus robur, Q. petraea, Q. cerris, Q. robur 'Fastigiata', Q. glandifera, Castanea sativa*	Asia ?/ today nearly global	1876 Portugal, 1906/1907 spreading throughout Europe; 1909 outbreak	Braun 1987	1907 (?)	Cejp et Skalický 1954, Příhoda1959

Powdery Mildew	Hosts	Origin/ distribution	First record from Europe	References	First record in the CR	References
***alphitoides* Griffon & Maubl.)**			Western Europe			
***Erysiphe arcuata* U. Braun, V.P.Heluta and S. Takam.**	*Carpinus betulus* (after revision; reported as a *E. carpinicola* previously)	Japan, Far East?	Germany, Hungary, Ukraine 2006	Braun et al. 2006 Pastirčáková et al. 2008	2004	Palovčíková et al. 2007
***Erysiphe azaleae* (U. Braun) U. Braun & S. Takam) (syn. *Microsphaera azaleae* U. Braun)**	*Rhododendron* spp.	North America	Germany 1997	Braun 1997, Inmann et al. 2000	2003	Palovčíková et Dančáková 2005, Lebeda et al. 2007, Bacigálová, Marková 2006
***Erysiphe berberidis* D.C. (syn. *Microsphaera berberidis* (DC.) Lév.**	*Berberis thunbergii 'Atropurpurea' B. vulgaris, B. vulgaris* x *rubra, Mahonia aquifolium*	- /all Europe, Central Asia, Turkey, Iran		Braun 1987 Paulech 1995	2004	Palovčíková et Dančáková 2005,
***Erysiphe elevata* (Burrill) U. Braun & S. Takam. (syn. *Microsphaera elevata* Burrill)**	*Catalpa bignonioides*	North America	2002	Vajna et al 2003, Ale-Agha et al. 2004	2005	Ale-Agha et al. 2004, Palovčíková et al. 2007
***Erysiphe euonymi* (DC.) (syn. *Microsphaera euonymi* (DC.) Sacc.)**	*Euonymus europaeus*	- /all Europe, Central Asia, Turkey		Braun 1987 Paulech 1995	2005	Palovčíková et al.2007
***Erysiphe euonymi-japonici* (Vienn.-Bourg.) U. Braun & S. Takamatsu (*Microsphaera euonymi-japonici* Vienn.-Bourg.)**	*Euonymus* spp.	Asia /Europe, Asia, N.America, S.America, Australia, New Zealand		Braun 1987 Paulech 1995	1931 Piskoř by Prague	Herb specimen M-0016258 The Erysiphales Collection at the Botanische Staatssammlung München,
***Erysiphe flexuosa* (Peck) U. Braun et S. Takamatsu (syn. *Uncinuliella flexuosa* (Peck) U. Braun)**	*Aesculus x carnea* *A. hippocastaneum,* *A. pavia*	North America/ North America	2000	Ale-Agha et al. 2000, Ing et Spooner 2002, Zimmermannova-Pastircakova et al. 2002	2004	Palovčíková et Dančáková 2005, Palovčíková et al 2007

Powdery Mildew	**Hosts**	**Origin/ distribution**	**First record from Europe**	**References**	**First record in the CR**	**References**
***Erysiphe hedwigii* (Lév.) U. Braun & S. Takam. (syn. *Microsphaera hedwigii* Lév.)**	*Viburnum lantana*	-/ Europe, Armenia, Siberia		Braun 1987 Paulech 1995	2004	Palovčíková et al. 2007
***Erysiphe lonicerae* (DC.) (syn. *Microsphaera lonicerae* (DC.) G. Winter)**	*Lonicera nigra*	- / all Europe, Central Asia, Japan		Braun 1987 Paulech 1995	2006	Palovčíková et al 2007
***Erysiphe ornata var. europaea* (U.Braun) U.Braun & S. Takam. (syn. *Microsphaera ornata var. europaea* U.Braun)**	*Alnus glutinosa* *Betula pendula* *Crataegus monogyna*	- / all Europe, Central Asia		Braun 1987 Paulech 1995	2005	Palovčíková et al 2007
***Erysiphe palczewskii* (Jacz.) Braun & Takamatsu (syn. *Microsphaera palczewskii* Jacz.)**	*Caragana* spp.	Asia/ Europe. Asia	Hungary 2005	Braun1995, Braun & Takamatsu 2000, Gelyuta et Minter 1998, Vajna 2006b	2006	Lebeda et al. 2008
***Erysiphe penicillata* (Wallr.) Link (syn. *Microsphaera penicillata* (Wallr.) Lév.)**	*Alnus glutinosa*	- / all Europe, Asia (Iran, Siberia, East USSR, Japan), N.America		Braun 1987 Sinclair 1987 Paulech 1995	2006	Palovčíková et al 2007
***Erysiphe syringae Schwein. (syn. *Microsphaera syringae* (Schwein) H. Magn.)**	*Syringa vulgaris, S. chinensis* *Viburnum opulus*	N.America/ N.America, Europa, Siberia, Australia		Braun 1987 Paulech 1995	2004	Palovčíková et Dančáková 2005, Palovčíková et al 2007
***Erysiphe tortillis* (Wallr.) Link (syn. *Microsphaera tortilis* (Wallr.) Speer**	*Cornus sanguinea*	- / all Europe		Braun 1987 Paulech 1995	2005	Palovčíková et al 2007
Erysiphe vanbruntiana* var. *sambuci-racemosae* (U.Braun) U. Braun & S. Takamatsu *(syn.	*Sambucus racemosa*	- / North America, Asie		Braun 1987 Paulech 1995	2005	Palovčíková et Dančáková 2005, Palovčíková et al 2007

Powdery Mildew	Hosts	Origin/ distribution	First record from Europe	References	First record in the CR	References
***Microsphaera vanbruntiana var.sambuci-racemosae* U.Braun)**						
Microsphaera sp. (Microsphaera penicillata s.l.)	*Sorbus intermedia*				2004	Palovčíková et al 2007
***Phyllactinia fraxini* (DC.) Fuss**	*F.excelsior, F.excelsior 'Hessei' F. angustifolia*	- / all Europe, Turkey, Asia, N.America, N.Africa		Braun 1987 Paulech 1995	2005	Palovčíková et al 2007
***Phyllactinia guttata* (Wallr.) Lév.**	*Betula pendula, B.verrucosa, B.verrucosa 'Yongii,, B. papyrifera, Cornus mas, Corylus avellana, C. avellana 'Concorta, C .avellana'Hetero phylla', C. colurna, C. maxima 'Purpurea', Crataegus monogyna , Fagus sylvatica, Salix* sp.	-/ global		Braun 1987 Paulech 1995		
***Phyllactinia mali* (Duby) U. Braun**	*Crataegus monogyna*	- / all Europe, Asia, N.America, N.Africa		Braun 1987 Paulech 1995	2006	Palovčíková et al 2007
***Phyllactinia roboris* (Gachet) Blumer**	*Quercus* spp. – not confirmed within past years; in red list	- / South Europe, Asia, S. America	1885 – Slovakia ??	Paulech 1995		
***Podosphaera clandestina* (Wallr.) Lév.**	*Sorbus intermedia*	- / all Europe, Asia, N.America		Braun 1987 Paulech 1995	2004	Palovčíková et al 2007
***Podosphaera clandestina var. clandestina* (Wallr.) Lév.**	*Crataegus oxyacantha*	- / all Europe, Asia, N.America		Braun 1987 Paulech 1995	2005	Palovčíková et al 2007
***Podosphaera pannosa* (Wallr.) de Bary (syn. *Sphaerotheca pannosa* (Wallr.) Lév.)**	*Rosa rugosa*	-/global		Braun 1987 Sinclair 1987		
***Podosphaera tridactyla* (Wallr.) de Bary**	*Padus avium*	- / all Europe, Asia, America, Australia, New Zealand		Braun 1987 Paulech 1995	2006	Palovčíková et al 2007

Powdery Mildew	Hosts	Origin/ distribution	First record from Europe	References	First record in the CR	References
***Sawadaea bicornis* (Wallr.) Homma**	*Acer campestre*, *A. ginnala, A. negundo,* *A. platanoides*, *A. pseudoplatanus,* *A. saccharinum*	- / all Europe, Asia, New Zealand		Braun 1987 Paulech 1995	2004	Palovčíková et Dančáková 2005,
***Sawadaea tulasnei* (Fuckel) Homma**	*Acer ginnala,* *A. palmatum 'Dissectum',* *A. platanoides*	- / all Europe, Asia		Braun 1987 Paulech 1995	2006	Palovčíková et al 2007
***Uncinula prunastri* var. *prunastri* (DC.) Sacc.**	*Prunus* sp.	- / Europe, Central Asia		Braun 1987 Paulech 1995	2006	Palovčíková et al 2007

Oak powdery mildew *Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam. (syn. *Microsphaera alphitoides* Griff.) is most important species for forestry as a naturalized species throughout Europe now. The origin of this species is unclear, although this species is widespread in Europe, Asia, North and South America, Australia and New Zealand. The first occurrence on this species is origin from limited area in Portugal from 1876 – 1877, in 1906 – 1907 were reported spreading of this species in many countries in Europe, in 1909 is mentioned outbreak of this species in Western Europe. Some sources note, that this species were introduced from Northern America (eg. Cejp et Skalický, 1954), some other authors assume, that this species were introduced to Asia, however recent genetic studies shows affinity to other species of powdery mildews in tropical areas in South East Asia (Limkaisang et al., 2006). Ufnalski et Przybyl (2004) show genetic diversity of oak powdery mildew.

Rhododendron powdery mildew *Erysiphe azaleae* (U. Braun) U. Braun & S. Takam. (syn. *Microsphaera azaleae* U. Braun), probably introduced from North America or Asia (Inman et al., 2000), has been from Europe recorded in England, Germany, Switzerland (Inman et al., 2000), and Poland (Piatek, 2003; Shin & Mulenko, 2004) over recent years. Rhododendron powdery mildew has been firstly recorded in the CR by in 2003 by Lebeda et al. (2007) and furthermore reported by Bacigálová and Marková (2006). Species is widespread in parks and gardens across CR actually. One of reasons of spreading is trade with plant material with combination of favorable climatic conditions within past years.

The catalpa powdery mildew *Microsphaera elevata* Burrill is a native species in North America (Braun 1987). From Europe was recorded in Europe by Ale-Agha et al. (2000). Actually is reported from the Germany, Hungary, Poland, Slovakia,

Switzerland, United Kingdom, etc. (Ale-Agha et al., 2004; Vajna et al., 2004; Pastirčáková et al., 2006; etc.). Is it widespread in plantation in towns in the CR now (Ale-Agha et al., 2004; Palovčíková et al., 2007). Some latest records are origin from Bulgaria (Denchev et al., 2008), Romania and from Italy (own observations, not published). Reasons of spreading are the same – trade with plant material and favorable climate. Some other species on Catalpa *Erysiphe catalpae* Simonyanis was not reported from CR up to date. This species is confused with *E. elevata* in some European countries frequently (Ale-Agha et al., 2004).

Horse chestnut mildew *E. flexuosa* (Peck) U. Braun & S. Takam (syn. *Uncinuliella flexuosa* (Peck) U. Braun.) on *Aesculus* spp., was firstly reported from Europe by Ale-Agha et al. (2000), Ing and Spooner (2002) and others. It is a common powdery mildew species infecting *Aesculus* trees in North America (Glawe and Dugan, 2007) and Europe - Croatia, France, Germany, Lithuania, Poland, Romania, Serbia, Slovakia, Slovenia, Switzerland, Ukraine, and United Kingdom (Braun, 1987; Heluta and Voytyuk, 2004; Pricop & Tănase, 2007; Zimmermannová-Pastirčáková et al., 2002; Kiss et al., 2004). From the Czech Republic is reported by Zimmermannová-Pastirčáková et al. (2002) and Palovčíková et al. (2007).

Some other new species for the CR is *Erysiphe arcuata* U. Braun, V. P. Heluta and S. Takam. on hornbeams *Carpinus betulus*, previously (Palovčíková et al., 2007) reported this species as *Erysiphe carpinicola* (Hara) U. Braun & S. Takam. Braun et al. (2006) re-examined European powdery mildew collections on *C. betulus* (including the anamorph *Oidium carpini*) from Germany, Hungary and Ukraine, and described them as a new species *E. arcuata*, contrary to previous records. Pastirčaková et al. (2008) reports this species from Slovakia. Previous records of *E. carpinicola* from Hungary (Vajna, 2006a) and from Poland (Wołczanska, 2007; Piatek, 2004) probably regarded as *E. arcuata* as well.

Caragana powdery mildew *Erysiphe palczewskii* Braun & Takamatsu (syn. *Microsphaera palczewskii* Jacz.) is native to Asia, however it has been introduced into many European countries (Gelyuta and Gorlenko, 1984; Braun, 1995; Gelyuta and Minter, 1998; Braun et al., 2006; Vajna, 2006b). During the summer of 2006 were severe *Erysiphe palczewskii* recorded in the CR into area of Central Moravia as well (Lebeda et al., 2008).

Erysiphe syringae Schwein. *(syn. Microsphaera syringae* (Schwein) H. Magn.) and *Erysiphe vanbruntiana* var. *sambuci-racemosae* (U. Braun) U. Braun & S. Takamatsu is common species across the CR actually and it was recorded in 2005 (Palovčíková et al., 2007).

Erysiphe euonymi-japonici (Vienn.-Bourg.) U. Braun & S. Takamatsu is reported from herbarium specimen No. M-0016258, collected in 1931 at Piskoř by Prague and specimen is deposited in powdery mildew collection at München, Germany (Botanische Staatssammlung München), although recent records are missing.

4. CONCLUSIONS

The largest number of newly discovered alien disease comes from the powdery mildew group *Erysiphales*. Newly recorded species are *Erysiphe arguata, E. azaleae, E. elevata, E. flexuosa, Erysiphe palczewskii, Erysiphe syringae, Erysiphe vanbruntiana* var. *sambuci-racemosae.* *Erysiphe euonymi-japonici* is reported from herbarium specimen in 1941 only.

Explication of its spreading could be climatic conditions within past years and also by the interest about this group within past 10 years. The occurrence of powdery mildew has not serious impact on health on observed woody plants actually. Powdery mildew is problem for nurseries and trade with plant material.

5. ACKNOWLEDGEMENT

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Czechoslovakia, *Microsphaera alphitoides* GriffonetMaublanc a *Phyllactinia roboris* (Gachet) Blumer]. *Preslia* **26**, 43–54.

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Abiotic Diseases And Other Diseases

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URBAN TREE HEALTH OF 49 GREEN SPACES IN MADRID (SPAIN)

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ABSTRACT

In order to improve the management of the urban tree health in Madrid, a sample of 49 green spaces was evaluated. Data were obtained by visual assessment in 6 different districts of the city, during a period of 3 or 4 years. Diseases, pests and other problems were identified for each species in every green area, without considering the number of trees. The health status of the trees declined along the period and there were few differences among the districts. The main problems in trees were stem injury, dead branches, epicormic shoots and decay, and the most damaged species were Siberian elm (*Ulmus pumila*), Black locust (*Robinia pseudoacacia*), Box elder (*Acer negundo*), and Plane tree (*Platanus hispanica*). Decay was related with stem injury. Biotic diseases were encountered less frequently than abiotic, and they affected a smaller rank of species. The results showed a positive association between naturalness of species and their health status, with a higher damage risk among the exotic ones. Based on their health features, a ranking of the less suitable species to be grown in Madrid is given.

Keywords: urban green space, tree health, shrub, naturalness, abiotic disease

1. INTRODUCTION

The city constitutes a hostile environment for ornamental vegetation because many adverse conditions may affect the plants living in it: from environmental factors such as urban heat island, air pollution or mechanical stem injury to biotic stresses such as pests and diseases. Several authors have reported different difficulties which are related with the urban environment (Impens and Delcarte, 1979; Rocray, 1983; Berrang et al., 1985). These factors not only injure the plants, but, some of them also predispose plants to suffer from another diseases (Kozlowski, 1985), interacting synergistically.

In order to improve vegetation management of the green spaces in Madrid, the city council commissioned a survey to the Forestry Pathology Department of the Technical University of Madrid, which was carried out from 2005 to 2008.

The study has four principal objectives in trees, shrubs, vines and seasonal flowers populations:

- Following up their health status.
- Identifying main problems and the most affected species.
- Evaluating if there would be an association between naturalness of trees species and their health condition.
- Proposing a ranking of the less suitable species, taking into consideration their health features.

2. THE STUDY SITE

The study was undertaken in six districts of Madrid. This city is the capital and the most populated city of Spain (about 3.2 million inhabitants). It is located at a latitude of 40°26′ N and a longitude of 3°41′ W with an altitude of 667m.a.s.l. The climate is temperate Mediterranean with a marked continentality. The monthly mean minimum temperature is 2.6º C in January and maximum, 31.2º C in July. August is the driest month with only 10mm of precipitation, being 436mm the annual value.

Madrid has more than 1,500 public green spaces which occupy more than 9% of its area. Besides, there are more than 300,000 street trees, without taking into account those of green spaces.

3. METHODOLOGY

The city is administratively divided into 21 districts, and the studied green spaces are distributed throughout six of them: Arganzuela, Barajas, Hortaleza, Retiro, Salamanca and Villa de Vallecas.

A randomized representative sample of 25% of the green spaces in each district was taken. Samples were collected for each year along the period 2005-2008, although in 2007 only the green spaces of Arganzuela were examined. Consequently, as a result of the random process, only a few green spaces were evaluated all the years. An interesting temporary evolution was expected, so, only the green spaces which were common to all the years in the period were analyzed in this survey.

Sampling is a more and more used technique because of the expensiveness of complete inventories, and it may be an accurate method for revealing the general patterns and trends in street tree populations (Jaenson et al., 1992). Complete inventories, such as the one which was carried out with 81,000 trees in Brussels (Impens and Delcarte, 1979), are much more precise, but, they would not be necessary if the management and conservation of green spaces was the target, since "*all data collected must be related to the goals of the inventory*" (Smiley and Baker, 1988).

To sum up, 49 green spaces were examined in the whole of the six districts and they represented about 10% of the total green spaces (Figure 1).

A periodicity of one or two years was thought as adequate (Harris et al., 2004). The seasons during which the inventories were compiled depended on the district, but in all cases, an inspection was conducted in autumn and, another one, in spring, so as to find out the different features than could be observed whether plants keep their foliage or not.

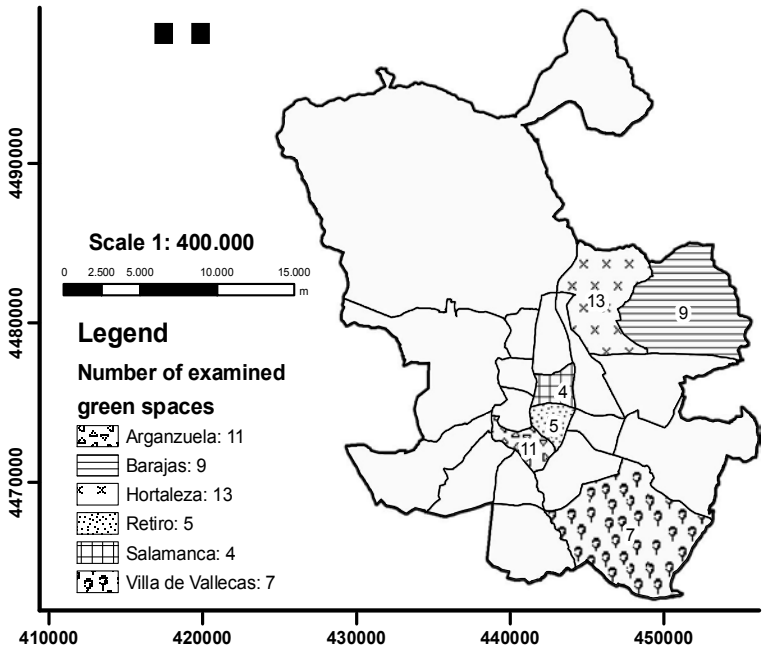

Figure 1. Number of examined green spaces in the inventoried districts.

Data were collected by professors and collaborators of the Forestry Pathology Department. They were trained so that they could recognize the major health problems of the ornamental species, before conducting the visual inspection in each area. Just in case they were not able to recognize something, both a photograph and a vegetal sample were taken, in order to identify them in lab, with the aid of bibliography. The information recorded in each green space and in each year consisted of: date; name of the green space; district and ward where it was located; problems affecting the soil; problems which were related with watering; all tree, shrub, vine or seasonal flowers species which were found in the green space; and all the diseases, pest and other problems or disturbances which were observed for each species.

Visual assessment was chosen as a tool for predicting the health status of the plants. Visual evaluation is frequently a controversial issue since some authors defend the idea that it can be a reliable means to predict internal decay (Kennard et al., 1996), whereas others think that this procedure is insufficient (Dunster, 1996). Therefore, the present study intended only to be a first approximation of the plant health condition, trying to predict failure situations, so that a possible further

survey could be focused on those riskiest cases. Nevertheless, predicting failure situations is a difficult task, as most guidelines have been developed after the failure has already occurred, and few analyses of the ability to predict have been performed (Harris et al., 2004).

All the green spaces were thoroughly inspected and the collected data were computerized in spreadsheets in order to facilitate their analysis. The unit for this analysis was defined as SYG, which stands Species-Year-Green space. It referred to one species which was observed in a certain year (2005, 2006, 2007 or 2008) and in a specific green space. From now on, it will be mentioned by simply using SYG.

4. RESULTS AND DISCUSSION

4.1 Tree and shrub condition declines.

The health condition of trees and shrubs declines. In 2005, 52.5% of SYGs were damaged, while in 2008 it accounted for 82.6% (Table 1). The figures for shrubs were smaller than those for trees, but they also increased, from 25.1% in 2005 to 58.1% in 2008 (Table 2). A rise in the number of the disturbances in vegetation is seen as the most likely cause in both cases. In fact, the number of these disturbances increased by four times in trees and three times in shrubs during the period. However, this higher level of disturbances is not attributed to more types of disturbances, since these did not increase in tune with the others. Therefore, some disturbances have become more frequent in the last years.

Regarding data collected in each district, there was a general decreasing tendency similar in the percentage of undamaged SYGs.

Table 1. Temporary evolution of damaged tree SYGs. Undamaged, damaged and percentage of damaged tree SYGs, in each district, for each year, and in all the inventoried green spaces.

Tree SYGs	2005			2006			2007			2008		
	Undamaged	Damaged	% of damaged	Undamaged	Damaged	% of damaged	Undamaged	Damaged	% of damaged	Undamaged	Damaged	% of damaged
Arganzuela	33	33	50.0	29	39	57.4	23	47	67.1	10	56	84.8
Barajas	25	24	49.0	20	46	69.7				13	56	81.2
Hortaleza	45	51	53.1	47	50	51.5				16	96	85.7
Retiro	19	11	36.7	14	12	46.2				7	21	75.0
Salamanca	4	9	69.2	5	9	64.3				2	13	86.7
Villa de Vallecas	18	31	63.3	14	44	75.9				14	53	79.1
TOTAL	144	159	52.5	129	200	60.8	23	47	67.1	62	295	82.6

Table 2. Temporary evolution of damaged shrub SYGs. Undamaged, damaged and percentage of damaged shrub SYGs, in each district, in each year, and in all the inventoried green spaces.

Shrub SYGs	2005			2006			2007			2008		
	Undamaged	Damaged	% of damaged	Undamaged	Damaged	% of damaged	Undamaged	Damaged	% of damaged	Undamaged	Damaged	% of damaged
Arganzuela	53	10	15.9	49	30	38.0	55	30	35.3	29	60	67.4
Barajas	38	13	25.5	21	42	66.7				33	34	50.7
Hortaleza	79	52	39.7	68	56	45.2				62	92	59.7
Retiro	17	3	15.0	17	14	45.2				18	10	35.7
Salamanca	24	2	7.7	16	10	38.5				9	20	69.0
Villa de Vallecas	51	8	13.6	23	45	66.2				31	36	53.7
TOTAL	262	88	25.1	194	197	50.4	55	30	35.3	182	252	58.1

4.2 Few differences are encountered among the districts.

There are few differences in the average percentage of damaged tree or shrub SYGs among the districts (Table 3). A ji-squared test shows that these figures are not significantly different at greater than the 90% level. This result was expected since there are not big differences in climate or in soil composition among the districts.

Table 3. Mean percentage of damaged SYGs. Mean figures of undamaged, damaged and percentage of damaged tree SYGs, in each district, and in all the green spaces.

SYGs	Tree mean 2005-2008			Shrub mean 2005-2008		
	Undamaged	Damaged	% of damaged	Undamaged	Damaged	% of damaged
Arganzuela	24	44	64.8	47	33	41.1
Barajas	19	42	68.5	31	30	49.2
Hortaleza	36	66	64.6	70	67	48.9
Retiro	13	15	52.4	17	9	34.2
Salamanca	4	10	73.8	16	11	39.5
Villa de Vallecas	15	43	73.7	35	30	45.9
All green spaces	111	219	66.3	216	178	45.3

Analysis of means shows that the districts which differ the most of the global mean are Retiro and Salamanca. In both of them, the areas of the studied green spaces reached lower figures than the rest of the districts. A possible explanation is that the bigger the area of green spaces observed is, the more representative of the district it becomes; however it is likely that an area threshold exists, so from this point the results will be stable.

4.3 Main problem in trees is stem wounds.

The major problem in trees is stem wounds, followed by dead branches, epicormic shoots and decay (Table 4).

Table 4. Percentage of affected SYGs by 4 main disturbances. In each year, during the whole period and in all the inventoried green spaces.

	% of SYGs				
Disturbances	2005	2006	2007	2008	All years
Stem wounds	13.2	22.8	31.4	36.4	25.2
Dead branches	10.2	8.2	18.6	33.6	18.0
Epicormic shoots	4.3	12.2	1.4	31.9	15.9
Decay	13.5	17.9	17.1	12.6	14.8

Stem wounds is also the most important problem identified in some districts, such as Arganzuela, Barajas, Hortaleza and Villa de Vallecas. The cause of this high frequency was mostly unknown, but some SYGs with stem wounds had, at the same time, sunburns lesions (11.2%) or human damages (9.0%) like those which were caused by lawn mowers, vehicles, tree shelters, stakes, and so forth. Therefore, these disturbances may have caused some of the stem wounds.

Stem wounds were also very common in several urban tree surveys (Rocray, 1983; Jaenson et al., 1992; Chacalo et al., 1994, Fostad and Pedersen, 1994; Cumming et al., 2001; Ayuntamiento de Madrid, 2009), besides, in the last three ones, the injuries were mainly caused by mechanized machinery and automobiles. Neither of them mentions any kind of sunburn lesion on the bark, nor the one conducted in street trees of Madrid.

Another consideration is the importance of SYGs which were damaged by decay. More than 60% of the SYGs with decay had, at the same time, stem wounds, which showed a possible relationship between these two variables. The explanation is that wounds can be penetrated by organisms which produce decay, such us fungi (Agrios, 2005). Therefore, the abundance of these two factors could indicate bigger internal defects of the trees and, trees could turn into hazardous because some decays might end up in failure. Besides, the strength loss due to decay is greater when it is produced by peripheral wounds, as in this survey (Kane et al., 2001). Any conifer SYG was affected by decay, maybe because of the existence of resin, as it was explained by Rodríguez Barreal et al referring to cypress (Rodríguez Barreal et al., 2000).

4.4 Main problem in shrubs, vines and seasonal flowers is dead plants.

The mayor problem is the high death rate. In shrubs, it is followed by aphids, powdery mildew and decaying plants.

4.5 The most damaged tree species are Siberian elm, black locust, box elder and plane tree.

Siberian elm (*Ulmus pumila*), black locust (*Robinia pseudoacacia*), box elder (*Acer negundo*) and plane tree (*Platanus x hispanica*) are the species which reach the greatest proportions of observed disturbances (Table 5). In fact, only the problems of 16 species accounted for 70% of the total. That would imply that some species are far more prone to problems; they would be the "key plants" (Raupp *et al*, 1985). Hence, these species require the biggest efforts in maintenance and conservation.

Table 5. Disturbances for the 20 most affected tree and shrub species. Relative abundance, percentage of total disturbances and frequency of SYGs with disturbances in each species, for all the years and for all the inventoried green spaces. (*) accumulated percentage until that species.

Tree species	% of total SYGs	% of total disturbances	% of SYGs with disturbances	Shrub species	% of total SYGs	% of total disturbances	% of SYGs with disturbances
Ulmus pumila	6.3	11.0	83.6	*Nerium oleander*	5.3	10.5	73.1
Robinia pseudoacacia	6	8.9	85.9	*Cotoneaster* sp.	6	7.5	60.0
Acer negundo	4.7	8.2	88.0	*Pittosporum tobira*	4.8	6.6	55.0
Platanus x hispanica	4.5	6.8	85.4	*Eonymus europaeus*	3	6.2	84.2
Prunus cerasifera var. *atropurpurea*	6.5	5.3	71.0	*Rosa* sp.	4.8	5.7	60.7
Populus alba var. *fastigiata*	2.2	4.8	95.7	*Viburnum tinus*	5.5	5.2	42.0
Tilia platyphillos	2.1	3.8	81.8	*Pyracantha* sp.	3.8	4.8	58.3
Cedrus sp.	4.7	3.6	66.0	*Berberis* sp.	2.1	4.1	70.4
Populus nigra	2.1	3.6	100.0	*Rosmarinus officinalis*	4.4	3.7	49.1
Morus alba	2.8	3.4	73.3	*Mahonia aquifolia*	2.2	3.0	50.0
Sophora japonica	2.5	3.1	77.8	*Laurus nobilis*	1.3	2.9	70.6
Acer sp.	2	3.0	85.7	*Juniperus* sp.	4.5	2.7	36.8
Cupressus sp.	6.4	2.7	38.2	*Photinia serrulata*	2.3	2.4	44.8
Olea europaea	1.9	2.6	70.0	*Lavandula latifolia*	3.3	2.3	41.5
Gleditsia triacanthos	1.8	2.4	84.2	*Cotoneaster horizontalis*	2.5	2.0	35.5
Pinus pinea	4.7	2.2 (75.5%*)	46.0	*Arbutus unedo*	1.6	1.9 (71.7%*)	35.0
Eleagnus angustifolia	0.9	2.2	100.0	*Spirea hypericifolia*	1.3	1.8	52.9
Populus alba	1.5	2.0	75.0	*Hibiscus syriacus*	1.5	1.8	52.6
Cercis siliquastrum	1.2	1.9	92.3	*Prunus laurocerasus*	2.5	1.8	34.4
Salix sp.	1.4	1.7	93.3	*Escallonia rubra*	2.3	1.7	41.4

There are some differences in health condition among tree species. For example, Siberian elm and black locust are very damaged as well as very abundant, whereas cypress (*Cupressus* sp.), umbrella pine (*Pinus pinea*) and cedar (*Cedrus* sp.) are very frequent but not so damaged.

The most important problem for Siberian elm is bad shaped trees. This term is used to describe the plants with bad structured crowns or trunks, for example, those trees with hanging small branches or leaning stems. Hanging branches seem to be very common in this species (Saiz de Omeñaca and Prieto Rodríguez, 2004).

Dead branches are frequent for black locust, and this was also showed in another survey (Rodríguez Barreal et al., 2000).

Box elder has stem wounds as its main problem. If this problem was associated with decay it could make trees fail, since the wood of box elder is very susceptible to decay and has a practical inability to close its wounds (Saiz de Omeñaca and Prieto Rodríguez, 2004). Powdery mildew is found as well in some green spaces, which is ordinary in this species (Saiz de Omeñaca and Prieto Rodríguez, 2004).

Anthracnose is observed in most of the green spaces where plane trees are grown. This fungal disease is produced by *Apiognomonia veneta* (*Sporonema platani*) and their first symptoms are not identified until the beginning of the 1970s in central Spain (Tello et al., 2000). Since that moment, the presence of the fungi has been known about in street trees in Madrid (Rodríguez Barreal, 1986). It remains unclear whether it affects less its precursor *Plantanus orientalis* (Villalva Quintana, 2005) than to the own hybrid (Rodríguez Barreal, 1986), although it seems that "*the different genotypes of the hybrids allow differences in disease severity among trees growing under the same environmental conditions, though eventually all trees are affected to some degree or another*" (Tello et al., 2000). The abundance of epicormic shoots and adventitious branches (3rd and 4th problem) could be related to this disease, since the infestation of the fungi in adventitious buds produces a much greater number of them (Tello et al.2000), however, it could be also associated with the tendency to create them when the planting conditions of plane trees are not appropriate (Saiz de Omeñaca and Prieto Rodríguez, 2004).

4.6 The most damaged shrub species are oleander, cotoneaster, Japanese cheesewood and European spindle.

Oleander (*Nerium oleander*), cotoneaster (*Cotoneaster* sp.), Japanese cheesewood (*Pittosporum tobira*) and European spindle (*Euonymus europaeus*) are the most damaged species (Table 5).

Main problem of oleander is the bacteria *Pseudomonas syringae* subsp. *savastanoi*, which affected about half of green spaces every year. The former produces galls in flower buds, so those plants become weaker and with aesthetic harms (Villalva Quintana, 2005). Dead plants are the principal problem affecting cotoneaster and Japanese cheesewood, the second regarding European spindle, and the third concerning oleander. Aphids were in more than 24% of SYGs of oleander,

cotoneaster and Japanese cheesewood, and their importance was lower in European spindle, with an affected 10.5%. The latter species was affected by powdery mildew in more than 70% of the SYGs where European spindle was found. This disease is induced by the fungi *Microsphaera euonymi-japonici* and it has a high incidence among this species in cold areas of the Iberian Peninsula (Villalva Quintana, 2005).

4.7 The most damaged vine species is ivy.

Ivy (*Hedera helix*) is the most affected vine species as well as the most abundant vine. Its most frequent problems are dead plants and sunburn lesions on its leaves. Sunburn lesions appeared because, even though it is a shade tolerant species which grows in the understorey layer in nature, in many of the cases it grew in green spaces under full sun situations.

4.8 Biotic diseases are encountered less frequently than abiotic in trees, although the result was the opposite in shrubs.

In this survey, biotic diseases were due to organisms such as viruses, bacteria, pests and fungi. On the contrary, abiotic diseases consisted of problems caused by meteorological and physical agents, by human activities and, by other factors whose origin is a priori unknown, such as cracks, stem wounds, tumours, etc. Dead plants were not included in any of the former types.

In trees, abiotic diseases comprised about 60% of total disturbances in each year (Table 6). The mayor contributions to the high incidence of abiotic diseases are due to stem wounds, dead branches and epicormic shoots, since they are the most frequent problems for trees. Similar results were also obtained in a survey in Quebec (Rocray, 1983).

Table 6. Percentage of abiotic diseases of the total of disturbances. In each year in trees and shrubs of all the inventoried green spaces.

% of abiotic diseases	2005	2006	2007	2008
Trees	58.8	69.1	56.6	79.7
Shrubs	19.7	47.9	27.6	23.7

The principal problem in green spaces is the abiotic stresses when they are compared with biotic diseases. This fact should lead to the consideration of the maintenance and conservation practise which is being applied mainly to the vegetation of green spaces, because most damages could be avoided if some recommendations were followed. Various efforts were observed about this issue in this survey, for instance, the use of stakes and tree shelters in new plantations, however, in many cases, the achieved effect was just the opposite of the expected one, since stems were leaned and the friction between the trunk and the object caused the wounds which were intended to be avoided. Not only the staff in charge of the conservation of green spaces are responsible for these problems, but also the

designers or planners of the green spaces, since some disturbances such as sunburn lesions or leaning stems by light deficit are generally due to inadequate designs. In fact, in natural environment most of these problems are very unusual. For all these reasons, the municipal green spaces management programmes should be look through in order to adapt conservation strategies to deal better with abiotic problems.

Besides, abiotic diseases were much less frequent in shrubs, with 20-48% of total disturbances depending on the considered year. Aphids were responsible of a great number of the biotic diseases, since it was the second problem, after dead plants. However, this result would have been pretty different if dead plants had been considered as an abiotic or biotic agent.

4.9 Abiotic diseases affect a greater rank of tree and shrubs species.

Abiotic diseases affect a greater variety of species (Table 7), which is reasonable if it is taken into account the fact that they are prompted by human activities or physical agents. However, biotic diseases seemed to be more specific in some plant species; for example, lace bugs (*Corythuca ciliata*), leaf beetle *Galerucella luteola* and *Pseudomonas syringae* subsp. *savastanoi* affected only one species (plane tree, Siberian elm and oleander, respectively). Concerning pests, this result would confirm the idea held by some authors who maintain that most herbivorous pests are specialized and only eat few taxa of plants (Rocray, 1983; Galvin, 1999; Raupp et al., 2001).

Table 7. Affected species by biotic and abiotic diseases. Tree and shrub species affected by biotic and abiotic diseases and the percentage of the total species, in all the years and in all the inventoried green spaces.

Trees	Total	Affected by biotic diseases	Affected by abiotic diseases	Shrubs	Total	Affected by biotic diseases	Affected by abiotic diseases
Species	73	38	57	Species	77	32	40
% of species	100	52.1	78.1	% of species	100	41,6	51,9

4.10 Positive association between naturalness of tree species and their health status.

Pyšek's criteria are used to define the naturalness of the tree (Pyšek , 1995). It is considered the national scale, therefore, all those species defined as natural of Spain according to those criteria, are classified as native in this survey. Bibliography is used to make easier this controversial task (López González, 2002; Real Jardín Botánico, 2009).

A ji-squared test on the data shows that the relationship between health status (damaged or undamaged SYG) and naturalness (native or exotic SYG) is significant at 95% level (Table 8). The probability for an exotic SYG to be damaged is greater (0.68) than the probability for a native SYG (0.59). However,

although these proportions were significantly different for a hypothesis test at 95% level, with a greater level, they were not different. Thus, the risk of being damaged is only slightly higher for exotic SYGs than for native SYGs.

Table 8. 2x2 contingency table of tree SYGs.

SYGs		Health status	
		Undamaged	Damaged
Naturalness	Native	92	134
	Exotic	266	567

This seems to be a coherent result since native species are expected to be better adapted, although there are authors who maintain that exotic species do better than native ones because their pests and diseases have not yet arrived from their home country (Schimdt and Kerenyine-Nemstothy, 1999). Similarly, Harris explains that native species sometimes do not perform as well as exotic ones (Harris et al., 2004). Therefore, as it remains unclear what kind of species should be planted more frequently, it should be consider that exotic flora affects native and overall species richness throughout the globe (Alvey, 2006) while native species improve the sustainability of urban forests (Clark et al., 1997). In any case, the recommendation of not planting invasive exotic species should be followed (Alvey, 2006).

4.11 Ranking of less suitable species to be grown in Madrid.

An algorithm to establish which tree and shrub species are less adapted to the urban environment of Madrid is created. On one hand, a quantitative scale is established to asses the severity of the observed disturbances, assigning greater coefficients to the most serious disturbances (Table 9). On the other, a qualitative scale takes into consideration: a) the number of disturbances which appeared within each species, and b) the proportion of damaged SYGs. The result of multiplying each severity coefficient by the number of disturbances (a) is weighted with the proportion of damaged SYGs (b) within each species. The outcome is a ranking with the least suitable species, where the species with the highest grade appear on top (Table 10 and Table 11).

Table 9. Severity classes of disturbances with coefficients.

Severity classes of disturbances	Coefficients
Hazardous	0.5
Pretty serious	0.3
Less serious	0.2
Acceptable	0.1

This kind of species suitability ranking has been observed in other surveys (Impens and Delcarte, 1979; Raupp and Noland, 1984; Nielsen et al., 1985; Ball,

1987; Rodríguez Barreal et al., 2000), although in some of them the way in which the results are obtained is not specified, so comparison is difficult. In spite of this, in the research carried out in the street trees of Madrid (Rodríguez Barreal et al., 2000), black locust appears as not very desirable species. Otherwise, this was considered very advisable species in another survey (Raupp and Noland, 1984) since it did not suffer from pest problems unlike the present survey, where many black locust SYGs were affected by aphids and wood borer insects.

Table 10. Ranking of ten least suitable tree and shrub species. Percentage of damaged SYGs, severity of disturbances (coefficient*number of disturbances) and final value, in all the years and in all the inventoried green spaces.

Order	Tree species	% of damaged SYGs	Severity of disturbances valuation	**Final value**	Tree species	% of damaged SYGs	Severity of disturbances valuation	**Final value**
1	*Robinia pseudoacacia*	85,9	64,3	**55,3**	*Nerium oleander*	73,1	20,6	**15,1**
2	*Ulmus pumila*	83,6	64,0	**53,5**	*Cotoneaster* sp.	60,0	20,9	**12,5**
3	*Acer negundo*	88,0	47,0	**41,4**	*Eonymus europaeus*	84,2	12,2	**10,3**
4	*Platanus x hispanica*	85,4	37,6	**32,1**	*Pyracantha* sp.	58,3	16,4	**9,6**
5	*Populus alba* var. *fastigiata*	95,7	32,0	**30,6**	*Pittosporum tobira*	55,0	16,7	**9,2**
6	*Populus nigra*	100,0	24,2	**24,2**	*Rosa* sp.	60,7	14,6	**8,9**
7	*Prunus cerasifera* var. *atropurpurea*	71,0	27,3	**19,4**	*Berberis thunbergii 'Atropurpurea'*	70,4	11,8	**8,3**
8	*Tilia platiphyllos*	81,8	23,2	**18,0**	*Rosmarinus officinalis*	49,1	14,5	**7,1**
9	*Sophora japonica*	77,8	22,0	**18,0**	*Viburnum tinus*	42,0	16,6	**7,0**
10	*Acer* sp.	85,7	19,8	**17,0**	*Mahonia aquifolia*	50,0	7,9	**4,0**

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CHARACTERISATION OF CZECH *Ophiostoma novo-ulmi* ISOLATES

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ABSTRACT

Ophiostoma novo-ulmi was recorded for the first time in the area of the Czech Republic in 2006, although it was suspected since early 1960´s. During the years 2006 and 2007, 58 isolates were collected. Isolated strains of the DED causal agent were determined by PCR and RFLP molecular methods. DNA of each sample was isolated from cultured mycelium, amplified at *cu*, and *col1* gene regions and restricted by *Hph* I and *Bfa* I endonucleases. Strains were determined according to both of these two gene regions. An old species *Ophiostoma ulmi* (Buism.) Nannf. was not noticed in any case. Every sample belongs to the species *Ophiostoma novo-ulmi* Bras., and both known subspecies *americana* (5 isolates) and *novo-ulmi* (29 isolates) were present. Additionally, 13 strains of *Ophiostoma novo-ulmi* were more particularly tested for vegetative compatibility type, mating type, fertility with both subspecies and cerato-ulmin production. Among these 13 strains only 3 seemed to be non-hybridized ssp. *novo-ulmi* and only one non-hybridized ssp. *americana*, the remainder (9 strains) was intraspecific hybrids. 5 of the tested strains were of mating type A and mating type B occurred 8-times. None of these strains was compatible with any other. High frequency of intraspecific hybrids (24 isolates) is remarkable and shows on a frequent occurrence of sexual hybridization. Cerato-ulmin production did not show any significant differences according to strain subspecies. Virulence of one *O. novo-ulmi* strain (M3) was tested on two Dutch resistant cultivars, 13-year-old elms ´Groeneveld´ and ´Dodoens´. Only 5 weeks after inoculation were sufficient period for 30 – 90% defoliation of cultivar ´Groeneveld´, ´Dodoens´ with only 0 – 5% seemed to be much more resistant.

Keywords: Dutch Elm Disease, PCR-RFLP, elm, infection tests

1. INTRODUCTION

Ophiostoma ulmi and *O. novo-ulmi* – causal agents of Dutch elm disease (DED) has been threatening elms in the Czech Republic since 1930's, as well as in the entire Northern Hemisphere. The first occurrence of Dutch elm disease in the Czech Republic caused by *Ophiostoma ulmi* (Buism.) Nannf. was noted by prof. Peklo (Polák, 1932) who found infected trees in elm alleys in Prague and Poděbrady in 1932.

A newer and more aggressive form was since 1970´s distinguished into two races - an Eurasian (EAN), probably originated in Moldavia and the Ukraine, and a North American race isolate (NAN). In 1991, the more aggressive form was described by Brasier as a new species *O. novo-ulmi* Bras (Brasier 1991). Ten years later, Brasier et al. (2001) designated races EAN and NAN as subspecies of *O. novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana*. Specific group of DED pathogens are hybrids of *O. novo-ulmi* subspecies.

O. novo-ulmi, its subspecies and their hybrids were for the first time recorded in the Czech Republic by Dvořák et al. (2006, 2007) . The aim of present study is a complex characterisation of strains, which are actually spreading in the area of the Czech Republic.

2. MATERIALS AND METHODS

2.1. Isolation of the pathogen: Coordinates of position and altitude of every infected elm with inner DED symptoms was achieved by GPS. Samples of twigs were cut from different parts of drying crowns and cultivated on cycloheximide 3% MEA medium. Each strain was deposited into the culture collection of MUAF Brno.

2.2. Molecular-biological characterisation: DNA was obtained from pure MEA cultures by PowerSoil DNA Kit (Mo Bio). Identification of species and subspecies was performed by the mean of Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) using two gene regions. The former is a cerato-ulmin (*cu*) gene region, described in more detail in Pipe et al. (1997) and the latter gene region is called *col1* and encodes colony type (Konrad et al., 2002). Amplified fragments of these two gene regions were restricted by endonucleases, *Hph* I used for *cu* gene region and *Bfa* I for *col1* region (Konrad et al., 2002). RFLP fragments were visualized on 3% agarose gel and evaluated. Sequencing was provided in a few cases and submitted to Gen Bank.

2.3. Production of cerato-ulmin: Amount of cerato-ulmin produced by liquid cultures of 15 isolates was determined by chromatography (Scala et al., 1994). The tested group of isolates was composed from 4 cultures of ssp. *novo-ulmi*, 2 cultures of ssp. *americana* and 9 strains of genetic hybrids of precedent strains. In this tested group, well known isolates H328 ssp. *novo-ulmi* and 182E ssp. *americana* were used as reference strains. Liquid cultures of dilutions 1:1, 1:2, 1:4 and 1:8 were stirred to obtain turbid milky solutions. The turbidity of the samples at the various dilutions was immediately assayed for optical density at 400 nm. Results of the dilution with the most variable values represent the best measurement for comparing the cu-production of the strains.

2.4. Mating type, vegetative compatibility and fertility tests: General characterisation of 13 isolates has been performed according to Brasier (1981). A special medium – Elm Sapwood Agar was used. Occurrence of elm sapwood in the

medium is necessary for obtaining synnemata and perithecia, essentially important for classifying into mating type (A or B), vegetative compatibility groups and fertility tests (identification of subspecies).

2.5. Infection tests: Isolate M3 was inoculated into sapwood of 10 living elms which represents *in vitro* cloned progenies of two elm hybrids – ´Groeneveld´ and ´Dodoens´ (Krajnakova and Longauer, 1996). The experimental plot is situated near to Banská Štiavnica (coordinates: N 48°28´ E 18°58´, 590 m a. s. l.) 5 representative trees per each hybrid were inoculated and observed according to Solla et al. (2005).

3. RESULTS

3.1. Ratios of *O. novo-ulmi* subspecies: Altogether 58 isolates were achieved from nearly the entire area of the Czech Republic. The isolated strains were identified as *O. novo-ulmi* and both subspecies *novo-ulmi* (29) and *americana* (5) were found, as well as their hybrids (24). Decreasing trend in occurrence of *Ophiostoma ulmi* confirms the results of other authors (Brasier, 1991; Hoegger et al., 1996; Konrad et al., 2002). They suppose that *Ophiostoma ulmi* disappeared by the end of the 1970's.

3.2. Production of cerato-ulmin: Absorbances of the samples were very variable. Dilution with most variable results was 1:4. Statistical analysis (z-test) did not show any differences in cerato-ulmin production between subspecies and hybrids. Interesting exception is M9, which produces no cerato-ulmin, although it was isolated from infected branch. Culture of M9 seems to be d-infected.

3.3. Mating type, vc-type and fertility response variability: Among 13 isolates investigated both mating types occur; 9 isolates are of B type, 4 of A type. Vc-tests showed on high diversity of strains. Any isolate was not compatible with any other, although two of them were collected from the same tree. Fertility tests revealed 3 subspecies *americana* and 10 ssp. *novo-ulmi*. In comparison with identification by molecular tools there is some relation with *cu*-region, isolate M10 is the only exception.

3.4. Infection tests: Isolate M3 caused extremely high defoliation on resistant elm hybrid ´Groeneveld´ and low defoliation on the hybrid ´Dodoens´.

4. DISCUSSION AND CONCLUSION

Methods which have been used proved to be useful in the recognition of diversity in DED causal agent populations. From the distribution of isolates is evident that we cannot draw any borders between areas which are occupied only by ssp. *novo-ulmi* or by ssp. *americana*. In many cases such areas were observed where both subspecies and their hybrids are overlapping and where more than one strain of pathogen is responsible for dying of a tree. This phenomenon was

concluded by negative vc-type tests of two isolates from one tree. This is confirmed by many authors (Brasier et al., 1998; Santini, unpublished). High variability of mating types and subspecies is an evidence of postepidemic phase of DED (Konrad et al., 2002; Brasier et al., 2004). Differences in cerato-ulmin production among subspecies which are described by Brasier et al. (2001) and others were not confirmed. Production of cerato-ulmin by hybrids and ssp. *novo-ulmi* is not different, production by ssp. *americana* is also not different, but there were only two isolates ssp. *americana* tested. Infection tests on living trees are useful for assessment of the resistance of each species or elm hybrid and are much more reliable by use of older plant material than common 3-year-old trees. (Solla et al., 2005). Cultivar ´Groeneveld´ showed very low resistance against currently spread strain of *Ophiostoma novo-ulmi.*

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HAIL DAMAGE OF FOREST TREES IN WESTERN CANADA

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ABSTRACT

Hail and hail storms damage agricultural and horticultural crops, as well as vehicles, houses and other buildings; these weather events also cause various degrees of damages to forest trees. Western Canada, with its diverse topography and varied weather systems, is known to have frequent hail storms. In the Canadian Rockies in the fall of 2008, a severe discoloration of foliage and many dead branches were observed over several hectares of a subalpine fir (*Abies laciocarpa* (Hook.)Nutt.) stand. After examining the symptoms and topographical features of the affected area, it was concluded that the damage was caused by a hail storm. Hail damage as well as other weather-related damage to forest trees, such as frost damage and drought damage, are often predisposing factors of branch and foliage symptoms associated with pathogenic fungi belonging to such genera as *Nectria*, *Cytospora*, and *Sphaeropsis*, and this weather-related damage should be considered when diagnosing forest tree health problems.

Keywords: hail damage, western Canada, non-biotic tree diseases, predisposition

1. INTRODUCTION

Hail or hail storms usually occur at the front of storm systems from spring to fall but rarely during cold winter weather. Hail is formed when updrafts in thunderclouds carry rain drops upward into extremely cold areas of the cloud and form ice particles; updrafts carry the ice particles to the cold region of the storm clouds, where the size of the ice particles increases. When the ice particles become too heavy to be supported by the updraft, they fall to the ground at speeds of up to 100 km/h. Hail storms can cause damage to crops, houses, and vehicles as well as injuries to people and animals. Hail damages to forest trees have often been reported (Benjamin, 1957; Grayburn, 1957; Hiratsuka and Zalasky, 1993; Laut and Elliott, 1966; Nelson, 2009; Riley, 1953).

Hail storms occur more frequently along mountain ranges because winds (moving in a horizontal direction) react to the change in topography, shifting upwards within thunderstorms and creating favorable conditions for the creation of hail and hail storms. Hail storms are known to occur throughout many parts of the

world mostly in temperate zones. Bangladesh has reported some of the largest hailstones ever measured and more hail-related human deaths than anywhere else in the world. In Canada, the foothills area of the Rocky Mountains, especially in the province of Alberta, is known to have more incidences of hail storms. Hail damage can be localized in small areas but areas as big as 10-km wide are also recorded.

2. OBSERVATIONS

In the fall of 2008, at Mount Assineboine Provincial Park, in British Columbia, Canada, I observed wide areas of natural forests of mature subalpine fir (*Abies laciocarpa* (Hook.)Nutt.) that were severely discoloured with many dead branches. No fungal or other biotic signs were present. After close examination of the damaged branches and topographical features of the area, I concluded that the symptoms were most likely caused by hail that occurred early in the summer, a few months before my visit. Symptoms included half- healed wounds on the upper side of the branches and many fallen branches on the ground. Needles positioned beyond the wounds on branches were often discoloured. Also, the damaged stands were facing a lake and the damage occurred mostly on the exposed side of the stand, indicating that the hail storm hit sideways from the direction of the lake.

3. DISCUSSION AND CONCLUSION

Hail damage as well as other harmful non-biotic conditions, such as severe drought and frost damage, will serve as predisposition factors to fungal and bacterial shoot and stem diseases. Trees in the affected areas need to be carefully examined and diagnosed. These climatic and physiological damages to trees initially do not have signs of fungi or bacteria, but they are often invaded by certain fungi or bacteria later, and they are often misdiagnosed as caused by secondary fungal invaders. Genera of fungi such as *Cytospora, Nectria,* and *Sphaeropsis* are known to be in this category of fungi on stems and branches of forest trees (Blodget et al., 1997; Zwolinski et al., 1995). In our recent publication of forest tree diseases of west-central Canada (Hiratsuka et al., 2004; Hiratsuka, 1987) we used expressions such as "*Nectria* and *Cytospor*a associated with canker of broad leaf trees—" rather than "*Nectria* canker" or "*Cyotspora* canker". So called "*Screloderris* canker" caused by *Gremmeniella abiteina* is also likely triggered by one or more non-biotic predisposition factor.

It is important to consider non-biotic factors, including hail damage, as important predisposing conditions when we diagnose forest tree health problems, especially in cold climatic regions such as those in Canada.

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Extended Abstracts

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THREATENING TREE DISEASE IN EAST AFRICA

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ABSTRACT

Severe and extensive outbreaks of a dieback and canker disease have recently been observed on *Grevillea robusta* in Kenya, Uganda and to a lesser extent in Ethiopia. Grevillea is an excellent agroforestry tree species grown intensively in east Africa to improve agricultural land use and rural livelihoods, and provide food security. Our recent studies on the disease indicate that 50-80% tree mortality occurs on severely infected farms. It is caused by *Botryosphaeria* spp., a fungal genus containing many species and more than one pathogenic species can occur in diseased trees. Samples were taken from Grevillea trees growing in different agroecological zones and from some other tree species with similar symptoms. Morphological and molecular methods were used to identify species and to study differences between populations in different agroecological zones as well as countries.

The disease is more severe in dry areas than wet ones, emphasizing the need for proper species-site matching. Several other tree species, including indigenous and exotics, were found infected by *Botryosphaeria* in the region. Especially alarming is the attack on different *Eucalyptus* species.

Such disease outbreaks may be attributed to increased tree planting in agroforestry and commercial tree plantations in the region. Increased acreage and number of trees/ha leads to an enlarged number of potential hosts, and a larger population size for pathogens to evolve genetically into more aggressive genotypes. Moreover, complex threats can arise when previously isolated fungal species brought together by human interference hybridize posing threats to tree hosts previously immune from their effects. Implications of the dieback and canker disease on the scaling up of agroforestry technologies and commercial forestry in the region are discussed.

Kewords: *Grevillea robusta*, *Eucalyptus* spp. *Botryosphaeria*, dieback and canker disease

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PREMATURE DEFOLIATION OF *Cedrus libani* IN SOUTH-WESTERN TURKEY

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Cedrus libani A. Rich 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. There are some 600 000 ha of *C. libani* forests in Turkey, most of which are mixed stands. Wood of *C. libani* is economically important as it is highly resistant to decay, durable and easy to process by hand tools and machines. In addition to economical value *C. libani* forests are significant from historical, cultural and aesthetic point of view, and therefore actions for sustainable usage and restoring degraded stands have been initiated.

In some *C. libani* stands in the lakes district of Turkey browning of needles occurred in spring, especially in the lower part of the canopy. The disease was observed both on saplings growing as understory in mixed forest as well as on approximately 10–m–tall trees in an even–aged stand. A close investigation revealed ascomata on 1–year–old needles still attached to the twigs. Often only the ascomata-bearing part of the needle was brown while other parts had remained green. In the lower part of the canopy of a sapling major part of the needles could be diseased. On a single branch showing signs of serious premature defoliation proportion of needles bearing ascomata was 49.3 % of the total dry weight of the needles, while the corresponding value for more or less healthy needles was 37.4%. Chlorotic or damaged needles without ascomata made up 13.2% of the total needle dry weight.

The morphological characteristics of the ascomata were very similar to those of *Ploioderma cedri* S. Singh, S.N. Khan & B. Misra occurring on *C. deodara* in India, but asci and ascospores were somewhat larger. The frequent fruiting on dead parts of an otherwise green needle indicates that the fungus is the causal agent of the disease.

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CONTRIBUTIONS TO THE PHYLOGENY OF EUROPEAN *Porodaedalea* SPECIES (BASIDIOMYCETES, HYMENOCHAETALES)

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ABSTRACT

The genus *Porodaedalea* is a taxonomically difficult complex of morphologically similar species causing white pocket rot of living conifers. The evolutionary relationships of European species were examined using sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA and of translation elongation factor 1 alpha (tefa). Our results confirm the occurrence of *Porodaedalea chrysoloma, P. pini* and *P. laricis* in Europe. *P. laricis* is newly reported in Fennoscandia on *Picea* and in the Central European mountains (Alps, High Tatras, and Bohemian Forest) on *Larix* and *Pinus* spp. These specimens had been previously identified as *Porodaedalea chrysoloma* or *Phellinus vorax* (an invalidly described species). Although frequently confused, *P. chrysoloma* and *P. laricis* can be distinguished on the basis of pore morphology. We also report our finding of *P. pini* on *Larix*. In general, the tefa sequences are more variable than the ITS sequences and reveal the remarkable affinity of some Scandinavian and Central European specimens to those from Central Asia.

1. INTRODUCTION

The genus *Porodaedalea* includes parasites on conifers, causing white pocket rot. The genus belongs to one of the most taxonomically difficult groups of hymenochaetoid pore fungi. The so-called *Phellinus pini* group was raised to the generic level by Fiasson and Niemelä (1984). The basidiocarps are perennial, effused-reflexed to pileate, solitary to imbricate, and corky to woody hardness. The colour is rust brown to dark grey on the upper surface, while the poroid surface is ochre brown or rust brown to umbre brown, and more or less shining. The pores are circular to angular, tending to split and becoming irregular to daedaleoid and labyrinthine. Setae are commonly present in the hymenium. In some areas the species are reported to be economically important pathogens of conifers (Lannenpaa et al., 2008; Černý, 1989; Lehtijärvi et al., 2007). The genus has previously been treated as part of a broadly conceived genus *Phellinus* s.l. (Ryvarden and Gilbertson, 1994), but molecular studies have revealed the heterogeneity of that genus (Wagner and Fischer, 2002). Therefore smaller, more homogeneous genera are currently accepted.

The genus *Porodaedalea* is comprised of a rather small number of species. Nevertheless, the substrate specificity and exact distribution of each species are poorly known due to the lack of microscopic characters suitable for exact identification at the species level (Fischer, 1996). In Europe, two species are traditionally recognized, *Porodaedalea pini* (Brot.) Murrill and *Porodaedalea chrysoloma* (Fr.) Fiasson & Niemelä, with the former species restricted to *Pinus* and the latter species restricted to *Picea* as specific hosts. A third species, *Porodaedalea laricis* (Jacz. ex Pilát) Niemelä (Niemelä et al., 2005), is distributed on *Larix* from the European part of Russia to Siberia and the Russian Far East and is probably present in China (Dai, 1999). Fischer (2000) discovered this species on a non-indigenous *Larix* trees in Southern Finland and proposed for it the new name *Porodaedalea niemelaei* M. Fischer. This name has been synonymised with *P. laricis* by Niemelä et al. (2005).

Indigenous stands of *Larix decidua*, *Pinus cembra* and *Pinus mugo* in the mountains of Central Europe (Alps, High Tatras) are inhabited by species differing from *P. chrysoloma* and *P. pini* (Černý, 1985; Fischer, 2000). These populations may belong to *P. laricis* or an undescribed species. The name *Phellinus vorax* has been applied to specimens from this region. However, the combination *Phellinus vorax* is based on incorrectly published basionym *Daedalea vorax* Harkness. Therefore, the name is unavailable, although it has been commonly used (Breitenbach and Kränzlin, 1986). The fungus named *Daedalea vorax,* a Western American species growing on *Pseudotsuga menziesii*, was later correctly described as *Phellinus gilbertsonii* M.J. Larsen (Larsen, 2000).

Molecular taxonomy methods are frequently used as tools for the identification of fungal taxa. Such methods as RFLP (Fischer, 1996) and sequencing of various regions of nuclear ribosomal DNA (Fischer, 2000; Wagner and Fischer, 2002) have been used to reveal the evolutionary relationships of the species. The sequence of the ITS region of the ribosomal DNA also completes the neotypification of *Phellinus chrysoloma* (Larsen and Stenlid, 1999). The aim of the present study is to elucidate the identification of *Porodaedalea* specimens occurring in various parts of Europe, using sequences of the ITS region of the nuclear ribosomal DNA (ITS) and of translation elongation factor 1 alpha (tefa). The study is focused mainly on specimens that have been previously identified as *Phellinus vorax*.

2. MATERIALS AND METHODS

In total, 30 specimens of *Porodaedalea* were included in the study. Either fungal cultures or herbarium specimens were used for DNA analyses.

DNA was isolated from dried fungal material or from fresh cultures that were grown on Petri dishes with MEA medium (3% Malt extract, 0.5% peptone, 1.5% agar; Himedia, Mumbai, India) using the PowerSoil™ DNA Isolation Kit (Mo-Bio, Carlsbad, USA). PCR reactions were set up according to standard protocols,

supplemented with 5% (v/v) bovine serum albumin (BSA) as a PCR enhancer. DNA fragments encompassing the ITS and tefa DNA regions were amplified using the primer combinations ITS1/ITS4 and EF595F/EF1160R, respectively (White et al., 1990; Kauserud and Schumacher, 2001). The DNA was PCR-amplified as in previous studies (Tomšovský et al., 2006) using a Mastercycler® ep thermocycler (Eppendorf, Hamburg, Germany). In cases when amplification of the ITS and tefa regions was difficult, the primer pairs ITS1F/ITS4B and EF-526F/EF-1567R, respectively, were used in nested PCR (for primer sequences, see Gardes and Bruns, 1993; O'Donnell et al., 1998).

The amplified fragments were sequenced by the DNA Sequencing Service of Macrogen Inc. (Seoul, Korea). We added an ITS sequence from the *Porodaedalea chrysoloma* neotype (Genbank acc. no. AF123440; Larsen and Stenlid, 1999) to the data set. ITS and tefa sequences of *Onnia leporina* were chosen as outgroups based on the results of Wagner and Fischer 2002.

Sequences of each individual marker were aligned using the Clustal W algorithm in BioEdit and adjusted manually. To determine whether the datasets from different genetic markers were in significant conflict, partition homogeneity tests were performed between the markers in all possible pair-wise combinations. The tests were done in PAUP 4.0b10 using 100 replicates and the heuristic general search option. The null hypothesis of congruence was rejected if $p < 0.01$

Phylogenies were generated in MrBayes version 3.1.2.The best-fit model and parameters given by MrModeltest were used in the analyses. Markov chains were initiated from a random tree and were run for 2,000.000 generations; the samples were taken every 100th generation. Posterior probabilities (PP) were used as Bayesian branch supports on the consensus trees. In addition, bootstrap branch support values (BP) were estimated in PAUP 4.0b10 under the maximum parsimony criterion using 1000 replicate datasets with random sequence addition during each heuristic search.

3. RESULTS

Partition homogeneity tests showed significant conflict between the two genetic markers used ($p \leq 0.01$). This did not allow us to perform a combined analysis of the ITS and tefa sequence data. The results of the phylogenetic analyses of the two datasets are ambiguous. The ITS phylogram shows three main groups, while the tefa phylogram shows four. In ITS data set, the most basal lineage within the ingroup is composed of *P. chrysoloma* specimens from the Czech Republic, Estonia, Romania, and Southern Sweden, including the *P. chrysoloma* neotype. This *P. chrysoloma* group is also consistent in the tefa phylogram.

The second group includes *P. pini* specimens from the Czech Republic, Estonia, Croatia, Lithuania, and Sweden, including a specimen growing on *Larix*. The position of this group is variable; it forms a well supported terminal clade in the ITS phylogram, but it is placed in the centre of the tefa phylogram.

In the ITS phylogram, the third clade is composed of *P. laricis* specimens from European part of Russia, Kazakhstan and the Russian Far East, Fennoscandia, and the Central European mountains (Alps, High Tatras, Bohemian Forest). However, this group is not consistent in both analyses. In the tefa phylogram, five sequences from the Czech Republic, Norway, Sweden, and Kazakhstan are excluded from this group due to the presence of unique nucleotide substitutions at positions 166 and 408 in the alignment. Nevertheless, this distant clade is poorly supported.

4. DISCUSSION

The topologies of the two gene regions (ITS, tefa) delimiting *Porodaedalea* species are inconcordant, so they do not follow phylogenetic species recognition (according to Taylor et al., 2000). Nevertheless, the phylogram topologies of these two regions have been found to be incongruent in other studies (Kauserud et al., 2007; Ota and Hattori, 2008). The most surprising result in our study is the division of the homogeneous *P. laricis* ITS group into two groups in the tefa dataset. However, the PP and BP values of the *P. laricis* B group in the tefa phylogram are low. Therefore, we suggest that the name *P. laricis* is adopted for all specimens included in the *P. laricis* ITS group that were originally identified as *P. chrysoloma*. Almost all sequenced specimens from Fennoscandia that were identified as *Phellinus chrysoloma* are unrelated to the neotype of that species and most likely belong to *P. laricis* instead. Our results resemble those of Černý (1985, 1989), who placed these Fennoscandian specimens growing on *Picea* in *Phellinus vorax*, an incorrectly published name synonymous with *Phellinus laricis* (Černý, 1985). In any case, the distribution of genuine *P. chrysoloma* in North Fennoscandia is questionable. The species occurs without doubt in southern Sweden and Finland (Larsen and Stenlid 1999; Fischer 2000), but the northern limit of its distribution is unclear.

The occurrence and host affinity of *Porodaedalea* is worth a detailed discussion. Niemelä et al. (2005) assumed strict host specificity of *Porodaedalea* species, and therefore they set the westernmost limits of *P. laricis* in accordance with the natural occurrence of *Larix sibirica* in Russia. Although Fischer (2000) examined a specimen growing on artificially planted *Larix* from Finland, it was reported to be an introduced fungus due to the non-indigenous state of its host. According to our results, *P. laricis* is widely distributed in Fennoscandia, using spruce as its host.

In Central Europe, the elevation (≥ 1400 m in the High Tatras in Slovakia) and native distribution of the hosts (*Pinus* and *Larix*) are reported to be crucial for the occurrence of *Phellinus vorax (= P. laricis)* (Černý, 1989). Our observations support this belief; *P. laricis* growing on *Pinus mugo* in the Bohemian Forest (= Šumava Mts., Czech Republic) occurs under different climatic conditions than *P. pini* inhabiting *Pinus sylvestris* in the adjacent area. While the only collection of *P. laricis* there was recorded at ca. 1300 m, the highest elevation recorded for *P. pini*

is 825 m, and the centre of distribution of *P. pini* is around 700 m or below (Tomšovský, 2002). Kotlaba (1984) confirms the distribution of *P. pini* between 155-780 m in elevation in the former Czechoslovakia. Jahn (1963) points out the occurrence of *P. chrysoloma* (under the name of *Phellinus pini* var. *abietis*) not only on *Picea*, but also on *Abies* and *Larix*. These host species are also cited by more recent publications (Kotlaba, 1984; Černý, 1989; Ryvarden and Gilbertson, 1994), but we did not obtain a specimen from *Abies*. Our results confirm that fungi on *Larix* may belong to either *P. laricis* or *P. pini*. The native status of the host trees, correlated with elevation, is crucial for occurrence of the respective species.

Collections of *P. pini* from *Larix* have been mentioned previously by Černý (1985, 1989) and Kotlaba (1984), while Ryvarden and Gilbertson (1994) reported *Pinus* as the only host genus of this fungus. Therefore, the host spectrum of *P. pini* might extend to non-indigenous conifers.

The complex phylogeographical structure of Euroasian *Porodaedalea* deserves to be studied in detail. Questions about the histories of local populations are posed by the occurrence of a common nucleotide substitution among geographically distant specimens from Scandinavia, the Czech Republic, and Kazakhstan (i.e., the "*P. laricis* B" group). Historical changes in host distributions probably led to gene-flow and introgression of new genotypes that may have survived in local areas.

5. ACKNOWLEDGEMENTS

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Abstracts

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EXAMINING THE GEOGRAPHIC DISTRIBUTION OF *Diplodia pinea* AND *D. scrobiculata*: A CASE STUDY FROM MINNESOTA, USA

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Although the shoot blight and canker pathogen *Diplodia pinea* is more commonly reported and is distributed in native and exotic pine stands in much of the world, a second very similar and closely related fungus, *D. scrobiculata*, has been detected in the USA, Mexico, France, Israel, Italy, and Spain, and is likely present in other countries. Both of these fungi are associated with red pine (*Pinus resinosa*) and jack pine (*P. banksiana*) in the northcentral and northeastern USA. Their distribution in Minnesota was studied by examination of seed cones (on which these fungi sporulate). 100 cones collected from the forest floor of each of 109 red pine stands and 28 jack pine stands were visually examined for *Diplodia* pycnidia and conidia. At least one of these fungi was detected from 106 of 109 red pine stands and from all jack pine stands. Mean frequencies of positive red and jack pine cones, respectively, were 27% (range 0-84%) and 12% (range 2-41%). PCR assays confirmed pathogen identity for subsets of cones. *D. pinea* was detected from cones collected at 102 of 109 red pine stands (69% of red pine cones tested), and 18 of 28 jack pine stands (18% of jack pine cones tested). In contrast, *D. scrobiculata* was detected from cones collected at only 26 of 109 red pine stands (7% of red pine cones tested), but 26 of 28 jack pine stands (79% of jack pine cones tested). These fungi sometimes co-occurred in stands of either host, and occasionally both were detected from individual cones of either host. Although differences between *D. pinea* and *D. scrobiculata* in host association, presence at a given location, and frequency of occurrence at a given location were apparent, each was found across the entire area surveyed.

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FOREST INVASIVE ALIEN FUNGAL SPECIES PRESENT IN LIVE PLANT MATERIAL

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An early warning system based on a random sampling of asymptomatic live plant material arriving in Canada is used to detect alien fungal pests. Forty-six sample lots collected by Canadian Food Inspection Agency (CFIA) inspectors from the province of Quebec were analyzed by cloning the fungal ribosomal ITS present in the plant tissues. We obtained 101 fungal species associated with 36 different host plants from the USA, France, the Netherlands and Thailand. Six fungal species found in this study could have a low to moderate potential impact and 11 could have a low potential impact for Canadian forests. Another 14 species could not be assessed given the limited scientific information available. In all cases, the potential impact evaluations of these 31 species originate from the fact that these species are new to science and/or belong to genera and families where pathogenic species are common. The alien fungal introductions with a potential to affect Canadian forests were found at a significant frequency (12.4%) and were present in every sample lot sent by CFIA. The 70 other species found in this study were non-pathogenic fungi; weak to moderately virulent, common and cosmopolitan species; or virulent species found on tropical hosts only.

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NEW ADVANCES IN THE STUDY OF THE TAXONOMY OF THE EUROPEAN RACE OF GREMMENIELLA ABIETINA

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In order to investigate the taxonomy of the European race of *Gremmeniella abietina* var. *abietina* ten Spanish isolates were randomly chosen among 91 to be analyzed genetically and compared with 7 Swiss isolates biotype Alpine, 10 Finnish isolates biotype A and 10 Finnish isolates biotype B randomly chosen in the same way. RAMS markers CCA, CGA and GAAA1000 supported by sequence analysis of the locus GAAA1000 were used in this report to study the genetic variability of Spanish and Swiss isolates in respect of the rest of European biotypes and, thus, to establish what biotype they belong to. Philogenic relationships among A, B, Alpine and Spanish sequences analyzed based on the neighbour-joining method showed that Alpine type, that is, Swiss sequences, is more closely related to B type, and Spanish isolates appear clearly separated from the rest of the biotypes.

Keywords: *Brunchorstia pinea* – European race – *Pinus halepensis* – RAMS – taxonomy.

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STUDIES ON THE SIGNIFICANCE, CAUSAL AGENTS AND CONTROL METHODS OF DAMPING- OFF DISEASE IN FOREST NURSERIES OF AEGEAN AND LAKES DISTRICT

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The survey of containerized and bare rooted seedlings of *Pinus brutia* (Ten.), *Pinus nigra* subsp. *pallasiana* (Lamb.)Holmboe, *Cedrus libani* (A. Rich), *Pinus pinea* (L.) and *Ailanthus glandulosa* (Desf.) showed that damping- off intensity was higher in the nurseries of Lakes District than that of Aegean District. The most prevalent fungi were found *Fusarium* spp., *Rhizoctonia solani* (Kühn.), *Pythium* spp., *Alternaria* spp. and *Macrophomina phaseolina* with the rates of 53, 19, 10, 10 and 6 %, respectively.

Five hundred fifty fungal isolates were collected from diseased seedlings and 108 of them were tested for their pathogenicity. Total 283 fungi and 200 bacteria were isolated from natural forest soil and among them, *Gliocladium virens*, *Trichoderma koningii*, *Penicillium ademetzi*, *Myrothecium verrucaria*, *Paecilomyces lilacinus*, 2 Streptomyces and 3 unidentified bacteria were found to have strong antibiotic activity against the selected pathogenic isolates. Five fungicides, propomocarp, hymexazole, thiram, PCNB and propineb, were evaluated under in vitro against the antagonists. Antagonistic isolates showed a very high degree of sensitivity to the fungicides with the exception of promomocarp and hymexazole.

In vivo studies were carried out as biological, chemical and integrated control of 5 damping- off pathogens on *P. brutia* and *P. nigra*. After soil infestation with pathogens, the fungicides were applied by soil drenching and the antagonist application was achived by seed coating. Propomocarp and hymexazole were found more effective than the other fungicides and gave the best results when they were used with the antagonists in combination.

Key words: *Pythium* spp. , *Macrophomina phaseolina*, *Rhizoctonia solani*, *Alternaria alternata*, *Fusarium* spp., Chemical control, biological control.

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A FOLIAR DISEASE OF *Celtis glabrata* IN THE LAKES REGION

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Celtis species are widely distributed in Turkey and has 4 species. One of them is *C. glabrata* (Steven ex Planchon) and is found in the Lakes region. A fungal disease was commonly observed on *Celtis* leaves with irregular, large, black spots on both sides and velvety apperance on the lower side. Disease incidence was so high that all the leaves of the trees showed symptoms with varying degrees. As a result of the microscopic examination of the lower leaf surfaces, the fungus was identified as *Sirosporium celtidis* (Biv., Bern. Ex Sprengel) M. B. Ellis. Since *C. glabrata* is not grown in forest nurseries in our country, the fungus is not economically important. However, it can cause defoliation and poor growth of trees in natural ecosystems.

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DETERMINATION OF MACROMYCETES IN THE REGION OF KOCAELI

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This study entitled "Determination of Macromycetes in the Region of Kocaeli" was carried out to identify macrofungi species growing in the province of Kocaeli. The aim of these types of studies can be summarized as to identify species, to determine its rank in the systematic and to delineate the species'in distribution area.

In the first step, the literature on the macrofungi was studied, and the relevant information was collected. The samples were collected following determining the field sites . All of the relevant information on the samples collected was recorded. Each sample was later carefully excavated, bagged separately and transferred to the lab.

The internal morphological characteristics of the reproductive organs including the cap and lamella of the samples were determined. The cap characteristics including shape, dimension, color, color change, centrality, and lamella or pore structure, and the odor and degree of density of the succulent part were determined. Various features of the stem including length, color, color change, and the existence of stem as well as the ring and residuals, if existed, were also noted.

Moreover, in order to determine spore print, one reproductive organ was sampled for each sample brought to the lab. The porous part of the cap was placed on a white sheet and protected from air currents that would sweep the fallen pollens. At least 12 hours later, spore print's color was captured on the sheet.

The macrofungi was identified after the information collected from the field sites and the lab study had been compared with the literature. At the end of the study, 89 macrofungi species, 79 of which belonged to the Basidiomycetes class and 10 of which were of the Ascomycetes class were indentified in the province of Kocaeli.

Keywords: Macrofungi, Kocaeli, forest, systematic, phytopatology.

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ARE SUBPOPULATIONS OF *Heterobasidion parviporum* DIFFERENTIATED BY LOCAL CLIMATE?

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We determined the decomposition rate (DR) of spruce wood by representatives of different subpopulations of *Heterobasidion parviporum* at various temperatures. Sixty three *H. parviporum* isolates originating from geographically distant and climatically varying environments (Finland, Denmark, Italy and Central Siberia) were cultivated at eight temperatures between 6°C and 33°C on Norway spruce saw dust as the only substrate. Decomposition activity was determined as the production of carbon dioxide. The optimal temperature for decomposition varied considerably between the isolates and ranged between 20°C and 30°C. The activity of all isolates decreased drastically at temperatures from 30°C to 33°C, being at 33°C only 7 % of that at 30°C. The highest between-isolate variations in DR were at the extremes of the applied temperature scale, at 33°C and 6°C.

The DR of the four subpopulations did not differ significantly from each other at any temperature, neither was found any variation according to the age of the cultures (0.3 - 16 y) (ANOVA). However, the Italian and Siberian isolates were collected from several locations in which the climate varied considerably, and the highest monthly average temperature of each district explained partly the DR of the isolates at 6°C ($p = 0.017$). When only the Italian isolates were included in the ANOVA, a similar significant variation was found ($p = 0.043$, $n = 15$). The highest monthly average temperature of the location correlated negatively with the DR of *H. parviporum* at 6°C. Hence, local climate affects significantly the DR of *H. parviporum*.

Gene variation of different isolates was studied with six microsatellites and by determining the DNA sequences of three sequence characterized amplified regions. Interestingly, no significant genetic variation was found between Italian, Danish and Finnish isolates. This suggests that there is significant gene flow between these subpopulations of *H. parviporum*, and that the variation in DR at 6°C between isolates from different localities may be a consequence of other factors than restricted gene flow.

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ATTEMPTS TO NATURALLY REGENERATE RED PINE CAN BE THREATENED BY DIPLODIA SHOOT BLIGHT DAMAGE TO UNDERSTORY SEEDLINGS

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Changes in red pine (Pinus resinosa) management, due to aesthetic and biodiversity concerns, include creation of harvest units with irregular edges, long borders of mature trees, and retention of some overstory trees within a harvested area. Also, in contrast to traditional even-aged management in which trees of one age class are grown, clearcut at final harvest, and replaced by planted seedlings, there is increasing interest in natural regeneration and developing multi-aged red pine stands. However, crowns of red pines can be sources of abundant inoculum of the shoot blight pathogen *Diplodia pinea*. To determine if Diplodia shoot blight threatens young, naturally regenerated red pine in the understory, six replicate plots were established in each of four mature plantations in central Wisconsin. The frequency of standing, dead seedlings bearing shoot blight symptoms or signs of the pathogen, and the incidence and severity of shoot blight damage to live seedlings were recorded. Mean seedling mortality ranged from 13-30% and mean incidence of blighted living seedlings ranged from 94-100% at all sites. The mean frequency of live seedlings with their terminal leaders killed in the past was from 55-94%. Mean severity of damage to live seedlings, on a 0-3 scale, was ÿ2.16 at all sites. Results of a PCR assay confirmed pathogen identity. These results support previous research and concern that shoot blight pathogens threaten young red pines in the understory.

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SOME FUNGAL SPECIES ON *Pinus pinaster* Ait. AND *Pinus radiata* D. Don PLANTATIONS IN MARMARA REGION OF TURKEY

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The global forest areas decrease with the growing world population. The protection of the natural forest is necessary and the most of wood production should be done from outside of the natural forest.

The industrial plantation give yields about 13,8-55,9 m3/ha/year for rotation between 10-30 years. The industrial plantations entrepreneurship gains importance as an effective sector in the countriers such as Chile, Brasil, USA, South African, New Zealand, Australia, South Korea

Regular afforestation began in 1955 in Turkey. The plantation of Forestry began in 1963. The studies and researchs on Fast Growing Forest trees were given to the Poplar Research Institute from General Directorate of Forestry in 1968

Marmara Region is located on the the Northwest of Turkey. The East Longitudes of region are 25^0 $50^{'}$ -30^0 $55^{'}$, the North latitudes of region are 39^0 $06^{'}$-42^0 $05^{'}$

The area of the Marmara Region is 67300 Km^2

Totally in the Turkey, there are 42 185 hectares plantation areas of Pinus pinaster and 949 hectares plantation areas of *Pinus radiata*

The aim of this study is to determine the harmful fungi on *P. pinaster* and *P. Radiata* plantations of the Marmara Region. The observation in plantation were done in 2003. Until now, the following fungal species have been identified: *Pluteus plautus, Lenzites striata, Pleurotus astreatus, Schizophyllum commune and Streum purpuretum*

Keywords: *Pinus pinaster*, *Pinus radiata* plantations, harmful fungi.

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RESPONSE OF *Alnus tenuifolia* TO INOCULATION WITH *Valsa melanodiscus*

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Valsa melanodiscus (anamorph *Cytospora umbrina*) is associated with cankered and killed alder (*Alnus*) stems in western North America from Colorado to Alaska. The responses of thinleaf alder (*A. tenuifolia*) stems to inoculation with each of two isolates of *V. melanodiscus* were studied in south-central Alaska. At each of two sites, eight stems per isolate were wounded to expose both inner bark and sapwood and inoculated in early May 2007 by placing a colonized agar plug over the wound. Sterile agar plugs were applied to wounded control stems. Sunken, elongated cankers similar to those with which *V. melanodiscus* has been associated resulted on inoculated stems. In contrast, wounded control stems exhibited strong callus production and wound closure. In September 2007, cankers were harvested and lengths were recorded. Mean canker lengths measured externally (data for both isolates pooled) at the two sites were 45 (range 20-156) mm and 73 (range 22-201) mm. Analysis of variance of log transformed data revealed strong support for effect of location ($P = 0.04$), but not that of isolate ($P = 0.12$) or interaction ($P = 0.20$) on canker length. The fungus was reisolated from each inoculated stem, but not from any control stem. The ability of *V. melanodiscus* to cause cankers on thinleaf alder stems is confirmed, and these results support the conclusion that this pathogen is a cause of alder dieback in western North America.

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GREMMENIELLA INFECTIONS ON SEEDLINGS AFTER REPLANTING SEVERELY INFECTED PINE FOREST

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During 1999 and 2001 the most severe *Gremmeniella abietina* epidemic ever appeared in Sweden. More than 300 000 ha forest were severely attacked and the forest industry lost milliards of SEK. Big forest areas, with at least 50 000 ha, needed to be clear cut in advance followed by replanting.

For the out-planting experiment pine seedlings were planted on three different locations in Dalarna in Sweden, that were clear-cut in advance due to severe *Gremmeniella* infection. The forest had been clear-cut in 2001 and this study was conducted during 2002-2005. Each site contained one clear cut area and a nearby *Gremmeniella* infected forest. Seedlings were planted on clear-cut areas with and without remaining twigs and branches, at the edge of the clear-cut areas, as well as in the adjacent forest. In total at least 200 seedlings were planted on each area. The areas were replanted every year with new one year old seedlings received from a forest nursery. The disease incident was determined visually the year after plantation and then the infection was confirmed with PCR using *Gremmeniella* specific primers.

For seedling planted at the clear cut areas, the infection decreased from 50 - 90% infected seedlings planted one year after felling to 0 - 55% planted the second year after felling and to 0 -38% for seedling planted three years after felling. After four years there was almost no infection on the clear-cut areas. The variation was big between the sites. Two and three years after felling there were almost no differences between seedlings planted on areas with or without twigs and branches. However seedling planted in the adjacent diseased forest became much more infected then seedlings planted on the clear cut areas. For seedlings planted in the forest one year after felling almost all seedlings became infected. Two and three years after felling they became infected to up to 50 % and even four years after felling 15-40 % of the seedlings became infected. The seedlings planted close to the forest edge were always more infected then the seedlings out on the clear-cut areas but less infected than the seedlings in the forest.

It is remarkable that the disease incidence on seedlings in the forest still is high after 3-4 years but we assume that the snow cover during winter have promoted the infections in small seedlings. From inventory in mature forest in the same areas it

has been found that the disease incidence decreased after some years after the severe outbreaks in 1999 and 2001. In spite of this it means that several years after a severe infection there is still a lot of viable spores left, particularly in the forest. More then two years after felling it seems to be a trend that seedlings planted in the middle of the clear-cut areas were less infected then seedling planted close to the forest. The variation between infections in the forest on the different sites was low compared to infections on the clear cut areas. The general conclusion is that it is advisable to wait with replanting with pine seedlings at least two years after felling severely *Gremmeniella* infected stands. Then the risk of infection form twigs and branches left on the clear cut area also is smaller.

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