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Proceedings of the First IUFRO Rusts of Forest Trees Working Party Conference, 2–7 Aug, 1998, Saariselkä, Finland

Edited by
Risto Jalkanen
Patricia (Pat) E. Crane
James (Jim) A. Walla
Tarmo Aalto

ROVANIEMI RESEARCH STATION

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The first conference of the IUFRO Rusts of Forest Trees Working Party (7.02.05), formerly the Rusts of Pines Working Party, was held on August 2–7, 1998, in Saariselkä, Lapland, Finland, with participants from 12 countries. The proceedings consists of 41 papers or abstracts, including those from the oral and poster presentations made at the conference and a few papers submitted by scientists who were unable to attend the conference. The articles cover various aspects of the taxonomy, epidemiology, disease resistance, control, genetics, molecular biology, distribution, ecology, and physiology of specific rusts of forest trees in Asia, Europe, North America, and South Africa. One paper provides a review of tree rusts in Hungary and another of tree rusts in Finland. A general paper discusses the systematics of rust fungi in the family Melampsoraceae and another deals with the taxonomy of autoecious pine stem rusts. Other papers concern one or more of the following rusts: *Melampsora* on *Populus* or *Salix* leaves, *Melampsora pinitorqua* on *Pinus* shoots, *Melampsoridium* on *Alnus* or *Betula* leaves, *Chrysomyxa* and *Thekopsora* on *Picea* needles or cones, autoecious stem rusts of *Pinus* (*Peridermium pini*, *Endocronartium* (*Peridermium*) *harknessii*), *Peridermium kurilense*), and heteroecious stem rusts of *Pinus* (*Cronartium flaccidum*, *Cronartium ribicola*, *Cronartium comandrae*, *Cronartium quercuum*).

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Contents

Preface 7

General

Szabó, I.
Occurrence and importance of rust fungi in Hungarian forests 9

Kurkela, T. and Jalkanen, R.
Checklist of the rust species recorded on trees in Finland 15

Berndt, R.
Haustorial characters as markers in the systematics of Melampsoraceae 19

Hiratsuka, Y.
Cytology, taxonomy and nomenclature of autoecious pine stem rusts..... 31

Melampsora on poplar

Bagyanarayana, G.
The species of *Melampsora* on *Populus* (Salicaceae) 37

Xu, M.
Leaf rust of *Populus* section Leuce in China 53

Pinon, J., Frey, P., Husson, C. and Schipfer, A.
Poplar rust (*Melampsora larici-populina*): the development of
E4 pathotypes in France since 1994 57

Bourassa, M., Bernier, L., Milligan, B. and Hamelin, R.
Sympatry between alternate hosts affects population structure of
poplar leaf rust 65

Cellerino, G.P. and Gennaro, M.
Microflora associated with the phylloplane of healthy cultivated poplars
and those attacked by *Melampsora* spp. 71

Gennaro, M. and Cellerino, G.P.
Assessment of the *in vivo* antagonism of phylloplane fungi and bacteria
against *Melampsora* on cultivated poplars 85

Steenackers, M., Steenackers, V., De Cuyper, B. and Michiels, B.
Breeding and selection of poplars for stable resistance to
Melampsora larici-populina 97

Hawke, G. and Laing, M.D.
Fungicide trials conducted to determine the potential for controlling
rust infections on poplars 105

Melampsora on willow

Nakamura, H., Kaneko, S., Yamaoka, Y. and Kakishima, M.
PCR-RFLP analysis of *Melampsora* spp. on willows in Japan 113

Hakulinen, J.
Variation in phenolic levels of willow leaves (*Salix myrsinifolia*) in relation to occurrence of *Melampsora* rust 121

Ramstedt, M.
Diversity of the *Melampsora* rust on willows — consequences for SRF planting strategies 123

Melampsoridium on alder and birch

Kurkela, T., Hanso, M. and Hantula, J.
Melampsoridium sp. on alder leaves 131

Poteri, M., Rousi, M. and Ryynänen, L.
Interaction between birch, *Betula* spp., and *Melampsoridium betulinum*, the leaf rust fungus 135

Elamo, P., Saloniemi, I., Helander, M. and Neuvonen, S.
Genetic and environmental variation in susceptibility of mature mountain birch trees to leaf rust 137

Chrysomyxa and *Thekopsora* on spruce

Crane, P.E., Hiratsuka, Y., Currah, R.S. and Jalkanen, R.
Systematics of spruce needle rusts in the *Chrysomyxa ledi* complex 139

Jalkanen, R. and Nikula, A.
Cone rusts on Norway spruce in northern Finland 145

Rusts on hard pines

Desprez-Loustau, M.-L., Capron, G., Dupuis, F. and Wagner, K.
Phenological synchronization in the twisting rust – maritime pine pathosystem 149

Hantula, J., Niemi, E.M., Kaitera, J., Moricca, S., Jalkanen, R. and Kurkela, T.
Genetic variation in *Cronartium flaccidum* and *Peridermium pini* 157

Kasanen, R.
The genetic variation within cankers of *Peridermium pini* 163

Moricca, S. and Ragazzi, A.
Occurrence and significance of *Cladosporium tenuissimum* on two-needle pine rust aeciospores 171

Walla, J.A., Wang, C., Schumann, C.M. and Tuskan, G.A.
Peridermium harknessii in the north-central U.S. may be a complex of taxa 183

van der Kamp, B.J.
Assessing gall rust resistance: the effect of local spore concentration and auto-infection 191

Bergdahl, D.R. and French, D.W.
Colonization of the primary tissues of *Pinus banksiana* by *Cronartium comandrae* 195

Miller, T., Gramacho, K.P., Schmidt, R.A., Amerson, H.V. and Kuhlman, E.G.
Responses of wound-inoculated seedlings of *Pinus elliottii* var. *elliottii* and *Pinus taeda* to mycelial cultures derived from multiple and single basidiospores of *Cronartium quercuum* f. sp. *fusiforme* 207

Schmidt, R.A. and Allen, J.E.
Spatial stability of fusiform rust resistance in slash pine in the coastal plain of the southeastern USA 219

Spaine, P.
Research on the biology of fusiform rust in the southeastern United States 231

Nakamura, H., Kaneko, S. and Spaine, P.
Differences in molecular characteristics between *Cronartium quercuum* from Japan and fusiform rust from USA 235

Yamamoto, F., Nakamura, K. and Hiratsuka, Y.
Is ethylene a trigger of stem hyperplasia caused by eastern gall rust in *Pinus densiflora*? 243

Rusts on soft pines

Imazu, M., Azbukina, Z.M., Kakishima, M., Ono, Y. and Kaneko, S.
Taxonomic reconsideration of *Cronartium ribicola* and *Peridermium kurilense* in the Russian Far East 253

Hamelin, R.
Molecular epidemiology of white pine blister rust 255

Cheng, D., Xue, Y., Liang, H., Pan, X. and Li, W.
Variation in isozymes, RAPDs and SEM-based morphology among three species of pine stem rust fungi from China 261

Kinloch, B.B., Jr., Westfall, R.D. and Dupper, G.E
Gene flow in *Cronartium ribicola* 269

Van Arsdel, E.P., Conklin, D.A., Popp, J.B. and Geils, B.W.
The distribution of white pine blister rust in the Sacramento Mountains of New Mexico 275

McDonald, G.I. and Decker-Robertson, D.L.
Long-term differential expression of blister rust resistance in western white pine 285

Zambino, P.J., Pijut, P.M., Echt, C.S. and Michler, C.H.
Dry-grinding miniprep method for extracting DNA from urediospores, aeciospores, and teliospores of white pine blister rust, *Cronartium ribicola* 297

Laflamme, G., Blais, R. and Desgagné, D.
Control of *Cronartium ribicola* in *Pinus strobus* plantations 299

Bérubé, J.A., Trudelle, J.G., Carisse, O. and Dessureault, M.
Endophytic fungal flora from eastern white pine needles and apple tree leaves as a means of biological control for white pine blister rust 305

Preface

A conference of the newly established Rusts of Forest Trees Working Party (7.02.05) of the International Union of Forest Research Organizations (IUFRO) was held in northern Finland from August 2 to 7, 1998. This was the first meeting of the former Rusts of Pine Working Party in its expanded format to include other tree rusts. Forty-two forest pathologists and mycologists from 12 countries, Belgium, Canada, China, Finland, France, Germany, Hungary, Italy, Japan, South Africa, Sweden, and USA, gathered in northern Finland.

The organizing committee, chaired by Risto Jalkanen of the Rovaniemi Research Station, Finnish Forest Research Institute, together with the committee members, Tarmo Aalto, Helena Poikajärvi, Pekka Närhi and Miia Marjaranta from the same station, did an excellent job of organizing and carrying out the conference.

The conference consisted of three days of paper presentations at the Hotel Riekonlinna with a half-day field trip on the 2nd day with Mr. Ari Siekkinen of the Forest and Park Service. Twenty-eight oral presentations and eight poster presentations were made. With the addition of papers on tree rusts other than pine, especially leaf rusts on poplar and willow, we had very informative sessions, including aspects of taxonomy, epidemiology, disease resistance, control and biotechnology of rusts of forest trees.



The participants of the field trip of the first IUFRO Rusts of Forest Trees Working Party Conference meeting Santa Claus outside his office in Rovaniemi, Lapland, Finland. August 6, 1998.

Travelling from Saariselkä southward to Rovaniemi on the Arctic Circle, we visited several field locations and also visited several culturally interesting places such as an old wooden church at Sodankylä, the Santa Claus Village, and Arktikum Museum in Rovaniemi. Risto's wife, who is a professional tourist guide, was very helpful and made our trip to these places very informative and interesting. Another day of field trips to Vanttauskoski and Kaihuavaara near Rovaniemi was organized to see various tree rusts. The very enjoyable preconference tour was done to North Cape, northern Norway.

Conference participants enjoyed and were all very impressed by the careful and tasteful arrangements prepared during the congress. Besides the scientific aspects of the conference, we were treated to many memorable events such as a Kuksa (wooden cup) initiation ceremony around an open campfire, excellent parties held at the Hotel Riekonlinna (Lapin Kulta Evening), Saariselän Panimo (Saariselkä Brewery), and the Rovaniemi Research Station, and a boat cruise on the Kemijoki River. During the five days, we were served many interesting and delicious Finnish foods and drinks, such as reindeer meat, salmon, cloudberry desserts, Lapin Kulta beer, and pine tar vodka.

At the business meeting, delegates elected James Walla of USA as the chairperson and Risto Jalkanen of Finland and Meiqing Xu of China as the co-chairpersons of the group for the period from 2000 to 2004. The Chinese delegates invited the group to have the next conference in Beijing with possible optional field trips to several forested areas of China in 2002 or 2003. Delegates from the western US expressed a willingness to consider hosting the next meeting if the arrangements cannot be made to meet in China.

We are thankful to Pat Crane, Jim Walla and Tarmo Aalto, who assisted Risto Jalkanen to edit the manuscripts for the proceedings. Risto Haikarainen is acknowledged for his technical help. Financial contributions to the conference from Enso Forest, Rovaniemi Wood Procurement Area; Finnish Forest and Park Service; Finnish Forest Research Institute; and the IUFRO committee in Finland are greatly appreciated.

Yasu Hiratsuka
Chairperson
IUFRO Working Party 7.02.05
Rusts of Forest Trees

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Occurrence and importance of rust fungi in Hungarian forests

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Summary

For trees and shrubs of forests in Hungary data are available on the occurrence of nearly 100 species of rust fungi belonging to 20 genera of 8 families. Rust fungi do not cause diseases of any practical importance on deciduous tree species such as oaks, black locust, beech, and hornbeam, which comprise most of the forests in our country. However, on poplars, which make up 9.3% of wooded areas, leaf rust due to *Melampsora* species is of major significance. Conifers are mainly represented by Scots and Austrian pine. Among their rust fungi, *Coleosporium* species, *Melampsora pinitorqua* and *Cronartium flaccidum* should be mentioned. On introduced white pine (*Pinus strobus*), *Cronartium ribicola* often causes significant damage.

Key words: review, rusts

1 Introduction

Hungary's geographical location, climatic conditions, and topography define the ratio of forests as well as their tree species composition. Environmental conditions are appropriate for afforestations on only 18% of the country's area. Wooded areas are to be found primarily in the northern and western parts of the country (Fig. 1).

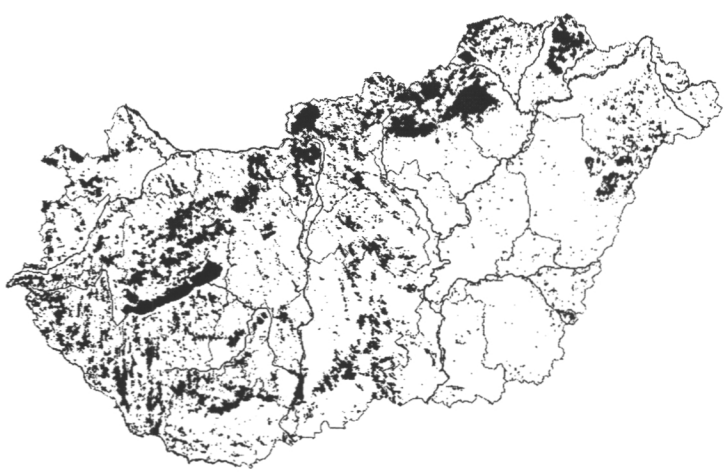

Figure 1. Location of forests in Hungary.

Among tree species (Fig. 2), deciduous ones dominate (84.5%): oaks 33.9% (mainly *Quercus petraea* (Matt.) Lieb., *Q. cerris* L., *Q. robur* L.), black locust (*Robinia pseudoacacia* L.) 19.6%, Euramerican hybrid and native poplars 9.3%, beech (*Fagus sylvatica* L.) 6.4%, and hornbeam (*Carpinus betulus* L.) 6.1%. The major conifers are Scots pine (9.3%) and Austrian pine (4.3%). Other Pinaceae occur in only small areas. Except for the narrow border strip in western Hungary they are not nature.

There are approximately 100 species of rust fungi found on trees and shrubs in Hungary (Ubrizsy 1968, Bánhegyi *et al.* 1985). They belong to 20 genera of 8 families (Table 1). These rust fungi are also known to occur in other areas of central Europe (Gäumann 1959, Blumer 1963). Some of them are rare in Hungary, the frequency of their occurrence being connected with that of their host plants. Apart from a few exceptions, rust fungi generally do not significantly the affect health of forests in Hungary (Varga and Szabó 1993).

2 Material and methods

Families, genera and major species of rust fungi present on forest trees and shrubs in Hungary are characterized according to their significance and the frequency of their occurrence. They are listed in Table 1 according to their systematic arrangement (Hawksworth *et al.* 1955).

3 Results and discussion

From the family Chaconiaceae *Ochropsora ariae* (Fuckel) Rambs. forms uredia and telia on the leaves of *Sorbus aucuparia* L. and *S. aria* (L.) Cr. In Hungary this rust fungus is fairly rare.

The aecia of *Chrysomyxa* species belonging to the family Coleosporiaceae attack the needles and cones of spruce. In our forests the number of planted spruce is low (1.5%) and they are located outside the natural range of the tree species at an altitude of only a few hundred meters. Under such conditions needle rust of spruce is rarely observed. Needle rust of *Pinus* caused by *Coleosporium* species is fairly common in our country, especially *C. senecionis* (Pers.) Fr. and *C. tussilaginis* (Pers.) Lév. In nurseries and young plantations they can be observed primarily on Scots pine. It is not known wether their severe occurrence leads to tree decay (Igmándy 1974).

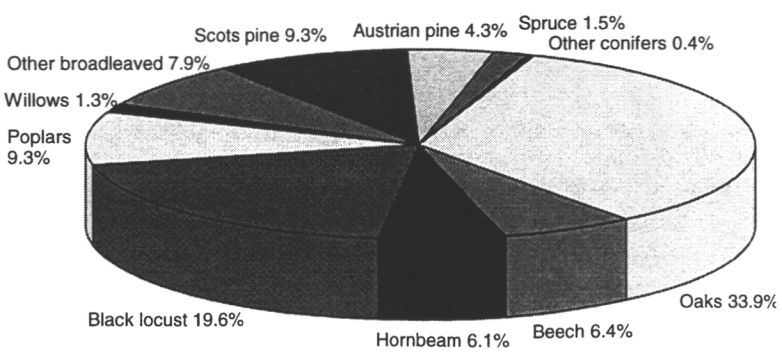

Figure 2. Proportion of tree species in Hungarian forests.

Table 1. Families and genera of rust fungi occurring on forest trees and shrubs in Hungary.

	Families	Genera	Nr. species on forest trees and shrubs	Obs.
1	*Chaconiaceae*	*Ochropsora*	1	rare
2	*Coleosporiaceae*	*Chrysomyxa*	5	rare
		Coleosporium	12	
3	*Cronartiaceae*	*Cronartium*	4	
4	*Melampsoraceae*	*Melampsora*	21	
5	*Phragmidiaceae*	*Gymnoconia*	1	very rare
		Kuehneola	1	rare
		Phragmidium	10	
6	*Pucciniaceae*	*Cumminsiella*	1	
		Gymnosporangium	7	
		Puccinia	9	
		Uromyces	2	
7	*Pucciniastraceae*	*Hyalopsora*	2	rare
		Melampsorella	2	
		Melampsoridium	2	
		Milesina	5	very rare
		Pucciniastrum	6	
		Thekopsora	4	rare
		Uredinopsis	2	very rare
8	*Uropyxidiaceae*	*Tranzschelia*	2	

Among the *Cronartium* species (Cronartiaceae) recorded in Hungary, *C. quercuum* (Berk.) Miy. is very rare, mentioned in the literature merely in two cases as uredia on *Quercus* leaves (Ubrizsy 1968). Neither is *C. flaccidum* (Alb. et Schw.) Wint. common on Scots pine. However, *C. ribicola* J.C. Fisch. is found in Hungary in all areas where *Pinus strobus* L. has been introduced. It leads to severe tree decay, which is the main limiting factor for major cultivation of this tree species (Varga and Szabó 1993).

In Hungary leaves of poplars and willows are occasionally affected severely by various *Melampsora* species (Melampsoraceae). On poplars, seven *Melampsora* species are known in Hungary (Bánhegyi *et al.* 1985, Ubrizsy 1968). On the poplars of section Aigeiros (*Populus nigra* L. and *P. x euramericana* (Dode) Guinier), *M. larici-populina* Kleb. and *M. allii-populina* Kleb. are common and of great significance. Breeding of poplars to develop improved resistance against the aforementioned *Melampsora* species has been carried on for nearly three decades (Gergácz 1975). To attain more effective selection, detecting the various races of the fungus species has been started in recent years in Hungary (Szántó and Steenackers 1998). On poplars of the section Leuce (*P. alba* L., *P. canescens* Sm., and *P. tremula* L.), *M. laricis* Hartig and *M. pinitorqua* Rostr. are more common. Besides attacking the leaves of poplars, the latter species is common in its pycnia and aecia form on *Pinus sylvestris* L., on which it causes damage such as lesions and distortion of sprouts. In nurseries and young plantations it sometimes occurs epidemically, leading to severe damage.

On willows, 14 *Melampsora* species have been recorded in Hungary (Ubrizsy 1968, Bánhegyi *et al.* 1985). *Melampsora salicis-albae* Kleb., *M.*

allii-fragilis Kleb., *M. amygdalinae* Kleb., *M. galanthi-fragilis* Kleb., *M. salici-caprearum* Thüm., and *M. ribesii-viminalis* Kleb. are the most frequent (Igmándy 1974).

On larch (*Larix decidua* Mill.) aecia of *M. larici-populina* Kleb., *M. laricis* Hartig, and *M. caprearum* Thüm. do not cause significant damage (Igmándy 1974).

Phragmidium species (Phragmidiaceae) occur on wild and cultivated *Rosa* and *Rubus* shrubs. Some of them are very common: *P. disciflorum* (Tode) James, *P. rubi* (Pers.)Wint., *P. rubi-idaei* (Pers.) Karst., *P. violaceum* (C.F. Schulz) Wint. In forestry they are insignificant.

Rust fungi belonging to the Pucciniaceae family primarily occur on herbs. Some species cause diseases on leaves and twigs of ornamental shrubs and native shrubs in forests. *Puccinia* spp. are known on the leaves of *Berberis, Mahonia, Rhamnus* and *Frangula* species. Telia of *Gymnosporangium* species develop on the branches of *Juniperus*, thus causing significant damage in natural juniper groves and in ornamental shrubbery as well. More common than the aforementioned species are *G. sabinae* (Dicks.) Wint. and *G. clavariiforme* (Pers.) DC. Their aecia also injure the leaves of trees and shrubs belonging to the Pomaceae. Among the members of the above family, *Cumminsiella mirabilissima* (Peck) Nannf., causing leaf rust of *Mahonia*, is fairly common.

Hyalopsora, Melampsorella, Milesina, Pucciniastrum and *Uredinopsis* (Pucciniastraceae) species are pathogens attacking fir (*Abies* spp.). However, as *A. alba* Mill. is present in Hungary only within a very small area and diseases caused by rust fungi are rare, these pathogens are not significant.

Among the species belonging to the genus *Melampsoridium* two are present in Hungary. *Melampsoridium betulinum* (Pers.) Kleb. is common, whereas *M. carpini* (Nees) Diet. is rare.

Thekopsora species attacking the cones and needles of spruce occur sporadically.

Tranzschelia pruni-spinosae (Pers.) Diet. included in the family Uropyxidaceae, often occurs on wild *Prunus* species as well as on cultivated ones.

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Checklist of the rust species recorded on trees in Finland

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Summary

A total of 28 species of rust fungi occur in the aecial, uredial and/or telial state on trees in Finland. They are listed, along with comments on their frequency and severity. Most of the fungi are host-alternating, although the aecial host is often uncertain in Finland. The checklist is based on field observations by the authors during the last few decades and also on some literature, especially on the monograph by Liro (1908) and the annotated checklist by Hylander *et al.* (1953).

The list

Species	Aecial hosts	Uredial and/or telial hosts
Hyalopsora aspidiotus	*Abies* spp. (not recorded)	*Gymnocarpium dryopteris*, common
Calyptospora goeppertianum	*Abies* spp.	*Vaccinium vitis-idaea*
Pucciniastrum epilobii	*Abies* spp. (not recorded)	*Epilobium angustifolium*, not recorded on other *Epilobium* species in Finland
Thekopsora areolata	*Picea abies*	*Prunus padus, P. virginiana*
P. abies, female cones are infected throughout Finland up to spruce timberline in central Lapland, vegetative shoots only in southern and central Finland; causes severe reductions in seed yields of *P. abies*, esp. in Lapland, where *P. abies* has good seed years once in 10 years (see Jalkanen and Nikula 1998, Lilja 1967).		
T. myrtillina, *T. vaccinuorum* (syn. *Pucciniastrum vaccinii*)	*Tsuga* spp., in North America (Ziller 1974), not recorded in Finland	*Vaccinium myrtillus*, *V. vitis-idaea*, *V. uliginosum*, *V. oxycoccos*, mostly as uredial state
T. sparsa	*Picea*, not recorded in Finland	*Arctostaphylos uva-ursi, A. alpina*, as uredial state only
Melampsoridium betulinum	*Larix* spp.	*Betula pendula, B. pubescens, B. nana, B. tortuosa*
In central Europe, larch has been shown to be the aecial host; inoculation tests conducted in Finland have not been successful (Liro 1908, Poteri 1998). The rust is very common esp. in rainy years; more common on *B. pubescens* than *B. pendula* in the field, a problem for *B. pendula* production in nurseries; two races (Poteri 1998). The rust may perennate on birches.		
M. hiratsukanum	*Larix dahurica*	*Alnus incana, A. glutinosa*
See Kurkela *et al.* 1998		

Cronartium flaccidum	*Pinus sylvestris*, resin top	*Pedicularis* spp., *Vincetoxicum* spp., *Paeonia* spp., *Melampyrum* spp.

Occurs throughout Finland but most common in southwestern part of the country and in Ahvenanmaa. Some *Melampyrum* species were recently recorded as new uredial and telial hosts for this rust (Kaitera, pers. comm.).

C. ribicola	*Pinus strobus, P. cembra, P. peuce*	*Ribes* spp.

White pine blister rust occurs up to the Arctic Circle on *Ribes*, less common on *Pinus*.

Peridermium pini	*Pinus sylvestris*, resin top	–

One of the most disastrous pathogens in Finland, causing severe losses in volume growth and value (Kaitera *et al.* 1994).

Coleosporium spp.	*Pinus sylvestris*, needle rust	Many hosts (*Melampyrum* spp., *Tussilago* spp., *Senecio* spp., *Campanula* spp., etc.)
Chrysomyxa abietis	– (microcyclic form)	*Picea abies*, on needles
C. ledi	*Picea abies, Picea* spp. needles	*Ledum palustre*

Very common in rainy years, especially in northern and eastern Finland; "golden" spruces with yellowed current-year needles in August; *C. ledi* (det. P. Crane) was found to sporulate in 1998 on needles of *P. glauca* (abundant aecia) and *P. mariana* (some aecia) in a tree species trial, in which *P. abies* was heavily infected in Siperia, Rovaniemi.

C. woroninii	*P. abies*, buds	*Ledum palustre*

Occurs in areas where *C. ledi* is most common, i.e. mainly in Lapland, infected buds can be seen before spruce flush, and sporulation is earlier than *C. ledi*.

C. empetri	*Picea* spp., not recorded in Finland	*Empetrum nigrum*
C. pirolata	*Picea abies*, on cone scales	*Pyrola* spp., *Orthi* spp., *Mone* spp.

Together with *T. areolata*, reduces significantly the seed germinability in infected cones of *P. abies* (see Jalkanen and Nikula 1998).

Chrysomyxa sp.	*P. abies*, needles	Several ericaceous species
Ochropsora ariae	*Anemone nemorosa*	*Sorbus aucuparia*

Common nearly every year on *Sorbus* foliage

Melampsora populnea

The following four rusts belong morphologically to *M. populnea* as physiological subspecies.

M. pinitorqua	*P. sylvestris*, shoots	*Populus* spp. (Leuce group)

One of the most serious pathogens, causing shoot dieback, and growth and quality losses in young *P. sylvestris* plantations every 4 to 7 years (Kurkela 1973, Jalkanen and Kurkela 1984).

M. larici-tremulae	*Larix*	*Populus* spp. (Leuce group)
M. rostrupii	*Mercurialis*	*Populus* spp. (Leuce group)

M. magnusiana	*Chelidonium majus*, *Corydalis*	*Populus* spp. (Leuce group)
Melampsora sp.	?	*Populus tremula*, perennial
M. larici-populina	*Larix spp.*	Several poplar species and hybrids
M. larici-pentandrae	*Larix* spp.	*Salix pentandra* etc.
M. larici-capraearum	*Larix* spp.	*S. caprea*, possibly perennating
M. epitea (*larici-epitea*)	*Larix* spp. (Petäistö and Kurkela 1996)	*Salix* spp.
M. epitea (*ribesii-purpureae*)	*Ribes* spp.	*Salix* spp.
M. amygdalinae	*Salix triandra* (autoecious)	*Salix triandra* (perennial)
Melampsorella caryophyllacearum	*Abies* spp. (not recorded in Finland)	*Cerastium* spp., *Stellaria* spp.
Tranzschelia pruni-spinosae	*Anemone ranunculoides*	*Prunus domestica, P. spinosa*
Gymnosporangium clavariiforme	*Crataegus* spp.	*Juniperus communis*
G. cornutum	*Sorbus* spp.	*J. communis*
Common nearly every year on *Sorbus* foliage.		
G. tremelloides	*Malus* spp.	*J. communis*

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Haustorial characters as markers in the systematics of Melampsoraceae

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Summary

The systematics of Melampsoraceae is based on morphological characters of the sori, mainly the telia. Whereas tribal subdivision of the family is comparatively clear, intertribal relationships are poorly understood and delimitation of certain genera is only weak. Therefore, ultrastructural and morphological characters of rust D-haustoria were investigated as possible tools for a better understanding of the systematics within Melampsoraceae. Two ultrastructural haustorial types, the "gymno-" and the "velopedunculate" type, were distinguished according to the form of the extrahaustorial membrane at the haustorial neck. In species of *Melampsora* and *Melampsoridium*, only the gymnopedunculate type was present. Velopedunculate haustoria were found in *Chrysomyxa*, *Coleosporium*, *Cronartium*, *Hyalopsora*, *Milesina*, and *Uredinopsis*. In *Melampsorella*, *Pucciniastrum*, and *Thekopsora*, species with gymno- or velopedunculate haustoria occurred. The haustorial bodies differed morphologically, and an extensively branched or "botryose" type and a more or less bulb-shaped or "vesicular" type were distinguished. Botryose haustoria were characteristic for *Milesina* and *Uredinopsis*. Members of the other genera had vesicular haustoria. Both morphological types occurred only in the genus *Melampsorella*. Because of the differences in haustorial characters *Pucciniastrum*, *Thekopsora*, and *Melampsorella* are considered to not be natural entities. *Melampsorella caryophyllacearum* may be more closely related to velopedunculate members of Melampsoraceae, especially the genera *Uredinopsis* and *Milesina*.

Key words: morphology, taxonomy, ultrastructure

1 Introduction

The Melampsoraceae are a group of rust fungi characterized by telial and spermogonial characters and by the host selection of the haplontic stage. The sessile teliospores form singly or in chains. They are produced in the host mesophyll or within epidermal cells, or they form crusts, gelatinous cushions, or threads (Dietel 1928). The spermogonia belong to group one (Hiratsuka and Hiratsuka 1980). Except in some *Melampsora* species, the haplontic stage of all Melampsoraceae is produced on coniferous hosts (Cummins and Hiratsuka 1983). Generally, the Melampsoraceae are regarded as an ancient group of rust fungi because of teliospore and telium morphology (Savile 1976) and the widespread occurrence of the species on phylogenetically old

host plants (Dietel 1904, 1938, Leppik 1953, 1955, 1965). The genera *Uredinopsis*, *Milesina*, and *Hyalopsora* are considered to be the most primitive as they parasitize ferns in their dikaryotic stage (Hiratsuka 1936, and references therein).

Dietel (1928) divided the Melampsoraceae into five tribes: Pucciniastreae, Cronartieae, Coleosporieae, Chrysomyxeae, and Melampsoreae, which are sometimes also treated as subfamilies or even families. Little is known about intertribal connections and even some of the melampsoraceous genera are only poorly delimited. Additional characters may help, therefore, to elucidate relationships among Melampsoraceae and to better understand certain genera.

Ultrastructural characters have hardly been used hitherto for systematic purposes above species level. Several works on spore surface structures show, however, that such characters can shed new light on the systematics of rust fungi (Hiratsuka 1971, Sato and Sato 1982, Imazu and Kakishima 1995). Recent studies on haustorial ultrastructure showed that two different types of D-haustoria occurred within Melampsoraceae (Berndt *et al.* 1994, Berndt and Oberwinkler 1995, 1997, Berndt 1996). Based on the form of the extrahaustorial membrane at the haustorial neck, they were designated "gymno-" and "velopedunculate" type (Fig. 1). In the present study, results are presented on the distribution of these haustorial types in all melampsoraceous genera, and the implications for the systematics of the group are discussed. Additionally, the potential use of morphological characters of the haustorial body for systematics is demonstrated.

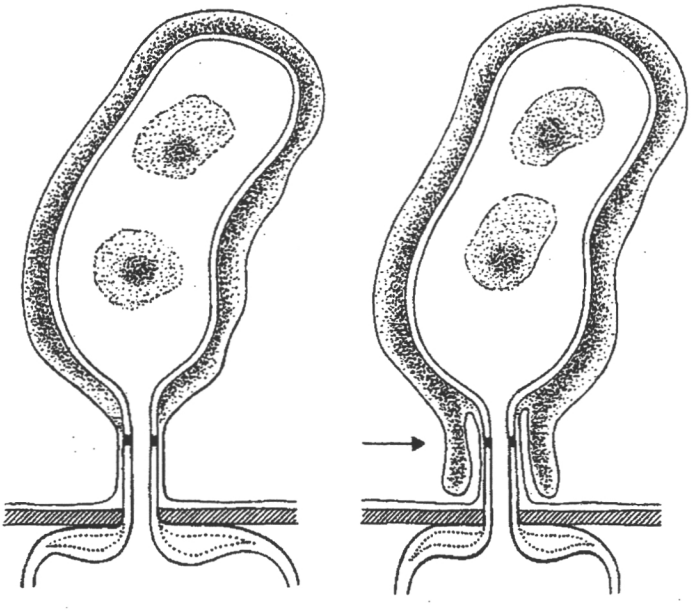

Figure 1. Schematic drawings of gymno- (left) and velopedunculate (right) D-haustoria. The velopedunculate haustorium differs from the gymnopedunculate one by the fold of the extrahaustorial membrane (arrow) which covers the haustorial neck.

2 Material and methods

2.1 Light microscopy

Freehand sections from herbarium specimens and fresh material were mounted in lactophenol and stained with cotton blue. Slides were subsequently gently heated to boiling. The preparations were observed with a Zeiss "Axioskop" with bright field or phase contrast.

2.2 Transmission electron microscopy

For transmission electron microscopy, small pieces of infected leaves were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for several days. After washing six times with 0.1 M cacodylate buffer, the material was postfixed for 1 h in 1% OsO_4 in cacodylate buffer. Afterwards, the specimens were washed three times in distilled water and stained with aqueous uranyl acetate for 1 h. After washing with distilled water (3x) and dehydration in an acetone series (10, 25, 50, 70, 95, and three times in 100%), samples were embedded in Spurr's resin (Spurr 1969). The embedded material was sectioned with a diamond knife on a Reichert Ultracut E microtome. Ultrathin sections (ca. 85 nm) were collected on formvar-coated copper slot grids and contrasted with lead citrate according to Reynolds (1963). Sections were observed with a Zeiss EM 9 S-2 and a Zeiss EM 10.

2.3 Specimens illustrated

Coleosporium asterum (Dietel) P. & H. Sydow. Taiwan (ROC), Taichung province, Ku Kwan recreation area, on *Aster formosana* Hayata, 21 Apr. 1990, leg. R. Berndt.

Coleosporium campanulae (Pers.) Lév. Germany, Tübingen, Hagelloch, on *Campanula* cf. *persicifolia* L., 20 Jul. 1992, leg. L. Kisimova-Horovitz.

Coleosporium euphrasiae (Schum.) Winter. Germany, Pfullingen, slopes of the Ursulaberg, on *Rhinanthus aristatus* (Celak) Hauskn., 25 Jul. 1987, leg. R. Berndt.

Melampsora cf. *allii-populina* Klebahn. Germany, Reutlingen, "Schafstall" area, on *Allium ursinum* L., 22 Apr. 1989, leg. R. Berndt.

Melampsora pinitorqua Rostr., leg. F. Oberwinkler (FO 29342).

Melampsoridium betulinum (Fr.) Kleb. Germany, Bavaria, Oberjoch, on *Betula pubescens* Ehrh., 11 Sep. 1987, leg. O. Ebinger.

Milesina blechni (P. & H. Sydow) P. & H. Sydow. Germany, Bavaria, Oberjoch, on *Blechnum spicant* Roth., Sep. 1989, leg. F. Oberwinkler (FO 36341).

Milesina vogesiaca (Faull) P. & H. Sydow ex Hirats. f. Switzerland, Jura, Doubs Valley at Soubey, on *Polystichum aculeatum* (L.) Roth., 20 May 1988, leg. R. Berndt.

Pucciniastrum hikosanense Hirats. f. Taiwan (ROC), Nantou province, Ho Huan San in Taroko Natl. Park, near ranger station, on *Acer kawakamii* Koidz., 5 Jul. 1990, leg. R. Berndt.

Thekopsora guttata (Schroeter) P. & H. Sydow. Germany, Donaueschingen, at the Schellenberg, on *Galium odoratum* (L.) Scop., 28 Aug. 1991, leg. R. Berndt.

A comprehensive list of all specimens studied can be found in Berndt *et al.* (1994), Berndt and Oberwinkler (1995, 1997), and Berndt (1996).

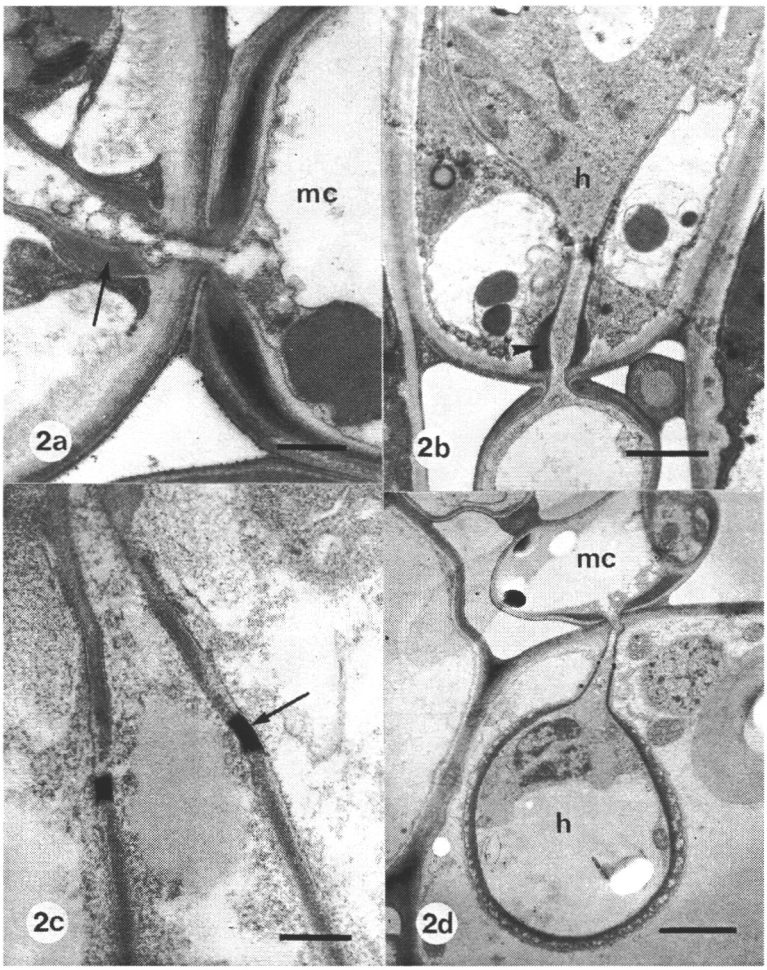

Figure 2. Gymnopedunculate D-haustoria. a. *Melampsora pinitorqua*. Haustorial mother cell (mc), penetration site and proximal part of the haustorial neck. At the base of the neck, a moderately electron-dense swelling (arrow) of the fungal wall can be seen. Scale bar = 0.5 µm. b. *Melampsoridium betulinum*. Haustorial mother cell and stalked haustorium (h). At the base of the haustorial neck, a prominent electron-dense swelling (arrowhead) of the fungal wall is present. Scale bar = 1 µm. c. *Thekopsora guttata*. Part of the haustorial neck with neckband (arrow). Scale bar = 0.2 µm. d. *T. guttata*. Overview of haustorial mother cell (mc) and vesicular pedicellate haustorium (h). Scale bar = 2 µm.

3 Results

3.1 Haustorial ultrastructure

In the genera *Melampsora* and *Melampsoridium*, only gymnopedunculate haustoria could be found (Figs. 2a, b). In *Melampsoridium betulinum*, a conspicuous blister-like and electron-dense swelling was present at the base of the haustorial neck (Fig. 2b). A similar but less prominent swelling could also be found in the haustoria of *Melampsora pinitorqua* (Fig. 2a). Only one

species of the genus *Naohidemyces*, *N. vaccinii* (Winter) Sato *et al.*, was studied, and it had gymnopedunculate haustoria. *Naohidemyces* may be included in *Pucciniastrum*, however. Species of *Pucciniastrum* s. str., *Thekopsora*, and *Melampsorella* had either gymno- (Figs. 2c, d) or velopedunculate haustoria. In members of *Chrysomyxa*, *Coleosporium* (Figs. 3c, d), *Cronartium*, *Hyalopsora*, *Milesina* (Figs. 3a, b), and *Uredinopsis*, only velopedunculate haustoria were found. Also, *Calyptospora goeppertiana* Kühn had velopedunculate haustoria. This species represents the monotypic genus *Calyptospora*, which may merely be a life cycle variant of *Pucciniastrum*. The distribution of haustorial types is summarized in Fig. 4.

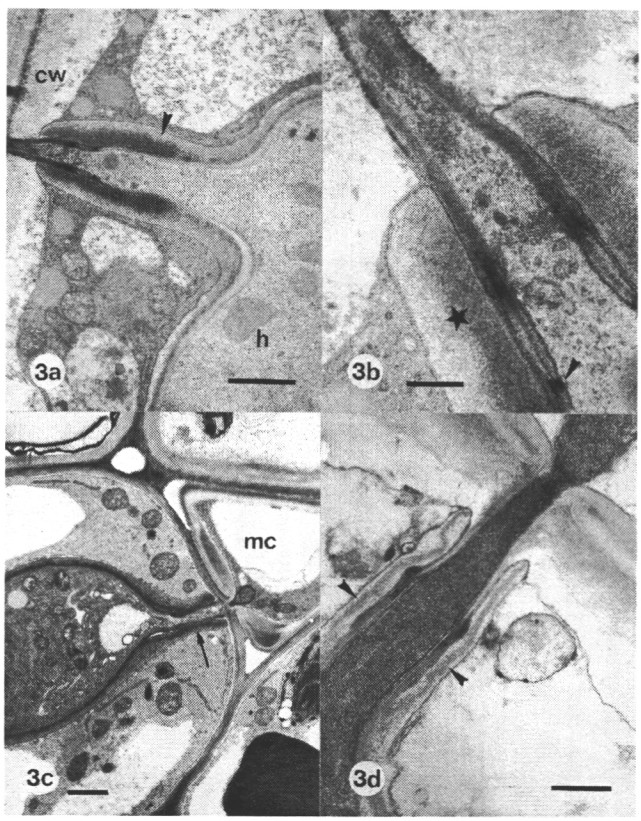

Figure 3. Velopedunculate D-haustoria. a. *Milesina blechni*. Haustorial neck and proximal part of the haustorial body (h). The neck is covered by a fold of the extrahaustorial membrane (arrowhead) which extends to the host cell wall (cw). Scale bar = 1 µm. b. *M. blechni*. Detail from Fig. 3a. Proximal part of the haustorial neck and fold of extrahaustorial membrane (star). The fungal wall reveals regions of different electron-density. The neckband is indicated by an arrowhead. Scale bar = 0.2 µm. c. *Coleosporium asterum*. Haustorial mother cell (mc) and stalked haustorium. The neck is covered by a fold of the extrahaustorial membrane (arrow). Scale bar = 1 µm. d. *C. euphrasiae*. Haustorial neck covered by a fold of the extrahaustorial membrane (arrowheads). A nucleus of the haustorial mother cell that probably migrates into the haustorial body fills the lumen of the haustorial neck. Scale bar = 0.5 µm.

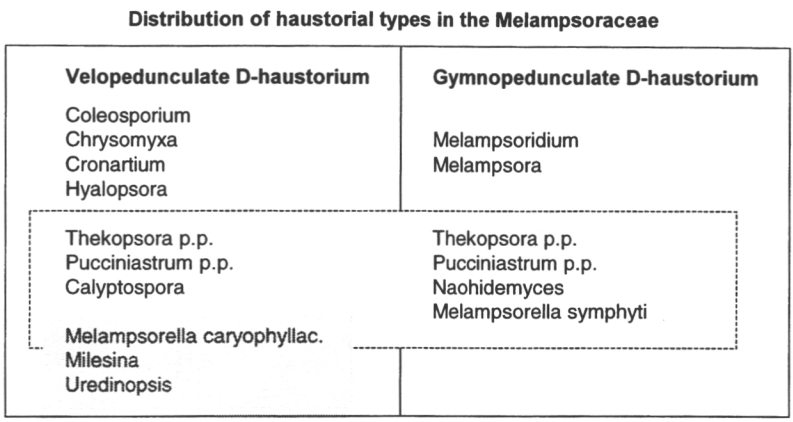

Figure 4. Distribution of ultrastructural and morphological types of D-haustoria in Melampsoraceae. The shaded area represents the presence of botryose haustoria. The dashed line circumscribes the *Pucciniastrum*-group.

3.2 Haustorial morphology

D-haustoria were delicately stalked and had a more or less inflated bulb-shaped or worm-like haustorial body (Figs. 2d, 3c, 5–7). The shape of the haustorial body varied considerably. In most genera it was simple or branching very sparingly (vesicular type; Figs. 2d, 3c, 5, 6). In *Milesina* and *Uredinopsis* species, however, and in *Melampsorella caryophyllacearum* Schroeter, the haustorial body branched extensively (botryose type; Fig. 7). Haustoria of all investigated members of *Pucciniastrum* and *Thekopsora* had vesicular haustorial bodies whether they were gymno- or velopedunculate. Sometimes these haustorial bodies branched but never exhibited the extensive apical branching characteristic of the botryose type. In *Melampsorella*, the situation was different. Haustoria of *M. caryophyllacearum* differed from *M. symphyti* Bubák, not only by velopedunculate haustorial ultrastructure, but also by the presence of botryose haustorial bodies.

Botryose haustoria similar to those of the mentioned rusts could be found in the haplontic stage of *Melampsora* cf. *allii-populina* (Fig. 8). These M-haustoria exhibited considerable morphological variability, however, which also comprised sparsely branched or filamentous forms. Furthermore, they showed typical features of M-haustoria and were plurinucleate.

4 Discussion

4.1 Ultrastructure and morphology of D-haustoria

4.1.1 Ultrastructure

Velopedunculate D-haustoria were first observed in *Cronartium* species by Gray *et al.* (1982) and Khan *et al.* (1982). The velopedunculate haustorium has since been found in species of *Calyptospora*, *Chrysomyxa*, *Coleosporium*, *Hyalopsora*, *Milesina*, *Pucciniastrum*, *Thekopsora*, and *Uredinopsis* (Berndt *et al.* 1994, Berndt and Oberwinkler 1995, 1997, Berndt

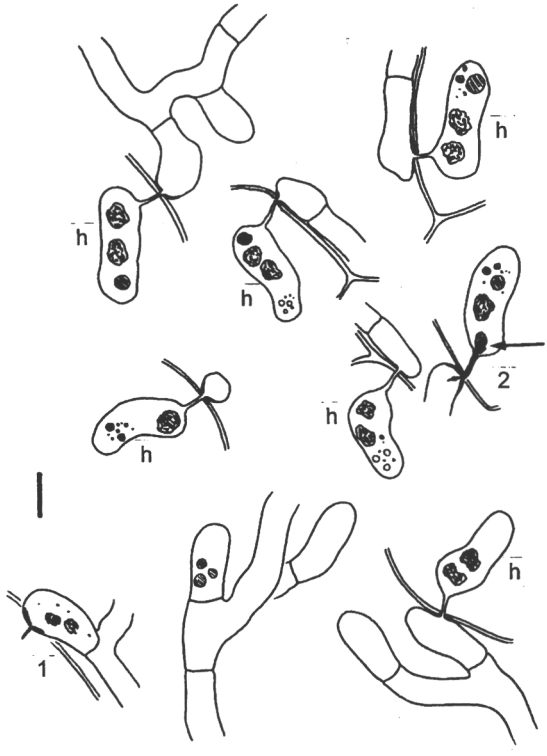

Figure 5. Vesicular D-haustoria (h) of *Coleosporium campanulae*. (1) represents a very early stage of haustorium formation with a nail-like peg formed by the haustorial mother cell. (2) shows a haustorium that has reached its final shape and contains one nucleus (arrow). The second nucleus is just migrating from the haustorial mother cell into the haustorium. Within the haustoria, nuclei and lipid droplets can be seen. Scale bar = 5 µm.

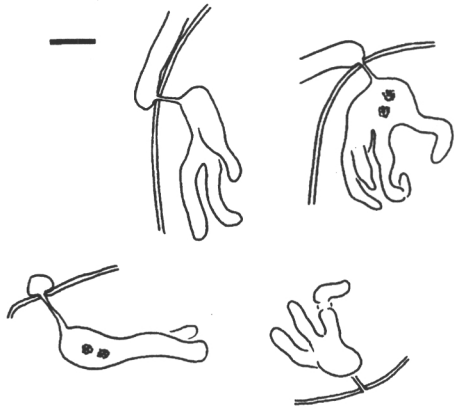

Figure 6. D-haustoria of *Pucciniastrum hikosanense* with sparsely branched haustorial bodies. Scale bar = 5 µm.

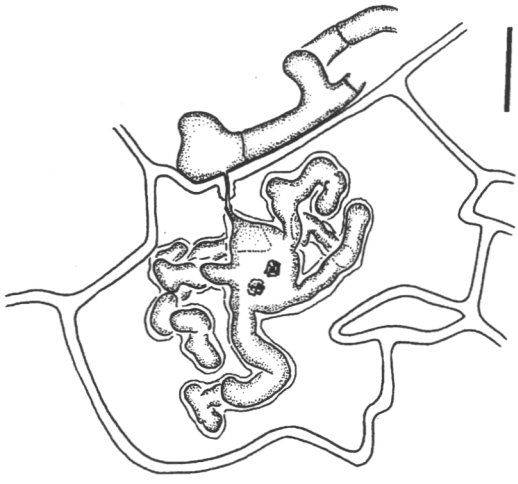

Figure 7. Botryose D-haustorium of *Milesina vogesiaca*. Scale bar = 5 µm.

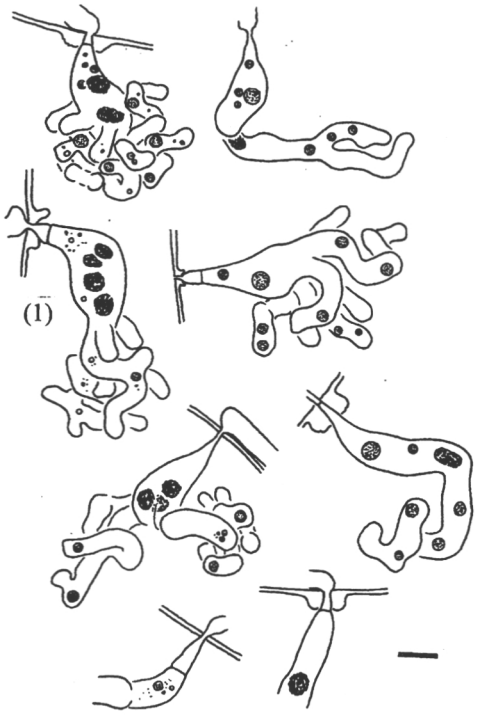

Figure 8. M-haustoria of *Melampsora* cf. *allii-populina*. The haustoria are variously shaped and comprise more or less botryose, branched, or filamentous forms. They are generally septate at their bases and not distinctly pedicellate. The haustoria contain up to four nuclei (1). Scale bar = 5 µm.

1996). This haustorial type seems to be restricted to members of Melampsoraceae, as there are no reports from rust fungi of other families. Haustorial ultrastructure appears to be a constant and unambiguous feature in most genera of Melampsoraceae (e.g., in *Melampsora*: Coffey *et al.* 1972, Rijkenberg 1972, Longo and Naldini-Longo 1975, Spiers and Hopcroft 1985) and in genera of other rust families that have been studied for this character (e.g., in *Puccinia*: Harder 1978, Borland and Mims 1980, Al Khesraji and Lösel 1981, Mims *et al.* 1989, Baka 1992, Berndt 1995). Only the genera *Pucciniastrum*, *Thekopsora* and *Melampsorella* were inhomogeneous with regard to haustorial ultrastructure (Berndt and Oberwinkler 1995, 1997).

4.1.2 Morphology

Extensive studies on the morphology of haustoria of rust fungi were carried out by Moss (1926), Rice (1927), and Hunter (1948), and by Rajendren (1972), who studied many tropical genera. While botryose (termed "coralloid" by Rajendren) or extensively branching haustoria occur quite widely in haplontic infections and some autoecious rusts (e.g., *Zaghouania*, *Botryorrhiza*), they seem to be rare in the dikaryotic stages of heteroecious rusts and appear to be almost restricted to members of Melampsoraceae (Moss 1926, Rice 1927, Rajendren 1972). The investigated species of *Milesina* and *Uredinopsis* had extensively branched or botryose haustoria, whereas simple vesicular (more or less tuber-shaped or allantoid) D-haustoria were characteristic for the remaining genera. Except in *Milesina* and *Uredinopsis*, botryose D-haustoria were only discovered in *Melampsorella caryophyllacearum*. The other known species of the genus, *M. symphyti* Schröter, had vesicular haustoria. One may assume, therefore, a closer relationship of *M. caryophyllacearum* with *Milesina* and *Uredinopsis* than with *M. symphyti*. This assumption is further corroborated by the different ultrastructure of the haustoria of *M. caryophyllacearum* and *M. symphyti*.

4.2 Teliosporic characters and delimitation of genera and tribes in Melampsoraceae

As in other rust relationships, sorus structure of the telia and morphology of the teliospore yield the most important characters for generic delimitation and tribal affiliation in Melampsoraceae (Cummins and Hiratsuka 1983). Additional characters come from the life cycle and uredinial morphology, which is typically pucciniastreous (i.e., with a more or less dome-shaped peridium) in most genera (Moss 1926), but caeomatoid in *Coleosporium* and *Chrysomyxa*. Unfortunately, teliospore morphology is very simple in several genera of Melampsoraceae, where the teliospores form irregular groups within or underneath the epidermis of the host leaves (Pady 1933). Especially in *Pucciniastrum* (incl. *Calyptospora* and *Naohidemyces*) and the allied genera *Thekopsora* and *Melampsorella*, only subtle teliospore characters such as location and septation of teliospores or location of germ pores and wall pigmentation are used to delimit genera (Dietel 1928, Cummins and Hiratsuka 1983). It is in the latter genera where species with ultrastructurally different haustoria occur. These differences may thus indicate that the mentioned genera are heterogeneous and not monophyletic.

4.3 Teliospore versus haustorial characters

One may ask why haustorial characters should be more important or stable than characters of teliospores. It should be emphasized here that teliospore characters are reliable and indispensable markers in many rust relationships and also that certain melampsoraceous genera can be satisfactorily delimited by them. In pucciniastroid genera, however, teliospore characters do not seem to suffice to reflect the relationships entirely. In this situation, haustorial ultrastructure and morphology may be more reliable characters that can help to recognize natural entities. The available investigations show that the ultrastructure of the haustorial neck is a stable character within a given rust species. The character does not change from one stage to another during haustorial ontogeny or by influence of the host, and there is no reason to assume that environmental factors could affect this special ultrastructural feature. Therefore, haustorial characters should take priority over telial characters in delimiting the mentioned pucciniastroid genera, although virtually nothing is known about the function and formation of the membrane fold in velopedunculate haustoria.

Morphological haustorial types appeared to be reasonably constant in a given rust species and are therefore regarded as stable features that can be used for systematic purposes. Some caution is necessary, however, with the use of haustorial morphology, as the shape of the haustorial body may vary to some extent even within a single parasitic mycelium. Furthermore, species with intermediate haustorial forms may exist that are difficult to assign to either the vesicular or botryose type. Thus, many haustoria must always be considered and the results are valid only in a restricted context (e.g., within *Melampsorella*, within Melampsoraceae).

Just a few of the ca. 7000 described rust species have been studied for haustorial morphology and ultrastructure so far. The results obtained from these species already show the structural diversity of rust haustoria, and it can be assumed that a wealth of information that could be used for systematics is still hidden. Thus, haustoria of more species should be investigated, especially in the diverse and poorly studied tropical rust fungi. In spite of the mentioned restrictions and difficulties connected with the use of haustorial morphology and ultrastructure in systematics, this may lead to a better understanding of rust relationships and will further increase the knowledge on haustorial diversity and function.

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Cytology, taxonomy, and nomenclature of autoecious pine stem rusts

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Summary

There are at least seven different species and varieties of autoecious pine stem rusts recognized in Europe, Asia, and North America. Their occurrence at high latitudes or high altitudes is consistent with a general tendency for short-cycle rusts. Two main types of germ tube morphology and cytological events are recognized. In one type, nuclear fusion occurs in the spore and subsequent meiosis occurs in the germ tube, and the resulting haploid nuclei are separated by septa. In another type, dikaryon nuclei enter the germ tube, nuclear fusion occurs in a clearly recognizable vesicle, and meiosis occurs in the vesicle or in the germ tube that grows out from the vesicle. These differences can be explained by the timing and place of nuclear fusion and meiosis in the germination process. Pine-to-pine stem rusts of both types exist in the United Kingdom in separate locations, and both types have been called *Peridermium pini* (*E. pini*), but may represent two distinct fungi. Although all autoecious species likely have been derived from parental heteroecious species and spore morphology is like the aeciospores of heteroecious species, they have endocyclic life cycles. They need to be treated as holomorphic fungi and cannot be included in the anamorphic genus *Peridermium*. The establishment of the genus *Endocronartium* is defended, and various nomenclatural options for this group of fungi are discussed.

Key words: *Endocronartium*, *Peridermium*, review, zymodeme

1 Introduction

Most of the pine stem rusts have host alternating (heteroecious) life cycles and are classified as species of the genus *Cronartium*. About 20 species of *Cronartium* are known (Peterson 1973, Cummins and Hiratsuka 1983). On the other hand, the following pine stem rusts species or forms have been recognized to have pine-to-pine (autoecious) life cycles.

1. *Endocronartium harknessii* (J.P. Moore) Y. Hiratsuka
   - °*Peridermium harknessii* J.P. Moore
   - °*Cronartium harknessii* (J.P. Moore) Meinecke
2. *E. pini* (Pers. emend Kleb.) Y. Hiratsuka
   - °*Peridermium pini* (Pers.) Lév. emend Kleb.
3. *E. sahoanum* Imazu & Kakishima var. *sahoanum*
   - °*Peridermium sahoanum* Imazu & Kakishima
4. *E. sahoanum* var. *hokkaidoense* Imazu & Kakishima
   - °*Peridermium sahoanum* var. *hokkaidoense* Imazu & Kakishima

5. *E. yamabense* (Saho & I. Takahashi) Paclt
   °*Peridermium yamabense* Saho & I. Takahashi
6. *Peridermium filamentosum* Pk. pp.
   "Inyo *Peridermium* "
7. *Peridermium filamentosum* Pk. pp.
   "Powell *Peridermium* "
8. A blister rust on *Pinus armandii* in Central China

Several other species and forms such as *Peridermium bethelii* Hedgcock & Long, a white-spored gall rust, and a yellow-spored gall rust, which are also considered to have pine-to-pine life cycles, have been reported and considered to be distinct from other autoecious species (Vogler and Bruns 1998).

Undoubtedly, these species and forms have been derived from heteroecious parental species but their cytology, interpretation of these events, and the resulting nomenclatural treatments are still in dispute (Epstein and Buurlage 1988, Hiratsuka 1968, 1969, 1991, Vogler and Bruns 1998, Vogler *et al.* 1997). In this presentation, I will discuss their cytology, taxonomy, and nomenclature based on the recognition of endocyclic life cycles with nuclear fusions and meiotic divisions in these rusts rather than consider them as imperfect rusts having mitotic rather than meiotic nuclear divisions in germ tubes. In *E. harknessii*, we have now observed the presence of synaptonemal complexes, which indicate nuclear fusion, in the spores just before germination (Y. Hiratsuka, unpubl.). This confirmed that nuclear fusion and meiosis are occurring in the process of germination in *E. harknessii*. I will present the evidences and discuss the matter in a separate paper.

2 Distribution and origin of pine-to-pine stem rusts

It is a well-known fact that the number of rust species with shortened life cycles are more abundant at high altitudes and high latitudes. Distribution of all known species of pine-to-pine stem rusts are restricted to high latitudes or high altitudes near the edges of their pine hosts' distribution. This indicates that these species were originally derived from their heteroecious parental species. Also, when ecologically separated from their alternate hosts, instead of becoming extinct, they managed to alter their life cycles by shortening their life cycles, and they acquired the ability to re-infect their pine host without going through life cycle stages on alternate hosts.

3 Germ tube morphology and cytological events

Two main types of germ tube morphology and cytological events are recognized among known pine-to-pine stem rust species. In one type, nuclear fusion occurs in the spore, and the subsequent meiosis occurs in the germ tube (*Endocronartium harknessii* and a part of *E. pini*) (Hiratsuka *et al.* 1966, Hiratsuka 1968, Gibbs *et al.* 1988). In the other type, the subsequent dikaryon

nuclei enter the germ tube, and nuclear fusion and the subsequent meiosis occurs in a clearly recognizable vesicle; then the nuclei migrate into a germ tube growing out from the vesicle (*E. sahoanum, E. yamabense*, a part of *E. pini* and possibly an undescribed species on *Pinus armandii* from central China) (Gibbs *et al.* 1988, Imazu and Kakishima 1992, 1995, Imazu *et al. 1991,* Kaneko and Harada 1995). It is interesting to note that there are two different populations of *"Peridermium pini"* with different types of germ tubes corresponding to the two types. When Dr. van der Kamp as a Ph. D. student at the University of Aberdeen studied *P. pini* around 1967, he published two significant papers on this disease. However, he never published a third manuscript (van der Kamp, B.J., Cytology of *Peridermium pini* (Pers.) Lev. aeciospore germination, unpublished manuscript), that he wrote on the cytology of germinating spores, because he could not clearly explain the cytological events in the germ tubes clearly at the time. However, his figures and observation of nuclei during germination were clearly those of the second type. These differences can be explained by the timing and place of nuclear fusion and meiosis in the germination process. This may mean that the timing and place of nuclear fusion are an indication of stability of endocyclic life cycle. Possibly the first type has more and the second type a less stable endocyclic life cycle.

Vogler *et al.* (1997) found two different populations of *E. harknessii*, zymodeme I and zymodeme II, and reported that mature spores of zymodeme II had one nucleus in an average of 93% of the spores while those of zymodeme I had one nucleus in only 21 to 29% of spores. It is also interesting that the same group of researchers (Epstein and Buurlage 1988) reported only 10% of the spores having one nucleus. Although they concluded from the observations that nuclear divisions in the germ tubes were mitotic, their data clearly indicate the occurrence of nuclear fusion and meiosis. Very high proportions of monokaryotic spores in zymodeme II can only be explained as the early occurrence of nuclear fusion in the spores of this race (zymodeme II) of western gall rust. Examination of nuclear conditions of hyphae in the pine tissue and very young spores will clarify the matter.

4 Taxonomy and nomenclature

If these autoecious species and forms are recognized as endocyclic species, four options are available for nomenclature:

1. Treat as species of *Cronartium*.

Morphological and molecular studies clearly indicate that these pine-to-pine forms are short-cycle variations of parental heteroecious species of *Cronartium* (Vogler and Bruns 1998). Vogler and Bruns (1998) stated that these autoecious forms are simply all *Cronartium* despite their altered life cycle and unusual cytology. This may be an acceptable option, but this will create confusion in nomenclature because the genus would need to be defined to have two different kinds of teleomorphs. Based on the morphology of teliospores, taxa of all other life cycle variations (macrocyclic, demicyclic, microcyclic) are called by the same generic name. However, some endocyclic species cannot be placed accurately to parental genera. For example, species

of *Endophyllum* are endocyclic rusts having *Aecidium*-type sorus and spores. Many rust genera have an *Aecidium*-type of aecia such as *Puccinia, Uromyces, Cumminsiella*, and *Ochropsora*, and many times the parental genera cannot be predicted.

2. Use the anamorphic generic name *Peridermium*.
If we do not recognize these pine-to-pine forms as having an endocyclic life cycle, the use of *Peridermium* may be a reasonable solution and many researchers are using this option. However, if the group of fungi is recognized to have endocyclic life cycles, they cannot be called by the anamorphic generic name *Peridermium*. Also, by definition, use of the name *Peridermium* means that the teleomorph or telial state of the fungus is still unknown, as described in one of the tables in Vogler and Bruns (1998). However, these fungi obviously do not have a separate telial state on alternate hosts because the whole life cycle is completed on the *Pinus* host. Use of *Peridermium* does not indicate the endocyclic nature of the life cycles of the taxa, and it may be confused with anamorphic taxa in which the life cycle is still unknown.

3. Put into one of the existing endo-genera.
This option is not acceptable because all endo-genera presently recognized are based on morphological types of their aecioid telia (or aecia of parental species).

4. Create a new endo-genus specifically for this group of rusts.
The author chose this option and established a new genus *Endocronartium* (Hiratsuka 1969) because this alternative has merits over the three previously mentioned options. However, the endocyclic form is an abnormal state obviously closely related to the parental genus, *Cronartium*. Nomenclature is a tool for convenience sanctioned by the International Code of Botanical Nomenclature to facilitate everyone referring to the same taxa by the same names. We need to keep our dialog open on the subject so that all concerned will call the same fungi by the same names and avoid confusion and misunderstanding.

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The species of *Melampsora* on *Populus* (Salicaceae)

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Summary

A systematic study of the species of *Melampsora* infecting the host genus *Populus* (Salicaceae) was made. *Melampsora abietis-populi, M. ciliata, M. cumminsii, M. larici-populina, M. medusae, M. microspora, M. occidentalis, M. osmaniensis, M. populnea, M. populnea* f. sp. *laricis, M. populnea* f. sp. *pinitorqua,* and *M. populnea* f. sp. *rostrupii* are recognized as valid. *M. populnea* f. sp. *allii-populina,* and *M. populnea* f. sp. *magnusiana* are made as new combinations. A key to the recognized species, detailed morphological characters of the spore forms, host range, and distribution are provided.

Key words: systematics, taxonomy

1 Introduction

The genus *Melampsora* was established by Castagne in 1843. Since then many species have been described showing either autoecious or heteroecious life-cycle pattern. The autoecious species parasitize various angiosperm hosts, whereas the heteroecious species produce telia on *Populus* and *Salix* of Salicaceae.

According to Cummins (1959) and Cummins and Hiratsuka (1983), "*Melampsora* is a genus of numerous described species but many of them are not distinct morphologically". Taking this into consideration, a systematic study of the entire genus has been taken up. The present paper deals with *Melampsora* species infecting the host genus *Populus* of Salicaceae.

2 Material and methods

This study is based on the morphological characters of the spermogonia, aecia, uredia and telia and their respective spore forms from rust-infected herbarium specimens obtained on loan from various international herbaria viz., The Botanical Museum and Herbarium, University of Copenhagen, Denmark (C); National Mycological Herbarium, Biosystematics Research Institute, Ottawa, Canada (DAOM); Canadian Forestry Service, Victoria, Canada (DAVFP); The Farlow Herbarium of Harvard University, Massachussets, USA (FH); Conservatoire et Jardin Botanique, Geneva, Switzerland (G); The Herbarium Cryptogamiae Indiae Orientalis, New Delhi, India (HCIO); Levin Mycological Herbarium, New Zealand (LEV); Instituto Di Botanica Orto Botanico, Pavia, Italy (PAV); Swedish Museum of Natural

History, Stockholm, Sweden (S); and Instytut Botaniczy, Universytetu Wroclawskiego, Wroclaw, Poland (WRSL).

The external morphology of the sori was observed under a Me-Opta stereo binocular dissecting microscope. The spores were scraped from the sori and mounted in a lactophenol – cotton blue mixture and observed under a Leitz Wetzlar microscope. For the study of pycnia, aecia, uredia, and telia either freehand sections or microtome sections were made using a Spencer 820 microtome. The microtome sections were prepared by the customary paraffin method of Johansen (1940). Camera lucida figures were drawn using a Leitz Wetzlar mirror type at the table level. The rust taxa were identified by using pertinent literature (Sydow and Sydow 1915, Arthur 1934, Savulescu 1953, Kuprevich and Tranzschel 1957, Gäumann 1959, Wilson and Henderson 1966, Ziller 1974, Hiratsuka *et al.* 1992). In addition to these, original publications and several papers pertaining to regional floras were also consulted.

3 Taxonomy and systematic location of the *Melampsora* species

***Melampsora* (Schub) Cast., Obs. Pl. Actoy. Fam. Ured. 1: 18. 1843.**

Pycnia subcuticular of type 3 or subepidermal type 2 of Hiratsuka and Cummins (1963). Aecia caeomoid. Uredia subepidermal, erumpent, paraphysate, paraphyses clavate to capitate. Urediospores pedicillate, borne singly on pedicels, colourless, echinulate, germ pores usually obscure. Telia subepidermal or subcuticular, indehiscent. Teliospores single celled, sessile, laterally adherent, appear like crusts of one spore deep.

Key to the species of *Melampsora* on *Populus* (Salicaceae)

1.	Urediospore wall thickened laterally	2
1.	Urediospore wall not thickened laterally	5
2.	Urediospore length more than 35 µm	3
2.	Urediospore length up to 35 µm	*M. medusae*
3.	Urediospore lateral thickness more than 3.5 µm	4
3.	Urediospore lateral thickness up to 3.5 µm	*M. osmaniensis*
4.	Teliospore apical thickness up to 3 µm	*M. larici-populina*
4.	Teliospore apical thickness up to 6 µm	*M. occidentalis*
5.	Urediospore length more than 20 µm	6
5.	Urediospore length less than 20 µm	*M. microspora*
6.	Teliospore length more than 30 µm	7
6.	Teliospore length up to 30 µm	*M. ciliata*
7.	Teliospores apically not thickened	8
7.	Teliospores apically thickened	*M. cumminsii*
8.	Urediospore length 20–35 µm	*M. abietis-populi*
8.	Urediospore length 30–40 µm	*M. populnea*

Melampsora populnea is a collective species consisting of five *formae speciales* on different aecial hosts

Aecia on *Allium, Arum, Muscari*	f. sp. *allii-populina* (Kleb.) comb. nov.
Aecia on *Chelidonium, Corydalis, Fumaria, Papaver*	f. sp. *magnusiana* (Wagn.) comb. nov.
Aecia on *Larix*	f. sp. *laricis* (Hart.) Boer. & Verh.
Aecia on *Mercurialis*	f. sp. *rostrupii* (Wagn. ex Kleb.) Boer. & Verh.
Aecia on *Pinus*	f. sp. *pinitorqua* (Hart.) Boer. & Verh.

Melampsora abietis-populi Imai, in Ito and Murayama, Trans. Sapporo Nat. Hist. Soc. 17: 164. 1943.

Caeoma abietis-mayrianae S. Imai. Ann. Phytopathol. Soc. Japan 12: 68–69. 1942.

Pycnia hypophyllous, type 3. Aecia hypophyllous, aeciospores globose, subglobose, obovate or broadly ellipsoid, 20–32.5 x 17.5–27 µm, wall 2–3.5 µm thick, densely verrucose. Uredia hypophyllous, urediospores ellipsoid, oblong or pyriform, 21–35 x 15–20 µm thick. Telia mostly hypophyllous, subepidermal; teliospores 25–47.5 x 7–12.5 µm, wall 1 µm thick.

Type: On *Populus nigra* L. var. *italica* Du Roi (Seiyohakoyanagi), Hokkaido, Bihoro-cho, Abashiri, 16 May 1942, S. Imai (Neotype: HH-78314, Isoneotype: SAPA).

Hosts and distribution: Pycnia and aecia on *Abies firma* Sieb. et Zucc, *A. homolepis* Sieb. et Zucc, *A. sachalinensis* Mast., *A. sachalinensis* var. *mayriana* Miyabe et Kudo, *A. veitchii* Lindl. Uredia and telia on *Populus koreana* Rehd., *P. maximowiczii* A. Henry, *P. nigra* L., *P. nigra* var. *italica* Du Roi, *P. simonii* Carr., and *P. euramericana* (Dode) Guinier. Japan.

M. abietis-populi almost resembles *M. populnea* in both the urediospore and teliospore morphology except for having slightly smaller urediospores. Further, the aecial stage is found on *Abies*, whereas none of the *formae speciales* of *M. populnea* have *Abies* as their aecial host. This species is so far reported from Japan only.

Melampsora ciliata Barcl., Ind. Journ. of the Asiatic Soc. Bengal 60: 223. 1891.

Pycnia and aecia not known. Uredia minute, hypophyllous, scattered, subepidermal, erumpent, pulverulent; urediospores 18–30 x 13–22 µm, ovate to ellipsoid, wall 1.5–2 µm thick, echinulate; paraphyses capitate, up to 50 µm long, 12–18 µm wide, apically 3–6 µm thick. Telia minute, hypophyllous, scattered, subepidermal, not erumpent, orange-brown to dark-brown; teliospores 30–34 x 8–9 µm, wall 1–2 µm thick.

Type: *Populus ciliata* Wall., Kashmir, India.

Hosts and distribution: On *Populus ciliata*, India

Specimens examined: On *Populus ciliata*, India, E.J. Butler, Kashmir, September 1908, HCIO.

Melampsora ciliata resembles *M. medusae* Thuem. in the urediospore morphology but differs from it in not having any lateral thickenings. In addition *M. ciliata* is confined to India.

Melampsora cumminsii Bagyanarayana and Ramachar, Curr. Sci. 53: 863–865. 1984 (Fig. 1)

Pycnia and aecia not known. Uredia minute, hypophyllous, scattered, subepidermal, erumpent, pulverulent, up to 0.2 mm, orange yellow; urediospores 21–32 x 12.5–21 µm, globose, subglobose, ovate to ellipsoid, wall 1.5–2 µm thick, echinulate; paraphyses clavate to spathulate, up to 45 µm long and 10–20 µm wide. Telia minute, hypophyllous, dense, very closely aggregated, subepidermal, not erumpent, 0.3 mm, or may be more due to fusion, dark cinnamon-brown; teliospores 24–40 x 8–16 µm, cylindraceo-prismatic, wall 1.5–2 µm thick, apically the corners are thickened prominently up to 3–5 µm, brown.

Holotype: On *Populus* sp., USA, Echo Lake North Conway, NH (Farlow Herbarium, Harv. Univ. JFC 16054), FH.

Hosts and distribution: On *Populus* sp., USA

Specimens examined: Type only.

The characteristic feature of this rust is the presence of conspicuously thickened apical corners of the teliospore wall; this feature is not seen in any of the other common *Melampsora* species.

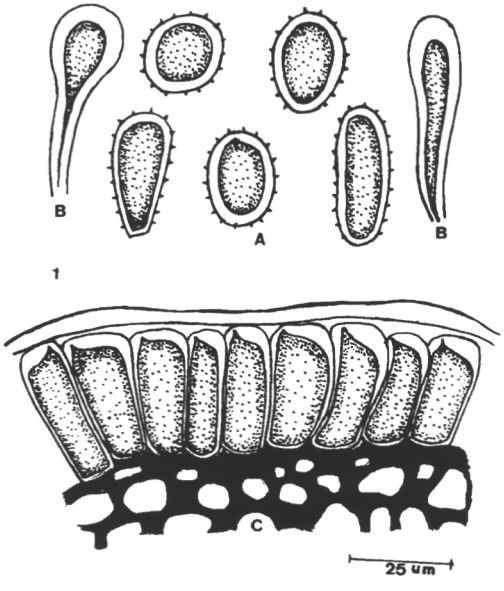

Figure 1. *Melampsora cumminsii*. A) urediospores, B) paraphyses C) vertical section of a telium showing teliospores.

Melampsora larici-populina Kleb., Zeitschr. f. Pfl. Krankh. 12: 43. 1902 (Fig. 2).

Uredo cylindrica Str., Ann. Wetter. Ges. 2: 92. 1910.

Caeoma laricis (West). Hartig, Wichtige Krankh. d. Waldbäume, p. 93. 1874.

M. cylindrica (Str.) Rostr., Tidsskr. f. Skovbrug. 2: 152. 1877.

U. larici-populina Arth., Result scient. Congr. Intern. bot. Vienne p. 338. 1905, 1906.

Pycnia amphigenous, subepidermal, up to 100 µm diam and 60 µm high. Aecia caeomoid, rarely pseudoperidiate, hypophyllous, 0.5–1 mm diam, orange, subepidermal, erumpent; aeciospores 16–24 x 15–20 µm, globose, subglobose, ovate, wall 1–2 µm thick, finely verruculose. Uredia hypophyllous, scattered or aggregated in small groups, 0.5–1 mm, subepidermal, initially covered by epidermis, later erumpent, pulverulent; urediospores 30–50 x 15–22 µm, broadly ellipsoid or oblong, wall 2 µm thick, thickened bilaterally up to 7 µm; paraphyses clavate to capitate, up to 80 µm long, and 15–20 µm wide, thickened apically up to 8 µm. Telia mostly epiphyllous, rarely hypophyllous, minute, aggregated in small groups, often fusing, initially light brown, later blackishbrown, 1 mm, subepidermal, not erumpent; teliospores 40–70 x 6–10 µm, rounded at both ends, wall 1–1.5 µm thick at sides, thickened apically up to 3 µm.

Hosts and distribution: Pycnia and aecia on *Larix decidua* Mill., *L. gmelini* Litv., *L. gmelini* var. *coreana* Ueki, *L. gmelini* var. *japonica* Maxim., *L. gmelini* var. *olgensis* Ostenfeld et Syrach-Larsen, *L. gmelini* var. *principis-rupprechtii* Pilger, *L. kaempferi* Sarg., *L. laricina* (DuRoi) K. Koch, *L. leptolepis* (Sieb. et Zucc.) Gord., *L. occidentalis* Nutt., and *L. sibirica* Led. Uredia and telia on *Populus alba* L., *P. adenopoda*, *P. angulata* Ait., *P. balsamifera* L., *P. canadensis* Moench, *P. candicans* Ait., *P. eugenei* Sim. Louis., *P. euramericana*, *P. eucalyptus*, *P. glerica* Hout., *P. generosa* Henry, *P. grandidentata* Michx., *P. koreana* Rehd., *P. laurifolia* Ldb., *P. lasiocarpa*, *P. marylandica* Moench., *P. maximowiczii*, *P. monilifera* Ait., *P. nigra*, *P. nigra* var. *italica*, *P. regenerata* Henry, *P. robusta* Schneider, *P. salicifolia* Rafin., *P. serotina* Hartig, *P. sieboldii* Miq., *P. simonii*, *P. suaveolens* Fisch., *P. trichocarpa* Torr. et Gray, *P. wettsteinia*, *P. wislizenii* (Wats.) Sarg., and *P. yunnanensis*.

China, Europe, India, Japan, Korea, Mongolia, New Zealand, South America, USA and USSR.

Type: On *Populus canadensis*, Borsteler Jager, Hamburg, Germany.

Specimens examined: G, LEV, S.

Melampsora larici-populina is similar to *M. occidentalis* Jacks. in its general morphology and the symptoms produced on the host. However, it differs from *M. occidentalis* in having subepidermal pycnia, and has a very limited aecial host range, confining itself to *Larix*.

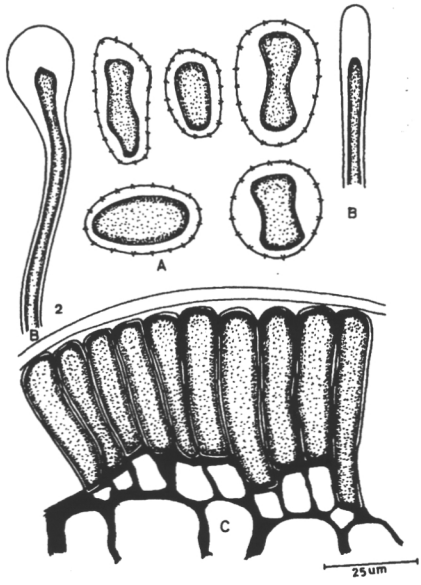

Figure 2. *Melampsora larici-populina*. A) urediospores, B) paraphyses, and C) vertical section of a telium showing teliospores.

Melampsora medusae Thuem., Bull. Torrey Bot. Club. VI: 216. 1885 (Fig. 3).

Caeoma abietis-canadensis Farl., Proc. Am. Acad. 20:323. 1885.
Uredo medusae Arth., Res. Sci. Congr. Bot. Vienne 338. 1906.
M. albertensis Arth., Bull. Torrey Bot. Club. 33: 517. 1906.
Peridermium fructigenum Arth., No. Am. Flora 37: 578. 1910.
C. pseudotsugae-douglasii Tub., Nat. Z. Forst. Landw. 12: 91. 1914.
M. abietis-canadensis (Farl.) C.A. Ludwig, Phytopath. 5: 279. 1915.
M. populi-tsugae J.J. Davis, Trans. Wis. Acad. 19: 676. 1919.
M. abietis-canadensis C.A. Ludwig ex Arth., No. Am. Flora 7: 664. 1924.
Caeoma faulliana Hunter, J. Arnold Arb. 17: 118. 1936.

Pycnia amphigenous, occasionally on cones, rarely on young shoots, punctiform, subcuticular, 40–80 µm in diameter.

Aecia mostly hypophyllous, on young stems and cones, scattered or slightly aggregated, pale yellow, subepidermal, erumpent, pulverulent; aeciospores 17–26 (32) x 13–20 (24) µm, globose or subglobose, broadly ellipsoid or obovate, wall colorless, verrucose, thickened bilaterally on opposite sides. Uredia minute, amphigenous, mostly hypophyllous, scattered or irregularly aggregated 0.3–0.5 mm in diam, erumpent, slightly pulverulent; urediospores 23–35 x 15–23 µm, ovate to ellipsoid, mostly laterally flattened, echinulate, wall 2.5–3 µm thick, thickened bilaterally on opposite sides up to 10 µm; paraphyses numerous, capitate, up to 65 µm long, 13–25 µm wide, wall 3–6 µm thick. Telia minute, amphigenous, mostly hypophyllous, scattered or aggregated, reddishbrown, subepidermal, not erumpent, up to 0.5 mm; teliospores 20–45 x 10–15 µm, prismatic, cinnamon brown, wall uniformly 1–1.6 µm thick, smooth.

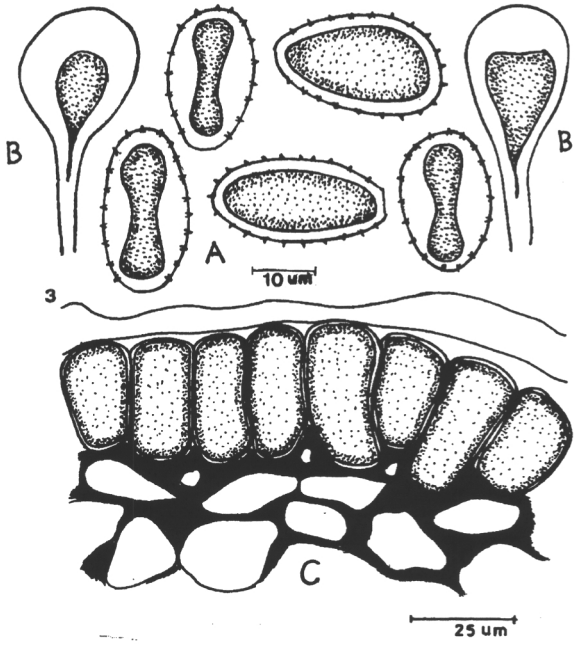

Figure 3. *Melampsora medusae*. A) urediospores, B) paraphyses, and C) vertical section of a telium showing teliospores.

Hosts and distribution: Pycnia and aecia on *Abies canadensis* Michx., *A. concolor* Hoops., *A. grandis* (Dougl.) Lindl., *A. lasiocarpa* (Hook.) Nutt., *Larix decidua*, *L. laricina*, *L. leptolepis*, *L. occidentalis*, *Picea sitchensis* (Bong.) Carr., *Pinus banksiana* Lamb., *P. contorta* Dougl., *P. lambertiana* Dougl., *P. ponderosa* Laws., *P. radiata* D. Don., *P. resinosa* Ait., *P. sylvestris* L., *Pseudotsuga menziesii* (Mirb.) Franco, *P. mucronata* (Raf.) *Sudw.*, *P. taxifolia* (Lamb.) Britt., *Tsuga canadensis* (L.) Carr., and *T. mertensiana* (Bong.) Carr. Uredia and telia on *Populus acuminata* Rydb., *P. angulata*, *P. angustifolia* James, *P. aurea* Tedstrom, *P. balsamifera*, *P. canadensis*, *P. candicans*, *P. carolinensis* Moench., *P. ciliata*, *P. dilatata* Ait., *P. grandidentata*, *P. heterophylla* L., *P. medusa* Benth., *P. monilifera*, *P. nigra*, *P. occidentalis* (Rydb.) Britt., *P. tremuloides* Michx., *P. trichocarpa*, *P. virginiana* Fouger, and *P. wislizenii*.

Argentina, Australia, Canada, France, India, Japan, and USA.

Type: On *Populus angulata* (*P. medusae* Benth.) Aiken - *Cardina australis* H.W. Ravenel. 1876.

Specimens examined: DAOM, DAVFP, FH, G, HCIO, S, WRSL.

Ziller (1974), based on his studies (Ziller 1965), reduced *M. albertensis* Arth. to a synonym of *M. medusae*, as they are "indistinguishable from each other in morphology, life history, host range and host reaction". In the present work *M. abietis-canadensis* is reduced to a synonym of *M. medusae*, as there is no difference in morphology or host range. Indeed, Arthur (1934) considered *M. abietis-canadensis* as a form of *M. medusae*

Melampsora microspora Tranz. et Eremeeva, Tranzschel Consp. Ured. URSS. Moscow, p 155. 1939.

Pycnia and aecia not known. Uredia minute, mostly hypophyllous, occasionally epiphyllous, scattered, often fusing; urediospores 11–17 x 11–13 µm, globoid or broadly ellipsoid, wall up to 4 µm thick, faintly verrucose, almost smooth; paraphyses clavate to capitate, 60 µm long, up to 15 µm wide, wall 5 µm thick. Telia mostly hypophyllous, scattered, often fusing, sub-epidermal, not erumpent, chestnut brown; teliospores 30–68 x 10–13 µm, apically not thickened.

Hosts and distribution: On *Populus nigra*, *P. usbekistanica* Kom., and *Populus* sp.

Iran and USSR.

Type: On *Populus nigra*, USSR.

This species is conspicuous in having the smallest urediospores among all the known poplar rusts. In addition the urediospore wall is mostly smooth except being faintly verrucose (Kuprevich and Tranzschel 1957).

Melampsora occidentalis Jacks., Phytopath. 7: 354. 1917 (Fig. 4).

Caeoma occidentalis Arth., Bull. Torrey Bot. Club 34: 591. 1907.

Pycnia on current year's needles on young shoots, subcuticular. Aecia on current year's needles, on young shoots; aeciospores 26–35 x 22–27 µm, globose, subglobose or broadly ellipsoid, wall thickenned bilaterally on opposite sides, verrucose. Uredia chiefly hypophyllous, scattered, round, 0.5–1.5 mm in diam, subepidermal, erumpent, pulverulent, in some cases the epidermis is persistent, orange yellow; urediniospores 30–50 x 17–30 µm, ellipsoid, oblong or pyriform, slightly flattened laterally, wall hyaline, 2–3 µm thick, laterally thickened up to 8 µm, echinulate; paraphyses numerous, capitate to clavate, up to 75 µm long and 15–25 µm wide, apically up to 6.5 µm thick. Telia minute, hypophyllous, scattered or circularly grouped, up to 0.6 mm in diam, subepidermal, not erumpent, cinnamon-brown to blackish brown; teliospores 40–66 x 10–20 µm, wall 1–2 µm thick at sides, cinnamon-brown, apically thickened up to 6 µm.

Hosts and distribution: Pycnia and aecia on *Abies concolor*, *Larix leptolepis*, *L. occidentalis*, *Picea sitchensis*, *Pinus contorta*, *P. lambertiana*, *P. monticola* Dougl., *P. ponderosa*, *P. radiata*, *Pseudotsuga menziesii*. Uredia and telia on *Populus alba, P. acuminata*, *P. angustifolia*, *P. aurea*, *P. balsamifera*, *P. candicans*, *P. candicantis* Ait., *P. carolinesis*, *P. dilatata*, *P. fermontii* Wats., *P. hastata* Dode, *P. monilifera*, *P. nigra*, and *P. occidentalis*.

Canada and USA.

Specimens examined: DAOM, FH, and HCIO.

Melampsora occidentalis closely resembles *M. larici-populina*, but differs in having a wide range of aecial hosts, possessing subcuticular pycnia, and teliospores which are more prominently apically thickened.

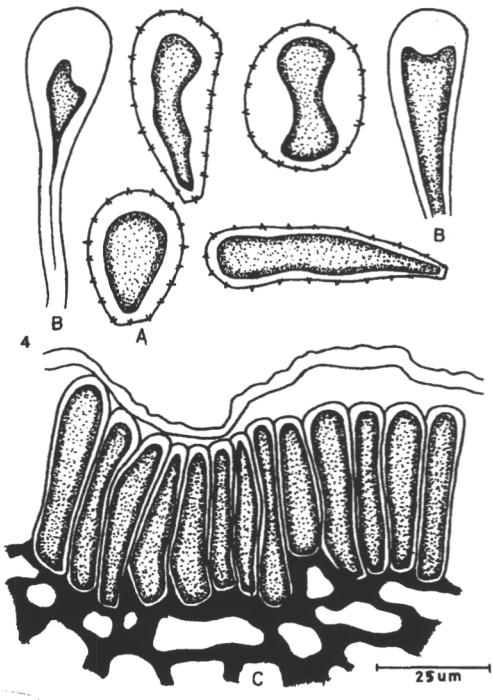

Figure 4. *Melampsora occidentalis*. A) urediospores, B) paraphyses, and C) vertical section of a telium showing teliospores.

Melampsora osmaniensis Bagyanarayana and Ramachar, Curr. Sci. 53: 863–865. 1984 (Fig. 5).

Pycnia and aecia not known. Uredia hypophyllous, dense, scattered over the entire leaf, subepidermal, erumpent, pulverulent, but sometimes epidermis is intact, 0.5–1.5 mm, may be due to fusion, pale orange; urediospores, 28–48 x 11–21 µm, ellipsoid, obovate to pyriform, wall 1–1.6 µm thick, side walls are thickened up to 3.2 µm, echinulate; paraphyses numerous, clavate to capitate, up to 60 µm long, 15–25 µm wide. Telia minute, epiphyllous, scattered, subepidermal, not erumpent, up to 0.3 mm, orange-brown to cinnamon-brown; teliospores 17.5–31.5 x 8–13 µm, cylindrical, round at both ends, wall 1 µm thick, pale brown.

Hosts and distribution: On *Populus sieboldii*.

Germany.

Holotype: On *Populus sieboldii*, Germany, P. Henning, Berlin Pr. Rixdorf, Sapthsche Baumschule, September 1895, Herb. Lugd. Bot. 10 Eur. As No. 910, 230–439.

Specimens examined: Type only.

Morphologically *M. osmaniensis* may resemble *M. larici-populina*, in the shape and size of the urediospores but differs in having thin urediospore side wall thickenings (up to 3.2 µm only). In addition, the teliospores are very small without any apical thickening.

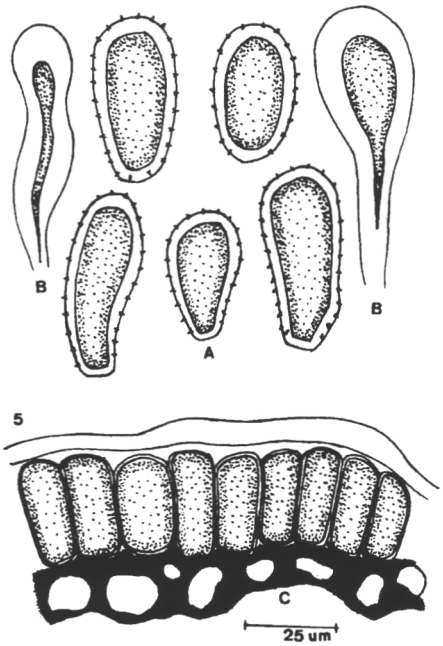

Figure 5. *Melampsora osmaniensis*. A) urediospores, B) paraphyses, and C) vertical section of a telium showing teliospores.

Melampsora populnea (Pers. ex Pers.) Karst., Bidr. Kenn. Finl. Nat. Folk 31: 53. 1879 (Fig. 6).

Sclerotium populneum Pers., Observationes mycol. 2: 25. 1799.
Sclerotium populneum Pers. ex Pers., Syn. Meth. Fung. p. 125. 1801.
Xyloma populinum Schum., Enum. Pl. Soell. 2: 179. 1803.
Uredo aecidioides D.C., Fl. Fr. 2: 236. 1805.
U. ovata a. *populi* Str., Ann. Wetter. Ges. 2: 92. 1810.
Sclerotium (Xyloma) populinum (Pers.) Fr., Syst. Myc. 2: 262. 1822.
Xyloma populinum (Pers.) Duby., Bot. Gall. p. 875. 1830.
Melampsora populina (Pers.) Lev., Ann. Sci. Nat. Bot. 3(8): 375. 1847.
M. tremulae Tul., Ann. Sci. Nat. Bot. 4(2): 95. 1854.
M. aecidioides (D.C.) Schroet., Cohn. Krypt. Fl. Schles. 3(1): 362. 1887.

Pycnia subepidermal, yellow, 140 µm high and 100 µm wide. Aecia on leaves and stems in small groups, subepidermal, erumpent, pulverulent, ruptured epidermis conspicuous, 1 mm in diam, orange yellow; aeciospores 17–25 x 14–19 µm, globose, ovato-globose or angulately globose, wall 2 µm, finely verruculose.Uredia hypophyllous, scattered or aggregated, subepidermal, usually erumpent, pulverulent, sometimes having intact epidermal covering, orange; urediospores 28–40 x 15–20 µm, elongated, ellipsoid or ovate, echinulate; paraphyses, numerous, capitate, up to 60 µm long, 16–20 µm wide. Telia amphigenous, scattered or aggregated, often fusing, subepidermal, not erumpent, reddishbrown to dark brown; teliospores 40–50 x 10–15 µm, cylindraceo-prismatic, pale brown.

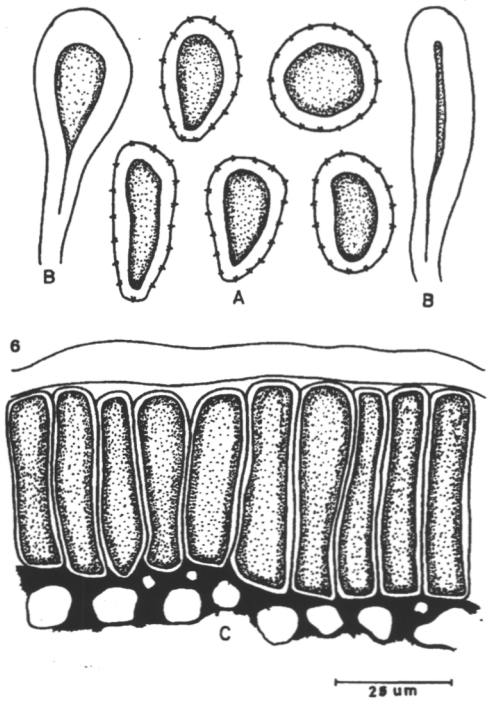

Figure 6. *Melampsora populnea*. A) urediospores, B) paraphyses, and C) vertical section of a telium showing teliospores.

Hosts and distribution: Pycnia and aecia on species of *Allium, Arum, Chelidonium, Corydalis, Larix, Mercurialis, Muscari, Papaver* and *Pinus*. Uredia and telia on species of *Populus*.

Cosmopolitan in distribution.

Type: On *Populus tremula*.

Europe.

Most poplar rusts are included under this collective species. This follows Hylander *et al.* (1953), Wilson and Henderson (1966), and Boerema and Verhoeven (1972), thus placing under *M. populnea* the various 'races' that do not differ essentially in morphology, but only in their aecial hosts. These are considered as *formae speciales*.

Melampsora populnea f. sp. ***allii-populina*** (Kleb.) Bagyanarayana comb. nov.

Caeoma allii-ursini Wint., Pilze Deutschl. p. 255. 1881.
C. ari-italici (Duby.) Wint., Pilze Deutschl. p. 256. 1882
Melampsora allii-populina Kleb., Zeitschr. f. Pfl. Krankh. 12: 25. 1902.
Uredo allii-populina Arth., Result. Scient. Congr. intern. bot. Vienne. p. 338. 1905, 1906.
M. allii-populina Kleb. f. sp. *muscaridis-populina* Viennot Bourgin Rev. Path. Veg. 24: 81. 1937.

Hosts and distribution: Pycnia and aecia on *Allium cepa* L., *A. ascalonicum* L., *A. carinatum* L., *A. oleraceum* L., *A. sativum* L., *A. schoenoprasum* L., *A. sphaerocephalum* L., *A. ursinum* L., *A. vineale* L., *Arum maculatum* L., *A. orientale* M.B., and *Muscari comosum* (L.) Mill. Uredia and telia on *Populus balsamifera*, *P. canadensis*, *P. laurifolia*, *P. monilifera*, *P. nigra*, *P. occidentalis*, and *P. suaveolens*.

Europe, USA and USSR.

Lectotype: On *Populus nigra*, Triglitz in der Prignitz, Brandenburg, Germany.

Specimens examined: FH, PAV, and S.

Klebahn (1902) created *M. allii-populina* to accommodate a poplar rust having its aecial stages on *Allium*. However, morphologically *M. allii-populina* closely resembles *M. populnea* (Pers. ex. Pers.) Karst. As such *M. allii-populina* is reduced to the status of a *forma specialis*.

Gäumann (1959) recognized two *formae speciales* in this species. One is *M. allii-populina* f. sp. *muscaridis-populina* Viennot Bourgin, having *Muscari* as its aecial host, and another is *M. allii-populina* f. sp. *allii-populina*, having *Allium*. Cruchet conducted experiments to prove *Arum* as an aecial host of *M. allii-populina*. From these studies it is clear that the *Melampsora* rust on *Populus* having *Allium, Arum* and *Muscari* as its aecial hosts is the same.

Melampsora populnea f. sp. ***laricis*** (Hart.) Boer. & Verh., Neth. J. Pl. Path. 78(1): 82-201. 1972.

M. laricis Hartig, Allg. Forst. u. Jagdz. 61: 326. 1885. (*nomen provisorium*); Hartig ex Kleb., Zeitschr. f. Pfl. Krankh. 7: 341. 1897.
M. tremulae f. *laricis* Hart., Lehrb. d. Baumkrankh. Ed. p. 193. 1889.
M. larici-tremulae Kleb., Forstl. Naturw. Zeitschr. 6: 470. 1897.
Zeitschr. f. Pfl. Krankh. 9: 146. 1899.

Hosts and distribution: Pycnia and aecia on *Larix decidua*, *L. leptolepis*, *L. sibirica*. Uredia and telia on *Populus alba*, *P. acuminata*, *P. balsamifera*, *P. canescens* Sm., *P. davidiana* Dode, *P. tomentosa* Carr., *P. tremula* L., and *P. tremula* var. *villosa*.

Europe, Japan, USA and USSR.

Type: On *Populus tremula*.

Specimens examined: S.

Melampsora laricis was reduced from the status of a species to a *forma specialis* by Boerema and Verhoeven (1972), as there was no morphological difference between *M. populnea*, and *M. laricis*. The author has followed Boerema and Verhoeven (1972). According to Gäumann (1959), this rust overwinters and perennates dikaryotic mycelium in the buds of the host.

Melampsora populnea f. sp. ***magnusiana*** (Wagn.) Bagyanarayana comb. nov.

Caeoma fumariae Link., Spec. Plant. VI. II: 24. 1825.
C. chelidonii Magn., Hedwigia 14: 20. 1875.

M. magnusiana Wagn., Österr. Bot. Zeitschr. 4f: 274. 1896.
(*nomen nudum*); Wagn. ex Kleb., Zeitschr. f. Pfl. Krankh. 7: 340–342. 1897.
M. klebahni Bubak, Sitz. ber. K. Bohm. Ges. Wiss., Math. nat. Cl. 28: 20. 1898 (*nomen nudum*); Zeitschr. f. Pfl. Krankh. 9: 28. 1899.

Hosts and distribution: Pycnia and aecia on *Chelidonium majus* L., *Corydalis ambigua* Ch. et Schl., *C. bracteata* L., *C. bulbosa* D.C., *C. cava* (Mill.) Schw. et K., *C. digitata* Pers., *C. fabacea* Pers., *C. laxa* Fries, *C. nobilis* (L.) Pers., *C. pumila* (Host.) Rchb., *C. remota* Fisch., *C. solida* (L.) Sw., *C. solida* var. *laxa* (Fr.) Mansf., *Fumaria officinalis* L., and *Papaver dubium* L. Uredia and telia on *Populus alba*, *P. canescens*, *P. geniculata* Michx., *P. grandidentata*, *P. nigra*, *P. rotundifolia*, *P. sieboldii*, *P. suaveolens*, *P. tomentosa*, *P. tremula*, and *P. trichocarpa*.

Canada, Czechoslovakia, China, Japan, Hungary, Korea, Norway, USA and USSR.

Type: On *Populus tremula*, Hernskretschen, Bohemia, Czechoslovakia.

Specimens examined: C, DAOM, FH, G, and S.

The poplar rust *M. magnusiana* is morphologically similar to *M. populnea*. The only difference is in the aecial hosts. Therefore it is reduced to the rank of a *forma specialis*.

Melampsora populnea f. sp. ***pinitorqua*** (Hart.) Boer. & Verh., Neth. J. Pl. Path. 78(1): 82–201. 1972.

Caeoma pinitorqum de Bary, Monatsber. K. Akad. Wiss. Berlin p. 264. 1863.
M. pinitorqum (Rostr.) Hartig, Allg. Forst u. Jagdz. 61: 326. 1885 (*nomen fortuium*; III not described).
M. tremulae f. *pinitorqum* Hartig, Lehrb. d. Baumkrankh. p. 140. 1889.
M. pinitorqua Rostr., De farligste Snyltere i Danmarks Skove. p. 10. 1889. Tidskr. f. Skovbrug 12: 177. 1890.
Uredo pinitorqua Arth., Result. scient. Congr. intern. bot., Vienne. p. 338. 1905, 1906.
M. pruinosae Tranz., Mycotheca rossica No. 265. 1912.

Hosts and distribution: Pycnia and aecia on *Pinus montana*, *P. mugo* Turra, *P. mugo* var. *rotundata* (Link.) Hoopes, *P. pinea* Habl., *P. ponderosa*, and *P. sylvestris*. Uredia and telia on *Populus alba*, *P. balsamifera*, *P. canescentis*, and *P. tremula*.

Europe, USSR and Japan.

Type: On *Populus tremula*, Denmark, E. Rostrup, Jylland Klelund Plantque, August, 1882, C.

Specimens examined: C, and S.

Boerema and Verhoeven (1972) reduced the species *M. pinitorqua* to the status of a *forma specialis* of the collective species *M. populnea* The author agrees with their treatment. According to Gäumann (1959), the basidiospores infect the pine sprouts in early spring and form a perennating intercellular mycelium in the bark-parenchyma and also in the

phloem and xylem vessels. Pycnia start developing at the end of May or early June. Aecia develop afterwards.

Melampsora populnea f. sp. ***rostrupii*** (Wagn. ex Kleb.) Boer. & Verh., Neth. J. Pl. Path. 78(1): 82–201, 1972.

Uredo mercurialis Mart., Prodr. fl. Mosq. II: 229. 1817.
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(*nomen nudum*); Wagn. ex Kleb., Zeitschr. f. Pfl. Krankh. 7: 342. 1897.
(*nomen provisorium*) et Die Wirtwechseldenen Rostpilze p. 407. 1904.
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Nat. Afrique du Nord, p. 139. 1915.

Hosts and distribution: Pycnia and aecia on *Mercurialis perennis* L. and *M. annua* var. *ambigua* (L.) Batt. Uredia and telia on *Populus alba*, *P. nivea* Wild., *P. balsamifera*, *P. canadensis*, *P. canescens*, *P. cordata* Hort. ex Poir., *P. euphratica* Oliv., *P. italica* (Dur.) Moench., *P. monilifera*, *P. nigra*, *P. rarius*, *P. subintegerrima* Lange, and *P. tremula*.

Europe, India, USA and USSR.

Type: On *Populus tremula*, Denmark, Rostrup, Overs K.D. Vidensk, Selsk, Forh. p. 14. 1884.

Specimens examined: C, FH, G, PAN, S, and WRSL.

The author has followed Boerema and Verhoeven (1972) in treating *M. rostrupii*, as a *forma specialis* of *M. populnea*.

Acknowledgements

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Leaf rust of *Populus* section Leuce in China

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Summary

Leaf rust of *Populus* section Leuce occurs widely in China. *Populus tomentosa* has been seriously injured by this kind of disease and seedling growth is affected. The main pathogens are *Melampsora magnusiana* and *M. rostrupii*, which differ in their alternate hosts. Mycelium of these rusts overwinters in the overwintering poplar buds. The following spring, the infected buds expand and a great quantity of yellow urediospores is formed, and this is the main source of infection. After germination, the urediospores penetrate the cuticle directly and infect the leaves. The incubation period is about 4–5 days; suitable germination temperature is 15–20° C and maximum temperature is 30° C. The pathogens infect only tree species of section Leuce and its hybrids. The rust tolerance of different species of section Leuce is different. *Populus tomentosa, P. hopeiensis* and *P. alba* are highly susceptible species; *P. davidiana* and *P. alba* var. *pyramidalis* are comparatively rust-resistant. Clonal tolerance varies significantly among geographical areas. The control measures are the following. (1) Clear away the diseased buds in spring. (2) Breed varieties with strong rust tolerance. (3) During rust incidence, spray 100 x 50% ambam or 500–1000 x 50% urbacide-ziram-thiram fungicide as a wettable powder. Triadimefon is effective in controlling the leaf rust on *P. alba*, but it will produce chemical burn on *P. tomentosa* leaves. (4) Clear away all the diseased trees for a distance of 1000 m from new nurseries.

Key words: leaf rust disease, *Melampsora magnusiana, Populus tomentosa*

1 Introduction

The cultivation of poplar in China has a long history. Poplars have been the main tree species planted in the plains of north China for many years. Since the founding of New China, they have been planted as windbreaks and to prevent soil erosion. A network of farmland and forest has been established and this has improved the environment and has also guaranteed high and steady agricultural production. According to statistics of the Chinese Poplar Commission in 1992, poplar plantations in China reached 18.47 million ha, accounting for 18.75% of the total area of plantations in China. Most of these are concentrated in central China, north China, northeast and northwest China (Zhao and Chen 1994). China is rich in poplar species. There are 18 poplar species in section Leuce, of which many are native tree species in China, such as *Populus tomentosa* Carr., *P. davidiana* Dode, and *P. simonii* Carr.

During the growing period, poplars are damaged by many kind of diseases

and insects. Leaf rusts of Chinese poplar in section Leuce are caused by several species of *Melampsora*. They are *M. abietis-canadensis* (Farl.) Ludw., *M. larici-populina* Kleb., *M. larici-tremulae* Kleb., *M. magnusiana* Wagner, *M. occidentalis* Jacks., *M. rostrupii* Wagner, and *M. pruinosae* Tranz. (Yuan 1984, Xu 1985). This paper discusses *M. magnusiana.*

2 Distribution and damage

The leaf rust on poplars of section Leuce occurs in most of their production areas; it is especially serious in Hebei, Henan, Shandong, Shanxi, Shaansi, and Xinjiang provinces (or autonomous regions). The rust mainly attacks young seedlings. When *P. tomentosa* is seriously infected, its new buds are wilted, leaves are twisted, and tender twigs are wilted. Thus, the height and diameter growth are affected and the time for transplanting is delayed. In Xinjiang, the seedling death rate of *P. alba* L. can reach over 10%. The rust causes less harm to big poplars.

Different *Populus* spp. vary in tolerance to *M. magnusiana*, which only infects poplars in section Leuce and its hybrids. The other poplars are immune or highly resistant. The rust tolerance of different poplars in section Leuce also varies: *P. tomentosa*, *P. hopeiensis* Hu & Chow, and *P. alba* are highly sensitive; *P. davidiana* and *P. alba* var. *pyramidalis* are relatively resistant. In China, *P. tomentosa* has a wide distribution and many varieties. Therefore clones of this species differ greatly in tolerance to *M. magnusiana*. The clones from southwest Shaanxi, southeast Gansu, and south-central Shanxi are highly resistant, whereas those from Henan, south-central Hebei, and the west part of Shandong are sensitive (Zhou *et al.* 1979).

3 Symptoms

The rust occurs on the leaves, pedicels, buds and young twigs. It causes scattered, round, yellow leaf spots that are the size of a pinhead or soybean. The diseased spots on pedicels and tender twigs are elliptical or shuttle-like. The infection can occur from leafing-out in early spring to autumn or winter when leaves fall. The infected winter buds usually sprout 2–3 days earlier than healthy buds. The symptoms on the infected winter buds differ according to the degree of infection. If seriously infected, the buds cannot burst normally; the folded tender leaves are covered with the yellow urediospore powder and soon die. If lightly infected, the tender leaves of the buds are wrinkled, thickened, and rolled oppositely to normal. Their surface is densely covered with uredia, creating the appearance of a yellow flower. If lightly infected, the buds can spread out normally with a small quantity of uredia on both sides of the tender leaves. Sometimes only an individual leaf from a bud is infected. The number of winter buds infected is usually very few, even in seriously infected areas, the amount being less than 0.2% of the total.

4 Pathogen

According to the records, there are three rust species on *P. tomentosa* in China (Ge *et al.* 1964, Zhou *et al.* 1979): *M. magnusiana*, *M. rostrupii* and *Uredo tholopsora* Cumm. So far, the former two species are common in China. No obvious differences in morphology and size between the urediospores, teliospores and paraphyses of these two species have been found. Uredia are yellow, scattered or aggregated. Urediospores are orange-yellow, round or elliptical, echinulate, and size 19.6–25.3 by 16.1–21.0 μm. Wall thickness is 2.8–3.5 μm. Paraphyses are head- or spoon-like, light yellow or colorless. Telia are located under the host's epidermis, and teliospores are nearly column-like, size 37–50 by 10–15 μm. These two species are classified according to their alternate host. The alternate hosts of *M. magnusiana* are plants of the genera *Corydalis* and *Chelidonium*, whereas those of *M. rostrupii* are plants of the genus *Mercurialis*. Based on the records, the spermogonia and aecia of *M. magnusiana* have been found on *Corydalis* in Hebei. Microscopic comparison of the morphology of urediospores and paraphyses of the three rust pathogens has been done. Artificial inoculations showed that they belong to the same species but there may be differences in pathogenicity (Zhou 1997).

5 Disease cycle

The mycelium overwinters in buds. In the following spring, the infected buds burst and form a big amount of uredia, which are the main sources for current-year infection. The mycelium in the diseased spots of the tender twigs can also overwinter and form uredia. The teliospores on the diseased fallen leaves can germinate and infect the alternate host but with the rise of temperature, their germination capacity decreases sharply. Before or after leaf fall, some teliospores are formed, but the alternate host is scarcely infected. Teliospores are not important in the infection cycle.

After germination, urediospores can penetrate directly through the cuticle. The incubation period is 4–15 days according to the temperature and age of the leaves. Leaves over two months are usually not infected. Suitable temperatures for urediospore germination are 15–20° C, and they will not germinate over 30° C (Shen *et al.* 1979). Thus, the peak of its incidence coincides with the peak of *P. tomentosa* growth. At this time, not only the temperature is important, but there are many tender leaves susceptible to infection. In a warm and humid summer, fungi in the genera *Cladosporium, Alternaria* and *Trichothecium* invade the rust spots (uredia and the neighbouring tissue), and a great deal of urediospores disappear. So in autumn when there are suitable temperature and tender leaves, the epidemic trend is not so important as it is in early summer. Research has shown that the infection of *P. tomentosa* leaves is in positive relation with the activities of polyphenol oxidase, peroxidase and phenylalaninase in the leaves (Shen *et al.* 1979). In the first stage of infection, the activities of the above-mentioned enzymes change obviously.

6 Control measures

1) Clear away the diseased buds in early spring. Since the diseased buds are fresh yellow, have an abnormal shape, and flush leaves earlier than the normal buds, it is easy to see them. This method is effective, but it is necessary to prevent the spores from spreading to other poplars.

2) Select poplar varieties with a strong tolerance. This is a simple and effective method.

3) During the incidence period, spray 100 x 50% ambam or 500–1000 x 50% urbacide-ziram-thiram fungicide as a wettable powder. Triadimefon is effective to control the leaf rust of *P. alba*, but it will produce chemical burn on *P. tomentosa* leaves.

4) Clear away all the diseased trees for a distance of 1000 m from new nurseries.

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Poplar rust (*Melampsora larici-populina*): the development of E4 pathotypes in France since 1994

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Summary

Soon after their description in Belgium, E4 pathotypes of *Melampsora larici-populina* were first detected at very low frequency in northern France in fall 1994. These pathotypes are able to infect some important cultivars, like *Populus deltoides* x *P. trichocarpa* 'Beaupré' or *P.* x *euramericana* 'Ghoy', which were selected for complete resistance. Surveys were conducted annually and at the end of 1997 these pathotypes were present in all the area of intensive poplar cultivation in France. Four different pathotypes were identified exhibiting between 3 and 6 of the 7 virulences described within *M. larici-populina*. Only wild stands of the native *P. nigra* located in SE France were free of these pathotypes.

Severe attacks were described in 1997 in the north and the east of the country. Complete defoliation occurred on some stands of 'Beaupré' at the beginning of July, with subsequent flushing and a second budding. *Melampsora allii-populina*, usually prevalent on this cultivar, disappeared. Some stands were chemically treated. Such severe damage resulted from a conjunction of several factors: larch (*Larix decidua*) trees planted close to poplar (allowing the alternation of the fungus), a wet spring, and valleys uniformly planted (up to 80%) with this susceptible cultivar. Even infected euramerican poplar showed better resistance. In spring 1998, larch was easily found infected in Picardie and Burgundy, while some 'Beaupré' stands already showed decline, like absence of flushing and bark necrosis (caused by *Discosporium populeum* and *Cytospora chrysosperma*) on the main stem.

Numerous cultivars were tested in the nursery and in the laboratory to establish their behavior towards the different pathotypes of *M. larici-populina*. Some new cultivars may be less susceptible, and completely resistant cultivars will be put on the European market despite the risk of a future breakdown of their resistance.

Key words: *Populus deltoides*, *Populus nigra, Populus trichocarpa,* virulence

1 Introduction

Poplar breeding has been very active and efficient in Europe for more than 40 years, mainly in Belgium, Italy, and the Netherlands. Very vigorous hybrids have been selected after crossing the American *Populus deltoides* Marsh. with the European *P. nigra* L. [= *P. x euramericana* (Dode) Guinier] or with balsam poplar (*P. trichocarpa* Torrey & Gray). Clones completely resistant to

the major leaf rust pathogen (*Melampsora larici-populina* Kleb.) were selected. While such cultivars accounted for around 20% of the cultivated poplars in 1987, they exceeded 50% in 1995 in France (Fig. 1). Unfortunately such complete resistance is unstable, the fungus being able to develop physiological races, as first demonstrated by van Vloten (1949). Nevertheless, breeders and growers did not pay attention to this risk despite recent warnings (Lemoine and Pinon 1978). In 1982 a new race was found in Belgium (Steenackers 1982) and in France (Pinon and Bachacou 1984). E3 races were discovered in France in 1986 (Pinon and Peulon 1989), while Pinon and Lefèvre (1994) described a new group in 1994. Until recently, only E3 races caused important damage, mainly on 'Luisa Avanzo', but this cultivar accounted for at most 5% of the plantations in France. In 1994, a new type of race (E4) was discovered in Belgium (Steenackers *et al.* 1994) and in France (Pinon 1995a). It was able to break down the complete resistance of cultivars like 'Beaupré', 'Boelare', 'Donk', 'Dorskamp', 'Flevo', and 'Ghoy', with 'Beaupré' being widely cultivated (up to 80 % in the northern stands). The aim of this paper is to present the current knowledge on the E4 races.

2 Material and methods

Several hundred samples have been collected in the field since 1994 mainly by nursery controllers (from the Ministry of Agriculture) and by observers from the Département Santé des Forêts or by the authors. Identification of *Melampsora* species was achieved, for each uredium, under the microscope and only samples from *M. larici-populina* were kept for further determination of virulences. This analysis was conducted as previously described (Pinon 1992) using the set of differential clones proposed for the seven known virulences (Pinon 1995b).

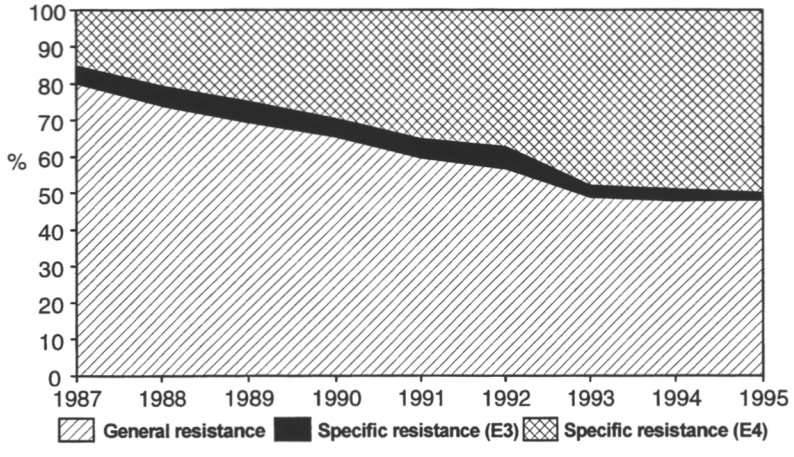

Figure 1. Evolution of the French populations of cultivars with general or specific resistance broken down by E3 or by E4.

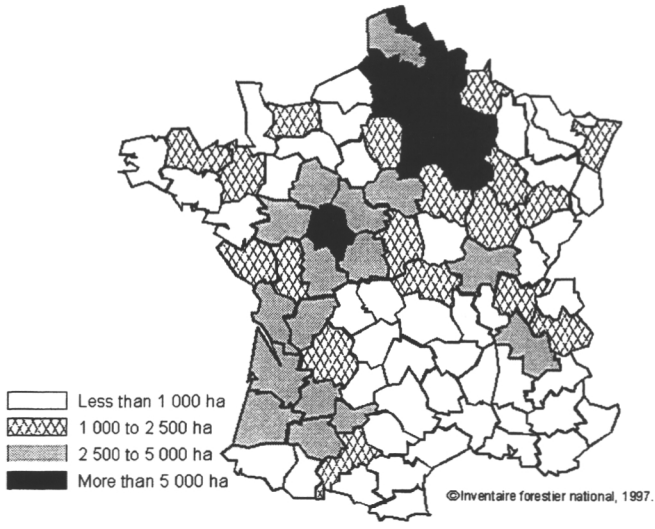

Figure 2. Distribution of poplar cultivation in France.

In the nursery, each plant was examined visually after 1997 flushing to detect the first infection. On 15–16 September 1997, all the clones susceptible only to E4 races were assessed for their symptoms with the method proposed by Pinon (1991) on a 0–100 scale, where 0 means healthy and 100 means all the leaves are completely covered with rust. The reaction of poplar clones to E4 was characterized *in vitro* and in the nursery. Cuttings were grown in the greenhouse to provide healthy leaves, which were collected when plants had developed 20 to 25 leaves. Disks were cut from the leaves inoculated by spraying spore suspensions, as already described (Pinon and Peulon 1989). Latency and the number of sori per disk were assessed.

Statistical tests were performed with StatGraphics software.

3 Results

3.1 Spread of E4 isolates in France between 1994 and 1997

A first survey was conducted in 1994, mainly close to the Belgian border where such isolates were first described. Although important infection was evident in the nursery and on adult trees in Geraardsbergen (Belgium) in September, the detection of E4 isolates in France was difficult because of their very low frequency and incidence. The first occurrence was found in three departments of northern France, and later in one in the east and one in central France. In our nursery near Nancy, all the clones susceptible to only E4 isolates were assessed leaf by leaf, but no infection with *M. larici-populina* was detected. In 1995 the area of the survey was enlarged and new cases of infection were found in nine other departments, while six others were apparently healthy. Two departments with infection were located in SW France far from the initial records. No real effect on the health of the poplar stands was observed. During the 1996 survey, many other departments were found contaminated by E4 isolates. Most of the important areas of poplar cultivation (Fig. 2) were affected by these isolates, while no trace of them

was found in 13 other departments. Last year (1997) most of the surveyed departments were declared contaminated even when poplar cultivation was insignificant (Fig. 3). Finally, E4 isolates had invaded most of the country within 4 years.

3.2 Temporal evolution of the epidemics due to E4 isolates

Although in 1994 E4 isolates were rare in northern and eastern France, in 1996 the first noticeable infection occurred on 'Beaupré'. In spring 1997, very early infections occurred followed by heavy infection and even complete defoliation at the beginning of July. Such epidemics were observed mainly on 'Beaupré' in locations where the alternate host (*Larix decidua* Mill.) was planted nearby. The epidemic was also favored by an unusually wet spring and early summer, and some stands were chemically treated. Although in the past, *M. allii-populina* was frequent on 'Beaupré in northern and eastern France, this fungus was totally absent there in 1997, being replaced by *M. larici-populina*, which developed numerous telia early. The following spring, the flushing was very limited in some young stands (about 5 years old) and bark infection due to secondary pathogens (*Discosporium populeum* (Sacc.) Sutton and *Cytospora chrysosperma* Fr.) was already obvious. Here again, the proximity of the alternate host was an important risk factor.

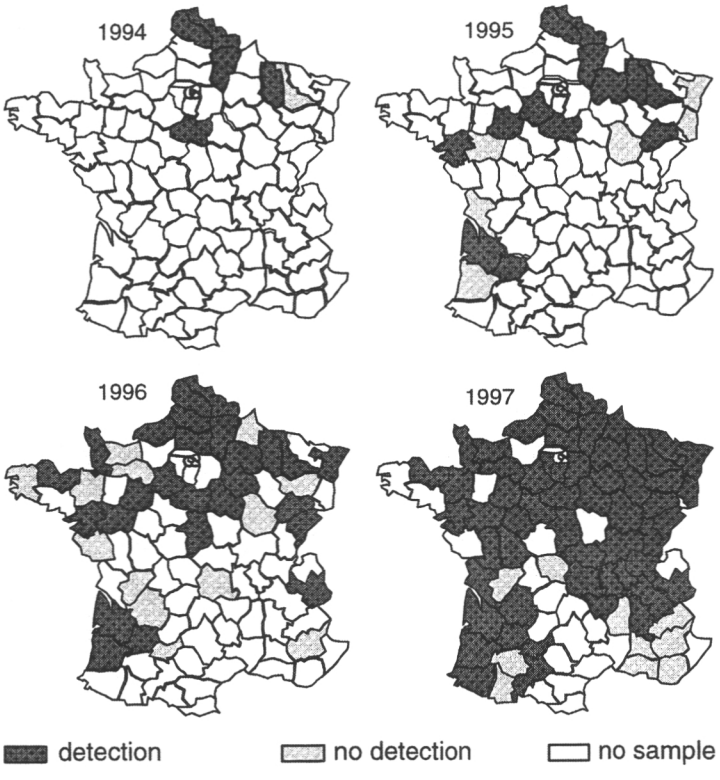

Figure 3. Spread of E4 isolates between 1994 and 1997.

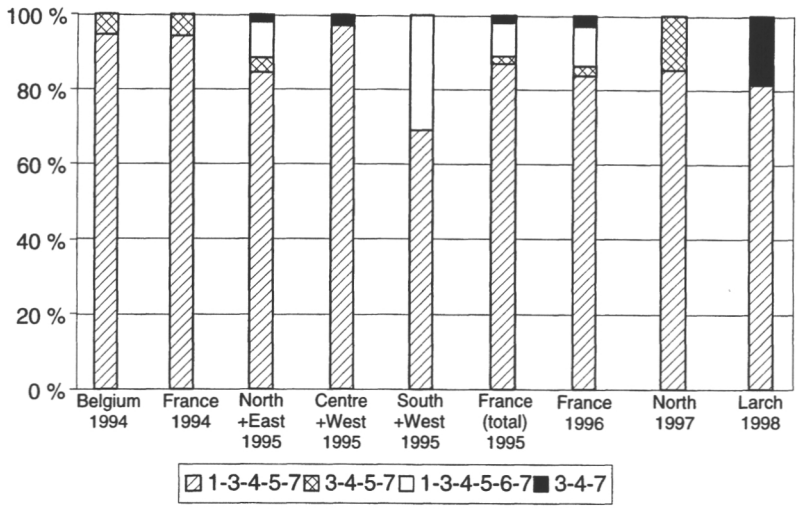

Figure 4. Frequencies of the four pathotypes from E4 isolates.

In our nursery near Nancy, where a large set of clones is present (some with general resistance and others with different specific resistance), we looked for E4 pathotypes on 'Robusta' (susceptible to all known isolates, including E4) and on European larch. In September, the frequencies of these isolates on 'Robusta' were successively 0/204 isolates in 1995, 2/508 in 1996, and 0/139 in 1997. On European larch, the frequencies were in spring 0/115 isolates in 1995, 1/192 in 1996, 0/81 in 1997 and 9/146 in 1998. Despite the fact that E4 isolates were not detected on 'Robusta' and larch samples in 1997, such isolates infected clones like 'Beaupré' and 'Boelare', which are susceptible only to these isolates.

3.3 Pathotypes among E4 isolates and their frequencies

All the E4 isolates have virulence in common 7. In 1994, both in Belgium and in northern France, we detected only two pathotypes: 1.3.4.5.7 (17 isolates out of 18 in Belgium, 16/17 in northern France) and 3.4.5.7. In 1995, it became possible to collect higher numbers of isolates and consequently to detect two other pathotypes: 3.4.7 and 1.3.4.5.6.7. Among more than 500 isolates identified from 1994 to 1998, none included virulence 2 (Fig. 4). Pathotype populations of the different years and areas were compared through a Chi-square test. Significant differences between years were found but they were mainly due to the inconstant frequency of pathotype 1.3.4.5.6.7. Nevertheless, 1.3.4.5.7 isolates were always the most common, whatever the year and the area of collection. Overall, the frequencies of this pathotype varied between 82 and 94%, but without any significant change between 1994 and spring 1998. In 1995 some differences were observed among the populations from different geographical origins (mainly isolates from 'Beaupré') without obvious reasons.

In a short rotation experiment, in which 'Raspalje' (susceptible to all known races) was planted near 'Beaupré', E4 pathotypes represented 26% of the isolates (93 identified) in 1997. Among these E4 isolates, there was 71% of 1.3.4.5.7, 17% of 3.4.5.7, and 12% of 1.3.4.5.6.7, confirming the

predominance of the 1.3.4.5.7 pathotype on clones susceptible only to E4 isolates.

3.4 Behavior of clones specifically susceptible to E4 pathotypes

During the summer 1997, infections developed well on clones susceptible only to E4 pathotypes. Therefore, detection of early infection and later assessment of the level of infection on such clones were conducted (Fig. 5). The first evidence of infection was detected on 'Boelare' on 25 July, and later the other clones became infected. For some clones, the first sori were found only at the end of the growing season, i.e. 84 days later ('YU S 120'). Infection progressed significantly in August and September, and on 15 and 16 September symptoms were assessed. Among the 29 clones present in our nursery that became susceptible to *M. larici-populina* after the outbreak of E4 pathotypes, a wide range of behavior was observed (Fig. 5). Some clones (mainly *Populus deltoides* x *P. trichocarpa*) were very susceptible (including cultivars like 'Boelare' and 'Beaupré'), whereas others exhibited a high level of general resistance. A very significant relationship ($r = -0.64$, $df = 29$) was established between the earliness of the first detection of rust in the season and the level of infection mid-September. Early detection was conducted again in spring 1998, when the frequency of E4 pathotypes became significant on larch (6%). 'Boelare' became infected earlier (9 June) than in 1997 and all clones (except the three more resistant ones, see Fig. 5) were infected by July 15.

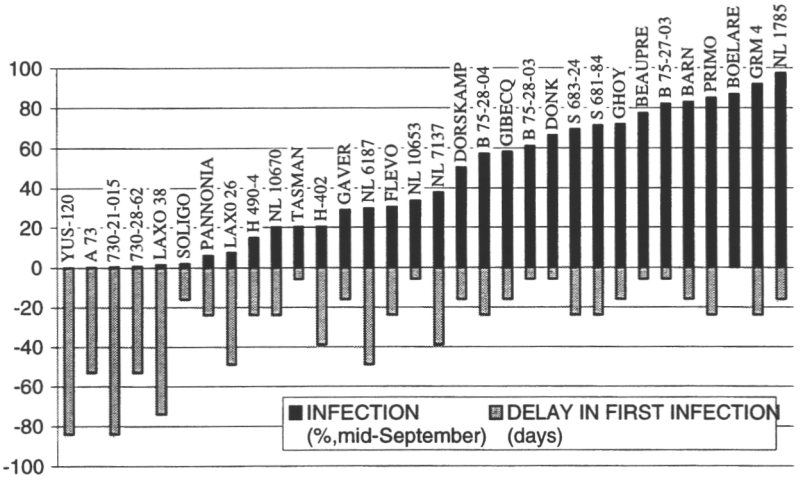

Figure 5. Behavior of clones susceptible only to E4 isolates in 1997 in a nursery near Nancy. Above: level of infection expressed as percentage related to the most susceptible clone ('NL 1785'). Below: delay (in days) in the appearance of first infection with reference to the date from 'Boelare' (25 July).

The most valuable clones were inoculated in the laboratory with one isolate of the most frequent pathotype (1.3.4.5.7). Latency and density of sori were estimated. By taking advantage of the 1997 assessment of infection on the same clones in the nursery, it was possible to compare the two sets of data. A multiple regression between the infection level in the nursery (expressed as its logarithm) and the data from the laboratory (latency and number of sori) was calculated. It provided a model explaining 75.6% of the variability of the infection level in the nursery. Highly significant correlation coefficients were established between the infection in the nursery and the data from the laboratory: –0.81 with latency and 0.79 with the number of sori.

4 Discussion

E4 pathotypes likely came from Belgium and began to establish in 1994 in France. Within 4 years they spread to all areas of poplar cultivation, and severe infection and damage are already known on 'Beaupré'. This is the first important epidemic due to new races. This may be explained by the extensive cultivation of a susceptible cultivar, the presence of the alternate host nearby, and a wet 1997 climate. However, the onset of E4 pathotypes was slower when the host population consisted of a mixture of clones (with different types of resistance). Four different pathotypes were described, which raises the question of their appearance: simultaneously or one appearing first (the most frequent) followed soon after by recombination. The pathotype 1.3.4.5.7 is the most frequent everywhere despite its two unnecessary virulences (1 and 5) for infection of the major host ('Beaupré). It does not seem to be counter-selected, which is in agreement with its high level of aggressiveness found in the laboratory (data not presented). The absence of virulence 2 may be explained by a phenomenon of allelism or by the absence of recombination with isolates having this virulence.

A wide range of behavior was found among clones, with a general tendency to insufficient general resistance in *P. deltoides* x *P. trichocarpa*. Specific resistance delayed the epidemic as long as E4 pathotypes were infrequent, as suggested for other diseases by van der Planck (1968). Laboratory tests reflect well the behavior of the clones in the field and provide a useful tool when discovering a new race.

We have identified clones that are still completely resistant, but the appearance of new races is continuing. In 1997 we found isolates able to infect 'Hoogvorst' and 'Hazendans'. The challenge for breeders is still to select for a fair level of general resistance and for growers to diversify the cultivars they plant.

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Sympatry between alternate hosts affects population structure of poplar leaf rust

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Summary

Poplars and larches are the telial and aecial hosts of *Melampsora medusae* f. sp. *deltoidae*. Epidemics of poplar leaf rust occur both within and outside the zone of sympatry of the two hosts. To test the hypothesis that epidemics originate from the zone of host sympatry, samples from 14 populations in sympatry and allopatry were tested with two PCR-based anonymous DNA markers using single-strand conformation polymorphism to allow codominant detection of alleles directly from uredia. The analysis revealed seven alleles for locus A in the zone of host sympatry, but only five in the zone of allopatry. For locus B, 18 alleles were found, 17 alleles in sympatry and only eight alleles in allopatry. Average gene diversity was larger in sympatry (0.65) than in allopatry (0.45). These results suggest that the sympatric area constitutes a center of genetic diversity from which epidemics originate. Allelic composition seems to indicate that there is gene flow from northern (sympatry) to southern (allopatry) rust populations.

Key words: *Melampsora medusae*, poplars

1 Introduction

Poplar leaf rust caused by *Melampsora medusae* Thuem. is considered among the most serious diseases affecting poplars. The biology of the rust in North America is characterized by a zone of sympatry, where the two hosts of the pathogen coexist and sexual reproduction can take place, and a zone of allopatry, where only one host is present and asexual reproduction presumably predominates. To date, however, there has been no test of this hypothesis and the origin of the epidemics has not been demonstrated.

The most efficient and least environmentally damaging method for poplar leaf rust control is planting resistant poplar clones or hybrids. However, knowledge concerning the source of inoculum is important to test resistant clones against all possible rust genotypes. This study was initiated to improve our understanding of poplar leaf rust epidemiology by using molecular approaches to test hypotheses concerning the epidemics of this disease. This paper reports on the development of the methods and preliminary results of the population study.

2 Material and methods

2.1 Development of markers

Random amplified polymorphic DNA (RAPD) markers were used to obtain DNA fingerprints. Mono-uredial isolates of *M. medusae* were generated and used for marker development to ensure genetic purity of the material. Differences in the RAPD profiles were investigated further using the single strand conformation polymorphism (SSCP) method, which makes it possible to distinguish alleles on the basis of single base pair mutations. Polymorphic loci were cloned and sequenced. The DNA sequence was analyzed and polymerase chain reaction (PCR) primers flanking polymorphic DNA regions were designed and synthesized. Two sets of primers amplifying two markers were developed using this approach.

2.2 Population analysis

Samples were obtained from throughout the range of the disease in eastern North America, from both the zones of sympatry and allopatry (Fig. 1). A total of 480 samples were collected and analyzed. DNA was extracted from single uredia. Individual uredia were cut out of the leaves and placed into a 1.5 mL Eppendorf microtube. Therefore, an individual was considered to be a single uredium. Following DNA extraction (Hamelin *et al.* 1996), the *Melampsora*-specific primers were used to amplify DNA markers directly from individual uredia without the need to develop single-urediospore cultures. Various alleles making up the populations were then assayed by SSCP analysis. Fourteen populations were analyzed; each population consisted of 30 samples collected from 10 trees. A total of 420 samples was tested.

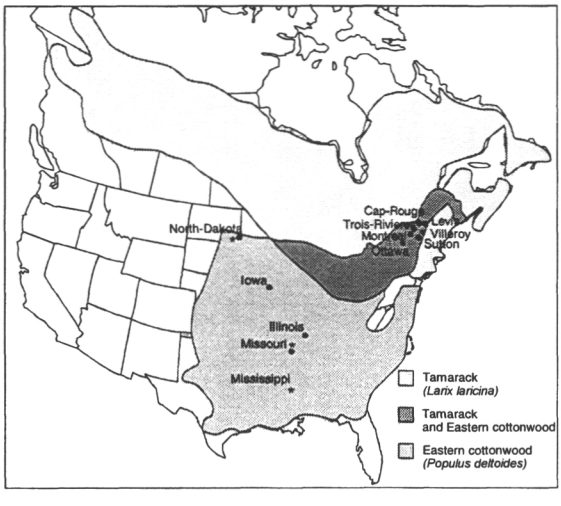

Figure 1. Distribution of the 14 populations under study throughout North America. Seven populations are located in sympatry in Canada. They are identified by the name of the city where the sampling was done. The other seven populations are located in allopatry in the USA. They are distributed within five states and are identified by the name of the state. Populations identified with a dot are natural stands, and populations with an asterisk are plantations.

3 Results and discussion

At locus A, seven alleles were detected, all of which were present in sympatry, but only five of which were detected in allopatry. At locus B, 18 alleles were found; 17 alleles were present in sympatry but only 8 of those alleles were present in allopatry (Table 1). Alleles at locus A were easier to detect because the migration on the SSCP gels gave clearly separated bands. Homokaryons were easily differentiated from heterokaryons by detecting the presence of two bands for homokaryons (one for each strand of the DNA fragment) and three or four bands for the heterokaryon (one or two for each strand of the two alleles, depending on the presence of co-migrating single strands) (Fig. 2).

The hypothesis that the aecial host, *Larix* spp., provides the primary inoculum for the poplar leaf rust epidemics in northeastern North America was tested by comparing allelic and gene diversity in the rust populations from the zone of allopatry between the telial and aecial hosts, and from the zones of sympatry. Gene diversity and allelic diversity were generally greater in the zones of sympatry than in allopatry, suggesting that the zone of sympatry constitutes a center of genetic diversity from which epidemics originate (Table 1). All but one allele found in allopatry were present in sympatry as well, suggesting that a gene flow exists from northern (sympatry) to southern (allopatry) rust populations.

Allelic distribution in the zone of allopatry seems to be highly clonal: similar allelic combinations occurred repeatedly in individual uredia from diverse sources. This suggests that gene flow occurs most probably from the asexual repeating urediospore stage in a similar but reverse fashion to the *Puccinia* path of cereal rusts (Roelfs 1989).

Table 1. Population parameters for the poplar leaf rust populations sampled in the zone of sympatry and the zone of allopatry of the aecial and telial hosts.

Population	Type of stand	Gene diversity	Number of alleles (locus A)	Number of alleles (locus B)
Ottawa	Natural stand	0.675	6	8
Montréal	Natural stand	0.484	5	7
Sutton	Natural stand	0.636	6	6
Trois-Rivières	Natural stand	0.645	5	11
Villeroy	Plantation	0.593	5	7
Cap-Rouge	Natural stand	0.628	5	11
Lévis	Natural stand	0.590	5	10
Average (sympatry)		0.650	5.29	9.00
North Dakota	Natural stand	0.657	5	3
North Dakota	Plantation	0.299	2	3
Iowa	Natural stand	0.478	5	4
Illinois	Natural stand	0.489	4	6
Missouri	Natural stand	0.327	3	3
Missouri	Plantation	0.451	4	3
Mississippi	Plantation	0.325	3	4
Average (allopatry)		0.450	3.71	3.86

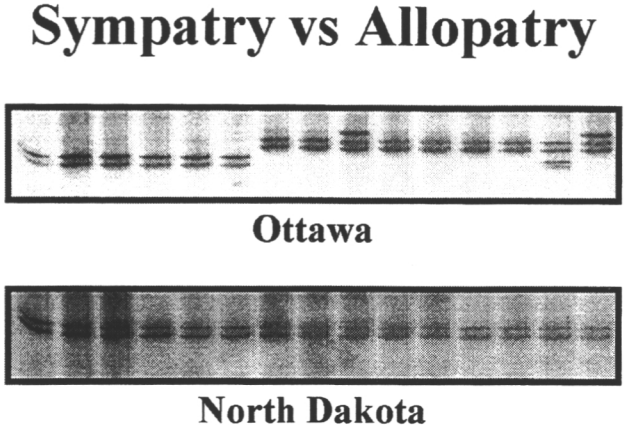

Figure 2. Comparison of a population located in sympatry (Ottawa, natural stand) and one located in allopatry (North Dakota, plantation). For each population, the result of genotyping of 15 samples on SSCP gels is shown to reveal differences between them. In Ottawa, five alleles are detected, whereas only one allele is detected in North Dakota. The alleles shown are from locus A, from which a total of seven alleles was identified.

Our results are consistent with the observation of earlier appearance of urediospores in spore traps in locations near the aecial hosts (Widin and Schipper 1980). However, some populations in the zone of allopatry (e.g. North Dakota, plantation) were highly polymorphic and possessed similar attributes to the populations in zones of sympatry. This observation could suggest that even in the zone of allopatry there could be local sources of overwintering. No coniferous alternate host was found in Kentucky in the zone of allopatry except for occasional ornamental larches (Shain 1988). Although winter urediospore survival has been reported in the Texas plain (Chitzanidis and Van Arsdel 1970), it appears that the spores did not survive the summer drought.

Our results suggest that the poplar leaf rust epidemics originate from the zone of sympatry between the aecial and telial hosts in northeastern North America and expand annually to the Gulf of Mexico. Circumstantial evidence of single-jump long-distance transport of *Melampsora* has been reported before (Van Kraayenoord *et al.* 1974, Wilkinson and Spiers 1976), and annual migrations from Mexico to the Canadian Prairies are well known (Stakman and Harrar 1957, Roelfs 1989).

The results of this study suggest that the source of inoculum of the epidemics might be distant from the sites where the tests for resistance screening of poplars are done; if the source is the aecial host, it would be expected that rust genotypes may be different from year to year. This could be important for poplar breeders in predicting rust population composition.

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Microflora associated with the phylloplane of healthy cultivated poplars and those attacked by *Melampsora* spp.

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Summary

The incidence of leaf rusts caused by some *Melampsora* species on fast-growing cultivated poplars has recently increased in Europe, both in nurseries and plantations. In Italy, this has been mainly due to the appearance and spread of the E3 race of *Melampsora larici-populina*. The use of the antagonistic action of fungi and/or bacteria associated with the poplar phylloplane for biological control under the usual conditions of cultivation has been proposed. A series of samplings was taken in 2-year-old polyclonal nurseries, at different times during the growing season and from healthy and infected leaves (*M. larici-populina* E3 on black and hybrid poplars, *M. rostrupii* on white poplar), to determine the composition of this microflora and determine the extent to which it is influenced by the presence of the pathogen. Several variability factors were considered and different sampling methods were used, some requiring selective treatment of the leaf surface.

The composition of the phylloplane microflora was not significantly affected by the location (northwestern and southern Italy) or the clone. However, changes were noticed as the growing season proceeded and also, though only in some cases, in relation to the presence of the parasite. With all sampling methods, a total of 23 mainly mitosporic and dematiaceous fungi were identified, with *Alternaria alternata* and *Epicoccum purpurascens* dominant. The relative frequencies of *E. purpurascens, Cladosporium herbarum* and *Acremonium* sp. and of the bacterium *Pseudomonas* sp. were correlated with the presence of the rust, but only under certain conditions and at certain times during the growing season.

Key words: *Alternaria, Cladosporium, Epicoccum, Melampsora larici-populina, Pseudomonas*

1 Introduction

The cultivation of fast-growing poplars for the manufacture of especially plywood and paper has recently been increasingly affected by leaf rusts caused by *Melampsora* species. This increased disease incidence is due to the high adaptability of the parasites, the ready diffusion of their propagules over long distances, and the occasional selection of unsuitable poplar clones, all factors

favoring the introduction of very virulent genotypes in regions previously characterized by pathosystems in fragile equilibrium. Damage in the field is quantitative, namely reduced annual growth owing to physiological stress, whereas very intense attacks in the nursery favored by a damp microclimate may even lead to the death of young poplar. The infected plants also become predisposed to weak parasites, mainly *Discosporium populeum* (Sacc.) Sutton, and hydrophysiological imbalance syndromes ("brown blotches").

Three *Melampsora* species are widespread in Europe on cultivated black poplars and hybrids [*Populus nigra* L., *P. deltoides* Bartr., *P. x euramericana* (Dode) Guinier]: 1) *M. medusae* Thüm. from North America, confined at present to Spain and southwestern France, perhaps because it is unable to complete its lifecycle on poplar in climates with a long winter, 2) *M. allii-populina* Kleb., a thermophilic species gravitating to central and southern Europe and preferring the southern parts of Italy, and 3) *M. larici-populina* Kleb., mainly distributed in central Europe and in the central and northern parts of Italy. Owing to the continuous proliferation of new physiological races compatible with a broad spectrum of clones, *M. larici-populina* is currently responsible for most of the damage referred to earlier. In Italy, it is almost solely present as physiological race E3, whose much greater virulence (Giorcelli *et al.* 1996) has relegated the old E1 population to brief appearances at the beginning of the summer.

The economic impracticability of chemical control and the long times needed to form a sound genetic improvement strategy have turned our attention to biological control, especially following the frequent observation in the field of uredia invaded by extraneous fungal hyphae and/or degraded in a more or less abnormal manner. It has thus appeared desirable to investigate the possibility of exploiting certain components of the microflora associated with the poplar phylloplane as biological control agents by relying on such antagonistic capacity as they may have and on their environmental adaptability (Blakeman 1985).

An approach of this kind must necessarily be preceded by a determination of the composition of this microflora, at different times during the growing season on leaves both attacked and not attacked by the parasite, in order to determine whether its presence is connected with the relative frequencies of phylloplane fungi or bacteria. Thus a sampling program was set up that would evaluate the influence of these factors on phylloplane colonization by a variety of microorganisms.

2 Material and methods

2.1 Variability factors

The microflora associated with the poplar phylloplane was sampled in 1996 and 1997 from 2-year-old polyclonal nurseries apparently free from diseases other than rust. They were not subjected to any form of chemical treatment and were regularly spray-irrigated. Most sites were at the experimental farm belonging to the Poplar Research Institute at Casale Monferrato (northwestern Italy) and one site was at the "Improsta" agricultural and forest farm at Battipaglia (southern Italy).

Five variables with different combinations were investigated over two years (Fig. 1): the geographical location, the time of sampling, the clone, the

presence/absence of the rust fungus (*M. larici-populina* E3 on black hybrid poplars or *M. rostrupii* Wagner on *P. alba* L.), and the position of the leaves on the stalk (three heights).

2.2 Sampling methods

Five methods for sampling phylloplane microflora were used in this study (Fig. 2): (I) leaf impression on agar, (IIa) serial washings of leaf disks and incubation on agar, (IIb) serial washings of leaf disks and incubation in a moist chamber (fungi only), (IIIa) sterilization of leaf disks and incubation on agar, and (IIIb) sterilization of leaf disks and incubation in a moist chamber (fungi only).

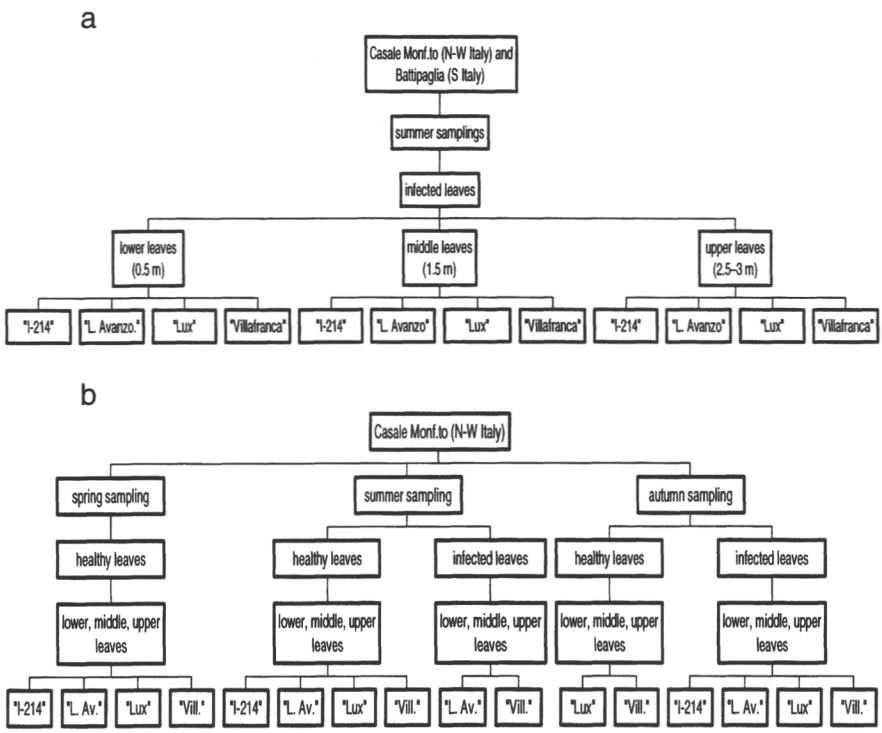

Figure 1. Samplings of the microflora associated with the poplar phylloplane made on leaves from 2-year-old polyclonal nurseries. a) Factors investigated in 1996 on infected leaves: geographical location (north-western and southern Italy, on 5th and 12th of August, respectively), position of the leaves on the stalk, different clone (*P. x euramericana* "I-214"and "Luisa Avanzo", *P. deltoides* "Lux" and *P. alba* "Villafranca"). b) Factors investigated in 1997: time of sampling in the growing season (28th of May, 5th of August and 29th of September), presence/absence of the rust fungus (*M. rostrupii* on "Villafranca", *M. larici-populina* E3 on the others), position of the leaves on the stalk, clone. The spring sampling was only made on the healthy leaves because of the complete absence of the parasite; in the summer sampling, "I-214" and "Lux" infected leaves were not yet found; in the autumn sampling, "I-214" and "L. Avanzo" healthy leaves were no longer found.

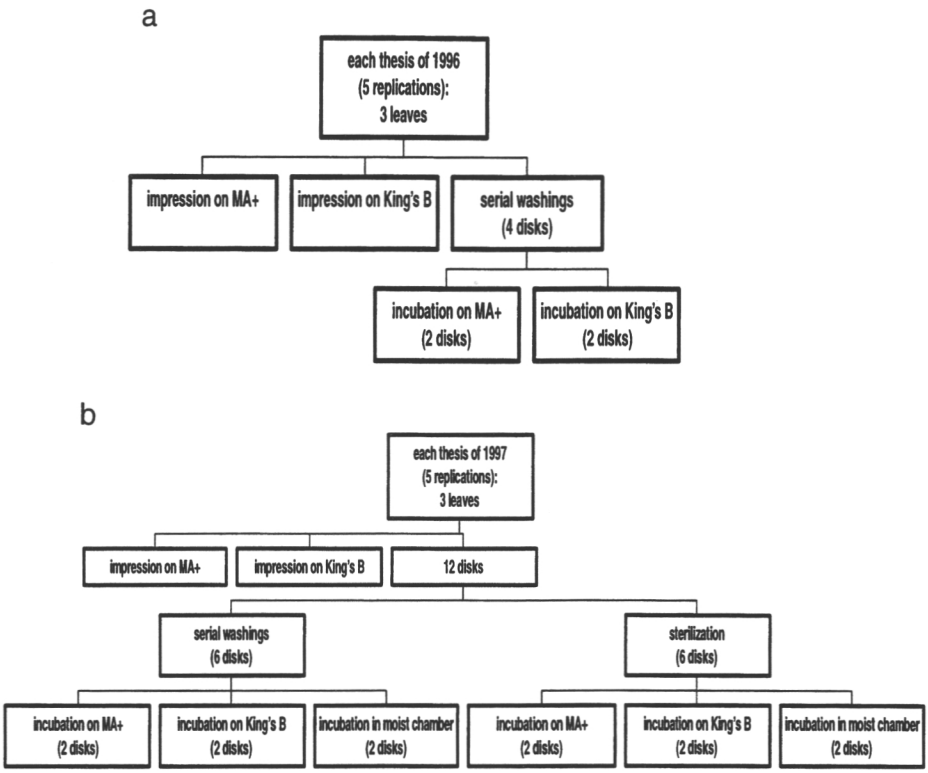

Figure 2. Methods used to sample the poplar phylloplane microflora. Impression or incubation on MA^+ (2% malt-gar medium supplemented with 50 mg/L aureomycin and 100 mg/L streptomycin) were made to detect fungi; impression or incubation on King's B medium were made to detect bacteria. In the "leaf impression" method, the abaxial surface of each leaf was pressed for a few seconds against the proper medium in a 9-cm petri dish; the"serial washings" method consisted of twenty 2-min washings in sterile distilled water of the leaf disks, afterwards dried and placed on the proper medium; in the "sterilization" method, seven serial washings were preceded by a 30-s immersion in a 0.1% (m/v) $AgNO_3$ solution of the disks, then rinsed twice for 2 min in two separate 0.5% (m/v) NaCl solutions to remove the sterilizer.

The first method, devised by Potter (1910) and used in a modified form in later investigations (Lamb and Brown 1970, Lindsey and Pugh 1976), was adopted to isolate all microorganisms on the phylloplane, including ephemerals and accidentals (e.g. fungi only present as propagules). The abaxial surface of each leaf was pressed for a few seconds, in a sterile environment, against a solid agar medium in a 9-cm petri dish and then removed. Fungi and bacteria were evaluated separately in all five methods: fungi on 2% malt-agar medium, regarded by many investigators as one of the best media for isolating a broad spectrum of saprotrophic fungi with no special nutritional requirements, supplemented with 50 mg/L aureomycin and 100 mg/L streptomycin (MA^+) to inhibit bacteria (Wildman and Parkinson 1979); bacteria on King's B medium to promote the proliferation of many prokaryotes.

Method (IIa) stems from works of Lindsey and Pugh (1976), Wildman and Parkinson (1979), and Vardavakis (1988); it allowed identification of active micro-organisms on the phylloplane, removing those present only as propagules. A corer, sterilized between each cutting, was used to extract disks (0.5 cm diam.) from each leaf in areas free from large veins. After twenty 2-min washings in sterile distilled water, these leaf disks were dried on sterile filter paper, and placed abaxial surface down, on agar (two leaf disks in each petri dish = one replication).

In the 2nd year, the methodology was extended to identify microorganisms colonizing the mesophyll and to isolate fungi whose slower mycelial growth places them at a disadvantage on malt agar (Vardavakis 1988). The first aim (corresponding to methods IIIa and IIIb) was pursued by sterilizing the leaf disks obtained by the previously described manner: each disk was dipped for 30 s in a 0.1% (m/v) $AgNO_3$ solution, rinsed twice in two separate 0.5% (m/v) NaCl solutions (2 min in each solution) to remove the sterilizer, then serially washed in seven changes of sterile distilled water (2 min per washing) and dried on sterile filter paper. The second aim (methods IIb and IIIb) was pursued by incubating some of the washed and some of the sterilized leaf disks in moist chambers, i.e. on sterile filter paper moistened with sterile distilled water in petri dishes; the humidity was periodically reestablished under sterile conditions. This incubation was used for fungi only, since it is not suitable for the isolation of bacteria.

2.3 Preparation and examination of the samples

All sampled leaves were collected from different plants for the replications, placed in separate sterilized polyethylene bags, maintaining them separated, stored at 5° C and treated within five days.

After all the treatments, the petri dishes and the moist chambers were incubated at 23–24° C under a 10 000 lx fluorescent light, irradiated with λ = 475 nm at 15 W m^{-2} (= 60 µE m^{-2} s^{-1}).

Fungal and bacterial colonies were recorded and described every 2–3 days for 21 days, then isolated for identification. Fungus sporulation was sometimes encouraged by using UV light, specific media, or media with a reduced nutrient content.

The relative frequency values, obtained from five replications per experiment, were transformed with the arcsine $x^{1/2}$ formula and subjected to analysis of variance (ANOVA), applying the Newman-Keuls test to determine the significance of differences between the means.

3 Results

3.1 Phylloplane mycoflora

Almost all the species recurring in the samplings are mitosporic fungi (teleomorphs, probably Ascomycetes, were never found), most of them dematiaceous or displaying pigmented structures; a few were moniliaceous; *Mucor hiemalis* Wehmer was the only Zygomycete. A good number of microfungi (9 of 23) were recorded in at least four of the five samplings, while others were sporadic and almost all found in the summer only.

Neither the geographical nor the clonal factor had any significant influence on the relative frequencies of the microfungi. As to the leaf height factor,

only *Alternaria alternata* (Fr.) Keissler on the sterilized leaf disks tended to be more frequent ($p < 0.05$) on the lower leaves (Table 1 and 2).

The species isolated varied considerably from one sampling method to another. Leaf impression on MA$^+$ provided the largest number of species, and showed major changes during the growing season. In fact, the composition of the phylloplane mycoflora recorded in this manner was always more complex and heterogeneous within the samplings carried out in the summer. Fungi were less diverse in the late spring sampling and the autumnal sampling (9 and 11 species, respectively, compared with 15–17 recorded in the summer), and the sporadic ones disappeared. The other methods were free from similar variations and, on the whole, gave quite equivalent results. They allowed isolation of only a part of the phylloplane components sampled by leaf impression on MA$^+$ and did not add any species to those isolated by leaf impression. The only difference of note between the washing and the washing plus sterilization methods was a slight quantitative rather than qualitative reduction of the fungi isolated on the sterilized leaf disks; there was no significant difference between the leaf disks incubated on MA$^+$ and those incubated in the moist chamber. It can therefore be stated that the mycoflora consistently associated with the cultivated poplar phylloplane, as shown by the methods applying some leaf treatment, consists of 5–6 species in varying frequency ratios.

Table 1. Relative frequencies of the components of phylloplane microflora from rust-infected leaves collected in 1996 at two different sites, from three different heights in 2-year-old polyclonal nurseries of *Populus x euramericana* "I-214" and "Luisa Avanzo", *P. deltoides* "Lux" and *P. alba* "Villafranca". The means with the same letters are not significantly different ($p<0.05$) according to Newman-Keuls test. The methods: see text.

	BATTIPAGLIA (S Italy)		CASALE MONF.TO (N-W Italy)	
Fungi	leaf impression on MA+	washed leaf disks on MA+	leaf impression on MA+	washed leaf disks on MA+
*Acremonium*sp.	4.89a	5.41a	3.13a	6.75a
Alternaria alternata	32.87a	42.93ab	29.75a	36.95a
Aspergillus clavatus	0.00a	0.00a	1.54a	0.00a
Aspergillus fumigatus	0.00a	0.00a	0.00a	0.00a
Aureobasidium pullulans	3.25a	3.17a	4.46a	0.00a
Cladosporium cladosporioides	1.62a	0.00a	2.62a	0.00a
Cladosporium herbarum	31.88b	8.30a	28.20b	10.50a
Epicoccum purpurascens	27.40a	33.33a	31.88a	39.68a
Fusarium heterosporum	0.00a	0.00a	0.00a	0.00a
Fusarium oxysporum	8.09b	0.00a	8.20b	5.15ab
Mycelia sterilia I	1.54a	0.00a	0.00a	1.60a
Mycelia sterilia II	1.73a	0.00a	0.00a	0.00a
Mycelia sterilia III	0.00a	0.00a	2.10a	0.00a
Mycelia sterilia IV	0.00a	0.00a	1.40a	0.00a
Mucor hiemalis	3.61a	0.00a	7.71a	0.00a
Nigrospora sphaerica	2.58a	7.74a	4.69a	12.09ab
Penicillium frequentans	2.76a	0.00a	1.34a	0.00a
Penicillium verrucosum	2.04a	0.96a	1.34a	1.38a
Phoma glomerata	0.00a	0.00a	1.46a	0.00a
Pithomyces chartarum	0.00a	0.00a	0.00a	0.00a
Rhinocladiella atrovirens	0.00a	0.00a	1.46a	0.00a
Sphaeropsis sp.	1.66a	0.00a	0.00a	0.00a
Trichoderma viride	0.00a	0.00a	1.42a	0.00a
Bacteria	on King's B	on King's B	on King's B	on King's B
Bacillus I	6.26b	0.00a	12.13b	2.92a
Bacillus II	52.56b	22.86a	51.53b	21.59a
Bacillus III	14.16b	0.00a	11.66b	0.00a
Pseudomonas sp.	22.86a	67.14b	25.85a	66.66b

Table 2. Relative frequencies of the components of phylloplane microflora from healthy and rust-infected leaves collected in 1997 at Casale Monferrato at three different times of the growing season in 2-year-old polyclonal nurseries of *Populus* X *euramericana* "I-214" and "Luisa Avanzo", *P. deltoides* "Lux" and *P. alba* "Villafranca. The means with the same letters are not significantly different ($p<0.05$) according to Newman-Keuls test. The methods: see text.

	LEAF IMPRESSION ON MA+* OR KING'S B**						WASHED LEAF DISKS ON MA?* OR KING'S B**						WASHED LEAF DISKS IN MOIST CHAMBER					
	spring		summer		autumn		spring		summer		autumn		spring		summer		autumn	
	healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected
Fungi																		
Acremonium sp.	1.62a	–	0.00a	0.00a	0.00a	2.95a	4.04ab	–	0.00a	0.00a	0.00a	5.08ab	3.72a	–	0.00a	0.00a	0.00a	9.68b
Alternaria alternata	36.97a	–	27.91a	29.05a	28.54a	28.67a	69.51b	–	49.95b	48.37b	31.34a	36.57a	61.62b	–	54.45b	46.73b	45.40b	48.93b
Aspergillus clavatus	0.00a	–	0.00a	0.00a	0.00a	2.74a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	2.47a	0.00a
Aspergillus fumigatus	0.00a	–	1.29a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Aureobasidium pullulans	9.81b	–	5.42ab	13.28b	6.47ab	0.00a	11.81b	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Cladosporium cladosporioides	7.29b	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a.	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Cladosporium herbarum	34.74c	–	31.97c	32.48c	32.61c	32.37c	8.25ab	–	5.27a	19.76b	13.42b	15.93b	14.69b	–	7.61ab	9.48ab	13.09b	14.02b
Epicoccum purpurascens	15.14b	–	31.66c	29.04c	33.89c	31.92c	1.93a	–	37.71c	34.50c	44.10c	42.20c	8.74b	–	32.57c	38.45c	30.83c	25.93c
Fusarium heterosporum	0.00a	–	1.46a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Fusarium oxysporum	3.09a	–	5.95ab	5.67ab	6.32ab	4.14a	0.00a	–	0.00a	3.25a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mycelia sterilia I	0.00a	–	1.29a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	2.69a	0.00a	0.00a	–	0.00a	0.00a	3.37b	0.00a
Mycelia sterilia II	0.00a	–	0.00a	0.00a	2.92a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mycelia sterilia III	0.00a	–	1.46a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mycelia sterilia IV	0.00a	–	0.00a	0.00a	3.25a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mucor hiemalis	6.21b	–	9.60b	8.38b	0.00a	7.58b	0.00a	–	0.00a	0.00a	2.38a	2.95a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Nigrospora sphaerica	0.00a	–	3.14a	5.80a	6.14a	3.51a	0.00a	–	0.00a	0.00a	14.02b	17.84b	0.00a	–	0.00a	0.00a	14.16b	11.96b
Penicillium frequentans	3.00a	–	6.06a	0.00a	5.85ab	2.48a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	4.75a	0.00a	0.00a	0.00a
Penicillium verrucosum	0.00a	–	0.00a	0.00a	0.00a	1.54a	0.00a	–	0.00a	0.00a	3.53a	1.29a	7.06b	–	0.00a	0.00a	2.70a	4.66ab
Phoma glomerata	0.00a	–	1.46a	6.27b	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Pithomyces chartarum	0.00a	–	1.54a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Rhinocladiella atrovirens	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Sphaeropsis sp.	0.00a	–	1.54a	0.00a	0.00a	1.56a	0.00a	–	2.01a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Trichoderma viride	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Bacteria																		
Bacillus I	34.06c	–	16.73b	19.06b	14.69b	7.49b	7.50b	–	10.00b	3.82a	0.00a	0.00a	–	–	–	–	–	–
Bacillus II	16.21b	–	21.59b	9.95ab	0.00a	2.94a	3.75a	–	4.56a	9.55ab	0.00a	0.00a	–	–	–	–	–	–
Bacillus III	1.75a	–	3.86a	4.43a	0.00a	2.21a	0.00a	–	0.00a	0.00a	0.00a	0.00a	–	–	–	–	–	–
Pseudomonas sp.	36.41b	–	54.82c	59.07c	75.32c	71.01c	33.75b	–	67.94c	78.79c	90.00d	90.00d	–	–	–	–	–	–

	STERILIZED LEAF DISKS ON MA+* OR KING'S B**						STERILIZED LEAF DISKS IN MOIST CHAMBER					
	spring		summer		autumn		spring		summer		autumn	
	healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected
Fungi												
Acremonium sp.	10.00b	–	0.00a	0.00a	0.00a	0.00a	4.35ab	–	0.00a	0.00a	0.00a	0.00a
Alternaria alternata	27.50a	–	58.35b	59.57b	37.16a	42.43b	46.46b	–	51.23b	54.79b	56.20b	54.85b
Aspergillus clavatus	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Aspergillus fumigatus	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Aureobasidium pullulans	0.00a	–	0.00a	0.00a	3.98ab	1.88a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Cladosporium cladosporioides	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Cladosporium herbarum	7.50ab	–	7.46ab	11.74b	0.00a	5.36ab	0.00a	–	6.93ab	6.44ab	13.83b	13.80b
Epicoccum purpurascens	7.50b	–	2.50a	10.88b	32.86c	40.40c	6.69b	–	2.30a	6.84b	21.90c	27.74c
Fusarium heterosporum	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Fusarium oxysporum	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mycelia sterilia I	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mycelia sterilia II	0.00a	–	4.59b	0.00a	0.00a	0.00a	0.00a	–	0.00a	7.10b	0.00a	0.00a
Mycelia sterilia III	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mycelia sterilia IV	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Mucor hiemalis	0.00a	–	0.00a	0.00a	3.52a	0.00a	0.00a	–	0.00a	0.00a	2.61a	1.35a
Nigrospora sphaerica	7.50ab	–	13.23b	0.00a	6.88ab	9.18b	2.50a	–	10.55b	0.00a	6.80ab	5.57a
Penicillium frequentans	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Penicillium verrucosum	0.00a	–	0.00a	0.00a	0.00a	1.73a	0.00a	–	0.00a	0.00a	0.00a	1.36a
Phoma glomerata	0.00a	–	0.00a	12.46c	0.00a	0.00a	0.00a	–	0.00a	6.86b	0.00a	0.00a
Pithomyces chartarum	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Rhinocladiella atrovirens	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Sphaeropsis sp.	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Trichoderma viride	0.00a	–	0.00a	0.00a	0.00a	0.00a	0.00a	–	0.00a	0.00a	0.00a	0.00a
Bacteria												
Bacillus I	0.00a	–	7.50b	0.00a	0.00a	0.00a	–	–	–	–	–	–
Bacillus II	0.00a	–	0.00a	0.00a	0.00a	0.00a	–	–	–	–	–	–
Bacillus III	0.00a	–	0.00a	0.00a	0.00a	0.00a	–	–	–	–	–	–
Pseudomonas sp.	0.00a	–	0.00a	7.50a	7.50a	45.00b	–	–	–	–	–	–

*MA+ medium (2% malt agar plus 50 mg/L aureomycin and 100 mg/L streptomycin) for isolation of fungi

**King's B medium for isolation of bacteria

Alternaria alternata, *Epicoccum purpurascens* Ehrenb. ex Schlecht. and *Cladosporium herbarum* (Pers.) Link were dominant in leaf impression, with quite similar relative frequencies, around 30% (Fig. 3a). Frequency of *A. alternata* increased substantially ($p < 0.05$) in the leaf treatment methods, particularly with incubation in the moist chamber, whereas *E. purpurascens*, while still dominant, was less consistent and tended to decrease after sterilization or incubation in the moist chamber. *Cladosporium herbarum* ceased to be dominant on washed and sterilized leaf disks and behaved in the same way as *Fusarium oxysporum* Schlecht.:Fr. and *Aureobasidium pullulans* (de Bary) G. Arnaud, which were reduced to a minimum except in leaf impression. *Nigrospora sphaerica* (Sacc.) E. Mason, on the contrary, totally subordinate in the leaf impression method, was not reduced by leaf washings or sterilization. It must be noted that *Penicillium frequentans* Westling was replaced by *P. verrucosum* Dierckx on the washed and sterilized leaf disks.

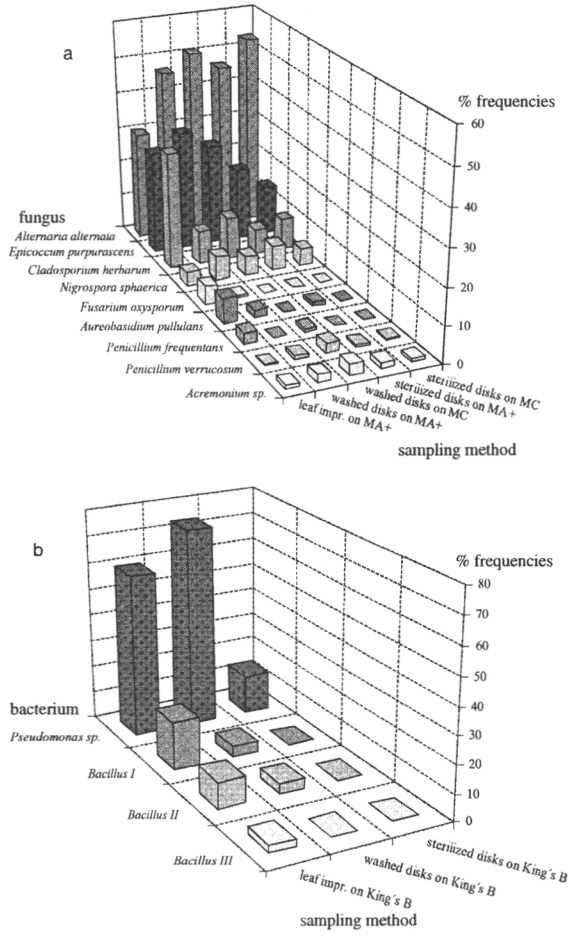

Figure 3. Influence of the sampling methodology on the relative frequencies of some components of the poplar phylloplane microflora (1997 data). a) fungi; b) bacteria.

Generally speaking, the presence of *Melampsora* spp. had some influence on the composition of phylloplane mycoflora, at least in the two 1997 samplings, which provided data for comparison with the uninfected leaves. The relative frequencies of the more representative fungi were in many cases the same on both healthy and infected leaves, but with some important exceptions: *C. herbarum* was more frequent ($p<0.05$) on infected leaf disks incubated on MA^+ in the summer sampling; *E. purpurascens* was more frequent on infected sterilized leaf disks ($p<0.05$); *N. sphaerica*, absent on the summer sterilized infected disks, reached values of more than 10% on the corresponding healthy disks; lastly, *Phoma glomerata* (Corda) Wollenw. & Hochapfel was more frequent ($p<0.05$) on infected impressed and sterilized disks than the healthy ones in the summer sampling.

The presence of the pathogen had little effect on the temporal succession of the main phylloplane fungi, as can be deduced from the three 1997 samplings carried out during the growing season when the disease was most frequent. *Alternaria alternata* was dominant throughout the season, and its values were always high, sometimes exceeding 60% in the washing or sterilisation methods. *Epicoccum purpurascens* displayed increased significantly in relative frequency ($p<0.05$) as the season advanced; its frequency values peaked to levels similar to those of *A. alternata* in summer in the case of leaf impression and washed disks, whereas on the sterilized disks frequency was always lower and increased the most in the autumn. *Cladosporium herbarum* was constantly present at frequencies similar to those of *A. alternata* in leaf impression, whereas, as already stated, it maintained its frequency but lost its dominance in the other methods. *Nigrospora sphaerica*, totally absent on the spring and summer washed leaf disks, reached frequencies of over 17%, i.e., similar to those of *C. herbarum* in the same treatment, in the autumn; it was constant on the sterilized disks. Lastly, a fungus provisionally identified as *Acremonium* sp. displayed a contradictory pattern: on the leaf impressions and the washed disks it was only found in spring and autumn, in this last case only on infected leaves, whereas on the sterilized disks it was found in spring only.

3.2 Phylloplane bacteria

On King's B medium four as yet unidentified species were isolated in all samplings (Table 1 and 2). One, indicated as *Pseudomonas* sp., is composed of small Gram-negative cells and forms fast-growing, yellowish colonies with distinct edges; three are *Bacillus* spp., almost indistinguishable microscopically, all larger Gram-positive rods but macroscopically each one with a characteristic in colony appearance, constant also after repeated transfers. Colonies formed by the bacterium, here denoted conventionally as *Bacillus I*, are similar to those of *Pseudomonas* sp., but ivory colored; *Bacillus II* forms crusted, whitish colonies with lobed edges; and *Bacillus III* forms floccose, poorly defined colonies with filamentous edges resembling cirri.

Their frequencies varied considerably from one method to another (Fig. 3b): *Pseudomonas* sp. always predominated but, whereas in leaf impression it was accompanied by the three other bacteria, these became sporadic on the washed leaf disks and were absent on the sterilized disks, on which *Pseudomonas* sp. was also sporadic. It is worth noting that sterilization

thinned out this bacterium more on the healthy disks (f = 2.5%) than on the infected disks (f = 26.3%).

In the 1996 samplings and especially at Battipaglia, *Bacillus II* was significantly more frequent ($p<0.05$) than in the 1997 samplings on both the impressed leaves and the washed disks. It is uncertain whether this was primarily due to the different season or the geographical factor.

Except for the fact that *Pseudomonas* sp. seemed less dominant on the impressed leaves and washed disks of the "Villafranca" white poplar clone in the 1997 spring and summer samplings, to the advantage of *Bacillus I* and *Bacillus II*, the clonal factor was of no apparent importance. There were no constant differences between the clones tested; the sudden variations in frequency observed in some experiments are to be regarded as coincidental.

The leaf position on the stalk was in no way related to the phylloplane bacterial microflora in any of the sampling methods.

The presence of *Melampsora* spp. had little effect on the time pattern of the presence of the four bacteria, apart from the already mentioned case of the sterilized leaf disks, though here one cannot even speak about bacterial communities. The frequencies of *Bacillus I* and *Bacillus II* on the impressed leaves and the washed disks decreased as the growing season advanced, whereas that of *Bacillus III* remained constant and very low. It is important to note that *Pseudomonas* sp. alone was found on the washed disks in the early autumn.

4 Discussion

The mycoflora associated with the phylloplane of cultivated poplars that emerges from an integration of these results, is in many respects similar to those described for communities on the leaves of other deciduous trees and shrubs reported in the literature (Lamb and Brown 1970, Pugh and Mulder 1971, Ruscoe 1971, Lindsey and Pugh 1976, Wildman and Parkinson 1979, Breeze and Dix 1981, Vardavakis 1988).

The differences in frequency that appear on comparing the sampling methods used in this study are related to the prevalent vegetative form present on the phylloplane. The many species observed only by the leaf impression method and usually with negligible frequencies are probably only present sporadically as inactive propagules, and hence have no influence on the real colonization of the phylloplane. That they were inactive and incidental, indeed, was confirmed by the disappearance of many of them when the same method was used in early autumn, a time when the probability of their isolation is compromised by an increase and the sporulation on the leaf surface of the colonies of dominant fungi.

Alternaria alternata and *E. purpurascens* were dominant in all methods. The first one appears to be the absolutely predominant species, since its relative frequency values were always the highest, and it could to colonize the phylloplane at an early stage, had a high propagation rate, and was able to differentiate resistance structures. Moreover, it seems to be able to penetrate the mesophyll of nonsenescent leaves as shown by its high frequencies on the sterilized leaf disks, though this phenomenon was more obvious on the more mature leaves.

Epicoccum purpurascens becomes dominant later in the growing season if compared with *A. alternata*. Its presence in the active form on the phylloplane increases rapidly during the summer, whereas it does not penetrate the mesophyll significantly until the autumn, and even more so in the tissues then massively invaded by *Melampsora* spp., when senescence of tissues is incipient and *A. alternata* has been in the endophytic stage for some time. It is uncertain whether this indicates a partial link between the phylloplane fungus and the rust, or whether it merely reflects the fact that penetration is facilitated by lacerations of the epidermis caused by eruption of the uredia. The same doubt can be extended also to *P. glomerata* behavior; it is not known why its frequencies were higher on infected leaves only in the summer and not in the autumn too, when the rust fungus is even more present.

It may seem surprising that *C. herbarum* ceases to be dominant with the methods involving selective treatment of the leaf surface. However, its mycelium, in addition to having a very high sporulation, can itself act as a means of propagation through the transformation of apical portions of hyphae into so-called ramoconidia. Thus, while still less stable on the leaf surface than the other two dominants, this fungus constitutes an equally stable member of the phylloplane mycoflora, if by stability one also means the ability to perpetuate its existence in the phylloplane and ensure its immigration through an adequate pressure of inoculum into the new "islands" (for the "island" concept applied to the phylloplane, see Andrews and Kinkel 1986) that gradually become available. In this case, too, it should be noted that there is a certain correlation, at least in the middle of the growing season, between the establishment of *C. herbarum* and the presence of *Melampsora* spp. The correlation could be ascribable to a link of some kind between these two fungi or to increased leaching, an important source of nutrients, once again due to tearing of the epidermis of the infected leaves.

The data also suggest that *N. sphaerica* is a stable member, though not dominant, of the phylloplane mycoflora, above all late in the growing season, and able to penetrate the mesophyll to some extent. It is interesting to observe that this penetration is somewhat inhibited in the summer by infection of the still fresh tissues by the parasite. Lastly, *Acremonium* sp. seems only present on infected leaves in the autumn.

In their investigation in England of the mycoflora of *Populus generosa* Henry leaves in an advanced stage of senescence, both healthy and infected by *M. larici-populina*, McKenzie and Hudson (1976) found that *Alternaria* spp., *Cladosporium* spp. and *E. purpurascens* were more frequent on the infected tissues.

Pseudomonas sp. seems to be clearly predominant over the three types of *Bacillus*, increasingly so as the growing season advances, in substantial agreement for example with the findings of Perissol *et al.* (1993) on holm-oak leaves. It is the only bacterium able to penetrate the mesophyll and, once again, this penetration seems to have a connection with the presence of the rust fungus.

In conclusion, the frequencies of the epiphytes associated with the phylloplane of cultivated poplars seem to be correlated, at least partially and mostly quantitatively, with the presence of *Melampsora* spp. as well as with the season. Possible links between the rust and the fungi or bacteria must be

investigated in experimental tests involving artificial inoculations, to see whether there are real interactions between them and whether these interactions result in an inhibition of the pathogen.

One fact of potential advantage, with regard to their use in a biological control strategy, is that the fungi or bacteria remarkable for their dominance or the partial correlations between their frequencies and the presence of the rust agent were already present in their active form in the late spring, i.e. before the pathogen appears.

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Assessment of the *in vivo* antagonism of phylloplane fungi and bacteria against *Melampsora* spp. on cultivated poplars

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Summary

Some fungi and bacteria associated with the phylloplane of cultivated black and hybrid poplars were chosen, because of their dominance and/or their relative frequencies on healthy and rust-infected leaves observed in field samplings, for experimental testing in the laboratory to see whether they were antagonistic to the physiological race E3 of *Melampsora larici-populina* and to *M. allii-populina*. Each microorganism and the rust fungus were inoculated jointly, but at different times according to four sequences, on leaf disks from rust-susceptible poplar clones. The progressive eruption of the uredia was then followed to determine three epidemiological parameters: the attack intensity, the mean presporulation period (MPP), and the percentage viability of the urediospores drawn from the same uredia.

All the fungi and bacteria tested significantly inhibited both rust agents to some extent. Those observed as dominant on the phylloplane, i.e. the fungi *Alternaria alternata*, *Epicoccum purpurascens* and *Cladosporium herbarum*, and the bacterium *Pseudomonas* sp., were the most effective antagonists. Substantial reductions of the attack intensity (a little over 70% on average for *M. larici-populina* E3, almost 80% for *M. allii-populina*), however, were only achieved when the presumed antagonists were sprayed on the leaves before or at the same time as the inoculation of the pathogen. Later applications had no significant inhibiting effect except that of *Pseudomonas* sp., which reduced the production of uredia by more than 60%. The MPP was scarcely affected by the presence of the antagonist, whatever the inoculation sequence, perhaps because the weakened urediospores were unable to originate normal infection centers producing uredia. On the contrary, the percentage viability of the urediospores taken from emerged uredia was greatly reduced, also in case of delayed inoculation of the antagonist. As far as can be gathered from the microscopic observation of these uredia, often composed of spores with decaying protoplasm, antibiosis or production of nonspecific secondary metabolites seems to be the main cause of the antagonism of most of the fungi and bacteria tested. In the case of *C. herbarum* and *Acremonium* sp., mycoparasitism may also be involved.

Key words: control, *Melampsora allii-populina*, *Melampsora larici-populina*

1 Introduction

The increasing incidence of rusts caused by *Melampsora allii-populina* Kleb. and *M. larici-populina* Kleb. is having a negative effect on the productivity of intensively cultivated black poplars and Euro-American hybrids. *Melampsora larici-populina*, especially, has differentiated some very virulent physiological races in recent years. Poor ecological and economic practicability of chemical control makes the development of a biological control strategy against these diseases an attractive proposition. The agents most suitable for this purpose would be microorganisms already present, though in a latent state, in the environment where the target pathogen vegetates and multiplies, and preferably those whose period of greatest activity coincides with that of the rust. There is thus an interest in conducting preliminary laboratory trials to assay the antagonistic potentials against *Melampsora* spp. of fungi and bacteria associated with the poplar phylloplane in the usual growing conditions. Successful experiments with other rust agents, mostly on herbaceous hosts, have been reported in the literature (Levine *et al.* 1936, French *et al.* 1964, Kapooria and Sinha 1969, McBride 1971, Hevesi and Mashaal 1975, Omar and Heather 1979, Manners 1981).

A previous paper (Cellerino and Gennaro 1998) described the outcome of samplings in 1996 and 1997 of this phylloplane microflora in a 2-year-old polyclonal nursery. In this study, several methods and variability factors were considered. The results showed that some fungi and bacteria were dominant and/or displayed definite trends in their relative frequencies of isolation on healthy leaves and those infected by *Melampsora* spp. Their possible antagonism against both *M. allii-populina* and *M. larici-populina* E3 has been experimentally investigated *in vivo* on suitable leaf material in the laboratory. An attempt was also made to determine the mechanisms of inhibition.

2 Material and methods

2.1 Fungi and bacteria tested

The following phylloplane fungi and bacteria were tested *in vivo* to investigate their possible antagonism to *Melampsora*:

Alternaria alternata (Fr.) Keissler, *Epicoccum purpurascens* Ehrenb. ex Schlecht., and *Cladosporium herbarum* (Pers.) Link as dominant fungi;

C. cladosporioides (Fresen.) de Vries, although negligible as a member of the poplar phylloplane mycoflora, for comparison with *C. herbarum*;

Fusarium oxysporum Schlecht., as a species that does not persist when leaf material is treated, but was recorded as an antagonist of other rust fungi (Kapooria and Sinha 1969);

Nigrospora sphaerica (Sacc.) Mason, as an active colonizer of the phylloplane;

Acremonium sp., on account of the partial link between its presence and that of the pathogen and its occurrence as a mycoparasite of other rust fungi (Jayapal Gowdu and Balasubramanian 1993);

In consideration of their small number, all isolated bacteria (still being identified, and here called *Pseudomonas* sp., *Bacillus I, Bacillus II,* and *Bacillus III*), among which *Pseudomonas* sp. was predominant.

The following antagonist/parasite combinations were investigated through joint inoculations on the same leaf material, giving preference to *M. larici-*

populina E3 on account of its currently greater epidemiological importance:

A. alternata, *E. purpurascens*, and *C. herbarum* on *M. allii-populina* and *M. larici-populina* E3;

F. oxysporum on *M. allii-populina*;

C. cladosporioides, *N. sphaerica*, and *Acremonium* sp. on *M. larici-populina* E3;

Pseudomonas sp. and *Bacillus I*, *II*, and *III* on *M. larici-populina* E3.

2.2 Preparation and execution of the *in vivo* antagonism tests

To eliminate the time factor in rust susceptibility (Giorcelli *et al.* 1996), leaves of average maturity (11–15 days after opening) were taken from the main shoots of healthy young plants of *Populus x euramericana* (Dode) Guinier, grown from potted scions provided by the Poplar Research Institute of Casale Monferrato (northwestern Italy) and cultivated in climatized growth chambers at DI.VA.P.R.A., Section of Plant Pathology. Leaf disks (diameter 2.5 cm), extracted with sterilized corers, were washed once for 3 min in a 0.6% NaClO solution, then four times (2 min each) in sterile distilled water, dried on sterile filter paper, and placed, abaxial side up, in multi-well petri dishes (3.5-cm diam.) containing a thin film of sterile distilled water. Each dish held five disks from five different leaves, representing five replications.

Melampsora allii-populina and *M. larici-populina* E3 were inoculated on two highly sensitive *P. x euramericana* clones, namely "Neva" [mother: 67-008 (*P. deltoides* 55-071 Illinois x ?); father: PI65-014 (*P. nigra* Lucca)] and "Luisa Avanzo" [mother: *P. deltoides* 3479/958 Stoneville 1; father: *P. nigra* (?)], respectively.

The inoculum consisted of a suspension containing 0.1 g/L agar (added to delay evaporation of the droplets from the leaf surface after inoculation) and 3 x 10^4 urediospores/mL characterized by high viability (about 90%), previously collected from mature uredia obtained from artificial rust multiplications carried out periodically in the laboratory. Their concentration was determined with a haemocytometer. Each inoculation was made by spraying tiny droplets of the suspension uniformly over the disks (two passages per well on average) by means of an electric micronizer; this was disinfected with 95% ethanol and repeatedly rinsed with sterile distilled water before each inoculation.

Two procedures were used to prepare the potential antagonist inocula. In the first one, fungi cultured on liquid 10 g/L malt extract were collected, homogenized in sterile distilled water, and filtered to remove large mycelium fragments; the suspensions thus obtained were diluted to a concentration of 3 x 10^5 conidia and/or mycelium fragments per milliliter (i.e., 10 times that of the urediospores). These conidial/mycelial suspensions were used as inocula and applied in the same way as the rust inocula. In the second method, young bacterial colonies cultured on King's B substrate were diluted in sterile distilled water to a concentration of 10^9 cells/mL (Docherty and Preece 1978) to provide the inoculum suspensions.

The inoculations of the rust fungus and of the supposed antagonist on the same leaf disks were performed according to four different sequences:

antagonist inoculated seven days before *Melampsora* spp. to determine its persistence on the leaf surface;

antagonist inoculated at the same time as *Melampsora* spp. to determine its influence on the urediospores and the germ tubes;

antagonist inoculated two days after *Melampsora* spp., when it was in the endophytic phase, to assess the possibility of inhibition at this stage;

antagonist inoculated seven days after *Melampsora* spp., when it was about to produce its first uredia.

These four sequences were compared with control inoculations of *Melampsora* spp. only.

The leaf disks inoculated with the rust fungus were incubated in a controlled environment, at first for 48 h in the dark at 16 ± 1° C, then exposed to 8 h of darkness at 16 ± 1° C and 16 h at 23 ± 1° C under fluorescent light (10 000 lx intensity, irradiated with λ = 475 nm at 15 W m^{-2} (= 60 µE m^{-2} s^{-1}) until the uredia were produced. When the antagonist was inoculated first, the leaf disks were immediately exposed to the above-mentioned photoperiod until *Melampsora* spp. was added.

2.3 Epidemiological parameters considered

The inoculated leaf disks were observed daily for 20 days to record the number of uredia that appeared, and when their production ceased. Three uredia randomly collected from each replication were placed on water agar dishes to determine the percentage viability of the respective urediospores.

The epidemiological parameters used to assess inhibition by the supposed antagonist were the following:

- the attack intensity, considered the main parameter, expressed as the number of uredia cm^2 of leaf surface;
- the mean presporulation period, representative of the latency period, i.e. the mean time between inoculation and eruption of the uredia, calculated as:

$$MPP(d) = \frac{\Sigma_i (u_i X_i)}{\Sigma_i u_i} \qquad (1)$$

where u_i is the number of uredia recorded on each disk i days after inoculation;

- the urediospore percentage viability, assessed after 24 h on water agar according to the criteria suggested by Manners (1966).

3 Results

All fungi, at different concentrations, inhibited both rust species, showing similar patterns in the four inoculation sequences (Table 1a). Substantial reductions of attack intensity only occurred when the supposed antagonist was inoculated before or at the same time as *Melampsora* spp., whereas later inoculations were totally ineffective. As a whole, *C. herbarum*, *A. alternata* and *E. purpurascens* were the most effective antagonists, decreasing the production of uredia by *M. allii-populina* by almost 80% and of *M. larici-populina* E3 by slightly more than 70%, when inoculated both before and at the same time as the rust fungus (Fig. 1a). The other species showed small, but still significant differences (p<0.05) between these two inoculation sequences: *F. oxysporum* was more effective when inoculated before *M. allii-populina*, whereas *N. sphaerica* and *Acremonium* sp. were more effective when inoculated at the same time as *M. larici-populina* E3. The first one did not inhibit the pathogen to the same extents as *E. purpurascens*, *A. alternata*

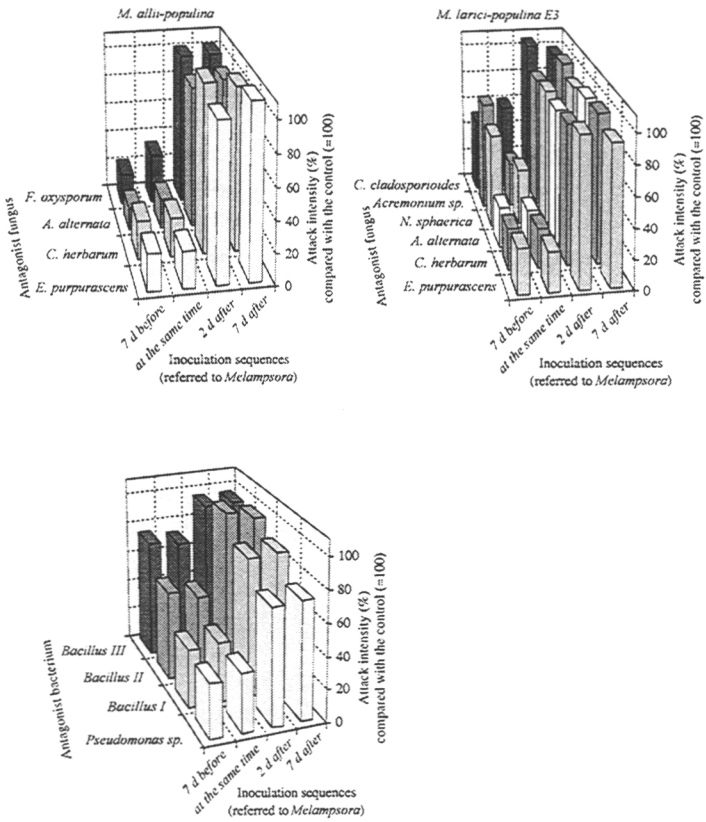

Figure 1. Variation of *Melampsora larici-populina* E3 and *M. allii-populina* E3 attack intensity on leaf disks of average maturity from *P. x euramericana* "Luisa Avanzo" and "Neva" clones respectively, recorded after joint inoculation with that one of possible antagonist phylloplane fungi and bacteria according to different sequences. Results are expressed as % of the respective controls (*Melampsora* only), considered equivalent to 100.

and *C. herbarum*. *Cladosporium herbarum* inhibited the attack intensity more than did *C. cladosporioides*, allowing the parasite to produce half of the uredia produced when the last one was inoculated under the same conditions.

Bacillus I, II and *III*, too, were only effective when inoculated before or at the same time as *M. larici-populina* E3. Their antagonism and that of *Pseudomonas* sp. were comparable with that of the dominant fungi, though less powerful in quantitative terms for most of them (Fig. 1b). There were no significant differences in the inhibition between *Bacillus I* and *Bacillus II*, whereas *Bacillus III* decreased uredia production by 25%, showing a limited antagonistic potential. *Pseudomonas* sp. was the most effective, and even caused a small amount of inhibition when inoculated after the pathogen.

Both the phylloplane fungi and bacteria had only a negligible effect on the MPP. Sometimes the MPP was occasionally increased slightly by prior or simultaneous inoculation, but never significantly by comparison with inoculation after the rust.

Table 1. Epidemiological parameters of *Melampsora allii-populina* or *M. larici-populina* E3 on leaf disks of average maturity (11–15 d after opening) from *P. x euramericana* "Neva" and "L. Avanzo" clones, respectively. Joint inoculation with possibly antagonistic phylloplane microorganisms was done at different times with reference to the rust inoculation. For each fungus or bacterium, the means with the same letters are not significantly different ($p<0.05$) according to Newman-Keuls test.

a) Fungi

	Inoculation times of the antagonist (referred to the parasite one)	M.P.P. (d) average	M.P.P. (d) confidence limit (P=0.05)	Attack intensity (uredinia/cm²) average	Attack intensity (uredinia/cm²) confidence limit (P=0.05)	% viability on water-agar
Epicoccum purpurascens on *M. allii-populina*	control (only *Melampsora*)	10.81	0.79	8.12	0.86	69.73 c
	7 d before	12.96	0.65	1.89	0.71	46.15 b
	at the same time	11.70	1.03	1.88	0.63	31.95 a
	2 d after	11.37	1.49	8.09	1.02	52.53 b
	7 d after	10.27	0.56	9.01	0.99	51.35 b
Epicoccum purpurascens on *M. larici-populina* E3	control (only *Melampsora*)	8.09	0.20	10.30	1.39	71.56 b
	7 d before	8.61	0.59	3.07	0.56	39.81 a
	at the same time	9.13	0.50	2.71	0.36	38.64 a
	2 d after	8.09	0.50	10.16	1.34	40.39 a
	7 d after	8.16	0.62	9.52	1.29	50.77 ab
Cladosporium herbarum on *M. allii-populina*	control (only *Melampsora*)	11.04	1.73	8.02	1.43	65.65 c
	7 d before	12.27	0.83	1.96	0.65	38.64 b
	at the same time	11.84	0.76	2.00	0.51	28.66 a
	2 d after	10.87	1.19	8.70	0.52	45.00 b
	7 d after	9.84	1.14	8.40	0.58	44.43 b
Cladosporium herbarum on *M. larici-populina* E3	control (only *Melampsora*)	8.09	0.38	10.57	0.72	72.54 b
	7 d before	8.92	0.39	2.87	0.65	47.02 a
	at the same time	8.96	0.40	2.77	0.74	44.21 a
	2 d after	8.47	0.41	9.83	0.80	46.14 a
	7 d after	7.92	0.24	10.46	1.10	40.70 a
Alternaria alternata on *M. allii-populina*	control (only *Melampsora*)	10.34	1.10	8.45	0.48	67.21 c
	7 d before	10.70	0.97	1.28	0.33	18.43 a
	at the same time	10.93	1.06	1.62	0.51	11.53 a
	2 d after	10.26	0.89	7.76	0.57	19.37 a
	7 d after	10.18	1.00	8.15	0.46	30.66 b
Alternaria alternata on *M. larici-populina* E3	control (only *Melampsora*)	8.12	0.33	10.69	0.94	70.63 b
	7 d before	8.60	0.59	2.90	1.14	22.78 a
	at the same time	8.88	0.61	2.54	1.22	21.13 a
	2 d after	8.08	0.38	9.70	1.27	33.21 a
	7 d after	8.12	0.42	10.48	1.19	30.66 a
F. oxysporum on *M. allii-populina*	control (only *Melampsora*)	10.23	0.42	8.74	0.55	71.56 c
	7 d before	10.97	1.08	1.80	0.42	28.66 a
	at the same time	11.36	0.69	2.86	0.62	34.75 a
	2 d after	10.10	1.32	8.75	0.76	48.44 b
	7 d after	9.98	1.04	8.80	0.58	44.43 b
C. cladospor. on *M. larici-populina* E3	control (only *Melampsora*)	8.14	0.56	10.45	0.86	73.57 b
	7 d before	8.47	0.61	5.21	1.03	58.37 b
	at the same time	8.65	0.41	6.24	0.94	44.43 a
	2 d after	8.22	0.74	10.98	0.87	59.23 b
	7 d after	8.11	0.63	10.25	0.74	60.66 b
N. sphaerica on *M. larici-populina* E3	control (only *Melampsora*)	8.21	1.02	11.04	0.68	77.08 ab
	7 d before	8.78	0.84	7.02	1.12	63.43 ab
	at the same time	8.64	0.91	4.25	0.86	51.35 a
	2 d after	8.01	0.87	10.25	0.71	68.03 ab
	7 d after	8.15	0.98	10.41	0.74	64.89 ab
Acremonium on *M. larici-populina* E3	control (only *Melampsora*)	7.98	0.68	10.76	1.02	75.82 b
	7 d before	8.69	0.84	7.89	1.14	59.67 ab
	at the same time	8.74	0.76	3.25	0.98	36.87 a
	2 d after	8.24	0.89	9.68	0.87	39.23 a
	7 d after	8.12	0.71	10.87	0.68	43.28 a

b) Bacteria

	Inoculation times of the antagonist (referred to the parasite one)	M.P.P. (d)		Attack intensity (uredinia/cm²)		% viability on water-agar
		average	confidence limit (P=0.05)	average	confidence limit (P=0.05)	
Pseudomonas on *M. larici-populina* E3	control (only *Melampsora*)	8.21	1.12	10.37	1.21	73.57 b
	7 d before	8.45	1.02	3.45	0.87	53.13 a
	at the same time	8.39	1.05	3.74	0.98	49.21 a
	2 d after	7.98	0.87	7.47	1.24	42.31 a
	7 d after	8.05	0.74	7.58	1.02	53.73 a
Bacillus I on *M. larici-populina* E3	control (only *Melampsora*)	8.11	0.78	10.76	0.94	72.05 b
	7 d before	8.76	0.97	3.89	1.14	50.13 a
	at the same time	8.89	0.84	4.02	1.23	54.33 a
	2 d after	8.23	0.64	9.36	1.02	58.05 a
	7 d after	8.09	0.74	9.45	0.87	42.47 a
Bacillus II on *M. larici-populina* E3	control (only *Melampsora*)	8.23	0.84	10.25	0.87	75.34 b
	7 d before	8.74	1.14	5.76	1.21	56.28 a
	at the same time	8.15	0.98	5.21	1.06	59.34 a
	2 d after	8.05	0.78	10.47	0.96	55.55 a
	7 d after	7.98	1.04	9.97	0.72	59.74 a
Bacillus III on *M. larici-populina* E3	control (only *Melampsora*)	8.01	0.94	11.02	0.76	72.54 a
	7 d before	8.45	1.05	8.24	1.14	60.53 a
	at the same time	8.23	0.99	7.94	1.23	58.05 a
	2 d after	8.54	0.87	10.48	0.94	63.87 a
	7 d after	8.26	0.74	10.54	0.87	59.27 a

The percentage viability of the urediospores collected from emerged uredia, on the other hand, was always appreciably reduced in all four sequences (Table 1a), and even halved by *E. purpurascens*, *C. herbarum*, and *F. oxysporum* on *M. allii-populina*, especially after simultaneous inoculation. *Alternaria alternata* was the most effective and reduced urediospore viability by more than two-thirds when inoculated either before or at the same time as either pathogen. *Cladosporium cladosporioides* and *N. sphaerica*, on the contrary, inhibited germination of urediospores very slightly. Bacteria were generally less effective on this parameter than fungi (Table 1b), confirming the limited antagonistic potential of *Bacillus III*; previously its slight influence on the attack intensity was observed.

Inoculation of fungi and bacteria was sometimes followed by the emergence of abnormal uredia, composed of hyaline urediospores that failed to germinate, but not intermingled with any extraneous hyphae (Fig. 2). On the contrary, uredia colonized by *C. herbarum* and *Acremonium* sp. often contained clumps of indistinct and decaying urediospores with conidiophores and conidia typical of these fungi on their surfaces (Figs. 3 and 4). Mycoparasitism by *C. herbarum* and *Acremonium* sp. is a possibility that will require more specific investigation.

4 Discussion

The fact that the intensity of *Melampsora* spp. was reduced largely by prior or simultaneous inoculations of the phylloplane micro-organisms shows — as expected — that their antagonistic action is directed against the epiphytic phase of the rust fungus, i.e. against its urediospores and germ tubes. This action can only take place when the fungus or the bacterium is already present or arrives on the leaf at the same time as of the urediospores, and not when the pathogen has reached the endophytic infection phase and has already differentiated its haustoria. Omar and Heather (1976) are of the

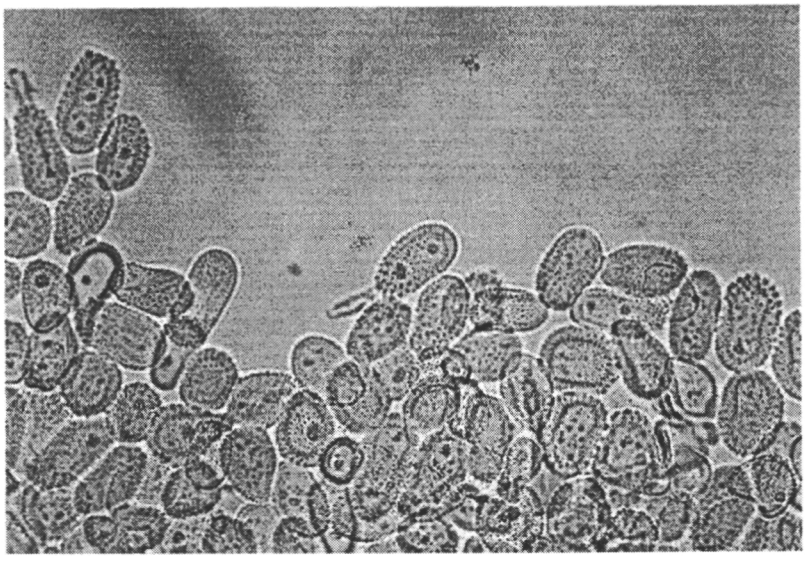

Figure 2. *Melampsora larici-populina* E3 urediospores collected from uredia produced on poplar leaf disks inoculated with various phylloplane microorganisms. They are apparently cell-less and unable to germinate (800 X).

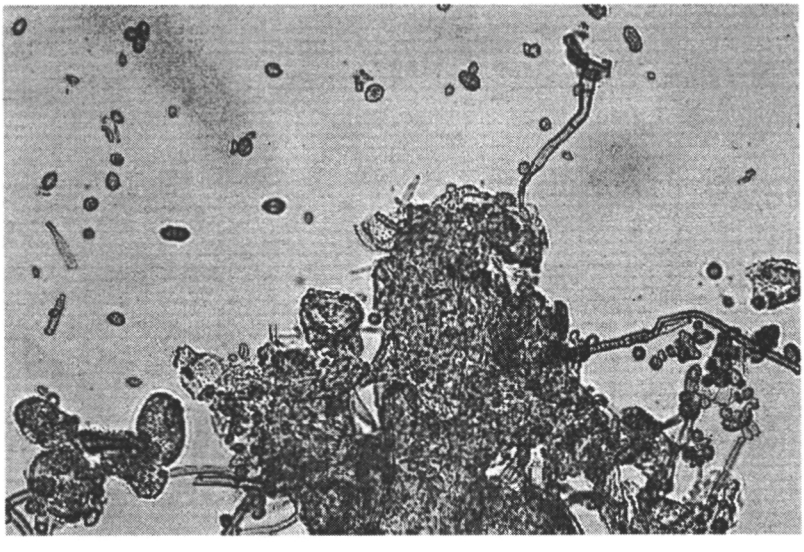

Figure 3. *Melampsora larici-populina* E3 urediospores collected from uredia produced on poplar leaf disks inoculated with, and apparently parasitized by, *Cladosporium herbarum* (800 X).

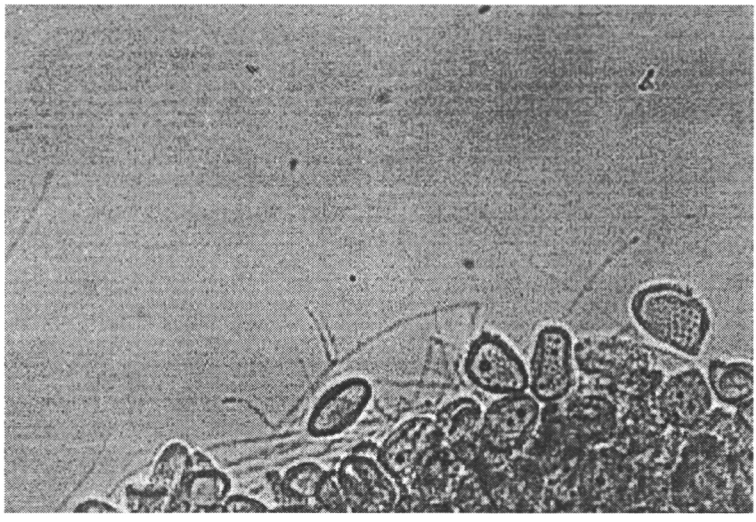

Figure 4. *Melampsora larici-populina* E3 urediospores collected from uredia produced on poplar leaf disks inoculated with *Acremonium* sp. Conidiophores of *Agremonium* sp. are present (800 X).

opinion that germination of urediospores begins within 30 min after inoculation, and penetration of the germ tube through the stoma occurs within 2–3 hours. It can be understood that when an antagonist is inoculated 2 to 7 days after the rust, the antagonist can no longer have any effect on the course of the infection; at this time the parasite is already feeding on mesophyll tissues. Even so, they should provide information concerning any epiphytes able to penetrate the mesophyll and thus potential antagonists of the internal infective structures in the endophytic phase. Among the tested microorganisms, only *Pseudomonas* sp. seems to have this ability, though its inhibiting effect was less than on the phylloplane. In another investigation the use of various methods for determining the phylloplane microflora (Cellerino and Gennaro 1998), it was the only bacterium isolated after sterilization of the leaf surface. Two dominant fungi, *A. alternata* and *E. purpurascens*, were also isolated after sterilization during that study, but in these antagonism tests their postponed inoculation proved ineffective.

The reduction of urediospore percentage viability following all four inoculation sequences is attributable to the protracted residence and action of the antagonists on the leaf surface; that is, they come into contact with the new propagules produced in the uredia even after several days. Moreover, the fact that this depression increases the longer the residence (i.e. following prior and simultaneous inoculations), points to a marked ability, especially typical of the dominant fungi, to proliferate independently on the leaf surface.

The inability of the antagonists to influence the MPP may be surprising, since even partial impairment of the urediospore and/or weakening of the germ tube could be expected to delay formation of the infective structures. It may be supposed that the relatively few urediospores that escape the effect of the antagonist manage to give rise to normal infection centers and thus the MPP is virtually unchanged, whereas the remaining ones may either form

centers incapable of reaching the reproduction stage, or else be totally unable to form them.

Our observations, during the inoculation tests, indicate that inhibition by these fungi and bacteria is similar to antibiosis or to production of nonspecific secondary metabolites, and this may explain the frequent observation of urediospores in the process of lysis or devoid of protoplasm. The further evidence needed to substantiate this suggestion could perhaps be provided by spraying culture filtrates instead of conidial/mycelial suspensions, provided they are readily diffusible in an aqueous medium. It is also possible that a mycoparasitic component contributes to the antagonistic action of *C. herbarum* and *Acremonium* sp.

The fact that all these phylloplane fungi and bacteria inhibited *Melampsora* spp. to some extent was expected, since many of them have already been described as antagonists of rusts when the concentrations of the inoculum suspensions were very high. Omar and Heather (1979) found that *Cladosporium* sp. and *Alternaria* sp. inhibited the viability of urediospores of *M. larici-populina* and reduced its production of uredia through a mycoparasitic mechanism. No evidence of mycoparasitism by *A. alternata*, however, appeared in our study. Our observation that *C. herbarum* was a more effective antagonist than *C. cladosporioides*, on the other hand, agrees with the results of Sharma and Heather (1981). They, however, found that both fungi were less effective against *M. larici-populina* than was *C. tenuissimum*, a species that has never been found in our phylloplane flora.

Our supposition about *Acremonium* sp. as a mycoparasite is equally supported by reports on other rusts, concerning afferent species or very closely related genera, such as *Verticillium* or *Aphanocladium* (Forrer 1977, Jayapal Gowdu and Balasubramanian 1993). *Nigrospora sphaerica* (Mishra and Tewari 1976) and *F. oxysporum* (Kapooria and Sinha 1969) are known to inhibit cereal rusts, while several bacteria, including *Pseudomonas* and *Bacillus* species, have been described as antagonists of various rust fungi (Levine *et al.* 1936, French *et al.* 1964, McBride 1971, Hevesi and Mashaal 1975, Tosi and Zazzerini 1994). In contrast, there have not been previous reports of the very active antagonism displayed by *E. purpurascens* in our experiments.

Adaptations of various kinds may have led the phylloplane fungi to develop a slight, nonspecific antibiotic power to be used as a form of defence against competitors. This would have been enhanced by some of the intrinsic conditions of our laboratory tests, namely the high inoculum density and hence the quick, massive proliferation promoted by preliminary washings of the leaf surface; the scene was thus set for the accumulation of secondary metabolites, with antibiotic action, at levels not reachable in nature and rendered them effective against the rust fungus. It may not be a coincidence that the dominant fungi (*C. herbarum*, *A. alternata* and *E. purpurascens*) and the dominant bacterium (*Pseudomonas* sp.), as emerged from our samplings, were also the most effective against *Melampsora* spp. Little is known about the real relations between them in nature. Moderate inhibition of *Melampsora* sp. by these epiphytes could, in fact, occur for long periods during growing season, but their existence, itself of no epidemiological consequence, would not be perceived during routine rust observations. *C. herbarum* alone, perhaps on account of its conspicuous mycoparasitism, is sometimes evident

by its extensive colonization of the uredia, especially at the end of the growing season.

Many of the microorganisms tested in the laboratory, on the basis of their proven inhibition of *Melampsora* sp. and their persistence on the leaf surface may substantially reduce the inoculum potential of the parasite over successive generations of urediospores. This reduction would occur even earlier and more extensively when the antagonist is already present at sufficient levels on the phylloplane when the rust propagules are deposited.

Application of these findings in the field must await further investigation about the nature of the antagonism of each fungus and bacterium and about the active principles involved. It can already be surmised, however, that this type of biological control is unlikely to block totally the production of uredia. A poplar, however, is able to maintain an optimum annual wood increment even when as much as 50% of its leaf surfaces is compromised. Complete elimination of rust, therefore, is unnecessary, whereas its confinement to levels that do not impair productivity would be a great advantage. In such context, the use of phylloplane fungi and bacteria as biological control agents would ensure sufficient and protracted protection, and they would be «natural dampers» of any new and more aggressive rust genotypes.

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Breeding and selection of poplars for durable resistance *to Melampsora larici-populina*

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Summary

Since the early 1980s, poplar culture in western Europe has encountered a breakdown of the resistance of commercialized clones to *Melampsora larici-populina*. The basis of the rust population currently observed on these clones is constituted by four physiological races. In 1997, a new physiological race was identified on several clones, including two that were released on the market only recently. The breakdown of this resistance, emanating from the *Populus deltoides* parent of these clones, is due to its monogenic nature. Therefore, selection of clones with a more stable, polygenic resistance to *M. larici-populina* has been an essential part of the selection and breeding program of poplar for some years. When creating new clones through controlled crosses, only clones showing a polygenic resistance to *M. larici-populina* should be selected. Consequently, the basic collection of genitors, built up since the early 1960s, must be screened for susceptibility to the different physiological races which have originated since then.

Key words: E4, *Populus deltoides*, *Populus nigra*, *Populus trichocarpa*

1 Introduction

Poplar culture in western Europe mainly aims at the production of fast growing clones producing high-quality timber for the match, veneer, and plywood industry. The quantity of timber produced annually, as well as its quality is largely dependent on the growing conditions, which in turn are affected by numerous factors, often changing from year to year. Within the selection and breeding program, several traits are applied as main criteria for selection: form, growth and vigor, rooting capacity, photoperiodic adaptation to latitude, frost hardiness, adaptation to a range of soil types including drought resistance, wood quality, wind resistance, and last, but not least, disease resistance. Through application of these criteria, fast growing interamericana clones, such as Boelare and Beaupré, could be selected, displaying an annual volume production which can exceed the production of old varieties like Robusta by 50 to 80%, depending on the soil type (Steenackers 1988).

Results obtained to date clearly show that wood of higher quality is produced by clones which are free of diseases, namely stem and bark diseases

(canker and blight), as well as foliar diseases. Wood quality is known to be particularly influenced by the *Melampsora* rusts. Consequently, resistance to *Melampsora* spp. has been adopted as one of the major selection criteria within the poplar breeding program at the Institute for Forestry and Game Management (IFG) in Geraardsbergen.

Since the start of the breeding program in 1948, a collection of many hundreds of individuals of *Populus deltoides* Marsh. (referred also to as D), *P. trichocarpa* Torrey & Grey (T), and *P. nigra* L. (N) has been gradually built up. Within these species individuals were selected that revealed high resistance to *Melampsora* rust. Within the species *P. deltoides* and *P. trichocarpa,* some individuals showing a high tolerance and some a high susceptibility were found. General resistance could only be observed within the species *P. deltoides*. These generally resistant individuals were selected and constituted the basis for further selection and breeding (Steenackers 1969).

The *P. nigra* collection has displayed a very high susceptibility to *Melampsora larici-populina* Kleb. However, because of their very high resistance to bacterial canker, caused by *Xanthomonas populi*, they were still involved in the breeding program as genitors for the production of the interspecific hybrids *P. deltoides* x *P. nigra* (Steenackers 1966). Starting from these basic collections, many inter- and intraspecific crosses were performed annually. At each time, within the interspecific crosses *P. deltoides* x *P. trichocarpa,* and *P. deltoides* x *P. nigra,* individual clones were selected because of their general resistance to the existing rust population.

In the early 1980s, poplar culture encountered a breakdown of the rust resistance of some of these clones. The loss of resistance of both these clones and other clones emanating from other European poplar breeding programs led to the discovery of physiological rust races. At present, seven virulence genes have been identified within the *M. larici-populina* population (Pinon 1995). The basis of the present rust population is constituted by four groups of races (Steenackers 1991, Steenackers 1994). The basic collections of the poplar species *P. deltoides, P. trichocarpa* and *P. nigra*, as well as the collection of hybrid clones have been subject to these ever changing rust populations since their establishment. For three years, the resistance level of these poplar species and their hybrids has been studied.

2 Material and methods

2.1 Identification of races of *Melampsora larici-populina* and of virulence genes

In order to assess the races present and their respective virulence genes, rust races were identified during three consecutive years (1996, 1997 and 1998). Identification was based on collections from natural infections on larch, the alternate host of *M. larici populina*. Earlier research proved that the rust races observed on larch in spring are a reflection of the races which will be found on poplar in summer (Pinon 1995).

From each aecium collected, a spore suspension was prepared and inoculated on healthy leaf disks from the clone Robusta, which is susceptible to all known races, and from clones showing race-specific reactions (Ogy, 76.004/10, Brabantica, Unal, 87B12, Rap and Boelare). During the 14 days

Table 1. Background of 443 selected poplar clones screened for susceptibility to *Melampsora larici-populina*.

Species/Hybrid [1)]	Number of clones	Number of *P. deltoides* clones used in the crosses
D	95	–
DxN	29	6
TxD or DxT	85	8
(TxD)xD	161	11 (TxD) and 23 D
TxT	73	–

[1)] D = *Populus deltoides*, T = *Populus trichocarpa*, N = *Populus nigra*

after inoculation, the appearance of symptoms and, later on, of sori was recorded on a daily basis. The presence and frequency of each virulence gene and of each race (each having between zero to five virulence genes) was established. Virulence genes were assessed for 100 sori of race E4.

2.2 Screening for susceptibility to *Melampsora larici-populina*

The rust susceptibility of 443 selected clones, often related through the *P. deltoides* parent, was assessed in the experimental nursery of the IFG. In the year of their selection, all clones, with the exception of the 73 *P. trichocarpa* clones, were selected for general resistance to *M. larici-populina*. At that time, the races E1–E4 were not present. For each clone, 20 two-year-old plants were raised beginning in 1995, using grafts for the *P. deltoides* clones and cuttings for the others (Table 1). Screening for susceptibility to *M. larici-populina* was carried out in October 1997 and was based on the observation of natural infections in the nursery. The scoring system used comprised 11 values between zero for trees entirely free of rust and five in cases of complete defoliation. Half values were included.

2.3 Race-specific susceptibility of *Populus deltoides* clones

The susceptibility to races E1, E2, E3, and E4 was assessed for 59 *P. deltoides* clones. Leaves were collected from nursery plants in June, before the appearance of any natural infection. Preparation of the inoculum and the inoculation method were adopted from Pinon (Steenackers 1991). Each clone-race combination was tested on four leaf disks of 1.5 cm diameter. Leaf disks were floated on water in closed multidisk plates and under continuous illumination. During the 14 days after inoculation, the appearance of flecks and, later on, of uredia was recorded daily.

3 Results

3.1 Identification of races and of virulence genes

Identification of races of *Melampsora larici-populina* (Fig. 1)

Since its appearance in the nursery in 1994, race E4 has shown a very rapid spread, from 20% in 1995 to nearly 70% in 1997–1998. This phenomenon can be accounted for by the presence in the nursery of a high number of one- and two-year-old trees of clones with a specific susceptibility to this race.

Races E2 and E3, which in 1995 still constituted 5.0% and 1.2%, respectively, of the rust population, were no longer observed in 1998, which can be explained by the presence of only a very restricted number of clones with a specific susceptibility to these races. Since the appearance of race E4, it was proved that clones which until then showed a specific susceptibility to race E2 are susceptible to race E4 as well. Finally, the share of race E1 in the rust population dropped from 70% in 1995 to 30% in 1998.

Identification of virulence genes from isolates belonging to race E4 (Fig. 2)
The most common virulence genes were 1, 3, 4, 5, and 7. Virulence genes 2 and 6 were observed in less than 5% of the isolates. This is explained by the fact that, in the nursery, less than 1% of the clones is marked by a specific susceptibility to those genes.

Number of virulence genes identified in isolates of race E4 (larch, 1998)
Nearly half (47%) of the isolates from larch had four virulence genes. The combination vir1vir3vir4vir7 appeared to be the most frequent. For the isolates for which vir5 was tested as well (30%), the combination vir3vir4vir5vir7 was observed in each case. Most (60%) of the isolates belonging to race E1 had the virulence genes 3 and 4, whereas 40% had virulence gene 4 only.

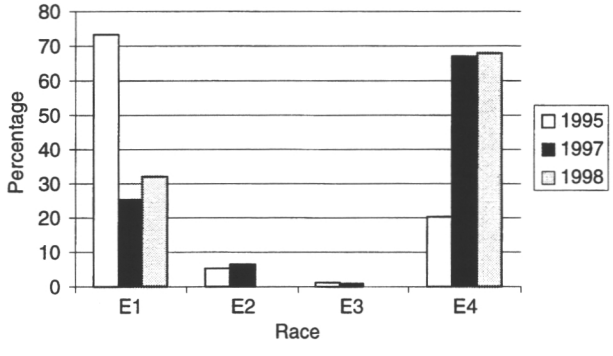

Figure 1. Incidence of races of *Melampsora larici-populina* on larch in the nursery of the IFG (Geraardsbergen, Belgium) during the years 1995, 1997, and 1998.

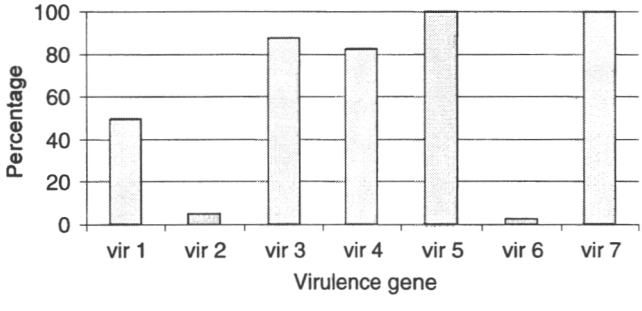

Figure 2. Identification of virulence genes from isolates belonging to race E4 of *Melampsora larici-populina* (larch, 1998). Virulence gene 5 was only tested for 30% of the E4 uredia.

3.2 Susceptibility of selected clones to *Melampsora larici-populina*

Susceptibility of *Populus deltoides* and *P. trichocarpa* clones (Fig. 3)

The rust susceptibility of *P. deltoides* clones varied between 0 and 3. Since the appearance of different physiological races in 1982, only 6% of the *P. deltoides* population maintained its general resistance. However, 90% of the *P. deltoides* clones still had a very satisfactory resistance level, between 0.5 and 2. Rust scores for *P. trichocarpa* clones varied between 1.5 and 3.5.

Susceptibility of DxN clones (Fig. 4)

From infection experiments in the laboratory, it appears that the breakdown of the resistance of these clones coincides with the appearance of race E4. One set of DxN clones showed a resistance level varying between scores 1 and 3. A second set of clones, genetically related through the *P. deltoides* mother, displayed a degree of susceptibility varying between scores 4 and 5. The different levels of resistance reflects the relative resistance of the *P. nigra* parent.

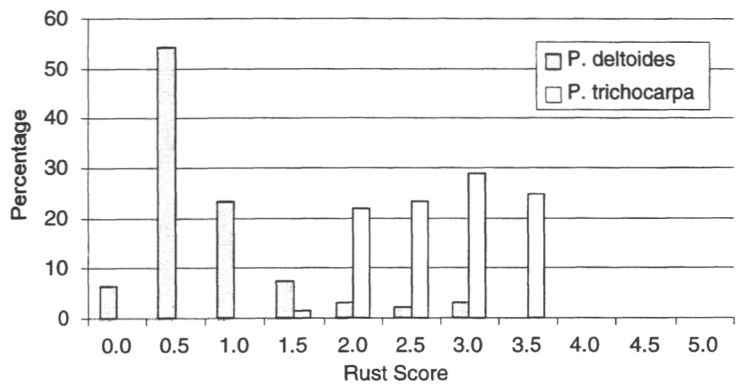

Figure 3. Susceptibility of selected *Populus deltoides* and *Populus trichocarpa* clones to *Melampsora larici-populina*.

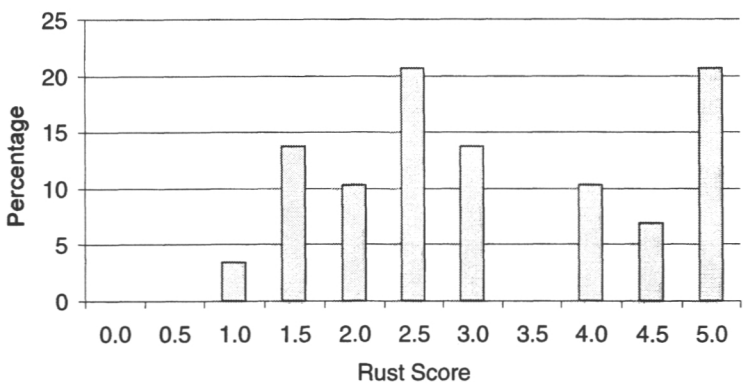

Figure 4. Susceptibility of selected DxN hybrid clones to *Melampsora larici-populina*.

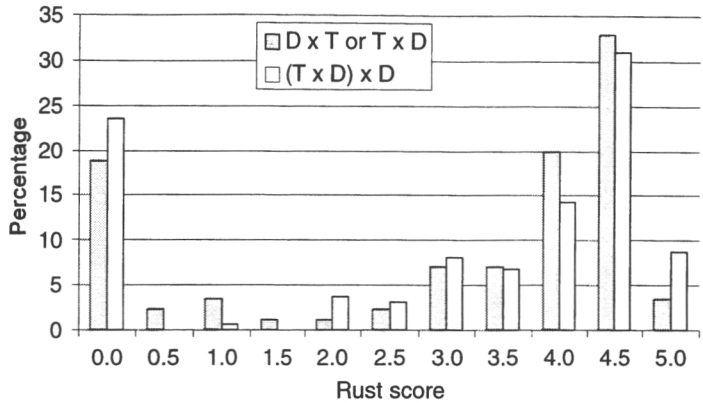

Figure 5. Susceptibility of DxT, TxD and (TxD)xD hybrids to *Melampsora larici-populina*.

Susceptibility of DxT, TxD and (TxD)xD clones (Fig. 5)

For all three sets of hybrids (*P. deltoides* x *P. trichocarpa*, *P. trichocarpa* x *P. deltoides*, (*P. trichocarpa* x *P. deltoides*) x *P. deltoides*), the degree of susceptibility covered the whole range from score zero (immunity to all four races) to score five (complete defoliation). A very low infection rate (scores 0.5 to 2) was observed for only a small percentage of the clones. On the other hand, more than half of the clones showed high degree of susceptibility, with scores between 3.5 and 5 and characterized by premature leaf shed.

3.3 Race-specific susceptibility of *Populus deltoides* clones

Merely 56% of the 59 clones could be infected in the laboratory. This might indicate the occurrence in the nursery of a race different from those known. A more plausible explanation, however, is that the resistance level of the *P. deltoides* clones is so high that artificial infection in the laboratory is difficult, if not impossible.

Each time a new race originates, the resistance of a larger number of clones is broken down, 8.5% of the clones became susceptible to race E1, 15.3% to race E2, 18.6% to race E3, and 32.3% to race E4. Some clones became susceptible to one race (E3 or E4), others to two races (E1 + E3 or E2 + E4), to three races (E1 + E2 + E3 or E1 + E3 + E4), or to four races (E1 + E2 + E3 + E4) (Fig. 6). Moreover, the highest degree of susceptibility is displayed by clones which are susceptible to all four races.

4 Discussion

Populus deltoides as a species will continue to play an important role in the selection and breeding of poplars for resistance to *M. larici-populina*. Not only is it the sole species with genes for race-specific resistance, it also displays a high level of resistance against newly developed races. Because of this high rust resistance, and also because of its high resistance to other foliar diseases such as *Marssonina populi* (Lib.) Magn. and *Venturia* sp. and of its high wood quality, this species is to be regarded as very valuable for building populations useful for the practice of poplar culture. In addition, individual

clones which can be involved in multiclonal plantations will be selected from existing and new populations. The continuous expansion of the basic collection should be considered as a major task, as both the building of populations and the selection of clones should start from a broad genetic basis.

One of the major criteria for *P. deltoides* clonal selection will probably be resistance to *Xanthomonas populi*. Research carried out in the early 1960s has revealed that 95% of the natural *P. deltoides* populations in the U.S. is susceptible to this bacterial disease. Nevertheless, susceptible individuals satisfying all other criteria can be involved as genitors in interspecific crosses with canker resistant *P. trichocarpa* and *P. nigra*.

Compared to the *P. deltoides* clones, most of the *P. trichocarpa* clones are more susceptible. Nevertheless, within the *P. trichocarpa* population, the selection of individuals with a very good level of resistance of 1.5 to 2 still remains possible. Depending on the *P. deltoides* and *P. nigra* parents used in the DxN interspecific hybrids, the creation of clones with a sufficiently high level of resistance to *M. larici-populina* is undoubtedly possible. Some 15 such clones are currently available. After further screening in plantations for growth properties, some of these clones will be released on the market.

The majority (76%) of the interspecific hybrids DxT and TxD and the back crosses (TxD)xD either show a general rust resistance (score zero) or a very high susceptibility (scores 4 to 5). Several new crosses are necessary in order to check on the possibility of creating hybrids marked by a level of resistance which is intermediate between that of the parents. A first indication is offered by some clones, DxT hybrids as well as back crosses, which show a resistance level of 2.0 to 2.5.

The first slight attacks by *M. larici-populina* (scores 0.5 to 1.5) were observed on some clones which until recently displayed a general resistance. The presence of another race remains to be proven.

Monoclonal culture of poplar clones with monogenic resistance is largely responsible for the actual rust problems in western Europe. New clones with a sufficient level of resistance should be released on the market as quickly as possible. Measures should be taken to cut back the present monoclonal culture and to make the use of multiclonal plantations compulsory.

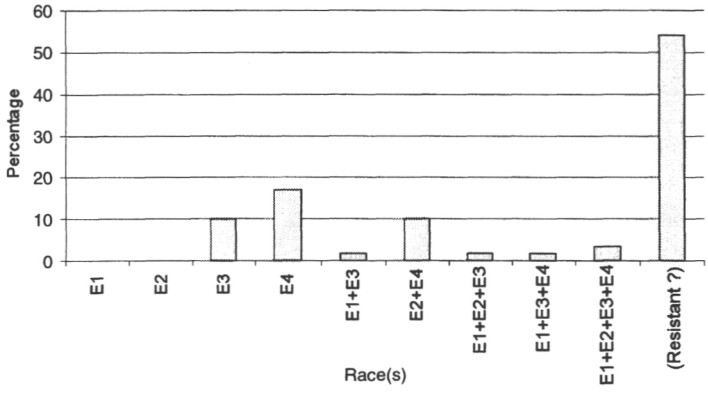

Figure 6. Race-specific susceptibility of 59 *Populus deltoides* clones.

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Fungicide trials conducted to determine the potential for controlling rust infections on poplars

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Summary

Four trials were conducted to determine the potential of fungicides to control rust infections on poplars. Sixteen different fungicides were tested. Naturally infected poplar trees of the clone 65/31, grown in pots, were used as test material. The first trial had 17 treatments (16 fungicides and one untreated control), applied as sprays. Four treatments were significantly more effective than other treatments: Alto (cyproconazole) (at 0.3 mL/L) with and without the adjuvant Armoblen 600 (at 0.75 mL/L), Anvil (hexaconazole) (at 0.2 mL/L) and Early Impact (flutriafol + carbendazim) (at 0.6 mL/L). Oxycarboxin appeared to enhance disease progression. Two experimental strobilurins, Stroby WG and Quadris FL (kresoxim-methyl (BASF) and azoxystrobin (Zeneca)) (at 0.12 mL/L and 0.4 mL/L, respectively), and Astor WG40 (experimental, Novartis) (at 2 g/L) controlled rust poorly. Four treatments were used in the second trial: Quadris as a foliar spray (0.4 mL/L), Impact applied on superphosphate granules ((1 mL + 5 g) per tree), superphosphate alone (5 g per tree), and untreated. The key finding of the second trial was that Impact gave complete control as a granular application over a 56-day period. Superphosphate alone enhanced rust development slightly. A third trial was conducted to confirm results of the first two trials. These were consistent with the first two trials, with Alto plus Armoblen 600 being the best treatment, Early Impact the next best, then Alto, Anvil, superphosphate coated with Impact, Impact alone, the untreated control, Duett, and lastly, superphosphate alone (which again slightly increased the disease levels). A fourth trial was conducted with different levels of Alto (0.1, 0.2, 0.3, 0.5, 0.7 mL/L, and an untreated control), applied with the standard rate of Armoblen 600. All rates of Alto gave control of the disease, the highest rate being the most effective.

Key words: control, *Melampsora medusae, Melampsora larici-populina, Populus deltoides*

1 Introduction

Of the many *Melampsora* species that cause leaf rust of poplars, *M. medusae* Thuem. and *M. larici-populina* Kleb. are the two most destructive, because each has a wide host range that includes many interspecific hybrids (Newcombe and Chastagner 1993). *Melampsora medusae* is native to North America but has been introduced throughout most of the world where poplars

are grown (Van Kraayenoord *et al.* 1974). Schipper and Dawson (1974) also report *M. medusae* as being one of the most serious diseases of the eastern cottonwood, *Populus deltoides* Bartr. In intensively managed plantations yield losses of up to 65% in volume were reported for hybrid poplars with eastern cottonwood parentage (Widin and Schipper 1981).

In attempts to control rusts, the development of resistant clones has played and will play an essential role. However, there are also shortcomings in breeding resistant varieties. For example, it is not always possible to combine resistance with other factors required (such as desirable wood qualities and fast growth), and new races of diseases may appear that successfully attack previously resistant varieties. Therefore, to protect against loss of resistance by emergence of new physiological races, the use of chemicals that effectively control poplar rusts is necessary (Buchenauer 1968).

The aim of this study was to test selected fungicides that could be expected to control rust development. Three trials were conducted. The second trial included treatments not used in the first due to the unavailability of some of the chemicals and time constraints. A follow-up fungicide trial was done to test the fungicides that were better in their action against the rust epidemic and to determine the reliability of the first two trials.

2 Material and methods

Naturally infected young poplar trees, 1.5–2 m in height, were used for all the following trials. For the preliminary fungicide trials (trial 1 and 2), 189 trees were arranged in groups of 3. Nine trees were allocated to each treatment. These were then divided into three groups, so that there were three replicates per treatment. Seventeen treatments were part of trial 1, and four treatments made up trial 2 (see Table 1). Trial 2 included treatments that were not available when trial 1 was begun. A suitable drip irrigation system was set up to water them regularly (twice a day for 4 min). A randomization program was used to randomize the treatments within the replicates and these were then labeled with numbers. The labeling with numbers was so that when rating was done visually, bias was not given to some treatments. The chemicals were measured using weigh balances (accurate to two decimal places) or 1 mL pipettes (depending on the formulation) and mixed with 1 L of water in 1.5-L hand spray bottles. Trial 1 and 2 had a second treatment application after 45 days, and sprays were done from the top of the trees to imitate aerial spraying as closely as possible. A cylindrical screen, placed around each replicate (group of three trees), was used to prevent drift from one treatment to the next during spraying. The effects of the fungicides were evaluated by rating the 10th and 11th leaves of each tree (i.e. the leaves are of the same age) every 2nd week. A visual rating scale (Fleming 1996) was used to improve the accuracy of this task.

The follow-up fungicide trial (trial 3), with nine treatments, was conducted at the Redclyff nursery site of the Lion match plantations, Seven Oaks (Table 1). Randomization of the treatments and replicates was done using a randomization program, and each treatment had a border of at least five trees between it and the adjacent treatment. Again, three trees were assigned to each replicate (i.e. nine trees per treatment). Again the effects of the

fungicides were evaluated by rating the 10th and 11th leaves of each tree every week with the rating scale.

Trial 4, also carried out at the Redclyff nursery, examined different concentrations of Alto with the same amount of Armoblen 600 (i.e. 0.75 mL/L). The treatments used were 0.1, 0.2, 0.3, 0.5, and 0.7 mL/L of Alto as well as an untreated control. In this trial five replicates were used again with three trees per replicate. The plots were randomized within the replicates, and at least five trees were used as a border between the treatments. Disease level was measured as before.

All data were placed into spreadsheets, and the AUDPC (area under disease progress curve) value was calculated. AUDPC values were analyzed using Statgraphics to determine the significance of the results obtained.

Table 1. Treatments used in the trials 1, 2, and 3.

Trial	Group	Active ingredients	Trade name	Rate/L
1,2,3	Control	N/A	N/A	N/A
1	Protectant	Mancozeb	DithaneWG	2 g
1	Piperazine	Triforine	Denarin	2 mL
1,3	Conazol	Flutriafol	Impact	1 mL
1,3	Triazole	Cyproconazole	Alto	0.3 mL
1	Triazole	Difenoconazole	Score	0.2 mL
1,3	Triazole	Hexaconazole	Anvil	0.2 mL
1	Triazole	Propiconazole	Tilt	0.2 mL
1	Anilinopyrimidine + protectant	Cyprodinil + Fludioxonil	Switch	0.4 g
1	not known	Benzothadiazole	Bion	0.15 g
1	Resistance stimulator	Not known	Astor WG 40	2 g
1	Morpholine	Tridemorph	Calixin	0.6 mL
1	Strobilurine	Kresoxim-methyl	Stroby WG	0.12 mL
2	Strobilurine	Kresoxim-methyl	Z1	0.4 mL
1	Carboxin	Oxycarboxin	Oxycarboxin	0.6 mL
2,3	Fertilizer	Fertilizer	Superphosphate	5 g/tree
3	Triazole	Epoxyconazol	Duett	0.4 mL
1,3	Triazole/ Benzimidazole	Flutriafol/ Carbendazim	Early Impact	0.6 mL
1	Triazole/ Benzimidazole	Flusilazole/ Carbendazim	Punch C	0.2 mL
2,3	Fertilizer + Triazole	Fertilizer + Flutriafol	Superphosphate + Impact	5 g +1 mL/tree
1,3	Triazole + Adjuvant	Cyproconazole + EN600*	Alto + EN600*	0.3 mL +0.75 mL

* Armoblen 600, a combination of alkoxylated fatty alkylamine polymer (500 g/L) and ethoxylated sorbitane ester (550 g/L).

3 Results

Trial 1

The results from trial 1 (Figs. 1–2) revealed that Early Impact was significantly better than Tilt, and all the treatments worse than that. Alto with Armoblen 600 and Alto alone were significantly better than Dithane, and all the treatments worse than that. Anvil was significantly better than Bion and worse treatments. The best five treatments were all triazoles. Early Impact had the lowest average AUDPC value.

Trial 2

The superphosphate coated with Impact (Fig. 3) was significantly better than all the other treatments and showed the best result (Fig. 4). Quadris FL controlled the rusts, but not significantly.

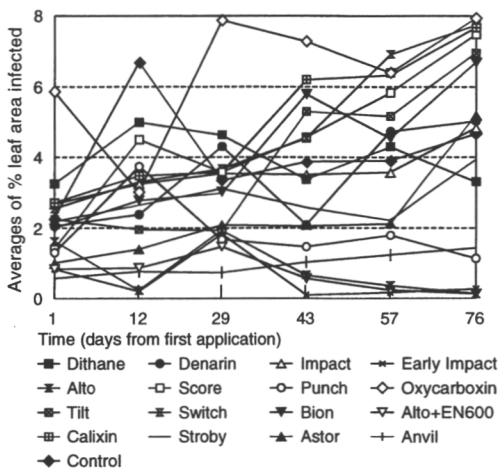

Figure 1. Disease progress curves of treatments, trial 1.

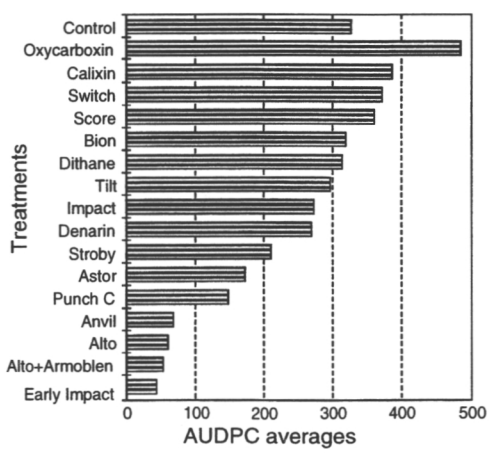

Figure 2. AUDPC averages of various fungicides, trial 1.

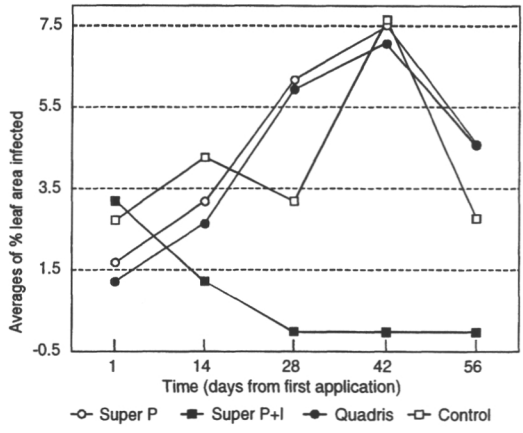

Figure 3. Disease progress curves of fungicide treatments, trial 2.

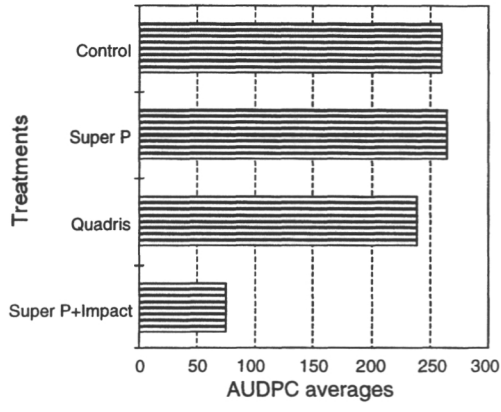

Figure 4. AUDPC averages of various fungicides, trial 2.

Trial 3

The results from trial 3 (the follow-up fungicide trial) were more or less consistent with the previous trials (Figs. 5–6); however, the superphosphate coated with Impact did not give complete control. Alto with Armoblen 600 was the best treatment, with Early Impact following closely. Superphosphate slightly enhanced disease progression. The Alto with Armoblen 600, as well as the Alto, reduced the disease level slightly. Superphosphate coated with Impact, and Impact alone (Fig. 5) both started control at slightly higher disease levels than that of the other treatments, and disease increase was slowed significantly with these treatments. Early Impact also slowed down the disease increase. Anvil maintained control until the 3rd week and then the treatment appeared to weaken slightly in its action. Duett increased the disease development.

Trial 4

All concentrations of Alto were significantly more effective than the control (Figs. 7–8). In general, the higher the concentration, the better was the control.

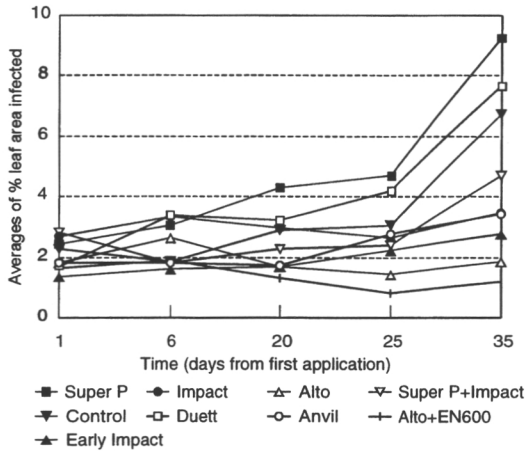

Figure 5. Disease progress curves of fungicide treatments, trial 3.

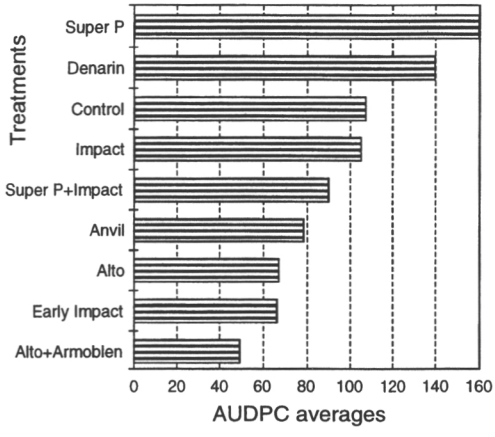

Figure 6. AUDPC averages of various fungicides, trial 3.

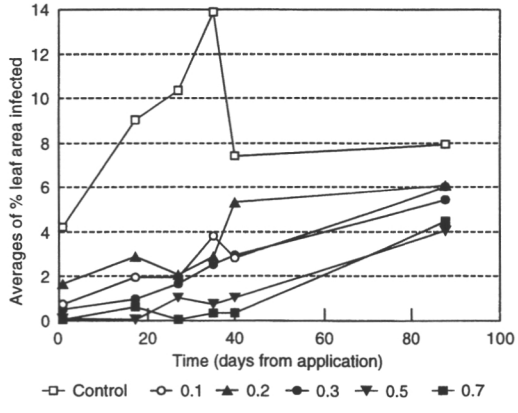

Figure 7. Disease progress curves at various Alto concentrations, trial 4.

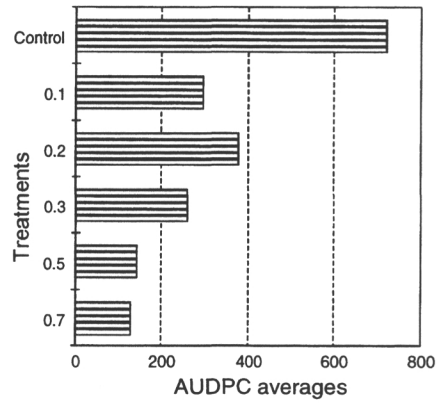

Figure 8. AUDPC averages of various Alto concentrations, trial 4.

4 Discussion

Trial 1

At day 43 (Fig. 1) there was a drastic decrease in disease severity with the Early Impact treatment. From then on, to day 76, control appeared to be consistently good, not reaching more than 0.5% leaf area infected (LAI). Alto and Alto + Armoblen 600 were still decreasing disease severity at day 76. Although Anvil gave good control, disease increased slowly, but consistently. The control showed less disease than expected, and this has been attributed to its positioning within the trial. In the trial layout each replicate of the control was coincidentally positioned on the outer edges. The Oxycarboxin seemed to enhance disease progression. The strobilurine (Stroby WG) did not rank very well (Fig. 2); however, its disease progress curve (Fig. 1) shows that disease did not increase significantly, nor did it decrease. Punch C has a mediocre ranking due to a high initial increase in disease on day 12 (Fig. 1); however, after this point the disease level dropped.

Trial 2

By the end of the trial superphosphate coated with Impact gave complete control (Fig. 3). Impact is a fungicide that moves acropetally through the plant (Lyr 1995). The application was to the roots, which explains why this treatment was better than the foliar application in the previous trial. This result could be very useful for poplar production because no costly aerial spraying would be involved. Application would be inexpensive using ground application.

Trial 3

The superphosphate coated with Impact treatment (Figs. 5–6) did not perform as well as expected. This was presumably because the treatment was not applied to a confined environment (as it was previously, within the bag) but to the ground, where it spread and was wasted, even though care was taken in placement of the treatment. This treatment may still have potential when applied on a large scale. Alto with Armoblen 600, as well as the Alto alone, reduced the disease level slightly (Fig. 5). This is because of their strong

curative action. The action of Duett was very disappointing, as this fungicide was the top wheat rust fungicide for aerial spraying at the time. No significant differences were obtained between any of the treatments and the control. This may be due to the short duration of the trial and the low disease pressure at the time the trial was conducted.

Trial 4

The results of this trial indicate the strong action of Alto, even at low concentrations. For economy, this product could be used at low concentrations, and good control would still be obtained.

Acknowledgements

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PCR-RFLP analysis of *Melampsora* spp. on willows in Japan

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Summary

PCR-RFLP analysis of internal transcribed spacer (ITS) regions including the 5.8S ribosomal DNA was done to differentiate 10 species of *Melampsora* rust fungi on willows in Japan. ITS regions of 54 collections of the 10 *Melampsora* species were amplified by PCR using primers ITS1-F and ITS4-B. The fragments from these collections varied in size (approximately 880 bp, 860 bp, and 840 bp). The restriction sites in the amplified DNA fragments were mapped after the RFLP analysis using four restriction enzymes, *Dra* I, *Eco* RI, *Ssp* I and *Taq* I. The *Melampsora* collections were divided into 11 RFLP types. In *M. capraearum*, *M. epiphylla*, *M. kamikotica*, *M. larici-urbaniana*, *M. microsora*, and *M. yezoensis*, the RFLP type was species-specific. The RFLP type of *M. chelidonii-pierotii* and *M. coleosporioides* was identical, with one exception. Three types were recognized in *M. epitea*. One of them was identical with the RFLP type of *M. humilis*. Results of inoculation experiments also suggested taxonomic reconsideration of the latter four species.

Key words: taxonomy

1 Introduction

Willows belonging to *Salix*, *Toisusu*, and *Chosenia* in Salicaceae are widely distributed in the world. They are an important source of biomass because of fast growth and easy propagation.

Rust diseases produced by *Melampsora* spp. cause premature defoliation of willows and sometimes result in serious damage. Fourteen *Melampsora* species have been reported to produce uredia and telia on willows in Japan (Hiratsuka and Kaneko 1982). Among them, seven species have heteroecious life cycles and produce spermogonia and aecia on larches, *Larix* spp., or on several herbaceous plants, *Chelidonium majus* L. var. *asiaticum* (Hara) Ohwi and *Corydalis* spp. The life cycles of the other species are unknown (Hiratsuka and Kaneko 1982).

Melampsora species have been identified based on morphology of both uredial and telial states and on host species. However, identification of the species is very difficult since morphological delimitation of the species is obscure. Furthermore, identification of the willow species is problematic because their morphological characteristics are highly variable. They also

easily intercross with one another in nature and produce many hybrids (Newsholme 1992). Additionally, it is difficult to identify the rust species based on aecial morphology or host species because the rusts are morphologically quite similar to each other in their aecial states and many rust species may occur on the same aecial host.

Molecular techniques based on the polymerase chain reaction (PCR), such as random amplified polymorphic DNA (RAPD) analysis and PCR-restriction fragment length polymorphism (RFLP) analysis, and utilizing ribosomal DNA (rDNA) or internal transcribed spacer (ITS) regions, have been used for identification and phylogenetic studies of many fungi (Bruns *et al.* 1991, Hibbet 1992, Foster *et al.* 1993, Hensen and French 1993) including rust fungi (Zambino and Szabo 1993, Kropp *et al.* 1997, Vogler and Bruns 1998). These studies showed that nucleotide sequences of ITS regions were useful for identifying the rust species.

We report here the differentiation of 10 species of *Melampsora* on willows in Japan by using PCR-RFLP analysis of ITS regions of rDNA. Additionally, we carried out inoculation experiments with urediospores to clarify the host range of four species which could not be distinguished by the above analysis.

2 Material and methods

2.1 Rust fungi

Forty six collections of *Melampsora* spp. producing uredia and/or telia were collected with host plant leaves at various locations in Japan from June 1995 to November 1996 as reported previously (Nakamura *et al.* 1998). For DNA extraction, field collections of the leaves with uredia or immature telia were stored at –80° C. For morphological observation, dry specimens from these collections were kept as vouchers in the Mycological Herbarium, Institute of Agriculture and Forestry, University of Tsukuba. These specimens were identified by morphological characteristics of both uredial and telial states according to Hiratsuka and Kaneko (1982). Ten species of *Melampsora* were identified among them (Table 1).

Axenic cultures from eight collections in six species of *Melampsora* growing on an artificial medium were also used (Table 1). These axenic cultures were established from uredial states by Yamaoka and Katsuya (1984a, b, c) and have been maintained as described previously (Nakamura *et al.* 1998).

2.2 DNA extraction

Total DNA was extracted from uredia and/or telia with host tissue (20 mg) and from axenic cultures (20 mg) by a modified method of Dellaporta *et al.* (1983) as described previously (Nakamura *et al.* 1998). The nucleic acids were suspended in 50 µL (100 µL for axenic culture samples) of RNase A solution (20 µg/mL in TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After incubation at 37° C for 30 min, the DNA preparations were stored at –20° C until used. Total DNA was extracted from rust-free plant leaves of 18 *Salix* spp., *Toisusu urbaniana* (Seemen) Kimura and *Chosenia arbutifolia* (Pall.) A. Skvortsov by the same methods as described above.

Table 1. The RFLP types of *Melampsora* collections.

Species	Collection No. [1]	RFLP type
M. capraearum	CAb-1. CAb-2, CAb-3, CAb-4, Cab-5, CAb-6, CAh-1, CAl-1, CA-ac1 [2]	1
M. chelidonii-pierotii	CHc-1, CHc-2, CHc-3, CHp-1, CHp-2	2
	CH-ac1 [2]	3
M. coleosporioides	COb-1, COb-2, CObr-1, Com-1, Com-2, CO-ac1 [2]	2
M. epiphylla	EPs-1, EPs-2, EPs-3, EPs-4, EPs-5, EPs-6, EP-ac1 [2], EP-ac2 [2]	4
M. epitea	ETg-1, ETg-2, ETg-3, ETg-4, ETm-1, ETr-1, ETr-2, ET-ac2 [2]	5
	ET-ac1 [2]	6
	ETf-1, ETj-1	7
M. humilis	HUi-1, HUi-2, HUi-3, HU-ac1 [2]	5
M. kamikotica	KAa-1	8
M. larici-urbaniana	LAu-1, LAu-2	9
M. microsora	MIs-1, MIs-2, MIs-3	10
M. yezoensis	YEj-1, YEj-2, YEs-1, YEs-2	11

[1] Host plant and locality of each collection were described previously (Nakamura *et al.* 1998)
[2] Axenic culture of uredial state

2.3 PCR amplification, RFLP analysis, and mapping of recognition sites

The primer pair ITS1-F and ITS4-B, designed by Gardes and Bruns (1993), was used for the amplification of ITS regions including the 5.8S rDNA and adjoining parts of the 3' end of the small subunit rDNA (approximately 70 bp in size) and the 5' end of the large subunit rDNA (approximately 200 bp in size) (Fig. 1). The amplified DNA fragments were obtained and partially purified as described previously (Nakamura *et al.* 1998).

The purified DNA fragments were digested with four restriction enzymes, *Dra* I, *Eco* RI, *Ssp* I, and *Taq* I, according to the manufacturer's instructions. The digested fragments were separated electrophoretically and restriction maps were made as described previously (Nakamura *et al.* 1998).

2.4 Inoculation experiments

Inoculation experiments with urediospores were conducted to examine the host range of four species, *M. chelidonii-pierotii* Matsumoto, *M. coleosporioides* Dietel, *M. humilis* Dietel, and *M. epitea* (Kunze et Schm.) Thümen. Urediospore collections of these species were maintained separately on detached host leaves on 1% (w/v) agar containing 40 mg ml^{-1} benzimidazole in petri dishes (9 cm in diameter) at about 20° C with fluorescent light (16 h per day). These urediospores were used as inocula.

In inoculation experiments, urediospores on small pieces of wet filter paper (3 mm x 3 mm) were placed onto the lower surface of detached leaves on agar in petri dishes. After inoculation, the petri dishes containing the leaves were placed in a dark moist chamber at about 20° C for two days and were then transferred into a growth chamber at about 20° C with fluorescent light (16 h per day). The inoculated leaves were kept for one month to observe whether uredia were produced on the leaves.

Salix species used for inoculation experiments were obtained from a Botanical Garden, Tohoku University, Sendai, Miyagi, Japan, or collected from various localities in Japan. The plants were maintained in a growth chamber at about 20 °C with fluorescent light (16 h per day).

In one experiment, four collections (RFLP type 2) of *M. chelidonii-pierotii* and four collections (RFLP type 2) of *M. coleosporioides* were used as inocula. Four *Salix* species were inoculated. *Salix chaenomeloides* Kimura and *S. pierotii* Miq. are host plants of *M. chelidonii-pierotii*, and *S. babylonica* L. and *S. matsudana* Koidz. f. *tortuosa* (Vilm.) Rehder are host plants of *M. coleosporioides* (Hiratsuka and Kaneko 1982).

In a second experiment, two collections (RFLP type 5) of *M. humilis* and four collections (two of RFLP type 5, two of type 7) of *M. epitea* were used as inocula. Eight *Salix* species were inoculated. *Salix integra* Thunb. ex Murray and *S. koriyanagi* Kimura are host plants of *M. humilis*, and *S. futura* Seemen, *S. gilgiana* Seemen, *S. gracilistyla* Miq., *S. japonica* Thunb., *S. miyabeana* Seemen, and *S. reinii* French. et Satvat. are host plants of *M. epitea* (Hiratsuka and Kaneko 1982).

3 Results

3.1 PCR amplification, RFLP analysis and mapping of recognition sites

The primer pair ITS1-F and ITS4-B permitted the amplification of a single DNA fragment from uredia and/or telia on host plant tissue and axenic cultures from 10 *Melampsora* species. This primer pair did not apmlify DNA fragments from rust-free host plants (data not shown). The amplified fragment sizes varied (approximately 840 bp, 860 bp, and 880 bp), but were uniform within each species except for *M. epitea* (Table 1, Fig. 1). In *M. epitea*, the fragments from eight collections of *M. epitea* were approximately 880 bp in size, and the fragments from three collections were 840 bp in size.

Three enzymes, *Dra* I, *Eco* RI, and *Ssp* I, had one or no recognition sites in the fragments, while *Taq* I had two or three sites (Fig. 1). All collections of the 10 species could be placed into 11 RFLP types based on RFLP patterns using the four enzymes (Table 1, Fig. 1). In all collections of eight species, *M. capraearum* (DC.) Thümen, *M. coleosporioides, M. epiphylla* Dietel, *M. humilis, M. kamikotica* Kaneko et Hiratsuka, *M. larici-urbaniana* Matsumoto, *M. microsora* Dietel, and *M. yezoensis* Miy. et Matsumoto, the RFLP pattern was identical within the species (Table 1). RFLP type 1 (*M. capraearum*), type 4 (*M. epiphylla*), type 8 (*M. kamikotica*), type 9 (*M. larici-urbaniana*), type 10 (*M. microsora*) and type 11 (*M. yezoensis*) were species-specific. The collections of *M. epitea* were separated into three RFLP types (types 5, 6, and 7). The collections of M. chelidonii-pierotii were separated into two RFLP types (types 2 and 3). The collections of *M. epitea* from which 840 bp fragments were amplified, contained two RFLP types (types 6 and 7). All the collections of *M. humilis* and eight collections of *M. epitea* from which 880 bp fragments were amplified showed the same RFLP type (type 5) (Table 1, Fig. 1). All the collections, except for collection CH-ac1 (type 3), of *M. chelidonii-pierotii* and *M. coleosporioides* showed the same RFLP type (type 2).

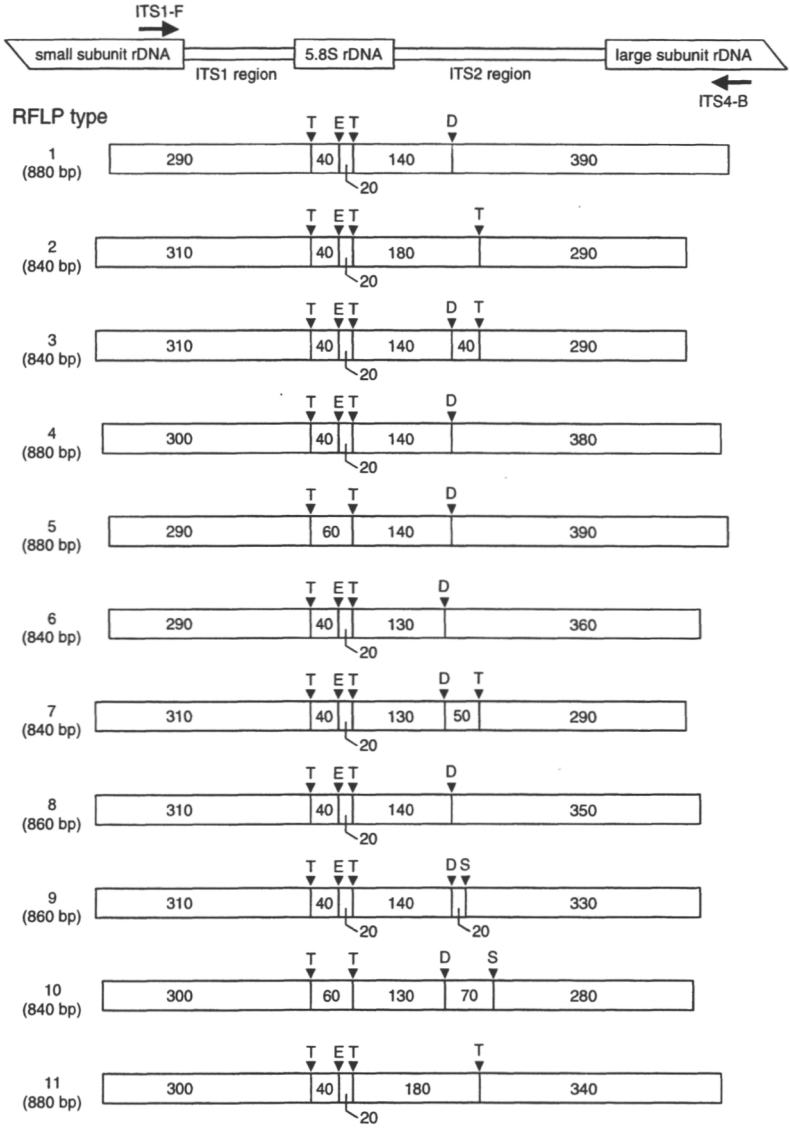

Figure 1. Representation of the ribosomal DNA (rDNA) repeat showing internal transcribed spacer (ITS) regions, including 5.8S rDNA, and restriction maps of ITS regions amplified by using the primer pair ITS1-F and ITS4-B. Locations of primers used for amplification are delineated as arrows. Estimated sizes of restriction fragments are indicated in base pairs. ▼ indicates recognition sites with each of four enzymes: D = *Dra* I, E = *Eco* RI, S = *Ssp* I, and T = *Taq* I. Sizes of intact DNA fragments amplified from *Melampsora* collections are in parenthesis under RFLP type number.

Table 2. Results of inoculation with urediospores of *Melampsora chelidonii-pierotii* and *M. coleosporioides* [1].

Plants inoculated	*M. chelidonii-pierotii* (RFLP type 2)	*M. coleosporioides* (RFLP type 2)
Salix babylonica		+
S. chaenomeloides	+	
S. matsudana f. *tortuosa*		+
S. pierotii	+	+

[1]+ -mark indicates production of uredia after inoculation

Table 3. Results of inoculation with urediospores of *Melampsora humilis* and *M. epitea* [1].

Plants inoculated	*M. humilis* (RFLP type 5)	*M. epitea* (RFLP type 5)	*M. epitea* (RFLP type 7)
Salix futura		+	+
S. gilgiana	+	+	
S. gracilistyla	+	+	
S. integra	+	+	
S. japonica	+	+	+
S. koriyanagi	+	+	
S. miyabeana			
S. reinii	+	+	

[1]+ -mark indicates production of uredia after inoculation

3.2 Host range

In the inoculations with the four collections of *M. chelidonii-pierotii*, uredia were produced on either *S. chaenomeloides* or *S. pierotii*. Two collections of *M. coleosporioides* produced uredia on *S. babylonica*, while the other two collections produced uredia on *S. pierotii, S. babylonica* and *S. matsudana* f. *tortuosa* (Table 2).

The two collections of *M. humilis* and the two collections of *M. epitea* of RFLP type 5 produced uredia on either seven or eight *Salix* species. On the other hand, the two collections of *M. epitea* of RFLP type 7 produced uredia only on two *Salix* species, *S. futura* and *S. japonica* (Table 3).

4 Discussion

In our experiments, the primer pair ITS1-F and ITS4-B permitted the amplification of a single DNA fragment both from leaves with rust sori and from axenic cultures, but no amplification of any DNA fragments from rust-free host plant leaves. Therefore, this primer pair is confirmed to be useful for the amplification of the DNA of rust fungi from rust-infected plants, as reported previously (Vogler and Bruns 1998).

Size of the amplified DNA fragments was constant within a species except for *M. epitea*. Fragment sizes were slightly different among the 10 species. However, these sizes could not be used for the identification of species because no significant difference was detected electrophoretically.

The rust collections of the 10 *Melampsora* species were divided into 11 RFLP types based on RFLP patterns using the four restriction enzymes. The collections of six species, *M. capraearum, M. epiphylla, M. kamikotica, M.*

larici-urbaniana, M. microsora, and *M. yezoensis*, revealed species-specific RFLP types. The RFLP type was identical within a species in spite of differences in host plants and localities. Therefore, the RFLP type is considered to be a useful character for distinguishing these species.

Between *M. chelidonii-pierotii* (except for one collection CH-ac1) and *M. coleosporioides*, however, no differences were found in the RFLP types. Also, no differences in their RFLP patterns were detected between the two species in preliminary experiments with three other enzymes, *Hae* III, *Hinf* I, and *Rsa* I. Uredial and telial states of these two species are also morphologically similar. Additionally, *M. coleosporioides* and *M. chelidonii-pierotii* produced uredia on some of the same host plants in inoculation experiments. Therefore, we suspected a close genetic relationship between the two species. These two species must be reconsidered taxonomically according to precise morphological observations and confirmation of aecial host(s).

The RFLP type of *M. chelidonii-pierotii* CH-ac1 differed from other collections of the same species. The CH-ac1 has been identified based on the morphology of urediospores and its host species. It is necessary to re-identify the collection by examining morphology of teliospores.

The RFLP type of all the collections was identical in *M. humilis* (type 5). The RFLP type of eight collections of *M. epitea* from which the 880 bp fragment was amplified was the same as that of *M. humilis*. In preliminary experiments with three other enzymes, *Hae* III, *Hinf* I, and *Rsa* I, no difference was found in the RFLP patterns between the two species. Hiratsuka and Kaneko (1982) reported that *M. humilis* was distinguished from *M. epitea* by only slight differences in morphology of teliospores and provisionally treated it as an independent species. Additionally, the host range of two collections of *M. humilis* and two collections of *M. epitea*, which showed the same RFLP type 5, were very similar to each other in the inoculation experiments. This suggests a close genetic relationship between *M. humilis* and these collections of *M. epitea*.

The collections of *M. epitea* were separated into three groups based on RFLP types. Neither RFLP types 6 or 7 in the collections of *M. epitea* from which 840 bp fragments were amplified were found in the other *Melampsora* species. In Europe and North America, *M. epitea* has been treated as a species complex because of a variety of aecial hosts and/or uredial and telial hosts (Hylander *et al.* 1953, Wilson and Henderson 1966, Ziller 1974). In our inoculation experiments, two collections of RFLP type 7 were apparently distinguished from two collections of type 5 based on the host range. These results indicate a clear difference in pathogenicity between the two groups. Consequently, our results suggest the necessity for taxonomical reconsideration of *M. epitea* RFLP type 5 and *M. humilis*. To clarify the taxonomic relationship between *M. epitea* and *M. humilis*, confirmation of their aecial hosts and more precise morphological observations are needed.

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Variation in phenolic levels of willow leaves (*Salix myrsinifolia*) in relation to occurrence of *Melampsora* rust

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Abstract

Phenolic levels in darkleaf willow (*Salix myrsinifolia)* were studied in relation to occurrence of *Melampsora* leaf rust. Field studies and greenhouse experiments showed that the phenolic concentrations vary significantly between willow clones. Moreover, in field studies phenolic levels were found to vary within and between growing seasons. There was marked clonal and temporal variation also in the occurrence of rust, but consistent correlations between individual phenolic glycosides and rust frequency were not found. In greenhouse-cultivated plants, nitrogen fertilization decreased the phenolic levels in several willow clones, but significant differences in rust frequency between the plants at low and optimum fertilization level were not found. Rust-induced changes in phenolic levels were detected at different stages of the infection process, but there was marked clonal, compound-specific, and temporal variation in the induced responses. The defensive importance of the observed rust-induced changes in phenolic levels is not yet clear. However, the results indicate that at least in some cases an individual phenolic compound, (+)-catechin, may have importance in constitutive and induced defence against rusts.

Key words: fertilization, phenols, resistance

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Diversity of the *Melampsora* rust on willows — consequences for SRF planting strategies

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Summary

Melampsora rusts on *Salix* spp. in plantations for short rotation forestry (SRF) represent a very heterogeneous population. In Sweden, over 30 different pathotypes were detected, possibly forming three *formae speciales*. The variable rust populations together with the long plantation cycles constitute a severe problem for both breeding work and when adopting crop mix theories for *Salix* spp.

Key words: *Salix*, short rotation forestry

1 Introduction

1.1 *Salix* rust

Melampsora rusts are the most serious fungal pathogens of *Salix* spp. (willows) in short rotation forestry (SRF) when cultivated as a monoclonal crop. *Melampsora* rusts on willows constitute a very heterogeneous population, containing some 20 possible species (Hubbes 1983), each with its own host preferences and virulence pattern. Although several species have been reported to occur (Pei *et al.* 1993a, b, Pei *et al.* 1996a), the main rust occurring on the willow species and clones used in SRF is *Melampsora epitea* Kunze & Schm. *M. epitea* is, however, considered to be a collective species that could be further divided into varieties (Hylander *et al.* 1953, Wilson and Henderson 1966), races, or subspecies. The common rust on *Salix* consists of a number of pathotypes that differ in virulence and aggressivity. Rust isolated from one willow species or clone is therefore not always compatible with another clone, even if the latter is usually heavily attacked by rust in the field. Most of the pathotypes are morphologically indistinguishable from each other, can use the same alternate host, and seem to be rather closely related.

1.2 Pathotypes and race formation

Early work on poplars showed that the poplar rust *Melampsora larici-populina* Kleb. contains several races with different virulence patterns (Pinon *et al.* 1987, Pinon 1992). The situation with willows appears to be similar based on field observations made mainly in the UK and Sweden. Inoculation experiments using a set of willow differentials (Ramstedt 1989) demonstrated a variety of pathogenicity patterns and indicated that the great variation in the

Melampsora rust population was caused by several pathotypes.

In further work, Pei *et al.* (1993a) standardized a set of willow differentials to allow comparison of the results from different countries. It was shown that the willow rust in the UK could be separated into several pathotypes including a stem-infecting form causing cankers on certain *Salix viminalis* L. clones (Royle and Hubbes 1992, Pei *et al.* 1993a, 1996a). The pathotypes could be classified into three main groups, or *formae speciales*, listed by Royle and Hubbes (1992) as: i) *larici-epitea typica*, LET (mainly infecting *S. viminalis* types), ii) *larici-retusae,* LR (*S. dasyclados* Wimmer types), and iii) *larici-daphnoides,* LD (*S. daphnoides* Vill. types).

In the genus *Melampsora*, no overwintering stage has been confirmed on willows in the countries with ongoing research on SRF pathogens. In Northern Ireland, and occasionally in England, however, there are some indications (Royle, pers. comm.) of overwintering mycelium in dormant buds of willow. This means that genetic recombination normally occurs every season, giving rise to new combinations of virulence genes the following year. The first infection of willow normally occurs in late spring when the aeciospores are spread from larch (*Larix* sp.) needles, while later infections are likely to result from secondary urediospores coming from these initial infections.

The time of disease development in early summer is apparently strongly dependent on the distance from larch. In a comparison of two similar fields with *S. viminalis*, a field with larch trees in and around the plantation was highly infected by rust approximately one month earlier than a field without larch in the vicinity (B. Samils, pers. comm.). This indicates that, when situated close to the larch source, primary infection is caused by aeciospores. Plantations farther from larch are infected later, presumably by urediospores when the spore load has increased in the air.

The present study was conducted to describe the composition and variability of the rust population on willows in SRF and to discuss possibilities to minimize disease development in the field.

2 Material and methods

Identification of the pathotypes was performed by using differential willow clones according to Royle and Hubbes (1992) and M.H. Pei (pers. comm.) in addition to a set of Swedish clones. For organizing and naming of the pathotypes, nine of these clones were selected for their ability to differentiate between the rust isolates tested (Table 1). Rust isolates from 90 different origins were used in the study.

Rusts were collected for pathotyping during 1993 to 1997 mainly 1993 to 1994. During 1993, one isolate was taken from each site/plantation/clone. The 1994 collection is represented by at least three isolates from each host unit. Single spore isolates used in the experiments were cultivated in the laboratory on detached leaves on wet filter paper in petri dishes. Spores were spread on the leaves by spraying or by a small brush. Inoculated leaves were incubated in growth chambers at 18° C in continuous light with a photon flux density of 200–250 µmol m^{-2} s^{-1} for about ten days before spores were harvested.

Table 1. Willow-differential clones used in pathotyping *Melampsora* rust.

Clone	Species	Sub-group	Virulence code[a]	*formae speciales* detected[b]
Mullatin	*S. viminalis*	a	1	LET
Q 83	*S. triandra x viminalis*	a	2	LET
Stipularis	*S. aurita x viminalis x caprea*	a	4	LET (LR)
Calodendron	*S. caprea x cinerea x viminalis*	b	1	LR // LET
Korso	*S. burjatica*	b	2	LR
Himalayas	*S. disperma*	b	4	LR (LET)
Bowles Hybrid	*S. viminalis*	c	1	LET
78149	*S. triandra x viminalis*	c	2	LET
78139	*S. daphnoides*	c	4	LD // LR // LET

[a]The figures for positive response are added together within each subgroup to give a three-digit code for virulence (e.g. 5.1.3)
[b]LET = *larici-epitea typica*, LR = *larici-retusae*, LD = *larici-daphnoides*

Pathotyping was done on detached leaves in petri dishes as above. The leaves were sprayed with spores suspended in water ($4x10^5$ spores mL^{-1}) with a few drops of tween added. After 10–14 days the reaction was recorded by counting the number and size of uredia as well as the number of necrotic spots. The combination of clones affected was designated as a pathotype code (Table 1).

3 Results and discussion

The sampled rust population consisted of more than 30 different pathotypes that vary in virulence and aggressivity (Table 2) and correlate with the pathotypes reported from the UK (Royle and Hubbes, 1992, Pei *et al.* 1996a, b) with certain modifications. The number of different pathotypes detected would likely increase if additional test willow clones were added.

The pathotypes formed three subgroups or possible *formae speciales*, as in the UK material (Royle and Hubbes 1992), with some degree of genetic barrier (Table 2). Rust isolates from *S. viminalis* (LET) were mainly compatible with willow differentials from the *viminalis* group, while the *dasyclados*-rust (LR) mainly infected the *dasyclados*-related clones. The positive responses outside their own group indicate that there is some recombination between the groups or that the groups are partly overlapping.

We have, however, not yet found any pathotype/isolate of the main *formae speciales* groups with virulence for both type species i.e., *S. viminalis* and *S. dasyclados* (clones Mullatin and Korso).

A problem when using the pathotype key in willow is the assumption of a straight qualitative response with a susceptible/nonsusceptible reaction. Willow leaves show a great deal of quantitative resistance that cannot be separated into different pathotypes using this system. Also, some of the "negative results" (minus reactions) are in fact a very weak infection, with

necrosis and retarded development of uredia instead of a lack of infection. Therefore, a pathotype is not concluded to be a specific genotype, a race in its proper meaning, or an isolated subpopulation. Rather, it is a heterogeneous group of different genotypes showing the same virulence pattern, but with internal differences in aggressivity and other properties (Lascoux *et al.* 1996). Among the isolates or strains belonging to the same pathotype, a significant magnitude of inconsistency in severity level of infection could be found. The heterogeneity of a pathotype was further supported by isozyme analysis demonstrating no correlation of the isozyme banding patterns and pathotype, as well as by ongoing RAPD and AFLP studies (B. Samils, unpubl.).

Table 2. Pathotypes found in Sweden 1993–1996.

Pathotype			Test clones								
			1	2	3	4	5	6	7	8	9
f. sp.	No.	code	Mull	Q83	Stip	B.H.	-149	Calod	Korso	Him.	-139
LD	1	0.0.0									
	2	0.0.4									+
	3	0.3.0						+	+		
	4	0.3.2					+	+	+		
	5	0.3.4						+	+		+
	6	0.7.0						+	+	+	
	7	0.7.2					+	+	+	+	
LR	8	0.7.4						+	+	+	+
	9	1.7.0	+					+	+	+	
	10	4.1.6			+		+	+			+
	11	4.3.0			+			+	+		
	12	4.7.0			+			+	+	+	
	13	4.7.2			+		+	+	+	+	
	14	4.7.4			+			+	+	+	+
	15	5.0.0	+		+	+					
	16	5.0.1	+		+	+					
	17	5.0.2	+		+		+				
	18	5.0.3	+		+	+	+				
	19	5.0.7	+		+	+	+				+
	20	5.1.2	+		+		+	+			
	21	5.1.3	+		+	+	+	+			
	22	5.1.6	+		+		+	+			+
	23	5.1.7	+		+	+	+	+			+
	24	5.4.3	+		+		+			+	
	25	6.1.0		+	+			+			
LET	26	6.1.6		+	+		+	+			+
	27	7.0.2	+	+	+		+				
	28	7.0.3	+	+	+	+	+				
	29	7.0.7	+	+	+	+	+				+
	30	7.1.1	+	+	+	+		+			
	31	7.1.3	+	+	+	+	+	+			
	32	7.1.5	+	+	+	+		+			+
	33	7.1.6	+	+	+		+	+			+
	34	7.1.7	+	+	+	+	+	+			+
	35	7.4.2	+	+	+		+			+	
	36	7.4.3	+	+	+	+	+			+	
	37	7.5.3	+	+	+	+	+	+		+	
virulence factor			**1**	**2**	**3**	**7**	**8**	**4**	**5**	**6**	**9**

+ = The pathotype shows virulence against the test clone.
Shadowed fields indicate recombinations between the main pathotype groups (*formae speciales*) LR and LET.

The *Melampsora* population on willow is very heterogeneous. The virulence factors detected with the willow differentials are, with some limitations, likely to occur in almost all different combinations that are theoretically possible (Table 2), thus forming many different pathotypes. Further work is needed with molecular tools such as RAPDs or AFLPs to study the relationship between different pathotypes and *formae speciales*.

In crossing experiments with selected isolates (Pei *et al.* 1996b), the hypothesis of three distinct populations or *formae speciales* of rust in SRF plantations is supported. Only crosses within each group were successful, and there was a very low percentage of viable offspring from crosses between pycnia of the LET and LR group in the UK. Only a few clones have been examined, and more extensive study is needed to evaluate gene exchange between the groups. How this will work in the field, with adjacent infections on the same larch needles, is not known. Wether the overlapping pattern are results of rare crosses or mutations remains to be shown and will probably be a task for molecular work.

The variable rust population with its high potential for adaptation and development of new pathotypes, constitutes a big problem for SRF plantations. New fairly resistant clones planted in large areas are at risk of suddenly being subjected to high disease levels. The risk is increased further by the way SRF is grown today, almost totally as homogeneous plantations of cloned material.

An approach for disease control that has been used in wheat and barley with some success is the so-called multi-line or mixed crop approach (Parlevliet 1979, Browning and Frey 1981, Wolfe 1985), in which different lines of a crop that have different resistance genes are used in random or organized mixtures. This approach is designed to avoid very quick spread and development of the disease within the crop due to uniform susceptibility of the crop to a single pathotype. Properly designed crop mixtures, with annual adjustments, have also been used in efforts to affect the pathogen population and to avoid development of complex races (Dileone and Mundt 1994).

For cloned willow, it is natural to ask if different clones could be used in plantations instead of single clones, so as to minimize pathogen attack. With the correct combinations of willow clones, one pathotype should not be able to spread all over the plantation, but only infect some of the clones in the mixture, while other pathotypes would be required to infect the other clones. McCracken and Dawson (1997) showed that, in two separate mixtures with five and six different species/clones, respectively, the mixture delayed the onset of disease, slowed its build-up, and resulted in a lower disease level at the end of the season. Similar experiments have also been reported from Long Ashton (Parker *et al.* 1996). These results indicate that the clonal mixture theory could also be adapted for willow. However, even if infection is initially reduced, there is a risk of increasing problems later with higher levels of infection that cannot be managed by the mixture of different clones. There are several questions to be addressed that are important for willow, limiting the conclusions to be drawn from other experiments concerning annual crops.

Even if the pathotyping of the rust, together with RAPD markers (B. Samils and V. Stepien, pers. comm.), gives information on how the rust population is composed, the overall amount of information would be small

compared to that for many annual crop systems. In several annuals, there is more knowledge concerning both population structures of the pathogens and resistance genes in the hosts, allowing the design of effective mixtures. For willow, there is still much to clarify before forecasting can be done to predict the effect on rust development of planting design and clone selection.

Results from an unintended clonal mixture of four *S. viminalis* clones in Sweden clearly show the importance of basic genetic knowledge about the components of a mixture to establish a durable plantation. One of the clones was heavily infected by *Melampsora* spp. for one year, while there were only slightly infected *S. viminalis* the following three years. After the first winter following the rust attack, the diseased clone suffered from severe shoot dieback, and three years later, 87% of the infected stools were dead while all non-infected stools were alive (Verwijst 1993). In trials or plantations with a one-year rotation, this problem would not be pronounced. With plantations to be harvested every 3–4 years, it is obviously a severe drawback. This clearly demonstrates that the rust in a crop mixture could strongly change the balance between the clones and mediate unwanted competition (Verwijst 1990, 1993).

The composition of the rust population is also problematic because the pathotypes can change from year to year as the hosts are changed. If the rust completes its sexual life cycle every year, recombination of the virulence genes must be considered. All of the rust isolates infect the same alternate host (larch), occurring as aecia, even on the same needle, and have the ability to recombine. After a few years, it is likely that there will be a pathotype that is able to affect all or most of the major clones in a given mixture, provided the mixture is used in large amounts. The point will thereby be reached where it is really not a clonal mixture anymore, as far as the resistance reactions are concerned. The use of a much larger number of clones in commercial SRF is, in any case, a necessity for the future development of willow plantations. How they should be planted, is, however, still to be solved. Since any single SRF plantation is supposed to last for many years, it would be hazardous to start larger commercial plantations with mixtures by chance.

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Melampsoridium sp. on alder leaves

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Summary

A *Melampsoridium* rust epidemic on alder (*Alnus* spp.) was recorded in 1996 in Estonia and one year later in Finland. In comparing the morphology of the rusts occurring on alder and birch (*Betula* spp.), we found clear differences in the length and roundness of uredospores. DNA sequences in the ITS region were also different. Based on these findings, we conclude that *Melampsoridium* rust found on alder is not conspecific with *M. betulinum* but is similar with *M. hiratsukanum*. This is the first record of *M. hiratsukanum* in Estonia and Finland.

Key words: *Alnus incana, Alnus glutinosa, Melampsoridium hiratsukanum,* PCR

1 Introduction

In 1996 *Melampsoridium* rust was observed on leaves of alder (*Alnus* spp.) in Estonia, and again in 1997 and 1998 in Estonia and Finland. A leaf rust on alder has been recorded only once in Finland, in 1953 (Jørstad and Nannfeldt 1958). In the literature, two species of *Melampsoridium*, *M. alni* (Thümen) Dietel and *M. hiratsukanum* Ito, have been described on alder (Gäumann 1959, Wilson and Henderson 1966).

Although *Melampsoridium* rusts on alder have been recorded in Europe, their identification seems to be problematic. According to Henderson and Bennell (1979), European records of *M. hiratsukanum* were doubtful. Roll-Hansen and Roll-Hansen (1981) were able to inoculate alder with uredospores obtained from both birch and alder leaves. They concluded that *M. alni* is conspecific with *M. betulinum* (Pers.) Kleb., which occurs on birch around the northern hemisphere (Gäumann 1959). *Melampsoridium* rusts on alder seem to be common in Siberia and eastern Asia (Kuprevicz and Tranzschel 1957). Hiratsuka and Kaneko (1981) described some characteristics that allow identification of alder rusts and their differentiation from *M. betulinum*. Surface structure and position of uredospores appeared to be useful in identication.

Different species usually have separate sequences in their internal transcribed spacer (ITS) regions (Gardes *et al.* 1990, Kasuga *et al.* 1993). This has made the ITS region (including the 5.8S rRNA gene) one of the most popular markers in mycological studies.

The denaturing gradient gel electrophoresis (DGGE) and constant denaturing gel electrophoresis (CDGE) efficiently separate DNA molecules not only according to their size but also sequence differences (Myers *et al.* 1986, Lessa 1992).

Recent *Melampsoridium* epidemics on alder supplied fresh material to study differences between the rusts occurring on birch and alder. In this study we tested whether the rusts observed on birch and alder can be separated by morphological methods and/or methods based on the polymerase chain reaction (PCR).

2 Material and methods

For morphological studies, infected leaves of *Betula pendula* Roth, *B. pubescens* Ehrh., *Alnus incana* (L.) Moench, and *A. glutinosa* (L.) Gaertner were collected in southern Finland and in Estonia. For spore measurements we used image analysis (Cambridge 500 by Leica). In addition to the length and width we also measured roundness of uredospores. Roundness is a shape factor that gives a value of 1 for a circle. This is calculated from the ratio of perimeter squared to area,

$$\text{Roundness} = \frac{\text{Perimeter}^2}{4 \times p \times \text{Area} \times 1.064}$$

The adjustment factor of 1.064 corrects the perimeter for the effect of the corners produced by the digitization of the image (Quantimet 500+, 1993). Statistical analysis was done with SYSTAT software.

The following samples were used:

1) ITS-analysis. Leaf discs, 1 mm^2 in size, containing single uredia were aseptically cut from *B. pendula*, *B. pubescens*, *A. incana,* and *A. glutinosa*.

2) DNA isolations. Total DNA was isolated from the leaf discs with uredia using a modification of the method described by Vainio *et al.* (1997).

3) PCR reactions. The fungal ITS DNA was amplified by the polymerase chain reaction (PCR) using the basidiomycete-specific primers ITS1-F and ITS4-B (Gardes and Bruns 1993).

4) Analysis of the amplification products. The PCR reactions were first analyzed by electrophoresis in agarose gels containing 1.0% SynerGel (Diversified Biotech) and 1.0% agarose (FMC BioProducts). The electrophoresis was run in TAE-buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA). The lengths of the amplification products were estimated by comparing them to a 100-bp DNA ladder (Gibco BRL). The CDGE was run according to the instructions of the manufacturer of the D-GENE denaturing gel system (BioRad). The denaturant (urea and formamide) concentration used was 30%. In both types of electrophoresis the amplification products were visualized using ethidium bromide under UV-light.

In 1998 we did inoculation tests to study the host range and life cycle of alder rust.

3 Results

In birch rust spores, echinulation was absent from the broader end, but in alder rust the surface of uredospores was uniformly echinulate. The germ pores were bizonate in position and equal in number in both birch and alder rusts in our samples.

The rust occurring on birch had longer uredospores than the rust collected from alder: the average lengths in all birch rust material were 32 and 34 µm on *B. pendula* and *B. pubescens,* respectively; the corresponding measures were 25 and 27 µm in *A. incana* and *A. glutinosa.*

The average roundness index varied in alder rusts from 1.33 (*A. incana*) to 1.41 (*A. glutinosa*) and in birch rusts from 1.46 (*B. pubescens*) to 1.60 (*B. pendula*). Thus the uredospores from *A. incana* were closest to a circle.

The PCR-amplification from rust uredia on birch or alder leaf discs resulted in one major 900-bp band and sometimes faint smaller molecular weight bands in agarose gel electrophoresis (not shown). We concluded that the major band corresponded to the ITS region, whereas the fainter bands were probably due to nonspecific priming or other PCR artefacts. No differences in the sizes of the major 900-bp bands were observed between the samples.

In CDGE the amplified ITS bands from birch migrated faster than those from alder. All samples from the two alder species were identical. Also the migration of amplified ITS samples from birch was very similar, although some slight differences were observed, suggesting that there may have been some intraspecific variation not properly resolved by CDGE.

We successfully inoculated *Larix dahurica* Turcz. needles with basidiospores developed on overwintered alder leaves. Aeciospores from *L. dahurica* infected both *A. incana* and *A. glutinosa.* Uredospores developed on alder leaves infected alder again but not *B. pendula.*

4 Discussion

The genetic and morphological data presented here indicate that the *Melampsoridium* species on birch and alder in Finland are different. The samples from both alder species were morphologically identical and they had identical ITS alleles. Among samples from birch we found no morphological differences. However, possible variation in ITS was observed, but most samples from both birch species had identical ITS alleles. These observations support the idea that only one *Melampsoridium* species is observed on birch, and another single species on alder in Finland.

To our knowledge this was the first study applying a denaturing gel electrophoresis of PCR-amplified ITS fragments to mycological studies. The method provided clearly interpretable results, suggesting that it could be used in further studies.

The rusts on *A. glutinosa* and *A. incana* are morphologically similar to *M. hiratsukanum,* which has been recorded in Asia and in America (e.g. Kuprevicz and Tranzschel 1957, Kaneko and Hiratsuka 1981) and now for the first time in Estonia and Finland. It is possible that *M. hiratsukanum* occasionally spreads from Asia to Europe and can survive several years only in locations where *L. dahurica* is present, e.g. in experimental forests.

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Interaction between birch, *Betula* spp., and *Melampsoridium betulinum*, the leaf rust fungus

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Abstract

The compatibility of the urediniospores of *Melampsoridium betulinum* collected from *Betula pendula* and *B. pubescens* in southern and central Finland was tested with detached leaves in the leaf disc assays and with attached leaves in the greenhouse and outdoors in the field. The clones and seedlings of *B. pendula* and *B. pubescens* had intra- and interspecific variation in their rust resistance responses measured as uredinia frequencies and uredinia cover-% of the leaves. *B. pubescens* had strong resistance to the isolates collected from *B. pendula*. The resistance was characterised as lowed pustule frequencies and hypersensitive reactions. *B. pendula* did not have clear resistance to any rust isolate collected from *B. pubescens*. The seedlings of *B. papyrifera* were also relatively resistant to the urediniospores of *B. pendula*. The physiological status (leaf nitrogen, protein, rubisco, chlorophyll content and C/N ratio) of the leaves did not correlate with the rust resistance, neither did the growth environment, greenhouse or outdoors, affect the rust resistance responses of the seedlings of five different birch species, *B. pendula*, *B. pubescens*, *B. platyphylla*, *B. papyrifera* and *B. resinifera* x *B. pendula*. The rust isolates germinated, formed appressoria and penetrated through stomata also on the sterile *in vitro* leaves of *B. pendula* and *B. pubescens*. The resistance response of *B. pubescens* to the *B. pendula* rust isolates was not related to the leaf surface structure (stomata) and appressorium formation of the urediniospore germlings. The resistance rankings of the clones of *B. pendula* obtained in the leaf disc assay correlated with the field resistance of the same clones. The highest correlations (r_s = 0.71–0.9) were, however, obtained with the clones grown in nutrient rich substrate which corresponded to the growth substrate of the used screening test material.

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Genetic and environmental variation in susceptibility of mature mountain birch trees to leaf rust

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Abstract

Genetic variation in resistance of deciduous trees against leaf rust diseases has been mainly studied on young saplings of *Populus* and *Salix*; generally, heritability of the resistance has been estimated to be high. We investigated whether genetic variation exists in the susceptibility of mature mountain birch (*Betula pubescens* subsp. *czerepanovii*) to the most important leaf disease of birch, caused by *Melampsoridium betulinum*. We studied the same mountain birch genotypes in two areas closely resembling natural growing habitats during three years by evaluating the frequency of natural birch rust infections in the field and by analyzing artificially infected detached leaves in the laboratory. The frequency of birch rust varied among the mountain birch genotypes. However, heritability of the birch rust resistance was found to be fairly low: with naturally infected birch the heritability was 0.27 in the year of the highest birch rust frequency. The frequency of birch rust varied greatly between the two study areas and among study years. Nevertheless, the relative frequency of birch rust among the individual trees and tree genotypes remained similar, so there was no notable genotype x environment interaction. The methods used to analyze birch rust frequency gave different results with respect to the ranking of birch genotypes by birch rust resistance. This study shows that the heritability of the resistance of mature forest trees against leaf rust diseases may not be as high as could be expected based on the earlier studies with *Populus* and *Salix* saplings. The results also stress the importance of the methods used in analyzing the resistance of trees.

Key words: *Betula pubescens* subsp. *czerepanovii*, *Melampsoridium betulinum*

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Systematics of spruce needle rusts in the *Chrysomyxa ledi* complex

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Summary

In 1879, Anton de Bary gave the name *Chrysomyxa ledi* to a rust that infected European *Ledum palustre* and needles of *Picea abies*. Later, similar rusts found on North American *Ledum* species and alternating to spruce needles were also assigned to *C. ledi*. In the 1950's D.B.O. Savile noted subtle differences in urediospore surface morphology among the rusts infecting various *Ledum* species and *Chamaedaphne*, *Rhododendron*, and *Vaccinium*. However, because of similarities in spore size and morphology on spruce needles, he felt that they did not warrant species status, and he created six varieties of *C. ledi* to accommodate these rusts. The purpose of this study is to reevaluate the *C. ledi* complex using modern microscopic methods. Scanning electron microscopy reveals clear morphological differences in the urediospores, aeciospores, and peridial cells of these varieties, especially in the surface ornamentation and in the nature and presence of a groove or smooth area on the spores. Surprisingly, a previously overlooked small-spored species has been found on North American *L. groenlandicum*, *Ledum* hybrids, and cultivated rhododendrons. Host specificity and morphological differences provide strong support for considering all of the *C. ledi* varieties to be separate species.

Key words: *Ledum, Picea abies*

1 Introduction

Rust fungi in the genus *Chrysomyxa* cause needle and cone diseases on spruce (*Picea* spp.). Most species of *Chrysomyxa* are heteroecious and alternate to ericaceous plants that have evergreen leaves, in which the rusts overwinter. Depending on the species, these fungi may have a circumboreal distribution wherever the two hosts occur together, or they may have a more restricted distribution on one continent. Infection of individual trees by *Chrysomyxa* needle rusts can be very heavy, and all or most of the current year's needles may be infected and drop prematurely. Heavy infection for two or more consecutive years could be economically significant in commercial stands or Christmas tree plantations (Hiratsuka *et al.* 1995).

There is confusion in the identity and relationship among *Chrysomyxa* species causing needle rusts on different continents and occurring on closely

related host plants. The name *C. ledi* de Bary was originally applied to a European rust occurring on *Ledum palustre* L. and *Picea abies* (L.) Karst. (de Bary 1879). Later the name was applied to rust fungi in North America with similar life cycles (Fraser 1911, 1912, Arthur 1912) but occurring on different *Ledum* and *Picea* species. Savile (1950, 1955) noted subtle differences in urediospore morphology among the rusts infecting *Ledum* spp. and other ericaceous plants, but contended that the aeciospores on spruce were indistinguishable and that they did not warrant species status. He therefore created six varieties of *C. ledi* de Bary to accommodate the rusts with similar spore size on *Rhododendron* spp., *Ledum* spp., *Chamaedaphne calyculata* (L.) Moench., and *Vaccinium parvifolium* Smith. Savile's variety names have been only partly accepted because telia were not described for all varieties. Variety names are often used for the uredinial states for several of these rusts, but the aecial states on spruce are called simply "*C. ledi* ". This leads to confusion in the identity of spruce needle diseases. There is a tendency to assume all rusts labelled *C. ledi* have the same origin. Basic biological studies and elucidation of species concepts are necessary so that outbreaks of needle rusts can be traced to their broadleaved hosts.

The purpose of this study is to reevaluate, using modern microscopic methods, the *C. ledi* complex, consisting of the rusts that occur on spruce needles and alternate to leaves of *Rhododendron* spp., *Ledum* spp., *Chamaedaphne calyculata*, or *Vaccinium parvifolium*.

2 Material and methods

Fresh material or dried specimens of the rusts included by Savile (1950, 1955) in the *C. ledi* complex were examined on *Ledum groenlandicum* Oeder, *L. decumbens* (Ait.) Lodd., *L. palustre*, *L. glandulosum* Nutt., *L. palustre* ssp. *diversipilosum* Hara, *Chamaedaphne calyculata*, *Rhododendron* spp., *Vaccinium parvifolium*, and *Picea* spp. These were from North America, Europe, and Asia, and were from the collections of the Northern Forestry Centre, Edmonton, Alberta (CFB) and Pacific Forestry Centre, Victoria, B.C. (DAVFP), Canadian Forest Service; the Arthur Herbarium, Purdue University, Lafayette, Indiana (PUR); and the Finnish Forestry Research Institute, Rovaniemi, Finland. For light microscopy, spores and hand sections of infected plant material were mounted in lactophenol or lactophenol-cotton blue. For scanning electron microscopy, aeciospores or urediospores were dusted onto adhesive-coated aluminum stubs, coated with gold, and examined with an Hitachi S-510 scanning electron microscope (SEM) operated at 15 or 25 kV.

For rusts available locally in Alberta, i.e., those on *C. calyculata*, *L. groenlandicum* (two rust species), and *L. glandulosum*, artificial inoculations were done onto spruce needles to obtain authentic spermogonia and aecia connected with a particular ericaceous host. In addition, field observations were made of the natural development of these rusts on both conifer and broad-leaved hosts for three years.

3 Results

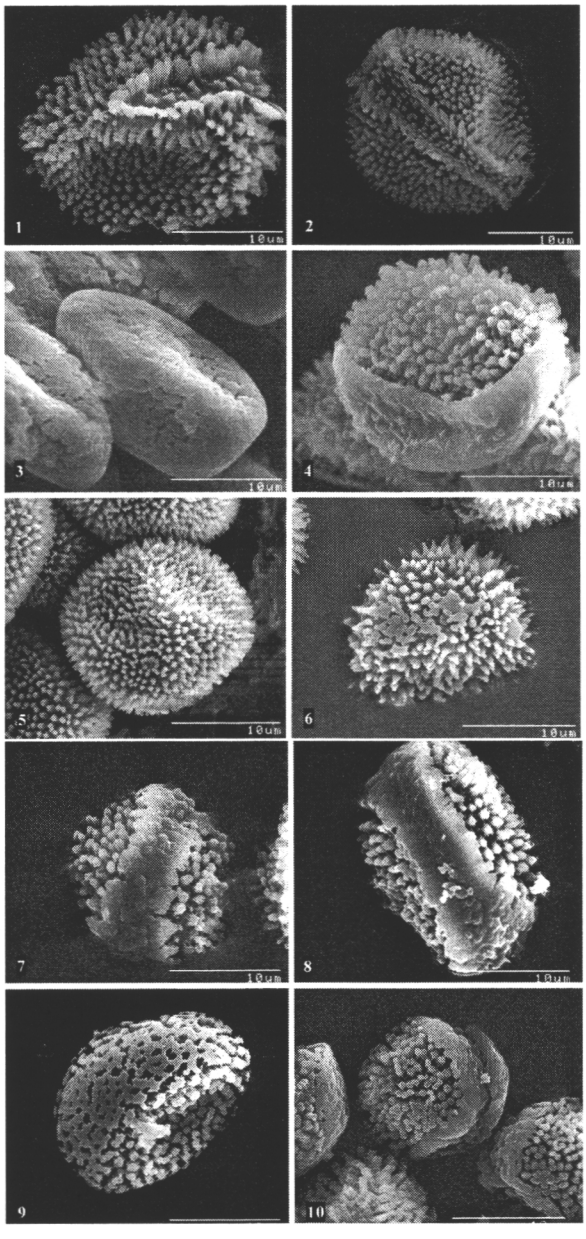
For a given taxon, morphology of aeciospores on spruce and urediospores on the ericaceous host are usually very similar. Consistent differences were found in spore morphology among the rusts on *L. groenlandicum* and *L. glandulosum* in North America and *L. palustre* in Europe (Figs. 1–6). Urediospores on *L. groenlandicum* have a smooth appearance except for small central areas where separate warts are visible; those on *L. glandulosum* have very tiny warts with indistinct annulations and no basal connections; and those on *L. palustre* have a narrow vertical groove and distinct tapering warts with prominent annulations. Besides these, an undescribed species was recognized on *L. groenlandicum* and cultivated rhododendrons in North America. The spores are smaller and more globose than those of *Chrysomyxa rhododendri* de Bary and have a reticulate vertical band. The aecial state was produced on spruce by artificial inoculation (Figs. 9 and 10). Spores of *C. rhododendri*, which occurs on native rhododendrons in northern and alpine regions of Canada, Europe, and Asia, tend to be more elongated and have an indistinct "smooth" band (Figs. 7 and 8). The Japanese rust on *L. palustre* ssp. *diversipilosum* appears to be most like the European one, but its spores have variable morphology. Examination and comparison of rusts on several *Ledum* subspecies and rhododendrons in Japan are needed to determine their identity. Spores of *Chrysomyxa cassandrae* (Peck & G.W. Clint) Tranz. on *Chamaedaphne calyculata* have distinct warts over most of the spore but also a broad flat area with a ragged edge and containing irregular shallow warts. On *V. parvifolium*, the urediospores have warts varying from coarse to fine, and sometimes joined at the spore ends. The aecial state is unknown.

4 Discussion

Characters useful in taxonomy of needle rusts in the genus *Chrysomyxa* are specificity of the ericaceous host, and size and surface morphology of aeciospores, urediospores, and peridial cells. On aeciospores and urediospores, the size and characteristics of surface warts, presence of basal connections between warts, and the nature and presence of a vertical groove or smoother area on spores are consistent characters separating the various taxa. Morphology of the sori (uredia, telia, aecia, and spermogonia) is less useful, as these structures are superficially similar in all species.

Host specificity and morphological differences strongly support the placement of members of the *C. ledi* complex in at least seven different species. Placing these rusts under one species, based mainly on similar spore size, leads to confusion.

The discovery of a unique small-spored rust on *Ledum* spp. and cultivated rhododendrons offers new insight into the identity of the rust that first appeared on cultivated rhododendrons in western North America in the 1950's. The disease may have originated from wild *Ledum* plants adjacent to nurseries, rather than from rhododendrons imported from Europe (Gould *et al.* 1955, Savile 1973). Because these obligate parasites are thought to have coevolved with their hosts, such a jump from *Ledum* to *Rhododendron* supports recent studies suggesting that the genus *Ledum* belongs in *Rhododendron* (Kron and Judd 1990, Harmaja 1991).



Figures 1–10. SEM images of urediospores (left) and the corresponding aeciospores (right) of some members of the *Chrysomyxa ledi* complex: 1) On *Ledum palustre* (Germany) and 2) *Picea abies* (Finland); 3) on *L. groenlandicum* (Alberta) and 4) *P. mariana* (Mill.) B.S.P. (Alberta, artificial inoculation); 5) on *L. glandulosum* (Alberta) and 6) *P. engelmannii* Parry ex Engelm. (Idaho); 7) on *R. lapponicum* (L.) Wahlenb. (Northwest Territories) and 8) *P. abies* (Italy); 9) undescribed species on *L. groenlandicum* (Alberta) and 10) *P. glauca* (Moench) Voss (Alberta, artificial inoculation).

Increasing movement of spruce species and other plants among continents increases the risk of introducing non-endemic rusts to new areas or of infection of introduced plants by endemic rusts. Clear species definitions, precision in the use of species names, and knowledge of rust life cycles are imperative so that outbreaks of spruce needle rusts can be traced to their broadleaved hosts.

Acknowledgements

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Cone rusts on Norway spruce in northern Finland

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Summary

Occurrence of two rust fungi infecting Norway spruce (*Picea abies*) female flowers was assessed after extremely good flowering and cone production in the summer of 1989 in northern Finland. *Thekopsora areolata* was more common than *Chrysomyxa pirolata*, with 18% and 2.5% of the cones infected by these fungi, respectively. An estimated 20.5% seed loss was caused by rust infection. The incidence level of *T. areolata* was considered to be exceptionally high because its alternate host, *Prunus padus*, is rare in the examined area.

Key words: *Chrysomyxa pirolata*, *Picea abies*, *Prunus padus*, *Thekopsora areolata*

1 Introduction

At high latitudes near conifer timberlines, such as in the northern boreal zone in northern Finland, natural regeneration of forests is very dependent on good seed production years. Such years occur sporadically and rarely. Norway spruce (*Picea abies* (L.) Karst.) had good seed years in 1958, 1967, 1970 (Sarvas 1968, Numminen 1989), and 1989. Spruce seed storage supplies for seedling production are based on collections made in good seed years.

When there is commercial harvesting of Norway spruce seed, the entire seed lot cannot be utilized due to seed problems. One of the main factors decreasing the seed potential is epidemics and outbreaks of pathogens and pests that infect or infest female flowers when they are blooming or soon thereafter. Seeds of ripening cones are eaten by insects or killed by fungal infections. Rust fungi may cause 100% loss in seed germinability (Roll-Hansen 1969). Because the decrease in germinability of seeds in good seed years is of economic importance, the occurrence and role of two rust fungi (*Thekopsora areolata* (Fr.) Magnus) and *Chrysomyxa pirolata* (Körn.) Wint.) in Norway spruce cones was studied in the good seed year of 1989 in northern Finland.

2 Material and methods

Norway spruce cones were sampled in 28 stands and three forest areas, summing 31, in northern Finland in late September to December, 1989, following an exceptionally good flowering year for Norway spruce. About 50 cones were collected per stand, providing a total of 2396 cones. The sampled stands were located between the latitudes 65°58'N and 68°12'N, the

northernmost sites reaching the northern timberline for Norway spruce (Fig. 1). For rust detection and identification, the cones were cut longitudinally into two halves, from which the presence of *Thekopsora areolata* and *Chrysomyxa pirolata* was assessed based on aecial occurrence on the cone scales.

The seed loss was estimated based on Rummukainen (1960), according to whom 75–100% of the seeds in a cone are lost if the cone is infected by either of the two rust fungi. No germination tests were carried out to verify this in our study.

3 Results

In the examined material, the proportion of cones infected by *T. areolata* and *C. pirolata* was 18% and 2.5%, respectively. Because there were no cones in which both rust fungi were fruiting, the proportion of rust-infected cones was 20.5%. Cones infected by *T. areolata* were recorded in 26 out of 31 stands, and its incidence per stand ranged from 2 to 71% in the infested stands. Infection by *C. pirolata* was less common, ranging from 1 to 13% of cones infected in the 12 infested stands.

Both *T. areolata* and *C. pirolata* were most common in the western part of northern Finland, where the thermal sum of the growing season is the highest. On average, 25% and 4% of the cones were infected by *T. areolata* and *C. pirolata*, respectively, in this area. Corresponding incidence was 10 to 12% and 0.4 to 1.3%, respectively, in other areas.

Based on the proportion of infected cones and the results of Rummukainen (1960), 20.5% of the seeds were ungerminable due to rust infection.

4 Discussion

Thekopsora areolata was clearly more common than *Chrysomyxa pirolata* in cones of Norway spruce in northern Finland. This conflicts with the earlier findings in Finland by Kangas (1940) and Rummukainen (1960), who found *C. pirolata* to be much more common than *T. areolata*. Further, the proportion of spruce cones infected by *T. areolata* (18%) was unexpectedly high, because even in a mixed stand where the alternate host *of T. areolata*, *Prunus padus* L., has been present, the proportion of infected cones has stayed below 20% (Lilja 1967). In the sampled area in northern Finland, *P. padus* or its subspecies is rare, occurring mainly in moist sites and especially along rivers. It is rarer than wintergreens (*Pyrola* spp.), the alternate hosts of *C. pirolata*, which are common in understory vegetation of spruce stands in the sampled area. Basing his opinion on Rainio (1926) and Kari (1936), Kujala (1950) explained the low infection level of *C. pirolata* by the fact that not all *Pyrola* species act as alternate hosts and by the difficulty of the fungal basidiospores to fly from undersides of the wintergreens in the low understory vegetation. An advantage for *T. areolata* is that infected cones remain in the crown for a longer time than is normal and some of the aeciospores stay viable for several years, providing a continual fungal reservoir that will be present whenever female flowers are formed.

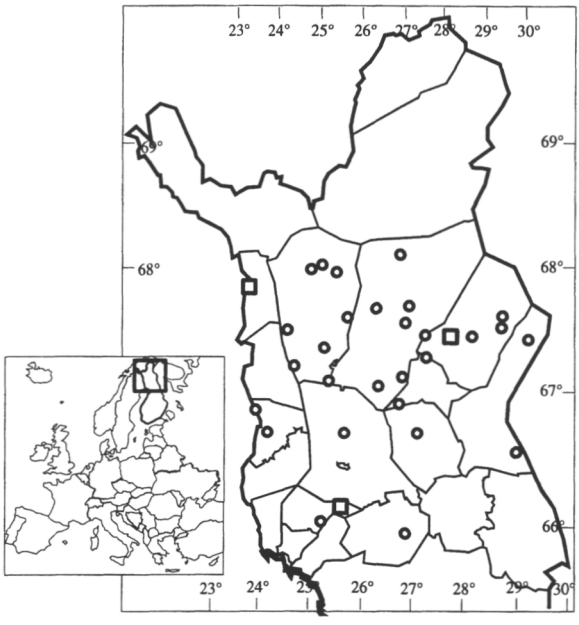

Figure 1. Location of the sampling sites in northern Finland. Symbols: □ = forest area; O = forest stand.

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Phenological synchronization in the twisting rust – maritime pine pathosystem

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Summary

Using simple phenological models, we were able to predict the period of teliospore maturity for twisting rust and the period of susceptibility for maritime pines, and thus simulate the overlap between them, corresponding to the potential infection window. According to our simulation in the Bordeaux area, teliospore maturity was nearly always reached before pines were susceptible. The expected consequence is a loss of inoculum potential in relation to the occurrence of rainy episodes during the lag period. The observed rust incidence in a 10-year period could be explained by the conjunction of the predicted inoculum potential and the weather conditions prevailing during the predicted period of pine susceptibility. The degree of synchrony between rust and pine phenologies, which determines inoculum potential, was predicted more often as a limiting factor for disease incidence than the weather conditions during the infection period in the Bordeaux area.

Key words: disease incidence, epidemiology, phenology, *Melampsora pinitorqua*, *Pinus pinaster*, synchrony, susceptibility

1 Introduction

Twisting rust, caused by *Melampsora pinitorqua* Rostr., is a common disease of pines throughout Europe, mainly on *Pinus sylvestris* L. and *P. pinaster* Ait. In France, it occurs in the large monospecific forest of "Les Landes", which yields a significant part of national wood production. The fungus is a macrocyclic heteroecious rust. Pine infections are initiated by the basidiospores, which are produced by germinating teliospores after their dormancy has been broken in spring. Pycnia and aecia are produced within a few weeks. Infections take place on elongating shoots of young trees and usually result in typical bending or withering of the thinnest shoots. From an epidemiological point of view, the disease on pines is monocyclic, with a single and short infection period each year. The multiplication (with urediniospores) and survival (with teliospores) of the fungus is achieved on the alternate host, i.e. poplars of the Leuce section, mainly aspens. The incidence and damage of twisting rust on maritime pines is very variable according to the site and year. Earlier work gave evidence that the target component, i.e. the amount of susceptible tissue at the time of spore dispersal, can be a major determinant of the variability in natural infection

levels. This was observed when comparing the susceptibility to *M. pinitorqua* of several geographic provenances of maritime pine that had different phenologies (Desprez-Loustau and Baradat 1991). The importance of the target effect was also demonstrated in a path analysis of components of pine susceptibility to twisting rust, using maritime pines which exhibited different growth patterns (Desprez-Loustau and Wagner 1997). Studies in other countries have also stressed the effect of pine phenology on the impact of twisting rust (Kurkela 1973, Naldini-Longo *et al.* 1991). The critical influence of the target effect has been reported for western gall rust on pines (Karlman *et al.* 1997). The importance of synchrony between particular stages of the host and of its aggressor has been especially analyzed for plant-insect interactions (Hunter 1990, Dewar and Watt 1992).

The purpose of our research has been to assess the importance of synchrony in the maritime pine–twisting rust interaction. In recent years, we have defined the critical phenological stages of both host and pathogen (Desprez-Loustau 1990, Desprez-Loustau and Dupuis 1992) and developed methods of prediction of these stages from meteorological variables (Desprez-Loustau and Dupuis 1994, Desprez-Loustau *et al.* 1998). The present study is a simulation of the degree of synchrony between pine and rust in SW France, based on the above-mentioned phenological models, with an analysis of the relationship between rust-pine synchrony and disease incidence.

2 Material and methods

2.1 Definition and prediction of critical phenological stages of pine and rust

2.1.1 Maritime pine susceptibility

The period of maritime pine susceptibility to *M. pinitorqua* was shown to run from stage B2 to stage B5, according to Debazac terminology (1966) (Desprez-Loustau 1990). This period is only a part of the shoot growing period, after the disjunction of bud-scales, allowing basidiospores to get onto the shoot surface, and before the full expansion of needles.

The prediction of the mean date of occurrence of these stages is based on the meeting of heat requirements. Heat sums are calculated from January 1 of each year, with a threshold value of 0° C (Desprez-Loustau and Dupuis 1994). The heat requirements observed for the Landes provenance of maritime pine were the following: 958 positive degree-days since January 1 for the B2 stage (beginning of the susceptibility period), 1407 positive degree-days for the B5 stage (end of the susceptibility period).

2.1.2 Basidiospore dispersal

The date of optimum germinability (DOG) of teliospores is used as the predictor of the beginning of potential basidiospore dispersal (Desprez-Loustau and Dupuis 1992). The variation in DOG in SW France was best explained by temperature variables (Desprez-Loustau *et al.* 1998). The two following equations gave the best predictions:

$$JDOG = 123.2 - 0.127 * Tjan \quad (1)$$

$$ST(Jan1–DOG) = 814 \text{ degree-days} \quad (2)$$

in which JDOG = number of days since January 1 to DOG, Tjan = heat sum accumulated in January, based on the sum of daily positive mean temperatures [(Tmin + Tmax)/2], and ST(Jan1–DOG) = heat sum accumulated since January 1 to DOG.

In our simulation, the first equation was used in most cases. However, the second equation was preferred for some years with extreme January heat sums (relative difference to the average greater than 20%), but heat sums in February–March were closer to the average.

2.2 Simulation and analysis of pine–rust synchrony

The simulation covers a 45-year period (1949–1995) at one site in SW France (Merignac, near Bordeaux). Standard meteorological data (daily temperatures and precipitation) were provided by Meteo-France.

From the predicted critical stages in rust and pine, the lag period was defined as the difference between DOG and the date of occurrence of the B2 stage. The period of pine susceptibility was predicted as the time between the predicted dates of occurrence of the B2 and B5 stages.

Since basidiospore release is expected to result from the occurrence of both active teliospores (i.e. after DOG) and rainfall (in addition to favorable temperature conditions), we examined weather data each year during the lag between DOG and B2, and during the period of pine susceptibility. Rainy episodes were defined as sets of successive days with at least 2 mm rainfall, since basidiospore dispersal was shown to occur with peaks related to such episodes (Kurkela 1973, Desprez-Loustau and Dupuis 1992).

No systematic records of twisting rust damage on maritime pine in SW France are available. Since 1988, when the Forest Health Department was created, the annual incidence (low/high) can be estimated (Département de Santé des Forêts, 1989, and subsequent years). We also have personal observations of high incidence of twisting rust in 1984 in the Bordeaux area.

3 Results and discussion

3.1 Simulation of the timing of rust dispersal and pine susceptibility in SW France over 45 years

As expected, since temperature sums were used to predict stages in both host and pathogen, a similar trend in year-to-year variability in the appearance of phenological stages was observed for pine and rust (Fig. 1). A significant correlation of 0.44 was obtained. A striking feature is that predicted teliospore maturity (DOG) was always reached before pines were predicted to be susceptible. DOG was usually predicted in early April, whereas the B2 stage occurred in late April or early May (Fig. 2). The lag between DOG and B2 was from 0 to 34 days, with 90% of the years showing a 1- to 4-week lag (Fig. 3).

This lag between critical phenological stages of *M. pinitorqua* and maritime pine might be considered as an imperfect adaptation of the rust to this pine species, as hypothesized by Moriondo (1957), as a result of the life history of rust at glacial and postglacial periods.

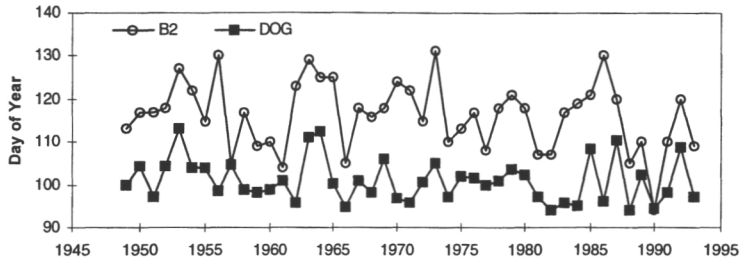

Figure 1. Predicted Date of Optimum Germinability (DOG) for *Melampsora pinitorqua* teliospores and date of occurrence of the B2 stage in maritime pines, Merignac 1949–1993.

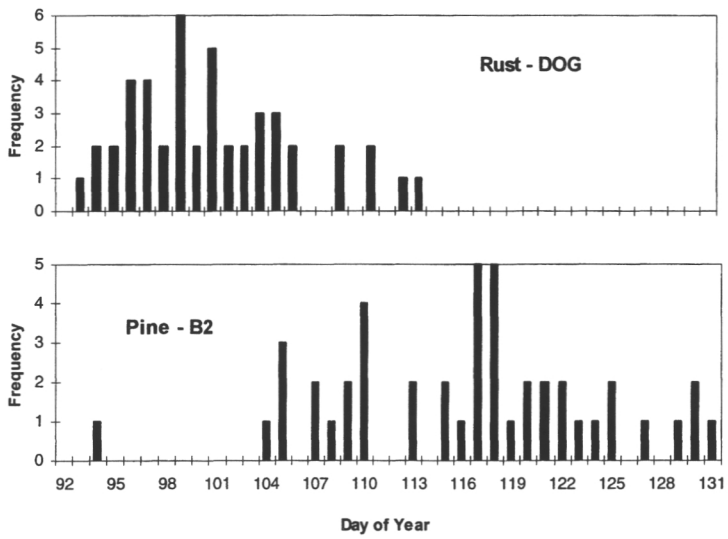

Figure 2. Frequency analysis of the Date of Optimum Germinability (DOG) for *Melampsora pinitorqua* teliospores and of the date of occurrence of the B2 stage in maritime pines, Merignac 1949–1993.

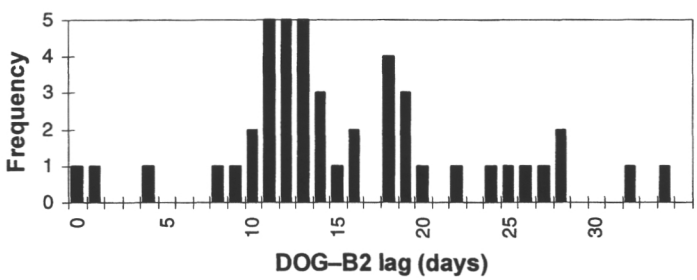

Figure 3. Frequency analysis of the length of lag between the Date of Optimum Germinability (DOG) for *Melampsora pinitorqua* teliospores and the date of occurrence of the B2 stage in maritime pines, Merignac 1949–1993.

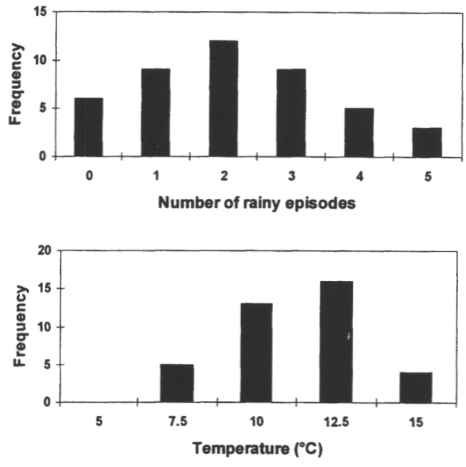

Figure 4. Frequency analysis of rainfall and temperature data during the lag between the Date of Optimum Germinability for *Melampsora pinitorqua* teliospores and the date of occurrence of the B2 stage in maritime pines, Merignac 1949–1993.

3.2 Relationship between pine-rust synchrony and damage

Conditions for basidiospore production during the DOG–B2 lag were met in most years, with mild temperatures and several rainy episodes (Fig. 4). This means that partial and even sometimes complete exhaustion of inoculum is likely before pines begin to be susceptible. A highly significant correlation between lag length (in days) and the number of rainy episodes was obtained (0.67). The inoculum potential thus statistically decreases with the degree of asynchrony between rust and pine.

For the 1984–1993 period, the 2 years with high disease incidence had a high predicted inoculum potential, related to only one rainy episode in the DOG–B2 lag (Table 1). Most years with low inoculum potential showed low incidence of the disease. However, 2 years (1990 and 1992) had high predicted inoculum potential and low incidence, and 1988 showed the reverse trend. We examined weather conditions for these years during the period of pine susceptibility (predicted stages from B2 to B5). For the 2 years with high rust incidence (1984 and 1993), weather conditions were very favorable for infections, especially numerous rainy episodes in 1984 and warm temperatures in 1993 (Table 2). Conversely, 1990 and 1992 showed the lowest temperature and number of rainy episodes, respectively. These might have been limiting factors for infections on pines in spite of a high predicted inoculum potential. The reverse was observed in 1988 with the most favorable conditions for infection (frequent rain and warm temperatures), which might have compensated for a lower inoculum potential. For the whole period 1949–1993, most years exhibited favorable conditions for infections during the period of predicted pine susceptibility: in 34 out of 45 years there were at least four rainy episodes and of these 34, 28 had a mean temperature higher than 12° C. Therefore, in the Bordeaux area, the asynchrony between twisting rust and maritime pine, which results in a loss of inoculum potential,

Table 1. Disease incidence and weather data during the predicted lag period between the date of optimum germinability of *Melampsora pinitorqua* basidiospores and the occurrence of the B2 stage in maritime pines, Merignac, 1984–1993.

Year	DOG–B2 lag (days)	Rainy days	Rainy episodes	Mean temperature in rainy days (°C)	Inoculum potential*	Observed incidence*
1984	24	2	1	10.2	+	+
1985	13	1	1	11.7	+	?
1986	34	16	10	9.3	–	–
1987	10	3	3	15.9	–	–
1988	11	7	3	12.1	–	(+)
1989	8	6	3	10.3	–	–
1990	0	0	0		+	–
1991	12	3	2	9.5	(–)	–
1992	11	2	1	11.9	+	–
1993	12	5	1	10.3	+	+

* + = high, (+) = moderately high, (–) = moderately low, – = low

Table 2. Disease incidence and weather data during the predicted period of maritime pine susceptibility to *Melampsora. pinitorqua*, Merignac, 1984–1993.

Year	Inoculum potential *	Observed incidence *	B2–B5 period (days)	Rainy days	Rainy episodes	Mean temperature in rainy days (°C)
1984	+	+	35	17	8	12.3
1985	+	?	32	12	5	12.0
1986	–	–	28	6	5	15.0
1987	–	–	32	5	3	11.3
1988	–	(+)	30	13	8	15.2
1989	–	–	30	4	3	10.8
1990	+	–	34	12	5	10.3
1991	(–)	–	36	9	6	11.2
1992	+	–	26	3	3	14.3
1993	+	+	31	12	4	14.4

* + = high, (+) = moderately high, (–) = moderately low, – = low

could be more frequently a limiting factor for disease incidence than the weather conditions during the infection period. Consequently, factors inducing earlier development in pines are likely to be associated with increased rust incidence.

Although the good agreement between actual rust incidence and predicted variables may give some support to the significance of our models, more functional models, based on physiological processes, are needed to make predictions in changing environments (Haninen 1995).

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Genetic variation in *Cronartium flaccidum* and *Peridermium pini*

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Summary

Analysis of random amplified microsatellite (RAMS) markers of aecia from pine trees showing symptoms of resin top disease or cronartium rust suggested that genetic differentiation between pathogen populations is low, and that *Cronartium flaccidum* and *Peridermium pini* are very closely related, probably two forms of a single species. Most of the aecia showed no heterozygosity, but heterozygotic aecia were observed in two populations. To further analyse this, a sequence-specific marker was developed and used to analyze Finnish and other rust populations. The results showed heterozygosity in aecia of *C. flaccidum,* but not in those of *P. pini.*

Key words: cronartium rust, RAMS, resin top disease, specific marker

1 Introduction

Cronartium rust and resin top are among the most severe diseases on Scots pine (*Pinus sylvestris* L.). The diseases are caused by two separate pathogens: *Cronartium flaccidum* (Alb. et Schw.) Wint. and *Peridermium pini* (Pers.) Lév. emend Kleb. (= *Endocronartium pini* (Pers. emend Kleb.) Y. Hirat.), respectively. *Cronartium flaccidum* is a host-alternating and *P. pini* an autoecious species. Their taxonomy is relatively complicated, as some aeciospores morphologically similar to those of *P. pini* are capable of causing infections on alternate hosts (Gibbs *et al.* 1988, Kaitera *et al.* 1998b). The two pathogens are indistinguishable in their aecial stages, but they have been reported to differ in morphological and cytological characteristics of their aeciospore germ tubes (Hiratsuka 1968). In Great Britain morphological, cytological, and cultural variation within *P. pini* has been found (Gibbs *et al.* 1988, Pei and Gibbs 1991). The internal transcribed spacer regions of different *P. pini* populations and *C. flaccidum* have been shown to share the same alleles (Moricca *et al.* 1996).

In Finland more than 20% of the trees in single stands may be affected by resin top disease, and in such cases the loss of timber value may be up to 7% (Kaitera *et al.* 1994). The causative agent in Finland is mostly *P. pini*, although *C. flaccidum* occurs in Åland archipelago and also sporadically in

the mainland (Liro 1908, Kaitera *et al.* 1998a). The rust populations have been shown to vary genetically in Finland (Pappinen *et al.* 1996).

We studied genetic variation and heterozygosity among single aecia of resin top and cronartium rust fungi using the RAMS technique (Hantula *et al.* 1996) and a specific molecular marker. The original results of the RAMS analysis are given in Hantula *et al.* (1998).

2 Material and methods

2.1 Samples

Spores from single aecia collected in June and July were dusted into microcentrifuge tubes. DNA was isolated from aeciospores collected in 18 locations in Finland. For most populations (including all of those analyzed for allele frequencies) one aecium was selected randomly among several aecia within a single lesion per tree, and the selected trees were scattered a few hundred metres apart within each location. In Kesälahti, Hossa, Iinattijärvi, Jokela, Juomukuru, and Kolari, aecia were collected from two such stands located a few kilometers apart from each other. In addition we studied aecia of *C. flaccidum* from Italy and *P. pini* from Thetford, UK.

2.2 DNA techniques

DNA was isolated from aeciospores with a protocol including cell disruption, two phenol-chloroform (1:1) extractions, a chloroform:isoamyl alcohol (24:1) extraction, precipitation with alcohol (or polyethylene glycol), and drying.

PCR amplifications were carried out in reaction conditions recommended by the manufacturer of the Dynazyme II DNA-polymerase (Finnzymes Ltd., Finland). Three RAMS primers were applied. In RAMS analysis, the amplification products were separated by electrophoresis in agarose gels. The electrophoresis was run in TAE-buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA), and the amplification products were visualized using ethidium bromide under UV light. The lengths of the amplification products were estimated by comparing them to a 100 bp DNA ladder (Gibco BRL).

A specific primer was developed by cloning and sequencing a RAMS marker not used in the RAMS analysis. The primer was designated based on the obtained sequence. The analysis of the amplification product using the specific primer was carried out in constant denaturing gel electrophoresis (CDGE), and the visualization of bands was done as described above.

2.3 Analysis of locus/allele relationships

The allelic RAMS markers (e.g., markers encoded by single loci) were identified either by heteroduplex analysis or by restriction enzyme analysis using *Alu* I, *Cfo* I, *Hae* III, and/or *Msp* I enzymes.

2.4 Hierarchial analysis of genetic diversity

The analysis of molecular variance was carried out using the AMOVA software (Excoffier *et al.* 1992). The analysis used data from populations of Kesälahti, Hossa, Iinattijärvi, Jokela, Juomukuru, and Kolari. Data from ACA580, CCA980, CCA1300, GAAA590, and GAAA1250 loci were used. A similar analysis used the data obtained with the specific marker.

3 Results

3.1 Variation observed with RAMS primers

Amplification with ACA-primer and GAAA-primer each resulted in three major and several minor bands. In the area of 1250 bp, three different markers were observed and shown to be of the same locus by heteroduplex analysis. In this GAAA1250 locus, both hetero- and homozygotic aecia were observed. Also, in the GAAA590 locus, heterozygotic aecia were observed. Amplification with CCA-primer produced a relatively complex pattern with five polymorphic markers.

3.1.1 Distribution of alleles in populations

The aecia studied represented 31 different genotypes based on the examined loci. The most common genotype was present in 43 aecia, whereas 19 genotypes were represented by single aecia.

The alleles were relatively evenly distributed in populations (not shown). All common alleles were observed throughout the studied area. Hierarchial analysis of genetic diversity indicated that approximately 80% of total genetic diversity was due to variation within the stands. Only 7.5% of the diversity was due to variation among closely related stands, whereas a somewhat higher proportion of variation (12%) was due to variation among distantly located populations.

3.1.2 Heterozygosity

In this study, heterozygotes were observed relatively rarely in GAAA1250 and GAAA590, and not at all in CCA980, CCA620, CCA600, or ACA580 loci. CCA1250 was difficult to interpret for heterozygosity and therefore was not considered in comparisons of heterozygosity. In GAAA1250 and GAAA590 loci, most heterozygotes were observed among aecia collected in Juomukuru and Kolari in northern Finland (in RAMS analysis, single aecia from Åland or Italy were not used).

3.1.3 Comparison of Finnish isolates to *C. flaccidum* and *P. pini* control samples

In order to determine the species of the Finnish rust samples, RAMS patterns were compared among a single aecium from Great Britain (Thetford) and a sample of mixed aecia from Italy, presumed to represent *P. pini* and *C. flaccidum*, respectively, and aecia from Finnish rust populations known to be *P. pini* based on inoculation experiments on alternate hosts (Kaitera *et al.* 1998a). However, the amplification markers were similar, and therefore no species identification (based on species specific markers) could be made.

3.2 Analysis of variation observed with a specific marker

Using a specific primer, 15 alleles were observed among the aecia studied. Of 10 alleles found among *C. flaccidum* aecia and 13 alleles found among *P. pini* aecia, eight were found in both species. The analysis of molecular variance indicated a low level of genetic differentiation, and heterozygotic aecia were observed only in areas where *C. flaccidum* occurs, e.g., in Italy, Åland, Juomukuru, and Kolari.

4 Discussion

In this investigation, genetic variation and differentiation among *P. pini* and *C. flaccidum* populations were studied. RAMS analysis indicated that variation in most amplified markers was evenly distributed in the studied area. Thus, genetic differentiation between the populations was low.

A thorough inspection of heterozygosity among aecia showed that heterozygotic aecia were observed only where *C. flaccidum* occurs. This indicates that the two fungi differ in their life cycle so that the heterozygotic condition in aecia is obtained only when part of the the life cycle occurs on the alternate host.

The similarity of RAMS markers among all aecia, including *P. pini* and *C. flaccidum* controls, suggests that the causative agents of the resin top and cronartium rust diseases are genetically very closely related, probably two forms of the same species. This is also supported by the observation of variation in ITS sequences (Moricca *et al.* 1996).

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The genetic variation within cankers of *Peridermium pini*

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Summary

The genetic composition of aecia of five perennial pine stem rust cankers was studied by screening SCAR markers with DGGE. Four cankers, identified as *Peridermium pini*, were each homogenic and contained single characteristic marker alleles, indicating that the fungus genotypes were homozygotic. One canker sampled from a site where *Cronartium flaccidum* has been identified was homogenic but heterozygotic. The homogeneity of the studied cankers indicated that the mechanism of genetic exchange via spermatia is not common. Recombination between genotypes in a canker could not be excluded by the observation of genetic structure, since selfing would create homogeneity and homozygosity. Since these fungi share the same alleles, it is possible that *P. pini* is an asexual mutant form of *C. flaccidum*.

Key words: *Cronartium flaccidum*, homogeneity, PCR, RAMS

1 Introduction

The mechanism of pairing between two *Cronartium* rust strains is spermatization within cankers, in which germinating spermatia permit plasmogamy with spermogonia to produce dikaryotic mycelium and aeciospores. There is some evidence that insects have a role in cross-fertilization of the macrocyclic *Cronartium ribicola* J.C. Fisch. by spreading spermatia within and between cankers (Hunt 1985). This hypothesis is further supported by the fact that cankers of *C. ribicola* contain several genotypes, and most of the genetic variation can be found within cankers and within sites (Hamelin 1996). On cankers with monomorphic spermatia, 75% of the aecia were a result of outcrossing, which indicates that *C. ribicola* has sexual recombination within cankers via spermatization. Moricca *et al.* (1996) suggested that several genotypes occur within canker samples of the macrocyclic *Cronartium flaccidum* Alb. et Schw. Thus, it appears that within an actively breeding population of a pine stem rust fungus, perennial cankers that produce spores successfully, contain several genotypes as a result of outcrossing.

The genetic composition of cankers is clearly one of the key factors in characterization of the sexuality of the *C. flaccidum-Peridermium pini* (Pers.) Lev. complex. Differences between the life cycles of *Cronartium* or *Peridermium* are still poorly described (i.e. Hiratsuka 1968, 1969, Laundon 1976, Epstein and Buurlage 1988, Gibbs *et al.* 1988, Vogler *et al.* 1997). If there are genetic differences among the aecia of *P. pini* within a canker, it is likely that the fungus recombines sexually within or between cankers.

Therefore, genetic variation within cankers and the possible hybrids between aecial genotypes of *P. pini* cankers could be considered as experimental evidence of a sexual life cycle. By using codominant DNA markers, one should be able to detect whether the different genotypes are results of recombination events between haploid branches of germ tubes from different aeciospores in a canker or of several infections without recombination, and to create a spatial map of the fungal genotypes in a canker. This information could be used to analyze whether the genetic composition of a canker is based on meiosis in the aecial germ tubes. Furthermore, aeciospores of *Cronartium quercuum* (Berk.) Miy. ex Shirai f. sp. *fusiforme* obtained from single cankers showed variation in pathogenicity (Kuhlman and Matthews 1993). Genetic variation among aecia in a single canker means that any variation that is studied between rust origins must also be determined within cankers. The aim of this study was to determine if the aecia of an individual canker are genetically different and to map the distribution of genotypes within a canker.

2 Material and methods

2.1 The fungal samples

Five perennial pine stem rust cankers from Finland were sampled, a map was drawn over the aecia collection points within each canker (Fig. 1), and 25–50 unruptured aecia per canker were collected (Table 1). The four cankers from eastern Finland (Lehmo and Kesälahti, abbreviations L1, L2, K1, and K2, respectively) were tentatively identified as *P. pini*, since no known alternate hosts were found within the collection sites. Northern samples (Juomukuru, J) are of an origin where macrocyclic spore stages of *Cronartium flaccidum* exist (J. Kaitera, pers. comm.). Previous studies of genetic variation within these stands show clearly that there are several fungal haplotypes present in the stands and even within individual pine trees (Hantula *et al.* 1998, Pappinen *et al.* 1996). Cankers L1, L2, K1, and K2 were perennial and also produced plentiful aecia during the previous (1995) and the following summer (1997). Because Vogler *et al.* (1997) observed that aeciospores of homozygous *Peridermium harknessii* J.P. Moore strains are predominantly binucleate, cankers L1, L2, K1, and K2 were examined and confirmed to contain dikaryotic aeciospores by fluorescence microscopy (R. Kasanen, unpubl.).

2.2 Marker design

Two polymorphic DNA markers were chosen by screening random amplified microsatellite (RAMS, Hantula *et al.* 1996) fingerprints for polymorphic marker sites. Markers Pp1 and Pp2 were converted into codominant sequence characterized amplified region (SCAR) markers; individual fingerprints containing several markers were cloned into a pCR 2.1 plasmid with Original TA Cloning Kit (Invitrogen). Transformants were PCR-screened with the RAMS primer used in fingerprinting. The plasmid DNA containing the marker was further isolated with miniprep columns (Qiagen). The sequencing was done with ABI Prism DNA Sequencers at the Institute of Biotechnology,

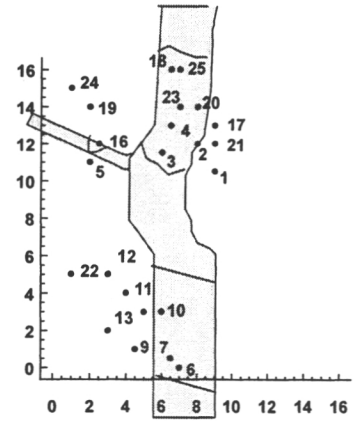

Figure 1. Example of a canker map. Dots with numbers represent collected aecia. As distance (cm) is measured along the bark surface, some dots are marked outside the stem.

Table 1. Collection data for aecial samples from single cankers.

Canker/ collector	Number of collected aecia/canker	Identification	Alleles present
Lehmo 1 R. Kasanen	30	No known alternate hosts were found: *P. pini*	L1
Lehmo 2 R. K.	30	"	L2
Kesälahti 1 R. K.	38	"	K1
Kesälahti 2 R. K.	25	"	K2
Juomukuru J. Kaitera	50	Collected from population which infects alternate hosts: *C. flaccidum* or *P. pini*	L2, K1

University of Helsinki and the A.I. Virtanen Institute, University of Kuopio. A pair of primers was designed in order to amplify each marker allele. The primer sequences were:

Pp1a 5'- CCCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGCCGGTGTATGATTGCTATGAT
Pp1b 5'- GTACCAGGCCAAAGG
Pp2/3 5'- CCCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGCTGGGTCAAGTCAAATCTCC
Pp2/2b 5'-GCCTTCGTCTCTGTCGTCTC

A 50 µL PCR reaction consisted of 2 µM of each primer, 0.2 mM dNTPs and 2U Dynazyme DNA Polymerase (Finnzymes Ltd, Finland). The PCR was started with denaturation of DNA template and primers for 10 min, followed by 39 cycles of 1 min denaturation, 45 sec at 50° C for Pp1 marker (or 54° C for Pp2) and 2 min at 72° C. The alleles of the marker loci were detected with denaturing gradient gel electrophoresis (DGGE), using a DGENE apparatus

(Bio-Rad). The separation of DGGE is based on differences in melting behaviour of DNA fragments (Fischer and Lerman 1983). PCR products were run with 70 V for 18 h at 58° C in 7.5% polyacrylamide gels containing 30% (or a gradient of 20–40%) denaturing solution (100% denaturing solution is a mixture of 40% formamide and 7 M urea).

3 Results

Based on the Pp1 marker, cankers K1, K2, L1, and L2 were homogenic, and each contained only one marker allele (Fig. 2b), indicating that the fungus genotype was homozygotic. In contrast, canker J was also homogenic, but contained two alleles, suggesting heterozygosity. Each canker had a different allele, except the alleles in canker J seem to have same mobility as alleles in cankers L2 and K1 (Fig. 2a). Presently, analysis of the Pp2 marker is in process. Preliminary results seem to support the identification of different fungal genotypes within each of the studied cankers since three different alleles have been found by amplifying DNA of single aecia from five cankers.

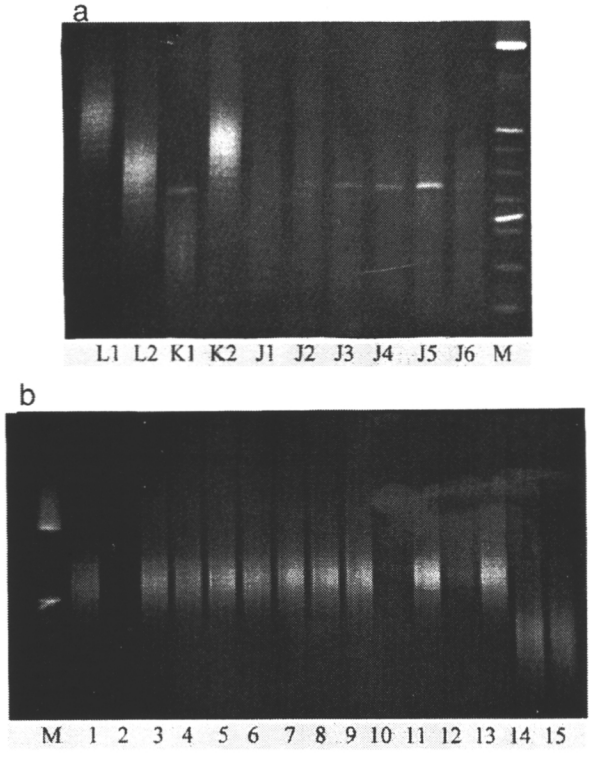

Figure 2. PCR products run in 7.5% polyacrylamide gels containing a gradient of 20–40% denaturing solution. a) Four marker alleles. Cankers are denoted as L1, L2, K1, K2, J. J1...J6 are individual aecia from canker J. M = DNA size standard; b) Example of canker homogeneity. Lanes 1–13 are aecia collected from K1 and 14–15 from K2 canker. M = DNA size standard.

4 Discussion

The interpretation of genetic homogeneity relies on polymorphism of the Pp1 marker. The same marker has been run on a larger data set of 150 aecia from 13 Finnish and Italian populations. With the Pp1 marker, 15 alleles were observed, resulting in 120 theoretically possible genotypes because of codominance (J. Hantula, pers. comm.). Further support for the resolution of individual fungal genotypes is that the distance between cankers L1 and L2, as well as K1 and K2, is less than 25 meters, and these cankers contained different fungus genotypes. The observed homozygosity is not a sampling artifact since heterozygotes at the Pp1 marker locus were not observed in the larger data set (J. Hantula, pers. comm.). Also, earlier studies with RAMS markers support this observation (Hantula *et al.* 1998).

If meiosis as described by Hiratsuka (1968) takes place within a germ tube, a dikaryotic, heterozygotic aeciospore should carry both alleles into one canker. Therefore, an aeciospore has three ways to produce a homogenic and homozygous canker: 1) the infecting aeciospore is a homozygote, 2) the germ tube branches containing one of the two alleles does not infect or grow successfully or 3) the genes get fixed later by some other process. Indeed, the obvious result of mitotic germination and growth as described by Epstein and Buurlage (1988) would be a homogenic and homozygous canker. However, meiosis can not be excluded only by the observation of canker homogeneity nor homozygosity since selfing of a homothallic fungus would be the fastest way to create homogeneity in perennial cankers and increase homozygosity in a population. It can be concluded that if the nuclei of *P. pini* go through meiosis, the cause of canker homogeneity and homozygosity would be excessive homozygosity in a population, which could be achieved by homothallism. Whether the nuclear cycle of a germ tube is mitotic or not, it is clear that if spermatia were functional, additional genotypes would be observed in perennial cankers as in the cases of *C. ribicola* and *C. flaccidum* (Hamelin 1996, Moricca *et al.* 1996). If the canker is a result of several infections originating from a polymorphic fungal population such as the studied ones, there would be a higher probability of finding several genotypes.

The homogeneity of the studied cankers indicated that the mechanism of genetic exchange via spermatia is not common. Although spermatia of some *Cronartium* spp. are functional, their ability to spread and cross-fertilize without insect transfer seem to be limited (Hunt 1985). Therefore, observed homogeneity can also be partly due to lack of suitable insect transfer. Hunt (1985) also observed that cross-fertilization of *C. ribicola* increased aecial production, and isolated cankers failed to produce aecia. If this is also the case in *P. pini*, the lack of cross-fertilization with insects could be an important factor regulating epidemics.

Since all heterozygous samples in the previously mentioned larger dataset have been identified as *C. flaccidum*, and heterozygotic *P. pini* cankers have not been found in this nor other studies (Hantula, pers. comm) detection of a heterozygotic, homogenic canker was unexpected. If a haploid basidiospore infects and produces a canker, it should contain only one allele. The role of spermatia can again be questioned; if the heterozygote is formed via spermatial crossing, parental genotypes should also be present in nearby

cankers, as was detected with *C. ribicola* (Hamelin 1996). The only way for two haploid hyphae to achieve the heterozygotic nuclear status is by anastomosis or a similar process. Since this observation is based on only one canker, more *C. flaccidum* data is needed to answer the question of whether such cankers are usually homogenic.

The similar mobility of alleles in *P. pini* and *C. flaccidum* samples raises the question of whether these fungi share the same alleles, since DGGE is a very sensitive method of screening for mutations. If several alleles were identical by descent, the only difference between these two types of pine stem rust would be zygosity or ploidy level. Other characters support the close relatedness. The ultrastructure of *P. pini* hyphae was studied by Walles (1974). Longo (1982) compared these results with the ultrastructure of *C. flaccidum*, and was not able to find any differences. Also, the spore surface of these species is indistinguishable (Kasanen 1997). The IGS and ITS regions of the nuclear DNA of this species complex are closely related (Moricca *et al.* 1996). As *P. pini* populations are also polymorphic (Pappinen *et al.* 1996, Hantula *et al.* 1998), it is unlikely that the fungus has only asexual, clonal reproduction. Since *P. pini* and *C. flaccidum* exist within the same sites (J. Kaitera, pers. comm.), it is possible that *P. pini* is an asexual mutant form of the sexual, macrocyclic *C. flaccidum*.

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Occurrence and significance of *Cladosporium tenuissimum* on two-needle pine rust aeciospores

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Summary

The rust hyperparasite *Cladosporium tenuissimum* was detected and identified using DNA-based techniques and classical methods from aeciospore samples collected at different pine-growing sites in Europe. Its antagonistic effect on the viability of rust propagula was tested on solid medium and in storage conditions. Conidia of the hyperparasite significantly reduced germination of spores of the two-needle pine rusts *Cronartium flaccidum* and *Peridermium pini*. Rust aeciospores mixed with conidia of the mycoparasite and stored at various temperatures exhibited low viability and often appeared disintegrated. Scanning electron microscopy revealed the ability of the hyperparasite to exert a mechanical force that allowed it to penetrate and proliferate within rust aeciospores, with or without the formation of appressoria. The apparent dissolution of the host cell wall when it came in contact with the mycelium of the hyperparasite also suggested enzymatic action. Although there is no evidence that *C. tenuissimum* affects the survival of rust fungi in their natural habitat, the investigations suggest that the role of the hyperparasite in the biological control of these rusts is potentially significant.

Key words: biological control, *Cronartium flaccidum*, *Peridermium pini*

1 Introduction

Rusts of hard pines caused by the heteroecious *Cronartium flaccidum* (Alb. et Schwein.) Wint. and by the autoecious *Peridermium pini* (Pers.) Lév. (≡ *Endocronartium pini* (Pers.) Y. Hirat.) (Kuprevicz and Tranzschel 1957, Gäumann 1959, Hiratsuka 1969) are among the most serious and destructive diseases of pines in Europe. *C. flaccidum* occurs on a number of aecial and telial hosts in a very wide range throughout Eurasia. It causes recurrent outbreaks in areas where both the primary and the secondary hosts are present, and where climatic conditions are favorable to the production and dispersal of the different spore types. *P. pini* is restricted to pine, specifically Scots pine (*Pinus sylvestris* L.), in a reduced life cycle. The ability of this rust to spread directly from pine to pine allows rapid intensification of outbreaks under favorable environmental conditions.

The most effective control strategy of pine rusts has hitherto mostly relied on the selective breeding of resistant pines, but the inefficacy of breeding programs for resistance to two-needle pine rust has generated a demand for alternative control strategies. An approach which has recently shown promise to forest pathologists is the use of hyperparasites to control the pine rusts (Tsuneda and Hiratsuka 1979, Ayer *et al.* 1980, Pickard *et al.* 1983, Hiratsuka 1991, Chakravarty and Hiratsuka 1995, Currie *et al.* 1995). A number of fungi have been found associated with conifer rusts. They have been reported to be parasites on the rust fungi, common saprophytes, or unrelated parasites which compete with the obligate parasites for host tissue colonization (Hubert 1935, Myren 1964, Peterson and Jewell 1968, Byler and Cobb 1969, Powell 1971).

The identity of most fungi associated with rust pustules and the nature of these associations are less known. Several of these nonrust fungi, however, have already been identified and investigated as potential control agents. Among these, a number of *Cladosporium* species have been reported to be hyperparasites of various spore states of rust fungi (Smith 1905, Keener 1954, Bolland 1973, McKenzie and Hudson 1976, Heather and Sharma 1977, Sharma and Heather 1978, Tsuneda and Hiratsuka 1979, Srivastava *et al.* 1985). We describe here the molecular and conventional procedures which have permitted the detection and positive identification of *Cladosporium tenuissimum* Cooke from field-contaminated aeciospores, and the assays we have developed to investigate its mode of parasitism. We chose this fungus for particular scrutiny among the mycoflora found associated with two-needle pine stem rusts because i) it is already reported to be a mycoparasite on the poplar rust *Melampsora larici-populina* Kleb. and ii) it has been isolated with high frequency from contaminated rust aeciospores.

2 Material and methods

2.1 Organisms

Aeciospores were collected over a three-year period from pustules of rust-infected pine shoots and stems at 13 sites in England, Finland, Greece, Italy, and Scotland (Table 1). Inoculum was collected with a cyclone collector, which sucks in spores and automatically deposits them in small gel capsules, or it was scraped from the aecia with a sterile spatula and placed in 0.5 mL Eppendorf tubes. The capsules/tubes were wrapped in aluminium foil to avoid light damage and stored at –20° C until use. The inoculum from each rust provenance was either used for molecular assays or seeded in a nutrient medium to obtain the growth of contaminants.

2.2 Molecular investigations

2.2.1 DNA preparation

Total genomic DNA was extracted using a modified version of the method of Lee and Taylor (1990). Aeciospore samples were crushed between two glass slides rendered water-repellent by pre-treatment with dimethyl-dichloro-silane at 180° C. The resulting homogenate was taken up into a micropipette tip in 50 µL of extraction buffer (0.089 M Tris base; 0.045 M boric acid; 0.05 µM EDTA; 1% β-mercaptoethanol), incubated at 75° C for 15 min,

resuspended by vortexing briefly, and then centrifuged to pellet cell debris. The amount of genomic DNA present in the supernatant was quantified using a TKO-100 Hoefer fluorometer (Hoefer Scientific Instruments, San Francisco, CA) and adjusted to 10 ng/µL in a TE buffer.

2.2.2 PCR amplification

Polymerase chain reactions were carried out in 0.5 mL microcentrifuge tubes, each with a total volume of 100 µL and containing 70 µM of dATP, dCTP, dGTP, and dTTP, 0.8 µM of each primer, 2 units of Taq DNA polymerase (Pharmacia), and 100 ng of DNA template in a PCR buffer (10 mM Tris-HCl; 50 mM KCl; 1.5 mM MgCl; 0.001% gelatin), using a Hybaid Omnigene temperature cycler (Hybaid, Middlesex, UK). Primers were supplied by Pharmacia LKB (Uppsala, Sweden). The temperature cycling conditions were: denaturation at 94° C for 5 min during the first cycle and for 1 min in subsequent cycles, primer annealing at 55° C for 1 min, and primer extension at 72° C for 3 min, with a total of 35 cycles; and a final extension at 72° C for 8 min.

Nested PCR assays were carried out to amplify the DNA of the pine rusts and their associated mycoflora. The ribosomal Internal Transcribed Spacer region (ITS) was amplified using the primer pair

ITS1 (5'- TCCGTAGGTGAACCTGCGG), and

ITS4 (5'- TCCTCCGCTTATTGATATGC) (White *et al.* 1990).

The PCR products obtained with the first amplification were diluted (1/40) with sterile deionized water and used as template DNA for a subsequent series of 35 PCR cycles using the primer pair

ITS3 (5'-GCATCGATGAAGAACGCAGC), and

ITS4 (White *et al.* 1990),

or the universal primer ITS4 and a primer specific for the two rusts, developed at our laboratory after sequencing the 5.8S gene of *C. flaccidum* and named

Rust3 (5'-ACATCGATGAAGAACACAGT) (Moricca *et al.* 1996).

Results of the nested PCR were checked by running 5 µL of the PCR products at 20 Volts for 15–16 h on 3-mm wide 3% Metaphor gel (Sigma).

Table 1. Aeciospore samples from two-needle pine rust provenances tested for the presence of the hyperparasite *Coleosporium tenuissimum* using both traditional and DNA-based assays.

Fungus	Host pine	Locality	Region	Country	Hyperparasite isolation	Supplier
C. flaccidum	*P. sylvestris*	Naz	Trentino A. Adige	Italy	+	S. Moricca
C. flaccidum	*P. pinea*	San Vincenzo	Tuscany	Italy	+	A. Ragazzi
C. flaccidum	*P. halepensis*	Mercadante	Apulia	Italy	+	N. Luisi
C. flaccidum	*P. halepensis*	Pulicchie	Apulia	Italy	+	N. Luisi
C. flaccidum	*P. pinea*	San Rossore	Tuscany	Italy	+	A. Ragazzi
C. flaccidum	*P. laricio*	San Gregorio	Campania	Italy	+	S. Moricca
C. flaccidum	*P. pinaster*	Monticiano	Tuscany	Italy	+	S. Moricca
C. flaccidum	*P. nigra*	Monte Vosgi	Tuscany	Italy	+	S. Moricca
P. pini	*P. sylvestris*	Thetford	East Anglia	England	+	K. Mitchelson
P. pini	*P. sylvestris*	Jokela	Rovaniemi	Finland	–	J. Kaitera
P. pini	*P. sylvestris*	Iinattijärvi	Pudasjärvi	Finland	–	J. Kaitera
P. pini	*P. sylvestris*	Mount Pieria	Macedonia	Greece	+	S. Diamandis
P. pini	*P. sylvestris*	Altyre	Morayshire	Scotland	–	K. Mitchelson

2.2.3 DNA sequencing

Products of amplified ITS and ITS2 regions were purified with a Prep-A-Gene kit (BioRad, Richmond, CA), and sequenced by the dideoxy method (Sanger *et al.* 1977) using a Sequenase 2.0 kit (USB, United States Biochemical Corporation, Cleveland, OH) and the primers ITS1, 2, 3 and 4 (White *et al.* 1990) for the ITS region, or the primers Rust3 and ITS4 for the ITS2 region. Strands were sequenced in both directions. PCR products were radiolabelled with ^{35}S following supplier's instructions. Sequence reactions were run for approximately 3 h on 5% polyacrylamide wedge gels in a TBE buffer. Gels were fixed, vacuum dried, and exposed to Kodak SB X-ray film for varying periods.

2.3 Growth of associated mycoflora

Aeciospores from each of the 13 rust provenances were seeded on 9-cm plastic petri dishes filled with 20 mL potato dextrose agar (PDA). Rusts are unable to grow on a such a simplified medium, but several fungal contaminants developed after a few days of incubation at 21° C. They were isolated in purity and identified as fungi belonging to the genera *Aspergillus* spp., *Penicillium* spp., and *Cladosporium* spp. The species of *Cladosporium* were subsequently identified at the Centraalbureau voor Schimmelcultures (CBS). Fungi known as common contaminants such as *Aspergillus* spp., *Penicillium* spp., and *Cladosporium cladosporioides* (Fresen.) de Vries were not considered. *Cladosporium tenuissimum* was successfully isolated from Thetford (England), Mount Pieria (Greece) and all the Italian rust provenances (Table 1) and was used for the antagonism assays.

2.4 Antagonism investigations

The antagonistic capabilities of the mycoparasite were investigated on one rust provenance from each country where the fungus was isolated: the English provenance Thetford (TH), the Greek provenance Mount Pieria (MP) and the Italian provenance Monticiano (MC). Prior to the experiments, the viability of the rust propagula was assessed using subsamples of 24 h rehydrated aeciospores.

2.4.1 Interaction between *C. tenuissimum* and rusts on solid medium

To obtain conidia for the *in vitro* antagonism assays, *C. tenuissimum* colonies were grown at 21° C in the dark. Conidia were harvested after incubation for about 25 days in sterile distilled water. Sterilized glass slides covered with a thin film of 2% water agar were inoculated with aeciospores of each rust and with 25 µL aliquots of conidial suspensions of *C. tenuissimum* (10^6 conidia/ mL of distilled water). To determine the optimum antagonistic effect, three treatments were used in the incubation tests: 1) inoculation with the parasite one hour before the saprophyte; 2) inoculation with the parasite one hour after the saprophyte; 3) inoculation with the parasite and saprophyte simultaneously. The inoculated slides were placed in humidity chambers at 21° C in the dark, and spore germination was checked under a Zeiss Lab microscope (x400) at 12, 18, and 24 h after inoculation. For the controls, rust aeciospores were incubated in 25 µL sterile water under the same conditions.

2.4.2 Effect of *C. tenuissimum* on the viability of rust spores in storage

Batches of 25 mg of aeciospores from each rust provenance were desiccated for 24 h at 5° C, and then inoculated with conidial suspensions (10^6 conidia/mL of distilled water) of the mycoparasite and incubated at –20° C, 4° C, and 20° C. An equal amount of spores from each rust provenance was inoculated in 25 µL sterile water as a control. The mixtures were put in 0.5 ml Eppendorf tubes, vortexed briefly to obtain a uniform mixture, and incubated at the three temperature regimes. The viability of the aeciospores was assessed on subsamples of these mixtures after 1, 11, 21, and 31 days. At each sampling, a small amount (2–3 mg) of the mixture was removed from the tubes with a sterile dissecting needle, placed on a sterilized glass slide covered with water agar and incubated in a humidity chamber represented by a glass petri dish (19 cm diam.) lined with water-saturated absorbent paper on the bottom to maintain moist conditions. After about a 5 h incubation period at room temperature (16–20° C) each slide was mounted with a drop of KOH and a cover slip. Spore germination was recorded again under a Zeiss Lab microscope (x400).

2.4.3 Ultrastructural analysis of host–parasite interaction

The inoculated Petri dishes from the first antagonism assay were used to investigate the host–parasite interface. Small square pieces of agar medium were cut out aseptically and prepared for SEM examination. Agar fragments were fixed in a 3% glutaraldehyde solution for 1 h, rinsed once in a phosphate buffer (pH 7.3) for 5 min, immersed in 1% OsO_4 in a phosphate buffer (pH 7.3) at room temperature for 1 h, rinsed three times in the same buffer for 5 min, and dried with a Bal-Tec 030 critical point drier in liquid CO_2. Samples were sputtured with gold palladium film using a S105A Edward sputter coater and examined with a Philips 505 scanning electron microscope at 20 kV.

3 Results

PCR amplifications of the ITS region in all rust provenances gave products of the expected size: roughly 650 bp, including the primer annealing sites and small terminal portions of 18S and 28S ribosomal genes. Electrophoretic migration of the products of nested-PCR on Metaphor gel revealed that the rust DNA was mixed with contaminant DNAs, whose molecular sizes were slightly different. Excision of these bands from the gel, separate amplification, and subsequent electrophoresis of the individual PCR products showed that the rust DNAs were contaminated by more than one extraneous DNA of a smaller size (Fig. 1). Sequencing of these fragments and a GenBank search for the greatest homologies conclusively demonstrated the presence of extraneous DNAs mixed in with the DNA of the two obligate parasites: a larger band of approximately 270 bp (Fig. 1, lane 3) displayed high identity with rusts of the genera *Cronartium* and *Puccinia*, the slightly smaller bands of approximately 255 bp had substantial sequence homology with species of *Cladosporium* (Fig. 1, lane 5–6), and the smallest bands (Fig. 1, lanes 2 and 4) revealed considerable sequence similarity with *Penicillium* spp.

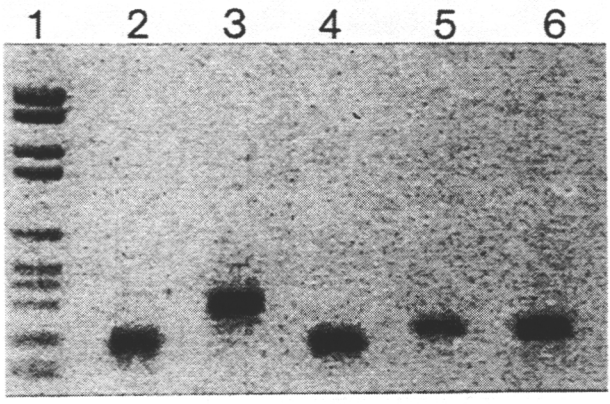

Figure 1. Ethidium-bromide stained agarose gel electrophoresis showing results of separate amplification of individual DNA bands excised from lanes with multiple bands containing rust DNA and contaminant DNA. Lane 1 is a molecular weight marker (marker VI, Boehringer) and lane 3 represents rust DNA; all the other lanes are PCR products from contaminant DNAs: *Penicillium* spp. (lanes 2 and 4) and *Cladosporium* spp. (lanes 5 and 6).

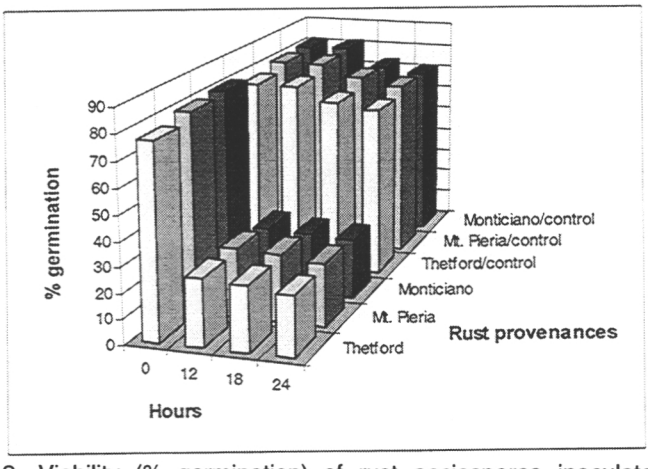

Figure 2. Viability (% germination) of rust aeciospores inoculated with conidia of *Cladosporium tenuissimum* in a humidity chamber at 21° C in the dark. The hyperparasite was inoculated 1 h before the rust fungi. Values represent the means of 100 spore counts.

The *in vitro* growth of fungal contaminants confirmed that the batches of rust aeciospores were heavily contaminated by *Cladosporium* spp. The known hyperparasite *C. tenuissimum* was then investigated for its antagonistic effect towards the rust spores.

Aeciospore germination was significantly reduced by conidia of *C. tenuissimum*. The maximum inhibitory effect was observed when conidia were inoculated one hour before the rust spores on solid medium (Fig. 2). The inhibitory effect was less marked when the conidia were inoculated at the same time as the rusts, and was still less when they were inoculated one hour

after the rusts. The three *C. tenuissimum* strains were equally parasitic on all the three rust provenances tested, with no difference in antagonistic effect between *C. tenuissimum* strains, nor any interaction between the source of *C. tenuissimum* and the country of origin of the rust, thereby excluding the possibility of host-specificity. Inhibition of rust spore germination was noted from the first inspection (12 hours after incubation) and reached a peak after 24 hours.

The germination ability (viability) of spores stored in mixtures with conidia of the hyperparasite consistently decreased with increasing storage temperature. Viability also decreased with increasing storage time (Fig. 3) so that inhibition of germination was greatest after 31 days incubation at 20° C. Storage at –20° C, on the other hand, preserved aeciospores from being parasitized by the mycoparasite, at least in the first days of mixed storage.

Microscopic examination confirmed the hyperparasitic nature of *C. tenuissimum*. It was strongly attracted to its host, producing long, straight hyphae which grew towards the rust spores and became attached to them. Therefore, the initial events of parasitization were the direct growth of the parasite hyphae onto the host surface and their adhesion to the rust spores. The parasite hyphae often coiled around the host spores, setting up an intimate relation with them (Fig. 4). The penetration of the spores by *C. tenuissimum* was also common. The fungus seemed to be able to penetrate directly, without forming infection structures but only as a result of mechanical pressure exerted on the host cell wall or through the formation of appressoria (Fig. 5). Once it had penetrated, the mycoparasite proliferated within and above the rust aeciospores (Fig. 6).

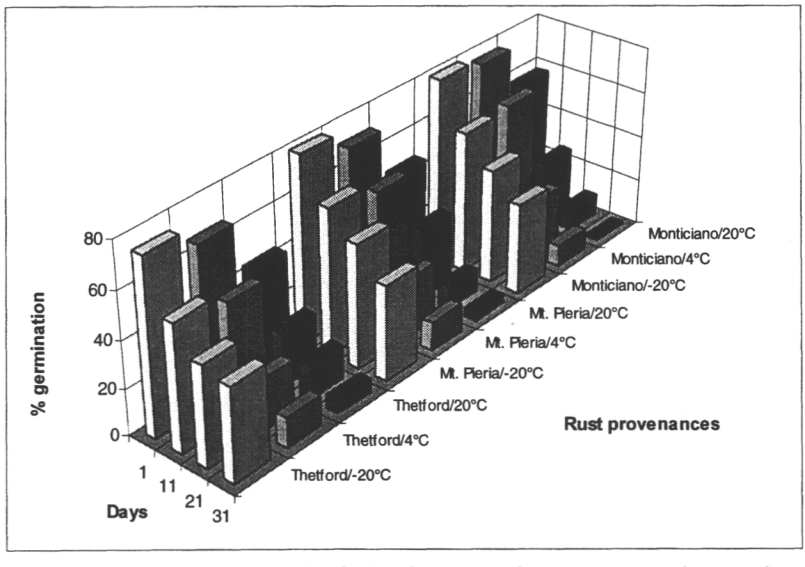

Figure 3. Viability (% germination) of rust aeciospores stored at various temperatures in mixtures with conidia of *Cladosporium tenuissimum* over a rather extended period. Values represent the means of 100 spore counts.

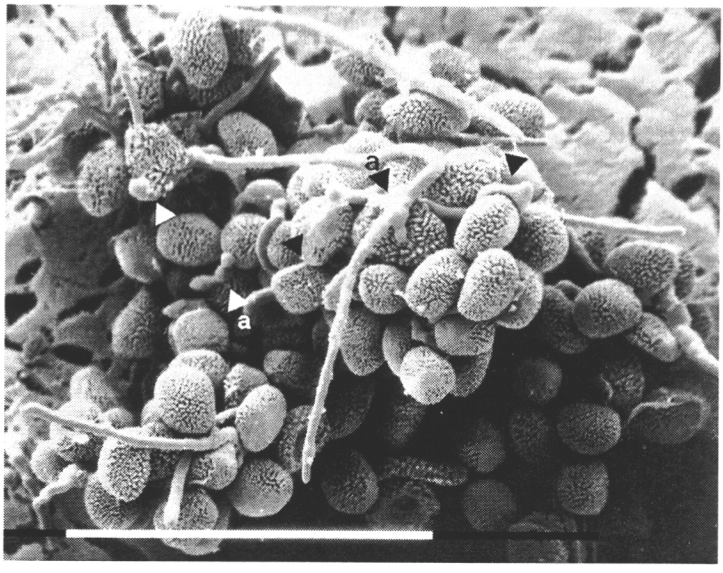

Figure 4. Scanning electron micrograph showing a group of aeciospores parasitised by *Cladosporium tenuissimum*. The mycelium of the hyperparasite grows appressed to the rust spores, coiling around them (arrows) and forming numerous appressoria (a). Bar scale = 100 μm.

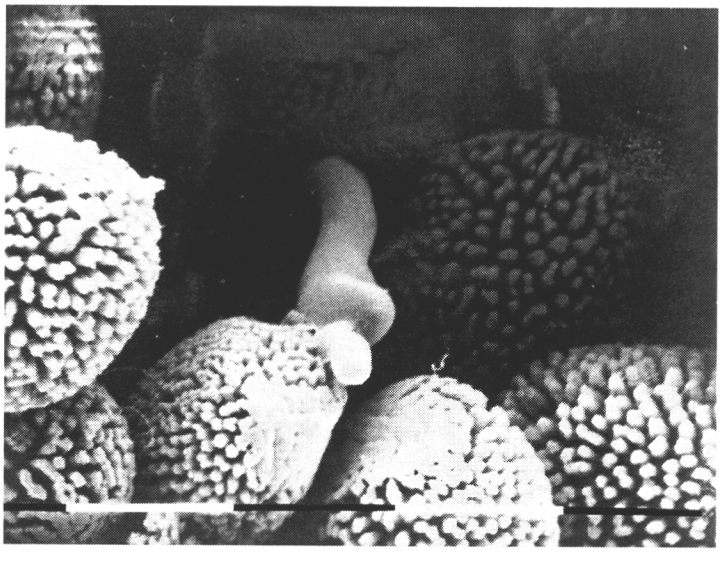

Figure 5. Scanning electron micrograph showing parasitisation of a rust spore by a hypha of *Cladosporium tenuissimum*. The hyperparasite adheres closely to the spore surface and forms an appressorium. Bar scale = 10 μm.

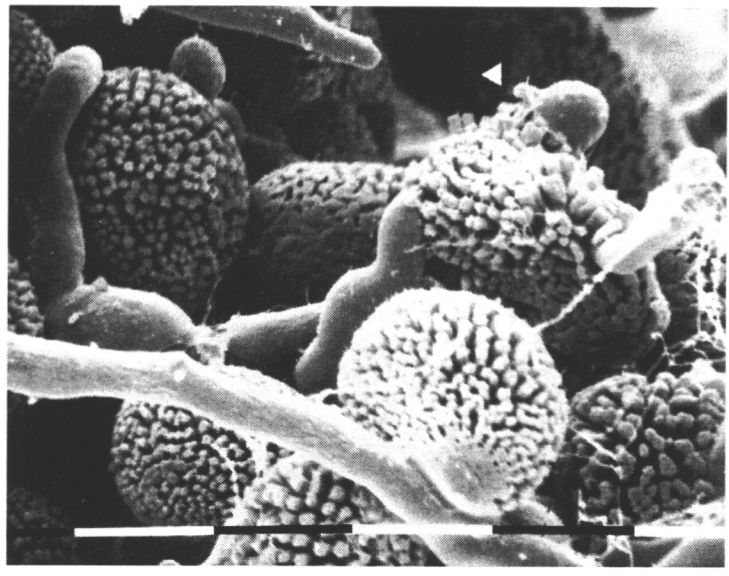

Figure 6. Scanning electron micrograph showing hyphae of *Cladosporium tenuissimum* which parasitise rust spores. Penetration of a rust spore by a hyphae of the hyperparasite, which swells the rust verrucae (arrow), is evident. The fungus proliferates within the spore and a conidiophore emerges from the spore carrying with it some verrucae on the apex. Secretion of an amorphous film consisting of a fibrous material is clearly visible. Bar scale = 10 μm.

4 Discussion

Molecular investigations focused attention on *C. tenuissimum* as a potential hyperparasite. The versatility of the PCR technique and a sequence database search made it possible to ascertain that the DNA of *C. flaccidum* and *P. pini* was heavily contaminated with the DNAs of various extraneous fungi, some of which belonged to the genus *Cladosporium*. Traditional identification of the mycoflora associated with the rust spores confirmed the molecular investigations and made it possible to identify one of the species of *Cladosporium* associated with the spores of the two-needle pine rusts as being indeed *C. tenuissimum*.

The large, heterogeneous genus *Cladosporium* comprises hundreds of species. While many taxa within this genus are common, plurivorous saprophytes, a number of *Cladosporium* species have been reported to be mycoparasites of rust fungi. These include *C. aecidiicola* Thüm., *C. gallicola* Sutton, *C. hemileiae* Steyaert, *C. tenuissimum* Cooke, *C. uredinicola* Speg., and *C. uredinophilum* Speg. (Smith 1905, Keener 1954, Bolland 1973, McKenzie and Hudson 1976, Heather and Sharma 1977, Sharma and Heather 1978).

Most of these *Cladosporium* species are parasitic on rusts of forest trees. Sutton (1973) reported a close association between *C. gallicola* and *Endocronartium harknessii* (J.P. Moore) Y. Hirat. on *Pinus banksiana* Lamb. and observed penetration of the rust aeciospores by hyphae of the

hyperparasite. Tsuneda and Hiratsuka (1979), observing the interaction between the same two organisms, reported that parasitism occurs by contact without penetration, or by penetration of the rust spores, with or without the formation of appressoria, and is followed by coagulation and the disappearance of host cytoplasm. *Cladosporium gallicola* also parasitised cankers caused by *Cronartium comandrae* Pk. on *P. contorta* Dougl. var. *latifolia* Engelm. (Powell 1971). Similar behaviour was reported for *Cladosporium aecidiicola* towards *E. harknessii* on *P. contorta*, *P. muricata* D. Don and *P. radiata* D. Don (Byler and Cobb 1969).

C. tenuissimum was first reported to be parasitic against uredospores of *Melampsora larici-populina* on *Populus* x *euroamericana* by Sharma and Heather (1981, 1983) in Australia. In an early account of such interfungus parasitism, Sharma and Heather (1983) observed both direct parasitism and antibiosis phenomena. Our preliminary assays demonstrate that this fungus, never before reported outside the Australian continent before, is also a destructive mycoparasite of the European pine rusts *C. flaccidum* and *P. pini*. The fungus seems to make its way into the host cell by using a variety of penetration mechanisms. It penetrates either by exerting a mechanical force with which it destroys the host cell structures (verrucae, cell wall), or by the formation of appressoria of varying shape. In some cases a type of direct penetration has been observed that suggests a possible biochemical mechanism based on the secretion of chemical substances. The lack of indentation of the host cell at the contact site and the minimal swelling sometimes observed at the infecting hyphal tip indicate that penetration may have been favoured by enzymatic action.

Mycoparasites have become adapted to colonizing other fungi as a source of nutrients. Penetration represents a crucial step in this process. *C. tenuissimum* seems to have evolved diverse invasion strategies to overcome host barriers. It is well known that in every host–pathogen interaction physiological changes such as the secretion of enzymes and an increase of pressure in the infection structure support the penetration process. Both these mechanisms seem to be employed by *C. tenuissimum*. It has also been observed that parasitization of rust aeciospores by *C. tenuissimum* is accompanied by secretion of an amorphous film consisting of a fibrous material; this may contribute to hyphal adhesion and preparation of the infection court.

C. tenuissimum has been found to colonize rust spores of diverse geographical origin and from different pine hosts. The fact that this fungus is widespread is not surprising considering that species of the genus *Cladosporium* represent a great part of the world air-spora (Ainsworth 1952). The wide diffusion of *C. tenuissimum* throughout Europe, taken together with its observed parasitism of the rust aeciospores, suggests that *C. tenuissimum* may have considerable epidemiological significance in the control of two-needle pine rusts caused by *C. flaccidum* and *P. pini*.

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Peridermium harknessii in the north-central United States may be a complex of taxa

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Summary

In the north-central United States, *Peridermium harknessii* populations appear to be from two geographic origins, one east and the other west of the Great Plains region. Phenotypic and molecular genetic markers and symptoms on inoculated hosts differ substantially between these populations. Isozyme analyses of aeciospores revealed a distance index between populations of 46 to 48% and within populations of 7 to 28%. Random amplified polymorphic DNA analysis of aeciospore collections revealed a diversity index between populations of 42% and within populations of 10 to 21%. Inoculations of *Pinus ponderosa* seedlings indicated that inoculum from the western population caused large fusoid galls, whereas inoculum from the eastern population caused small subglobose galls. The level of differences found between these *P. harknessii* populations is comparable to that between species of other fungi. It is not known whether the differences correspond with taxonomic rank, and, if so, at what level, e.g., race, *forma specialis*, species. However, enough data are now available that it should be considered a possibility that *P. harknessii* as known in the north-central United States comprises a complex of taxa.

Key words: *Pinus ponderosa*, taxonomy

1 Introduction

The taxonomy of fungi causing autoecious pine gall rusts in North America has never been clearly established (Peterson 1967), in part because they have relatively few morphological traits. *Peridermium harknessii* J.P. Moore [= *Endocronartium harknessii* (J.P. Moore) Y. Hirat.] has often been termed a species complex or a variable species. What is now named *P. harknessii* was described in western North America. The Woodgate *Peridermium* was considered a separate pine gall rust that occurs in eastern North America (Merrill and Wenner 1985). These and other taxa were later included in *Cronartium coleosporioides* Arth. Then the rusts causing galls on pines in North America were placed together in *P. harknessii*. Now, autoecious white-spored and yellow-spored *Peridermium* species causing pine galls in the southwestern United States have been described as genetically distinct from

P. harknessii (Vogler and Bruns 1998), and considerable genetic variation within the rest of what is currently known as *P. harknessii* has been described (Vogler *et al.* 1996). Variability in *P. harknessii* needs to be considered in biological work with this taxon. In our case, we are studying resistance to *P. harknessii* and need a better understanding of the pathogen population. If the variability fits within one taxonomic level (e.g., race), then testing will be different than if the variability is at a scale expected of a different taxonomic level (e.g., *forma specialis*, species).

In the north-central United States, *P. harknessii* occurs naturally in only a few isolated stands on the eastern edge of the natural range of *Pinus ponderosa* Dougl. ex Laws. and on the western edge of the natural United States range of *P. banksiana* Lamb. The pathogen also occurs in plantings of these and other pines in this region. The occurrence of pathogen variation within and among stands was anticipated in these studies and has been confirmed (Tuskan *et al.* 1990). After a number of genetic analyses and inoculations, a pattern is appearing that suggests taxonomic-level variation in the sampled area of North Dakota, western Minnesota, and eastern Nebraska. That variation is the subject of this paper.

2 Background of available data

2.1 Variation in isozyme and RAPD markers

In a population study of *Peridermium harknessii* (Tuskan *et al.* 1990), isozyme analysis was used to compare 192 single-gall spore collections from 10 geographic locations in the north-central United States (Fig. 1a). In this study, 13 putative isozyme loci were assessed, and 22 isozyme biotypes were identified. Cluster analysis indicated the presence of two major clusters (termed populations when referring to the pathogen), one associated with isolates that likely originated east of the sampled area and the other west of the sampled area. The minimum hierarchical distance between the two populations was about 48%. Distances within populations were 28% for the eastern population and 12% for the western population. Three of the 22 isozyme biotypes occurred in both populations, while 14 were unique to the eastern population and five to the western population. Of 13 allozymes in polymorphic isozyme loci, three were unique to the eastern population and none were unique to the western population. These results suggested that *P. harknessii* in the sampled area was diverse, possibly representing *P. harknessii* as the western population and the Woodgate *Peridermium* as the eastern population, or indicating divergent evolution between eastern and western populations of the same pathogen.

In a similar study (Wang 1995), isozyme analysis was used to compare 285 additional spore collections from eight geographic locations from the same or nearby locations as the previous study (Fig. 1b). In this study, 15 putative isozyme loci were assessed, with most of the isozymes the same as those used by Tuskan *et al.* (1990). Eleven isozyme biotypes were identified. Again, cluster analysis indicated the presence of two major populations, each associated with isolates that likely originated east or west of the sampled area. The minimum hierarchical distance between the populations was about 46%. Distances within populations were 28% for the eastern population and

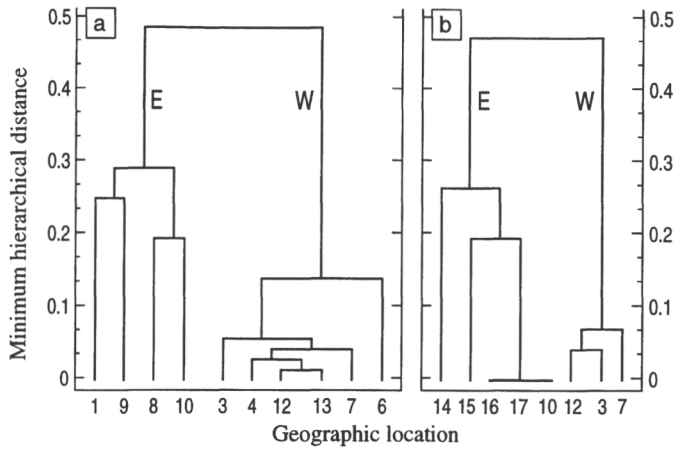

Figure 1. Cluster analysis of genetic distances based on isozyme analysis of geographic sources of *Peridermium harknessii* from the north-central United States. a: 10 sources (Tuskan *et al.* 1990), b: 8 sources (Wang 1995). Sources are from the eastern (E) or the western (W) population. Locations with the same number in a and b are the same.

7% for the western population. There was no overlap of isozyme biotypes between the two populations. Of 23 allozymes in polymorphic isozyme loci, 10 were unique to the eastern population and two were unique to the western population. The number of allozymes unique to the eastern population and the lack of overlap of isozyme biotypes between the populations is a further indication of the distinction between the populations.

Random amplified polymorphic DNA (RAPD) analysis using eight single-gall spore collections from *Pinus ponderosa* at three locations in the north-central United States was done to assess further the variation in *Peridermium harknessii* among and within the collection locations (C.M. Schumann and J.A. Walla, unpubl.). Sixty-nine of the 100 primers included in the analysis revealed polymorphism among isolates, with a total of 183 bands being polymorphic. The reverse of Jaccard similarity coefficients (1-S, as a diversity index) was used to produce a dendrogram using average linkage between groups (Fig. 2). The dendrogram showed two main clusters, again each associated with isolates that likely originated east or west of the sampled area. The diversity index between the populations was 42%. Diversity indices within populations were 10% for the eastern isolates and 21% for the western isolates.

2.2 Variation in disease symptoms

Nine single-gall *P. harknessii* spore collections (six from the western population, i.e., eastern Nebraska and southwestern and north-central North Dakota; three from the eastern population, i.e., northeastern North Dakota) collected from *Pinus ponderosa* were used to inoculate seedlings of each of three bulk-collected *P. ponderosa* seed sources from Montana, Nebraska, and South Dakota (Wang 1995). There were significant inoculum effects between the western (inocula 1 to 6) and eastern (inocula 7 to 9) populations for several symptoms from soon after inoculation through gall development.

Infection of seedlings ranged from 80% to 97% by inocula 1 to 6 and from 45% to 70% by inocula 7 to 9. There were significant inoculum effects on gall width, gall length, and gall volume (Fig. 3). Inocula 1 to 6, individually and as a group, caused large fusoid galls, whereas inocula 7 to 9 caused small subglobose galls. The average ratio of gall width to gall length for inocula 1 to 6 ranged from 0.23 to 0.28, indicating fusoid galls. The average ratios for inocula 7 to 9 were 0.63 to 0.66, indicating subglobose galls. The more fusoid shape appeared to be the most damaging form, i.e., greater needle and seedling death, suggesting that inocula 1 to 6 may be more virulent than inocula 7 to 9.

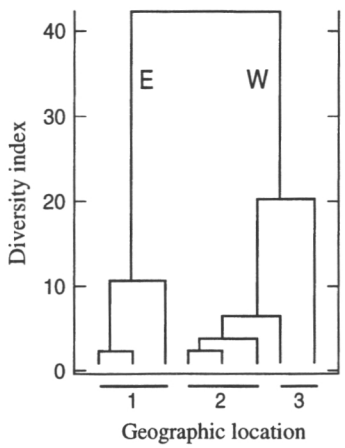

Figure 2. Diversity index based on RAPD analysis of eight *Peridermium harknessii* collections from two locations for the western (W) and one for the eastern (E) population (C.M. Schumann and J.A. Walla, unpubl.).

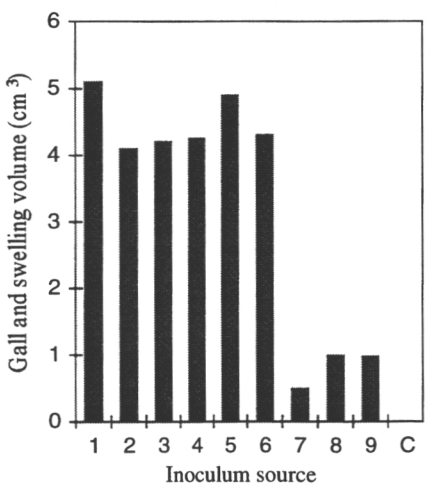

Figure 3. Gall volume caused by nine inocula of *Peridermium harknessii* (Wang 1995).

3 Discussion

Isozymes are phenotypic characters of individuals that can be used to delimit fungal species (Harrington and Rizzo 1999), and can help solve taxonomic problems when there are few available morphological parameters or when other characters are plastic among isolates (Micales *et al.* 1986). Isozyme analysis has been used to examine differences among and within a wide variety of fungal species, but use of different statistical analyses complicates comparisons among studies of similarities among and within taxa. For comparison of results from other reports with the results reported here, when results of statistical analyses were reported as similarity indices, the reverse (1-S) was calculated to obtain a diversity index. The diversity index between species of *Leptographium* Lagerb. & Melin ranged from about 48% to 90%, and indices of less than 40% were associated with isolates of individual species, suggesting that groups with a higher diversity index might be distinct taxa (Zambino and Harrington 1992). The distance between two *Alternaria* spp. clusters was 69%, and distances within species were 35% to 38% (Petrunak and Christ 1992). The diversity between clusters of *Uromyces appendiculatus* (Pers.:Pers.) Unger was 60% or more and within clusters was 17% or less; Linde *et al.* (1990) suggested that the clusters may represent different taxa. Vogler *et al.* (1996) used pairwise distance analysis to assess similarities among nine species and forms of hard pine stem rusts. Notably, two groups of *P. harknessii* were identified in that study, with one group being more similar to *formae speciales* of *C. quercuum* (Berk.) Miy. ex Shirai than to the other *P. harknessii* group. Their analysis identified clusters (possibly taxa) within what had previously been included in single species (including one that had been included with *P. harknessii*) and two other groups that may be previously unknown taxa. The range and type of distances between and within species in these reports is similar to that between and within the *P. harknessii* populations in our studies.

Results of our RAPD analysis of *P. harknessii* were similar to those using isozyme analysis, identifying clusters corresponding to the eastern and western populations. In contrast to our results using RAPDs, Hubbes and Lin (1995) did not identify geographic variation among *P. harknessii* isolates from Ontario, Canada. Hamelin *et al.* (1994) identified relatively minor geographic divergence among *C. quercuum* f. sp. *fusiforme* isolates sampled across the southeastern United States. Thus, within pine rusts, our results using RAPDs are an exception, supporting the existence of two populations of *P. harknessii* in the north-central United States.

Certain allozymes of isozyme loci have often been found useful in differentiating species of various fungi. Powers *et al.* (1989) identified two isozymes that differentiated each *forma specialis* of *C. quercuum*, and one of those was also differential for *P. harknessii*, although the small sample size and use of some bulk spore collections may have hidden some of the variability. Each of 12 allozymes of six polymorphic isozyme loci scored in *P. harknessii* from the western United States was unique to one of two populations (Vogler *et al.* 1991). Vogler *et al.* (1996) differentiated nine species and forms of hard pine stem rusts using isozyme analysis, and banding patterns from just two loci allowed differentiation of all species and most forms or races examined. These types of population- and species-level

differences were also present in the north-central United States (Tuskan *et al.* 1990, Wang 1995).

Vogler and Bruns (1998), using sequences of the internal transcribed spacer region from the nuclear ribosomal RNA genes, examined the relationship of pine rusts, including several sources of autoecious pine gall rusts that had been included in or were similar to *P. harknessii*. Nucleotide analysis indicated that a white-spored form (distinctive nuclear condition and gall shape) and a yellow-spored form (distinctive sporulation pattern and gall shape) in the southwestern United States were distinct from *P. harknessii*. This indicates that *P. harknessii*, as currently defined, is a species complex and that gall shape is an important taxonomic character. The data also indicated that the *formae speciales* of *C. quercuum* were genetically distinct, further supporting gall shape as a useful taxonomic character.

Gall shape caused by the nine inocula used by Wang (1995) was of two types. Because the inoculated seedlings were from bulk collections from the same geographic origins, the different gall size and shape reveals variation in virulence between the two inoculum sources. The consistent conformity to this pattern indicates that *P. harknessii* occurs as western and eastern populations. Work by Wenner and Merrill (1987) may be an earlier indication of these differences. They inoculated *Pinus sylvestris* L. with *Peridermium harknessii* inoculum from *Pinus sylvestris* and *P. ponderosa*. Inoculum from *P. ponderosa* resulted in fewer obvious galls and more stem cracking with or without swelling. There is a likelihood that their inoculum from *P. ponderosa* represented the western population and that from *P. sylvestris* the eastern population. Others have reported differences in virulence of or in host reaction to sources of *Peridermium harknessii* inoculum from different geographic locations (van der Kamp 1988). Because *P. harknessii* has been distributed among regions in North America on nursery stock, it is possible that what we have termed the eastern and western populations have been introduced throughout North America (Merrill and Wenner 1985). If the difference between the populations proves to be at a taxonomic level, and introductions from one region to another have occurred, researchers will need to carefully characterize the pathogen source with which they are working.

Burdsall and Snow (1977) chose to reduce the *Cronartium* species that alternate between pine and oak in the eastern United States to *formae speciales* (parasites with physiological, but little or no morphological, variation). There are similarities between that situation and the situation presently known with *P. harknessii*. The most common means for distinguishing *C. quercuum* f. sp. *banksianae* and *C. quercuum* f. sp. *fusiforme* is shape of galls (Burdsall and Snow 1977), with those of *C. quercuum* f. sp. *fusiforme* reported to be fusiform and those of *C. quercuum* f. sp. *banksianae* reported to be globose to subglobose. Gall shape is not entirely dependable because each can produce galls that fit the expected range of the other. The ranges of differences in the reported "races" of *C. quercuum* f. sp. *banksianae* were similar to those previously used to separate *C. quercuum* and *C. fusiforme* (Hedgc. & Hunt ex Cumm.). In comparison, morphological differences sufficient to justify taxon rank have not been identified in *P. harknessii* in the north-central United States, but appropriate morphological comparisons have not been made. As with *C. quercuum*, the

populations cause galls of different shape (one fusoid, the other subglobose) on young seedlings.

The level of variation in *P. harknessii* in the north-central United States indicates taxon level differences, at least *forma specialis* level, and possibly species level. Sample size and sampling area were small, and morphological comparisons were not made, so it is inappropriate to propose taxon rank for the two populations. Nonetheless, the results 1) indicate a need for caution in designing resistance studies, and 2) provide a clue to populations that should be examined in future taxonomic investigations. Results of this and other studies (Vogler *et al.* 1996, Vogler and Bruns 1998) may provide a basis from which biological and taxonomic investigations can lead to a better understanding of the *P. harknessii* species complex.

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Assessing gall rust resistance: the effect of local spore concentration and auto-infection

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Summary

In disease resistance trials in which trees are exposed to several generations of a pathogen, part of the variation in infection severity between individuals may be due to local high inoculum concentrations. This works as follows: once the first few infections are established, the inoculum load in the immediate vicinity of these infections may be higher than the mean for the trial. This in turn leads to higher infection rates in that vicinity, etc. This paper presents results from a lodgepole pine trial planted near Fort St. James, B.C. in 1986, which has experienced two distinct infection cycles by western gall rust (*Endocronartium harknessii* (J.P. Moore) Y. Hirat.), first in 1990 and again during 1993 and 1994. Thirteen 64-tree blocks (tree spacing of 2 by 2 m) were inspected in 1997, and the number of galls per tree were counted in two classes: on 1990 internodes ('old') and on 1992 to 1994 internodes ('new'). The mean number of old and new galls per tree was 3.16 and 3.20 respectively. Regression of the number of new infections per tree (y) on old infections (x) gave a slope of 0.9085, less than but not significantly different from the expectation (given no effect of local inoculum concentration) of 1.0127. Hence variation in local inoculum concentration leading to exaggerated new infection in the more susceptible trees was not detected in this trial.

Key words: *Endocronartium harknessii*, *Pinus contorta*

1 Introduction

Western gall rust caused by *Endocronartium harknessii* (J.P. Moore) Y. Hirat. is a common stem rust of hard pines in North America. In B.C. the disease is usually found on lodgepole pine (*Pinus contorta* Loud.) wherever this host occurs in the province. The rust induces the formation of woody galls at the point of infection, and on these galls aeciospores are produced annually starting 2 or 3 years after infection. Infection takes place directly through the epidermis of the elongating shoots (or, less commonly, through the female flower stem) in spring, and so the age of a gall can be dated by the age of the internode on which it occurs. Evidence for this infection site consists of numerous inoculation experiments and gall dissection. The xylem in galls is easily distinguishable from normal xylem, and that altered xylem normally extents to the pith. Small galls are occasionally found on older internodes that, on dissection, appear to be younger than that internode. It

may be that such cases represent infection through needle bases, but direct evidence of such infection does not exist. At any rate, such infections are both rare and, at least when they are young, easily distinguished from normal infections. When the original epidermis on the shoot surface is replaced by a periderm, usually within two weeks of the end of shoot elongation, the shoot is no longer susceptible to new infections. That periderm starts to form at the base of the shoot and then extends slowly up the shoot. The last tissue to remain susceptible is the stem of the female flower at the top of the new shoot.

Gall rust spore production in central B.C. starts about the end of May and is virtually complete by mid June. The amount of infection varies considerably from year to year. During years of heavy infection (known as 'wave years') the infection rate may exceed the average long-term rate by a factor of 10. I believe that infection during wave years occurs mostly as a single infection event lasting no more than one day. The main evidence for this is that galls dating from such years are mostly found on a particular part of the internode. For instance, during a wave year around Prince George in 1982, over 75 percent of infection occurred on female flower stems, and the remainder were always near the upper end of internodes. During the following major wave year in the area (1990), infections were almost always located just below the middle of the shoot.

When first developing, plantations or naturally regenerated stands of lodgepole pine typically have few or no rust galls because the rust can only survive on living hosts. The first infections in such areas are therefore derived from what might be called the regional spore cloud. Two or three years later, such infections begin to produce spores, and at that time the spore concentration in the immediate vicinity of these early infections will presumably be higher than in the remainder of the area. Spore concentration (and therefore deposition) around a continuously emitting point source declines with distance to the 1.6–2.0 power. So the 'immediate vicinity' mentioned above (the space in which the spore concentration is well above the regional average) will have a spatial extent of a few meters at best, and certainly less than half the average distance between infections. It is likely, therefore, that the major effect will be limited to single trees or small groups of trees. The problem, then, is that trees that happen to be among the first infected, not because they are particularly susceptible, but merely by chance, will be subject to significant rates of auto-infection, and will therefore appear to be more susceptible than they really are.

2 Methods

An experiment was previously established jointly by a group of Swedish and Canadian geneticists and pathologists (Lindgren and Lindgren 1990) to compare growth and susceptibility to various pathogens of Scots (*Pinus sylvestris* L.) and lodgepole pine in Sweden and northern Canada. One of the study sites provided an ideal place to test the phenomenon of variation in infection due to variation in local spore concentration. The site, at Fort St. James B.C. (54°26'N, 124°2'W) consisted of fifty-seven 64-tree blocks of either Scots or lodgepole pine, Siberian larch (*Larix sibirica* L.), or mixtures of these. The site was located on a flat, low hill top and was planted in the

spring of 1986. The spacing was 2 by 2 m throughout. In 1987 seedlings artificially inoculated with gall rust were planted at 4 by 4 m spacing, the lowest density that would result in all trees being equidistant from such inoculated seedlings. These seedlings started to produce spores in 1988, and most were dead by 1993, although some survive even today. The gall rust infection history on this site is described in detail elsewhere (van der Kamp *et al.* 1995). In brief, there was virtually no infection on lodgepole pine until 1990 (average number of galls per tree established in 1987, 1988, 1989 and 1990 was 0.01, 0.01, 0.0, and 2.61, respectively, averaged across the whole site).

In 1997 the number of galls per tree, as well as the age of these galls, was measured for thirteen 64-tree blocks of lodgepole pine. In 1991 and 1992 there was virtually no new infection, but total infection during 1993 and 1994 was nearly equal to that in 1990 (3.20 and 3.16 galls per tree, respectively). Galls established in 1990 started to produce spores in 1993, and presumably most of the inoculum giving rise to the 1993 and 1994 galls was derived from them.

3 Results and discussion

It is presumed in this paper that the 1990 infection event resulted from rather uniformly distributed inoculum. That inoculum would have been derived from two sources: the planted inoculated seedlings and the regional spore cloud. If the phenomenon postulated above had a significant effect, then trees with large numbers of infections in 1990 ('old infections') should have an even larger proportion of infections in 1993 and 1994 ('new infections'). This is because during the 1993 and 1994 infection events, inoculum would not be evenly distributed, but rather concentrated in and around trees that were heavily infected in 1990.

Several tests were conducted. First, if there was no effect, regression of new (y) on old (x) infections should have a slope of new/old, or 1.013. If the phenomenon was significant, the slope should be greater (indicating trees heavily infected in 1990 have a disproportionately large number of new infections). In fact, regression yielded:

$$y = 0.329 + 0.9085x$$

a slope of 0.9085, lower than but not significantly different from the null expectation.

Second, trees were divided into two groups, those with less than five old (1990) galls and those with more than four old galls. The number of new galls for each of these groups was then tallied. The outcome (Table 1) supports the previous analysis: new infection on trees that were heavily infected in 1990 was slightly less than expected. However, variation was considerable. On trees with less than five galls per tree in 1990, new infection ranged from zero to 23 galls per tree, and on trees with more than four galls in 1990 from zero to 55.

In conclusion, no evidence was found of excessive rates of re-infection on trees that were heavily infected during the first (1990) major infection event. Thus distortion of western gall rust infection rates due to variation in local spore concentration does not appear to be a significant phenomenon.

Table 1. 'Old' (1990) and 'new' (1993 and 1994) gall rust infection of lodgepole pine on lightly and heavily infected trees.

	Infection in 1990, galls per tree	
	< 5	> 4
Number of trees	609	187
'old' galls/tree	1.003	10.166
'new' galls/tree	1.197	9.706

References

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van der Kamp, B.J., Karlman, M. & Witzell, J. 1995. Relative frequency of bole and branch infection of lodgepole pine by western gall rust. Canadian Journal of Forest Research 25: 1962–1968.

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Colonization of the primary tissues of *Pinus banksiana* by *Cronartium comandrae*

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Summary

Seedlings of *Pinus banksiana* were artificially inoculated with basidiospores of *Cronartium comandrae* and incubated in a mist chamber for 36 h. Cotyledon, primary needle, hypocotyl and developing epicotyl tissues were then harvested and processed for light and scanning electron microscopy. Basidiospore germ tubes were only observed to penetrate host tissues via stomata followed by vesicle formation in the substomatal chamber. Vesicles varied in shape but were most often fusiform with the distal portion extended to form an intercellular hypha. Intercellular hyphae were primarily restricted to mesophyll of cotyledons and primary needles but also grew along the leaf trace into specialized mesophyll of the hypocotyl. Ten days after infection, rust hyphae were restricted to spongy mesophyll-like tissues of the hypocotyl; however, after 36 days, these hyphae were found in outer cortical tissues and in medullary rays. After 67 days, rust hyphae had extensively colonized all hypocotyl tissues and were beginning to grow into the developing epicotyl. Also, pycnia were present and producing spores. Intracellular haustoria were observed in all rust-infected tissues following cell wall penetration by infection hyphae. Infection hyphae penetrated host cell walls directly but were observed penetrating via bordered pits of xylem tracheids when adjacent to infected ray tissues. All haustoria were uninucleate, aseptate, and either simple, bi-lobed, or multi-lobed. A single haustorium per host cell was most common; however, as many as nine were observed. Most haustoria were closely associated with host cell nuclei.

Key words: infection, seedlings

1 Introduction

Comandra blister rust caused by *Cronartium comandrae* Peck is native to North America (NA) and is not known to occur elsewhere in the world. This fungus is a macrocyclic, heteroecious rust and an obligate parasite that grows perennially in some species of hard pines and annually infects subspecies of *Comandra umbellata* (L.) Nutt. (comandra, bastard toadflax or false toadflax) or *Geocaulon lividum* (Richards) Fern. (northern comandra) (Arthur 1929). This rust is destructive in some areas of western NA and can cause damage to *Pinus ponderosa* Dougl. ex Laws. (ponderosa pine) and *P. contorta* Dougl. ex Loud. (lodgepole pine) (Mielke 1957, Childs 1968, Powell 1970).

Comandra rust on pines in eastern NA has only been reported to cause local disease problems on *P. banksiana* Lamb. (jack pine) (Bergdahl and French 1973), *P. echinata* Mill. (shortleaf pine), and *P. taeda* L. (loblolly pine) (Powers *et al.* 1967, Wolfe 1970); however, many other hard pines are known to be susceptible (Mead *et al.* 1978).

The comandra rust fungus can infect hard pines of all ages, but young seedlings are considered very susceptible. Bergdahl and French (1976) have shown that 4-week-old jack pine seedlings had over four times the number of infections as 11-week-old seedlings when exposed to natural levels of inoculum under the same field conditions.

Kirkwood (1915) was the first to suggest that the comandra rust fungus penetrated the pine host either directly through the bark or through wounds; however, Bergdahl and French (1985) showed that germ tubes of basidiospores only penetrate the pine host through stomatal pores, which is similar to the mode of infection described for *C. ribicola* J.C. Fisch., the causal agent of white pine blister rust (Patton and Johnson 1970).

Histological observations of the growth and development of comandra rust in secondary tissues of ponderosa pine have been reported by Krebill (1968). Also, Tainter (1985) has described intercellular and intracellular structures formed by *C. comandrae* in secondary (aeciospore-producing) tissues of both loblolly and shortleaf pines. However, there have been no histological studies on early colonization of *C. comandrae* in young primary tissues of the pine host. Therefore, the main objective of this paper is to detail early colonization of the primary tissues of *P. banksiana* by *C. comandrae.* A preliminary report on the colonization of these tissues has been prepared by Bergdahl (1979).

2 Material and methods

Jack pine seedlings were grown in plastic tubes (1x7.6 cm) for 30 days and then inoculated in a mist chamber with basidiospores of *C. comandrae.* The inoculation period was 36 h. Cotyledons, primary needles, hypocotyl and epicotyl tissues were excised and processed for light, scanning (SEM), and transmission electron microscopy (TEM) immediately following inoculation and at intervals of 10, 26, 36, 67, and 97 days thereafter.

For light microscopy, the selected host tissues were fixed in formalin acetic acid (FAA), dehydrated, and embedded with paraffin (Johansen 1940). These tissues were mounted and then sectioned at 12 µm thick using a rotary microtome. All sectioned tissues were differentially stained with orseillin BB and aniline blue using a modification of Jewell (1958) (Bergdahl 1979). The stained tissues were mounted in Permount and examined with a compound microscope.

All tissues selected for SEM were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and critical point dried using carbon dioxide (Bergdahl 1979). The specimens were mounted on stubs, transferred to a vacuum evaporator, and coated with a gold-palladium alloy. All specimens were examined with a Cambridge Stereoscan electron microscope. In addition, infected tissues were processed for TEM using a procedure similar to that reported by Tainter (1985).

3 Results

Upon studying the infection process for *C. comandrae* on young jack pine seedlings, we observed that germ tubes from basidiospores grew randomly across and appressed to the host epidermis. These germ tubes penetrated the host through stomatal pores of cotyledons, primary needles, or hypocotyl tissues (Fig. 1). Following penetration, a fusiform-shaped vesicle formed in the substomatal chamber (Fig. 2). The distal part of the vesicle was extended to initiate development of intercellular hyphae. These hyphae were initially restricted to the mesophyll tissues of cotyledons and primary needles and to the specialized mesophyll of the hypocotyl (Fig. 3). Continued colonization of these mesophyll tissues was best described as a loose network of hyphae growing between cells, with haustoria formed within cells (Figs. 4 and 5). In some areas, these hyphae appeared firmly attached or cemented to the host cell wall but the nature of this attachment was unknown. The intercellular hyphae in cotyledons and primary needles eventually grew through the mesophyll into the cortical tissues of the hypocotyl and epicotyl. Haustoria were formed in all tissues.

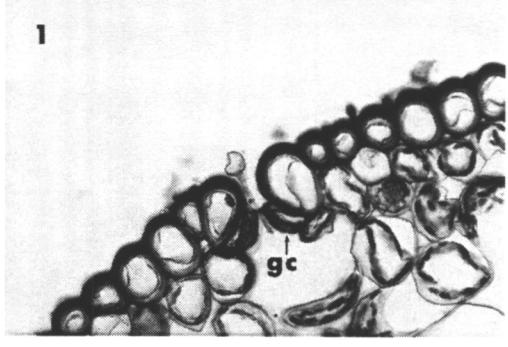

Figure 1. Basidiospore germ tubes of *Cronartium comandrae* penetrating between the guard cells (gc) in the hypocotyl of *Pinus banksiana*. Basidiospore diameter is about 10 µm.

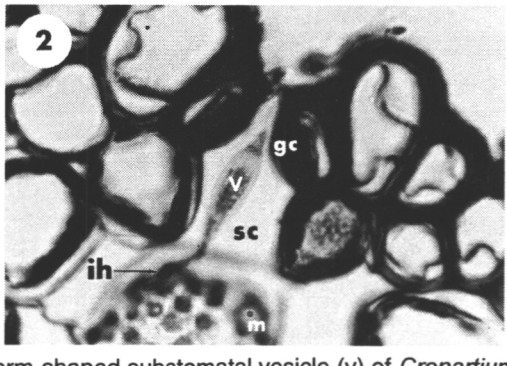

Figure 2. Fusiform-shaped substomatal vesicle (v) of *Cronartium comandrae* in the substomatal chamber (sc) in the hypocotyl of *Pinus banksiana*. The distal portion of the vesicle is extended to form an intercellular hypha (ih). Note: m = mesophyll cell, gc = guard cell.

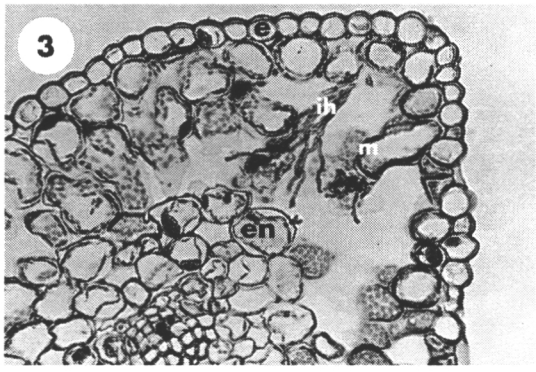

Figure 3. Intercellular hyphae (ih) of *Cronartium comandrae* in the mesophyll tissue of a primary needle of *Pinus banksiana*. Note: e = epidermis, en = endodermis, m = mesophyll cell.

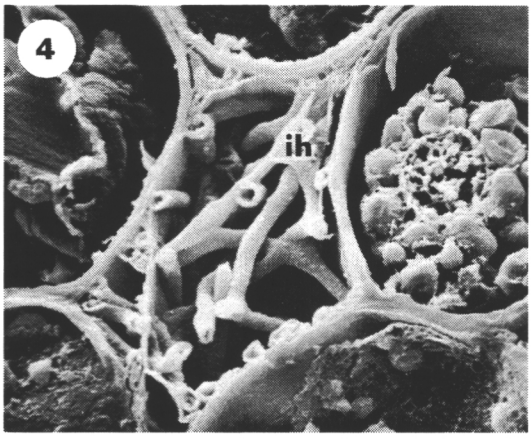

Figure 4. Intercellular hyphae (ih) of *Cronartium comandrae* between the cortical cells in the hypocotyl of *Pinus banksiana*.

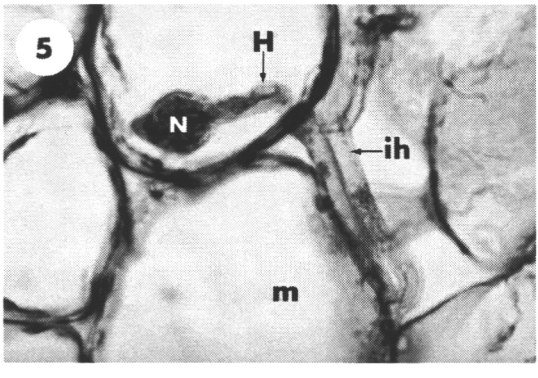

Figure 5. Intercellular hyphae (ih) and haustorium (H) in the specialized mesophyll (m) of the hypocotyl. Note: haustorium in contact with the host cell nucleus (N).

Advanced colonization of the cotyledons and primary needles was most extensive in the mesophyll. There appeared to be a general breakdown of the structural integrity of the mesophyll and a loss of chloroplasts as early as 36 days postinfection. Infected needles appeared chlorotic, and haustoria were occasionally found in the endodermal and epidermal cells. By 67 days postinfection, chlorosis worsened, and by 97 days, most cotyledons and some primary needles were dead.

Colonization of the hypocotyl resulted either from germ tube penetration through stomata (Fig. 6) or from the growth of intercellular hyphae along the leaf trace of cotyledons or primary needles into the mesophyll and cortical tissues. Colonization via the leaf trace occurred as early as 10 days postinoculation.

Following basidiospore penetration of the hypocotyl, intercellular hyphae of *C. comandrae* were restricted to mesophyll cells, but within 10 days these hyphae extended through the tissues as far as 560 μm above and 490 μm below the initial point of penetration. Based on these measurements, the average longitudinal growth of the fungus was calculated to be about 53 μm per day. Radial growth of *C. comandrae* was also restricted to the mesophyll and only extended about 95 μm below the initial point of infection during these first 10 days (Fig. 6).

By 26 days postinfection, the intercellular hyphae ramified through the intercellular spaces in the mesophyll and began to colonize cortical cells of the hypocotyl. Haustoria were well-developed and many were associated with host cell nuclei. There was no evidence of ray or pith tissues being colonized at this time.

At 36 days postinfection, rust hyphae were still concentrated in the outer mesophyll and cortical tissues of the hypocotyl. Occasionally, hyphae were found along the leaf trace near the phloem and in the medullary rays, but the fungus was not extensive in these tissues. However, by 67 days, the rust was extensively colonizing the cortical, phloem, medullary ray and pith tissues of the hypocotyl (Figs. 7–9) and was continuing to grow into the developing epicotyl. The ray parenchyma tissues were also colonized and haustoria were occasionally seen in the epithelial cells that line resin canals. The intercellular hyphae appeared to enter the ray tissues in the region of the cortex and then proceeded to grow between rays cells, through the cambial zone, and into the xylem (Fig. 9). Many small haustoria were found in the xylem, but only when adjacent to infected ray parenchyma.

Pycnia were not apparent 36 days postinfection but were well-developed and producing spores on both the hypocotyl and epicotyl at 67 days (Fig. 10). These pycnia developed in the region between the specialized mesophyll and the true cortical tissues, in an area near the developing periderm. In older tissues, this area would be between the periderm and the underlying cortex. Development of the pycnium was preceded by a massing of hyphae between the cortical cells and by the formation of a hyphal cushion upon which pycniophores and then pycniospores were produced. The tissues overlying the developing pycnia eventually ruptured, allowing pycniospores to escape. Pycnial development on some seedlings exceeded 50% of the stem circumference at 67 days. Aecia or aecial initials were never observed and most infected seedlings died within 1 year after inoculation.

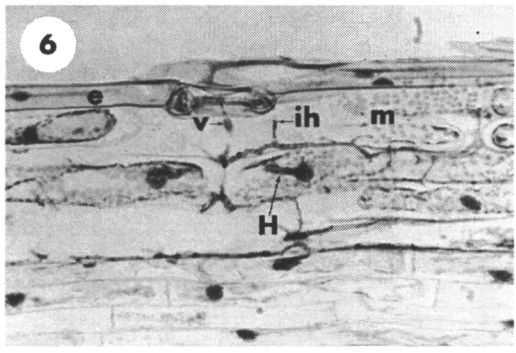

Figure 6. Initial penetration and colonization of the specialized mesophyll (m) of the hypocotyl of *Pinus banksiana* by *Cronartium comandrae* 10 days postinfection. Note: e = epidermis, H = haustorium, ih = intercellular hyphae, v = vesicle.

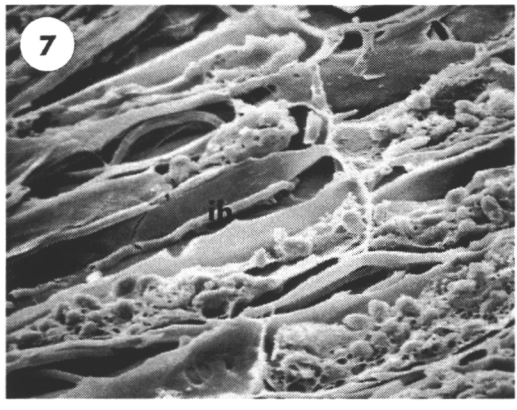

Figure 7. Intercellular hyphae (ih) of *Cronartium comandrae* growing between the cortical cells of *Pinus banksiana*, 67 days postinoculation.

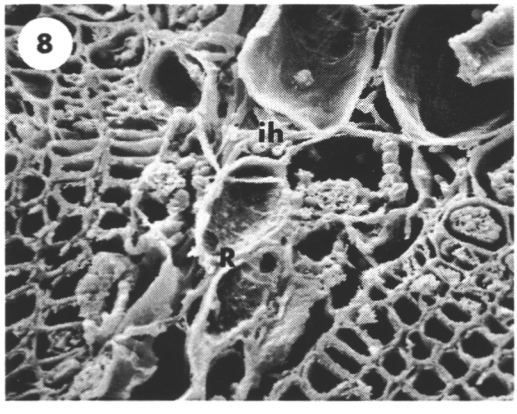

Figure 8. Intercellular hyphae (ih) of *Cronartium comandrae* in the medullary ray (R) of *Pinus banksiana*, 67 days postinoculation.

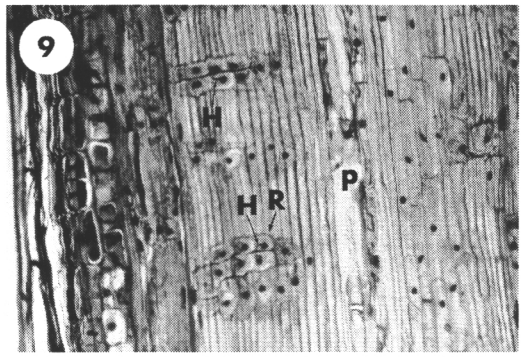

Figure 9. Intercellular hyphae and haustoria (H) of *Cronartium comandrae* in hypocotyl tissues of *Pinus banksiana*. Note: P= pith, R = ray.

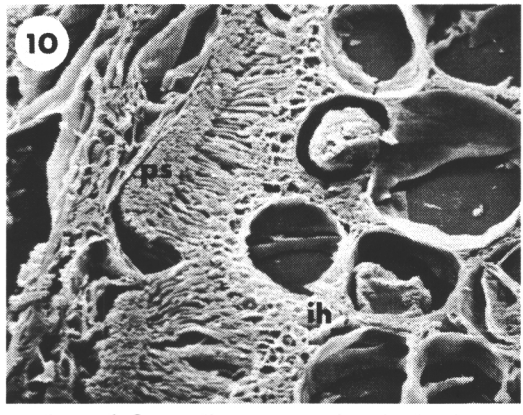

Figure 10. A pycnium of *Cronartium comandrae* in the hypocotyl of *Pinus banksiana*, 67 days postinfection. Note: ih = intercellular hyphae, ps = pycniospores.

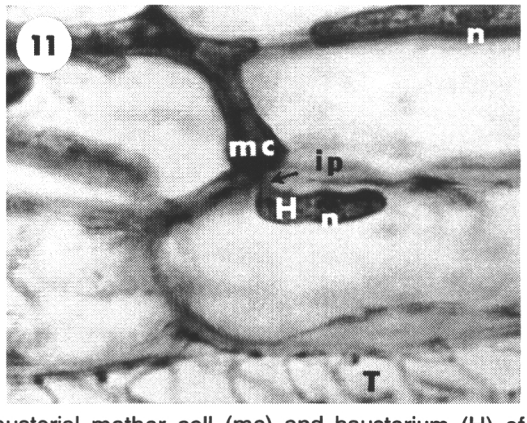

Figure 11. Haustorial mother cell (mc) and haustorium (H) of *Cronartium comandrae* in a parenchyma cell of the hypocotyl adjacent to xylem tracheids of *Pinus banksiana,* 67 days postinoculation. Note: ip = infection peg, n = nucleus of haustorium.

Intercellular hyphae of *C. comandrae* colonized all host tissues and infection hyphae were formed where these intercellular hyphae came in contact with host cell walls. Haustorial mother cells (Fig. 11) formed along these points of contact, and infection hyphae (infection pegs) penetrated the host cell wall either via direct penetration or by growing through natural openings (bordered pits) (Fig. 12). However, penetration through bordered pits was only observed in xylem tissues and only when adjacent to infected ray tissues.

Once the infection peg completed penetration, it rapidly expanded within the host cell to form a haustorium, which appeared to invaginate the host plasmalemma (Figs. 13–14). These haustoria were delimited from infection pegs by a single septum (Fig. 14) and all appeared to be uninucleate and non-septate (Figs. 13–15). A single haustorium per host cell was most common, but as many as nine haustoria were observed in a cell near the pith.

The following three distinct haustorial forms or shapes were observed for *C. comandrae*: simple, bilobed or branched, and multilobed. These terms have been previously used to describe the haustorial forms of *C. comandrae* as well as haustoria of other *Cronartium* species (Colley 1918, Krebill 1968, Miller 1972, Tainter 1985). The simple form was quite variable in size and shape (Figs. 11–14) and was the most common form observed in all tissues except the cortical tissues of the hypocotyl. The bilobed haustorium was usually quite large and most prominent in the parenchyma cells of the cortex (Fig. 15). This form was rarely observed in ray parenchyma and never observed in xylem tracheids. The multilobed form was least common and not seen in cotyledons and only occasionally seen in the mesophyll cells of primary needles and in parenchyma tissues of the hypocotyl.

All haustorial forms, except simple forms in xylem tracheids, were frequently associated with host cell nuclei (Figs. 5, 6, 9, 13, and 14). These haustoria did not appear to penetrate host nuclei, but nuclei often appeared somewhat distorted, which may be the result of physical pressure exerted by the fungus. In the mesophyll cells of the cotyledons and primary needles and in ray tissues, about 85% of all cells with haustoria had this kind of nuclear association.

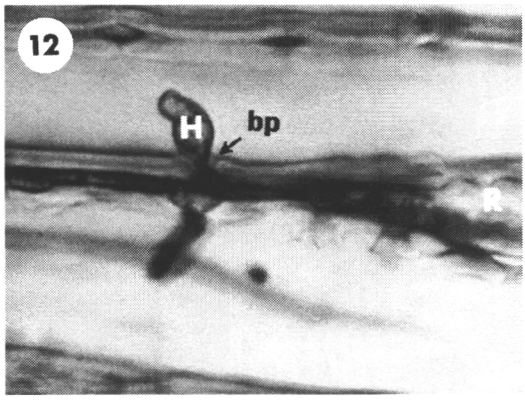

Figure 12. Simple haustorium (H) of *Cronartium comandrae* in xylem tracheids of *Pinus banksiana* 67 days postinoculation. Tangential view of infected ray (R) with bordered pits (bp).

The surface texture of all three forms of haustoria was smooth as seen by light microscopy (Figs. 11–13 and 15). However, haustoria appeared to be covered with a sheathlike material that most likely was host plasmalemma (Fig. 14). Observation by SEM revealed surface morphology of the haustorium to range from almost smooth to very rugose (Figs. 14 and 16). However, this rugose texture was believed to be an artifact of the fixation or dehydration process and only appeared to involve the invaginated host plasmalemma, as confirmed by TEM (Fig. 14).

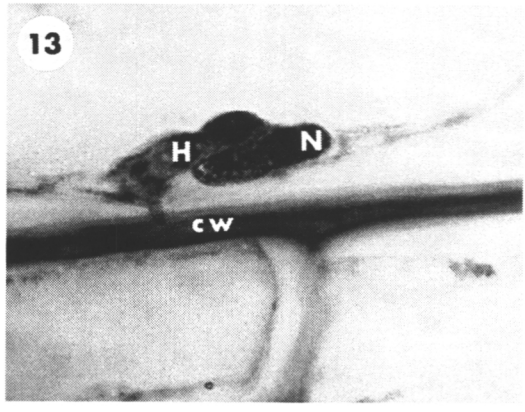

Figure 13. Simple haustorium (H) of *Cronartium comandrae* associated with the host cell nucleus (N) of *Pinus banksiana*. Note: infection peg penetrating the host cell wall (cw).

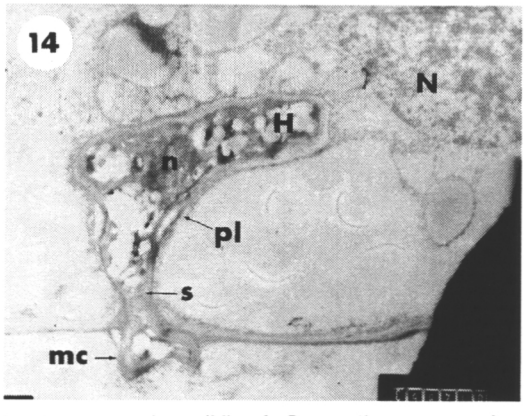

Figure 14. Simple haustorium (H) of *Cronartium comandrae* in a cell of *Pinus banksiana*. Note: invaginated host plasmalemma (pl), haustorial association with host cell nucleus (N), haustorial mother cell (mc), septum (s), and single nucleus of haustorium (n).

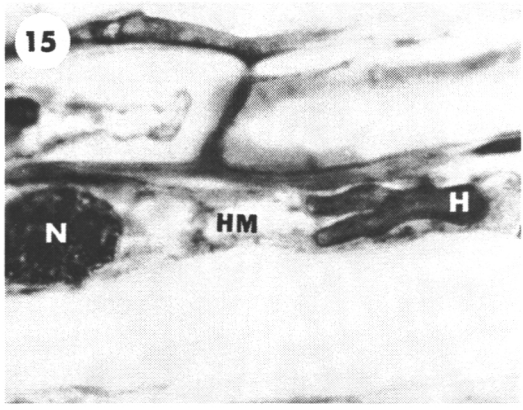

Figure 15. Bilobed haustorium (H) of *Cronartium comandrae* in the specialized mesophyll tissues (HM) of the hypocotyl of *Pinus banksiana*. Note: position of haustorium extended toward the host cell nucleus.

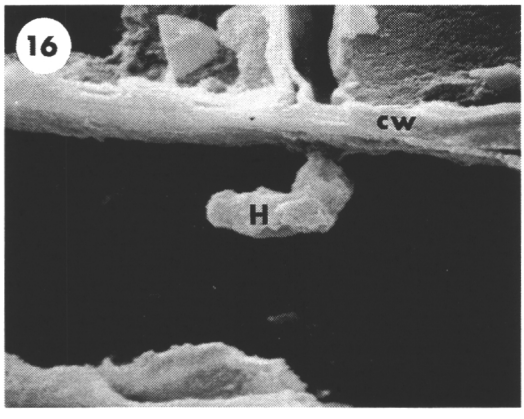

Figure 16. Simple haustorium (H) of *Cronartium comandrae* in a specialized mesophyll cell in the hypocotyl of *Pinus banksiana*. Note: rugose surface of the haustorium, cw = host cell wall.

4 Discussion

The *Cronartium* rusts attack susceptible pines of all ages, and both direct and indirect modes of penetration have been described for different *Cronartium* rusts (Patton and Johnson 1970, Miller 1972). However, basidiospore infection by *C. comandrae* of primary tissues of jack pine seedlings was only through stomata, and there was no evidence to suggest infection could happen in any other way (Bergdahl and French 1985).

Following infection by *C. comandrae*, initial colonization of *P. banksiana* differed from that reported for *C. fusiforme* (Miller 1970), but was very similar to that reported for *C. ribicola* (Patton and Johnson 1970). According to Miller (1970), the basic difference between *C. fusiforme* and these other two species of *Cronartium,* is in the way the fungus initially enters and

begins to colonize the host. *Cronartium fusiforme* first directly penetrates the outer wall of an epidermal cell, colonizes it, penetrates the inner wall and then continues to colonize the host. Continued colonization of needle and stem tissues involves the growth of intercellular hyphae through the mesophyll; hyphae eventually entering the vascular tissues within the needle prior to entering the main stem (Miller 1970, Jewell 1975). For *C. ribicola*, indirect penetration is quickly followed by the formation of intercellular hyphae and infection hyphae in the mesophyll tissues near the substomatal chamber (Patton and Johnson 1970). Continued colonization by *C. ribicola* follows the pattern of colonization described for *C. fusiforme*. In contrast, colonization by *C. comandrae* involved growth of intercellular hyphae through the mesophyll tissues of cotyledons or primary needles. Eventually, these hyphae entered the leaf trace before growing into the main stem. Only rarely did we observe hyphae of *C. comandrae* within the vascular tissues of these needles prior to entry into the main stem, and this differs from the reports on *C. ribicola* (Patton and Johnson 1970) and *C. fusiforme* (Miller 1970).

In the rusts, the term haustorium refers to an intracellular structure that is produced following penetration of the host cell wall by infection hyphae. These haustoria invaginate the host plasmalemma and are believed to serve as the main organ of nutrient uptake by the rusts (Bushnell 1972). Littlefield and Heath (1979) refer to haustoria of rust fungi as being either dikaryotic (d-type) or monokaryotic (m-type). For the *Cronartium* rusts, it is the m-haustorium that forms following infection by basidiospores. Tainter (1985) has described the ultrastructure of m-haustoria of *C. comandrae* in the secondary tissues of shortleaf pine using TEM.

The m-haustoria of *C. comandrae* we observed were in the primary tissues of jack pine as viewed through light microscopy, SEM and TEM. These haustoria were of three different shapes (simple, bilobed, or mulilobed) and their frequency of occurrence was dependent on host cell type. Also, we noted infection hyphae appeared to slim down during penetration through the host cell wall, and some infection hyphae actually penetrated host cell walls via bordered pits. The reports by Krebill (1968) and Tainter (1985) did not mention these above observations. In addition, we documented the high incidence of haustoria in close association with host cell nuclei, especially in the ray parenchyma tissues; however, the true nature and significance of this nuclear association awaits additional study.

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Responses of wound-inoculated seedlings of *Pinus elliottii* var. *elliottii* and *Pinus taeda* to mycelial cultures derived from multiple and single basidiospores of *Cronartium quercuum* f. sp. *fusiforme*

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Summary

In 1991, a series of experiments was initiated to examine the effectiveness and research value of inoculating 6-week-old seedlings of slash (*Pinus elliottii* var. *elliottii*) and loblolly pine (*P. taeda*) with suspensions of basidiospores of *Cronartium quercuum* f. sp. *fusiforme* onto wounds made by severing the upper approximately 1 cm of the stems. These experiments were to evaluate the wound technique as an alternative to the standard spray inoculations with basidiospore suspensions, particularly as a means of identifying potential mechanisms of resistance and to facilitate single-spore inoculations. The success of the wound inoculation technique led to experiments in which seedlings of slash pine were wound-inoculated with mycelia from cultures derived from multiplespace and single-basidiospore isolates. These experiments produced what is believed to be the first *in vivo* infection of pine seedlings by single-basidiospore isolates of *C. q. fusiforme*. In the most successful inoculation test, galls were produced on 11 of 58 slash pine seedlings inoculated with six different single-spore isolates. Evaluation of single-spore inoculations was continued with mycelium grown from virulent and avirulent cultures of *C. q. fusiforme*. Fifty seedlings of a half-sib family of loblolly pine were inoculated with each of five single-spore isolates. One V single-spore isolate resulted in galls (8/50). Twelve months after inoculation a cube of tissue was removed from the upper portion of each gall, embedded, sectioned, stained and examined microscopically to evaluate the patterns of colonization and host reactions to the one virulent isolate. Overall, the pattern of colonization was similar to seedlings inoculated with the standard spray technique. However, differences were noted in intensity of colonization and characteristics of spermogonial development.

Key words: basidiospores, inoculation techniques, loblolly pine, slash pine

1 Introduction

Over the past 40 yr, the economic losses in the southern United States (US) to the fusiform rust disease, caused by *Cronartium quercuum* Miy. ex Shirai f. sp. *fusiforme* on *Pinus elliottii* (Engelm.) var. *elliottii* and *P. taeda* L. have been reduced significantly (Pye *et al.* 1997). This success has been largely due to the practical application of the research accomplishments of forest pathologists, forest geneticists and tree breeders in finding the means of reducing the incidence of this destructive disease through deployment of pine families and geographic seed sources with genetic resistance to *C. q. fusiforme*.

In spite of the relative success of using genetic resistance, fusiform rust continues to cause unacceptable losses in many parts of the southern US. Much of this continuing problem may be associated with the pathogenic variability of *C. q. fusiforme* (Snow *et al.* 1975, Powers *et al.* 1977, Kuhlman 1992). Since little is known about the genetics of *C. q. fusiforme*, such research is a prerequisite to progress in understanding the host/parasite interaction and in developing additional strategies for management of the disease.

A major requirement for investigating the genetics of *C. q. fusiforme* is to determine if the *Pinus* host can be infected by a single basidiospore, by mycelium produced from a single basidiospore or with masses of basidiospores, and whether these methods will induce symptoms typical of those occurring in nature.

Research that resulted in the routine production of axenic cultures of *C. q. fusiforme* from multiple basidiospores has been underway for nearly 30 yr (Amerson *et al.* 1985). Success at obtaining a culture from a single basidiospore was first reported in 1984 by Frampton. However, techniques for routinely producing cultures from single basidiospores was reported in 1991 by Hu and Amerson. Their techniques involved casting basidiospores onto cellulose nitrate-acetate membranes that were suspended on media in petri dishes. After small colonies of mycelium were formed the membrane was removed and single basidiospores of *C. q. fusiforme* were transferred to this conditioned "nurse" medium. The medium (HG)was an altered version of that developed by Harvey and Grasham (1974). The final medium (HGYP) consisted of HG plus 0.1 g/L of $CaCO_3$ and 1 g/L each of yeast extract (Y) and peptone (P). Bovine serum albumin (10g/L)(BSA) was added to produce a medium identified as HGYP + BSA. Hu and Amerson (1991) were able to obtain cultures from up to 40% of single basidiospores of *C. q. fusiforme*. The conditioned "nurse" medium was essential for growth of single-spore (SS) cultures.

The SS culture technique of Hu and Amerson (1991) was used by Wang (1991) for inoculation experiments using multispore (MS) and SS cultures to inoculate seedlings of *P. taeda*. The pine seedlings were maintained with their radicles inserted into either modified gresshof and doy solution +Y+P (GDYP) or one-half strength GD. Seedlings were inoculated by placing segments of mycelia with media onto the seedling hypocotyls. Infection of hypocotyls was obtained with MS and SS cultures with and without wounding. There are no reported *in vivo* infections of pine seedlings with SS cultures of *C. q. fusiforme* under greenhouse conditions.

Miller and Matthews (1984) reported that potted 1-yr-old *P. taeda* seedlings became infected and produced typical galls when basidiospores were applied to wounds created by severing the upper portion of the stems. Galls also developed following inoculation of wounds created by pruning limbs from 5-yr-old *P. taeda* seedlings in a plantation.

In 1992 studies were initiated to evaluate the feasibility of routinely inoculating 6-week-old seedlings of *P. elliottii* var. *elliottii* and *P. taeda* through wounds created by severing the upper ca 1 cm from the seedling apices (Miller and Schmidt 1994, 1997). The success of the wound inoculations on seedlings of *P. elliottii* var. *elliottii* and *P. taeda* encouraged us to evaluate the technique to infect pine seedlings with mycelial cultures derived from single basidiospores of *C. q. fusiforme*. Results of recent research using genomic mapping procedures (Wilcox *et al.* 1996) and inoculation studies with virulent (V) and avirulent (A) isolates of *C. q. fusiforme* (Kuhlman *et al.* 1997) have demonstrated a resistant locus or gene (Fr1) in progeny of an open-pollinated family (10-5) of *P. taeda*. The Fr1 resistance in family 10-5 is specific (gene-for-gene), such that V isolates will overcome resistance whereas A isolates should not (Wilcox *et al.* 1996). The avoilability of seed of *P. taeda* family 10-5 and the V and A isolates of *C. quercuum fusiforme* provided us the opportunity to investigate the host-parasite interactions in this system using single-basidiospore cultures developed from the A and V isolates.

This paper summarizes the results of our studies on inoculating *P. elliottii* var. *elliottii* and *P. taeda* seedlings with MS and SS cultures through stem wounds.

2 Material and methods

2.1 Selection and culture of *Pinus* host families

The seedlings of *P. elliottii* var. *elliottii* inoculated in these experiments were from two half-sib families: highly susceptible and susceptible. Seedlings of a single half-sib family of *P. taeda* were inoculated. Seeds of *P. elliottii* var. *elliottii* were germinated, and the germlings were transplanted into leach tubes containing Vermiculite:Perlite:peat moss (3:1:1 by volume). Before inoculation, seedlings were kept in a growth chamber at 25–28° C. Light was from cool-white fluorescent bulbs with a photoperiod of 12 h. Seedlings were fertilized once with a liquid fertilizer (N-P-K, 15-30-15) before inoculation at 6 weeks. The culture of the *P. taeda* seedlings was similar except that seedlings were grown in a greenhouse after transplanting.

2.2 Axenic culture procedures

Collection and processing of basidiospores and growth of cultures are described by Amerson *et al.* (1985). Methods to produce SS cultures are described by Hu and Amerson (1991).

Large quantities of both MS and SS cultures were grown following a procedure used at the Institute of Forest Genetics, Placerville, CA, USA (G. Dupper, pers. comm.). This technique involves blending axenic cultures in GDYP medium for 1 min, and pipetting the resulting suspension into liquid GDYP medium in 250-mL flasks. Liquid cultures were maintained at

20° C without shaking and with no specific light requirement. After several weeks of growth, liquid was decanted, a small portion of fresh liquid medium was added, and the mycelium was homogenized. This suspension was pipetted into HGYP + BSA medium (Hu and Amerson 1991) in 60-mm plastic culture dishes and placed in an incubated chamber at 20–24° C. Maximum mycelial growth was obtained after 2 to 4 months, depending on the volume of mycelial suspension added.

2.3 Source of inocula

The basidiospore inoculum used on seedlings of *P. elliottii* var. *elliottii* was derived from aeciospores collected from galls at several locations in the states of Georgia and Florida. The inoculum for *P. taeda* was derived from virulent and avirulent single-urediniospore isolates of the pathogen from North Carolina and South Carolina (Kuhlman *et al.* 1997). The basidiospores were collected from germinating telia on leaves of *Quercus rubra* L. Single spores were isolated and cultured using a modification of the technique of Amerson *et al.* (1985).

2.4 Pine seedling inoculation

The procedures for inoculating both *P. elliottii* var. *elliottii* and *P. taeda* were the same. Seedlings were inoculated at about age 6 weeks. The apex of each seedling was severed a few millimeters below the base of the plumule with surgical scissors. Inoculum was applied quickly to the freshly cut stem surface. Inoculated seedlings were moved quickly into a mist chamber for periods varying from 24 to 72 h. Seedlings were then returned to a growth chamber or headhouse and finally to a greenhouse. Seedlings were typically observed for symptoms at 3, 6 and 9 months after inoculation.

A series of inoculation tests was initiated in 1993 using MS and SS axenic cultures of *C. q. fusiforme* on seedlings of both *P. elliottii* var. *elliottii* and *P. taeda*. Several different inoculation techniques were tried on wounds on *P. elliottii* var. *elliottii* seedlings.

The basic technique used a segment of mycelium with adhering medium (MAS). Small colonies (clumps) of mycelium (< ca 3 mm diameter) were used intact. Larger colonies were cut into ca 3–4 mm^2 segments using a surface-sterilized razor blade. The mycelial side of the segment was placed on the wound.

To determine if the resin exudate produced at the cut stem surface might inhibit infection, we blotted the exudate with a piece of sterile filter paper just before inoculation. To reduce desiccation of the inoculum segment, the water-holding compound Viterra® (Aglukon Agri-Products, Congers, N.Y., USA) was applied to the wound just before the inoculum. In another method, we tried in an effort to reduce desiccation of the inoculum segment on the seedling by inverting a micropipette tip over the mycelium–agar segment and upper part of the stem.

2.5 Inoculation of *P. taeda* seedlings having the Fr1 locus/gene

Axenic cultures, both MS and SS, were established from basidiospores produced on leaves of *Q. rubra* by *C. q. fusiforme* virulent (V) cultures NC2-36 and avirulent (A) cultures 5C35-5 (Kuhlman *et al.* 1997). Culture procedures yielded 7 SS isolates and 3 MS isolates from the V source and 18 SS isolates and 2 MS isolates from the A source.

Seedlings of the *P. taeda* family 10-5 (Kuhlman *et al.* 1997) were grown to age 8 weeks in a greenhouse at Athens, GA, USA. Sufficient quantities of the V and A cultures of *C. q. fusiforme* were produced to inoculate 324 seedlings: 20 seedlings with each of 2 A MS isolates; 44, 60 and 80 seedlings, respectively, with 3 A SS isolates, SCG, SCD and SCH; and 50 seedlings with each of 2 V SS isolates. The MAS inoculation procedure was used for all seedlings.

After inoculation, seedlings were moved into a humidity chamber at 20° C for 48 h and were kept in a headhouse for 24 h, then moved into a greenhouse. Seedlings were examined for symptoms after 3, 6 and 9 months. Seedlings with galls at nine months were taken to Gainesville, FL, USA, repotted, and placed in a greenhouse. Succulent stem tissues and needle samples were collected from the infected seedlings of family 10-5. The samples were subjected to DNA analysis to establish the presence or absence of the RAPD marker band J7-485A, which is tightly linked with resistance locus/gene Fr1 in family 10-5 (Wilcox *et al.* 1996, Kuhlman *et al.* 1997).

A second experiment with *P. taeda* family 10-5 and A and V cultures was established at the Resistance Screening Center (RSC), US Forest Service, Asheville, NC, USA, in September 1997. Seedlings were grown at the RSC and inoculated at age 10 weeks. The inoculum used was three V SS and two MS isolates and five A SS isolates. The total number of seedlings inoculated was 512, with the number per mycelial culture ranging from eight seedlings to 140 seedlings, depending on the quantity of different cultures. Seedlings were examined for symptoms after nine months. Those with galls were taken to Gainesville, repotted, and placed in a greenhouse.

2.6 Histological observations of infected seedlings of *P. taeda* family 10-5

Fourteen months after inoculation, samples were taken from galls on seedlings of *P. taeda* family 10-5 that were inoculated with V culture NCE. Blocks of tissue about 5 x 10 mm were cut from galls on each of eight seedlings. Samples were fixed in formalin - propionic acid - ethyl alcohol, embedded and sectioned by standard procedures, and stained in Pianeze IIIb (Vaughn 1914).

3 Results

3.1 Infection of seedlings of *P. elliottii* var. *elliottii*

The initial test using MS cultures to inoculate a highly susceptible and a susceptible half-sib family of *P. elliottii* var. *elliottii* was encouraging. Using homogenized mycelia in aqueous suspension or mycelial-agar segments (MAS), the infection of the two families was 22% and 50%, respectively.

Table 1. Results of inoculation experiments on seedlings of *P. elliottii* var. *elliottii* using single-basidiospore (SS) and multi-basidiospore (MS) axenic cultures of *C. q. fusiforme*.

			Axenic cultures			SS cultures			MS cultures	
Test	Date	Host families	SS	MS	Inoculation methods[1]	Number infecting	Seedlings inoculated No.	Seedlings infected No.	Seedlings inoculated No.	Seedlings infected No.
1	1/93	A	7	0	MAS	0	82	0	–	–
		B	7	0	MAS	0	21	0	–	–
2	3/93	A	6	1	MAS	6	38	8	10	2
					MAS + (Vt)	2	20	3	10	0
		B	6	1	MAS	1	17	1	20	2
					MAS + (Vt)	0	13	0	20	3
3	7/94	A	3	0	MAS	3	48	7	–	–
					MAS+B	3	24	5	–	–
4	10/94	A	4	0	MAS	0	48	0	–	–
					MAS+B	0	16	0	–	–
					MAS+T	0	16	0	–	–
					MAS+B	1	16	2	–	–
5	2/95	A	3	1	MAS+B	0	96	0	24	1
6	5/95	A	3	1	MAS+B	0	88	0	22	0

[1] MAS = mycelium-agar segment; Vt = Viterra; B = blot; T = micropipette tip (tube)

Over the next 3 years, we initiated inoculation experiments using both SS and MS axenic cultures of *C. q. fusiforme*. The results of these tests were highly variable (Table 1). Relative to the SS cultures, all cultures infected one or more seedlings in tests 2 and 3. In test 4, only one of the four SS cultures resulted in infection.

The most infection by MS cultures occurred in test 2, where the best results also were obtained with the SS cultures (Table 1). None of the variations in inoculation methods used was an improvement over using entire, small colonies or small segments cut from cultures (Table 1) with the mycelial surface placed onto the stem wound.

3.2 Infection of seedlings of *P. taeda* family 10-5 having the Fr1 locus/gene

At 9 months after inoculation of 324 seedlings of half-sib family 10-5, 11 seedlings had typical fusiform rust galls. Eight of the galls were on the 50 seedlings that were inoculated with a SS culture (NC-E) grown from the virulent *C. q. fusiforme* isolate NC2-36 (Kuhlman *et al.* 1997). An SS culture (NC-B) from the NC2-36 isolate produced a gall on a single seedling of 50. An MS culture (SC-MS-B) grown from the avirulent isolate of *C. q. fusiforme* (SC-35-S) infected and produced galls on 2 of 50 inoculated seedlings of family 10-5.

Of the 11 seedlings with galls that were genotyped, six were positive for the Frl locus/gene for resistance and five were negative (Table 2). Both of the seedlings with galls resulting from inoculation with the A MS-SC culture were negative. Among the nine seedlings with galls resulting from the two V cultures, six were positive for the Frl locus/gene and three were negative. The DNA profiles of the 11 infected 10-5 seedlings were compared with the profiles of the samples of the family collected from field plots in 1994 (H.V. Amerson, pers. comm.). The analysis showed that the 11 samples were definitely from *P. taeda* family 10-5.

The previous experiment was repeated using three SS and one MS culture of the V isolate NC-2-36 and five SS cultures of the A isolate SC 5-35 to inoculate 512 seedlings of *P. taeda* family 10-5. After nine months, only four seedlings had galls: one from A-SC-SSG, two from V-NC-SSD, and one from V-NCMS. These infected seedlings were not genotyped.

Table 2. Presence (+) or absence (–) of the locus/gene (Fr1) in the infected seedlings of *Pinus taeda* half-sib family 10-5.

Seedling	Inoculum	Source and virulence of SS and MS cultures	Fr-1 locus/gene presence
A	SSNC-E	NC2-36 V	+
B	SSNC-E	NC2-36 V	+
C	MS-SC-B	SC35-5 AV	–
D	SSNC-E	NC2-36 V	–
E	SSNC-E	NC2-36 V	+
F	SSNC-E	NC2-36 V	–
G	SSNC-E	NC2-36 V	–
H	SSNC-E	NC2-36 V	+
I	SSNC-E	NC2-36 V	+
J	SSNC-B	NC2-36 V	+
K	MS-SC-B	SC35-5 AV	–

3.3 Histological analysis of gall tissues from seedlings infected by a virulent, single-spore culture

The outward morphology of the galls produced on the seedlings was quite typical in size and shape for the period after inoculation (Fig. 1).

Examination of the stained thin sections from the samples taken from the eight galls induced by V-SS culture NC-E showed both similarities to and differences from typical fusiform rust galls.

The pattern of colonization of the SS isolate in gall tissue was very similar to that previously reported and illustrated by Jewell *et al.* (1962) and Miller *et al.* (1976) for typical fusiform rust galls. A difference was the less intensive development of the fungus in the areas colonized (Fig. 2). Also, most of the pathogen-induced rays were only a single cell wide (Fig. 3), whereas the rays of fusiform rust galls induced by basidiospores were many cellswide.

A major difference in these galls was the unusual development of the spermogonial layers. Most of the samples from the eight galls had layers of what appeared to be nearly mature spermogonia (Fig. 4A). However, none of these spermogonial had ruptured the epidermis. Also, the cortical cells underlying the spermogonia layers were necrotic, based on the staining reaction, and a periderm had formed between the necrotic cortical cells and the living cortical cells (Fig. 2). A similar zone of darkly stained cortical cells and a newly formed periderm were also apparent even in the seedlings that had not developed spermogonia. In typical fusiform rust galls resulting from infection by masses of basidiospores of *C. q. fusiforme*, spermogonia and underlying cortical cells are identifiable (Fig. 4b). The spermogonia are releasing spermatia through the ruptured epidermis, large quantities of apparently vigorous hyphae are present connecting the spermogonia and the cortical tissues, and the cortical tissue contains large numpers of both hyphae and haustoria.



Figure 1. Fusiform rust galls on seedlings of *Pinus taeda* (family 10-5) at 15 months after wound inoculation with an axenic single-spore culture of a virulent isolate of *Cronartium quercuum* f. sp. *fusiforme*.

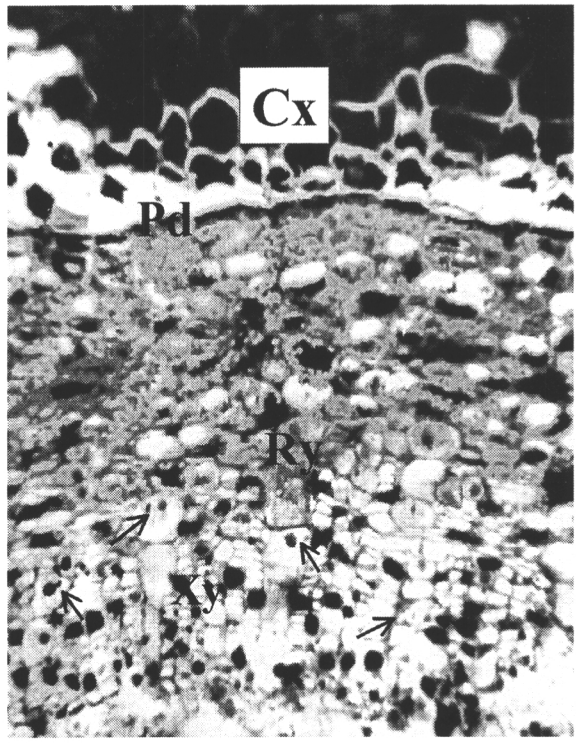

Figure 2. Portion of a fusiform rust gall on seedling of *Pinus taeda* (family 10-5) at 15 months after inoculation showing intensively stained and necrotic cortical cells (Cx), secondary periderm (Pd), and abnormal cells induced by *Cronartium quercuum* f. sp. *fusiforme*. Note the enlarged ray cells (Ry) and abnormal xylem (Xy). Arrows indicate hyphae and haustoria.

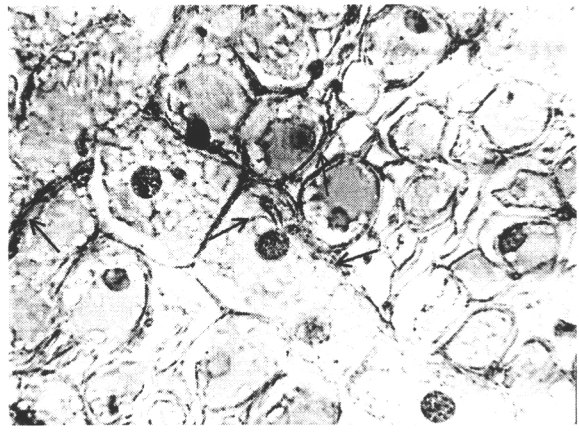

Figure 3. Typical pathogen-induced ray cells in a fusiform rust gall on wound-inoculated seedling of *Pinus taeda* (family 10-5), showing hyphae and haustoria of *Cronartium quercuum* f. sp. *fusiforme*.

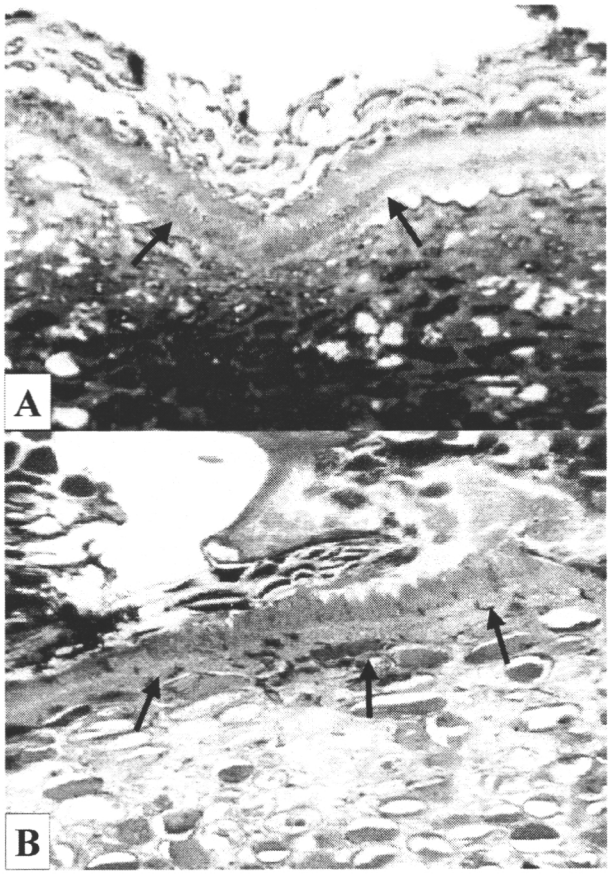

Figure 4. Photomicrographs of spermogonia and underlying cortical cells. (A) Galls induced by a single-basidiospore culture of *Cronartium quercuum* f. sp. *fusiforme* showing no release of spermatia and necrosis of cortical cells. (B) Example of a spermogonial layer, release of spermatia, dense areas of hyphae supporting the spermogonia (arrows), and living cortical cells with numerous hyphae and haustoria in a gall produced following inoculation with mass basidiospores.

4 Discussion

We have demonstrated for the first time that it is possible to obtain infection and typical galls on seedlings of *P. elliottii* var. *elliottii* and *P. taeda* with haploid, single genotypes of *C. q. fusiforme*. Fourteen of the cultures grown from single basidiospores of *C. q. fusiforme* induced typical galls on one or more seedlings inoculated through stem wounds. What we can not explain is why such a small percentage of inoculated seedlings was infected and why the results were so variable from test to test.

A completely negative response to the inoculations would clearly indicate either that axenic cultures of *C. q. fusiforme* can not infect wounded tissues of susceptible *Pinus* seedlings or that the inoculation and/or incubation procedures were inappropriate. In fact, few of the inoculation experiments

with either *Pinus* species were complete failures. Although pathogen variability among the SS cultures is a possible, a frequent occurrence was infection on 1 or 2 seedlings out of 10–24 inoculated with the same culture. Also, there was a similar inconsistency with the use of MS cultures, a technique that would greatly reduce the probability of the culture being avirulent. Also, the half-sib family of *P. elliottii* var. *elliottii* inoculated most frequently was the most fusiform rust susceptible family we have tested. In fact, this family is consistently infected at over 95%, and frequently has 100% galls with both spray and wound inoculation with mixed basidiospores. These facts would suggest a lack of any genes for resistance.

The results obtained with the inoculation of *P. taeda* half-sib family 10-5 with the SS and MS cultures derived from the V and A isolates of *C. q. fusiforme* were similar to those on *P. elliottii* var. *elliottii*. Only 11 of 324 inoculated seedlings (3%) developed galls after nine months. Eight of those galls resulted from one V SS mycelial culture. However, even in this case the percent gall development was relatively low (8/50 = 16%). Since all 50 seedlings were inoculated at the same time with the same V mycelial culture and incubated under the same conditions, it is difficult to explain the low percentage of gall development.

The second inoculation experiment with V and A SS mycelial cultures on family 10-5 resulted in even less success: only four seedlings out of the 512 inoculated developed galls after nine months. None of the three isolates that infected 10-5 in the first experiment infected any seedlings in this experiment. The 512 seedlings inoculated in the second 10-5 test were far more vigorous and succulent and, based on previous experience, should have been infected.

Inappropriate or ineffective inoculation techniques may account for the lack of consistency in this research. However, as was true with the infectivity of the SS cultures, the same techniques that were successful in one test failed in the next.

The histological examinations of the gall tissue on *P. taeda* family 10-5 are the first of seedlings infected by a single genotype of *C. q. fusiforme* under greenhouse conditions. Although it is inappropriate to make any definitive conclusions from the examination of six galls induced by one single basidiospore isolate on a single half-sib family, the interactions observed on these seedlings warrant further study. One interesting response of the seedlings examined was the formation of the large areas of apparently normal spermogonia that ceased development just before they would normally emerge through the host epidermis. Whether or not this is an artifact of this particular test or an important characteristic of host reactions to a haploid culture of *C. q. fusiforme* should be determined.

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Spatial stability of fusiform rust resistance in slash pine in the coastal plain of the southeastern USA

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Summary

A study to characterize spatial and temporal stability of fusiform rust resistance among selected resistant families and susceptible family checks of slash and loblolly pine was established in the coastal plain of the southeastern USA in 1987, with subsequent plantings in 1989, 1991, and 1994. This report summarizes results of the 1987 plantings of slash pine at age 5 yr established at 14 locations. At each location, progeny from four rust-resistant and two rust-susceptible open-pollinated families were planted in five 10-tree-row plots. The number of branch and stem galls and total tree height were recorded annually for each tree through age 5 yr. Rust incidence was normally low in years 1 and 2 but increased significantly by age 5 yr as potentially susceptible tissue increased or conditions became more favorable for infection. Among locations, the percentage of trees infected and number of galls/plot on the two rust-susceptible families averaged 66.1 and 23.7, respectively. Among locations, the percentage of trees infected and number of galls per plot on the four resistant families averaged 15.6 and 3.2, respectively. Among resistant families the effects of families, locations, and family-by-location interaction were significant for rust incidence variables. Differential height growth and variation in rust incidence did not appear to cause the spatial instability of resistant pine families.

Key words: *Cronartium quercuum* f. sp. *fusiforme,* disease resistance, pest management, *Pinus elliottii* var. *elliottii*

1 Introduction

Fusiform rust, caused by *Cronartium quercuum* (Berk.) Miy. ex Shirai f. sp. *fusiforme*, is the most important disease of intensively managed slash (*Pinus elliottii* Engelm. var. *elliottii*) and loblolly pine (*P. taeda* L.) in extensive areas of the southeastern USA and causes millions of dollars of loss annually (Powers *et al.* 1975, Anderson *et al.* 1986). Genetically rust-resistant slash and loblolly pines are planted to significantly reduce losses (Schmidt *et al.* 1981, 1985). The benefit/cost ratio to develop resistance to fusiform rust is estimated to vary from 5 to 20, depending on forest management options (Pye *et al.* 1997). Notwithstanding the successes, much basic information is needed on the nature of resistance. Specifically, the quantity and quality of genes influencing resistance are largely unknown, as is the temporal and

spatial variation in the heterogeneous pathogen population. This information is needed to develop effective breeding and gene deployment strategies. Recently, Wilcox *et al.* (1996) identified a major gene for fusiform rust resistance in loblolly pine. Several additional genes associated with fusiform rust resistance in loblolly pine have been identified (H.V. Amerson, pers. comm.). Major gene resistance has also been described in other tree rust pathosystems, e.g. white pine blister rust (Kinloch *et al.* 1970) and poplar rust (Newcombe *et al.* 1996). There is abundant evidence of substantial pathogenic variability in *C. q. fusiforme* (Snow and Kais 1970, Snow *et al.* 1975, Powers 1980, Snow and Griggs 1980, Powers 1985, Kuhlman 1990), but additional knowledge is needed to understand better the temporal and spatial variation in pathotypes and the stability of resistance in pine.

A study to characterize temporal and spatial variability in the fusiform rust pathogen and stability of resistant slash and loblolly pine families was established in the coastal plain of the southeastern USA in 1987, with subsequent plantings in 1989, 1991 and 1994. This report summarizes the spatial stability at age 5 yr of slash pine established at 14 locations in 1987.

2 Methods

2.1 Plantation establishment

Slash pines were planted at 14 locations in the coastal plain in AL, FL, GA and MS (Fig. 1). Most locations had well- to moderately well-drained soils most often associated with the presence of the alternate oak host and high rust incidence (Froelich and Snow 1986, Schmidt *et al.* 1990). Plantings were mostly on previously forested sites of moderate to high site quality. Site preparation varied, depending on soil characteristics and previous land use; sites did not receive cultural treatments.

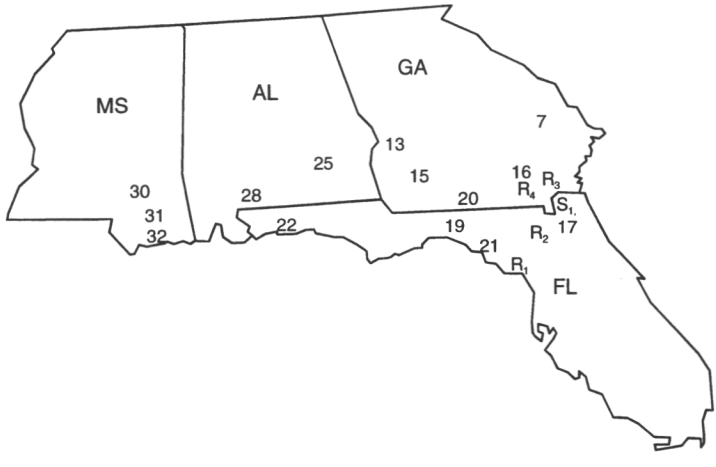

Figure 1. Approximate location and number of 14 sites to study the geographic variation in fusiform rust incidence among progeny of six open-pollinated slash pine families planted in the coastal plain of the southeastern USA. Locations where the four rust-resistant (R1-R4) and two rust-susceptible (S1 and S2) maternal parents were selected are shown.

Seedlings were grown at one nursery in Georgia. Seeds were sown in the spring of 1986 and bare-root seedlings were planted at 1.8x3.0 m spacing (6x10 ft) the following December–January. Progeny from six open-pollinated families, four rust-resistant (R1–R4) and two rust-susceptible (S1 and S2), were planted at each location. The maternal parents were originally selected in SE Georgia and NE Florida, and the seeds were collected from orchards in those areas. At each location, five blocks were established, and in each block, progeny of each family were planted in random10-tree-row plots. A total of 300 seedlings (50 per family) was planted at each location.

2.2 Data collection

The number of branch and stem galls and tree height were recorded for each tree annually for 5 yr. Normally data were collected in the winter after gall formation and before the initiation of height growth. The percentage of trees with one or more galls (% rust), the number of galls per 10-tree-row plot (# galls per plot), the percentage of rust in each resistant family as a proportion of the mean percentage rust in the two susceptible families (R/S ratio), and average tree height per 10-tree-row plot (tree ht) were calculated. The R/S ratio was an attempt to standardize % rust in resistant families among locations, which varied in rust incidence on susceptible check families.

2.3 Data analyses

An analysis of variance (randomized block) assessed the fixed effects of locations (L) and families (F) and the FxL interactions on % rust, # galls per plot, R/S ratio, and tree height. The % rust was transformed to the inverse sine of the square root of the proportion of trees infected: the # galls per plot was transformed to the square root of the number of galls per plot to stabilize the experimental error variance. In addition to the analysis of variance, family performance across locations was examined by correlation and regression analyses and also by pairwise comparisons between locations. Since the experimental error variance likely was not constant among locations, an adjusted critical value from Tukey's honestly significant test ($p = 0.10$) was used in the pairwise comparisons between locations. This adjusted critical value provided a conservative test of significance and minimized Type I errors.

3 Results

3.1 Rust incidence

3.1.1 Location effects

There were statistically significant location (L) effects for cumulative 5-yr rust incidence variables (% rust, # galls per plot, and R/S ratio) for resistant and susceptible families (Table 1). Among resistant families, average rust ranged from 1.4% at L22 to 31.3% at L19, whereas that for susceptible families ranged from 37.9% at L32 to 91.8% at L20 (Fig. 2). The average cumulative # of galls per plot at age of 5 yr in the two susceptible families varied from 7.9 to 68.6 across locations and overall averaged 23.7. The average # of galls per plot at age of 5 yr in resistant families varied from 0.1 to 10.9 and averaged 3.2. The average R/S ratio in resistant families varied from 0.034 to 0.375 across locations and averaged 0.223.

Table 1. Analysis of variance at age of 5 yr for cumulative fusiform rust incidence and pine height in progeny of four rust-resistant and two rust-susceptible open-pollinated slash pine families planted at 14 locations in the coastal plain of the southeastern USA.

		Mean squares			
Source	df	% trees infected	# galls/plot	R/S ratio[1]	Total tree height[2]
Resistant families					
Location	13	0.2777***[3]	72.62***	0.1725***	1768.7***
Block (Loc)	56	0.0316***	5.03	0.0468***	33.0***
Family	3	0.1890***	23.96***	0.2505***	87.0***
Fam*Loc	39	0.0313***	8.16***	0.0437**	14.1**
Error	153	0.0186	4.27	0.0264	9.07
Susceptible families					
Location	13	0.3768***	173.67***		712.8***
Block (Loc)	55	0.033	18.48		16.9
Family	1	0.1369**	77.16**		45.8*
Fam*Loc	11	0.0382	12.39		14.2
Error	39	0.0297	16.35		14.57

[1] R/S ratio represents the ratio of proportion of trees infected with fusiform rust in resistant families to the average proportion of trees infected in the two susceptible families at each location.
[2] Average total height was weighted by the total number of living trees on the plot at age 5 yr. Plots with less than five living trees at age 5 yr were deleted from all analyses.
[3] *, **, and *** indicate statistical significance at p=0.10, 0.05, and 0.01, respectively.

Table 2. Pairwise comparisons of fusiform rust incidence (% rust and R/S ratio)[1] within resistant family R3, among six locations with high percentage rust on susceptible families, for progeny of open-pollinated slash pine planted in the coastal plain of the southeastern USA.

	Susceptible families S1 and S2	Resistant family (R3)	
Location[1]	Average % rust	% rust[2]	R/S ratio[2]
19	90.1	50.9a[3]	0.56a[3]
20	91.8	35.9a	0.39a
25	80.9	32.0a	0.40a
13	77.2	24.4b	0.32a
15	83.0	22.0b	0.27a
28	78.9	5.9c	0.07b

[1] Refer to Fig. 1 for location.
[2] % rust is percentage trees with rust and R/S ratio is (percentage oftrees with rust on resistant family R3)/(percentage trees with rust on the average of susceptible families S1 and S2 for each location).
[3] Within columns, values with the same letter are not significantly different (p= 0.10).

A significant main effect of location for % rust, # of galls per plot, and the R/S ratio (Table 1) may be expected, since it reflects the variation in rust hazard of the location. However, pairwise contrasts within families also identified significant location effects for rust incidence variables among locations having similar high rust incidence. For example, in resistant family R3, % rust and R/S ratio varied significantly among six high-rust incidence locations (Table 2, see also family R3 in Fig. 3). There were similar significant location effects for family R1, but not for families R2 and R4.

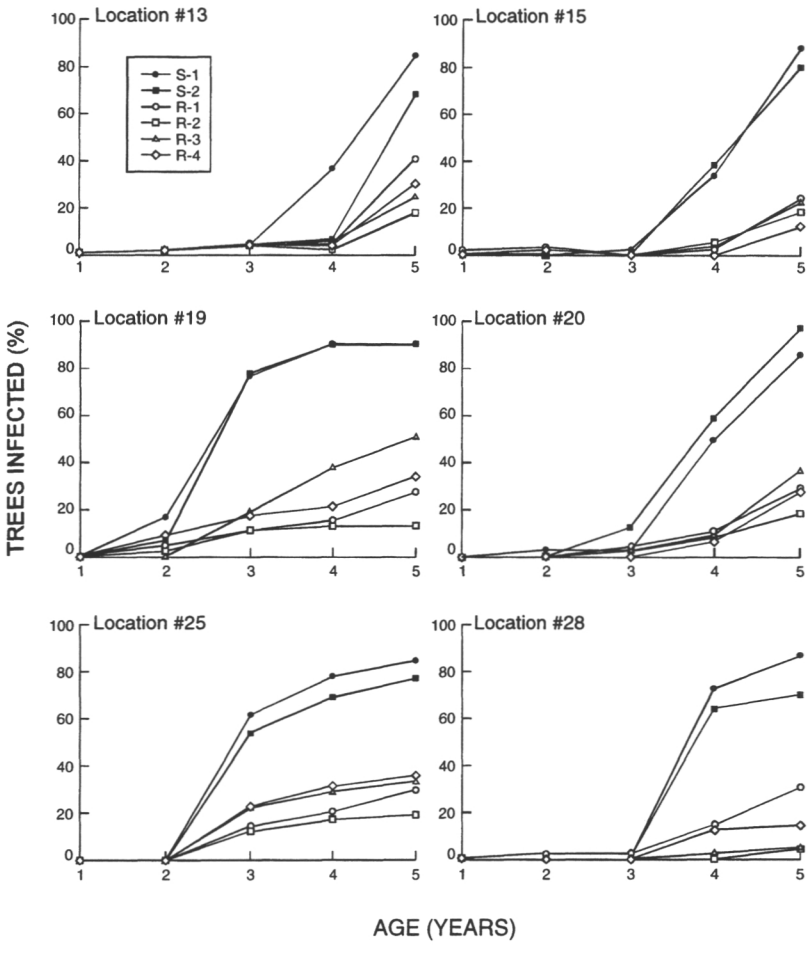

Figure 2. Average fusiform rust incidence (% trees infected) at age 5 yr on progeny of two rust-susceptible (S1 and S2) and four rust-resistant (R1-R4) open-pollinated slash pine families planted at 14 locations in the coastal plain of the southeastern USA. Location numbers refer to Fig. 1 and are arranged in order of increasing average rust incidence on susceptible families. Each datum point is the average of five 10-tree-row plots, when there were no missing trees or plots.

3.1.2 Family effects

There were statistically significant family effects for cumulative 5-yr rust incidence variables for resistant and susceptible families (Table 1). Cumulative % rust averaged 69.1 and 62.4 for families S1 and S2, respectively, and 66.1 overall. Percentage rust averaged 19.0, 9.7, 17.1 and 16.0 for families R1–R4, respectively, and 15.6 overall. Cumulative # of galls per plot averaged 29.3 and 20.6 for families S1 and S2, respectively, and 23.7 overall. The # of galls per plot averaged 3.9, 2.0, 4.4, and 2.9 for families R1–R4, respectively, and 3.2 overall. The R/S ratio averaged 0.28, 0.14, 0.24, and 0.23 for families R1–R4, respectively.

Percent rust in resistant families increased in proportion to that in susceptible families (Figs. 2–3). The regression analyses indicate that family R2 was most stable and family R3 was least stable, especially at locations with high rust incidence. Families R1 and R4 were also relatively unstable across locations (Fig. 3).

Cumulative disease progress curves for all families at six locations (Fig. 4) indicate that typically % rust was low in years (age) 1 and 2 but increased significantly in years 3 through 5, as potentially susceptible tissue increased. However, at some locations % rust deviated from this typical pattern, likely influenced by weather.

3.1.3 Family-by-location interactions

The FxL interaction was statistically significant for the three rust incidence variables among resistant families, but was not statistically significant for susceptible families (Table 1). There are numerous FxL interactions among resistant families, and also between resistant and susceptible families (Fig. 2; ordered by increasing % rust on susceptible families). FxL interactions are evident as "family lines" cross between adjacent locations. For example, between L22 and L30 there are changes in % rust among resistant families and between resistant and susceptible families, especially for family R1. Between L15 and L19, and L19 and L20, the % rust increases on some resistant families, but decreases on other resistant families. Also % rust increases slightly on susceptible families between L13 and L28, whereas that of resistant families decreases.

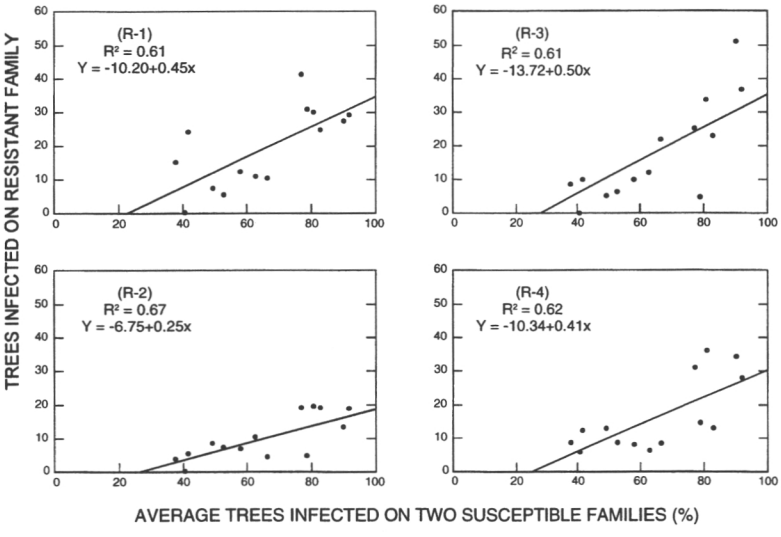

Figure 3. Fusiform rust incidence (% trees infected) at age 5 yr on progeny of four rust-resistant open-pollinated slash pine families (R1-R4) in relation to average rust incidence on two rust-susceptible open-pollinated slash pine families (S1 and S2) planted at 14 locations in the coastal plain of the southeastern USA. When there were no missing trees or plots, each datum point for a resistant family is the average of five 10-tree-row plots. Averages for susceptible families are from five 10-tree-row plots for each of two families.

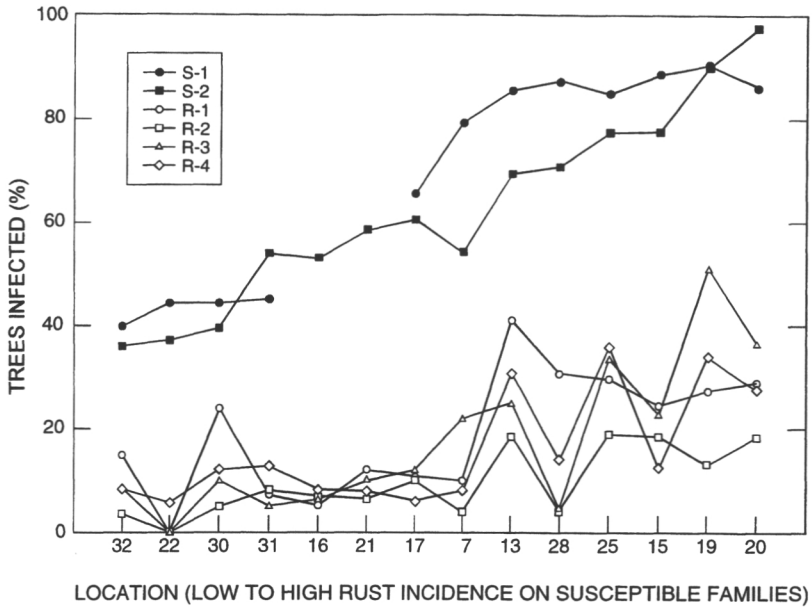

Figure 4. Cumulative disease progress (% trees infected) for progeny of fusiform rust susceptible (S1 and S2) and resistant (R1–R4) open-pollinated slash pine families planted at six locations in the coastal plain of the southeastern USA. Location numbers refer to Fig. 1.

A strong FxL interaction in indicated by % rust, # of galls per plot, and R/S ratio, which differ greatly between families R1 and R3 at L19 and L28 (Fig. 5). The height data (Fig. 5) suggest that the amount of susceptible tissue was not the cause of the FxL interaction.

3.2 Pine height

Among resistant families, total pine height at age 5 yr was significant for locations, families, and the FxL interaction (Table 1). Among susceptible families, height varied significantly for locations and families. There was no strong correlation between height and % rust among locations or families, suggesting that the amount of susceptible tissue (as judged by height) was not responsible for the FxL interactions.

4 Discussion

The regional pattern of rust incidence was similar to that reported in recent surveys (Anderson *et al.* 1988, Starkey *et al.* 1997); the few exceptions were likely due to local conditions, i.e., alternate host concentrations (Froelich and Snow 1986). The pattern of disease increase was typical of that reported by Froelich and Snow (1986) and Schmidt and Allen (1997), i.e., rust incidence was low in year 1 and 2, and increased in years 3–5 as potentially susceptible tissue increased. Exceptions to this pattern may have been conditioned by local weather.

Rust incidence on resistant families increased in proportion to that on susceptible families, i.e., as inoculum potential increased. Similar trends for

slash pine have been reported (Goddard and Schmidt 1979, Sohn and Goddard 1979, Hodge *et al.* 1993, Schmidt and Allen 1997). Families R1, R3, and R4 were relatively unstable, but the most resistant family (R2) was stable across locations and rust incidence levels.

The statistically significant main effects of the location for % rust and # galls per plot are, in part, the result of varyable rust incidence among locations, as measured by the two susceptible check families. However, the significant difference among locations for % rust and R/S ratio, identified by the pairwise comparisons among locations with similar high rust incidence, shows that some families, e.g., R3, were unstable across locations with similar high rust incidence. The cause of this family instability was not due to variation in rust incidence.

Of primary importance were the significant FxL interactions for all rust incidence variables. The cause of the FxL interactions cannot be conclusively determined from these field studies, where potential causal factors (e.g. climate, phenology, amount of susceptible tissue, rust incidence, host resistance mechanisms, and pathogen variability) are confounded. However, our data indicate that the amount of susceptible tissue (as indicated by height) and the level of rust incidence were not responsible for the FxL interactions. We suggest that host-pathogen variability is the primary cause of the FxL interaction and we will attempt to confirm this with artificial inoculations using inocula collected from the locations and families represented here.

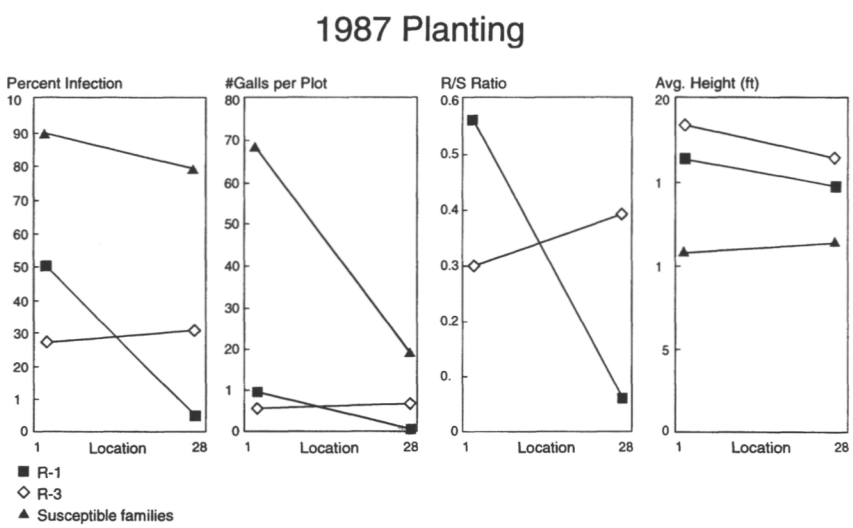

Figure 5. Family-by-location (FxL) interaction of % fusiform rust, # galls per plot and R/S ratio for progeny of two rust-resistant slash pine families (R1 and R3) planted at two locations (19 and 28) in the coastal plain of the southeastern USA. Location numbers refer to Fig. 1. R/S ratio = % rust on resistant families (R) as a proportion of the average % rust on the two susceptible families (S). Data on rust-susceptible families and pine height are shown for comparison.

In conclusion, resistance of slash pine families has demonstrated significant family and location effects and FxL interactions for rust incidence in our field studies. One family was stable across locations and levels of rust incidence; other families were unstable across one or both of these variables. Other studies have identified location, family, FxL, and family-by-inoculum effects in field or laboratory studies (Snow and Kais 1970, Snow *et al.* 1975, Sohn *et al.* 1975, Goddard and Schmidt 1979, Snow and Griggs 1980, Walkinshaw and Bey 1981, Powers and Zoerb 1982), and most have concluded that the primary cause was pathogen variability. Whatever the cause of this spatial instability of the rust-resistant families, a better understanding of spatial and temporal variability of host and pathogen in the field will result in more effective pine breeding and deployment strategies for fusiform rust management. This is especially important as intensive forestry moves toward clonal plantations.

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Research on the biology of fusiform rust in the southeastern United States

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Summary

The incidence of fusiform rust throughout the southeastern United States has continued to be one of the major forest disease problems in the southeastern United States. In the past, much of the research has concentrated on field studies with provenance selection and genetic breeding of pine families to increase resistance in the host. In the last ten years, there has been an increased interest in the actual biology of the fusiform rust fungus. Three areas of research on-going in the south are overlapping the areas of molecular, cellular, and population biology. In order to control the disease, a combination of host genetics will be valuable, but so will a biological understanding of the survival mechanisms and ecology of *Cronartium* species. In the last decade, research has started in the southeastern United States with collaborations between federal and state agencies concentrated on the biology of fusiform rust.

Key words: *Cronartium quercuum* f. sp. *fusiforme*, *Pinus elliottii*, *Pinus taeda*

1 Introduction

Fusiform rust incidence has increased over time with the intensive culture of loblolly (*Pinus elliottii* Engelm. var. *elliottii*) and slash (*P. taeda* L.) pines. At present, the annual losses to fusiform rust have been estimated at $35 million in the five southern states of Florida, Georgia, South Carolina, North Carolina, and Virginia, with significant losses occurring in Alabama, Mississippi, and east Texas as well (Schmidt 1998). The incidence of fusiform rust from the causal fungal pathogen *Cronartium quercuum* (Berk.) Miy. ex Shirai f. sp. *fusiforme* has increased as land use patterns have changed in the southeastern United States (Pye *et al.* 1997). Former agricultural lands used for the production of crops, such as cotton and tobacco, have been increasingly planted for forestry crops since the turn of the century. Current estimates of slash and loblolly pine acreage in the southern United States is 47.9 million acres (Starkey *et al.* 1997). Although a recent study of forest inventory data for four states (Mississippi, North Carolina, South Carolina, and Virginia) has shown a downward trend in rust infection, there are few long-term studies assessing whether it is management changes or improved genetics that may be influencing changes on long-term performance. Although many years and much federal and state funding have been spent on examining provenance studies and the incidence of rust, far less research has actually been done on what conditions and genetics favor or

slow fusiform rust spread in the field, and on the biology, ecology, and genetics of the organism itself.

2 Population biology

Three areas of research concentration in the southern United States have been in the population, molecular, and cellular definition of resistance responses. Initial population studies on the *C. q. fusiforme* pathogen were undertaken in the USDA, Forest Service, Southern Institute of Forest Genetics in Mississippi. Pycnial samples were collected over a wide range of the southern and southeastern United States to determine patterns of genetic differentiation among and within field populations using RAPD markers. In the results of this initial research, there appeared to be some genetic differences in populations east and west of the Mississippi River (Hamelin *et al.* 1994). There appeared to be more variation in certain regions, and research was extended to a more comprehensive sampling of pycnial samples. A larger collection has been taken of pycnial populations on twenty or more loblolly pines at 25 geographic locations throughout the southern and southeastern United States. This study was joined by the researchers at the USDA Forest Service in Athens, Georgia, and the Daniel B. Warnell School of Forest Resources at the University of Georgia. At present, the Southern Institute Forest Genetics has identified 21 potential markers that are polymorphic and showed consistent bands. These markers were identified by Doudrick *et al.* (1993), and correspond to RAPD markers that either have been found in genetic maps constructed for a segregating population of *C. q. fusiforme* infecting loblolly pine or were unlinked to map loci. The goals are to examine the amount of relatedness among the different geographical locations of the populations (Roberds *et al.* 1997). This research will provide a broader picture of the genetic variability present in the pathogen population. Samples were taken to measure variability within small natural stands and between stands. Sampling these sites over time will present a temporal view of the pathogen's population changes in natural stands.

3 Molecular biology

Differential display of cDNAs is being used to unravel the interaction between *C. q. fusiforme* and pine. Researchers in Sarah Covert's Lab at the University of Georgia are examining the differential transcription of genes in healthy and *C. q. fusiforme* infected pine (Covert *et al.* 1997). Their research goals are two-fold. First, they want to improve our basic understanding of how *C. q. fusiforme* causes galls to form on pine trees. Ultimately, they want to use the information gained from these basic studies to design genetic engineering strategies that will create resistant pines. The structural and biochemical characteristics of fusiform rust galls all indicate that *C. q. fusiforme* is interfering with the normal growth and development of pine xylem. Their working hypothesis is that the fungus causes at least some of these effects by altering pine gene expression at the level of transcription. To determine if pine genes in galls exhibit different patterns of transcription than pine genes in healthy tissue, and to identify particular genes that are altered in their transcription, they are using the polymerase chain reaction (PCR)-based

technique known as differential display. They chose this technique in part because it will allow them to clone and characterize the affected genes. It also will allow them to identify fungal transcripts that may play a role in gall formation. The infected and healthy seedlings used in this study are from a rust susceptible seed lot and are provided by Carol Young at the USDA, Forest Service, Rust Screening Center, Asheville, NC. For the differential display experiments, each infected tree is paired with a healthy tree, and RNA is extracted from three different types of tissue: galled, asymptomatic, and heathy. Each RNA sample is reverse transcribed to produce cDNA. A subset of each cDNA sample is then amplified in the PCR by a variable combination of arbitrary and/or oligo-dT anchored primers. The resulting products are separated by size on a polyacrylamide gel. After the differential display reactions, the differentially transcribed cDNAs are cut from the acrylamide gel, reamplified, and cloned. The clones are sequenced and compared to sequences filed at GenBank, at the National Center for Biotechnology Information.

To date, Dr. Covert's lab has cloned 22 interesting cDNA fragments that appear to be differentially transcribed in galled and healthy pine tissues. Their current work focuses on verifying the differential transcription of the genes encoding these clones and determining if they are from the fungal or the pine genome. In the near future, they plan to select a few of these clones for more detailed analysis in an effort to determine what role, if any, they play in gall formation.

4 Cellular biology

The final area of concentration is in cellular responses of early events in germination and infection. In work by Wilcox *et al.* (1996), in which genomic mapping was used to identify a region of the loblolly pine genome that determines resistance to fusiform rust, random amplified polymorphic DNA (RAPD) markers used for mapping were obtained by genotyping haploid megagametophytes. The genotype of the megagametophyte is identical to the contribution of the seed parent to the diploid seedling. The seedling was then transplanted and grown. In this case, we know the genetics of the individual seedling. Working with progeny sets inoculated with *C. q. fusiforme*, segregating RAPD markers amplified from megagametophyte DNA were used to construct genomic maps for the parent families. Genomic maps were then used to detect associations between markers and symptom types in a factorial experiment where full-sib progeny were screened in the greenhouse against multiple pathogen isolates and scored for disease resistance. They found the resistance locus due to its very high association with field resistance. Having this advanced knowledge of whether a seedling carries a major gene for resistance to a known pathogen isolate allows us to plan some future cellular studies looking at how seedlings of known resistance respond at the early stages of inoculation and infection. The question that we would like to ask with our research is whether there are early resistance and susceptible responses that can be characterized when genotypes of seedling and fungal sources are held constant.

5 Guidelines for the future

Defining fusiform rust biology is an intricately woven act because we are trying to understand the biology of this organism foremost without the overlapping complexities contributed by its obligate host. After understanding the biology of the organism, our next area of interest is that of deployment. Wilcox *et al.* (1996) provided an open window to begin sorting the resistance genes in families. Since we have made breakthroughs on being able to designate a host as having or carrying a major gene for resistance (Wilcox *et al.* 1996), we can begin to set up a stable of tester plants. In the future we would like to forecast the deployment of known genotypes to monitor pathogen virulence.

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Differences in molecular characteristics between *Cronartium quercuum* from Japan and fusiform rust from USA

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Summary

The molecular characteristics were compared among *Cronartium quercuum* f. sp. *densiflorae* and *C. quercuum* f. sp. *thunbergii* from Japan and *C. quercuum* f.sp. *fusiforme*, fusiform rust from the USA. We examined the PCR-amplifield internal transcribed spacer (ITS) regions of ribosomal DNA from Japanese and American sources of aeciospores by restriction fragment length polymorphism (RFLP) analysis and nucleotide sequences. The ITS regions of six collections from *Pinus densiflora* and four collections from *P. thunbergii* from Japan and five collections from the native American hosts *P. taeda* or *P. elliottii* were amplified by PCR. The RFLP patterns with the three enzymes *Dra* I, *Hinf* I and *Taq* I showed clear differences between the two Japanese *formae speciales* and the American *C. quercuum* f. sp. *fusiforme*. No differences were found in the RFLP patterns between ff. spp. *densiflorae* and *thunbergii*. Nucleotide sequences of the ITS2 region confirmed the distinction between ff. sp. *densiflorae* and *fusiforme*. Comparisons with sequence data obtained from GenBank database on the other *formae speciales* in *C. quercuum* also comfirmed the distinctiveness of f. sp. *densiflorae*. These molecular data supported the morphological differences reported earlier between the Asian and American forms in the *C. quercuum* complex.

Key words: *Pinus densiflora*, *Pinus elliottii*, *Pinus taeda*

1 Introduction

In the *Cronartium quercuum* (Berk.) Miy. ex Shirai complex, Kaneko *et al.* (1991) and Kuhlman and Kaneko (1991) found apparent distinctions in basidiospore morphology between Asian and American collections. They also reported slight morphological differences among the four North American *formae speciales*. Others have reported distinctions within the North American *formae speciales* by isozyme analysis (Powers *et al.* 1989, 1991) and nucleotide sequences (Vogler and Bruns 1998).

Within the Japanese sources, Kondo (1975) suggested the existence of two physiological strains of the rust, a *Pinus densiflora* Sieb. et Zucc. (Japanese red pine) – *Quercus serrata* Thunb. strain and a *P. thunbergii* Parl. (Japanese black pine) – *Q. acutissima* Carr. strain, based on inoculation experiments.

The two strains in Japan were designated as *C. quercuum* f. sp. *densiflorae* and f. sp. *thunbergii* by Kuhlman and Kaneko (1991).

To compare differences in the molecular characteristics between *C. quercuum* ff. spp. *densiflorae* and *thunbergii* from Japan, and also those between Japanese forms and the fusiform rust, *C. quercuum* f. sp. *fusiforme* in the USA, we examined the PCR-amplified internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) from aeciospores by restriction fragment length polymorphism (RFLP) analysis and nucleotide sequences.

2 Material and methods

2.1 Fungal collections

Ten aeciospore samples of *C. quercuum* were collected from various localities in Japan (Table 1). Six aeciospore collections were from *P. densiflora* galls and four aeciospore collections from *P. thunbergii* galls. The aeciospores of each collection were sampled from a single gall and were stored at –80° C without drying until use. Additionally, five vacuum-dried aeciospore isolates of *C. q. fusiforme* collected in Georgia and South Carolina, USA, were used.

2.2 DNA extraction and PCR amplification

Template DNA for PCR amplification was extracted from aeciospores following the procedures of Suyama *et al.* (1996). Two to three hundred aeciospores were crushed in a microtube with a pipette tip under an optical microscope. Twenty microliters of extraction buffer [10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl; 0.01% Proteinase-K; 0.01% (w/v) sodium dodecyl sulfate (SDS)] were added into the PCR tube, incubated for 60 min at 37° C, and heated for 10 min at 95° C. The extract was used directly as template.

The primer pair ITS1-F and ITS4-B designed by Gardes and Bruns (1993) was used for the amplification of ITS regions including 5.8S rDNA and for joining parts of the 3' end of the small subunit rDNA (approximately 70 bp in size) and the 5' end of the large subunit rDNA (approximately 200 bp in size). PCR amplification was performed in 100 µL of reaction mixture containing 200 nM concentrations of each primer, 200 µM concentrations of each deoxynucleotide triphosphate, two units of TaKaRa Ex Taq™ (Takara Biochemicals, Japan), and 1 X TaKaRa Ex Taq™ buffer. Two microliters of the above DNA extract was used as a template per reaction. The mixtures were covered with 40 µL of mineral oil to prevent evaporation. Reaction cycles were repeated 35 times with the following program: 95° C, 30 s; 55° C, 1 min; 72° C, 1 min. After amplification, aliquots (6 µL) of the reaction mixtures were subjected to electrophoresis on 1% (w/v) agarose gels containing 0.5 µg/mL of ethidium bromide in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4).

Table 1. Fungal collections.

Forma specialis	Collection No.	Host pine species	Locality
densiflorae	MO1	*P. densiflora*	Iwate, JPN
	MO2	*P. densiflora*	Iwate, JPN
	MO3	*P. densiflora*	Iwate, JPN
	NB1	*P. densiflora*	Nagano, JPN
	TK1	*P. densiflora*	Ibaraki, JPN
	TK2	*P. densiflora*	Ibaraki, JPN
thunbergii	TK3	*P. thunbergii*	Ibaraki, JPN
	TK4	*P. thunbergii*	Ibaraki, JPN
	TT1	*P. thunbergii*	Tottori, JPN
	TT2	*P. thunbergii*	Tottori, JPN
	BL2	*P. taeda*	Georgia, USA
	BL3	*P. taeda*	Georgia, USA
fusiforme	I-83	*P. taeda*	Georgia, USA
	LOB88	*P. taeda*	Georgia, USA
	SCSC2	*P. elliottii*	South Carolina, USA

2.3 RFLP analysis, sequencing and sequence analysis

The amplified fragments were digested with 10 restriction enzymes, *Alw* 44 I, *Dra* I, *EcoR* I, *Hae* III, *Hha* I, *Hinf* I, *Msp* I, *Sau* 3A I, *Ssp* I, and *Taq* I, according to the manufacturer's instructions. The digested fragments were separated electrophoretically on 4% (w/v) agarose X (Nippon Gene, Japan) gel in TAE buffer and stained with ethidium bromide.

The amplified fragments and the ITS4 primer pair designed by White *et al.* (1990) was used for sequencing the ITS2 region using DyeDeoxy Terminator Cycle Sequencing Kit and 377 DNA Sequencing System (Perkin Elmer). The nucleotide sequence data were aligned and analyzed using GENETYX-MAC (Software Development, Japan). Dendrograms were constructed using the unweighted pair-group method based on arithmetic average (UPGMA) (Sneath and Sokal 1973) and the neighbor-joining method (Saitou and Nei 1987).

3 Results

3.1 RFLP analysis of amplified ITS regions

The primer pair ITS1-F and ITS4-B permitted the amplification of a single DNA fragment (approximately 900 bp in size) from all the collections tested. From the 10 enzymes used for digesting DNA fragments, the three enzymes *Hae* III, *Hha* I, and *Msp* I had no recognition site in the fragments. The RFLP patterns with the four enzymes *Alw* 44 I, *EcoR* I, *Sau* 3A I, and *Ssp* I were identical among all the collections. On the other hand, the number of recognition sites with the three enzymes *Dra* I, *Hinf* I, and *Taq* I differed from each other in the fragments, and DNA polymorphism in the amplified ITS regions was detected among the collections (Fig. 1).

The three enzymes *Dra* I, *Hinf* I, and *Taq* I produced the RFLP patterns for *C. q. densiflorae* that were identical with those of *C. q. thunbergii*. The RFLP patterns with the three enzymes for *Cronartium* collections from Japan were different from those of *C. q. fusiforme* collections from North America (Fig.

1). No differences were found in the RFLP patterns among the five collections of *C. q. fusiforme*.

3.2 Sequencing and sequence analysis

Nucleotide sequences of the ITS2 region were determined for one representative collection of each of *C. q. densiflorae* (MO1) from Japan and *C. q. fusiforme* (BL2) from the USA. The size of the ITS2 region in *C. q. densiflorae* (MO1) was 220 bp pairs and that in *C. q. fusiforme* (BL2) was 225 bp pairs (Fig. 2).

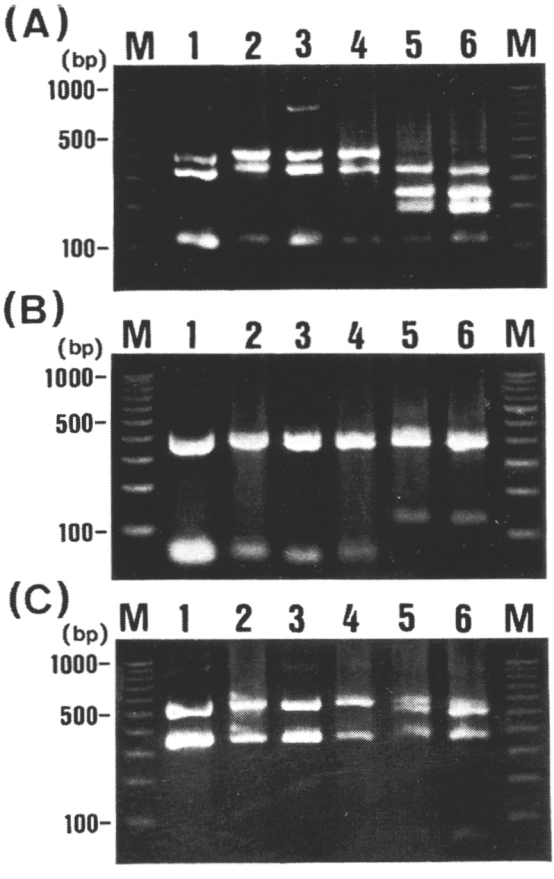

Figure 1. Restriction patterns of internal transcribed spacer regions of ribosomal DNA amplified from six collections in three *formae speciales* of *Cronartium quercuum* with *Dra* I (A), *Hinf* I (B), *Taq* I (C). Lane 1, *C. quercuum* f. sp. *densiflorae* (MO1); lane 2, *C. quercuum* f. sp. *densiflorae* (TK1); lane 3, *C. quercuum* f. sp. *thunbergii* (TK4); lane 4, *C. quercuum* f. sp. *thunbergii* (TK3); lane 5, *C. quercuum* f. sp. *fusiforme* (BL2); lane 6, *C. quercuum* f. sp. *fusiforme* (LOB88); M, fragment size markers (100 bp DNA ladder).

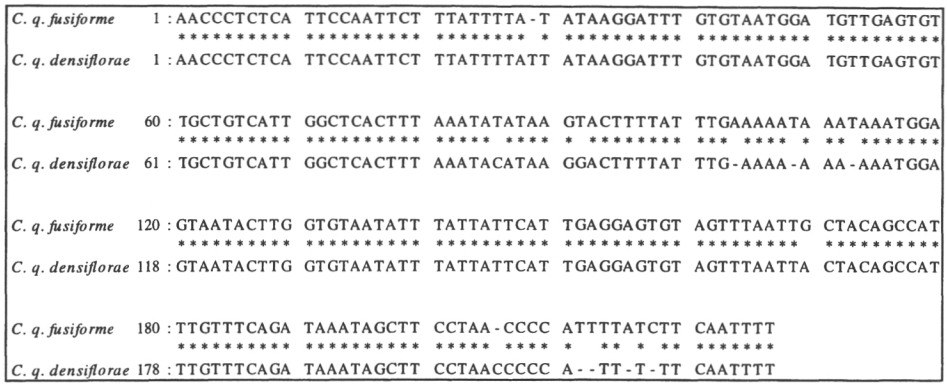

Figure 2. Nucleotide sequence alignment of ITS2 region in *Cronartium quercuum* f. sp. *densiflorae* (MO1) from Japan and f. sp. *fusiforme* (BL2) from the USA. The sequence of f. sp. *fusiforme* BL2 was identical with that of the same form from GenBank (No. L76494). Alignment gaps, insertations, or deletions are indicated by dashes.

For the UPGMA analysis, the ITS2 region sequences of 10 *Cronartium* species were obtained from the GenBank database (Vogler and Bruns 1998): *C. coleosporioides* Arth. (GenBank accession No. L76500), *C. comandrae* Peck (L76485), *C. conigenum* Hedgc. et N. Hunt (L76486), *C. flaccidum* (Alb. et Schw.) Wint. (X83901), *C. q. banksianae* (L76491), *C. q. echinatae* (L76493), *C. q. fusiforme* (L76494), *C. q. virginianae* (L76495), *C. ribicola* J.C. Fisch. (L76496), *C. strobilinum* Hedgc. et Hahn (L76482). In these alignments, the sequence of *Chrysomyxa arctostaphyli* Diet. (GenBank accession No. L76488) was used as outgroup.

The sequence of *C. q. fusiforme* collection BL2 by us was identical with that of *C. q. fusiforme* from the GenBank database. In the UPGMA similarity dendrogram derived from the sequence data of the ITS2 region, the *Cronartium* species formed a single cluster. The collection MO1 of *C. q. densiflorae* from Japan was placed in a different lineage from the other *formae speciales* in *C. quercuum* and was placed between *C. conigenum* and *C. ribicola*.

In the dendrogram produced by the neighbor-joining method, the *Cronartium* species consisted of three main clusters. Three species, *C. conigenum*, *C. quercuum* including the five *formae speciales*, and *C. strobilinum*, were included in one cluster. In this cluster, *C. q. densiflorae* from Japan and *C. conigenum* were included in a distinct lineage from the other four *formae speciales* in the USA.

4 Discussion

Clear distinctions were found in RFLP patterns between the two *formae speciales* of *C. quercuum* from Japan and *C. q. fusiforme* from the USA. No distinctions were found in the patterns among the collections from Japan, consisting of the two *formae speciales* f. sp. *densiflorae* and f. sp. *thunbergii*. The analysis of the nucleotide sequences of the ITS2 region confirmed the distinction between f. sp. *densiflorae* and f. sp. *fusiforme*.

Comparisons with the sequence data obtained from the GenBank database recorded by Vogler and Bruns (1998) for the other *formae speciales* in *C. quercuum* also indicated a separate position for f. sp. *densiflorae* from the other *formae speciales*. These molecular data support a previous report based on the morphological examinations (Kaneko 1992) suggesting that the Asian forms of *C. quercuum* may be different from the North American *C. quercuum*. In this paper, however, the nucleotide sequences of only the ITS2 region were examined. Comparisons of the whole ITS region between the Japanese forms and the North American forms are needed to resolve the phylogenetic position of the Japanese forms in the *C. quercuum* complex.

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Is ethylene a trigger of stem hyperplasia caused by eastern gall rust in *Pinus densiflora*?

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Summary

Ethylene release by segments of branch galls caused by eastern gall rust (*Cronartium quercuum*) in 22–45-year-old *Pinus densiflora* trees was determined in July and October, 1996, and April, 1997. Ethylene production was the most vigorous in April in comparison with the other two seasons. Anatomical characteristics of bark and xylem in the rust-induced galls used for ethylene detection were also studied. In transverse sections of galls, thickness of bark, radial number of tracheids, and density of resin ducts and rays were greater than those of uninfected branch portions. Ethrel, an ethylene-releasing substance, applied in lanolin paste at a concentration of 1% to current-season shoots of 30-year-old *Pinus densiflora* trees in June, 1996, greatly altered stem anatomy within four months. The application of ethrel stimulated increase in bark thickness and the production of resin ducts in xylem. The application of 1% IBA-S also affected stem growth. The newly-formed tracheids in ethrel-treated stems were similar to those of rust-induced galls, whereas newly-formed xylem in auxin-applied stems was composed of compression wood tracheids. The role of ethylene in stem hyperplasia caused by eastern gall rust in *P. densiflora* is discussed.

Key words: *Cronartium quercuum*, resin duct

1 Introduction

Eastern gall rust (pine-oak gall rust) is caused by the rust fungus *Cronartium quercuum* (Berk.) Miy. ex Shirai and produces perennial globular galls on stems and branches of *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parl. in Japan. The disease cycle of *C. quercuum* has been elucidated by Kondo (1975). However, there is little information about the physiological mechanisms of gall development.

The formation of pine galls is derived from partially-accelerated cambial activity under the stimulus of infection by rust fungi such as *C. quercuum* or *Endocronartium harknessii* (J.P. Moore) Y. Hirat. (Allen *et al.* 1990). In general, cambial activity has been attributed to the regulatory effects of several kinds of endogenous plant hormones including auxin, gibberellins, cytokinins, abscisic acid, and ethylene (Little and Pharis 1995). Various

investigations have concluded that, in addition to other plant hormones, ethylene plays a contributory role in the acceleration of cambial activity (Little and Pharis 1995).

Ethylene production by stems of woody plants increases as a result of various kinds of stresses including flooding (Reid and Bradford 1984), gravity (Robitaille 1975, Nelson and Hillis 1978, Yamamoto and Kozlowski 1987a), mechanical bending (Leopold *et al.* 1972, Brown and Leopold 1973, Robitaille and Leopold 1974), injection of chemicals (Wolter 1977), insect attack (Shain and Hillis 1972) and wounding (Yamanaka 1985, 1986, Yamamoto *et al.* 1990). In flooded woody plants, for instance, increased ethylene production in submerged stem bases is reliably associated with hypertrophic or hyperplastic stem growth as has been shown for several species (Newsome *et al.* 1982, Tang and Kozlowski 1982, Yamamoto and Kozlowski 1987a, b, c, d, Yamamoto *et al.* 1987, 1995a, b).

Various investigations have shown that plants infected with fungi produced large quantities of ethylene (Abeles 1973). However, very little information has been obtained on the behavior and role of ethylene in stem gall formation caused by rust fungi. In the present study, a potential role of ethylene in gall formation caused by eastern gall rust in *P. densiflora* is discussed.

2 Material and methods

2.1 Seasonal changes in ethylene emanation from galls

Twelve rust-infected, 22 to 45-year-old trees (*P. densiflora*) with small-sized branch galls were selected in the Mizoguchi Forest of the Tottori University forests. Six galls in each of three trees, three galls in each of six other trees, and six galls in each of the remaining three trees were harvested in July 1996, October 1996, and April 1997, respectively. The harvested galls were about 1 cm in diameter. Also, segments of uninfected stems were sampled from 20 cm below the galls at the same times. Six small segments about 0.1 cm^3 containing inner bark, cambium, and xylem were prepared from each gall or stem segment. Each small segment was placed in a 10-mL vial and incubated at 30° C for 4 h. Aliquots (1 mL) of head space gas were taken and analyzed for ethylene with a Hitachi 263-50 gas chromatograph equipped with a flame ionization detector (FID) and a spiral glass column (0.35 x 200 cm) packed with 60/80 mesh activated alumina. Column, injector, and detector temperatures were 70, 80, and 100° C, respectively, the carrier gas was N2, and the flow rate was 35 mL min^{-1}. Amounts of ethylene released were determined from standard curves obtained from known concentrations of ethylene-nitrogen mixtures (10.0 and 49.7 ppm) obtained from Seitetsu-kagaku Co. Ltd (Tokyo, Japan). The amount of ethylene released by the small segments (nmol g DW^{-1}) was calculated as described by Yamamoto *et al.* (1987).

2.2 Histological observations and measurements of galls

Six branch galls, about 1 cm in diameter, were sampled from one 22-year-old tree (*P. densiflora*) that was 5.0 m tall and 10.0 cm DBH. Also, segments of uninfected stems about 1 cm in length were sampled from 10 cm below the

galls. The galls or stem segments were fixed in FAA solution (formaldehyde/acetic acid/ethanol/water, 5/5/60/30) for 24 h, rinsed in water, dehydrated in ethanol, and sealed in a paraffin block. Samples were sectioned transversely at 12–15 μm on a sliding microtome, stained with safranin-fast green solution, and mounted with Diatex. For each section, the thickness of inner bark, the width of xylem produced in 1996, the density of resin ducts (number per mm^2), and size of spaces between rays near the cambium in tangential direction were analyzed by light microscopy.

2.3 Effects of the application of ethrel and IBA-S on the growth and anatomy of stems

Two 30-year-old clones of a many-stemmed *P. densiflora* cultivar with crowded upright stems were used in this experiment. To the current-season shoots of the clones, 1% ethrel (2-chloroethylphosphonic acid), an ethylene-releasing substance, or 1% IBA-S (indolebutric acid-sodium chloride), a synthetic auxin in lanolin paste, was applied in a 2.0 cm band 2–3 cm above the current-season branch whorl in June 1996. Plain lanolin applied to current-season shoots was used as a control. Ten shoots were used for each treatment. The treated shoots were harvested in October after four months for determination of diameter growth and study of stem anatomy. Histological measurements were performed using the method described in Section 2.2.

3 Results

3.1 Seasonal changes in ethylene emanation from galls

In rust-infected branches, the amount of ethylene released by the segments of galls was consistently higher than that released by segments of uninfected branches in all three seasons. Ethylene production was the most vigorous in April in comparison with the other two seasons (Fig. 1).

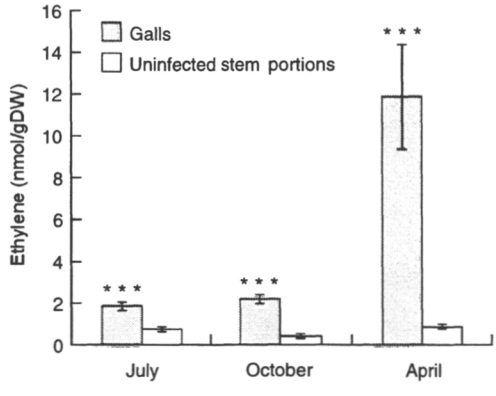

Figure 1. Ethylene production by branch galls and uninfected stem portions 20 cm below the galls in July 1996 (in the middle of the growing season), October 1996 (at the end of the growing season), and April 1997 (at the beginning of the growing season). Error bars represent standard errors (n=108). Symbols: *** = significantly different from the uninfected portions at the 0.1% level.

Table 1. Anatomical characteristics of branch galls caused by *Cronartium quercuum* and uninfected portions 10 cm below the galls in transverse sections of 22-year-old *Pinus densiflora*. The values are means ± SE (n=6).

	Bark thickness mm	Width of xylem produced in 1996 mm	Resin duct density number mm^{-2}	Spaces between rays mm
Branch galls	1.32±0.08***	1.41±0.25**	1.7±0.1**	0.07±0.01***
Uninfected portions	0.62±0.04	0.19±0.04	1.3±0.1	0.25±0.04

** = significantly different from the uninfected portions at the 1% level,
*** = at the 0.1% level.

Table 2. Anatomical characteristics of artificial galls induced on current shoots of *Pinus densiflora* with the application of 1% ethrel or 1% IBA-S in lanolin paste. The values are means ± SE (n=10).

	Bark thickness mm	Xylem width mm	Resin duct density number mm^{-2}	Spaces between rays mm
Ethrel	1.30±0.07*	0.78±0.08 ns	4.1±0.2**	0.13±0.02 ns
IBA-S	1.12±0.10 ns	1.06±0.10**	2.1±0.1**	0.17±0.01 ns
Control	0.97±0.11	0.62±0.07	3.0±0.2	0.14±0.02

* = significantly different from control at the 5% level,
** = at the 1% level,
ns = not significant.

3.2 Histological observations and measurements of galls

In the transverse sections of galls, thickness of bark, radial number of tracheids, density of longitudinal resin ducts per unit area, and tangential number of rays were significantly greater than those of uninfected branch portions (Fig. 2, Table 1).

3.3 Effects of the application of ethrel and IBA-S on the growth and anatomy of stems

Application of ethrel and IBA-S to the current-season shoots of the 30-year-old *P. densiflora* trees greatly altered stem growth and anatomy within four months. Stem diameters on treated portions were significantly increased by both chemicals (Fig. 3). The application of ethrel stimulated an increase in bark thickness and the production of longitudinal resin ducts in xylem. The application of IBA-S also affected stem growth. The newly-formed tracheids in ethrel-treated stems were similar to those of rust-induced galls, whereas newly-formed xylem in IBA-S-treared stems was composed of compression wood tracheids (Fig. 4, Table 2).

4 Discussion

Various investigations have demonstrated that ethylene has an important regulatory role in certain aspects of cambial growth in coniferous species (Yamamoto and Kozlowski 1987a, e, Savidge 1988, Little and Pharis 1995, Eklund and Little 1995, 1996, 1998).

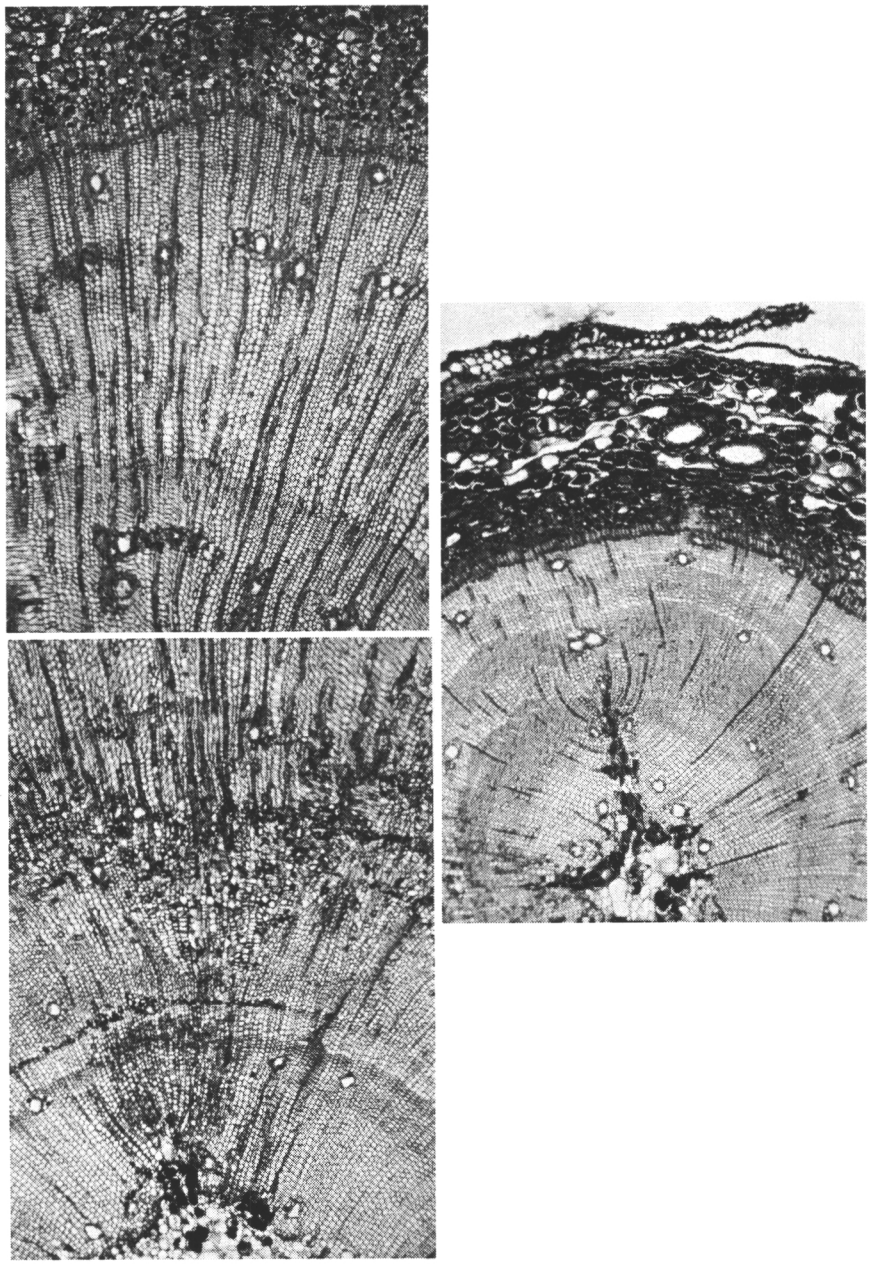

Figure 2. Transverse sections of a branch gall (left) and an uninfected portion (right) 10 cm below the gall. Note the enhanced xylem formation and the increased density of resin ducts and rays in the gall of *Pinus densiflora*.

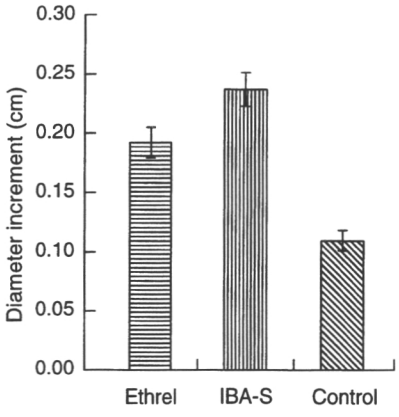

Figure 3. Effects of the application of 1% ethrel or 1% IBA-S diluted in lanolin on diameter increment of current-season shoots. Symbol: *** = significantly different from the untreated portions at the 0.1% level.

In the present study, hyperplastic gall development and the formation of abundant tracheids, thickened bark, and increased density of resin ducts and rays were associated with high ethylene production by gall segments in all seasons. The application of ethrel also increased bark thickness and density of resin ducts in current-season shoots. In the stems of *Pinus halepensis* Mill. seedlings (Yamamoto and Kozlowski 1987e), the application of ethrel significantly increased bark thickness, the number of tracheids, and the density of resin ducts and rays. An increased density of resin ducts was also observed in the ethrel-treated stems of *P. densiflora* seedlings, suggesting that ethylene may be the most important factor controlling resin duct formation (Yamamoto and Kozlowski 1987a). These observations and the present results suggest that ethylene is involved in gall formation caused by the eastern gall rust fungus. However, various cambial responses attributed to ethylene involve synergistic effects of auxin and other plant hormones (Savidge 1988, Eklund and Little 1995, 1996, 1998, Little and Pharis 1995). For instance, Eklund and Little (1996) reported that laterally applied ethrel locally increased the IAA concentration in the cambial region of *Abies balsamea* (L.) Mill. shoots. We have also observed that blocking basipetal auxin transport with NPA (1-N-naphthylphthalamic acid) suppressed hyperplastic stem growth of flooded *Fraxinus mandshurica* Rubr. and *Alnus japonica* (Thunb.) Stend. seedlings (unpubl.), suggesting that both ethylene and auxin may be essential in the activation of cambial growth. Further investigations are needed to study synergistic effects of plant hormones in gall formation.

When plants are infected with fungi, the host tissue usually produces the ethylene, although in some cases the fungus is the source of ethylene production (Abeles 1973). In *Abies balsamea* shoots, ethylene is mainly produced from the cambial region (Eklund and Little 1998). The present results also suggest that the major source of ethylene produced by galls may be in the cambial region. However, additional research is required to determine the mechanism of ethylene production caused by the eastern gall rust fungus in the cambial region.

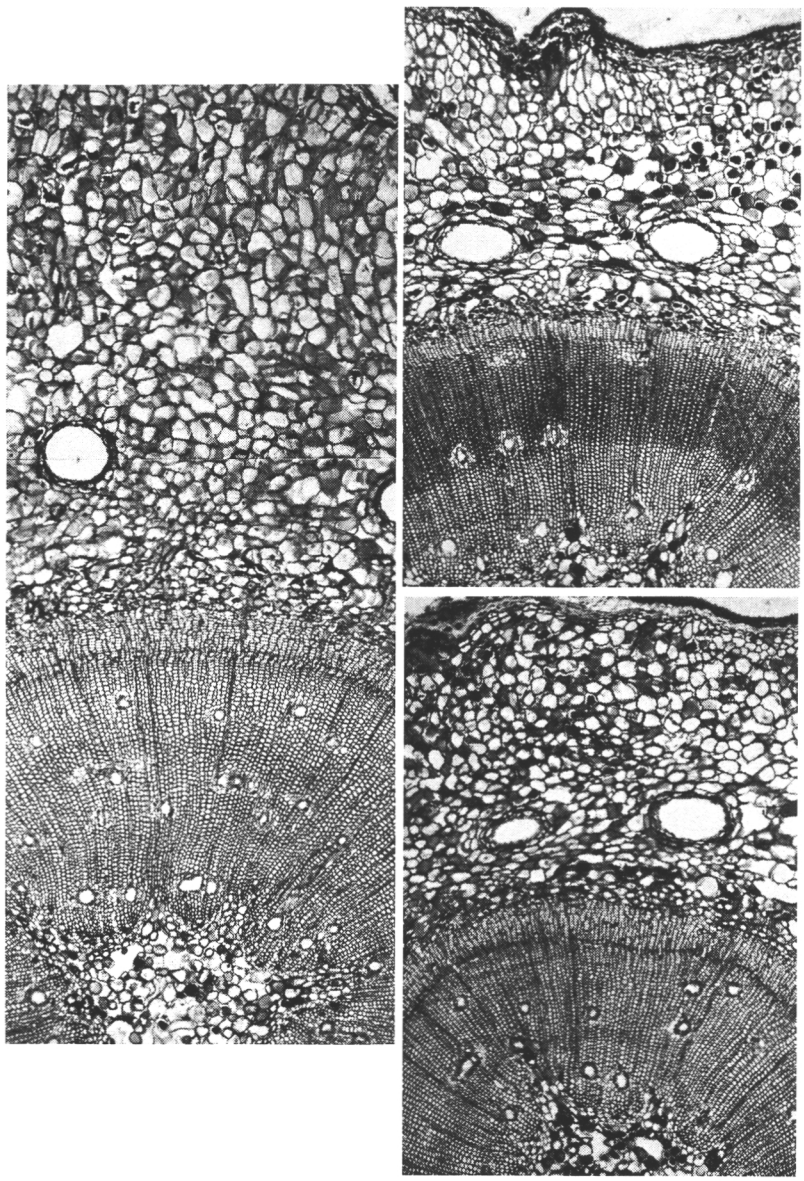

Figure 4. Transverse sections of ethrel-treated (left), IBA-S-treated (right, upper), and control (right, lower) shoots of *Pinus densiflora*. Note the thickened bark and the increased density of resin ducts in the ethrel-treated shoot and compression wood in the IBA-S-treated shoot.

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Finnish Forest Research Institute, Research Papers 712: 253, 1998

Taxonomic reconsideration of *Cronartium ribicola* and *Peridermium kurilense* in the Russian Far East

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Abstract

Cronartium ribicola, the causal fungus of white pine blister rust, is believed to have originated in northeastern Asia. It is, however, now widely distributed in Asia, Europe, and North America. In the Kuriles, an additional blister-causing fungus, *Peridermium kurilense*, was once recorded on *Pinus pumila*. The latter fungus has often been treated as synonymous with *C. kamtschaticum* (= *C. ribicola*), but biological and taxonomic relationships between the two fungi have remained uncertain. To clarify taxonomic relationships of these fungi, we made several collections of blister-causing fungi on *P. pumila* in the Kuriles and Kamchatka in 1996–1997. Field surveys and morphological examinations of these collections and additional specimens from various herbaria suggested that the blister-causing fungus in the Kuriles is endocyclic in life cycle and taxonomically identical with *Endocronartium sahoanum* var. *hokkaidoense*. However, the other fungus in the Russian Far East was revealed to be morphologically identical with *C. ribicola*.

Key words: *Endocronartium sahoanum*, *Pinus pumila*, taxonomy

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Molecular epidemiology of white pine blister rust

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Summary

Several aspects of the epidemiology of *Cronartium ribicola*, the causal agent of white pine blister rust, are still not clearly understood. Molecular approaches are used to complement traditional approaches and test specific hypotheses concerning the population genetics and epidemiology of this pathogen. DNA analysis of *C. ribicola* populations in eastern Canada revealed that there was no correlation between genetic and geographic distance, but that there was a small but statistically significant genetic differentiation among populations within regions. This result is consistent with the effect of genetic drift following colonization events. However, genetic differentiation was very high (53%) between *C. ribicola* populations from British Columbia and eastern Canada. Similar results were obtained using aeciospores, urediospores, and spermatia. A genetic distance analysis clearly indicates the presence of two distinct epidemiological units in North America, one in the east and the other in the west. There seems to be extensive gene flow within epidemiological units, but few if any exchanges between them. The absence of hosts over the Prairies and the Great Plains is probably responsible for the isolation between eastern and western populations.

Key words: *Cronartium ribicola*, gene flow, *Pinus strobus*, RAPD

1 Introduction

The epidemiology of white pine blister rust, caused by *Cronartium ribicola* J.C. Fisch. on *Pinus strobus* L., has been studied in North America since the beginning of the twentieth century. Much knowledge has been gathered concerning the various spore stages and the function of spores in the life cycle. However, there are still several unknown parameters in the epidemiology of this disease, in particular regarding the functional importance of the various spore types, the distance these spores are disseminated, and the genetic composition of the pathogen population. In recent years, the development of molecular characterization methods, in particular those based on the polymerase chain reaction, has allowed epidemiologists to reconsider old questions concerning the epidemiology of white pine blister rust. In this paper, results are presented on the molecular epidemiology of white pine blister rust, in particular on the definition of epidemiological units in North America based on the genetic structure of the pathogen.

2 Material and methods

2.1 Sample collection

Spermatia and aeciospore collections were made as reported previously (Hamelin *et al.* 1995, Hamelin 1996). For comparison among populations from eastern Canada, spores from 30 individual aecia were collected from each of nine *C. ribicola* populations from Newfoundland (3), New Brunswick (1), Nova Scotia (1), Quebec (1), and Ontario (3). For western collections, aeciospores were collected by Rich Hunt and Gary Jensen (Canadian Forest Service, Victoria, British Columbia) from four locations on Vancouver Island and in the B.C. interior. In addition, 23 single urediospore cultures derived from collections from throughout the United States were obtained from Paul Zambino (U.S. Forest Service, Rhinelander, WI).

2.2 Sample processing

Aeciospores and spermatia were processed as previously described for DNA extraction (Hamelin *et al.* 1995, Hamelin 1996) and urediospores were processed according to a protocol described by Zambino *et al.* (1998).

2.3 DNA markers

RAPD markers previously characterized for *C. ribicola* were used in this study (OPA01, OPC08) (Hamelin *et al.* 1995, Hamelin 1996). In addition, three new primers (OPE15, OPK11, OPK19) were used after a screening of 100 new primers. This yielded a total of nine polymorphic RAPD markers that were characterized in *C. ribicola* populations for the various spore types.

2.4 Data analysis

Markers were scored as present or absent and compiled in a matrix of samples x markers. Haplotype frequency was calculated, and a matrix of genetic distance among the various haplotypes was calculated. This matrix was used to perform an analysis of molecular variance (AMOVA) to partition the genetic diversity into hierarchical components (Excoffier *et al.* 1992). F_{st} analogs were calculated and their statistical significance was assessed by performing 1000 randomizations of the dataset.

To assess the relationship between genetic and geographic distances, a distance matrix based on pairwise F_{st} among all pairs of populations was calculated and a dendrogram was constructed by using a neighbor-joining tree algorithm (PHYLIP software version 3.56).

3 Results and discussion

Most of the genetic diversity (93%) in the eastern populations of *C. ribicola* was present within populations. The level of genetic differentiation among regions (maritimes: Newfoundland, New Brunswick, Nova Scotia vs center: Ontario, Quebec) or among provinces was small and not significantly different from zero (Fig. 1). The level of genetic differentiation among populations within regions was also small (7%) but significantly different from zero. This result is consistent with previous rust surveys indicating a high level of genetic diversity within populations or even within cankers, but

a low level of genetic differentiation among geographically separated populations (Hamelin 1996, White *et al.* 1996). Genetic differentiation in *C. ribicola* populations among regions within Quebec was not significantly different from zero, yet F_{st} was 11% among populations within regions (Hamelin *et al.* 1995). Similarly low levels of genetic differentiation were also reported for *Cronartium quercuum* (Berk.) Miy. ex Shirai f. sp. *fusiforme* in the southern U. S. (Hamelin *et al.* 1994).

The most likely explanation for this genetic structure in the *C. ribicola* populations in eastern Canada is that extensive gene flow takes place over large geographic distances. However, it also appears that genetic drift within local populations is responsible for the observed genetic differentiation among populations within regions. This genetic drift could be caused when new populations are established from long-distance spore dispersal or by the introduction of a small number of individuals on infected nursery material.

By contrast, genetic differentiation between populations from western and eastern Canada was large (53%) and significantly different from zero (Fig. 2). Some DNA markers (e.g., OPK11-550) were fixed in the western populations, but were polymorphic in the eastern populations. Others (e.g., OPA01-1700) were polymorphic in eastern populations, but were almost always absent in western populations. The analysis of genetic distance separated the eastern and western populations into two clades (Fig. 3). However, there was no correlation between genetic distance and geographic origin within clades. For example, the Quebec and Newfoundland populations were more closely related than the Quebec and Ontario or the Newfoundland and New Brunswick populations.



Figure 1. Analysis of molecular variance with RAPD markers for *Cronartium ribicola* populations from eastern Canada (Newfoundland, Nova Scotia, New Brunswick, Quebec, and Ontario).

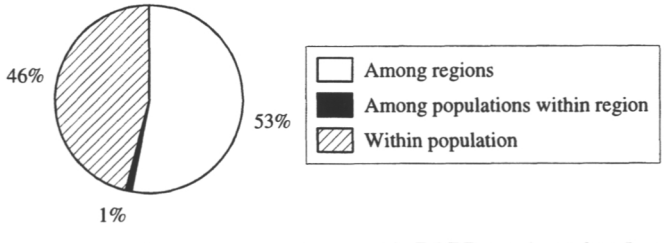

Figure 2. Analysis of molecular variance with RAPD markers for *Cronartium ribicola* populations from eastern and western Canada (British Columbia, Ontario, Quebec, and Newfoundland).

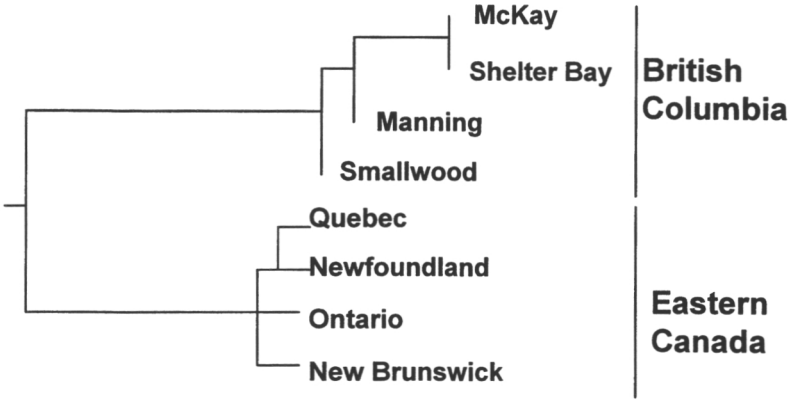

Figure 3. Dendrogram of *Cronartium ribicola* populations from eastern and western Canada based on genetic distances between populations.

To confirm that the levels of genetic differentiation observed with aeciospore samples were not attributable to sampling or processing artefacts, spermoatia and urediospore samples were analyzed using the same sets of RAPD markers as those used for the aeciospores. In both cases, high levels of genetic differentiation were observed between eastern and western populations of *C. ribicola* (Table 1). The large differences observed among spore types (e.g., genetic differentiation was 28%, $p<0.001$, between eastern and western spermatia and 38%, $p<0.001$, between eastern and western urediospores) is most likely attibutable to the difference in sampling intensity among the spore types. In particular, the sample sizes were very small for spermatia and urediospores. More intensive sampling is planned to analyze a more representative sample of urediospores from North America.

The results presented here are consistent with the presence of two distinct epidemiological units within North America, one in the east and the other in the west, with extensive genetic exchanges and no evidence of isolation by distance within the epidemiological units. It appears that either the physical barrier constituted by the Rocky Mountains or most likely, the absence of hosts over the Canadian Prairies and the American Great Plains prevents the movement of inoculum between eastern and western North America. The recent appearance of white pine blister rust in New Mexico could suggest that the absence of hosts, and not the Rocky Mountains, is the barrier to spore movement. Our current two samples from New Mexico belong to the western haplotype and are most likely part of the western epidemiological unit.

Table 1. Genetic differentiation among eastern and western populations of *Cronartium ribicola* for spermogonial, aecial and uredial samples using RAPD markers.

	Spermatia	Aeciospores	Urediospores
ϕ_{ST}	0.28	0.53	0.38
p	<0.001	<0.001	<0.001
N	46	220	23

Because of the nature of the markers used in the current study, it is not known whether there are biologically or epidemiologically significant differences between the two epidemiological units. The total number of genetic markers differing between eastern and western populations is very small; approximately 7% of the RAPD primers screened yielded polymorphisms. Yet, some virulence genotypes have been observed in western North America; the existence of a race of *C. ribicola* that was virulent on previously resistant sugar pines was reported (Kinloch and Comstock 1981, Kinloch and Dupper 1987). The genetic differentiation detected in our study indicates that, should there be adaptive or virulence differences between eastern and western populations, a quarantine could become important to prevent dissemination across the epidemiological units.

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Variation in isozymes, RAPDs, and SEM-based morphology among three species of pine stem rust fungi from China

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Summary

Genetic and morphologic variation among three pine stem rust fungi, *Cronartium ribicola, C. flaccidum,* and *C. quercuum,* from China was examined using isozyme, randomly amplified polymorphic DNAs (RAPD), and scanning electron microscope (SEM) techniques. Aeciospores representing different isolates from diverse pine hosts and geographic origins were studied. The isozyme patterns of both esterase (EST) and malate dehydrogenase (MDH) had no common bands among the three rusts, thus clearly distinguishing the three species. Pairwise similarity analysis on nine isolates using RAPD showed that the three pathogens, despite their intraspecific variations, are genetically divergent from one another. Also, *C. ribicola* and *C. flaccidum* were more closely related to each other than to *C. quercuum*. Under SEM, the most prominent feature of aeciospore surfaces was the verrucose areas occupied by many warts varying greatly in morphology. However, none of the varied wart characters were species-specific; thus they failed to distinguish these three rust fungi.

Key words: *Cronartium*, differentiation

1 Introduction

There are three pine stem rusts damaging pine forests in most parts of China: *Cronartium ribicola* J.C. Fisch., *C. flaccidum* (Alb. & Schw.) Winter, and *C. quercuum* (Berk.) Miy. & Shirai. Morphologically the three species are very similar, and their classification so far has been based on host ranges and the symptoms they caused on pines, though these phenotypic characters are overlapping and confusing. For example, *C. ribicola* and *C. flaccidum* induce similar canker symptoms in pine, and cannot be distinguished unless by their pine hosts: the former attacks five-needle pines and the latter two-needle ones. *Cronartium quercuum* causes galls but has the same pine host range as *C. flaccidum*. Although several studies tried to distinguish the three rusts by morphological characters of aeciospores (Tong *et al.* 1979, Liu and Teng 1986), no studies have been done to differentiate these rusts genetically.

Isozyme and randomly amplified polymorphic DNAs (RAPD) are two useful molecular techniques for identifying species, races or isolates; they are especially powerful for resolving taxonomic problems where morphology is confusing, by providing insights into the genetic background of the taxonomic groups in question (Murphy *et al.* 1990, Williams *et al.* 1990). The authors used isozymes to identify the three Chinese *Cronartium* species (Cheng *et al.* 1995) and analyzed their population structures (Cheng *et al.* 1997, 1998a), tried the RAPD technique to assess their genetic relationship (Cheng *et al.* 1998b), and also examined morphological variation of the spores using a scanning electron microscope (SEM). This paper compares the usefulness of isozyme data, RAPD data, and SEM-based spore morphology in combination with phenotypic characters, in assessing the variation among the three Chinese pine stem rust fungi.

2 Materials and methods

2.1 Isolate collection

Each sample of aeciospores, considered one isolate, was collected from a single canker lesion or a single gall from one tree. The sampled spores were kept at –15° C before use. During 1993–1997, 134 isolates representing three *Cronartium* species were collected from different pines and locations across middle and northeastern China (Table 1).

2.2 Isozyme analysis

Twenty milligrams of spores per isolate were hand-ground with a bit of quartz sand in an ice-cooled mortar for about 30 s. The resultant powder was thoroughly blended into a slurry with 0.2 mL 0.1M Tris-HCl buffer (pH 7.0) amended with 10 mM dithiothreitol (DTT) and 10 mM vitamin C. The supernatant from the centrifuged slurry was subjected to electrophoresis immediately. Vertical polyacrylamide slab gels and a discontinuous gel buffer system were used. After electrophoresis the gels were stained to detect the isozyme banding patterns. Thirteen enzyme systems were stained following the recipes of Murphy *et al.* (1990). The above process was triplicated for certainty.

Table 1. Origins of the *Cronartium* spp. isolates of pine stem rust.

Species	Isolate	Host	Location
Cronartium	Cr1	*Pinus koraiensis*	Dunhua, Jilin
ribicola	Cr2	*P. koraiensis*	Caohekou, Liaoning
	Cr3	*P. koraiensis*	Jigongshan, Liaoning
Cronartium	Cf1	*P. sylvestris* var. *mongolica*	Jiagedaqi, Inner Mongolica
flaccidum	Cf2	*P. massoniana*	Nanzheng, Shanxi
	Cf3	*P. massoniana*	Yuexi, Anhui
Cronartium	Cq1	*P. sylvestris* var. *mongolica*	Mishan, Heilongjiang
quercuum	Cq2	*P. takahashi*	Mishan, Heilongjiang
	Cq3	*P. taiwanensis*	Yuexi, Anhui
	Cq4	*P. massoniana*	Ankang, Shanxi
	Cq5	*P. densiflora*	Tsukuba, Japan
	Cq6	*P. massoniana*	Ningshan, Shanxi

2.3 RAPD analysis

Nine isolates for the three *Cronartium* species were used for RAPD analysis, with an autoecious rust isolate of *Endocronartium sahoanum* Imazu & Kakishima on *Pinus pumila* (Pallas) Regel (Imazu *et al.* 1989) added as an outgroup. DNA extraction was conducted following Zhu *et al.* (1994). The protocol was unigue in that its lysis buffer contained 40% (v/v) benzyl chloride, which enhances the DNA isolation by disrupting the fungal cell walls. For each isolate, 25 mg of aeciospores was used to extract DNA. The resultant genomic DNA was stored in 50 µL TE (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA) at –20° C until use. PCR amplification was performed on a Minicycler system (M.J. Research, USA) with a temperature profile of 46 cycles. Each cycle consisted of 94° C for 20 s (the first cycle for 1 min), 37° C for 1 min, 72° C for 1 min (the last cycle for 10 min). Each 25 µL of reaction contained 0.1 µM of 10-bases primer (Operon), 100 µM of each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.5 µL Taq DNA polymerase (Shanghai Biotech.), and genomic DNA at a pretested concentration of x250 dilution of the original DNA extract. The amplified product was resolved in 1.5% agarose and visualized by UV-fluorescent staining with ethidium bromide. The amplification was repeated at least twice to assure the reproducibility. Based on the amplified DNA fragments, pairwise similarity analysis was conducted and a cluster tree was constructed using (UPGMA) the unweighted pair-grup method based on arithmetic average to assess the relatedness of the isolates.

2.4 Examination of spore morphology

Aeciospores of each isolate were spread on double-sided tape attached to a specimen holder, coated with gold, and examined with a SEM (JEOL JSM-T330, Japan). At least 30 spores were examined for each isolate to determine their surface structures, which have been reported to be useful for pine stem rust identification (Hiratsuka 1971, Tong *et al.* 1979, Liu and Teng 1986). At least three isolates with different host and/or geographic origins were examined for each rust species.

3 Results and discussion

3.1 Isozyme analysis

Of the 13 enzyme systems tested, 8 gave positive staining reaction and among them esterase (EST) and malate dehydrogenase (MDH) produced the most clear and species-specific isozyme patterns (Fig. 1). The three *Cronartium* species could be easily distinguished by the different mobilities of the banding regions. Although banding variation was detected in some isolates, it was very small and did not affect the species specificity of the patterns as a whole. The authors earlier conducted an allozyme analysis on 134 *Cronartium* isolates at six putative isozyme loci including those of EST and MDH (Cheng *et al.* 1998a). The result suggested an obvious genetic isolation among the three *Cronartium* species, which possessed few loci in common. The three rusts have no common EST or MDH allozyme bands at all (Fig. 1).

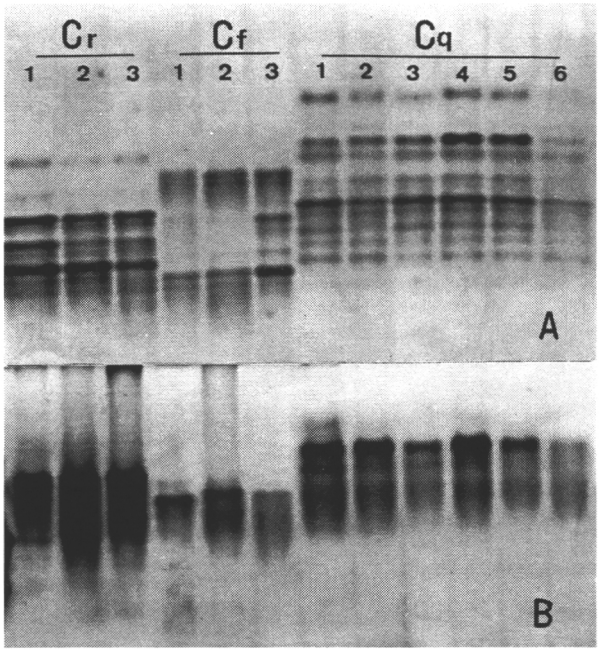

Figure 1. Esterase (A) and malate dehydrogenase (B) isozyme patterns of 12 isolates of *Cronartium ribicola* (Cr), *C. flaccidum* (Cf), and *C. quercuum* (Cq). These isolates were from different pine species and/or geographical locations across middle and northeastern China.

The EST isozyme patterns were multibanded and the MDH were monobanded. The banding mobility of different rust species differed more distinctively in EST than in MDH. Therefore, for identifyication purposes, EST patterns would be more reliable than MDH patterns. On the other hand, these isozyme patterns were not sensitive enough to detect intraspecific variations of these rust fungi.

3.2 RAPD analysis

Five primers (OPE03, OPE07, OPR20, OPV08, and OPW06) screened out of 54 produced 51 reproducible polymorphic fragments useful for genetic variation analysis. A cluster tree (Fig. 2) was constructed with these fragments that substantially represented 51 unknown loci scattered within the genomes of the tested rusts.

The cluster tree yielded two distinct main branches, each holding two sub-branches. The three *Cronartium* species, at the sub-branch level, were clustered into apparently different groups, demonstrating an obvious genetic divergence among them. At the main branch level, *C. ribicola* and *C. flaccidum* were clustered into one group, and *C. quercuum* into another together with *E. sahoanum*. Such a dendrogram suggests that *C. ribicola* and *C. flaccidum* are more closely related to each other than to *C. quercuum*. This coincides with the similarity relationship reflected by symptomatic and host-origin characters of the three rusts. However, *E. sahoanum* being grouped

with *C. quercuum* instead of *C. ribicola* was unexpected, because phenotypically *E. sahoanum* is closer to *C. ribicola* but distant from *C. quercuum. Endocronartium sahoanum* and *C. ribicola* induce identical canker symptoms on five-needle pines. Considering that only one isolate of *E. sahoanum* was analyzed, the location of *E. sahoanum* on the cluster tree remains uncertain.

The similarity coefficients among isolates were small for both *C. flaccidum* and *C. quercuum*, as compared with *C. ribicola,* which formed a quite homogenous group. The obvious heterogeneity between two *C. flaccidum* isolates is possibly associated with their host specificity. Inoculation tests have shown that *C. flaccidum* on *Pinus sylvestris* L. var. *mongolica* and that on *Pinus massoniana* Lama. have different telial hosts and are considered as two *formae speciales* (Ju *et al.* 1984, Jing and Wang 1988). Also, the high level of variation in four *C. quercuum* isolates from different pines suggests that *C. quercuum* in China may consist of several pine host-specific groups like that known in North America (Powers *et al.* 1989).

Compared with isozyme data, RAPD appeared more suitable for detecting the intraspecific variation and examining the relatedness among these rust fungi.

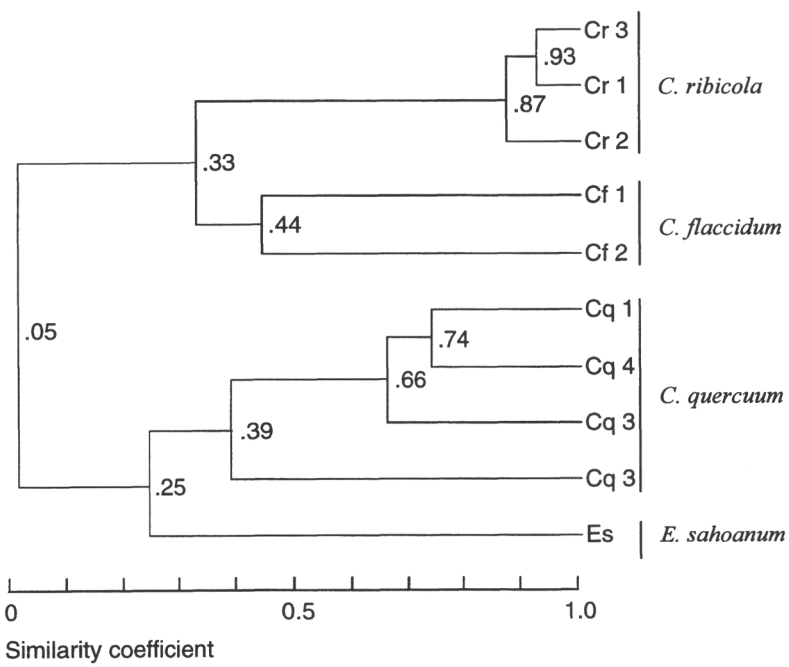

Figure 2. Cluster tree showing relatedness of 10 isolates of *Cronartium ribicola, C. flaccidum, C. quercuum,* and *Endocronartium sahoanum*, based on analysis of 51 RAPD fragments yielded by five 10-base primers (OPE03, OPE07, OPR20, OPV08 and OPW06).

3.3 Spore morphology

The surface of aeciospores consisted of smooth areas and verrucose areas (Fig. 3a), being generally similar to that reported earlier (Tong 1979, Liu and Teng 1986). However, the shape of the warts varied greatly with respect to the following characters: the degree of wart tapering (wart width narrows towards the tip), the degree of annulation on the warts, the degree of longitudinal ditch formation on the warts, and the height/width ratio of the warts. All these characters could be conveniently divided into different types. For example, the wart tapering could be divided as clearly tapered, slightly tapered or no tapering at all (uniform) (Fig.3a and c), the annulation as clearly annulated (Fig. 3a, c and d), slightly annulated or no annulation at all (smooth) (Fig. 3a), and so on. For the annulated warts, the annulation number per wart ranged from 3 to 7 across all the spores examined. The different tapering combined with different height/width ratio of the warts made some warts look slender (Fig. 3c) and others stumpy (Fig. 3d).

Most importantly, however, none of the above-described characters were found to be species-specific or isolate-specific, because different types of the varied characters could be observed in the same species or the same isolate. Frequently, even the same single spore could possess different wart types.

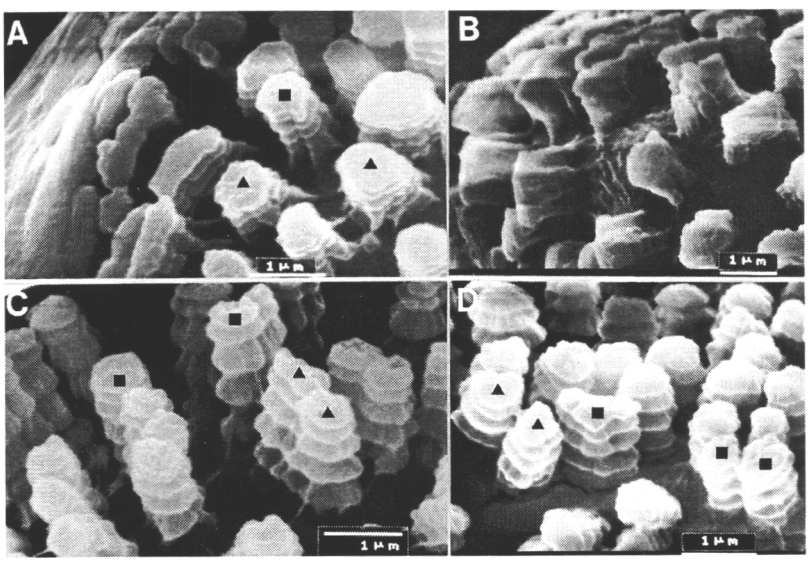

Figure 3. Aeciospore surfaces observed under SEM for some isolates of *Cronartium* species. A: Smooth area and wart-occupied area on a spore of a *C. ribicola* isolate. Tapered warts (black triangle) and uniform warts (black square) coexist on the same spore. Longitudinal ditches are visible on some warts. B: Smooth warts (without annulations) on a spore of another *C. ribicola* isolate. C: Tapered warts (black triangle) and uniform warts (black square) coexist on a spore of a *C. flaccidum* isolate. These warts have a slender shape. D: Tapered warts (black triangle) and uniform warts (black square) on a spore of another *C. flaccidum* isolate. These warts have a stumpy shape.

For example, clearly tapered warts and uniform ones often coexisted on the same spore (Fig. 3a, c and d). Only the frequency of a particular type of character differed among species, isolates, or spores.

The usefulness of the spore surface morphology in distinguishing *Cronartium* species reported so far is controversial. For example, some *Cronartium* species from Canada (Hiratsuka 1971) and Japan (Imazu *et al.* 1989) show differences in spore surface characters, but those from North America (Grand and Moore 1972) do not, nor do European species (Kasanen 1997). For Chinese *Cronartium* species, Tong (1979) claimed differences in wart tapering based on their limited samples. Liu and Teng (1986) reported that whereas *C. ribicola* and *C. flaccidum* generally differed from each other in wart tapering and in annulation number per wart, there were also many exceptions. The authors' observations, coupled with the inconsistency in earlier reports, suggest that for some *Cronartium* species, including the three Chinese ones, wart morphology of aeciospores is a highly variable character that is unsuitable for identification purposes.

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Gene flow in *Cronartium ribicola*

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Summary

Alleles for specific virulence/avirulence (vR/AVR) to major gene resistance (MGR) to white pine blister rust (*Cronartium ribicola*) in sugar pine (*Pinus lambertiana*) are clearly marked by contrasting phenotypes on foliage of hosts carrying the dominant resistance allele (R). We monitored the frequency of vR in five small sub-demes in and around its site of origin in a small plantation of R– genotypes near Happy Camp in northern California for nine years in order to assess rates of gene flow. In 1987, a few infections appeared on two of three known Rr parents on Thompson Ridge, 4 km downwind and 330 m higher in elevation from Happy Camp, in a stand of old growth sugar pine suffering chronic and heavy infection from rust. Assays for vR in the ambient basidiospore cloud were made annually by collecting infected *Ribes* leaves bearing mature telia in plots surrounding the three trees, and using them to inoculate R– seedlings. Inocula from Happy Camp and Poker Flat, another plantation with known R– progenies, 9 km west of Happy Camp, were similarly sampled. Frequencies of vR/AVR in the five demes were estimated by simple counts of spot types. Relatively constant (and nearly fixed) frequencies of vR at Happy Camp, and constant and nearly fixed frequencies for AVR at Poker Flat were observed over the years. Frequencies on Thompson Ridge fluctuated greatly, both among years and the three demes, ranging between 0.89 and 0.00, the last estimate at one site. The data show the interplay of selection, migration, and drift in the metapopulation structure of this pathogen. Gene flow is erratic, often depending on founder populations of small size, which are vulnerable to extinction.

Key words: monitoring, *Pinus lambertiana*, virulence

1 Introduction

Rare genetic markers provide unusual opportunities to measure gene flow from a point source. *Cronartium ribicola* J.C. Fisch., the causal agent of white pine blister rust, is dimorphic for a gene with specific virulence/avirulence (vR/AVR) to a dominant gene, R, conditioning hypersensitive necrosis resistance in sugar pine (*Pinus lambertiana* Dougl.) (Kinloch and Comstock 1981). The wild type in western North American populations is avirulent (Kinloch and Dupper 1987), but a mutation to virulence in the rust occurred in a small test plantation of sugar pine progenies from phenotypically resistant parents in the Siskyou Mountains near Happy Camp, California, around 1976. Because most of the sugar pine genotypes remaining at that time were resistant, selection pressure on the rust was intense, and vR had already approached fixation on alternate host *Ribes*

spp. in the plantation by the time monitoring started. Yet, it could not be detected on *Ribes* immediately outside the plantation, or anywhere else, for almost a decade (Kinloch and Dupper 1987). Discovery of a few new infections in 1987 on two known Rr genotypes on Thompson Ridge, an old growth sugar pine stand with a long history of severe and chronic infection from blister rust about 4 km downwind and 330 m higher in elevation from Happy Camp, suggested a recent migration of vR from Happy Camp. To learn the fate and dynamics of this founder population, we have monitored the relative frequencies of vR in the basidiospore population derived from *Ribes* on Thompson Ridge for nine years, and compared them with those at Happy Camp and a nearby site with a small planting of sugar pines with major gene resistance, but without any evidence of vR.

2 Material and methods

Inoculum was sampled from blister rust-infected *Ribes sanguineum* Pursh. bushes at the Happy Camp test site and three plots on Thompson Ridge (Fig. 1). These plots were within 1.5 km of each other, and each had an Rr sugar pine genotype at plot center, two of which were infected. A fifth location at Poker Flat, about 9 km west and 650 m higher in elevation than Happy Camp was also chosen because of its proximity to both Happy Camp and Thompson Ridge, and also because it supported a small plantation of sugar pines of comparable pedigree to those at Happy Camp. These trees, planted in 1983, had been subjected to repeated and severe attacks of rust. All susceptible controls were killed within a few years, but resistant (R–) genotypes remained uninfected, indicating no change of virulence in the rust. The plantation was surrounded by natural forest containing a substantial number of mature, infected sugar pine and *Ribes* spp.

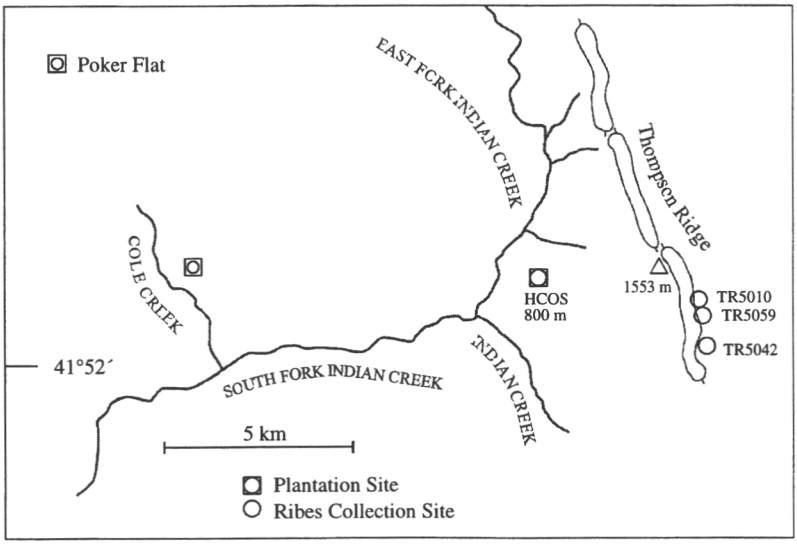

Figure 1. Vicinity around Happy Camp outplanting site (HCOS), showing features discussed in text. Squares, plantations of sugar pine with major gene resistance; circles, collection sites for *Ribes* inoculum.

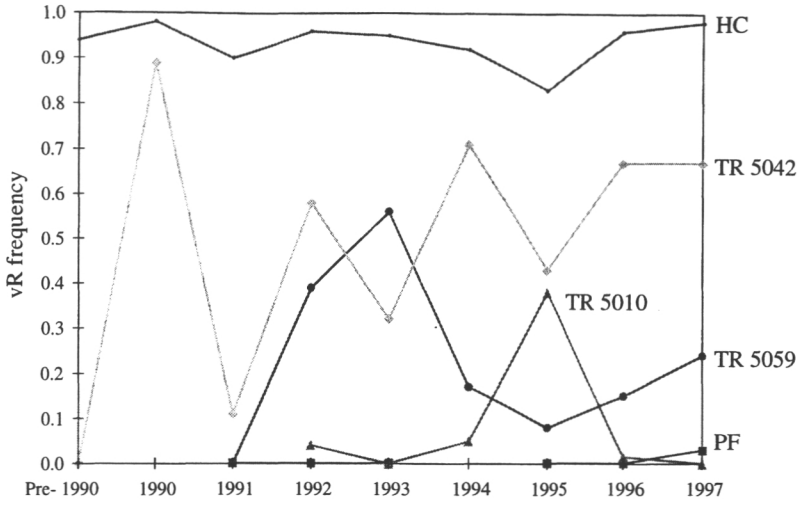

Figure 2. Annual frequency of the virulent allele (vR) of *Cronartium ribicola* to major gene resistance in sugar pine at five sites in the neighborhood of the Happy Camp outplanting site, 1990–1997.

Infected *Ribes* leaves were sampled when telia were mature, in late summer or early autumn. Usually, one leaf was sampled from each of 20 to 50 bushes on a site; in the case of Thompson Ridge, bushes were sampled in an approximate 30 m radius around each Rr sugar pine at plot center. Leaves were kept fresh until used (within three days), when they were well mixed and then suspended over recently germinated R– sugar pine seedlings in a dew chamber for 48 hours at 16° C in the dark, allowing basidiospores to be produced and cast (Kinloch and Dupper 1987).

The virulent phenotype on R– seedlings is characterized by bright yellow (or red) spots, the same as wildtype on rr seedlings. In multiple infections, these continue to enlarge until they coalesce. The avirulent phenotype, by contrast, is a necrotic spot that is self limiting. Spot phenotypes could be resolved within a few weeks after inoculation, when frequencies of each phenotype were counted (and before yellow spots began to coalesce). Each spot was presumed to result from infection by a single haploid basidiospore. Thus, each phenotype denoted the genotype of the spore that caused it, and the counts provided direct estimates of the relative allele frequencies in sample populations. Usually, all spots induced from a given inoculum source were counted, unless numbers were very large. Two samples had counts under 20, but the rest were usually much greater (median: 143).

3 Results and discussion

Over the years, allele frequencies were stable and almost fixed for vR at Happy Camp (0.83–0.98), and for AVR at Poker Flat (Fig. 2). vR appeared for the first time at Poker Flat in low frequency (0.03) in 1997. Frequencies of vR on Thompson Ridge fluctuated widely (0.00 to 0.89) at each of the three sites, but were not synchronous among the sites. These fluctuations attenuated considerably over the years until reaching relative stability

between 1996 and 1997. This trend was most conspicuous at TR5042. At TR5010, vR went from a peak at 0.38 in 1995 to 0.015 in 1996 and was undetectable in 1997 (though sample size in this instance was only 45).

Fluctuating allele frequencies indicate low effective population size (Ne), and low Ne's are highly vulnerable to genetic drift. Calculated Ne is inversely proportional to the standardized binomial variance of allele frequencies and sample sizes, measured at two different times (Waples 1989). Conversely, stable allele frequencies over time indicate high Ne.

The data show, in interesting contrasts, the interplay of selection, migration, and drift on this pathogen that portray a typical metapopulation structure. We have presumed that vR arose as a mutation at Happy Camp that was rapidly selected. R– sugar pine genotypes exert absolute selectivity for vR rust genotypes; they were almost the only pines remaining on the site after virulence was detected in 1978, and the only ones planted since that time for the purpose of secondary screening for slow-rusting traits (Kinloch and Davis 1996). This has apparently been sufficient to maintain vR near fixation within this site. In contrast, inoculum at Poker Flat has been nearly fixed for the alternate allele, AVR, even though blister rust hazard is much higher at this site than at Happy Camp, and even though a similar cohort of planted resistant progenies has been there since 1983 (though in smaller numbers than at Happy Camp). Apparently, chance has not yet favored vR here.

On Thompson Ridge, three infections on tree TR5042, a known Rr genotype, were observed by binocular inspection in 1987, and in 1997 the tree was climbed and 18 were counted. These were distributed in three different cohorts, representing known wave years in 1983, 1989, and 1993. This increase of infection on TR5042, as well as the tendency towards stabilization of vR at intermediate frequencies on surrounding *Ribes*, is evidence that some degree of selection was operating. This is interesting, because, unlike Happy Camp, the overwhelming majority of sugar pines in this natural stand are susceptible genotypes. Whether selection is a factor at TR5059 and TR5010 is more ambiguous.

Although blister rust is capable of long distance spread (Mielke 1943, Smith 1996), such events are infrequent. Our data show that even in a local neighborhood, migration is sporadic and limited to quite short distances. For example, vR could not be detected as close as 0.5 km from the periphery of the test site at Happy Camp for years after it appeared within the site (Kinloch and Dupper 1987), and in 1993, the last year of measurement, was at a frequency of only 0.19 outside the site (unpublished data), compared with 0.95 inside (Fig. 2). The weak level of immigration of wild type (i.e., avirulent) blister rust back into the site is perhaps more impressive, considering that the test site is only about 2 ha and surrounded on all sides by natural forest or brush of both alternate hosts; AVR ranged from only 0.02 to 0.17 over the nine years of measurement (Fig. 2; reciprocal of vR at Happy Camp). Two other small plantations in the area at Cole Creek and Poker Flat (Fig. 1), both with R– sugar pine genotypes to monitor changes in virulence, have shown no change. We consider the initial appearance of vR on Thompson Ridge to represent a small founder population that migrated from Happy Camp, because of the proximity of Thompson Ridge downwind from Happy Camp, and because of closer affinities between these two sites than other nearby sites based on molecular markers (Kinloch *et al.* 1998). No

evidence of virulence had been found here on either alternate host prior to 1987 (Kinloch and Dupper 1987).

Evidence of genetic drift is quite clear in fluctuations of vR frequencies at each of the three plots on Thompson Ridge. Even in this small area, however, interesting and dramatic differences are apparent. Initially, variance of vR was greatest at TR5042, but later trended towards stabilization at high frequency. Spores at TR5010 also have high variance, but appear to be trending towards loss of vR. In all plots, trends are asynchronous. This suggests that in spite of their proximity, they may have become three separate demes.

We recently showed that populations of *C. ribicola* in North America were highly differentiated from each other (G_{st} = 0.21), based on multivariate analysis of molecular markers (Kinloch *et al.* 1998). Happy Camp had the greatest genetic distance from all the rest, which we attributed to natural selection of a small founder population of spontaneous mutants with specific virulence to R. Selection would have been essentially neutral with respect to molecular marker loci, however, subjecting these loci to random drift. Data of the present study, using only a single locus (vR/AVR), but a finer geographic scale, is thus consistent with the earlier results on a much coarser scale. Both show that gene flow in this pathogen is usually slow, erratic, and largely controlled by genetic drift. Extinction of a gene, even for virulence, is a real possibility if selection is not too strong, as the case of vR at TR5010 shows.

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The distribution of white pine blister rust in the Sacramento Mountains of New Mexico

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Summary

White pine blister rust, *Cronartium ribicola*, was discovered by F.G. Hawksworth in 1990 on southwestern white pine, *Pinus strobiformis*, in the Sacramento Mountains near Cloudcroft, NM. Informal surveys that year by pathologists of the U.S. Forest Service Southwestern Region and Rocky Mountain Research Station showed that the rust had already spread throughout the Sacramento Mountains. The rust distribution within the Sacramento Mountains reported here was determined from three plot series established by Conklin (1990 to 1998) with 41 to 52 trees in each plot, by Van Arsdel and Popp (1996 to 1998) with 8 to 37 trees in each plot, and by all four authors in 1998. Various site features, including elevation, aspect, and *Ribes* species and abundance were noted for each plot. The trees have been observed from one to three times to determine the presence and location of rust cankers and the appearance of new infections. Data from each plot series indicated that the rust in the Sacramento Mountains was increasing and was influenced by *Ribes* distribution, elevation, and topography. Rust incidence was greater where *Ribes pinetorum* was abundant, where the rust had been present longer, and at higher elevations. Of the six species of *Ribes* reported in the area of the Sacramento Mountains, only *R. pinetorum* and *R. cereum* were common and widespread. Wherever the incidence of blister rust on pine was high, *R. pinetorum* bushes were present, and rust incidence was greatest near these bushes. The presence of *R. cereum* had little effect on the distribution of the rust on pines. At lower elevations, *R. pinetorum* was found on north slopes and in some canyon bottoms; it was widespread above 2740 m (9000 ft.). Below 2590 m (8500 ft.) there was a local topographic effect upon the rust distribution, and infection of pines was greatest at the base of the slope. At higher elevations, for example above 2740 m (9000 ft.), rust was widespread and local topographic effects were not evident.

Key words: *Cronartium ribicola*, *Pinus strobiformis*, *Ribes*

1 Introduction

The introduction of white pine blister rust (*Cronartium ribicola* J.C. Fisch.) in the Sacramento and White Mountains of New Mexico has provided an opportunity to study its introduction and distribution in a relatively small and discrete area where the effects of a variety of climatic and environmental factors could be studied in detail (Geils 1993). Surveys immediately after

Hawksworth's discovery in 1990 showed that the rust was already widespread throughout these mountains (Hawksworth 1990).

The importance of southwestern white pine, *Pinus strobiformis* Engelm., has been understated in the literature. There are about 202 000 ha (500 000 acres) of forests in the Sacramento Mountains, the adjoining White Mountains, and the nearby Capitan Mountains that contain southwestern white pine (Conklin 1994). In roughly half of this area, it is a significant stand component. Here, the species grows with a range in elevation from about 2130 m (7000 ft.) to over 3050 m (10 000 ft.). This population seems to be the largest in the southwestern USA. Smaller populations occur in nearly all the higher ranges of mountains in New Mexico and Arizona. It has a wide range of occurrence in Mexico. The general form of the tree is much more like that of the western white pine (*P. monticola* Dougl. ex D. Don) than limber pine (*P. flexilis* E. James), with which it has been taxonomically confused. Compared to limber pine, southwestern white pine has a larger straighter form, much longer thinner cones with thinner recurved scales, longer needles, and a lack of stomata on the outer surfaces of the leaf bundles that makes the tree looks greener (Andresen and Steinhoff 1971, Little *et al.* 1980). There are populations of intermediate forms on isolated mountains between the ranges shown on map number 8 in (Critchfield and Little 1966). These intermediate forms are generally shown as one pine or the other. Both species do occur in the Sangre de Christo Mountains, which are shown as having overlapping ranges on that map.

There were six *Ribes* species found in this region, and a seventh has been reported. *Ribes pinetorum* Greene (orange gooseberry) was common in the mixed conifer forest above 2440 m. It typically became more abundant with increasing elevation, and was generally widespread above 2740 m. Below 2440 m it was found on north slopes and in canyon bottoms. *Ribes cereum* Dougl. (wax currant) was more widespread than *R. pinetorum*. It was much more common at lower elevations than *R. pinetorum*, but it was found at higher elevations, too. It was generally found on warmer sites than *R. pinetorum*, but it was found on cooler sites, too. *Ribes mescalerium* Cor. (Apache currant), *R. inerme* Rybd. (white stem gooseberry), and *R. wolfii* Rothr. (black currant) were much less common than the above species. *Ribes aureum* Pursh (golden currant) was found in stream bottoms below the elevation of the pines. *Ribes montigenum* McClatchie has been reported in these mountains, but we did not find it in the field or in the local herbaria. It may be present at higher elevations on Sierra Blanca, the highest peak in the White Mountains.

Introduction of the rust into the Sacramento Mountains

There are two major hypotheses on the spread of white pine blister rust into the Sacramento Mountains. It is possible that the rust was introduced on planted white pine stock, but old infections indicate occurrence of the rust in at least three sites in the early 1970s, prior to the first recorded plantings made in 1977, indicating a natural spread. The rust is old and abundant in Bradford Canyon, Silver Springs Canyon, and Karr Canyon. The rust is definitely on 1975 wood and some is probably older. These sites are on the west flank of the mountains at approximately 2440 m in wind passes. The oldest heavy infections are remote from residential development, so the

introduction of the rust from ornamental plantings at homes seems unlikely. The spread of rust into the mountains by planted infected *Ribes* plants is unlikely because of the low chances for spread from *Ribes* to pine. Wave years for conditions favoring pine infections are infrequent. The possibility of rust having been introduced to this area at the former Cloud Croft Experimental Forest in the 1960s or 1970s on planted white pines needs to be investigated by checking for the presence of white pines that could have been planted there. Records from the forest do not indicate that there was any tree planting there.

Experience in tracing the annual introduction of peanut rust into Texas from the Dominican Republic and the West Indies at an 2440 m transport level from where the spores were brought down by frontal rains, suggests that natural introduction of white pine blister rust was most likely. In the case of the peanut rust, spores were trapped on slides in an airplane at 2440 m above ground, spores trapped in rain barrels preceded field infection by the proper 10–12 day intervals, and the spores from the rain traps were used to inoculate and infect peanuts (Van Arsdel and Harrison 1972, Van Arsdel 1973, 1974). The spread of white pine blister rust into Indiana further augments the conviction that this type of spread was likely. There, rust was found on *Ribes* in 1936, 1938, 1943, 1945, 1946, and 1947. There was no rust in the intervening years. The rust was found on 1944 wood on two or three pine trees in 1947. The rust on *Ribes* in Indiana was 483 to 644 km (300 to 400 miles) away from the rust on pines in Michigan and Wisconsin (Indiana white pine blister rust control, annual report, 1949, E.P. Van Arsdel). Mielke (1943) provided a much better known reference on distribution of the rust. He documented the spread of the rust from pine to *Ribes* for more than 483 km in several directions. The documented transport from the coastal forest of British Columbia, Canada to the mountains of north central Idaho, USA would be comparable to the spread from California to the Sacramento Mountains. Mielke (1943) also cites references noting the high elevation spread of rust spores and pollen. The natural spread of the rust into the Sacramento Mountains likely was from the mountains of southern California where it was first found in the mid-1960s. The rust would probably have infected *Ribes* several times before pine infection occurred; such might be confirmed by examination of herbarium specimens. All of the older infection sites were at about 2440 m, the elevation at which clouds frequently flow into these mountains. There would be no need for rain to bring the spores down, because clouds flow right into the forests at this elevation and frequently bathe the trees in fog. It would take about 4.3 hours for the spores to travel the 1047 km (650 miles) from California in a 242 km per hour (150 mph) jet stream wind. Both peanut rust urediospores and blister rust aeciospores are fairly large (25 μm long), thick walled, pigmented, daytime spores (aeciospores, urediospores) that can easily withstand a flight of that duration.

2 Material and methods

Three series of sample plots were established. The first series of sample plots established by Conklin in 1990 to 1998 was rechecked for *Ribes* by Popp and Van Arsdel in 1998. The same trees on each plot have been checked every three years up to three times for incidence of rust. These plots were located

throughout the white pine range in the Sacramento and White Mountains and include a wide range of site conditions. Each plot sampled 41 to 52 representative "crop trees" (trees of relatively good form and position selected without regard for rust infection). The sample on each plot included size classes from saplings to mature trees, selected over an indefinite area of 0.4 to 1.2 ha (1 to 3 acres).

A second set of 16 plots was established by Van Arsdel and Popp in 1996 to 1998 and contained 8 to 37 trees each. Infection and environmental data were recorded in a slightly different form and *Ribes* data were included. All trees above 0.3 m high were recorded in plots of about 0.04 ha (0.1 acre), but some plots were larger or smaller to sample approximately 10 trees. These plot locations were designed to sample different microclimatic sites, and some were made in a transect across Spudpatch Valley.

3 Results

The data from the first series of 12 plots, the Conklin plots, showed that there was generally an increase in the amount of rust with the amount of *R. pinetorum* nearby (Tables 1 and 2), the elevation (Fig. 1), and the length of time the rust had been present in the vicinity (Fig. 1). The Silver Springs plot had the most rust because of its elevation, the relatively long time since the first infection (older than 1980), and the presence of *R. pinetorum* (Tables 1 and 2). Much *R. pinetorum* occurred just outside the boundaries of this plot. Wills Canyon, the plot with the highest elevation, had the elevation and *Ribes* presence, but had not had a long enough time since infection to insure a high level of rust infection. It had the greatest rate of increase in the amount of rust. The amount of *R. pinetorum* had been increasingin this plot in the aftermath of logging that occurred less than two years before the plot was established. In the 12 plots, the amount of *R. pinetorum* generally, but not always, increased with elevation (Fig. 2). The amount of rust could also increase with elevation, but, as in the case of the James Ridge plot, when *R. pinetorum* was not present, then the rust was not abundant (Tables 1 and 2). The size of the 16 plots in the Van Arsdel/Popp series was not big enough to draw major conclusions, but there was more rust at the higher elevations, and less when *Ribes* was absent or rare on the plot.

Random sampling of white pines within six study areas (= airsheds; a mountain valley or canyon whose nocturnal drainage winds make a more or less closed system) is in progress. To date, 1168 trees over 1.4 m (4.5 ft.) tall and 391 trees less than 1.4 m have been sampled on 96 plots. Rust was found on 32% of the taller trees and 8% of the seedlings. Preliminary analysis suggests that elevation, habitat type (series), and slope position are predictors of rust incidence and severity in the Sacramento Mountains. Stands above 2440 m typically harbored more rust than lower elevation stands. Conversely, on a given slope, rust incidence was generally highest near the bottom and lowest near the top (Table 3). At similar elevations, white fir (*Abies concolor* (Gordon & Glend.) Lindl. ex Hildebr.) and blue spruce (*Picea pungens* Engelm.) habitat types usually had more rust than Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) habitat types (Table 3).

Table 1. Amount of blister rust infection on 12 Conklin plots in order of elevation.

Elevation order	Field plot No.	Plot name	Elevation in feet	Year sampled	Number of trees	Number infected	Cankers per 100 trees	Date of oldest infection
1	8	Sixteen Springs	7200	1995	45	0	0	1992
				1998		4	9	
2	9	Bonito Lake	7500	1995	48	1	2	1990
				1998		13	40	
3	3	Poison Spring	7500	1991	52	0	0	1989
				1993		2	4	
				1996		4	8	
4	11	Lower Fence	7550	1997	49	1	2	1989
5	7	Monjeau	7800	1995	48	0	0	1990
				1998		2	4	
6	5	Hoosier	7900	1994	41	23	268	1985
				1997		26	390	
7	6	Long Canyon Carisa Lookout	8100	1995	46	3	7	1989
				1998		12	35	
8	12	Upper Fence	8100	1997	48	25	354	1985
9	4	James Ridge	8500	1994	45	3	11	1985
				1997		7	27	
10	10	Little Apache	8700	1995	47	34	434	1985
				1998		34	515	
11	1	Silver Springs	8700	1991	41	39	1049	1980
				1993		39	1220	
				1996		39	1585	
12	2	Wills Canyon	9300	1991	45	3	7	1985
				1993		6	49	
				1996		26	256	

Table 2. Amount of *Ribes* on 12 Conklin plots in feet of living stem (FLS) per acre.

Elevation order number	Establish-ment plot number	Plot name	Elevation in feet	*Ribes cereum* FLS/acre	*Ribes pinetorum* FLS/acre
1	8	Sixteen Springs	7200	0	0
2	9	Bonito Lake	7500	310	0
3	3	Poison Spring	7500	85	55
4	11	Lower Fence	7550	2870	0
5	7	Monjeau	7800	0	0
6	5	Hoosier	7900	1350	75*
7	6	Long Canyon, Carisa L.O.	8100	0	0
8	12	Upper Fence	8100	630	1200
9	4	James Ridge	8500	210	0
10	10	Little Apache	8700	1230	875
11	1	Silver Springs	8700	1100	10*
12	2	Wills Canyon	9300	550	1825

* About 10 000 FLS per acre *R. pinetorum* occurred just off the sampled area.

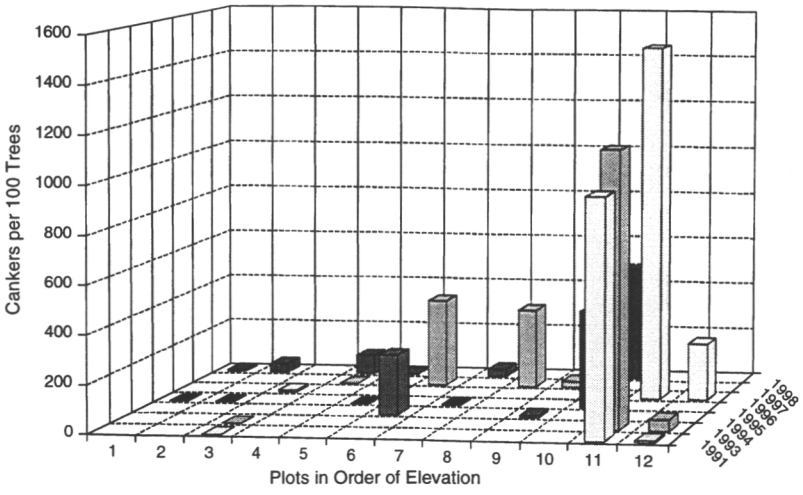

Figure 1. The amount of blister rust infection on pines in the 12 Conklin Plots. See Table 1 for respective plot numbers.

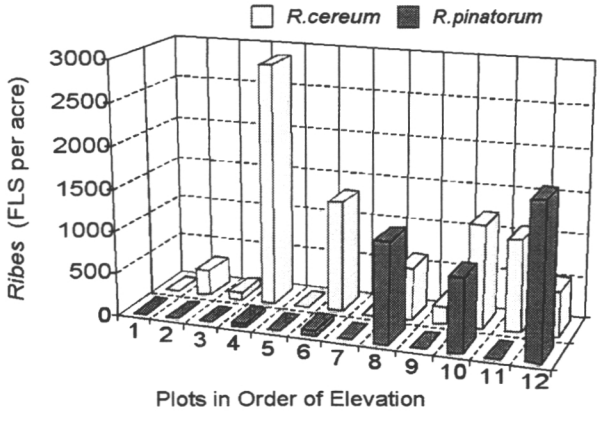

Figure 2. The amount of *Ribes* per acre on the 12 Conklin Plots. See Table 1 for respective plot numbers. FLS = feet of living stem.

Table 3. Amount of rust on pines over 1.4 m tall in Spudpatch study area by slope position and forest type series.

Slope position or type series	Number of trees	Number of infected trees	Percent infected trees	Number of cankers per 100 trees
Lower slope	99	71	72	340
Mid slope	110	64	58	410
Upper slope	169	65	38	220
White fir/blue spruce type	165	108	65	490
Douglas-fir type	213	92	43	170

Throughout these mountains, *R. pinetorum* was a major contributor to the spread of rust to nearby pines. *Ribes cereum* was rarely infected by *C. ribicola* and it did not seem to be important in the spread of rust to nearby pines. The high coverage of the leaves by another rust fungus, *Coleosporium ribicola* Arth. (= *C. jonesii* (Peck) Arth.) could possibly be limiting the amount of blister rust that can infect *R. cereum*, but this does not seem to account for the absence of blister rust in all areas. *Ribes inerme* was occasionally found infected with a little blister rust in some of the stream bottom patches, but this was rare. Blister rust has not been found on *R. aureum* along the stream banks below the elevation of the pines.

4 Discussion

The introduction of white pine blister rust into the Sacramento Mountains is an opportunity, a challenge, and a disaster. An opportunity because it gives a unique chance to study the development of an epidemic and hazard class zones for blister rust. A challenge because we need to learn to control and manage this situation. A disaster because a great many wonderful southwestern white pine trees are dying and more will die as more trees become infected.

The hazard classes are beginning to show that there are *Ribes*-free areas, especially at the lower elevations where many pines will survive by escaping the rust. There is a low elevation zone that has pine but no *Ribes pinetorum*. Above that there is a fairly low elevation zone where there are susceptible *Ribes* but little rust infection on the pine because it is not a favorable climate for rust infection. The next higher zone is one in which the rust is found where there are *Ribes* and locally favorable microclimates to benefit the disease. The next higher zone is one in which the rust is found within about 305 m (1000 ft.) of susceptible *Ribes* plants. Lastly there is a zone so climatically favorable to the rust that the rust can spread long distances. In this climatically high hazard zone at high elevations there are *Ribes* free zones and zones with only *R. cereum* nearby that have only low percentages of trees infected. In such areas an important number of trees can survive. However, there are vast areas where climatic conditions and the populations of *R. pinetorum* are very favorable to spread of the rust, and many trees will die. Disease management practices can be developed using well known control principles augmented by new knowledge we can gain to reduce these losses to manageable levels in many areas.

Control of the rust (or management) would require site selection and pruning. The pruning is complicated by the local rust infection habit of infecting high-up in the trees, unlike in other regions where most of the rust is close to the ground. This is probably due to the transported moisture in the clouds being the main source of moisture permitting infection rather than rainfall being evaporated from the soil as it is in the Great Lakes region. The selection and breeding of resistant trees should be a long term goal in control of the rust. There appear to be resistant individuals present in the infected areas in these mountains. However, multiple sources should be obtained in genetic resistance breeding programs.

The transmission of rust by only one species of *Ribes* makes the Sacramento Mountains a better place than some others for control by

alternate host eradication. Sampling of the rust where *R. pinetorum* is present and where it is not has already indicated that pines can be grown in the absence of this *Ribes* species. Local control by *Ribes* eradication has been shown to be effective in reducing the amount of rust infections on pines (Van Arsdel 1968). To paraphrase part of the paper: Of Honey's and Putnam's 35 plots maintained from 1935 to 1932, 29 had eradication treatment. I found that during the four years before eradication (eradication years varied), 5174 cankers were initiated; during the four years after eradication, 103 cankers were initiated. This was 72.4 cankers per 100 trees before eradication compared to 1.4 cankers per 100 trees after eradication (Van Arsdel 1968). The eradication problem is less in *P. strobiformis* stands than in some other areas because only one species of *Ribes* is important in spreading the rust, and *R. pinetorum* has a limited range. The eradication of those bushes within the stand will greatly reduce the amount of rust infection, but important amounts of rust will come in from bushes in surrounding margins. The most effective control system for this region would probably involve *Ribes* eradication by airsheds. Studies of the drainage winds that move spores and the patterns of rust infection associated with them are needed to clarify the best ways to manage the rust. Pilot studies testing eradication procedures could be initiated before the results of the studies are in.

Ribes control may be a more viable option in the intensively managed forests of the Mescalero-Apache Indian Reservation than on the Lincoln National Forest. White pine has been a prefered crop in many mixed species stands in the reservation because of dwarf mistletoe on the ponderosa pine *P. ponderosa* Dougl. ex P. Laws. & C. Laws.) and/or Douglas-fir. Most of the white pine in the reservation occurs at or below 2440 m; overall, rust is less severe than in the higher elevation forests of the adjacent national forest. Moreover, compared to the Lincoln National Forest, there is relatively little *R. pinetorum* on reservation lands (*R. cereum* and/or *R. mescalerium* are much more common than *R. pinetorum* in most areas). It is conceivable that a relatively limited amount of *Ribes* control work could significantly reduce potential rust impact on the reservation.

While stopping all infection is not a reasonable goal, management strategies can be developed to limit rust spread.

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Long-term differential expression of blister rust resistance in western white pine

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Summary

Between 1950 and 1970, western white pines (*Pinus monticola*) resistant to the blister rust fungus (*Cronartium ribicola*) were bred for the northern Rocky Mountains of the western United States. F_1 and F_2 seedlings were selected using a program of full-sib crossing with intense artificial inoculation. Since selected resistance was hypothesized to be vertical, long-term exposure to racially stable rust was predicted to yield 33% canker-free trees for F_1 and 66% for F_2. Epidemics observed in an experimental plantation established in 1970 were compared to test this hypothesis. Poor early survival, unevenly distributed throughout the plantation, negated the original complete block design. Subsequently, data were stratified into two site-types, one characterized by a hot site-preparation burn (brush) and the other by a normal burn (forest). Rust incidence (proportion cankered) was measured on cohorts of about 100 trees at exposure times of 2, 4, 6, 12, and 26 years. The nonlinear monomolecular model of disease progress was used to analyze rust incidence and absolute infection rate. Rust incidence in epidemics of control stock and one resistant stock attained unity, indicating that K (incidence asymptote parameter) should equal one. Incidence data were bootstrapped 20x to generate confidence limits. Of six epidemics (two sites x three stocks), only F_1-forest and F_2-brush mean infection rates were not significantly different. The finding that brush to forest infection-rate ratios were nearly constant over stocks indicated that environmental factors other than sporidial density influenced the infection rate for all stocks. Finally, all combinations of resistant stock and site exceeded expected incidence thresholds after 26 years. Was racial variation of the rust responsible, or did selection focus on horizontal resistance?

Key words: *Cronartium ribicola*, *Pinus monticola*

1 Introduction

The blister rust (BR) fungus (*Cronartium ribicola* J.C. Fisch.) was introduced into western North America in 1910 and promptly caused widespread mortality. Particularly large losses occurred in extensive stands of commercially valuable western white pine (WWP; *Pinus monticola* Dougl.) growing in eastern Washington, northern Idaho, and western Montana, a region known locally as the Inland Empire. The pathogen reached the Inland Empire about 1925, where the initial response was to eradicate alternate

hosts, species of the genus *Ribes*. Sufficient eradication was impossible, given that five million acres of mountainous terrain needed attention. Soon, the need for alternatives became evident.

Resistant phenotypes of WWP were well known by 1947, and breeding for resistance began in 1950 (Bingham 1983). Reproductively mature and canker-free trees were selected from stands exposed to severe rust hazard. Ultimately, 400 candidate trees were located, crossed in a partial-diallel mating scheme, exposed to artificial inoculations, and screened for canker-free seedlings four years after inoculation (Bingham 1972). Large progeny tests were analyzed for evidence of resistance mechanisms based on signs, symptoms, and disease cycle. Two mechanisms located in needle tissue, premature-needle-shed and reduced-needle-lesion frequency, were found (McDonald and Hoff 1970, Hoff and McDonald 1980b). Fungicidal-short-shoot and canker elimination (Hoff and McDonald 1971, 1972a) were located in stem tissue. General combining ability (GCA) for resistance based on these mechanisms was found in about 100 candidates. These GCA-selected individuals produced increased levels of canker-free progenies in all crosses (Bingham *et al.* 1969).

GCA-selected trees were crossed to yield an F_1 that produced 25% spots-only (combined premature-needle-shed and fungicidal-short-shoot) and 8% canker elimination resistance after exposure to intense artificial inoculation. Thus, 33% of trees known to have needle infections were expected to remain free of cankers (Hoff *et al.* 1973). Canker-free trees selected from the artificially inoculated F_1 population became the parents of the second generation (F_2) of resistant material. When exposed to artificial inoculation, F_2 produced 59% spots-only and 7% canker elimination resistance, or a 66% canker-free expectation (Hoff *et al.* 1973). Simple inheritance (McDonald and Hoff 1971) and assumed blockage of further rust development (Hoff and McDonald 1980a) in plants with these resistance mechanisms formed the basis of a vertical resistance hypothesis. Comparative analysis of epidemics can provide a critical test.

Disease progress curves allow comparisons of epidemics based on a strong theoretical foundation (van der Plank 1963, 1975, Zadoks and Schein 1979, Madden and Campbell 1990). BR epidemics are known to follow the nonlinear monomolecular model (Kinloch and Byler 1981, McDonald and Hoff 1982). Madden and Campbell's (1990) equation,

$$y = K\,(\,1 - e^{-rt}), \qquad (1)$$

is appropriate, where y = incidence at time t, K = maximum incidence, e = base of natural logarithm, r = absolute infection rate in incidence/year, and t = age of the epidemic in years. The model assumes r is constant (unbiased variation) over t and that disease occurrence and pathogen spread are random processes. Rate of incidence increase is assumed proportional to the amount of healthy tissue remaining (Campbell 1986) or the number of healthy plants (Waggoner 1981). When initial amount of disease is zero, as in WWP plantations, the infection rate r is

$$r = \ln\,(K/(K - y)/t), \qquad (2)$$

(McDonald and Hoff 1982), where r, K, y, and t are as above and ln = natural logarithm.

Infection is an ecological process, and resistance mechanisms can be either resistance to infection or resistance to colonization (Nelson 1978). Mechanisms preventing infection are vertical, hypersensitive, race-specific, or major-gene resistance. These mechanisms in BR, along with environmental factors, control the magnitude of the spores-to-spots ratio. At least one classic vertical mechanism is known in the BR pathosystem (Kinloch and Comstock 1981). Nelson (1978) defined mechanisms that impede colonization of the invader as horizontal, generalized, minor-gene, or race-nonspecific resistance. In BR, these mechanisms and environmental factors control the magnitude of the spots-to-canker ratio. Finally, additional horizontal mechanisms in the BR pathosystem could influence canker size and shape, spore-production rates, and growth rates of cankers (Hoff 1984).

Resistance definitions stated in terms of the monomolecular model provide quantitative points useful for epidemic comparison. If resistance is vertical in a portion of a population, the ratio K_S/K_R (susceptible/resistant) is greater than one. Three factors that depress K are clumped inoculum distribution, heterogeneous infection microclimate, and incomplete vertical resistance (McDonald *et al.* 1991). K determined by actual observation is needed to use equation (1) (Neher and Campbell 1992). When K_S measured on-site is equal to one, the assumption of random distribution of disease is validated. Then, K_R can be used to compare resistant material. In epidemics involving vertical resistance, the infection-rate ratio, r_R/r_S, will equal one (van der Plank 1975). Here, the hypothesized canker-free WWP proportions of 0.33 (F_1) and 0.66 (F_2) become K = 0.67 and 0.34, respectively. When vertical resistance is complete, K_R equals zero and K_S/K_R is undefined. However, racially-caused breakdown of resistance would move K_S/K_R toward one. Alternatively, if resistance is horizontal, K_S/K_R equals one and all action shifts to the r parameter. Epidemics in different WWP stocks would have unequal r and equal K_S/K_R. Ratios of r parameters associated with differing levels of resistance would be stable over time and not equal to one. Thus, variation of infection rate distinguishes populations expressing horizontal resistance (Nelson 1978). This paper reports a preliminary test of the hypothesis that BR resistance in Inland Empire WWP is vertical.

2 Material and methods

Bingham *et al.* (1973) presented the layout and experimental design of the 14.6 ha Merry Creek plantation located in northern Idaho, USA. After the second growing season, seedlings were selected from control, F_1, and F_2 stock. A metal tag was applied to 25 trees from each of nine 0.4 ha plots planted to each stock. Seedlings were inspected each fall at 2, 4, 6, 12, and 26 years after planting, except that the 26-year inspection required an entire field season. Cankers/tree, tree height, and tree condition (living or dead) were recorded to provide disease severity (cankers/tree) and disease incidence (proportion of population infected) assessments. Cankers were counted while inspectors were standing at the base of crowns. All 26-year-old trees designated canker-free from the ground inspection were climbed to verify their status.

Uneven mortality, not caused by *C. ribicola*, negated original design objectives and forced creation of a new design. A boundary delineated by

density of a brush species afforded an opportunity to divide the plantation into two site-types. Density of evergreen ceanothus (*Ceanothus velutinus* Dougl.) is directly related to intensity of the heat pulse generated by a forest fire or site-preparation burn (Noste and Bushey 1987). Twenty-eight years after burning, a boundary seen as a change from low to high brush density was mapped using GPS technology. Then, location of individual sample trees was determined by an offset technique (McDonald *et al.* 1998). Next, plot trees were assigned to brush and forest site-types based on their mapped location. Nutrient and biophysical verification that brush and forest site-types were different was planned but not completed for this preliminary study.

Random distribution of infections is required for comparative analysis of epidemics (Madden and Campbell 1990). The three components of infection distribution are distribution of spores, infection microclimate, and resistance genes in the host population (McDonald *et al.* 1991). Distribution of sporidial concentrations was not measured at Merry Creek. However, much is known about sporidial distribution. Pine infection is caused by windborne sporidia (5 μm in diameter) produced on the alternate host. Five spore-stages function in clearly defined annual cycles on two woody perennials to produce long-lived epidemics. Annual sporidia concentrations can vary 130x (McDonald *et al.* 1991). At Merry Creek, 3000 sticky currant (*Ribes viscosissimum* Pursh.) per hectare (unpubl.) should produce about 150 spores cm^{-2} in a "good" weather season (based on extrapolation of Figure 3 in McDonald *et al.* 1991). At such densities, average distance from spore source to target is 2 m (McDonald *et al.* 1981). An empirical disease-distance curve constructed for BR (Buchanan and Kimmey 1938) shows that about 90% of spores produced by a bush will be deposited within 2 m of that bush. The relationship assumed that the ratio of needle infections to canker appearance was not distant-dependent. We assumed that high *Ribes* bush densities evenly distributed across brush and forest site-types would provide random horizontal distribution of sporidia.

Growth of host tissue may cause problems with application of disease progress models (Madden and Campbell 1990). These problems mostly pertain to polycyclic diseases where inoculum is being produced on the growing hosts. Available host tissue can increase faster than available inoculum. In the case of BR, inoculum production is not related to amount of needle tissue except to the extent that WWP canopies shade *Ribes* bushes. Such shading can result in significant reductions of inoculum load (Hagle *et al.* 1989), thereby reducing infection rate as trees grow. In addition, high population densities of *Ribes* produce such immense volumes of sporidia that trees can reach a large size before available infection sites exceed the number of available spores. For example, a 25-year-old WWP growing on a good site has a projected crown area of about 49×10^3 cm^{-2} (calculated from data in McDonald *et al.* 1981). Thus, 7.36×10^6 sporidia (49×10^3 cm^{-2} x 150 sporidia cm^{-2}) should be available in the 25th season to infect 1.1×10^6 cm^2 of infective needle surface (calculated from data in McDonald *et al.* 1981). On this theoretical basis, we assumed that host tissue had not saturated inoculum availability by the 26th year of the epidemics.

Heterogeneous infection microclimate could be another major cause of clumped infection. Microclimate data were not collected at Merry Creek, but microclimate could be modeled for the 26-year duration of the experiment.

However, in the Inland Empire, conditions for pine to become infected appear to be uniform within infection cycles (season) because most pine infections develop during the passage of stable slow-moving cool and wet air masses (McDonald *et al.* 1981). Thus, the assumption was made that infection microclimate remained uniform across brush and forest sites within an infection season.

Rust incidence was determined three times for control populations planted on brush (91 trees) and forest (80 trees) site-types. Incidence was determined five times on F_1-brush (54 trees), F_1-forest (91 trees), F_2-brush (36 trees), and F_2-forest (68). To supply estimates of confidence limits, these samples were bootstrapped 20 times using a sampling with replacement algorithm in Systat 7.0 (Wilkinson and Engelman 1997). Using the bootstrapped samples, r was computed by equation (2), where t = 2, 4, and 6 years for controls and 2, 4, 6, 12, and 26 years for the remaining populations. Thus, 20 estimates of r became available for each combination of measurement-year, stock, and site-type. Means and confidence limits (95%) of rust incidence were computed from the bootstrapped data for each inspection year x site-type x stock combination. These observed values were compared to expected incidence (y in equation (1) where r = mean infection rate, and K = 1).

3 Results

Attainment of 100% rust incidence by control stocks at Merry Creek validated the assumption of random distribution of disease (Table 1). The BR epidemic in the F_1-brush pine population progressed to unity as well. The remaining epidemics, F_1-forest, F_2-brush, and F_2-forest nearly achieved unity after 26 years of exposure (Table 1). On this basis, we tentatively concluded that K = 1 in all Merry Creek epidemics and continued with the analysis.

The ranking of epidemics with respect to observed rust incidence was mostly consistent (Table 1). Two exceptions were evident in F_1-forest and F_2-brush after six and 12 years (Table 1). Agreement between observed and expected rust incidence within individual epidemics and across years appeared to be unbiased for all epidemics (Table 2). This finding means that infection rates could be pooled over years for each stock and site combination. Four of the combinations differed significantly from mean infection rate and two, F_1-forest and F_2-brush, did not differ (Table 3).

Table 1. Observed incidence of blister rust (number of cankered trees/total number of trees) in six populations of western white pine grown at the Merry Creek plantation located in northern Idaho, USA.

	Years of exposure				
Epidemic	2	4	6	12	26
C-Brush	0.83	0.89	1	–	–
C-Forest	0.71	0.84	0.94	1	–
F_1-Brush	0.45	0.58	0.71	0.81	1
F_1-Forest	0.37	0.45	0.58	0.67	0.96
F_2-Brush	0.29	0.41	0.63	0.72	0.97
F_2-Forest	0.20	0.23	0.42	0.50	0.89

Table 2. Residual of observed blister rust incidence minus predicted incidence[1] in six populations of western white pine grown at the Merry Creek plantation located in northern Idaho, USA.

	Years of exposure = *t*				
Epidemic	2	4	6	12	26
C-Brush	+0.05	–0.06	0.0	–	–
C-Forest	+0.08	–0.02	0.01	0.0	–
F_1-Brush	+0.01	+0.01	0.01	–0.11	0.0
F_1-Forest	+0.12	–0.02	–0.04	–0.18	–0.02
F_2-Brush	+0.04	–0.03	+0.05	–0.10	0.0
F_2-Forest	+0.05	–0.06	+0.02	–0.14	0.0

[1] predicted incidence = $(1-e^{-rt})$ where r = mean rate in Table 3

Table 3. Means of absolute rates of blister rust infection calculated from bootstrapped (20x) data and the monomolecular model of disease progress as observed in six populations of western white pine grown at the Mary Creek plantation located in northern Idaho, USA.

	Mean	95% confidence limit		Brush to	C/F_1 & C/F_2	C/F_1 & C/F_2
Epidemic	Rate	Lower	Upper	Forest ratio	Ratios (brush)	Ratios (forest)
C-Brush	0.753	0.707	0.799			
C-Forest	0.504	0.48	0.528	1.49		
F_1-Brush	0.212	0.199	0.224		3.7	
F_1-Forest	0.162	0.147[1]	0.177	1.31		3.1
F_2-Brush	0.144	0.135	0.153[1]		5.2	
F_2-Forest	0.084	0.079	0.089	1.71		6.0

[1] confidence limits overlap, therefore F1-Forest and F2-Brush are not significantly different

The various ratios of infection rates provide clues about relationships between resistance and environmental factors. A nearly constant brush-to-forest ratio of 1.5 over the three stocks (Table 3) indicated that the brush site was more suitable for expression of cankers than the forest site. Control to F_1 ratio was larger on the brush site than on the forest site, as expected. Control to F_2 ratios diverged unexpectedly, where the brush ratio was smaller than the forest ratio (Table 3).

4 Discussion

The primary objective of this paper was to ascertain if long-term performance of Inland Empire blister rust (BR) resistant western white pine (WWP) followed the model of vertical resistance. If resistance in the F_1 and F_2 populations was vertical, maximum rust incidences would be 0.67 and 0.34, respectively. Also, according to theory, the susceptible portions of these two populations should exhibit equal infection rates and that rate should be equal to the susceptible population. Results showed that rust incidence for one of the four possible combinations of resistant populations and sites had already achieved 100% infection. Two of the remaining three exceeded 95% infection and the best combination (F_2-forest) was 90% infected. Since this experiment was not replicated, chance cannot be ruled out as an explanation. However, F_2 stock has been planted in many locations in the Inland Empire and critical

determination of rust incidence has shown several cases where *K* exceeds 0.34 (unpubl.). One noteworthy case of rust incidence above 34% in F_2 stock was attributed to mislabeled planting stock (McDonald *et al.* 1994); now this analysis should be revisited. One could argue that the 5% and 11% proportion canker-free trees at this time represent *K* values of 0.95 and 0.89. The following discussion about rate of infection will add evidence with which to judge this question.

Another important aspect of horizontal resistance is its relative nature (Shaner and Finney 1977). Horizontal resistance to a leaf pathogen of wheat could only be measured in relation to a susceptible cultivar. As conditions for disease improved (more soil nitrogen or better weather), the relative performance of the resistant cultivar improved (Shaner and Finney 1977). At Merry Creek, four of six combinations of stocks and sites exhibited significantly different infection rates. More importantly, the ratios of infection rates from brush site-type to those from forest sites-types of all stocks were about 1.5. Thus, the brush site provided the highest relative infection rate in both controls and resistant stock. As expected from the wheat experience, relative F_1 performance was better on the brush site-type than on the forest site-type. This trend did not carry through to the F_2, where the forest site gave the best relative performance. Overall, analysis of rates of infection also suggests that resistance in Inland Empire WWP is horizontal.

Since a thorough understanding of infection rate is crucial, this area should receive future emphasis. One potentially productive approach would be to study soil biophysical differences between brush and forest sites at Merry Creek to verify they differ. On the other hand, the difference between brush and forest may have been caused by a consistent difference in sporidial concentration that was assumed to be absent. A careful analysis of canker frequency on a large sample of surviving trees at Merry Creek may provide some answers about relative hazard. Additional evidence about the Merry Creek epidemics might be obtained by applying the needle-trace method of assaying needle retention in pines (Jalkanen *et al.* 1994). If signatures of expression of needle-spots-only and/or canker elimination resistance remain in WWP needle traces, then the frequency of such host reactions could be measured on living and dead white pine. In addition, this method has potential to track amounts of needle tissue over time to validate pine target models.

In a thorough review of horizontal resistance in crop plants, Simmonds (1991) listed seven characteristics of horizontal resistance. These are: polygenic inheritance, continuous distributions of infection rates, resistance rather than immunity, pathotype non-specificity, durability over time, and resistance composed of several components. Finally, genetic evidence is necessary for critical demonstration of horizontal resistance. The population of rust-resistant WWP selected by the Inland Empire program fits most of the seven characteristics.

Infection rate was continuously distributed over several levels of selection where early experimental plantations contained a spectrum of stock from susceptible to F_1 (Goddard *et al.* 1985). Inland Empire WWP has expressed resistance rather than immunity. Pathotype non-specificity in the BR pathosystem was critically investigated once (McDonald *et al.* 1984). Materials from a selection program in Oregon were used to delineate the

Champion-Mine (CM) pathotype. Selected and non-selected WWP were compared after infection with wild type and CM inoculum. The CM pathotype was equally more aggressive than the wild type toward reduced-needle-spot-frequency and needle-spots-only mechanisms on both selected and non-selected families (McDonald *et al.* 1984). This finding is consistent with the horizontal resistance hypothesis because differences were relative. Durability over time can be currently assessed only by looking at the correspondence of mechanisms and their durability in related five-needle pine growing in native associations with *C. ribicola.* There is some evidence that BR-resistant Inland Empire white pine has these durable mechanisms (Hoff and McDonald 1972b, Hoff *et al.* 1980). The single hypothesis constant with results reported in this paper and the existing literature is that the Inland Empire BR breeding program selected for classic horizontal resistance instead of vertical resistance.

Regarding critical genetic evidence, many of the tests suggested by Simmonds (1991) could be applied. Foremost among these are generation means and variance analysis, analysis of specific and general combining ability, offspring-parent regressions, and response to selection. The last test has been applied in that response from susceptible to F_1 was 0.33 and from F_1 to F_2 was another 0.33. Horizontal resistance typically yields a linear response to selection (Simmonds 1991). These critical tests, as well as other avenues, should be explored, because breeding and integrated management programs alike depend on determination of the true nature of resistance in Inland Empire WWP. These investigations will provide an improved foundation for breeding and blister rust management of all five-needle pines.

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Dry-grinding miniprep method for extracting DNA from urediospores, aeciospores, and teliospores of white pine blister rust, *Cronartium ribicola*

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Abstract

The ability to extract high quality DNA from small quantities of spores is necessary for studies of DNA marker diversity in tree rusts. In this study, a method was developed that uses the rapid sample processing of a reciprocating action shaker (BIO101 Savant FP120 Fast Prep machine) to extract DNA from spores desiccated to 20 RH and viably stored at –80° C prior to extraction. Five to 50 mg urediospores were placed in 2 ml tubes containing each of seven commercially available grinding matrixes (BIO101) with or without the addition of 5 to 50 mg acid washed diatomaceous earth (DE) (Sigma). Tubes were held on wet ice prior to being shaken for 10–30 sec at machine setting 5, with or without the inclusion of extraction buffers before shaking. DNA was then recovered using commercial "FastPrep" protocols with "CLS-F" incubation buffer (Bio101), "Pure Gene" protocols (Gentra Systems, Inc.), and a more rigorous SDS extraction protocol. In the SDS protocol, total nucleic acids were extracted by mixing 525 µL fresh SDS buffer (50 mM TRIS pH 7.2, 50 mM EDTA pH 8.0, 3% SDS, 1% beta-mercaptoethanol) with the ground spores, incubating 1 hr at 65° C with occasional mixing, followed immediately by phenol:chloroform and chloroform:isoamyl alcohol cleanups. Nucleic acids were precipitated using sodium acetate/ethanol, resuspended in 100 µL TE buffer (10 mM TRIS, 0.1 mM EDTA, pH 8.0), and treated with 5 µL of 10 mg/mL RNAase. The resulting solution was purified twice using Strataclean resin (Stratagene). DNA was then selectively precipitated using 0.43 volume 5M NaCl and 2 volumes cold ethanol.

Nucleic acids were of high quantity and molecular weight (MW) when spores were shaken dry for 20 sec in matrices that included 0.8 or 1.0 mm beads and DE, provided the SDS extraction protocol was used, but low in quantity and MW whenever spores were ground in buffer. In our hands, extractions using FastPrep and Pure Gene protocols yielded little DNA. Under optimum extraction conditions (10 mg desiccated urediospores shaken 20 sec in 0.8+2.0 mm zircon bead matrix (Bio101 #6550-225) + 25 mg DE), yields from SDS extractions were 0.7–1.0 µg DNA/mg spores, DNA size was >23 Kb, and PCR amplification yielded consistent RAPD bands. Ten mg quantities of desiccated aeciospores and teliospores shaken 10 sec in tubes of

zircon + DE matrix at setting 5.0 also yielded high quality, unsheared DNA. Successful isolation of high molecular weight DNA from these three rust spore types as well as from ustospores of corn smut *Ustilago maydis* (DC) Corda, basidiospores from a spore print of the mushroom *Amanita muscaria* (L. ex Fr.) Hook. var. *formosa*, and basidiospores from dry puffballs of *Lycoperdon echinatum* Pers. and *L. perlatum* Pers. indicate that these methods may be generally useful for extracting DNA from desiccated fungal spores.

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Key words: molecular biology, techniques

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Control of *Cronartium ribicola* in *Pinus strobus* plantations

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Summary

After the introduction of white pine blister rust (*Cronartium ribicola*) into North America at the beginning of the century, reforestation with white pine (*Pinus strobus*) became hazardous; the disease caused a high rate of mortality and it was a limiting factor for reforestation. In 1933, a plantation with 300 000 white pines in Valcartier near Quebec City was a complete failure; 60% of the seedlings were killed by the pathogen in less than 20 years. The main control measure applied in the United States during the first half of the century was the eradication of the alternate host, *Ribes* spp. When applied by forest owners in eastern Canada, eradication did not control the disease. Later, pruning was considered as an alternative to eradication. Recent epidemiological studies indicate that the age of the plantation is the critical factor when deciding when it is time to prune. Under conditions prevailing in the Appalachian Mountains in eastern Canada, pruning trials in 11 plantations ranging from 8 to 12 years old indicate very good control of the disease, as very few new infections were observed three years after pruning. In the 14 unpruned plantations, the rate of infection varied from 3% to 35% after three years of observation. It is interesting to note that the diseased trees are located near the *Ribes*, indicating that the short distance infection is very important in the epidemiology of the disease. On these old farm lands, it is recommended to prune white pine 7 to 12 years after planting and to eradicate the *Ribes* that are usually located on old piles of stones.

Key words: *Ribes*

1 Introduction

White pine blister rust (WPBR) caused by *Cronartium ribicola* J.C. Fisch. was observed for the first time in North America in 1906. The disease was introduced from Europe on eastern white pine (*Pinus strobus* L.) seedlings imported mainly from Germany (Maloy 1997). In the province of Quebec, the disease was first observed in 1916 at Macdonald College near Montreal (Pomerleau and Bard 1969). After almost one century on this continent, this disease seems to discourage foresters from growing white pine in eastern Canada. In Quebec, WPBR is the main problem that explains part of the failure of white pine regeneration. In spite of the importance and the great impact of the disease, research on WPBR has decreased during the last fifty

years (Maloy 1997). How do we explain this situation? Is it hopeless to work on this disease or are we waiting for an instant solution that may be provided by new technology?

At the end of this century, in spite of the presence of WPBR, white pine is still present in most of its geographic range, but there are fewer white pines than 100 years ago because of the harvesting of this high value species. It does not regenerate well and when it does, the rust can kill a good portion of the regeneration. Establishment of white pine plantations can help solve the regeneration problem, but again, WPBR is devastating in some regions. Lavallée (1986) divided the province of Quebec into hazard zones based on the work of Van Arsdel *et al.* (1956). However, there are local variations within the hazard zones that can be greater than variations between zones. For example, in hazard zone 1, a northern slope or a humid depression can be as favorable for the rust as in hazard zone 3. Also, there is always the possibility of having "very good years" for the rust as was reported in the first half of the century (Mielke 1943; see Maloy 1997). This means that in northeastern Canada, great care of white pine plantations is needed to achieve success with this species. Foresters and entomologists have to deal with competitive vegetation and white pine weevil, respectively. As pathologists, we have to deal with white pine's most important problem, white pine blister rust, which behaves like a "predator" of this species.

One control measure widely used in the past in the United States was eradication of the alternate host, *Ribes* spp. Its use began in 1909 around tree nurseries and then on a large scale on wild *Ribes* as well as on cultivated ones (Maloy 1997). In Canada, only a few trials were done, like the one reported by Pomerleau and Bard (1969). The efficacy of this treatment was questioned, and in the 1950s, it became less popular with the hope that genetic resistance and antibiotics would solve the problem once and for all. Nevertheless, after 70 years of *Ribes* eradication in the state of Maine, WPBR was reduced significantly (Ostrofsky *et al.* 1988).

Pruning infected branches or systematic pruning is another method to control WPBR (Hunt 1982). When applied in eastern Canada, though, failures to control the disease were commonly recorded. Epidemiological studies showed that under local conditions, age is the critical factor (Lavallée 1992). The incidence starts to increase at the age of 6, 7, or 8 years, and then increases at an exponential rate shortly after that in hazard zone 3, which is very conducive to WPBR.

The objectives of this work were 1) to use systematic pruning to control WPBR in white pine plantations prior to the exponential increase of the disease, and 2) to look at the impact of *Ribes* population in or near a plantation.

2 Material and methods

In 1994, twenty-four white pine plantations located in the Appalachian mountains were selected for this study. Their age varied from 7 to 12 years. These plantations contained between 500 and 6500 stems (mean of 1700) and were all in hazard zone 3 (Lavallée 1986). Since 1994, the incidence of the disease was measured annually, as was the height of the highest infection on the 100 pines sampled at random in each plantation.

In 1995, all the white pines in 10 plantations were pruned up to the height where infection was recorded that year in that particular plantation. Pruning was done with manual pruners. Up to 50% of the whorls were pruned, and at least two whorls were left at the top of the tree in smaller trees. Pines with infection on the trunk were cut down. The other 14 plantations were used as controls. From 1996 on, the incidence of the disease was measured every year the same way in all 24 plantations.

In 1997, all pines of one plantation (A-1-7) were mapped using the software ArcView and infected pines were identified. Using a *Ribes* colony as the center of a circle, percentages of infected trees inside circles of increasing size were calculated.

3 Results

In 1996 and 1997, the incidence of the disease in pruned plantations was stable with nearly 3% of the pines infected; all these infected trees had cankers on the trunk or branches which had not been detected in 1995 (Fig. 1). New infections on the shoots began to appear in 1998; the incidence went up to 10% that year. The increase would have been around 6% but two of the 10 plantations were responsible for the overall high rate of infection. In unpruned (control) plantations (Fig. 1), the incidence rate is continuously increasing, leveling in 1996 and 1997 at 11%, but reaching 18% in 1998.

In the unpruned plantations, the incidence of the disease was higher in plantation number A-1-7 than in the other ones, reaching 35% in 1998 (Fig. 2). There are more infected trees near the infection centre, and the infection rate decreases with distance from the centre (Fig. 3).

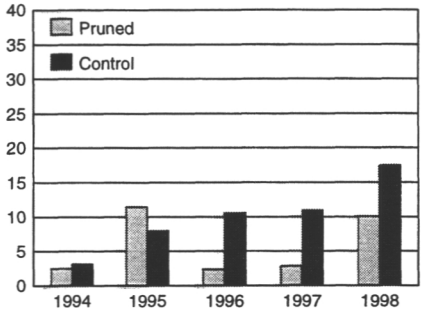

Figure 1. Incidence of white pine blister rust expressed in percentage from 1994 to 1998. The means were calculated for the 10 pruned plantations and for the 14 unpruned (control) ones.

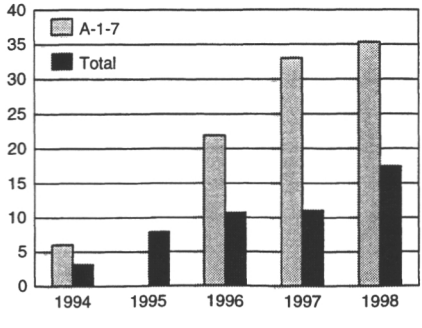

Figure 2. Incidence of white pine blister rust expressed in percentage from 1994 to 1998 in the unpruned plantation A-1-7 as compared with the means of all unpruned (control) plantations.

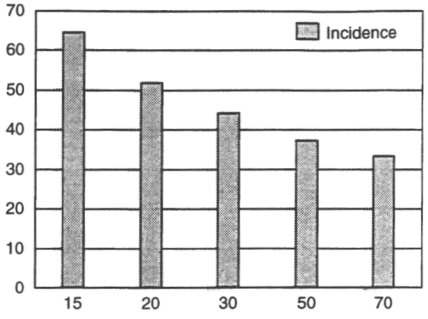

Figure 3. Incidence of white pine blister rust expressed in percentage in plantation A-1-7 with the inside circles increasing in size (meters) from the infection center.

4 Discussion

To be efficient, pruning must be used when white pine plantations are young. The incidence of WPBR does not increase at the same rate everywhere inside a hazard zone; local conditions are very important to determine the age to prune. This is why we recommend evaluating the incidence and severity of the disease in a plantation before any intervention, to help make a decision on the year and height of pruning.

Eradication of *Ribes* may not appear to be a very efficient way of controlling the disease throughout a country. On the scale of a plantation, it is clear that *Ribes* promotes a sharp increase in the incidence of WPBR; the destruction of the alternate host will reduce the annual rate of infection. On old farm land in the Appalachian region, we recommend destroying *Ribes* in and around plantations; *Ribes* is relatively easy to find because it is restricted to stone piles or fences.

Finally, a combination of both pruning and eradication is a very promising tandem in the control of WPBR. Successive pruning may be necessary to keep the incidence of the disease at a low rate. This is not an obstacle, as pruning increases the quality of the logs and it prevents decay by *Fomitopsis pinicola* (Sw.:Fr.) P. Karst., which causes wood decay after entering through branch stubs.

There are other promising paths to control WPBR in forest management practices. In tree nurseries, chemical control can be efficient (Bérubé 1996). The choice of the plantation site is also a very important preventive measure (Lavallée 1986). Biological control is not to be neglected, as thousands of endophytic fungi are not known, offering an enormous source of potential antagonist fungi to pathogens.

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Endophytic fungal flora from eastern white pine needles and apple tree leaves as a means of biological control for white pine blister rust

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Summary

We are studying the biodiversity of the endophytic fungal flora from eastern white pine (*Pinus strobus*) across its Canadian range. 3200 needles from 6 Canadian ecoregions were plated on growth media to determine qualitatively and quantitatively the biodiversity of its endophytic flora. Preliminary results indicate that more than 80 species of fungal endophytes are to be isolated from eastern white pine. Another large collection of previously isolated endophytic fungi from white pine needles and apple tree (*Malus domestica*) leaves were tested for their potential to inhibit *Cronartium ribicola* the causal agent of white blister rust. *Coniothyrium* spp. isolates from apple leaves was very effective in controlling *C. ribicola* on *Ribes* leaves, the alternate host of the disease. *Coniothyrium* caused a dramatic reduction in the number of teliospores, infecting and killing the fungus at the uridial stage. From the 99 white pine needle endophytes tested, three have shown potential to inhibit the infection of *C. ribicola* basidiospores on white pine needles. Preliminary results of these tests will be presented and discussed.

Key words: *Cronartium ribicola,* endophytes, *Microsphaeropsis arundinis, Pinus strobus*

1 Introduction

Eastern white pine (*Pinus strobus* L.) is one of the most valuable timber species in eastern Canada, and it is highly susceptible to white pine blister rust caused by *Cronartium ribicola* J.C. Fisch. Seedlings and young saplings are most susceptible to infection, especially under cool, wet environmental conditions such as those found in the northeastern American continent.

A biocontrol agent effective against *C. ribicola* would be a useful tool for the management of young white pine plantations and naturally regenerating stands. Since teliospores of *C. ribicola* produce the infectious propagules

leading to blister rust on white pine, inhibition of teliospore production or its uredium precursor could be an effective way to control this disease. Alternatively, inhibition of blister rust in the early infection stages on pine needles would yield the same result.

We report here on the biodiversity of fungal endophytes found in needles of *P. strobus*, nursery tests of 63 isolates of needles endophytes to inhibit infection on white pine seedlings, and *in vivo* tests of two isolates of *Microsphaeropsis arundinis* (Ahmad) Sutton, against *C. ribicola* on *Ribes glandulosum* Grauer leaves.

2 Biodiversity of fungal endophytes of *Pinus* strobus

2.1 Material and methods

To evaluate the biodiversity of fungal endophytes of *P. strobus*, 320 needles on eight trees were sampled in four stands in the region near Québec City. Needles were surface sterilized and plated on potato dextrose agar (PDA) and maltextract agar (MA). Fungal endophytes usually emerged from needles after one week but up to one month.

2.2 Results

Pinus strobus has a very diverse endophytic fungal flora. Some 73 putative species of endophytes were isolated. After sampling three sites, 95% of the fungal endophytes of a given region had been found. The most common endophytes found were *Leptostroma* spp. (32.7%), *Pseudotypella translucens* (Gordon) Reid & Minter, which is a parasite of *Leptostroma* spp (17.8%), Dematiaceous 1 (15.5%), *Hormonema* spp. (12.4%), and *Acremonium kiliense* Grutz (6.2%) (Table 1). *Apiospora montagnei* Sacc., *Pestaliopsis funerea* Steyert, *Phacidiopycnis* spp., *Myrioconium* spp., *Phacidiopycnis* spp., *Cladosporium* spp., and *Coniothyrium* spp. were found at lesser frequencies.

Table 1. Frequency of the most common fungal endophytes of *Pinus strobus*.

Isolate number	Name	Frequency
QS-10	*Pseudotypella. translucens*	17.8%
QS-24	Dematiaceous 1	15.5%
QS-7	*Hormonema* 1	8.5%
QS-18	*Leptostroma* 6	7.0%
QS-1	*Leptostroma* 2	6.2%
QS-40	*Leptostroma* 1	6.2%
QS-88	*Acremonium kiliense*	6.2%
QS-85	*Leptostroma* 3	4.7%
QS-108	*Leptostroma* 4	4.7%
QS-6-3	*Leptostroma* 5	3.9%
QS-6-1	*Hormonema* 4	3.9%

3 Inhibition of *C. ribicola* on *Pinus strobus* needles using fungal endophytes

3.1 Material and methods

A collection of 63 fungal endophyte isolates previously collected from healthy asymptomatic needles of white pine were tested for their biocontrol potential. The isolates were grown on PDA in the dark at 20° C until the media was covered with spores. A spore suspension was made by gently crushing the collected surface mycelium in sterile distilled water containing Tween 20.

The 63 fungal endophyte isolates were inoculated onto 2000 one-year-old seedlings in July 1997. Thirty days later, in August 1997, the seedlings were exposed to *C. ribicola* in a nursery compound and in a wooded lot where the disease is common. The seedlings were placed in a greenhouse in December 1997 to accelerate symptom expression. Results were scored by counting yellow spots on treated and control seedlings as an indicator of blister rust infection.

3.2 Results

Fourteen isolates representing some seven putative fungal endophyte species (Table 2) were found to inhibit infection by *C. ribicola*. The most interesting of fungal endophyte species, labeled Species A, is represented by three isolates in our collection. Control seedlings developed an average of more than 10 yellow spots, whereas seedlings treated with Species A averaged less than 0.2 spots per seedling.

Table 2. Potential* for needle endophyte isolates to inhibit infection by *Cronartium ribicola* on white pine seedlings.

S-1	–	Species E	?	S-13	–	S-18	–
S-22	–	S-26	–	S-32	–	S-40	–
S-41	–	S-43	–	S-52	–	S-68	–
S-73	–	S-89	–	S-95	–	S-96	–
Species B	?	S-101	–	S-102	–	S-104	–
Species G	?	Species C	?	S-115	–	S-116	–
Species C	?	S-142	–	S-143	–	S-155	–
S-162	–	S-164	–	Species F	+	S-175	–
S-186	–	S-192	–	S-195	–	S-198	–
Species C	?	S-209	–	Species C	+	S-229	–
S-230	–	S-233	–	S-234	–	S-237	–
S-238	–	Species B	?	S-254	–	Species D	?
S-268	–	S-271	–	S-276	–	S-277	–
Species C	++	S-286	–	S-288	–	S-290	–
Species A	++	Species A	+	S302-1	–	S-314	–
Species A	++	S-318	–	S-321	–		

*Almost complete elimination (+++), major reduction (+), marginal reduction (?), and no difference (–) in number of yellowspots on white pine needles, as compared with controls.

4 Inhibition of *C. ribicola* on *Ribes* spp. using *Microsphaeropsis arundinis*

4.1 Material and methods

Isolates P-176 and P-130 of *Microsphaeropsis arundinis*, which have shown great potential in controlling apple scab (Bernier *et al.* 1996), were isolated and grown on PDA in the dark at 20° C until the media was covered with mycelium. A mycelial suspension was made by gently crushing the collected surface mycelium in sterile distilled water containing Tween 20.

Mature leaves of red currant (*R. glandulosum*) were surface sterilized by dipping them for 2 min in 20% Javex, then 1 min in 70% EtOH, and then rinsed in distilled water. Leaf disks, 37 mm in diameter, were punched from the leaves and transferred onto 2% water agar in petri dishes. The disks were then inoculated with previously frozen (–80° C) *C. ribicola* aeciospores. Inoculation of biocontrol agents P-176 and P-130 was done 0, 7, and 14 days following inoculation with *C. ribicola*. The inoculum density of P-130 was 8.1 colony-forming-units (cfu) per cm^2 and 9.2 cfu for P-176.

Leaf disks were transferred to a growth chamber at 18° C with eight hours of light for 21 days. At this time, uredia with urediospores were present on the surface of leaf disks. The disks were then transferred to another growth chamber at 13° C with eight hours of light for 14 days.

4.2 Results

Microsphaeropsis arundinis strains P-176 and P-130 showed great potential to inhibit *C. ribicola* on red currant leaves. The controls developed an average of 440.3 (±80.5) uredia. Strain P-130 infected an average of 253.8 (96.4%) uredia per leaf disk, leading to an average of only three healthy uredia per leaf disk (Table 3). Strain P-176 was similarly effective, with 148.1 infected uredia per leaf (89.2%) and only 7.7 healthy uredia per leaf disk.

Table 3. Incidence of symptoms of two *Microsphaeropsis arundinis* isolates on *Cronartium ribicola* uredia grown on *Ribes* leaf discs inoculated 0, 7, and 14 days after inoculation with *C. ribicola*.

	14 days	7 days	0 days
P-130	0.6% healthy (1.6±1.6)*	0.0% healthy	2.2% healthy (7.4±6.6)
	0.7% infected (1.7±1.3)	5.8% infected (11.8±11.8)	1.4% infected (4.6±3.4)
	98.7% dead (246.1±48.9)	94.2% dead (191.1±53)	96.4% dead (324.5±33.9)
P-176	5.0% healthy (8.7±4.5)	10.5% healthy (14.9±13.7)	0.0% healthy
	4.7% infected (8±5.4)	6.0% infected (8.5±3.8)	6.2% infected (11.2±7.3)
	90.3% dead (155.2±30.2)	83.5% dead (118.4±17)	93.8% dead (170.8±24)
Controls	94.3% healthy		
	5.7% infected		
	0.0% dead		

*Condition of *C. ribicola* uredia.

5 Discussion

Pinus strobus needles have a very diversified endophytic fungal flora, with some 73 putative species isolated from its needles in this study.

Those fungal endophytes were tested for their capacity to inhibit infection by *C. ribicola*. Seven species of endophytes were shown to have potential for biocontrol of white pine blister rust. Fungal endophyte Species A successfully inhibited the development of yellow needles spots on white pine seedlings, showing efficacy similar to the fungicide triadimefon (Bérubé 1996). Potential use includes systematic inoculation of seedlings in nurseries to protect them during the production period. Annual inoculation for the first six years in the field would allow seedlings to escape blister rust in their most vulnerable life stage. Field trials are planned for the 1998–2004 period.

Since the telia originating from uredia are the structures responsible for producing basidiospores, which are the propagules for infection of white pine, we wanted to see if the biocontrol agent *Microsphaeropsis arundinis* was able to inhibit their production. Isolates P-179 and P-130 inoculated after infection by *C. ribicola* efficiently inhibited formation of uredia and telia on *Ribes*. In practical field treatments, this means that biocontrol agents would have to be applied in late June or early July for maximum efficacy. Since basidiospores can travel a few kilometers, the area around the plantation must also be treated. *Microsphaeropsis arundinis* makes possible the biocontrol of *C. ribicola* on *Ribes* spp., an important control option for commercial currant growers in areas where there is quarantine restriction on such a culture.

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38

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