From Forest Nursery Notes, Summer 2013

80. Distribution and genetic diversity of root-rot pathogen *Neonectria macrodidyma* in forest nursery. Menkis, A. and Vasaitis, R. Proceedings of the 7th Meeting of IUFRO Working Party 7.03.04, p. 113-117. USDA Forest Service, Southern Region, Forest Health Protection Report 10-01-01. 2010.

DISTRIBUTION AND GENETIC DIVERSITY OF ROOT-ROT PATHOGEN *NEONECTRIA MACRODIDYMA* IN FOREST NURSERY

Audrius Menkis and Rimvydas Vasaitis
Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden (Audrius.Menkis@mykopat.slu.se)

ABSTRACT

The aim of this study was to assess fungal communities in fine living roots of *Pinus sylvestris* seedlings in a forest nursery and to determine genetic diversity and spatial distribution of common fungal symbiont. Root systems of 100 seedlings were collected using 1.5m x 1.5m grid design in the area of 225 m^2. From each root system, 20 individual fine roots were sampled randomly, surface sterilized and plated on MMN media for fungal isolation. Isolation yielded 606 pure cultures, which using morphological and molecular methods, were identified as 71 distinct taxa. Root-rot pathogen *Neonectria macrodidyma* was the most common isolate and comprised 20.3% of the total fungal community. As ITS rDNA sequences were 100% identical for all strains, polymorphism of intergenic spacer (IGS) of rDNA was studied by means of double restriction digestion of 3.8 kbp-long PCR amplicons to determine genetic diversity of *N. macrodidyma* isolates. Restriction digestion showed that among 123 strains of *N. macrodidyma* only two distinct IGS types were present at the frequency 40:83. Mapping data and estimates on spatial distribution using nearest neighbor method revealed overlapping occurrence and even distribution of both IGS types in the study area. Results of this study indicate that *N. macrodidyma* is commonly associated with fine living roots of pine seedlings, is largely disseminated by vegetative means of local genotypes and has even distribution in forest nursery soils. Furthermore, in living roots *N. macrodidyma* is likely present as dormant propagules but under favorable conditions it may develop rapidly and have a significant negative effect on plant health and productivity.

INTRODUCTION

In forest nurseries, seedling production may often be limited by root diseases caused by fungal pathogens. Typical symptoms of root infections in conifer seedlings are stunted growth, discoloration of needles and partial or complete death of the root systems (Lilja and others 1992), which essentially may lead to a significant decrease in plant quality. In some cases, due to the intensive management practices, aboveground disease symptoms in the nursery may be absent but seedlings, infected with root pathogens, may exhibit reduced survival rates following their outplanting in the field.

Fungi from the genera *Fusarium*, *Neonectria*, *Rhizoctonia*, and *Pythium* have been reported as the main causal agents of the root dieback in forest nurseries (Galaaen and Venn 1979, Lilja and others 1992, Kope and others 1996, Lilja and Rikala 2000, Menkis and others 2006). A majority of these fungi are considered to be opportunistic, necrotic pathogens that produce toxins to invade and kill the plant tissues (Unestam and others 1989, Beyer-Ericson

and others 1991). They may often act as saprotrophs while being attached to the surface of living roots, but stress and reduction in seedling vitality may induce a rapid pathogenic response by these fungi (Unestam and others 1989). Although there is a substantial amount of information on incidence of root pathogens in bare-root forest nurseries, information on genetic diversity, distribution on the local scale and potential importance of those fungi on asymptotic seedling roots is largely unavailable. Such information is also of practical importance allowing further optimization of management practices in forest nurseries.

In this study, fungi associated with seedling roots were assessed in a confined nursery plot using systematically sampled seedlings of *Pinus sylvestris*. Combining fungal isolation into pure culture and molecular fingerprinting, we determined both communities of root inhabiting fungi and genetic diversity and spatial distribution of common fungal symbiont.

METHODS

The study site was located in a forest nursery situated in the vicinity of the Baltic Sea coast in western Lithuania. This nursery produces seedlings using standardized bare-root cultivation in beds. Two-year old seedlings of *Pinus sylvestris* were sampled after the growing season in October 2007. The sampling area was 225 m^2 in size and included four adjacent beds each 1.5 m width and 37.5 m long. In total, 100 seedlings were sampled using a systematic grid design at a spacing of 1.5 m x 1.5 m. Seedlings were gently excavated to preserve fine roots, individually labeled, packed into plastic bags, transported to the laboratory and kept at 4°C until analysis.

The isolation of fungi into pure culture was attempted from 2000 individual root tips, which were obtained by randomly sampling 20 individual root tips from each root system of 100 plants. Before isolation, root tips were placed in 10 × 20 mm net bags (mesh size 0.2×0.2 mm), sterilized in 33% hydrogen peroxide for 30 s, and then rinsed three times in sterile deionised water. About ten tips per each Petri dish were plated onto modified Melin Norkrans medium (Marx 1969) and incubated at room temperature in the dark. Dishes were checked daily and any newly growing mycelia was immediately subcultures onto fresh agar media. Isolated cultures were examined under a microscope (Carl Zeiss Axioplan, Oberkochen, Germany) equipped with 10× ocular and 25× long distance objective magnification, and grouped into mycelial morphotypes.

For identification, the internal transcribed spacer of the fungal ribosomal DNA (ITS rDNA) was sequenced for representatives of each mycelial morphotype using primers ITS1F and ITS4 (White and others 1990). Extraction of DNA, amplification and sequencing followed established methods described by Rosling and others (2003). Raw sequence data were analyzed using the SeqMan version 5.01 software from DNASTAR package (DNASTAR, Madison, WI, USA) and BioEdit version 7.0.5.2 (Hall 1999). Databases at GenBank (Altschul and others 1997), UNITE (Koljalg and others 2005) and at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala were used to determine the identity of sequences.

Genetic diversity of fungal isolates was studied by using PCR based restriction fragment length polymorphism (PCR-RFLP) of intergenic spacer (IGS) of the nuclear ribosomal DNA. Amplification of the entire IGS region, situated in between 26S and 18S genes of rDNA, was done by primer pair LR20R and SR7. Description of those primers is available at http://www.lutzonilab.net/primers/index.shtml. Double digestion of amplified PCR products was done by using restriction enzymes HinfI and HhaI (Fermentas Life Sciences, Germany) according to the manufacturer's recommendations. Restriction fragments were separated by electrophoresis on 1% agarose gels (Agarose D1,Conda, Spain) in 1x SB buffer (Brody and Kern 2004) for 3h at 150V. The gels were stained with ethidium bromide and obtained images were analyzed by Quantity One version 4.6.3 (Bio-Rad laboratories, CA, USA) software. Clark – Evans nearest neighbor statistics (Clark and Evans 1954) were used to estimate whether distribution of isolated fungi in nursery plot was random, even or clustered.

RESULTS AND DISCUSSION

Of 2000 fine roots used for isolation of fungi into pure culture, 606 (30.3%) resulted in fungal growth, and the remaining 1394 (69.7%) either remained sterile following surface sterilization or were colonized by bacteria and/or rapidly growing fungi from neighboring roots plated in the same dishes. Therefore, isolation in this study yielded 606 pure cultures, which following morphological and molecular identification, were recognized as 71 distinct taxa. Of those, 50 (70.4%) were identified at least to genus level. For unidentified taxa, only 4 (5.6%) could be matched to ITS rDNA sequences available in the databases and 17 (23.9%) showed unique sequences. Of the total isolated fungal community, 89.3% were ascomycetes and deuteromycetes and 10.7% were basidiomycetes. Frequently isolated fungi were ascomycetes and deuteromycetes *Neonectria macrodidyma* (20.3%), *Phialocephala fortinii* (13.5%), Unidentified sp. PM29C (4.8%) and basidiomycete *Hebeloma cavipes* (4.6%).

Neonectria macrodidyma appeared to be the most commonly isolated taxon from the healthy-looking root tips of bareroot nursery cultivated pine seedlings. This indicated that *N. macrodidyma* play an important role in determining plant health and productivity and may be of great economic importance. To gain more specific information on this species, both genetic diversity of the isolates and their spatial distribution in the confined nursery plot was studied. Firstly, we identified sequence variation within ITS rDNA for 123 isolates of *N. macrodidyma.* As ITS rDNA sequences were 100% identical for all strains, polymorphism of IGS rDNA was studied by means of double restriction digestion of PCR amplicons. In *N. macrodidyma* a total size of amplified IGS rDNA region was ca. 3.8kb in size. Restriction digestion of amplified products showed that among all strains of *N. macrodidyma* isolated in this study, only two distinct IGS types were present at the frequency 40:83. Mapping data for isolates of each IGS type and estimates on their spatial distribution revealed that both IGS types were intermingled and evenly distributed in the nursery plot.

Neonectria macrodidyma was recently described as a new species (Halleen and others 2004), commonly associated with black foot disease of wine grapes in a wide geographic area (Alaniz and others 2007, Auger and others 2007). Disease symptoms included drying and

dying shoots, abnormal development and necrosis of roots, black discoloration of the wood and overall reduction in root biomass. In our previous studies, this species was also commonly isolated from healthy-looking and diseased roots of nursery grown conifer seedlings (Menkis and others 2005, Menkis and others 2006). This suggests that *N. macrodidyma*, similar to many other *Neonectria* spp., is a plant pathogen well adapted to a wide range of hosts and habitats.

The results of the present study confirmed an earlier observation on intimate association of *N. macrodidyma* with living tree roots. More importantly, information on both limited genetic diversity of isolates and their even distribution in the nursery plot was acquired. The latter results suggests that *N. macrodidyma* is largely disseminated by vegetative means of local genotypes and that soil cultivation practices is likely contribute to dissemination of this species in the forest nursery soils. Often isolation of this species from the asymptotic fine roots may further suggest that in living roots *N. macrodidyma* is present as dormant propagules, but under favorable conditions it may develop rapidly and have a significant negative effect on plant health and productivity.

ACKNOWLEDGEMENTS

Financial support is greatly acknowledged to Helge Ax:son Johnsons Stiftelse, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) and European Commission Marie Curie Transfer of Knowledge Project No. 042622 LITCOAST.

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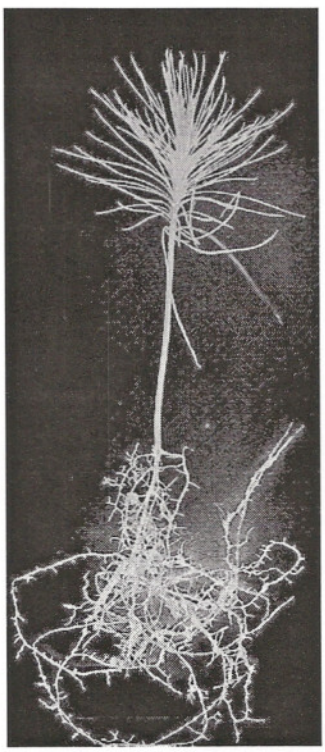
United States
Department
of Agriculture

Forest Service

Southern Region

Forest Health Protection
Report 10-01-01

Proceedings of the 7th Meeting of IUFRO Working Party 7.03.04 Diseases and Insects in Forest Nurseries



2010

Proceedings of the Seventh Meeting of IUFRO Working Party 7.03.04

Diseases and Insects in Forest Nurseries

Hilo, Hawai'i USA
July 13 to 17, 2009

Edited by
Michelle M. Cram

Forest Health Protection
United States Department of Agriculture
Forestry Service, Southern Region
Forest Health Protection Report 10-01-01

2010