METSÄNTUTKIMUSLAITOKSEN TIEDONANTOJA 689, 1998
FINNISH FOREST RESEARCH INSTITUTE, RESEARCH PAPERS 689, 1998

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to be presented, with the permission of the Faculty of Agriculture and Forestry
of the University of Helsinki, for public criticism in the lecture room II in Metsätalo,
Unioninkatu 40 B, on August 17th, 1998, at 12 o'clock noon.

SUONENJOEN TUTKIMUSASEMA

Marja Poteri 1998. Screening of birch, *Betula* spp., for rust resistance to *Melampsoridium betulinum.*

Finnish Forest Research Institute. Research papers 689, 1998.
45 + 57 p.
Metsäntutkimuslaitoksen tiedonantoja 689, 1998. 45+ 57 s.

ISBN 951-40-1630-0
ISSN 0358-4283

Key words: clone, hypersensitive reaction, leaf disc assay, micropropagation

Publisher: Finnish Forest Research Institute, Suonenjoki Research Station. Accepted by Matti Kärkkäinen, Research Director, 15.5.1998

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Suonenjoen Kirjapaino Oy
Suonenjoki 1998

Preface

This study was started in 1989 at the Section of Forest Pathology, Finnish Forest Research Institute. During 1992–1997 the work continued at the Department of Plant Biology, University of Helsinki.

I am grateful to Professor Timo Kurkela who introduced and encouraged me in the field of rust research. In the beginning of the study I had helpful discussions with Mr. Sakari Lilja and Dr. Arja Lilja, whom I would also like to thank.

Co-operation with the co-authors was fruitful and enjoyable. At Punkaharju Research Station Dr. Matti Rousi facilitated the field and greenhouse experiments and Mrs. Leena Ryynänen cultured suitable birch clones for laboratory studies. I wish to thank them for the useful discussions and support during the years. I would also like to thank Dr. Marjo Helander, Dr. Kari Saikkonen and Mrs. Pirjo Elamo at the University of Turku for the Ruissalo field experiment and for their participation in the rust discussions. At the University of Helsinki I collaborated with Mr. Gao Zhi-he, and I wish to thank him for his help and advice in the different stages of the study.

My warmest thanks go to the many people who have taken part and helped in my studies at the Finnish Forest Research Institute and at the University of Helsinki. My special thanks at the Finnish Forest Research Institute go to Kerttu Rainio and Rauni Valjakka at the Vantaa Research Centre and to Kari Kuokkanen, Hanni Sikanen and Tiina Ylioja at the Punkaharju Research Station, to Pentti Heinänen, Tuula Laine, Hannele Mäkinen, Jouko Närhi, Lahja Pesonen and Hanna Rantala at the Department of Plant Pathology, University of Helsinki, and to Marja-Leena Lahtinen at the University of Turku.

Finally, I wish to thank Professor Kim von Weissenberg and Docent Heikki Smolander for their support in completing my thesis. I would like to express my thanks to the two reviewers for the pre-examination and constructive comments on the manuscript. I also thank Mr. John Derome for revising the language.

The study was financed by the Ministry of Agriculture and Forestry in Finland, Niemi Foundation, the Finnish Society of Forest Science and Suomalainen Konkordia Liitto.

Contents

Literature review

Abstract

Resistance to *Melampsoridium betulinum* leaf rust fungus was studied in clones of *Betula pendula* and *B. pubescens,* and with seedlings of *B. pendula, B. papyrifera*, *B. platyphylla*, *B. pubescens* and *B. resinifera* x *B. pendula* in a leaf disc assay, in the greenhouse and in field conditions. The urediniospores used in the inoculation experiments were collected from *B. pendula* at two locations in southern and at one location in central Finland; the urediniospores from *B. pubescens* were collected at one location in central Finland. Field experiments with natural rust populations were carried out at two different locations, one in south-western and one in eastern Finland.

The two clones of *B. pubescens* grown in the greenhouse and the seedlings of one genotype grown in the greenhouse and outdoors were highly resistant to the rust isolates collected from *B. pendula*. The resistance of *B. pubescens* consisted of lowered pustule frequencies and hypersensitive reactions. The *B. pendula* clones and seedlings, totalling 23 different genotypes, did not show hypersensitive reactions to any of the rust isolates, and consistently significantly lowered pustule frequencies to the *B. pubescens* rust isolate were not observed.

An investigation of the germination and pre-penetration process on greenhouse-grown and microcultured, sterile *in vitro* leaves of *B. pendula* and *B. pubescens* clones did not reveal any differences in the frequency of appressorium formation among the *B. pendula* and *B. pubescens* rust isolates. On the *in vitro* leaves, the germlings were more easily detached from the leaf surfaces probably because of poor cuticle formation.

A higher nitrogen level in the growth substrate increased the natural rust infection rate among eight clones of *B. pendula*. However, the observed interactions revealed that the response of the clones to an increased nitrogen level in the growth substrate was not the same. The Spearman rank correlations (r_s) between the infection rates obtained in the leaf disc assays and in field conditions ranged between 0.46 and 0.93; the lowest correlations were obtained with the clones grown in the least fertile growth substrate. There were signif-

icant differences in many of the nutritional parameters of the greenhouse and outdoor grown leaves of seedlings of *B. platyphylla*, *B. papyrifera*, *B. pendula*, *B. pubescens* and *B. resinifera* x *B. pendula*. The birch species also varied significantly in leaf nitrogen, carbon, total soluble protein, rubisco and chlorophyll content and C/N ratio. However, none of the leaf analysis parameters correlated with the rust resistance of the seedlings against two rust isolates, *B. pendula* and *B. pubescens*, in a leaf disc assay.

Leaf age had a significant effect on rust incidence in a field trial with ten *B. pendula* clones inoculated with a mixture of two *B. pendula* rust isolates, and also in a natural rust infection by the local rust strain. The older leaves appeared to be slightly less susceptible to rust infection, despite the fact that the studied *B. pendula* clones showed interclonal variation in this respect.

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I) Poteri, M. 1992.
Screening of clones of *Betula pendula* and *B. pubescens* against two forms of *Melampsoridium betulinum* leaf rust fungus. European Journal of Forest Pathology 22:166–173.

II) Poteri, M. and Rousi, M. 1996.
Variation in *Melampsoridium* resistance among European white birch clones grown in different fertilization treatments. European Journal of Forest Pathology 26:171–181.

III) Poteri, M., Rousi, M. and Zhi-He Gao. 1997.
Differences in the rust resistance of greenhouse and outdoor -grown white birch species. European Journal of Forest Pathology 27:363–372.

IV) Poteri, M. and Ryynänen, L. 1998.
Pre-penetration behaviour of the urediniospores of birch rust, *Melampsoridium betulinum,* on greenhouse-grown and microcultured leaves of *Betula* spp. In print for European Journal of Forest Pathology.

V) Poteri, M., Helander, M., Saikkonen., K. and Elamo, P. 199x.
Effect of *Betula pendula* clone and leaf age on *Melampsoridium betulinum* rust infection in a field trial (submitted).

Screening of birch, *Betula spp.*, for rust resistance to *Melampsoridium betulinum*

1. Introduction

Rust diseases are caused by obligate plant parasites that are a considerable challenge to plant and forest pathological studies. As biotrophs, rusts derive their nutrition from living plant cells (Lewis 1973). Large scale experiments with pure cultures of rust fungi are still not a routine practice, although progress has been made in the axenic culturing of rusts (Lane and Shaw 1974, Hare 1978, Maclean 1982, Pei and Pawsey 1990, Fasters et al. 1993). The rusts used in experimental studies are mainly cultured and maintained in their living host plant tissue, *in planta*. The mechanism involved in the establishment of rust fungus in its host plant resembles the nutrition and colonisation of beneficial fungal symbionts, e.g. mycorrhiza. The plant-rust interaction is, however, entirely parasitic and causes malformations in the plant tissue or leads to the premature death of infected host cells. The economic losses caused by rust species are considered to be the greatest of all the Basidiomycota species (Littlefield 1981, Alexopoulos et al. 1996).

Another challenge to plant and forest pathology is the complex life cycle of rust fungi (Fig. 1). Macrocyclic rusts change their host plant and, in some cases, an alternate host is a prerequisite for the survival of the fungus. In the case of microcyclic rusts, an alternate host is not necessary for completion of the rust life cycle or the alternate host may be totally lacking.

In the case of macrocyclic rusts, disease epidemics can in some cases be controlled by eliminating the alternate host, as has been successfully done with the pine twisting rust, *Melampsora pinitorqua* (Braun) Rostr., through the eradication of aspen, *Populus tremula* L. (Kurkela 1973, 1994). On the other hand, the macrocyclic type of life cycle can become a serious problem, e.g. in the case of *Cronartium ribicola* J.C.

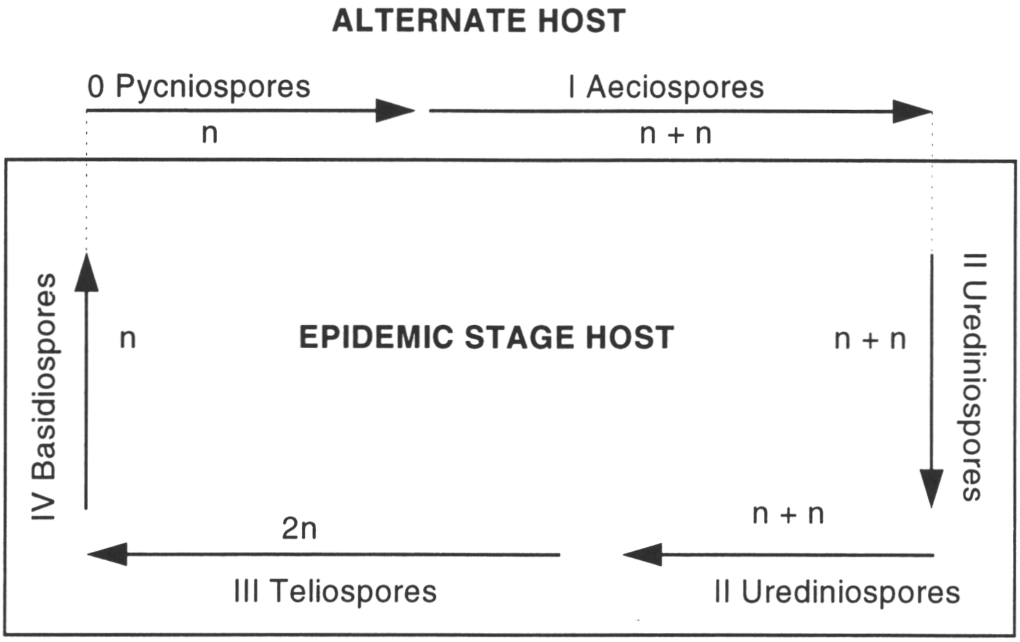

Fig. 1. Schematic diagramme of the different spore stages of rust fungi with broad-leaved trees as their telial hosts. The different spore stages are: 0 = pycniospores (spermatia), I = aeciospores, II = urediniospores, III = teliospores and IV = basidiospores. The nuclear condition and the ploidy level of the rust fungus varies in the different spore stages. (Modified from Littlefield 1981 and Alexopoulos et al. 1996).

Fischer ex Rabenh., the rust attacking five-needle pines in the western and north-eastern parts of United States. Eradication of *Ribes* spp., the alternate host of the ribicola rust, has proved to be an impossible task in the local environment and the white pine blister rust has continued to spread (Littlefield 1981, Manion 1991).

1.1 Birch rust

Birch rust, *Melampsoridium betulinum* (Fr.) Kleb., was described in Central Europe as a macrocyclic rust (Fig. 2). Its telial host is in Betulaceae, and *Larix decidua* Miller (*L. europea* DC.) is its aecial host (Klebahn 1904). However, it is presumed that *M. betulinum* can also survive without host alternation by overwintering in the bud scales and twigs

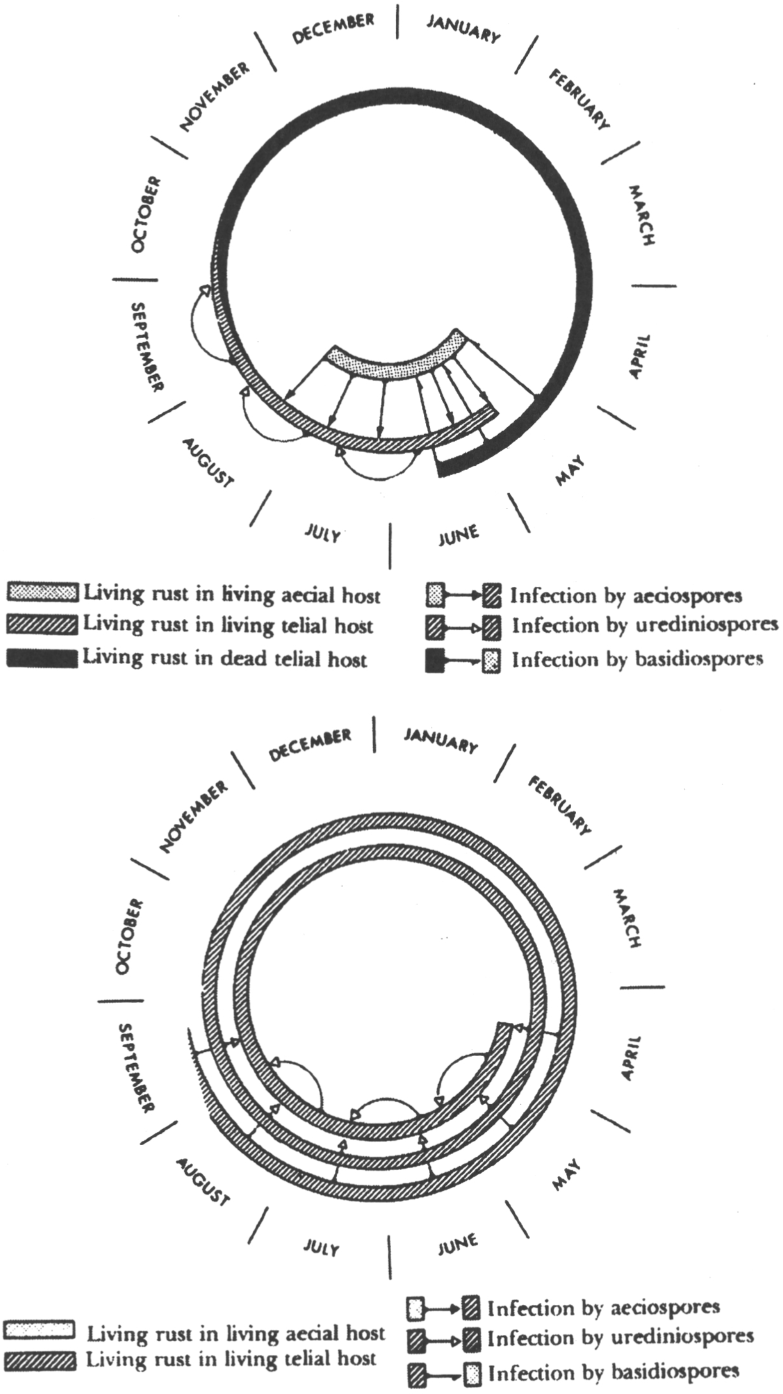

Fig. 2. Life cycle of *M. betulinum* according to Ziller (1974).

(Dooley 1984). Small birch seedlings that retain their green leaves late in autumn up until they become covered with snow, have been reported to bear viable urediniospores (Liro 1906). The same phenomenon, i.e. the overwintering of rust urediniospores, has been reported for *Melampsora larici-populina* Kleb. on poplar leaves in Japan (Chiba and Zinno 1960).

Birch rust has been found in forests on all the continents in the northern hemisphere: in the Nordic countries (Liro 1908, Roll-Hansen and Roll-Hansen 1981), including Lapland (Helander 1994), and also in Asia and North America (Gäumann 1959, Arthur 1962, Kuprevich and Transhel 1970).

M. betulinum belongs to the family Pucciniaceae (Cummins and Hiratsuka 1983). It is taxonomically separated from the other common rusts of broad-leaved trees, *Melampsora* spp., which attack aspen, poplars and willows in similar geographical areas. However, the disease symptomology and epidemiology of the *Melampsoridium* rust, as well as the losses caused to plant material, resemble those of *Melampsora* rusts.

Birch rust is considered to be mainly harmful in the nurseries (Kurkela 1994). In some cases the leaves of large forest trees may become heavily infected too, but the disease has no fatal effects on the tree. Under optimum growing conditions in nurseries birch rust develops rapidly and forms several urediniospore generations, causing premature leaf fall on the birch seedlings. Further frost damage during the winter or in cold storage are common in rust-infected birch shoots. The same phenomenon has also been reported for other broad-leaved trees that lack winter hardiness as a result of heavy rust attacks (Dawson 1988, Gullberg 1989, Verwijst 1990, Pohjonen 1991). Birch rust infection is also reported to decrease the growth of the seedlings during the next spring (Lilja 1973).

1.2 Factors affecting the growth and development of leaf rusts

Many of the leaf rusts have an epidemic stage in their uredinial stage when they occupy the green leaves of a host plant. The time between infection and sporulation is relatively short, about one week, which enables the production of several urediniospore generations in optimum conditions during the growing season. The factors essential for the development of a disease can be arranged in a disease triangle, or a disease tetraedry (Fig. 3).

The genotypes of rusts and hosts form an interaction between the virulence of the pathogen and the resistance responses of the host. The environment includes complex direct and indirect factors in the pathosystem, which may also be affected by human interventions. Rust epidemics of broadleaved trees have occurred following the introduction of rust diseases into new areas, e.g. *Melampsora* rusts were reported in Austral-Asia for the first time some 25–30 years ago (Walker et al. 1974). On a smaller scale, the attacks of local rust populations have greatly increased in short- rotation

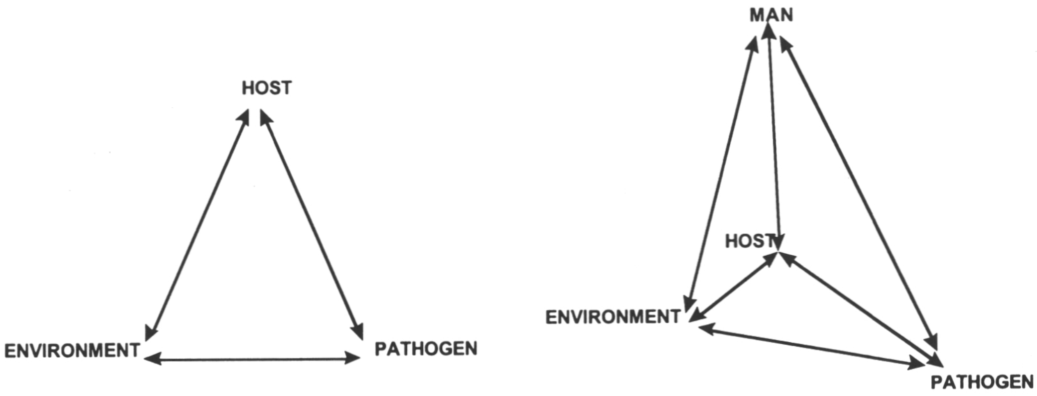

Fig. 3. Disease triangle and disease tetraedry on the right (modified from Zadoks and Schein 1979).

cultivations established for bioenergy projects (Pohjonen 1991, Parker et al. 1993). Investigations carried out in different parts of North America have shown that heavy rust attacks by different *Melampsora* spp. significantly reduce the biomass yield among poplar clones (e.g. Thielges and Adams 1975, Widin and Schipper 1981, Wang and van der Kamp 1992, Hsiang et al. 1993).

Year-to-year variation in the rust epidemics of broad-leaved trees is usually considerable large, but once rust epidemics have started early in the growing season the rate of rust multiplication is very high (e.g. Kurkela 1973, Widin and Schipper 1980, Hamelin et al. 1993). Leaf rusts are sensitive at their initial germination point on the leaf surface. Water is essential for urediniospore germination (Manners 1981, Beckett et al. 1990). The leaf wetness time needed for a successful rust infection is rather short, e.g. for poplars a minimum of eight hours' wetness is enough for the start of rust epidemics (Hamelin et al.1992b). On alfalfa leaves, *Uromyces striatus* J. Schröt., infections increased linearly when the duration of leaf wetness increased from 4 to 24 hours (Webb and Nutter 1997).

Germination is also sensitive to elevated temperatures. Birch rust urediniospores did not germinate at temperatures over 25 °C, the optimum temperature being 10 °C (Dooley 1984). Temperature, on the other hand, also affects the host plant and can change resistance reactions. The resistance of poplar clones varied when different temperature regimes were applied before inoculation and during the incubation period. Poplars kept at higher temperatures before inoculation were more resistant to *M. medusae* rust than poplars kept at lower temperatures (Prakash and Heather 1986a). However, the resistance was not persistent among the rust population and rust races in those interactions where tolerance was found (Prakash and Heather 1986b). Light intensity also had an effect on the development of the rust (Prakash and Heather 1986b). Opposite results have been obtained in other plant-pathogen interactions, i.e. susceptibility has increased after heat treatments (Sanden and Moore 1978, Thebud and Santarius 1982). Maize plants maintained at high temperatures before inoculation lacked resistance to a T-race of *Bipolaris maydis* (Garraway et al. 1989). One proposed explanation for this is that high temperatures may block peroxidation and lignification, leading to reduced hypersensitive reactions in plant tissues (Zacheo et al. 1995).

For germination, especially in urediniospores, there are reservoirs mainly as lipids for the several cell differentiations needed at the prepenetration phase. The nutrition of rusts is naturally dependent on the condition of its host. Rust fungi take up nutrients through specialised hyphal structures, haustoria (Bushnell 1972, Hahn et al. 1997). The haustorium penetrates the plant cell wall and ramifies in the cell lumen. The haustoria remain invaginated in the plant plasma membrane, which is called the extrahaustorial membrane. The extrahaustorial matrix and the fungal cell wall, which together separate the two organisms, are located between the fungal plasma membrane and the plant extrahaustorial membrane. The haustorium neck of a rust hypha is equipped with a special structure that resembles the Casparian strip in vascular plants (Heath 1976a). This structure is assumed to prevent the escape of apoplastic fluid and to favour effective nutrient flow in the haustorial neck in one direction, i.e. from the plant cell into the rust hypha. Nutrient uptake is also promoted by the electrochemical gradient maintained in haustoria by H+-ATPase activity (Struck et al. 1997). Rust fungi absorb carbohydrates, minerals and amino acids from the host plant cells (Mendgen 1981). All amino acids are taken mainly from the host plant cells, and only a small proportion is derived from fungal biosynthesis (Jäger and Reisener 1969). Hahn et al. (1997) reported that the biotrophic growth stage and haustoria formation are essential for amino acid uptake by rust fungi.

1.3 Resistance mechanisms to leaf rusts

Plant resistance can be classified as preformed and induced resistance (Isaac 1992). In crop plant breeding, a distinction is drawn between seedling and adult plant resistance when screening plant varieties against diseases (Parlevliet and van Ommeren 1975, Mares 1979). This classification takes into account the differences in plant physiology and plant age which may also cause variation in the resistance responses. With biotrophs a distinction is also drawn between prehaustorial and posthaustorial resistance (Heath 1981). In resistance breeding, quantitative resistance, or horizontal resistance, is characterised by the ability of a plant to resist to some extent all the genotypes of the pathogen, while in qualitative, or vertical resistance, plant varieties or cultivars are resistant only to certain genotypes of the pathogen (Vanderplank 1968).

1.3.1 Preformed resistance

Pre-existing resistance is based on the different mechanical barriers located at the plant surface. Among poplar clones, the rate of epidermal sculpturing and robust venation was higher on the leaves of the susceptible *P. trichocarpa* Torrey & A. Gray ex Hooker than on the leaves of the resistant *P.* x 'Serotina de Poitou' clones (Mlodzianowski et al. 1978). For rust fungi, the formation of a cuticular wax layer is essential for the attachment of urediniospores (Wynn 1981). Thus, natural or artificial changes in the cuticle component may decrease urediniospore germination and infection. Variation in cuticle formation can occur between different species or between different plant varieties (Staples et al. 1985). Some of the pesticides used to control rust infections contain film-forming compounds that manipulate the plant cuticle (Zekaria-Oren and Eyal 1991).

In leaf-rust interactions, the functioning and density of stomata may play a role as a resistance mechanism because the urediniospores penetrate the host via the stomata; only in a very few cases can urediniospore germlings penetrate the epidermis (Hunt 1968). The stomatal density of poplar clones did not correlate with the *Melampsora larici-populina* Kleb. resistance (Werner 1982). In studies on stomatal functioning it was found that poplar clones that opened later in the morning were also more resistant to *M. larici-populina* (Siwecki and Przybyl 1981, Siwecki et al. 1982).

In some plant species, e.g. Gramineaceae, the stomata are located regularly in straight lines, but in most dicotyledeons and broad-leaved trees the stomata are sparsely located over the leaf surface. The stomatal architecture appears to be more important than the stomatal density. There are several reports that the height of the guard cells or the structure of guard cell lids (e.g. Staples et al. 1985, Allen et al. 1991) can serve as topographic signals for the formation of appressorium.

The inter- and intracellular growth of rust fungi can be restricted by the plant cell wall structure and by the cell contents and fluids which may chemically inhibit the growth of rust hyphae. These resistance barriers can be preformed or induced. The preformed secondary metabolites extractable from the shoots of *Pinus pinaster* Ait. contained compounds that inhibited the basidiospore germination of *M. pinitorqua* (Desprez-Loustau 1986). In rust infections the formation of haustoria is one of the crucial points that distinguish between resistant and susceptible host reactions (Heath 1981). Tissue measurements on the leaves of poplar clones showed that a more compact mesophyll structure increased resistance to *M. larici-populina* (Werner 1982). Also a high density of the thick-walled vascular bundles in leaves may restrict the growth of rust fungi (Kurkela 1973, Werner 1982).

1.3.2 Induced resistance

Induced resistance proceeds as a cascade of predetermined biochemical reactions. Induced changes in the plant cell contents and plant cell wall structures are a result of a specific or a non-specific response of plant receptors to an external stimulus which can be biotic or abiotic in nature. Investigations revealing the very first stages in induced resistance responses have greatly expanded our knowledge of plant disease resistance.

For a relatively long time it has been known that plants are able to kill some of their own cells in order to prevent the spread of a pathogen. This suicide effect, or determined cell death, was described as a hypersensitive reaction (HR) for the first time by Ward (1902) and Stakman (1915). In their reports hypersensitive reactions were related to infection by obligate parasites, rusts. In rust infections, death of the host cells is the most common induced, or post-haustorial, resistance response (Heath 1981). It is widely accepted that the biochemical reactions associated with hypersentivity are non-specific, and can be induced by several biotic and abiotic stress factors, wounding, and even by natural plant cell senescence (Isaac 1992, Goodman and Novacky 1994). However, Heath (1976b) suggested on the basis of ultrastructural studies that the formation, as well as the ultrastructural, and probably the biochemical nature of hypersensitive reactions may differ according to the agent involved.

The search for the very first stages of induced resistance have revealed an oxidation burst at the plant cell membranes, which is thought to be a prerequisite for further reactions (Sutherland 1991, Goodman and Novacky 1994). In the later stages enzyme activities are turned on. These include several enzymes of the phenyl-propanoid pathway, which result in the lignification of cell walls and the formation of various phenolic containing phytoalexins (e.g. Vance et al. 1980, Hahlbrock and Scheel 1989). Peroxidase activity, an essential step in lignification, was reported to increase in the leaves of *B. papyrifera* Marsh. seedlings after SO_2 fumigation, but the same increase was also detected in ageing foliar tissues (Khan and Malhotra 1982).

In addition to the processes of the phenyl-propanoid pathway, the accumulation of hydroxyproline-rich glycoproteins (HRGP) has been identified as a defence response. Proteins containing hydroxyproline are essential for the active growth of plant cell walls (Lamport and Northcote 1960, Cooper et al. 1987). HRGPs were also secreted in the cell walls of melon after a *Colletotrichum lagenarium* attack (Esquerré-Tugayé et al. 1979). Later on, the defensive role of HRGPs was associated with the lignification process in the cell walls of *Cladosporium cucumerinum* Ell and Arth. resistant cucumber plants (Hammerschmidt et al. 1984).

PR proteins (pathogenesis related proteins) is a general name for a diverse group of enzymes and proteins characterised in the course of plant resistance responses. Some of them belong to the enzymes synthesised in the phenyl-propanoid pathway, and some are plant chitinases and ß-1,3-glucanases which accumulate in incompatible interactions (Joosten and DeWit 1989).

1.4 Host-rust interactions

1.4.1 Recognition in the host-rust interaction

Host-rust interactions have been used in plant pathology to study experimentally the genetic basis of plant-pathogen interactions. The gene-for-gene theory was developed in the course of inoculation experiments of flax with its rust, *Melampsora lini* (Pers.) Lév. (Flor 1954). Already earlier Biffen (1905) proposed that the plant-pathogen interaction is controlled according to Mendelian laws.

The concept of recognition is connected closely with the gene-for-gene theory. One of the crucial questions is whether pathogenicity results from the recognition or non-recognition of the pathogen by the host. The classical gene-for-gene theory states that resistance genes in a plant have corresponding pathogenicity or virulence genes in a pathogen (Flor 1942, 1953, 1956, 1971; Vanderplank 1991). However, in host-pathogen studies, particularly with the leaf spot causing bacteria, it has become more evident that the products of avirulence genes are the molecules which are recognised by the plant's defence system (Newton and Andrivon 1995). Especially the broad assumption that recognition and induction of plant resistance is induced by those gene products which enable the fungus to utilise a host as a substrate system has been questioned (Newton and Andrivon 1995). More support has accumulated for the hypothesis that resistance genes generally allow the recognition of specific elicitors (Heath 1991, Schiller et al. 1993).

Very little is known about the extracellular enzymes of rust fungi during their intercellular growth phase and haustoria formation. One of the model rusts, broad bean rust *Uromyces viciae-fabae* (Pers.) Schröt., has been shown to secrete different extracellular enzymes at various stages of the infection process. For adhesion on the leaf surface, serine esterases with cutinase activity are secreted. After entering the stomatal cavity, chitin deacetylase activity increases in the fungal hyphae and several other enzymes are secreted at a later stage of intercellular growth (Deising et al. 1994). The production of all these cell wall degrading enzymes is connected with the various steps in the successful differentiation of the rust hyphae (Deising et al. 1995).

1.4.2 Physiological rust races

The classification of different resistance responses among the host lines is the basis for distinguishing physiological rust races. In agriculture, resistance breeding is an important factor in the formation of physiological races (Zadoks 1959). The classical example of this is the wheat-rust interaction in which new races of the pathogen appear when new wheat varieties are deployed. Variation in the pathogenicity of rust isolates against different wheat cultivars was reported for the first time by Stakman and Piemeisel (1917).

Physiological races have been reported among poplar and willow clones. In Europe, a more virulent race E2 of *M. larici-populina* was reported to attack previously resistant poplar clones in Belgium and Netherlands (Pinon et al. 1987), and the concept of three races occurring among *M. larici-*

populina in Europe has since been proposed (Pinon 1992). Another poplar rust, *M. allii-populina* Kleb., is also reported to comprise a number of physiological races in Europe (Frey and Pinon 1997). In North America, *M. medusae* Thüm. is reported to have geographical races with varying virulence to poplar clones (e.g. Thielges and Adams 1975, Prakash and Thielges 1987, Hamelin et al. 1992a). Geographically different cottonwood *P. trichocarpa* Torr. & Gray clones, originating from interior and coastal locations in British Columbia, differed in their resistance to *M. occidentalis* H. Jacks. leaf rust (Hsiang and van der Kamp1985, Wang and van der Kamp 1992). However, in the analysis of the responses of different clones to a number of *M. occidentalis* rust isolates, no rust specialization on the host genotypes was found (Hsiang and van der Kamp1985). In Australia, variation in *Melampsora* resistance has been reported, although the pathogen is relatively new in the continent (Sharma and Heather 1976, Prakash 1985). In Great Britain, differences have also been found in the resistance against *Melampsora* rusts between willow clones (Pei et al. 1996). The multiplication of rust urediniospores during the epidemic stage may increase the aggressiveness of a rust isolate within a relatively short time. In the laboratory environment, the number of *M. medusae* uredinia increased significantly when the infection rates obtained with the first and 11th generations were compared with two *Populus* x *euramericana* (Dode) Guinier cultivars (Prakash 1985).

1.5 The aim of the studies

The aim of this study was to examine experimentally the responses of a number of birch species and genotypes to different birch rust isolates originating from *B. pendula* and *B. pubescens.* To aid the evaluation, a screening protocol for birch was developed. The infection biology of different birch rust isolates and the quality of birch leaves in relation to the rust infections was studied in detail in the original papers as follows:

- birch rust resistance responses of different birch species and clones (I)
- birch rust resistance expressed in leaf disc assays, in the greenhouse and under field conditions (II, III)
- processes involved in the pre-penetration stage of the rust

fungus on birch leaves (IV)

- the effect of leaf physiology and leaf age on the responses of the birch species and clones to different rust isolates (III, V)

2. Material and methods

2.1 Rust isolates

The rust isolates used in the inoculation experiments were collected as urediniospores from *Betula pendula* Roth and *B. pubescens* Ehrh. separately. Detached leaves bearing rust pustules were incubated in plastic boxes in humid conditions at 5–8 °C for 2–4 days. The urediniospores were collected with a cyclone device, dried in desiccators at + 5 °C and stored in plastic bags over silica gel at -25 °C. The rust isolates were collected in southern and central Finland (Fig. 4 and Table 1.).

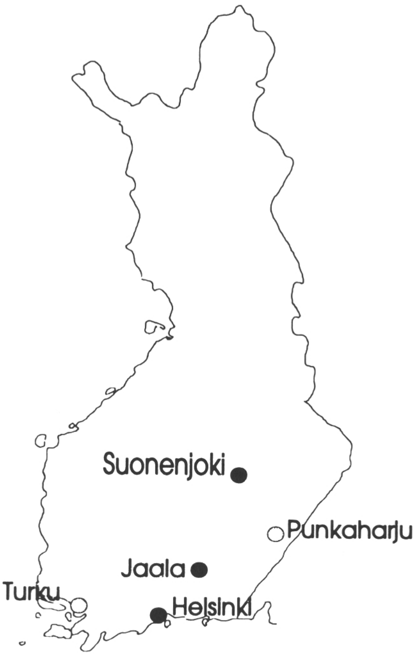

Fig. 4. The origin of the *M. betulinum* rust isolates used in Exp. I–V. The rust isolates were collected from Suonenjoki (62° 38′ N, 27° 04′ E), Jaala (61° 03′N, 26° 30′E) and Helsinki (Viikki: 60° 13′N, 25° 01′E). The field experiments were performed in Punkaharju (61° 45′N, 29° 08′E) and in Turku (Ruissalo: 60° 26′N, 22° 10′E).

able 1. The *M. betulinum* rust isolates collected from *B. pendula* and *B. pubescens* and used in Exp. I–V.

lost source	Collection site	Collection time	Urediniospore source	Experiment
pendula	Suonenjoki	July 1990	Fresh, multiplied	I
	Suonenjoki	July 1990	- 25 °C storage, multiplied	II
	Suonenjoki	August 1991	Lyophilised, multiplied	III, IV
	Jaala	July 1996	Fresh, bulk collection	V
	Viikki	July 1996	Fresh, bulk collection	V
pubescens	Suonenjoki	July 1990	Fresh, multiplied	I
	Suonenjoki	August 1991	Lyophilised, multiplied	III, IV
etula spp.	Punkaharju	July-August 1991	Natural rust infection	II
	Turku	July-August 1996	Natural rust infection	II

2.2 Birch clones and species

The birch clones of *B. pendula* and *B. pubescens* were micropropagated either at the Punkaharju Research Station (Ryynänen and Ryynänen 1986) or at the nursery of Enso Gutzeit company (Jokinen et al. 1991). Seedlings of *B. pendula*, *B. pubescens*, *B. platyphylla* var. *japonica* Hara, *B. papyrifera* and *B. resinifera* Britton x *B. pendula* were also used in the experiments. The origins of the different birch clones and seedlings used in the individual experiments are summarised in Table 2. At the time of inoculation the plantlets and seedlings were one-year-old (I, IV), two-year-old (II, III) and five-year-old (V). The sterile *in vitro* plantlets used in Exp. IV were subcultured in culture vials for 2–3 months.

The greenhouse plantlets were transplanted in 2–3 litre pots and grown in the greenhouse (I, IV). The clones in Exp. II and the seedlings in Exp. III were grown as pot plants. During the growing season the plantlets were watered and fertilised with 0.01 % Superex-5^{TM} solution. In the greenhouse, mites (Kelthane W^{TM}) and aphids (Decis^{TM}) were controlled when necessary. The field grown seedlings (II, V) were not fertilised. The *in vitro* plantlets (IV) were grown in growth chambers with a 16-hour photoperiod (100–120 μmol m^{-2}s^{-1}) at 18–20 °C.

2.3 Inoculation experiments

Urediniospore suspensions prepared in 0.01 % Tween 20^{TM} solution were used as inoculum. Control leaves were treated with the Tween solution only. The spore concentrations in

Table 2. The birch clones and seedlings used in Exp. I–V.

Species	ID Number	Provenance	Type	Experiment
B. papyrifera		Midland, Michigan, USA	Seedling	III
B. platyphylla		Jyozankei, Japan	"	III
B. pendula	V59562	Valkeakoski	Clone	V
	0154	Punkaharju x Punkaharju	"	V
	V5818	Loppi x Rautalampi	"	V
	V50080	Keuruu	"	V
	V5920	Varkaus	"	V
	V50079	Keuruu	"	V
	K1659	Eno	"	V
	K1898	Pielavesi	"	V
	K1932	Pielavesi	"	V
	E9661	Kihniö	"	V
	E4212	Ristiina	"	I, IV
	E4213	Ristiina	"	I
	E4214	Ristiina	"	I, IV
	E4215	Ristiina	"	I
	E5387	Punkaharju	"	I, IV
	E5398	Punkaharju	"	I, IV
	JR1/1	Kangasala x Nummi	"	II
	JR1/4	Kangasala x Nummi	"	II
	36	Ristiina	"	II
	39	Valkeakoski	"	II
	5818	Loppi x Rautalampi	"	II
	5832	Rautalampi x Keuruu	"	II
	2674	Eno	"	II
B. pendula JR		Kangasala x Nummi	F_1 seedling	III
B. pendula PH		Punkaharju	Seedling	III
B. pubescens	V5940	Somero	Clone	I, IV
	V5944	Somero	"	I, IV
B. pubescens			Seedling	III
B. resinifera x *B. pendula*		Alaska, USA	Hybrid seedling	III

Exps I–IV varied from 1.2 x 10^4 to 5.5 x 10^5 spores ml^{-1}. In Exp. V the spore concentration was the highest, 1.8 x 10^6 spores ml^{-1}. For inoculation of the *in vitro* plantlets and in the leaf disc assays, a 10 ml aliquot of the spore suspension was spread on the leaf surface with a glass rod after inserting a suspension droplet on the leaf surface. In the field inoculations (II, V) the inoculum was sprayed on the abaxial surface of the leaves.

2.3.1 Leaf disc screening

Leaf disc screening was conducted in Petri dishes on 1 % water agar, amended with 10 ppm gibberellic acid as a senescence-delaying agent. The leaves were either greenhouse grown mature leaves (I, II, III, IV), outdoor grown mature leaves (III) or *in vitro* leaves cut from the sterile shoots (IV). Mature leaves were surface sterilised using 0.35–0.5 % sodium hypochlorite. The leaves were rinsed in cold tap water before surface sterilising. The leaves were subjected to the sterilising treatment for 2 minutes, followed by rinsing three times with sterile distilled water. The discs (diameter 1.5 cm) were cut with a cork borer and placed, abaxial surface upwards, on the water agar in the Petri dishes. Incubation was carried out in the growth chambers with a 16-hour photoperiod (100–120 μmol $m^{-2}s^{-1}$) at 18–20 °C.

2.3.2 Greenhouse and field inoculations

In the greenhouse the leaves on the shoots were inoculated using a brush on the abaxial leaf surfaces (IV). After inoculation the leaves were enclosed overnight in plastic bags. In the field the suspension was sprayed on the abaxial surfaces of the topmost leaves (II, V). In Exp. V whole shoots bearing the treated leaves were enclosed in plastic bags overnight.

2.4 Leaf structure and biochemical studies

2.4.1 Scanning electron microscopy studies

For scanning electron microscopy the leaves were fixed in sodium-phosphate buffer containing 2.5 % glutaralhehyde (pH 7.0) (Robinson et al. 1987). The fixed samples were dehydrated and stored in absolute ethanol before critical point drying (Balzars) and coating with either gold or platinum. The encoded samples were studied under an electron microscope (JEOL JSM-800) at an acceleration voltage of 5–8 kV.

2.4.2 Biochemical leaf analysis

The leaf samples collected for the biochemical and nutrient analysis were stored at -80 °C. The samples were ground in liquid nitrogen and the leaf powder was suspended in an extraction buffer (Gezelius and Hallen 1980, Pääkkönen et al.

1996). From the supernatant the amount of rubisco enzyme was determined by polyacrylamide gel electrophoresis according to Rintamäki et al. (1988). Total soluble proteins were measured spectrophotometrically (Bradford 1976) using bovine serum albumin as a standard. The total chlorophyll content and chlorophyll a/b ratio were determined according to the method of Arnon (1949). The excess leaf powder was dried in an oven (60–70 °C) and used to determine the nitrogen and carbon concentration and carbon/nitrogen ratio in the leaves (Leco CHN 900).

2.5 Analysis of data

Infection rates on the leaf discs were determined as pustule frequencies (I, II, III). To describe infections over time Area Under Disease Progress Curves (AUDPC) were calculated (II, III). Non-parametric Kruskall-Wallis analysis (BMDP 1992) was used to compare the pustule frequencies between the birch clones and between the birch species (I). A non-parametric Mann-Whitney U-test (BMDP 1992) was used to compare the biochemical and nutrient parameters between the greenhouse and outdoor grown seedlings (III). Student-Neumann-Keuls multiple comparisons (BMDP 1992) were used to compare the AUDPC of the different birch clones (II–III). Contingency tables and the G^2-analysis were applied to compare the different appressoria formation frequencies on different leaf surfaces (IV). Analysis of variance (BMDP 1992, SAS 1989) was used in experiments II and V to search for interactions and to analyse the effect of inoculation methods on the different birch clones. Spearman rank correlations (BMDP 1992) were used to determine the relationships between the leaf disc method and field inoculation (II), and to describe the relationship between rust infection rate and leaf age (V).

3. Results and discussion

3.1 Screening of the rust resistance of the birch species

The leaf disc assay was applied to four different birch species, *B. pendula*, *B. papyrifera*, *B. platyphylla*, *B. pubescens,* and to a hybrid *B. resinifera* x *B. pendula*. On the leaf discs the first symptoms of uredinia formation were visible on the *B. pendula* and *B. pubescens* clones after 6–9 days (I). The time period from inoculation to the appearance of the first symptoms was in the same range for the other birch genotypes in the other experiments, too.

The first sign of a developing pustule was the collapse of the epidermal cells and the degradation of chlorophyll, giving a pale or whitish colour to the affected tissue. Under optimum conditions these whitish spots turned yellow in one day, and a clear orange-yellow pustule was formed. The rupture of uredinia and sporulation took place the following day. In the field, uredinia were usually observed at the stage where sporulation had started, which means at least 7–10 days after infection.

Depending on the extent of leaf venation, rust hypha were able to grow over a relatively large area and thus form several new pustules around the original uredinium. These secondary pustules were usually smaller and were formed a few days later than the first one.

The response on the leaf discs depended on the birch genotype and on the rust isolates used. The number of rust pustules varied significantly between the different host-rust combinations (I–III). On the two clones of *B. pubescens*, significantly lower levels of rust infection were found with the *B. pendula* rust isolates than with the *B. pubescens* isolates (I). Immune reactions occurred on the leaves of *B. pubescens* and on outdoor grown *B. papyrifera* leaves when the leaf discs were inoculated with a *B. pendula* rust isolate (III). It is possible that the infections observed on the leaf discs of *B. pubescens* in Exp. I were caused by urediniospores from *B. pubescens*. In contrast to the other leaf disc assays with *B. pubescens* (III and IV), rust multiplication in Exp. I was performed with fresh urediniospore material and not with lyophilised material, which usually has a lower germination and infection rate. The use of fresh spores may have allowed more diversity in the rust population. The isolates used in the other inoculation experiments also represented another collection year (1991), instead of 1990 used in Exp. I, eventhough the same collection site was used. The possibility of contamination of the *B. pendula* rust isolate with urediniospores of *B. pubescens* cannot be excluded either. However, the occurrence of necrotic reactions in these leaf discs suggests to some other type of infections that differs from those caused by the pure *B. pubescens* genotype.

In addition to the immune reactions, hypersensitive reactions also occurred in the interactions between the *B. pendula* rust isolate and the *B. pubescens* clones (I). In these interactions lower infection levels were found on the leaf discs of the *B. pubescens* clones. The reduced pustule frequencies were partly caused by the necrosis that inhibited sporulation or, in the later stage, prevented further sporulation (I). Although *B. papyrifera* had an immune reaction to the *B. pendula* rust isolate when the outdoor grown leaves were inoculated, no hypersensitive reactions were detected in these leaf discs (III). The number of *B. pendula* genotypes screened was the highest (23), but no hypersensitive reactions or immunity were found, although there were some significant differences in the responses between the different combinations of *B. pendula* genotypes and the rust isolates (I–III).

The type of resistance reaction with *B. pubescens* was similar to the adult plant resistance reported for crop plants. For example, in the interaction between wheat and yellow rust *P. striiformis* West., adult plant resistance reduced sporulation and, at the same time, sporulation was inhibited by the death of haustoria at the sites of cell necrosis (Mares 1979). A high susceptibility of diploid *B. pendula* to *M. betulinum* compared to triploid hybrids or crossings of *B. pendula* x *B. pubescens* was reported by Eifler (1960). Klebahn (1904) reported that the resistance responses to birch rust were diverse with *B. pubescens*, *B. pendula* and *B. nana* L. seedlings, and that rust rankings between the species varied considerably during the three observation years.

Leaf disc bioassays are widely used for screening in resistance studies, although the properties of detached leaves are known to differ from those of intact leaves. The first physiological changes in detached birch leaves are obviously connected to the tissue water content and photosynthesis. Photosynthesis decreased in detached *B. pubescens* leaf discs after 6 hours (Skre 1993). Chlorophyll degradation resulting from increased peroxidase activity takes place in natural leaf senescence processes, but increased peroxidase levels have also been reported in detached leaves (Farkas et al. 1964).

3.2 The effect of leaf physiology on rust infections

In Exp. II, the three different fertilisation treatments of the *B. pendula* clones had a significant (ANOVA, F = 809.8, df = 2, $p<0.001$) effect on height growth, which was 89.5, 139.9 and 151.3 cm for the fertilisation levels 0.01 %, 0.05 % and 0.1 %, respectively. No analysis of leaf nutrient concentrations was performed on these clones. In the inoculation studies, birch rust resistance seemed to be connected with the nutrition level of the *B. pendula* clones (II). However, the fertilisation effect of the three different nitrogen levels was not uniformly the same for the eight *B. pendula* genotypes studied, since significant interaction was found between the responses of the birch clones and the nutrient levels. Moreover, the analysis of different leaf nutrients did not clearly show any correlations between the nutrient concentrations of the birch leaves and the rust susceptibility among the six different birch genotypes consisting of five different birch species (III). The foliar nitrogen, carbon, soluble protein and chlorophyll concentrations as well as the C/N ratio, were determined.

Resistance reactions obtained in leaf disc assays should always be verified under field conditions. The correlations in rust infection rates between the leaf disc assay and field observations were relatively high ($r_s = 0.46–0.93$, df = 7), but depended on the fertilisation level of the *B. pendula* clones (II). No qualitative traits, such as hypersensitive reactions, were observed. The interpretation of the results obtained in the field experiments may be difficult because of the heterogeneity of the growing environments. The clonal differences in rust resistance among the willow clones were significant, but the soil type also had a significant effect on the *Melampsora* resistance of *Salix* spp. (Rönnberg-Wästljung and Thorsén 1988).

Nutritional aspects are difficult to study in the host - rust interactions. It is obvious that the preformed nutrient level of plant tissues has an effect on the rust infections while, at the same time, the rust itself is also able to manipulate nutrient flow in the plant tissue. In several reports rust infection has resulted in nutrient accumulation in infected leaves (Paul and Ayres 1988, Roberts and Walters 1988, Paul et al. 1990). Hormonal changes in the leaves due to infections by biotrophs have also been observed (Heath 1981).

In poplar clones, higher nutrient content in the leaves led to higher susceptibility to *Melampsora* leaf rust (Suzuki 1973). The environment, like nutrition, can affect rust resistance traits in a number of ways. In leaves of *Senecio vulgaris* L., pustules of *Puccinia lagenophorae* Cooke were more frequent under poor nutrient conditions; while fertilised plants had fewer pustules, the pustule size was greater (Paul et al. 1990). High Mn and Cl concentrations in bean leaves also increased susceptibility to *Uromyces appendiculatus* (Pers.) Unger measured as rust pustule diameter (Zaiter et al. 1991).

In the birch leaves there were also suggestions that high nutrient levels increased uredinium size and thus also urediniospore production. Furthermore, a high nutrient level usually increases individual leaf size thus enlarging the interveinal area of birch leaves. Thus, in larger leaves there is probably more space for the formation of several rust pustules. With birch leaves it seems that the increase in the infection rates of fertilised plants was caused both by the higher number and larger size of the rust pustules.

The effect of the nutritional status of the growth substrate on rust infections becomes even more complicated when its effects on the microclimate are considered. The more vigorous growth at high nutrient levels increases e.g. the amount of moisture on the leaf surfaces. Thus, in Exp. II and in some other rust studies (Vuorinen 1992) it has not been possible to clearly separate the effect of leaf nutrient content from environmental factors.

3.3 The effect of leaf structure and age on rust infection

3.3.1 Microcultured *in vitro* leaves

The leaf structure of the mature greenhouse-grown leaves and that of *in vitro* leaves differed according to the SEM micrographs (IV). The *in vitro* leaves had an atypical stomatal anatomy and there were also indications that the cuticle formation of plantlets grown in microculture vials differed from that of greenhouse grown plantlets. The stomata on the *in vitro* leaves had a more raised structure and also their guard cell architecture differed from that of mature stomatal structures. The stomata of *in vitro* leaves seemed to be open more frequently. This was most probably due to the poor acclimation of the *in vitro* leaves when transferred from the culture vials onto the water agar in Petri dishes. The poorly formed cuticle and wax layer on the *in vitro* leaves also appeared to be the cause of the detachment of sporelings and germ hyphae from the *in vitro* leaf surfaces (IV). However, at the prepenetration stage the germlings were able to form appressoria on *in vitro* leaves in all the host-pathogen combinations. The ratios between stomatal appressoria and failed appressoria did not vary significantly between the different leaf types or different host - rust interactions (IV).

Because Tween 20™-solution was used in the inoculation, urediniospore germination and the growth of germ tubes most likely took place inside the water droplet. Thus, there was probably no direct chemical contact with the leaf surface at the germination stage, which may also explain why some of the germ hyphae were detached. Another reason for the observed detachment could be the effect of the fixation treatments needed for SEM. It is not known at which stage appressorium formation was induced; whether it took place in the presence of excess water or if the appressoria were formed in dry conditions either outside the water source or after the water had evaporated. According to the SEM micrographs, the appressoria were in most cases attached to the leaf surface eventhough the germ hyphae were detached from the leaf surface. This probably means that the appressoria were mainly formed in drier conditions than germ tube development.

Atypical forms in the development of plant organs of tissue-cultured plants has been described for many plants. Essential factors involved in rust infection and also reported for *in vitro* plants are a more raised and open stomatal structure, reduced cuticle and wax formation, and extensive intercellular and substomatal air spaces (Wetzstein 1986). In the *B. pendula* and *B. pubescens* clones used in the experiment the stomatal structure of the *in vitro* plants had the same atypical characteristics as reported for other broad-leaved trees (Smith et al. 1986). Adhesion onto the leaf surface is one of the early pre-penetration events (Kunoh et al. 1991) and is dependent on proper cuticle formation. At the post-penetration stage the nutritional quality of *in vitro* leaves may also be altered because the photosynthesis rate of microcultured plantlets is reported to be reduced compared to that of *in vivo* plants (Lee et al. 1985).

3.3.2 Leaf age

The effect of leaf age on the rust resistance of the *B. pendula* clones seemed to be connected with the birch genotype (V). Furthermore, the rust isolate affected the responses of leaves of different age, thus indicating genotypic variation among the different rust isolates with respect to their ability to infect and colonise birch leaves. In Exp. V, a set of ten leaves per shoot was studied. This can be considered to fully cover the range of physiological differences found in the leaves, especially as the rust inoculation was performed at the end of the summer in August. In late summer the photosynthesis rate starts to decrease, and nutrients, various sugars and nitrogen bound in the soluble proteins, are translocated from the leaves to other plant parts for winter storage (Friedrich and Huffaker 1980).

The age of a plant organ has importance for biotrophs that do not produce the same array of plant cell wall degrading enzymes as necrotrophs. For example, an ageing leaf affects the rust resistance because older leaves tend to have thicker walled and more lignified veins. *Melampsora* rust hyphae were restricted to areas bordered by thick veins in the leaves of aspen and poplar (Kurkela 1973, Werner 1982). Successful infection by basidiospores of *M. pinitorqua* is also critically dependent on the growth stage of pine shoots and the amount of shoot suberisation (Kurkela 1973, 1990; Desprez-Loustau 1990). The most susceptible stage of one-year-old Scots pine seedlings to *M. pinitorqua* was estimated to be between 550 and 620 degree days (von Weissenberg 1978, 1980).

3.4 Genotypic variation in the rust resistance of birch species

In the experiments there was altogether 23 different genotypes of *B. pendula* and three genotypes of *B. pubescens*

(Table 1). The exotic species *B. platyphylla*, *B. papyrifera* and *B. resinifera* x *B. pendula* were all seedlings. Variation in the rust resistance between species and between clones within species was determined quantitatively either on the basis of pustule frequency or as the percentage of leaf area covered by pustules. Some intraspecific variation was found for *B. pendula* (I, II, V) and *B. pubescens* (I) as the infection rates varied significantly between the clones. With *B. pendula* this variation occurred both in the leaf disc assays (I, II) and in the field experiments (II, V).

The rust resistance of the birch genotypes did not show any clear patterns related to geographic origin. One reason for this is probably the fact that the Finnish birch genotypes and the rust isolates used came from a relatively small area. In these experiments there were three different *B. pendula* rust isolates, two from southern Finland (Jaala and Viikki) and one from central Finland (Suonenjoki). The birch genotypes also originated from southern and central Finland. Some field comparisons between the different rust strains could be made in Exp. V. According to the rust infection levels in the control leaves of the ten *B. pendula* clones, the pathogenicity of the local Turku rust strain seemed to differ from that of the other two rust isolates used in the inoculum mixture and collected from southern Finland (Helsinki and Jaala). In a field experiment in Scotland the susceptibility of *B. pendula* provenances to *M. betulinum* increased with latitude when the rust incidence was estimated as the coverage-% of uredinia on the leaves (Mason et al. 1982).

The exotic birch species exhibited diverse responses to the *B. pendula* and *B. pubescens* rust isolates. The immune reactions of *B. papyrifera* to the *B. pendula* rust isolate were as expected because it is tetra- or pentaploid like *B. pubescens,* which also has strong resistance to *B. pendula* rust isolates (I, III). The mechanism involved in the resistance responses to *B. pendula* rust isolates is not known. The question seems to be rather complex as the seedlings of *B. papyrifera* were infected by the *B. pendula* rust isolate when grown in the greenhouse in Exp. III. Kechel and Böden (1984) reported interspecific differences in birch rust resistance in a field experiment in which North American *B. papyrifera* and *B. alleghaniensis* Britton were more resistant than the European *B. pendula* and *B. pubescens*.

3.5 Conclusions

Birch rust resistance is genetic in nature, as indicated by the constant resistance reactions to *B. pendula* rust isolates among the *B. pubescens* clones and seedlings. The resistance responses of *B. pubescens* clones to *B. pendula* rust isolates can be characterised as partial resistance including hypersensitive reactions. The reactions of *B. papyrifera* to *B. pendula* were more inconsistent. The *B. pendula* clones and seedlings also varied in their infection rates to different *B. pendula* and *B. pubescens* rust isolates.

The experiments were performed with a restricted number of birch genotypes. The *B. pendula* genotypes came from a rather limited area in southern and central Finland; thus it is probable that a large proportion of the variation in the rust resistance of *B. pendula* was not revealed in these studies. In some of the experiments the resistance reactions could also be masked because of the use of a mixed rust inoculum.

The mechanisms involved in the resistance process are not known. In these experiments the measured physiological traits and the processes involved in the pre-penetration stage of infection did not explain the observed variation in rust resistance. Neither did the growth environment, greenhouse or outdoors, affect the rust resistance. On the other hand, sugars were not analysed and they are also important for the growth of biotrophs like rust fungi.

Relatively low irradiances were used in the leaf disc assays and this most likely affected the leaf carbohydrate content and may also have created for the rust isolates an environment that is different from the natural one. According to the results, however, leaf disc screening for birch rust resistance can be reliable if the nutrition of the tested birch plantlets or seedlings corresponds to the conditions prevailing in natural growing sites.

In order to screen birch for rust resistance in a leaf disc assay, vigorously growing host material and young, fully flushed leaves should be used. With outdoor grown material the screening can be performed in June. Because of the risk of natural rust infections in July and August, the material to be tested in late summer screenings should be grown in rust free conditions (in the greenhouse). To avoid contamination in the leaf discs, fresh urediniospores should be used in preparing the water suspension. Urediniospores can also be multiplied on leaf discs, but attention should be paid to avoid pre-adaptation of the rust. Multiplication should be made using different host genotypes than the ones to be used for the tests. Incubation of leaf discs in growth chambers will give the most uniform results and will permit the comparisons to be made between different years. The most accurate method for rust assessment is to count pustule frequencies in the leaf discs.

In Finland there is no birch breeding program for rust resistance, because so far the heavy rust attacks and economic losses have occurred only in nurseries where rust infections can be controlled by fungicides. Only adult trees are chosen for breeding work and they usually no longer suffer from rust infections and their vulnerability to rust is thus also unknown. Variation in the rust susceptibility of *B. pendula* and *B. pubescens* indicates that new birch rust races may evolve, thus leading to more serious rust attacks, for example when introducing new birch provenances to (trans)plantation sites.

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Eur. J. For. Path. 22 (1992) 166–173
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ISSN 0300-1237

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Screening of clones of Betula pendula and B. pubescens against two forms of Melampsoridium betulinum leaf rust fungus

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Abstract

Leaf discs from seedlings of six clones of *B. pendula* Roth and two clones of *B. pubescens* Ehrh. were inoculated with two urediniospore isolates of *M. betulinum* Kleb. The field collection isolates were obtained from *B. pendula* and *B. pubescens* growing in the field. The *B. pendula* rust was more specialized than the *B. pubescens* rust. The clones of *B. pubescens* showed partial resistance against the *B. pendula* rust, while the *B. pubescens* isolate was compatible to both birch species. Some interclonal variation was also found in both birch species. The results support an earlier suggestion by Klebahn that *M. betulinum* has two *formae speciales*.

1 Introduction

The two birch species of silvicultural importance in Finland are European white birch, *Betula pendula* Roth, and pubescent birch, *B. pubescens* Ehrh. Propagation of these species by means of tissue culture has been applied in the production of valuable seedlings (Simola 1985; Ryynänen and Ryynänen 1986), and cloned seedlings have been used in research as well (Rousi 1990; Särkilahti 1990).

Melampsoridium betulinum (Pers.) Kleb. is a heteroecious rust which has its uredinial stage and telia on *Betula* spp. and on some *Alnus* spp. (Roll-Hansen and Roll-Hansen 1981), and its aecial stage on *Larix* spp., if available. The epidemic phase occurs on birch in late summer (July–August), when the urediniospores infect the leaves. The fungus occurs throughout the whole country, but the disease expression varies among the different *Betula* spp. Klebahn (1904) has proposed that *M. betulinum* has two *formae speciales* which differ in their host preference. According to Klebahn, f. sp. *betulae-verrucosae* Kleb. (i. e. *B. pendula*) affects *B. pendula* and *B. nana* L., but *B. pubescens* hardly at all, while f. sp. *betulae-pubescentis* Kleb. infects *B. pubescens* and *B. nana* but hardly or only slightly *B. pendula*.

In agriculture *formae speciales* have traditionally been established for many rust fungi on the basis of their reactions on the telial host. The formation of teliospores is considered to correlate with the level of adaptation of the pathogen, and this phenomenon has been used in the study of the leaf rusts of trees, too (Hsiang and van der Kamp 1985). In North America two *formae speciales* have been distinguished for *Melampsora medusae* Thuem., a leaf rust fungus which has either *Populus deltoides* Bartr. or *P. tremuloides* Michx. as its uredinial-telial hosts (Shain 1988).

The compatibility of the urediniospores of *M. betulinum* derived from naturally growing *B. pendula* and *B. pubescens* hosts was determined in this study. Six clones of *B. pendula* and two clones of *B. pubescens* were screened for their resistance against these two isolates.

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2 Material and methods

2.1 Clones

B. pendula clones E4212–E4215, E5387 and E5398 (plustrees originating from South Finland) and *B. pubescens* clones V5940 and V5944 (trees of second generation selection) were tissue cultured at Punkaharju Research Station (RYYNÄNEN and RYYNÄNEN 1986). In 1989–1990 the clones, each consisting of 8–15 seedlings, were transferred to the Department of Forest Pathology at Tikkurila near Helsinki. The one-year-old seedlings were transplanted into pots (5 l) containing a peat: vermiculate mixture (3:1) and grown in the greenhouse in order to prevent rust infections. During the growing season the seedlings were fertilised once a week with 0.1 % Superex-5 water suspension and sprayed against leaf damaging aphids and mites with cypermetrin every third week. In the spring the shoots were cut back and leaves from the one-year-old shoots were used in the experiments.

2.2 Urediniospores

In July 1990 rust isolates were collected in a forest next to the seedling nursery of Suonenjoki Research Station (62° 38′N, 27°04′E) in Central Finland. Birch leaves bearing the first pustules were detached from the shoots of *B. pendula* and *B. pubescens* and stored separately. The urediniospore collections were pured from other fungal epiphytes in the laboratory, and multiplied on the sterilized leaf discs of nursery seedlings in Petri dishes (see 2.3).

2.3 Inoculation experiments

The fourth or fifth full-grown leaf from the shoot tip was detached and surface sterilized in 0.35 % NaOCl for 2 min and rinsed 3× in sterile water. A leaf disc assay and inoculation with water suspension (SHAIN and CORNELIUS 1979) were carried out. Leaf discs (1.77 cm^2) were cut off aseptically with a cork borer and plated on 1 % water agar containing 10 ppm gibberellic acid. There were six to eight leaf discs with their abaxial surface upwards in each Petri dish. For the inoculum 0.6–1.0 mg of fresh urediniospores and 8 ml of 0.1 % Tween 20 water were mixed in a 10 ml bottle using an Ultra Turrax at half power for 20–30 seconds. The discs were inoculated immediately with 10 μl of spore suspension, the drop being spread over the leaf surface with a glass rod. The Petri dishes were sealed with parafilm and incubated in a growth chamber at 18 °C day temperature and 16 °C night temperature with a 16 hours photoperiod. The spore concentrations were determined with haematocytometer after the inoculations, and varied between $1.2–19 \times 10^4$ spores/ml (120–1900 spores in the inoculum for each leaf disc). Experiments no. 1, 2 and 3 were carried out on August 30, on September 11 and on September 28, respectively. The total number of leaf discs used in the screening was 762.

2.4 Inspection of the syptoms and disease rating

The responses of the clones to both isolates were assessed under a stereomicroscope starting 5 days from inoculation and continuing for 14 days postinoculation. The number of pustules on the leaf discs was counted and the duration of the latent period (days from inoculation to the production of new spores) and the appearance of necrotic symptoms were observed. The frequency data were analysed using BMDP software (BMDP 1988). Multiple comparisons of the infection frequency means of the *B. pendula* clones were made using a Kruskal-Wallis test. A Kruskal-Wallis one-way analysis of variance, based on the ranks of the means, was used to compare the infection frequency means of *B. pubescens* clones and the infection frequency means of the two birch species overall.

3 Results

3.1 Differences in response between the species and the expression of partial resistance

Comparison of the reactions between the species indicated host specialization of the *B. pendula* rust (Table 1). As well as having a significantly ($p < 0.001$) lower infection level, the leaf discs of *B. pubescens* also had a longer latent period when inoculated with the *B. pendula* rust. The latent period was 6–9 days for the clones of *B. pendula*. The pustules

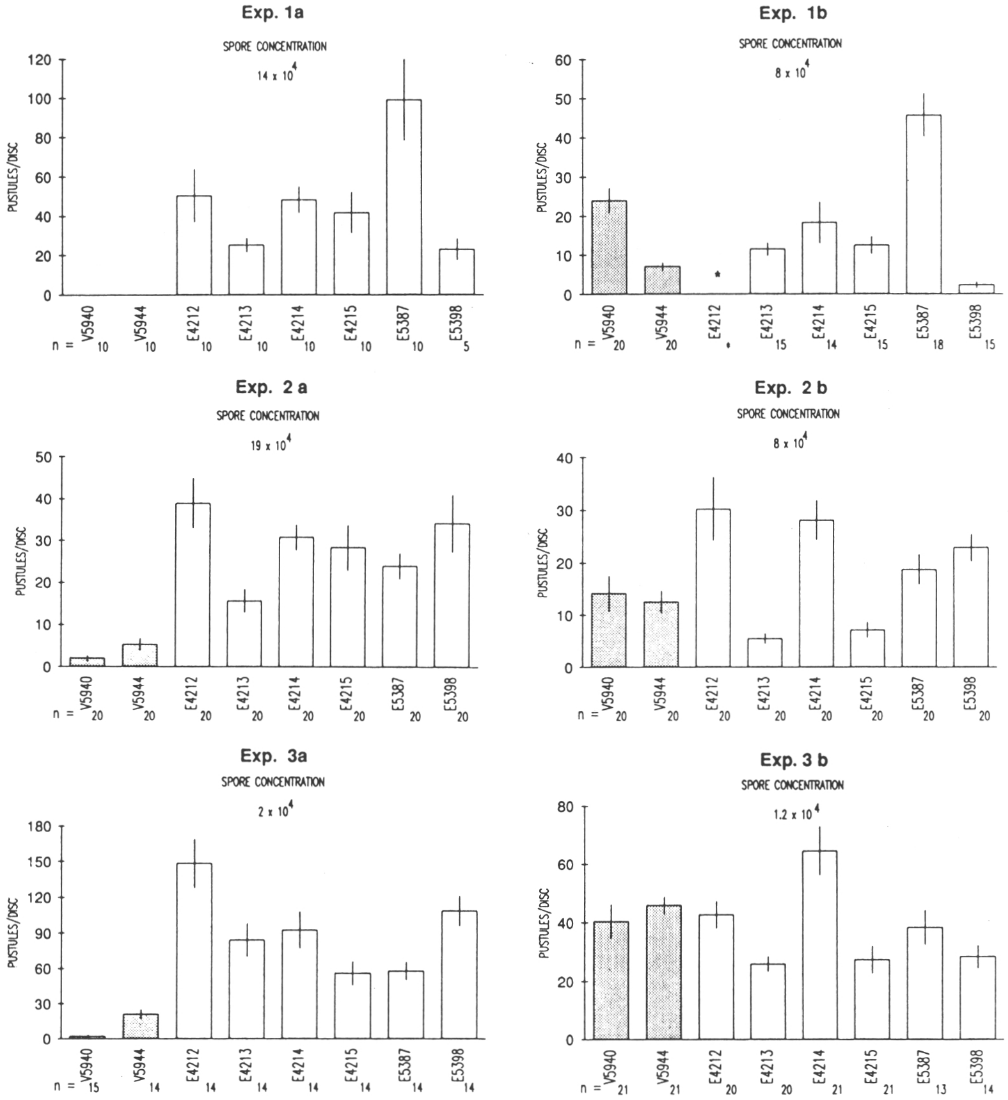

Fig. 1. Infection frequencies in the three different experiments defined as the means of sporulating pustules on the leaf discs of the birch clones inoculated with the *B. pendula* rust (1a, 2a, 3a) and with the *B. pubescens* rust (1b, 2b, 3b)
V5940 and V5944 = *B. pubescens* clones (shaded columns)
E4212–E5398 = *B. pendula* clones
Bars indicate ± SE, n = number of leaf discs in each treatment, * = missing clone

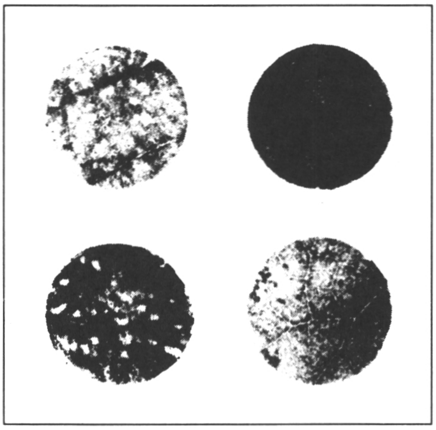

Fig. 2. The adaxial surfaces of the leaf discs of the *B. pubescens* clone V5940. The two discs on the left inoculated with the *B. pubescens* rust isolate are susceptible. The two discs on the right inoculated with *B. pendula* rust isolate show partial resistance, expressed as necrotic lesions in the infection sites. The responses were photographed three weeks after inoculation

became visible on the leaf discs of *B. pubescens* species and ruptured 9–13 days after inoculation. The size of the pustules appeared to be smaller in the *B. pubescens* clones-*B. pendula* rust combination. In Experiments 2 and 3, where the *B. pendula* rust showed compatibility (in Experiment 1 no infection occurred), *B. pubescens* developed necrotic lesions around the infection sites (Fig. 2). The necrosis that developed in all infection sites reduced or completely stopped the production of spores.

No differences were found between the species as regards their response to the *B. pubescens* rust (Table 1).

Table 1. **Kruskal-Wallis one-way analysis of variance for the means of the infection frequencies of B. pendula and B. pubescens species in three different inoculation experiments: Exp. 1, Exp. 2 and Exp. 3. The sources of the variance are the two rust isolates originating from B. pendula and B. pubescens**

Source	Exp. 1		Exp. 2		Exp. 3	
	H	p	H	p	H	p
B. pendula rust	44.54	<0.0001	68.2	<0.0001	55.55	<0.0001
B. pubescens rust	0.43	0.5106	2.53	0.112	3.88	0.0487

H = value of Kruskal-Wallis test statistics.
p = level of significance.

3.2 Variation in infection level between the clones of B. pendula

The variation displayed by the clones in the different experiments prevents more detailed analysis of the reactions among the clones. However, interclonal variation was at a lower level with the *B. pendula* rust (Table 3). The resistance showed by clones E4213 and E4215 was more strongly expressed in the inoculations made with *B. pubescens* rust in all three experiments (Fig. 1). In Experiment 1 the extreme values of clones E5387 and E5398 separated them ($p<0.05$, $p<0.10$) from the other clones when inoculated with *B. pubescens* rust (Fig. 1, Exp. 1b).

3.3 Variation in infection level between the clones of B. pubescens

In Experiment 1 neither clone became infected with the *B. pendula* rust. Later on they both showed susceptibility, and in Experiment 3 it was significantly higher in clone V5944 (Table 2). However, V5944 was less infected ($p<0.0001$) by the *B. pubescens* rust in Experiment 1. In the further inoculations their responses were at the same level (Fig. 1, Exp. 1b–3b).

Table 2. **Kruskal-Wallis one-way analysis of variance for the means of the infection frequencies of the two B. pubescens species in three different inoculation experiments: Exp. 1, Exp. 2 and Exp. 3. The sources of the variance are the two rust isolates originating from B. pendula and B. pubescens**

Source	Exp. 1		Exp. 2		Exp. 3	
	H	p	H	p	H	p
B. pendula rust	0	0	3.56	0.0591	17.77	< 0.0001
B. pubescens rust	21.48	< 0.0001	0.03	0.8709	2.17	0.1408

H = value of Kruskal-Wallis test statistics.
p = level of significance.

Table 3. **Multiple comparisons for the means of the frequencies of six clones of B. pendula inoculated with B. pendula and B. pubescens rust isolate. Multiple comparisons performed separately for the three different inoculation experiments: Exp. 1, Exp. 2 and Exp. 3**

Comparison	Exp. 1		Exp. 2		Exp. 3	
	rust isolate		rust isolate		rust isolate	
	B. pendula	*B. pubescens*	*B. pendula*	*B. pubescens*	*B. pendula*	*B. pubescens*
4212–4213	ns	a	**	**	ns	ns
–4214	ns	a	ns	ns	ns	ns
–4215	ns	a	ns	**	**	ns
–5387	ns	a	ns	ns	**	ns
–5398	ns	a	ns	ns	ns	ns
4213–4214	ns	ns	*	**	ns	**
–4215	ns	ns	ns	ns	ns	ns
–5387	**	**	ns	**	ns	ns
–5398	ns	*	ns	**	ns	ns
4214–4215	ns	ns	ns	**	ns	**
–5387	ns	**	ns	ns	ns	ns
–5398	ns	**	ns	ns	ns	*
4215–5387	ns	**	ns	*	ns	ns
–5398	ns	*	ns	**	*	ns
5387–5398	*	**	ns	ns	ns	ns

Kruskal-Wallis multiple comparisons
ns = not significant
* = $p < 0.10$, critical value Z = 2.91
** = $p < 0.05$, critical value Z = 3.12
a = missing clone

4 Discussion

The two rust isolates were classified according to their host plant and they represented a heterogeneous field collection type. Their virulence may have changed during multiplication in laboratory conditions on their original hosts. Whether this was the reason for the increase in the virulence of the *B. pendula* rust is unclear. However, the variation in spore concentrations was most likely the cause of the variation between the repeated inoculations.

In general, the clones showed similar reactions to both rust isolates and to repeated inoculations. Clones E5387 and E5398 in Experiment 1 (Fig. 1, Exp. 1a and 1b) deviated the most from this pattern. This may be caused by differences in their physiological status or because of a somaclonal variation. Differences in the disease resistance of trees due to somaclonal variation have recently been reported with *Populus* spp. (OSTRY and SKILLING 1988; PRAKASH and THIELGES 1989).

The results of the inoculations agree with the observations of KLEBAHN (1904) and GÄUMANN (1959). The wider virulence of *B. pubescens* rust was expected, but it was not possible to distinguish between the responses of the two birch species to this isolate. However, the results of laboratory experiments and infection levels in the natural environment are not directly comparable. Field resistance is usually stronger, as has been reported in the interactions between poplars and their leaf rusts (SHAIN and JÄRLFORS 1987; SHAIN 1988). Environmental factors seem to be more significant in determining the degree of disease than the virulence of races or the resistance expressed by cultivars in the poplar-*Melampsora* leaf rust system (HEATHER and CHANDRASHEKAR 1982).

In rust and mildew pathosystems the forma specialis-genus specifity may have progressed from broadly pathogenic, non-aggressive forms to highly specialized, aggressive forms (GREEN 1971; TOSA et al. 1990). In the co-existence of the pathogen and its host, the genetic diversity of the plant sets more requirements on the adaptation of pathogen. Thus, the concept of *formae speciales* for *M. betulinum* reflects so far the ability of birches to occupy different habitats. A wider range of geographical sites and environments can be occupied by *B. pubescens* because of its tetraploidy (2n = 56) compared to the diploid *B. pendula* (2n = 28) (KUJALA 1946; JACKSON 1976; SÄRKILAHTI 1990).

The inoculations of *B. pubescens* with *B. pendula* rust revealed the existence of partial resistance, including necrosis of the infection sites. This type of resistance has been considered to be race-nonspecific and long-lasting because of its horizontal nature (PARLEVLIET 1979; VAN DER PLANK 1968). Some observations suggest that partial resistance in certain cultivars of *P. deltoides* is not durable against all the races of *M. medusae* (PRAKASH and HEATHER 1989). The extended growing of *B. pubescens* in nurseries may accelerate the appearance of more virulent races of *M. betulinum* rust.

Acknowledgements

I am grateful to Prof. TIMO KURKELA, Dr. REIJO KARJALAINEN, Mrs. ARJA LILJA and Mr. SAKARI LILJA for their comments on the manuscript. Mr. JOHN DEROME revised the English text. The study was supported by the Ministry of Agriculture and Forestry.

Summary

The resistance of six clones of *Betula pendula* and two clones of *B. pubescens* was screened against two field collection isolates of *Melampsoridium betulinum*. The clones of *B. pendula* were derived from plus trees growing in South Finland, and the clones of *B. pubescens* represented a second selection generation. The urediniospore isolates originated from *B. pendula* and *B. pubescens* growing in Central Finland. The screening was performed three times using a leaf disc assay and inoculation with water suspension. The responses of the clones were determined as the number of pustules that developed on the leaf discs, and the components of partial resistance were observed. The variation between the birch species confirmed the stronger host specialization of the *B. pendula* rust; lower infection frequency and the expression of partial resistance among the clones of *B. pubescens*. Partial resistance was apparent as a delayed latent period, and necrotic lesions developed in the infection sites. It was not possible to distinguish the responses of *B. pendula* from those of *B. pubescens* with respect to the inoculations with the *B. pubescens* rust isolate. Interclonal variation among the *B. pendula* clones was greater in the inoculations with the *B. pubescens* isolate than with the *B. pendula* rust. The clones of *B. pubescens* differed from each other in their infection level with both rust isolates. The concept of *formae speciales* for the *M. betulinum* rust is considered to reflect the different range of natural habitats of the two birch species.

Résumé

Tri de clones de Betula pendula et B. pubescens vis-à-vis de deux formes de Melampsoridium betulinum

La résistance de six clones de *B. pendula* et deux clones de *B. pubescens* a été éprouvée vis-à-vis de deux isolats de *M. betulinum*. Les clones de *B. pendula* provenaient d'arbres-plus du Sud de la Finlande et ceux de *B. pubescens* représentaient une sélection de seconde génération. Les isolats d'urédiniospores provenaient de *B. pendula* et *B. pubescens* de Finlande centrale. Le tri a été effectué à trois reprises par des tests sur disques foliaires inoculés par une suspension aqueuse. La réponse des clones a été déterminée par le nombre de pustules développées sur les disques, et les composantes de la résistance partielle ont été observées. Les variations entre les espèces de bouleau ont confirmé la plus forte spécialisation d'hôte chez la rouille de *B. pendula*, une plus faible fréquence infectieuse et l'expression d'une résistance partielle chez les clones de *B. pubescens*. La résistance partielle se manifestait par une période de latence plus longue et par des lésions nécrotiques. Il n'a pas été possible de distinguer les résponses entre *B. pendula* et *B. pubescens* inoculés par les isolats issus de *B. pubescens*. Les variations interclonales chez les clones de *B. pendula* étaient plus grandes avec les isolats de *B. pubescens* qu'avec ceux de *B. pendula*. Les clones de *B. pubescens* différaient entre eux par leur degré d'infection des deux isolats. On considère que le concept de *formae speciales* chez *M. betulinum* reflète les différents habitats naturels des deux espèces de bouleaux.

Zusammenfassung

Anfälligkeit von Betula pendula- und B. pubescens-Klonen gegen zwei Formen des Birkenblattrostes Melampsoridium betulinum

Sechs *Betula pendula*-Klone und zwei *B. pubescens*-Klone wurden auf ihre Resistenz gegen zwei Freiland-Isolate von *Melampsoridium betulinum* getestet. Die Klone von *B. pendula* stammten von Plus-Bäumen aus Südfinnland und die Klone von *B. pubescens* waren in der zweiten Generation selektiert. Die Uredosporenisolate des Rostes stammten von *B. pendula* und *B. pubescens* aus Zentralfinnland. Der Infektionstest wurde dreimal durchgeführt, der Pilz wurde dabei in wässeriger Suspension auf Blattstücke inokuliert. Die Reaktion der Birkenklone wurde anhand der Anzahl der Pusteln bestimmt, welche sich auf den Blattstücken entwickelten. Daneben wurden Symptome beobachtet, die für partielle Resistenz charakteristisch sind. Die Variation zwischen den Birkenarten bestätigte die stärkere Wirtsspezifität des *B. pendula*-Rostes; diese äußerte sich in niedrigeren Infektionsraten und partieller Resistenz bei den Klonen von *B. pubescens*. Die teilweise Resistenz manifestierte sich in einer verlängerten Latenzperiode und in nekrotischen Läsionen an den Infektionsstellen. Es war nicht möglich, die Reaktionen von *B. pendula* und *B. pubescens* auf Inokulationen mit dem *B. pubescens*-Rost voneinander zu unterscheiden. Die interklonale Variation bei *B. pendula* war nach Inokulation mit dem *B. pubescens*-Isolat größer als nach Inokulation mit dem *B. pendula*-Rost. Die Klone von *B. pubescens* unterschieden sich voneinander in ihrer Anfälligkeit gegen beide Rost-Isolate. Es wird angenommen, daß das Konzept der *formae speciales* für den *M. betulinum*-Rost die unterschiedlichen natürlichen Habitate der beiden Birkenarten reflektiert.

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Receipt of ms.: 20.7.1991

Eur. J. For. Path. 26 (1996) 171–181
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ISSN 0300–1237

Variation in *Melampsoridium* resistance among European white-birch clones grown in different fertilization treatments

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Summary

Resistance of 2-year-old plantlets of seven European white birch, *Betula pendula,* clones to birch rust, *Melampsoridium betulinum*, was studied in field experiments and in a leaf-disc bioassay. In addition, rust resistance of plantlets growing in a nursery under three fertilization treatments was tested. The birch clones clearly varied in their levels of resistance. One of the clones was consistently the most resistant, and two were very susceptible. Plantlets growing in the lowest fertility treatment were the most resistant. Clone × fertilization interaction was small. The plantlets grown in the lowest fertilization treatment, in particular, deviated from the other treatments. Generally good rust-resistance correlations were obtained between different experiments. The leaf-disc bioassay was an effective way of determining the field rust resistance of birch clones. The possibility of trade-offs between rust resistance and tree growth is discussed.

1 Introduction

Birch-leaf rust caused by *Melampsoridium betulinum* is the most important leaf disease in natural birch forests and in plantations in Fennoscandia. Interspecific variation in resistance to birch rust has been observed in natural rust infections among European and North-American birch species (KLEBAHN 1904; KECHEL and BÖDEN 1984). In artificial inoculation experiments, *Betula pendula* Roth exhibited slightly more susceptibility than *B. pubescens* Ehrh. (POTERI 1992). *B. pendula* is commonly used in silviculture and, consequently, screening clones for intraspecific resistance in this species may have important practical implications. Inter- and intra-specific variation in the resistance to *Melampsora* rusts have been reported both in *Populus* spp. (ELDRIDGE et al. 1973; THIELGES and ADAMS 1975; GALLO et al. 1985; PINON 1992) and in *Salix* spp. (VERWIJST 1990).

In resistance breeding, there is a demand for efficient and accurate screening methods. Detached leaves have been widely used in the study of plant disease resistance (BENEDIKZ et al. 1981; BUSSEY 1991). This method is especially useful for obligate plant parasites, as incubation can be carried out in controlled and replicable conditions. Poplar rust resistance has mainly been screened in leaf-disc bioassays (SHAIN and CORNELIUS 1979; CHANDRASHEKAR and HEATHER 1980). There are, however, only a few reports concerning correlations between screening results and field resistance among the leaf rusts of broad-leaved trees (PICHOT and TEISSIER DU CROS 1993; HAMELIN et al. 1994).

The progress of an epidemic is affected not only by the genotypes of the host and the pathogen but also by the environment. Fertilizers, especially nitrogen, may increase infections by obligate plant parasites such as rusts (ROWAN 1977; LAM and LEWIS 1982; BOQUET and JOHNSON 1987) and powdery mildews (BROSCIOUS et al. 1985; BOQUET and JOHNSON

Received: 28.11.1995; accepted: 05.02.1996

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1987). However, nutritional effects can be indirect. For example, changes in localized humidity at higher fertilization levels, owing to enlarged leaf area alone, may increase rust infection (VUORINEN 1992). In addition to variations in resistance exhibited by a single genotype in different environments, interactions between environments and host plants can be found. Such interactions have been reported between growing sites and rust infections of *Salix* clones (RÖNNBERG-WÄSTLJUNG and THORSÉN 1988) and between soil sites and fusiform rust susceptibility of loblolly- and slash-pine families (ROWAN 1977; GRIGGS et al. 1978). Conversely, no cultivar–environment effect has been found in barley-rust infections (PARLEVLIET and VAN OMMEREN 1975; PARLEVLIET 1979).

In this study, clones of European silver birch, *B. pendula*, were tested when grown in the field, potted outdoors, and by leaf-disc bioassay to determine whether: 1. Birch genotypes differ in their resistance to *M. betulinum*; 2. Rust resistance is affected by fertilization level; and 3. The responses of the clones in the leaf-disc bioassay correlate with artificial inoculations of intact plants and natural infections.

2 Materials and methods

2.1 Birch clones and rust isolates

Two-year-old micropropagated *B. pendula* clonal plantlets were used (JOKINEN et al. 1991). The parental trees were plus trees growing in southern Finland and selected because of their good quality and growth.

M. betulinum was collected in August 1990 from *B. pendula* in the Suonenjoki forest nursery (62°38′N, 27°04′E). Urediniospores were stored at −25°C over the winter and increased during June–July 1991 using inoculated leaf cultures and greenhouse seedlings. Urediniospores from Suonenjoki were used in leaf-disc bioassays and in the artificial inoculation in the field. In the nursery experiment, the rust infections were natural (i.e. probably 'local' Punkaharju (61°45′N, 29°08′E) rust strain).

2.2 Leaf-disc bioassay

Six 2-year-old birch plantlets from each of seven clones were grown outdoors in peat–sand mixture (3 : 1 by volume) in 2-l pots in 1991 at the Finnish Forest Research Institute, Vantaa (60°30′N, 25°00′E). The randomized plantlets were watered and fertilized once a week with 0.1% Superex 5 solution (Kekkilä®: N 10.9% (NO_3-N 9.1%), P 4%, K 25.3%, Mg 1.5%). On July 26, the third and the fourth leaves from the shoot tip of each plantlet were detached for a leaf-disc bioassay (SHAIN and CORNELIUS 1979; POTERI 1992). In total, 66–73 leaf discs (disc area 1.77 cm^2) of each birch clone were plated on 1% water agar amended with 10 mg/l gibberellic acid. In each petri dish, 6–7 discs were plated with their abaxial surfaces upwards. The concentration of the spore suspension was 1.5×10^5 spores/ml and a 10 μl aliquot of spore suspension was spread on a disc. Discs were incubated at 18–20°C in a growth chamber (CKS 2000, Kryoservice, Finland) with a 16-h photoperiod and irradiance of 120 μmol/m^2/s.

2.3 Field experiment

The seven 2-year-old clones included in the field experiment were planted in May 1991 at Punkaharju Research Station in a randomized block design with six replications of four plantlets in each 2 × 2 m plot. The field was previously planted with hay, but rather poor plantlet growth (20–30 cm/year) indicated leaching of nutrients and suboptimal condition for birches (M. ROUSI unpubl. data). Plants were inoculated on July 29 by spraying 4 ml of urediniospore suspension (concentration 1.3 × 10^5 spores/ml) on the lower surfaces of the leaves in the upper third part of the plantlet length.

2.4 Pot fertilization experiment

At Punkaharju Research Station, the seven 2-year-old birch clones were grown outdoors in plastic trays. Each 18-l tray was divided in four parts for four plantlets (4.5 l/plantlet) and the trays were randomized into four replications. The three growing media for different fertilization levels were obtained as follows: 1. 0.01% fertilization (30% peat + 70% sand, by volume); 2. 0.05% fertilization (70% peat + 30% sand, by volume); and 3. 0.1% fertilization (100% peat, by volume). The composition of fertilization was changed to ensure the winter hardening of the plantlets. The plants were fertilized six times between June 13 and July 11 using Superex 9 (Kekkilä®): N 19.4% (NO_3-N 7.2%), P 5.3%, K 20%, Mg 0.2%; five times using Superex 5, July 19–August 2; six times using Superex 7 August 16–September 5: N 0%, P 6.9%, K 31.9%, Mg 1% (plantlets in the growth medium 1 obtained only four Superex 7 fertilizations).

2.5 Assessment of rust infection

The incidence of infection was monitored four times after inoculation in the leaf bioassay and in the field trials. In the leaf bioassay, the number of rust pustules was counted under a stereo-microscope on days 6, 9, 11 and 14 after inoculation. In the field experiment, rust was estimated as a percentage of leaf area diseased (PLAD) on a scale: $1 = 0$, $2 = > 0\text{–}< 5$, $3 = 5\text{–}< 25$, $4 = 25\text{–}< 75$, $5 = 75\text{–}100$ (SLOPEK 1989). The recording was taken for the same two leaves, which were the third full-grown leaf from the shoot tip and the leaf next to the main shoot on the second upper-side shoot. Plantlets were scored 7, 9, 14 and 21 days after the inoculation. In the pot experiment, PLAD was recorded on August 20 when the natural rust infection in the nursery had developed for 3 weeks. As in the field experiment, two leaves of four seedlings in each tray (clone) were scored.

2.6 Analysis of data

The estimated PLAD classes were transformed to continuous disease indices by counting an arithmetic average of arbitrary classes 1–4 according to a formula $(f_2 \cdot 1 + f_3 \cdot 2 + f_4 \cdot 3 + f_5 \cdot 4) \cdot n^{-1}$, where f_2–f_5 = frequencies in each PLAD class 2–5 (the class 1 = no infections was excluded) and n = total number of countings. The means of disease indices or pustule frequencies were plotted against time to obtain disease-progress curves and areas under the disease-progress curve (AUDPC) were determined. In order to calculate correlations between the field tests and the bioassay, the pustules counted in the leaf bioassay were converted to a 1–5 PLAD scale, assuming that 300 pustules/disc corresponded to a 100% infection. An analysis of variance was performed (BMDP 1990). Data was tested for normality and equality of variances. A fixed-effects model was based on the formula: $y_{ijk} = \mu + \alpha_i + \gamma_j + (\alpha\gamma)_{ij} + \varepsilon_{ijk}$, where α_i was the effect of clone, γ_j was the effect of fertilization, $(\alpha\gamma)_{ij}$ the possible interaction of clone and fertilization, and ε_{ijk} was the error term. A significance level $\alpha = 0.05$ was specified for the Student-Newman-Keuls multiple range tests and for the Spearman rank-correlation coefficients.

3 Results

3.1 Differences in rust resistance among the clones

The first scoring dates, 6 and 7 days after inoculation, revealed considerable variation in the appearance of rust symptoms (latent periods; Figs 1, 2). Three of the clones (JR1/1, 5832 and 39) had the longest latent periods both in the field and in the leaf bioassay. In the leaf bioassay, clone (2674) formed sporulating pustules most quickly (Fig. 1). In the field, clones (5818 and 36) and (2674) had the highest disease indices in the first scoring (Fig. 2). Clone

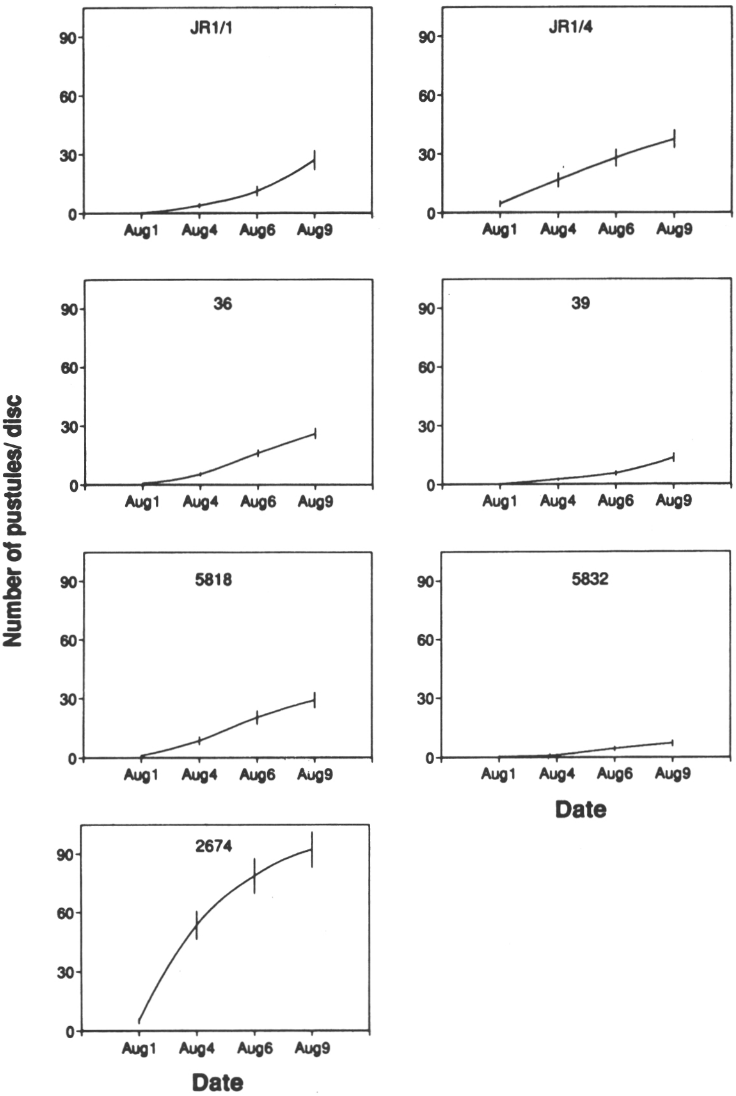

Fig. 1. Disease-progress curves for *Melampsoridium betulinum* on seven *Betula pendula* clones in the leaf-disc assay. Date of inoculation is July 26. The curves are based on pustule frequencies and mean ± 1 SE are shown

(5832) was very resistant both in the field and in leaf-disc assay (Table 1). The progress of the rust infection indicated significant differences in the resistance of the clones.

3.2 Effect of fertilization on the resistance of the clones

Fertilization increased plantlet susceptibility (Fig. 3; Table 2), although the difference in the disease indices between the two highest fertilization treatments within the clones was generally small and statistically non-significant (Table 3). As expected, clone (5832) was the most resistant within all fertilization treatments. At the two highest fertilization levels, it

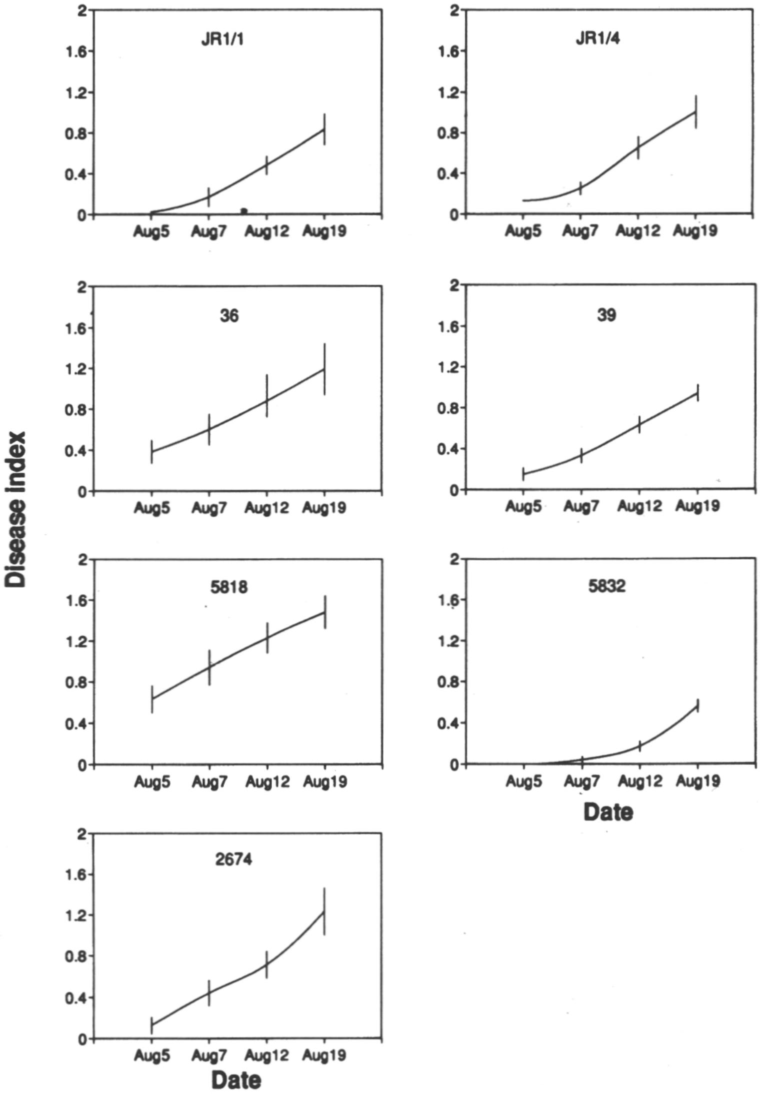

Fig. 2. Disease-progress curves for *Melampsoridium betulinum* on seven *Betula pendula* clones in the field experiment. Date of inoculation is July 29. The curves are based on disease indices and mean ±1 SE are shown

Table 1. **Area under the disease-progress curve, AUDPC, for the *B. pendula* clones in the leaf disc assay and in the field experiment**

Clone	AUDPC[a] Field experiment[b]	 Leaf-disc assay[c]
JR1/1	0.23^{ab}	60.11^{a}
JR1/4	0.29^{ab}	142.03^{b}
36	0.66^{ab}	71.94^{a}
39	0.22^{ab}	30.64^{a}
5818	0.9^{b}	93.18^{ab}
5832	0.08^{a}	19.42^{a}
2674	0.55^{ab}	390.39^{c}

[a] AUDPC followed by different letters in the columns differ significantly ($p < 0.05$) according to the Student-Newman-Keuls multiple-range test

[b] Calculations based on disease index

[c] Calculations based on pustule frequencies

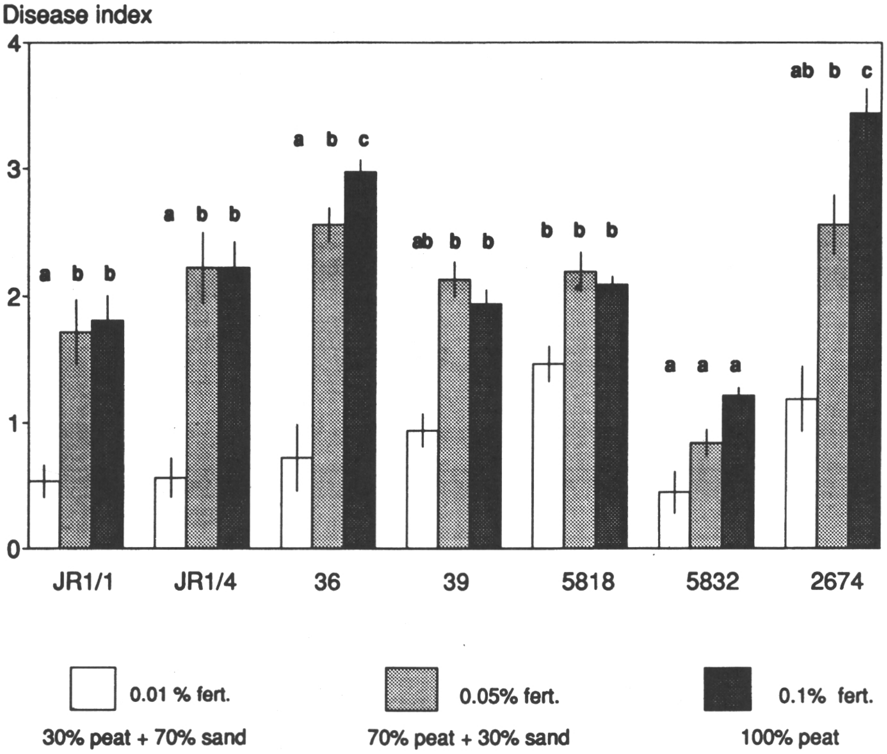

Fig. 3. Disease index of seven *Betula pendula* clones grown under different fertilization treatments (0.01, 0.05 and 0.1%) in pot experiments. Bars indicate ±1 SE. Statistically significant differences between the clones within each fertilization treatment are indicated by different letters above the columns (Student-Newman-Keuls multiple-range test, $\alpha = 0.05$)

Table 2. **ANOVA table for the disease index of seven *B. pendula* clones tested against three different fertilization levels (0.01, 0.05 and 0.1%) in the pot experiment**

Source	df	MS	F	p
Clone	6	3.1	25.34	<0.0001
Fertilization	2	16.11	131.12	<0.0001
Interaction	12	0.56	4.52	<0.0001

Table 3. **Mean disease indices ±1 SE for the *B. pendula* clones grown under different fertilization levels (0.01, 0.05 and 0.1%). The indices followed by different letters in the rows differ significantly between the fertilization levels within the clone according to the Student-Newman-Keuls multiple-range test ($\alpha = 0.05$)**

	Fertilization treatment					
	0.01%		0.05%		0.1%	
Clones	mean	±SE	mean	±SE	mean	±SE
JR1/1	0.53^{a}	0.13	1.72^{b}	0.25	1.81^{b}	0.19
JR1/4	0.56^{a}	0.16	2.22^{b}	0.22	2.22^{b}	0.2
36	0.72^{a}	0.27	2.56^{b}	0.13	2.97^{b}	0.09
39	0.94^{a}	0.13	2.13^{b}	0.14	1.94^{b}	0.11
5818	1.47^{a}	0.14	2.19^{b}	0.15	2.09^{b}	0.06
5832	0.44^{a}	0.17	0.84^{b}	0.11	1.22^{b}	0.06
2674	1.19^{a}	0.26	2.56^{a}	0.23	3.44^{a}	0.19

differed significantly from the other clones. Clone (5818) was highly susceptible at the lowest fertilization treatment (Fig. 3). The clones did not respond similarly to the changes in the growth substrate, as indicated by a significant clone × fertilization interaction, although the interaction was the smallest contributor to the variation (Table 2).

3.3 Comparison between the leaf bioassay and the field and pot experiments

Results from the leaf-disc inoculations were in good accordance with those of the field experiment (Fig. 4). Disease indices measured from inoculated leaf discs were also strongly correlated with the disease indices measured in the pot experiments. However, the correlation coefficient between the leaf bioassay and the disease indices measured at the lowest fertilization level was quite low and statistically non-significant.

4 Discussion

Plantlets were tested in different environments with natural rust infections and artificial inoculations. In all experimental settings, one of the clones (5832) was the most resistant to rust infection. Conversely, some of the tested clones were highly susceptible to rust infection (especially clone 2674).

The rust strain used in the artificial inoculation was collected some 150 km from the Punkaharju nursery where the natural infection by rust took place. Although there was some difference in the resistance ranking of the clones between different experiments, the results indicated that there were clear genetic differences in susceptibility to rust among European white birches.

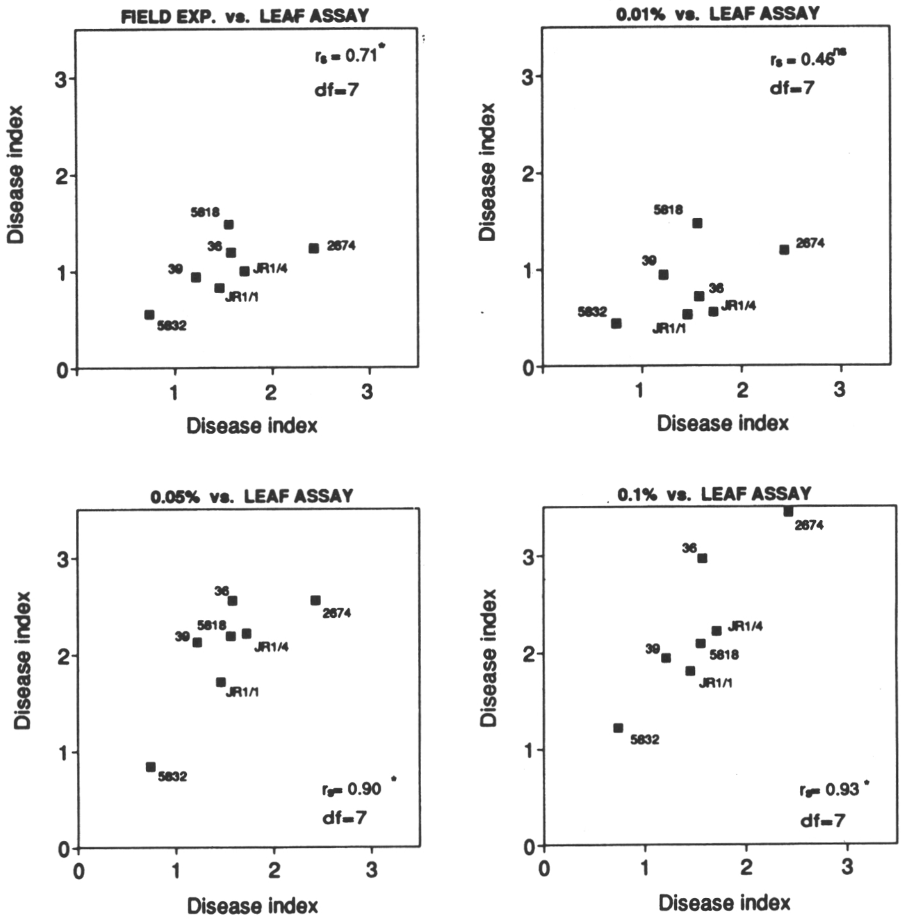

Fig. 4. Relationships between *Melampsoridium betulinum* infections in the leaf disc assay (x) and in the field and pot experiments (y). The infection levels are expressed as disease indices. r_s = Spearman rank correlation coefficient, $\alpha = 0.05$

In addition to plant genotype, environment may also be important in the determination of plant resistance. In grain crops, soil nitrogen content increases the amount of rust infection (BOQUET and JOHNSON 1987), although the effect of different nutrient components can be very complex (ANDERSON and DEAN 1986). Similarly, nitrogen and phosphorous deficiencies increased poplar resistance to *Melampsora* leaf rust (SUZUKI 1973). In the experiment reported here, clones grown in very low nitrogen were more resistant than those grown in more fertile soil (Fig. 3; Table 2). Plantlets grown in the highest fertilization treatment were clearly the tallest. There was, however, no significant difference in rust resistance between the two highest fertilization levels. Vigorous plant growth and density under high nitrogen supply may extend the time of moisture on the leaf surface and thus favour urediniospore germination (SHARP et al. 1958; MANNERS 1981). Moisture may partly explain differences between the low and higher fertilization treatments. The relative susceptibility of some clones (e.g. 36, 5818 and 2674) varied in different fertilization treatments (Fig. 3, Table 2), indicating that the adaptability of different birch genotypes to different soils may also be important in the determination of plant resistance. Because

European white birch is adapted to good sites (OIKARINEN 1983), the lowest fertilization level (30% peat + 70% sand) should be considered very poor substrate for growing birch. Such levels are not used in nurseries and low fertility sites are also avoided in birch-plantation forestry. The results of this study are, however, based on only seven clones. Moreover, birch also often occupies poor sites in natural conditions.

The number of rust pustules can be affected by environmental factors (DAS et al. 1993), and the size of the rust pustules may depend on the nutritional status of the plant (PAUL et al. 1990). In this study, rust infections were analysed as percentage of leaf area diseased (PLAD); this measure can be regarded as including both the number and size of rust pustules. More detailed investigation of birch-rust symptoms (the number and size of uredinia recorded separately) might have revealed whether some of those traits changed under different growing conditions. The number of uredinia on the leaf discs of the bioassay correlated well with the PLAD in the natural rust infections of the pot experiments, excluding the lowest fertilization level, and the correlation to the PLAD in the artificial inoculation in the field was also high. In those years when birch-rust infections appear very late or not at all, leaf bioassays may provide reliable results for further field testing.

Physiological constraints have been suggested to result in trade-offs between the high rate of growth and plant secondary chemistry, making plants more resistant to biotic threats (HERMS and MATTSON 1992). At the end of the growing season, there were substantial differences in the size of plantlets of different clones in our nursery-fertilization experiment. After heavy rust attack, the plantlets of the most resistant clone were the tallest (clone 5832) whereas the plantlets of susceptible clones tended to be small (especially clone 2674; M. ROUSI unpubl. data). Generally, the growth of a susceptible clone (2674) has been somewhat better in several field experiments than that of a resistant clone (5832; M. ROUSI unpubl. data) where there was no relationship between susceptibility to rust infection (Fig. 3) and average growth in those experiments ($r_s = -0.18$, $p = 0.70$). No ecological trade-offs in the form of negative correlations in resistance to different biotic and abiotic threats was found, although there was some tendency for late-flushing clones to be slightly more resistant to rust infection (M. ROUSI unpubl. data). Owing to the rather poor field growth of the exceptionally resistant clone (5832), one cannot exclude the possibility of a trade-off between growth and resistance.

Acknowledgements

The authors thank Timo KURKELA, Veikko KOSKI and Jorma TAHVANAINEN for commenting on the manuscript. Thanks also to Kari KUOKKANEN and Hanni SIKANEN for their assistance with the field and pot experiments at Punkaharju Research Station, and Robert SCHROEYERS for assisting with the laboratory work at the Forest Research Institute in Vantaa. Linguistic correction was made by Edward KAMRIN. The study was supported by the Ministry of Agriculture and Forestry in Finland.

Résumé

Variations dans la résistance à Melampsoridium chez des clones de Betula pendula soumis à des fertilisations

Des plants de 2 ans de 7 clones de *Betula pendula*, ont été étudiés pour leur résistance à *Melampsoridium betulinum*, par des essais en nature et par des tests sur disques foliaires. De plus, trois types de fertilisation ont été étudiés en pépinière. Les clones de bouleau variaient nettement pour la résistance. L'un d'eux était constamment le plus résistant et les deux autres étaient très sensibles. Les plants soumis à la plus faible fertilisation étaient les plus résistants. L'interaction clone × fertilisation était faible, mais les plants soumis à la plus faible fertilisation se distinguaient. Nous avons généralement eu de bonnes corrélations pour la résistance entre les différents essais. Le test sur disques foliaires était une méthode efficace pour déterminer la résistance au champ des clones. Les possibilités de compromis entre la résistance et la croissance sont discutées.

Zusammenfassung

Variation der Resistenz gegen Melampsoridium bei unterschiedlich gedüngten Betula pendula-Klonen

Die Resistenz 2jähriger Pflanzen von sieben Klonen der Hängebirke (*Betula pendula*) gegenüber dem Birkenrost (*Melampsoridium betulinum*) wurde in Freilanduntersuchungen und einem Biotest an Blattstücken geprüft. Zusätzlich wurde die Resistenz von Jungpflanzen in einer Baumschule bei drei verschiedenen Düngestufen untersucht. Die Birkenklone unterschieden sich klar in ihrer Resistenz. Einer der Klone war durchweg der resistenteste, zwei waren sehr anfällig. Die Pflanzen mit der niedrigsten Düngung waren am resistentesten. Die Wechselwirkung zwischen Klon und Düngung war gering. Insbesondere die Pflanzen der niedrigsten Düngestufe unterschieden sich von den anderen Behandlungen. Zwischen den einzelnen Experimenten und der Rostresistenz bestanden gute Korrelationen. Mit dem Test an Blattstücken konnte die Feldresistenz der Birkenklone effizient bestimmt werden. Die möglichen Wechselwirkungen zwischen Rostresistenz und Baumwachstum werden diskutiert.

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III

Eur. J. For. Path. 27 (1997) 363–372

ISSN 0300–1237

Differences in the rust resistance of greenhouse and outdoor-grown white birch species, *Betula* spp.

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Summary

The 2-year-old seedlings of five different white birch species (*Betula platyphylla*, *Betula papyrifera*, *Betula pubescens*, *Betula pendula* (two types) and *Betula resinifera* × *Betula pendula*) grown both in a greenhouse and outdoors, were inoculated in a leaf disc assay with two different birch rust (*Melampsoridium betulinum*) isolates from *B. pendula* and *B. pubescens*. The resistance of these birch species varied significantly. Resistance to the *B. pubescens* rust isolate was not related to the resistance of the *B. pendula* rust isolate. The behaviour of a birch genotype grown in the greenhouse did not correspond to the behaviour of the same genotype grown outdoors.

The outdoor growth environment greatly increased the contents of soluble proteins, rubisco, chlorophyll and nitrogen in the leaves of diploid birch species (*B. platyphylla, B. pendula* and *B. resinifera* × *B. pendula*). For tetraploid and pentaploid species (*B. pubescens* and *B. papyrifera*, respectively) there was no such clear difference in the leaf physiological status between the seedlings grown outdoors and in the greenhouse. The C:N ratio was higher for the greenhouse-grown seedlings in all the birch species, but the difference was significant only with the diploid species. The incidence of rust in the birch species did not correlate with any of the leaf physiological parameters studied. The adaptability of birch genotypes to the environment in relation to their resistance to birch leaf rust is discussed.

1 Introduction

Birch rust, *Melampsoridium betulinum* (Fr.) Kleb., is reported to infect all birch species (Gäumann 1959) and in the family of Betulaceae *Alnus* (Roll-Hansen and Roll-Hansen 1981) and *Carpinus* (Gäumann 1959) can also be infected. Interspecific resistance to birch rust among different birch species has, however, received little study. Kechel and Böden (1984) reported North American *Betula papyrifera* Marsh. and *B. alleghaniensis* Britton to be more resistant to *M. betulinum* than *B. pendula* Roth and *B. pubescens* Ehrh. in a field trial in Germany. In a leaf disc assay *B. pendula* and *B. pubescens* differed in their resistance to the Finnish isolates of *M. betulinum* (Poteri 1992).

The environment has an indirect effect on the nutrient uptake and development of rust fungi. Early rust studies showed that *Puccinia coronata* infections were delayed in oat plants that were kept in the dark (Fromme 1913). Light is the main factor controlling photosynthesis and the availability of carbohydrates, which are essential for the fungal growth. The rate of photosynthesis is also positively correlated with the leaf nitrogen content (Evans 1989), and generally high nitrogen levels are known to favour rust infections (Manners 1993; p. 196). A significant portion of the organic nitrogen in the leaf is bound to a chloroplast enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco). Rubisco also serves as an important storage protein and is among the first enzymes to be degraded in the senescence process in the autumn (Friedrich and Huffaker 1980).

Received 23.12.1996; accepted 4.4.1997

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Different species of trees, and genotypes within species, are adapted to varying lengths of growth periods, which is, for example, indicated by the different timings of leaf senescence. Leaf rusts usually appear in late summer, which may also cause genotypes to vary in their rust resistance owing to differences in their senescence processes. In resistance screenings such variation in the leaves is generally minimized by using leaves of similar ages in the inoculations (SHARMA et al. 1980). However, the timing of the cessation of growth of species and origins depends on the environmental factors the genotype is adapted to (KOSKI and SIEVÄNEN 1985). The difference in the timing of the cessation of growth can also be seen in the time course of photosynthesis (KOIKE 1995). Consequently, decline in the nitrogen content of leaves, for example, probably depends on the origin of a genotype.

The aim of this study was (1) to determine the variation in resistance to Finnish *M. betulinum* isolates among six different birch species or genotypes, (2) to compare the susceptibility of the seedlings of different species grown outdoors and in a greenhouse and (3) to relate the infection rates of the seedlings to the physiological status of the leaves.

2 Materials and methods

2.1 Plant material

Seeds of four different white birch species were sown: *B. platyphylla* var. *japonica* Hara (origin Jyozankei, Japan), *B. papyrifera* (origin Midland, Michigan), *B. pubescens* (local), *B. pendula* (loc.) and *B. pendula* (JR, a fast growing Finnish F_1 family). In addition, seeds of one controlled crossing between a single tree of *B. resinifera* Britton (origin Alaska) and *B. pendula* were sown. The *B. pendula* genotype (clone no. 39) involved in the crossing was found to have medium resistance to *Melampsoridium* in the clonal test carried out by POTERI and ROUSI (1996).

The seeds were sown May 25, 1993, in 0.28-l nursery pots at the Punkaharju Research Station, Finland (61°45′N, 29°08′E). The first summer the seedlings were grown in a greenhouse, in prefertilized peat, obtaining normal greenhouse fertilization (for details see ROUSI et al. 1991, 1993). In May, 1994, the seedlings were randomly divided into two groups: half of the seedlings were transferred outdoors, half kept indoors. In both places the seedlings were divided into three replicates, 14 seedlings per replicate.

The early spring of 1994 was sunny but the nights were cold. To avoid the effect of exceptionally low temperatures on developing tissues, the seedlings were transferred from the experimental field to a greenhouse every night from May 19 to June 1.

The seedlings were fertilized using Superex-5 (Kekkilä^{TM}, Kekkilä, Hyrylä, Finland: N 10.9% (NO_3-N 9.1%), P 4%, K 25.3%, Mg 1.5%) on July 25 (0.17% solution) and on July 26 to August 16 once per week (0.26% solution), and with Superex-7 (0% N, P 6.9%, K 31.9%, Mg 1%) on August 24 to September 6 (0.13% solution). The fertilization regime was changed to ensure the winter hardening of seedlings.

2.2 Inoculation

The two rust isolates of *M. betulinum* originating from *B. pendula* and *B. pubescens* were collected in 1991 at the Suonenjoki Research Station (62°38′N, 27°04′E). Urediniospores were multiplied on leaf discs and lyophilized for further use.

On August 18, 1994, 12 leaves for a leaf disc assay were collected from each birch species, both in the greenhouse and outdoors. A third or fourth leaf was detached from the top of 12 seedlings, which represented four seedlings in three replicate blocks. Sampled leaves were surface sterilized with 0.35% NaOCl, rinsed three times in sterile water and cut into leaf discs to obtain 60 discs for each birch genotype, both in the greenhouse and outdoors. A total of 720 discs were cut. The discs were plated in Petri dishes on 1% water agar amended with 10 mg/l gibberellic acid and the Petri dishes were stored overnight in the cold room (+ 8–10°C). The following day the discs were inoculated with a 10 μl aliquot of urediniospore suspension which was spread with a glass bar over the discs (Poteri 1992). For the inoculation 30 discs were treated with the rust isolate of *B. pendula* and the other 30 discs with the rust isolate of *B. pubescens*. The concentration of spore suspension was adjusted to 3.7×10^5 spores/ml for both rust isolates. After the inoculation at the Punkaharju Research Station, Petri dishes were transported in ice boxes to the Department of Plant Biology at the University of Helsinki, Finland, where the inoculated leaf discs were incubated at 18–20°C in a growth chamber (Weiss) with a photoperiod of 16 h and an irradiance of 100 μmol/m^2 s^{-1}.

2.3 Rust assessment

The rust infections were counted under a stereomicroscope as pustule frequencies on the leaf discs 8, 9, 10 and 12 days after the inoculation. The pustule frequencies were plotted over time and the area under disease progress curve (AUDPC) was calculated for each treatment.

The occurrence of natural rust infections in seedlings growing outdoors was checked macroscopically on August 25, 7 days after the inoculation of the leaf discs. No rust pustules were detected in the sampled leaves, which were the same leaves collected for physiological and biochemical analysis (see below).

2.4 Leaf analyses

For the leaf analyses, one leaf from each of 10 seedlings of five birch species (*B. pendula* JR was excluded) was removed both in the greenhouse and outdoors. The leaves were chosen from the same leaf position as the leaves that were collected for the leaf disc assay. Detached leaves were frozen in liquid nitrogen and stored in dry ice during the transport from the Punkaharju Research Station to the Department of Plant Biology in Helsinki, where the samples were stored at –70°C.

For the analysis of total soluble proteins, rubisco and chlorophyll content, leaves were first ground in liquid nitrogen and a 100 mg sample of leaf powder was mixed with 3.0 ml of ice-cold extraction buffer (50 mM MES pH 6.8, 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1% Tween-20) (Gezelius and Hallen 1980; Pääkkönen et al. 1996). The leaf extract was stored in Eppendorf tubes at –20°C. The amount of rubisco was analysed from the supernatant of leaf extract by running a polyacrylamide gel electrophoresis with a 4% concentration gel and a 6% separation gel (Rintamäki et al. 1988). Purified ribulose-1,5-bisphosphatecarboxylase-oxygenase (Sigma R8000, Sigma, Deisenhofen, Germany) was used as a standard rubisco. Electrophoresis (Bio-Rad Minigel II, Bio-Rad, Hercules, CA, USA) was done at room temperature using 10 mA for a gel in the concentration stage and 20 mA for a gel in the separation stage. The area and intensity of rubisco bands were recorded with a scanner (Pharmacia, Uppsala, Sweden) and analysed with a Pharmacia Image Master program. The concentration of the rubisco standard was estimated by the Bradford (1976) method, using bovine serum albumin (BSA, Sigma) as a reference. Soluble proteins were measured spectrophotometrically by the same method, using Bio-Rad Reagent Dye to stain the proteins. A standard curve was obtained with BSA (Sigma). The chlorophyll a and b content was analysed by the Arnon (1949) method using a spectrophotometer (Perkin-Elmer, Überlingen, Germany).

The remains of the ground sample leaves were oven-dried at 60°C. The total nitrogen and carbon contents of the dried samples were determined in a carbon nitrogen analyser (Leco CHN 900, St. Joseph, MI, USA).

2.5 Statistical analysis

The differences between the AUDPC of the birch genotypes were analysed with a Student–Neuman–Keuls multiple range test. A comparison between the AUDPC and leaf physiological data of greenhouse and outdoor-grown seedlings was made using a non-parametric Mann–Whitney *U*-test. AUDPC correlations between greenhouse and outdoor-grown birch species were calculated by applying a Spearman rank correlation test. The statistical analyses were made by using a BMDP (1990) program package.

3 Results

3.1 *Melampsoridium betulinum* infections

The birch species differed significantly in their AUDPC in all four experiments (Fig. 1). The hybrids of *B. resinifera* × *B. pendula* were resistant to both rust isolates, and the

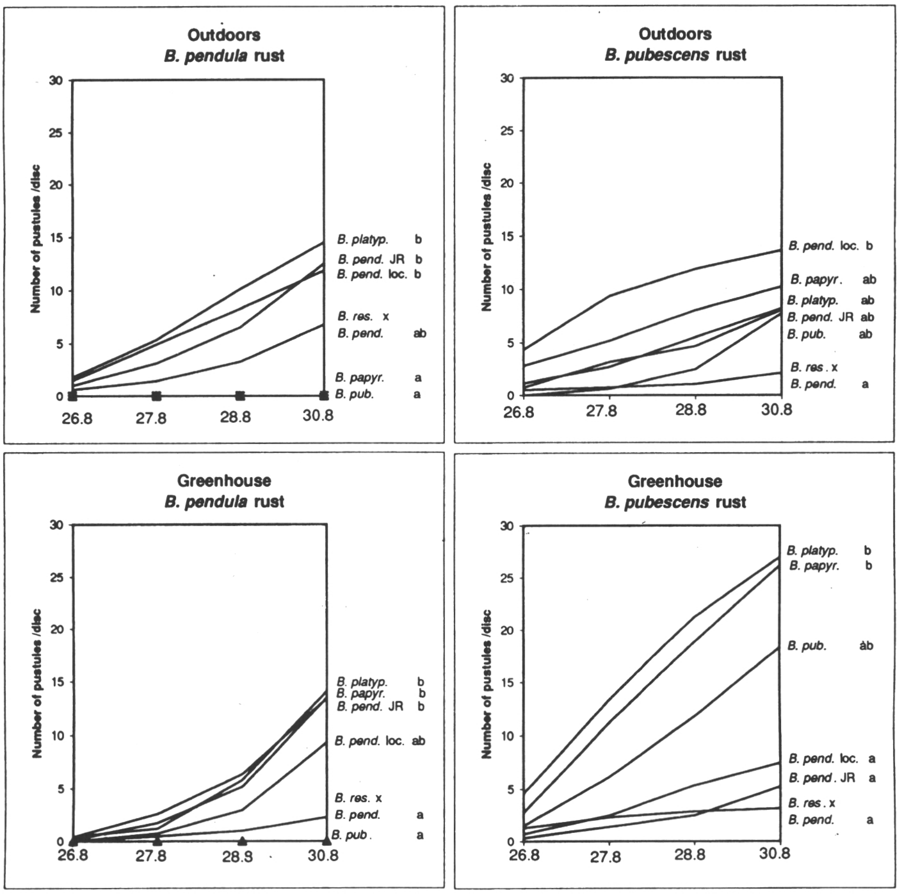

Fig. 1. Disease progress curves for the six birch genotypes grown in the greenhouse and outdoors inoculated with the *B. pendula* and *B. pubescens* rust isolates. Birch genotypes marked with different letters differ in their AUDPC according to Student–Neuman–Keuls multiple range test ($\alpha = 0.05$, n = 30). *B. platyp.* = *B. platyphylla*, *B. papyr.* = *B. papyrifera*, *B. pub.* = *B. pubescens*, *B. pend.* JR = *B. pendula* JR, *B. pend.* loc. = *B. pendula* local, *B. res.* × *B. pend.* = *B. resinifera* × *B. pendula*

outdoor-grown seedlings of *B. papyrifera* were not infected by the *B. pendula* rust isolate (Fig. 1).

Resistance of the birch species varied between both the growing environments (Table 1) and the rust isolates (Table 2). Outdoor-grown *B. papyrifera* seedlings were totally immune to the *B. pendula* rust isolate (Table 1). However, the seedlings grown outdoors were generally more susceptible to the rust inoculations of the *B. pendula* isolate, although the difference was significant only in the case of *B. resinifera* × *B. pendula* seedlings. In contrast, seedlings grown indoors were generally more susceptible when inoculated with the *B. pubescens* rust isolate. This was seen clearly in *B. platyphylla* and *B. pubescens*, the exception being the seedlings of local *B. pendula*, which were significantly more susceptible when grown outdoors (Table 1).

In both environments *B. papyrifera*, *B. pubescens* and the local *B. pendula* were more susceptible to the *B. pubescens* rust isolate than to the *B. pendula* rust isolate. *B. platyphylla* and *B. resinifera* × *B. pendula* reacted inconsistently to the rust isolates in different environments (Table 2).

Table 1. **Effect of the growing environment (greenhouse or outdoors) on the susceptibility (AUDPC) of the six birch genotypes inoculated with the *Betula pendula* and *B. pubescens* rust isolates. Differences in the AUDPC of the growing environments tested according to Mann–Whitney *U*-test, n = 30**

	B. pendula rust				*B. pubescens* rust			
	Greenhouse		Outdoors		Greenhouse		Outdoors	
	mean	(±sem)	mean	(±sem)	mean	(±sem)	mean	(±sem)
B. platyphylla	24.3	(±4.01)	36.02	(±8.91)	74.48**	(±19.12)	19.77	(±5.69)
B. papyrifera	23.1**	(±5.57)	0	(±0)	66.98	(±20.64)	28.87	(±8.59)
B. pubescens	0	(±0)	0	(±0)	42.88**	(±8.32)	12.08	(±2.71)
B. pendula JR	25.87	(±7.44)	26.02	(±5.64)	10.47**	(±3.42)	18.62	(±4.24)
B. pendula loc.	14.55	(±4.61)	29.95	(±11.72)	18.37	(±9.28)	43.02	(±19.31)
B. resinifera × *B. pendula*	4.22*	(±1.11)	13.52	(±3.75)	10.4	(±6.84)	4.8	(±2.66)

* = $p < 0.05$, ** = $p < 0.01$.

Table 2. **Effect of the rust isolate (*Betula pendula* and *B. pubescens* rust isolates) on the susceptibility (AUDPC) of the six birch genotypes grown in the greenhouse and outdoors. Differences in the AUDPC of the rust isolates tested according to Mann–Whitney U-test, n = 30**

	Greenhouse				Outdoors			
	B. pendula rust		*B. pubescens* rust		*B. pendula* rust		*B. pubescens* rust	
	mean	(±sem)	mean	(±sem)	mean	(±sem)	mean	(±sem)
B. platyphylla	24.3	(±4.01)	74.48	(±19.12)	36.02*	(±8.91)	19.77	(±5.69)
B. papyrifera	23.1	(±5.57)	66.98	(±20.64)	0***	(±0)	28.87	(±8.59)
B. pubescens	0***	(±0)	42.88	(±8.32)	0***	(±0)	12.08	(±2.71)
B. pendula JR	25.87*	(±7.44)	10.47	(±3.42)	26.02	(±5.64)	18.62	(±4.24)
B. pendula loc.	14.55	(±4.61)	18.37	(±9.28)	29.95	(±11.72)	43.02	(±19.31)
B. resinifera × *B. pendula*	4.22**	(±1.11)	10.4	(±6.84)	13.52**	(±3.75)	4.8	(±2.66)

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.2 Physiological status of the leaves

Nitrogen content in the leaves was significantly higher for all the birch species when grown outdoors, except for *B. pubescens* (Fig. 2). Differences in carbon content were small. All the species had a higher C:N ratio in the greenhouse than outdoors (Fig. 2).

The concentration of soluble proteins in the leaves was significantly higher in the seedlings grown outdoors; the only exception was *B. papyrifera*, with no significant difference in its protein content between the greenhouse and outdoor-grown seedlings (Fig. 3). The seedlings had significantly more rubisco in the leaves when grown outdoors, except for *B. pubescens*, whose leaves had the same rubisco content both outdoors and in the greenhouse (Fig. 3). Among the birch species studied rubisco yielded from 28.3% to 41.8% of the soluble proteins. The clear exception, in which the rubisco level was too low to be detected, was the greenhouse-grown *B. papyrifera* leaves. In the outdoor-grown seedlings of *B. papyrifera*

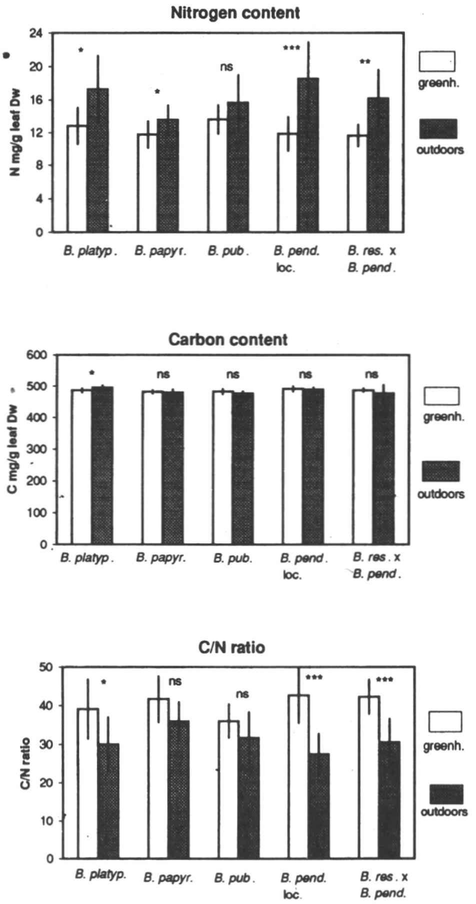

Fig. 2. Total nitrogen and carbon content (mg/g dry weight) and C:N ratio in the leaves of the seedlings of six birch genotypes grown in the greenhouse or outdoors. Bars indicate standard error of mean, n = 10. Mann–Whitney *U*-test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. ns = non-significant). Abbreviations see Fig. 1

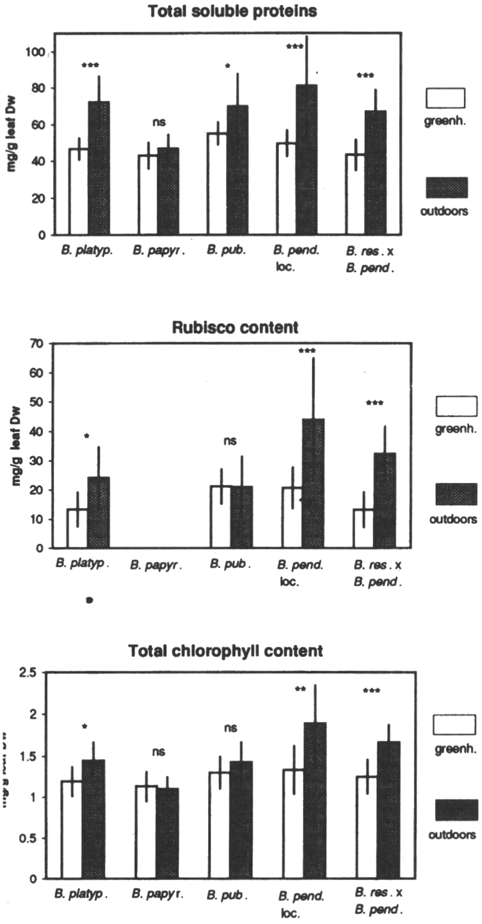

Fig. 3. Total soluble protein, rubisco and total chlorophyll content (mg/g dry weight) in the leaves of the seedlings of six birch genotypes grown in the greenhouse and outdoors. Bars indicate standard error of mean, n = 10. Mann-Whitney *U*-test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. ns = non-significant). Abbreviations see Fig. 1

the extraction succeeded in only half of the leaves. Total chlorophyll content was variable; in the leaves of *B. papyrifera* and *B. pubescens* seedlings grown outdoors the content was nearly the same (Fig. 3). The chlorophyll a/b ratio was the highest in outdoor-grown leaves (results not shown). However, the chlorophyll a/b ratio was at the same level for all species (3.59–4.14), with no significant differences between greenhouse and outdoor-grown species.

The AUDPC of the outdoor and greenhouse-grown seedlings of birch types did not correlate with either of the rust isolates according to the Spearman rank correlation test. The correlation was higher for the rust isolate of *B. pendula*: $r_s = 0.494$ ($df = 6$, $\alpha = 0.05$) compared with the rust isolate of *B. pubescens* where $r_s = 0.115$ ($df = 6$, $\alpha = 0.05$) (Fig. 4). The correlations between the AUDPC of the species and the leaf substances (total soluble proteins, rubisco, chlorophyll, nitrogen and carbon content and C:N-ratio) were not significant with either of the rust isolates used (results not shown).

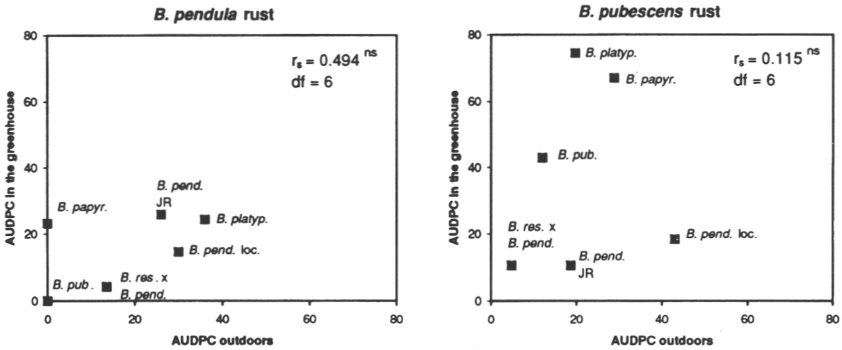

Fig. 4. Relationships of the rust infections (AUDPC) of the seedlings of six birch genotypes grown outdoors (x) and in the greenhouse (y) and inoculated with *Betula pendula* and *B. pubescens* rust isolates. r_s = Spearman rank correlation coefficient. ns = non-significant. Abbreviations see Fig. 1

4 Discussion

B. platyphylla, *B. pendula* and *B. resinifera* are closely related birch species (DUGLE 1966). As expected, the seedlings of all these three species were susceptible to the *B. pendula* rust isolate in both environments. The hybrids between *B. resinifera* and *B. pendula* were more resistant to the rust isolate of *B. pendula* than either of the *B. pendula* types (JR and local), which suggests that *B. resinifera* is a relatively resistant species. Because we had only one cross and no seedlings of pure *B. resinifera* available, we should be cautious in drawing any generalizations from this result.

Tetraploid and pentaploid *B. pubescens* and *B. papyrifera*, respectively, form another group of birch species that can be distinguished from the previous diploid groups. When we inoculated these birch species with *B. pendula* rust, very variable results were obtained. The seedlings of *B. pubescens* in both environments were not infected with the rust isolate derived from *B. pendula*, which was in agreement with earlier inoculation experiments (POTERI 1992). Greenhouse-grown *B. papyrifera* was susceptible to the *B. pendula* rust type. However, the striking immunity of the outdoor-grown seedlings of *B. papyrifera* to the *B. pendula* rust isolate was evident, as the uredinial frequencies on the leaf discs were counted under a stereomicroscope, where even minimum-sized rust pustules could be observed. The rust isolated from *B. pubescens* had clearly a broader host range than the *B. pendula* rust, which has also been reported previously (KLEBAHN 1904; POTERI 1992).

Fertilization and subsequent high nitrogen content in host plants generally increase fungal infections. For example, the leaf nitrogen content and the susceptibility to poplar leaf rust correlated positively with the greenhouse-grown poplars (SUZUKI 1973). In our experiments there was more nitrogen, protein and rubisco in the leaves of the outdoor-grown seedlings, but outdoor-grown seedlings were generally no more susceptible than the seedlings grown indoors. Moreover, the susceptibility of the greenhouse or outdoor-grown birch types was not related to the nitrogen level of the leaves.

The host recognition and the prehaustorial stage is sensitive to the anatomical differences of host leaves in rust infections (FERREIRA and RIJKENBERG 1991; RUBIALES and NIKS 1992). Consequently, structural resistance of leaves, like the thickness of leaf mesophyll layers, lignification of cell walls, number of stomata, etc., is modified by the growing environment. The effect of the environment on the leaf structure is probably species and genotype specific. This is also indicated by the low correlations between AUDPC of the seedlings grown in the greenhouse and outdoors (Fig. 4).

Resistance of a plant is based on mechanical barriers, plant nutrient content or chemical defence. The resistance mechanism against birch rust is not known and may be based on a combination of diverse factors; thus, the changes in growth environment may have complicated effects on plant resistance. In this experiment, the white birch species used were adapted to very different growing conditions, such as the short Alaskan summer (*B. resinifera*) or the long, hot and humid Lake States growing period (*B. papyrifera*). Not surprisingly, environment strongly modified the resistance of these exotic birch species and the effect was species specific (Fig. 1, Table 1). Growth environment also had a strong influence on the resistance of both *B. pendula* and *B. pubescens*, which were growing in a climate they had adapted to (Fig. 1, Table 1). On the basis of our results it is evident that no simple factor, such as leaf nitrogen content alone, can explain the susceptibility of birch species to leaf rust. Our results suggest the need for caution in interpreting the results of pathogenicity studies made using only one rust type and one genotype of host species adapted to different growing conditions.

Acknowledgements

The authors thank Arja PENNANEN for commenting on the manuscript and Nancy SEIDEL for linguistic corrections. Prof. Daniel A. HERMS is acknowledged for supplying the seeds of *B. papyrifera* and *B. resinifera*, and Prof. Marjatta RAUDASKOSKI for supplying the scanner and software to analyse rubisco gels. The study was supported by the Ministry of Agriculture and Forestry in Finland.

Résumé

Différences de résistance à la rouille de plusieurs espèces de bouleau élevées en conditions de serre ou d'extérieur

Des semis de deux ans de cinq espèces de bouleau (*Betula platyphylla, B. papyrifera, B. pubescens, B. pendula* et *B. resinifera* × *B. pendula*) élevés soit en serre soit à l'extérieur, ont été utilisés pour un essai d'inoculation sur disques foliaires. Deux isolats de *Melampsoridium betulinum* ont été utilisés, obtenus de *B. pendula* et de *B. pubescens*. La résistance de ces espèces de bouleau variait significativement. La résistance à l'isolat issu de *B. pubescens* n'était pas liée à la résistance à l'autre isolat. Le comportement d'un génotype donné de bouleau élevé en serre ne correspondait pas non plus à celui du même génotype élevé à l'extérieur. Les conditions de l'extérieur augmentaient beaucoup le contenu en protéines, rubisco, chlorophylle et azote des feuilles des espèces diploïdes (*B. platyphylla, B. pendula* et *B. resinifera* × *B. pendula*). Chez les espèces tétra- et pentaploïdes (*B. pubescens* et *B. papyrifera*, respectivement) il n'y avait pas de différences aussi nettes. Le rapport C:N était plus élevé chez toutes les espèces élevées en serre mais la différence n'était significative que chez les espèces diploïdes. La gravité de la rouille selon l'espèce de bouleau n'était corrélée avec aucun des paramètres physiologiques étudiés. L'adaptabilité des génotypes de bouleau à l'environnement en relation avec leur résistance à la rouille foliaire est discutée.

Zusammenfassung

Resistenzunterschiede von Weissbirkenarten gegenüber Birkenrost bei Gewächshaus- und Freilandkultur

Zweijährige Sämlinge von fünf Birkenarten (*Betula platyphylla, Betula papyrifera, Betula pubescens, Betula pendula* (zwei Typen) und *Betula resinifera* × *B. pendula*) aus Gewächshaus- und Freilandkultur wurden in einem Biotest mit Blattstücken mit zwei verschiedenen Birkenrostisolaten (*Melampsoridium betulinum*) von *B. pendula* und *B. pubescens* inokuliert. Die Resistenz dieser Birkenarten variierte signifikant. Die Resistenz gegenüber dem *B. pubescens*-Rostisolat stand in keiner Beziehung zu der Resistenz gegenüber dem *B. pendula*-Rostisolat. Ebenso stimmte das Verhalten derselben Birkengenotypen bei Freiland- und Gewächshauskultur nicht überein. Bei den diploiden Birkenarten (*B. platyphylla, B. pendula, B. resinifera* × *B. pendula*) waren unter Freilandbedingungen die Gehalte an löslichem Protein, Rubisco, Chlorophyll und Stickstoff in den Blättern deutlich erhöht. Bei den tetra- und pentaploiden Arten (*B. pubescens* bzw. *B. papyrifera*) waren keine solchen klaren Unterschiede im physiologischen Zustand der Blätter von Gewächshaus- und Freilandsämlingen zu beobachten. Das C/N Verhältnis war bei allen Birkenarten bei Gewächshauspflanzen grösser, Unterschiede liessen sich jedoch nur bei den diploiden Arten statistisch sichern. Die Rostanfälligkeit der Birkenarten war mit keinem der untersuchten physiologischen Parameter korreliert. Die Anpassungsfähigkeit der Birkengenotypen an unterschiedliche Umweltbedingungen wird in Bezug auf ihr Resistenzverhalten gegenüber *M. betulinum* diskutiert.

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00373 Blackwell XEJFP (BSL No. 96) Ms. 096 Typeset from Disk February 1998.

Eur. J. For. Path. 28 (1998) 000–000

ISSN 0300–1237

Pre-penetration behaviour of the urediniospores of birch rust, *Melampsoridium betulinum*, on greenhouse-grown and microcultured leaves of *Betula* spp.

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Summary

In two different experiments the urediniospores of *Melampsoridium betulinum* from *Betula pendula* and *Betula pubescens* germinated both on mature leaves of greenhouse-grown plants and on sterile *in vitro* leaves of micropropagated plantlets, which were cloned from the same *B. pendula* and *B. pubescens* genotypes. The urediniospores and germ tubes were more easily detached from the leaf surfaces of *in vitro* leaves. If germination took place on a leaf vein, the growth continued across the veinal ridges; otherwise, no determined growth towards the stomata could be observed with either of the leaf types studied. In both experiments on the *in vitro* leaves of *B. pubescens* clone V5944, the germ tubes of the rust isolate from *B. pendula* mislocated appressoria significantly more often than the germ tubes of the rust isolate from *B. pubescens*. On the mature leaves of *B. pendula* clone E4214 and *B. pubescens* clone V5940 there were also significant differences in appressorial locations between the two rust isolates but the clonal responses were inconsistent. The results of the inoculations suggest that the incompatibility of the rust isolates from *B. pendula* on the leaves of *B. pubescens* is not related to the significantly higher ratio of failures in locating appressoria in this host–rust combination.

1 Introduction

The urediniospores of the birch rust fungus, *Melampsoridium betulinum* (Fr.) Kleb., infect the leaves of the tree species in Betulaceae (Gäumann 1959). However, the susceptibility of different birch species to different birch rust isolates varies. In inoculation studies, various Finnish birch rust isolates originating from *Betula pendula* Roth have been shown to be incompatible on the leaves of *Betula pubescens* Ehrh. (Poteri 1992).

Stomata on the leaf surfaces serve as entrance pathways for the majority of the urediniospores of rust fungi. The processes that a spore germling undergoes before penetration into the stomatal cavity have been studied with the aid of light microscopy and various scanning electron microscopy (SEM) techniques. Spore adhesion onto the leaf cuticle is one of the first events in infection process (Kunoh et al. 1991). The tip of a germ tube is able to sense the topography of a leaf's epidermal layer and start appressorium formation after receiving a suitable height signal (Hoch et al. 1987; Allen et al. 1991). For some rust fungi, the height of the lips of the stomatal guard cells is the correct signal when germinating on a host species (Wynn 1976; Terhune et al. 1991). Appressorium formation is, however, a nonspecific reaction that can be induced by topographic signals other than stomatal ones. High proportions of failures in locating appressoria over stomata have been correlated with the rust resistance of *Gladiolus* sp. (Ferreira and Rijkenberg 1991) and *Hordeum chilense* lines (Rubiales and Niks 1992).

Received 26.8.1997; accepted 11.11.1997

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B. pendula and *B. pubescens* clones have been used in rust resistance studies (POTERI 1992). The micropropagation technique (RYYNÄNEN and RYYNÄNEN 1986) provides genotypically homogeneous plant material, which is advantageous when investigating the behaviour of rust isolates on different host species. In addition, the use of leaves from microcultures reduces the time and cost of screening. The rust infections of microcultured *in vitro* leaves of *B. pendula* and *B. pubescens* appear to correspond to those of mature leaves (M. POTERI, unpublished data). There is, however, no information on how the different leaf anatomy and surface properties of microcultured plants (SMITH et al. 1986; ZIV 1995) affect birch rust germination and infection.

The aims of this study were to investigate, with a scanning electron microscope, the prepenetration stages of the urediniospores of *M. betulinum* on the leaves of the clones of *B. pendula* and *B. pubescens* when using (1) two different rust isolates derived from *B. pendula* and from *B. pubescens*, and (2) two different leaf types: mature leaves from greenhouse-grown plants and *in vitro* leaves from microcultures. It was of special interest to confirm whether the incompatible rust isolate from *B. pendula* has more failures in locating appressoria when germinating on the leaves of *B. pubescens* than when germinating on the leaves of its host.

2 Materials and methods

Micropropagation of birch was started from dormant vegetative buds of selected genotypes. One of the *B. pendula* clones, E4214, was growing in a natural silver birch stand at Ristiina, in eastern Finland (61°24′N, 27°24′E; 90 m above sea level), and the other two *B. pendula* clones, E5387 and E5398, in a planted silver birch stand at Punkaharju, in eastern Finland (61°49′N, 29°18′E; 90 m above sea level). Both of the *B. pubescens* clones, V5940 and V5944, were growing in a progeny test at Somero, in central Finland (60°40′N, 23°34′E; 112 m above sea level). Cultivation was done according to RYYNÄNEN ~~(1996)~~ using woody plant medium WPM (WPM; LLOYD and MCCOWN 1980) with 4.4 µM 6-benzylaminopurine (BAP) as a growth regulator. The plantlets were then subcultured for 3 weeks in a half-strength MS medium (MURASHIGE and SKOOG 1962) containing 2.2 µM BAP and 2.85 µM indole-3-acetic acid (IAA) in order to obtain bigger leaves for inoculations.

The greenhouse plants for mature leaf material were produced according to RYYNÄNEN ~~(1996)~~. The *in vitro* shoots were rooted in WPM without any growth regulators. The rooted shoots were potted in peat–perlite (1:1), and the plants were grown for 2 weeks in propagators in decreasing relative air humidity before their transfer to the greenhouse. After about 4 weeks the plants were transferred ~~in~~ 2-l pots. The plants were fertilized during the growing season with 0.1% Superex-5 solution: N 10.9% (NO_3-N 9.1%), P 4%, K 25.3%, Mg 1.5% (Kekkilä™, Hyrylä, Finland).

The urediniospores of the two rust isolates were collected separately in 1991 from *B. pendula* and *B. pubescens* at the Suonenjoki Research Station (62°38′N, 27°04′E) and lyophilized for further usage. For the inoculation experiments the lyophilized urediniospores were multiplied on surface-sterilized leaf discs cut from the leaves of ordinary nursery seedlings. Urediniospores were harvested from 10–15-day-old uredinia. For the inoculation, a water suspension was prepared from freshly harvested urediniospores (8.6×10^4 to 5.5×10^5 spore/ml). The suspensions were spread with a brush on the lower surfaces of mature leaves of greenhouse-grown pot plants and the inoculated leaves were enclosed in a plastic bag to maintain the moisture. Two to three leaves of each clone were treated. The detached sterile *in vitro* leaves were placed with abaxial surfaces facing upwards in Petri dishes on water agar (7 g/l) and inoculated with a droplet of spore suspension which was spread on the leaves with a sterile glass rod. Eight to 10 *in vitro* leaves of each clone were plated and treated. The inoculated pot plants and *in vitro* leaves in Petri dishes were incubated in a growth chamber (Weiss™, Reiskirchen, Germany) at 18–20°C with a 16-h photoperiod (100 ~~mol/m^2 s~~). Two experiments were performed: experiment A in 1992 and experiment B in 1996.

The samples were collected 14–20 h after the inoculation. Pieces 5 mm × 5 mm, in the central part of the leaf, were cut from the mature leaves. The *in vitro* leaves were, in most cases, small enough to be fixed whole. Samples were fixed in 6% glutaraldehyde in 0.1 m sodium-phosphate buffer (pH 7.0) and dehydrated in ethanol. After critical point drying, using liquid CO_2 as a transition solvent, the specimens were fastened onto metal stubs and coated either with gold (Experiment A) or with platinum (Experiment B). To ensure objective evaluation, the samples were coded. A minimum of 33 germinated spores were recorded for each sample, excluding the spores that were found to germinate on the leaf veins. The samples were studied on a JEOL JSM-820 (Akishima, Tokyo, Japan) scanning electron microscope operating at an acceleration voltage of 5–8 kV.

Fourfold contingency tables were applied for the statistical analysis. The appressorial frequencies over stomata or failures were tested and a comparison made between the two rust isolates and the two leaf types by calculating a log-likelihood test value G^2.

3 Results

Because of the bizonate arrangement of the germ pores on the urediniospores of *M. betulinum*, germ tubes emerged from both ends of the spores. There were usually 4–6 germ pores initiating growth, but only 1–2 tubes elongated and formed a germ tube. The germ tubes were, in many cases, extensively branched. The growth of the germ tube depended on where the spore had been deposited. If germination took place on a leaf vein, the growth continued across the veinal ridges (Fig. 1a). No such pattern could be found in the interveinal regions and no clear orientation of germ tubes towards stomata was observed. Both of the birch species had hairs on the abaxial leaf surfaces, which changed the growth habit of the germ tubes; in some cases growth orientated upwards along the hair, or the appressorium formation was initiated close to the hair. The attachment of the germ tube was enabled by the release of extracellular matrix (EM), which was visible beneath the germinating spores and germ tubes. The EM was particularly evident in the vicinity of the germ tube tips (Fig. 1b). Both rust isolates produced EM independent of the host species used in the inoculation. Germinating spores also released EM on the *in vitro* leaves. However, on the leaf surface of the *in vitro* plantlets, the germlings appeared to be easily detachable (Fig. 1c,d).

Appressorium formation was initiated in different places on the leaf surface and not only over the stomata (Fig. 1e). The septum separating the appressorium from the germ tubes was sometimes visible (Fig. 1e). Appressoria had different shapes: in most cases they were cushion-like swellings of the tube (Fig. 1f). Appressorium formation also took place on *in vitro* leaves (Fig. 1f). In a few cases germ tubes grew into the stomata without forming any appressoria (Fig. 1h).

The proportions of stomatal appressoria on the mature leaves of *B. pendula* clones ranged from 63 to 74% with the *B. pendula* rust isolate and from 47 to 75% with the *B. pubescens* rust isolate. In experiment A (Fig. 2a), the *B. pendula* rust isolate formed significantly more stomatal appressoria than the *B. pubescens* rust isolate on the mature leaves of the *B. pendula* clone E4214. The proportions of stomatal appressoria on the mature leaves of *B. pubescens* clones ranged from 40 to 71% with the *B. pendula* rust isolate and from 38 to 80% with the *B. pubescens* rust isolate. The frequencies in the appressorium locations with the two different rust isolates were similar on the mature leaves of *B. pubescens* clones in experiment A. In experiment B, with *B. pubescens* clone V5940, the *B. pubescens* rust isolate had significantly more stomatal appressoria than the *B. pendula* rust isolate (Fig. 2a).

The proportions of stomatal appressoria on the *in vitro* leaves of *B. pendula* clones ranged from 62 to 76% with the *B. pendula* rust isolate and from 60 to 81% with the *B. pubescens* rust isolate. No significant differences in the appressorial ratios between the two rust isolates

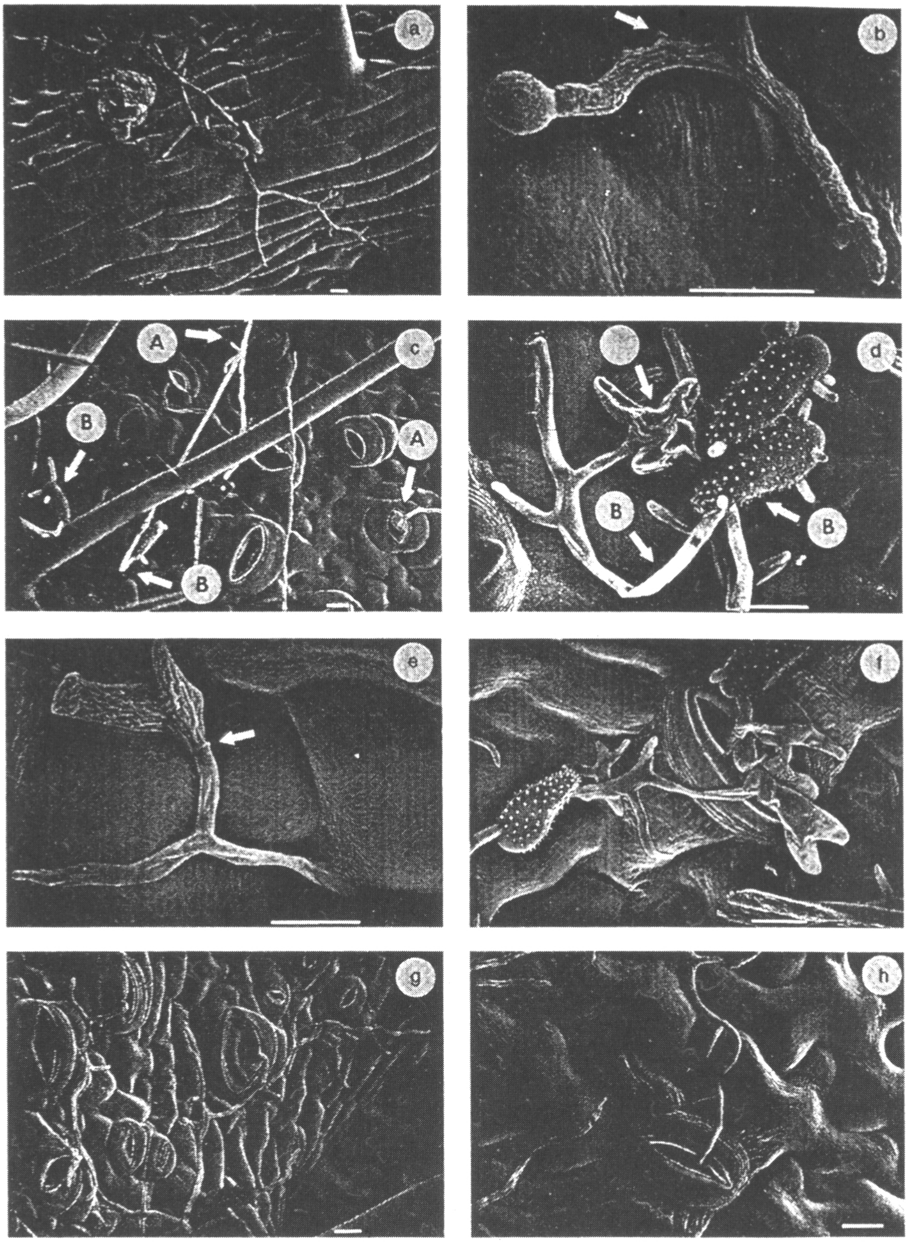
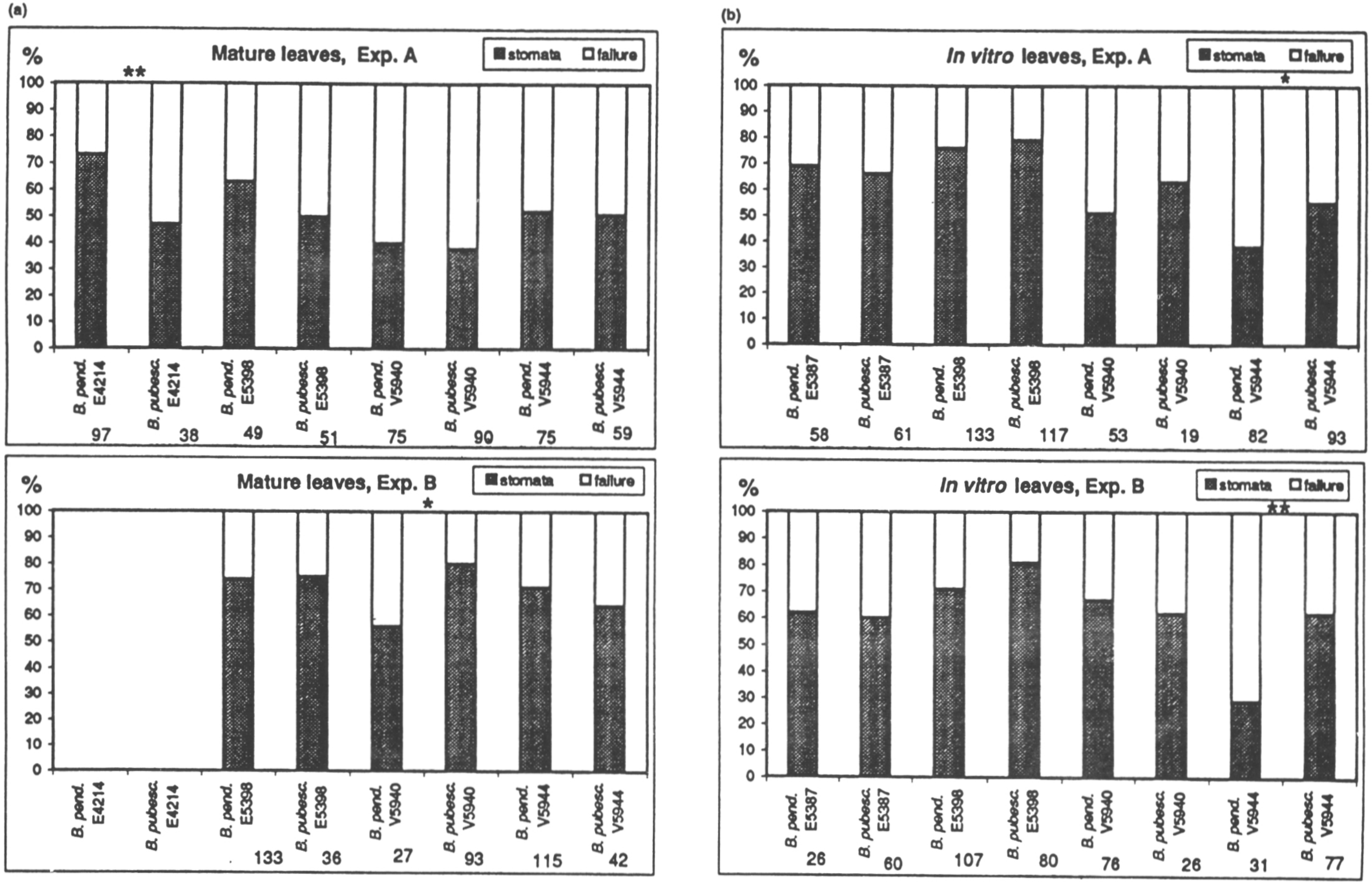


Fig. 1. Scanning electron micrographs of the pre-penetration stages of the urediniospores and germ tubes of ***Melampsoridium betulinum***. Bars = 10 µm. a. Germ tubes of the ***Betula pubescens*** rust isolate grow across the veins on the mature leaf of ***B. pubescens*** clone V5944. b. The tip of the germ tube of the ***B. pubescens*** rust isolate attached with extracellular matrix (EM) onto the leaf cuticle of the mature leaf of ***B. pendula*** clone E4214 (arrow). c,d. Detached germ tubes of the ***B. pendula*** and ***B. pubescens*** rust isolates on the ***in vitro*** leaf surfaces of ***B. pubescens*** clone V5940 and ***B. pendula*** clone E5387, respectively; appressoria formation (arrows A) and detached germlings and germ tubes (arrows B). e. A mislocated appressorium of the ***B. pubescens*** rust isolate on the mature leaf of ***B. pubescens*** clone V5940; appressorium separated from the germ tube by a septum (arrow). f. Appressoria formed over stomata by the rust isolate from ***B. pubescens*** on the mature leaf of ***B. pubescens*** clone V5940. g. Germ tubes and appressoria of the ***B. pendula*** rust isolate on the ***in vitro*** leaf of ***B. pendula*** clone E5387. h. The germ tube of the ***B. pubescens*** rust isolate grown into the stoma without forming an appressorium on the mature leaf of ***B. pubescens*** clone 5944



** $G^2 = 7.87$ $(X^2_{0.01\,(1)} = 6.635)$
* $G^2 = 5.84$ $(X^2_{0.05\,(1)} = 3.841)$

* $G^2 = 5.11$ $(X^2_{0.05\,(1)} = 3.841)$
** $G^2 = 10.03$ $(X^2_{0.01\,(1)} = 6.635)$

Fig. 2. Percentages of the appressoria formed over stomata or failures with the mature leaves (a) and *in vitro* leaves (b) of ***Betula pendula*** and ***B. pubescens*** clones in ***Melampsoridium betulinum*** inoculation experiments A and B. Significant differences in appressorial ratios between the *B. pendula* and *B. pubescens* rust isolates are indicated by an asterisk. The total number of appressoria is indicated separately below the columns for each rust–clone interaction

were found with the *B. pendula* clones in experiments A and B. The proportions of stomatal appressoria on the *in vitro* leaves of *B. pubescens* clones ranged from 29 to 67% with the *B. pendula* rust isolate and from 55 to 63% with the *B. pubescens* rust isolate. In both experiments A and B (Fig. 2b) the *B. pubescens* rust isolate had significantly more stomatal appressoria than the rust isolate from *B. pendula* with the *B. pubescens* clone V5944. The two rust isolates did not differ in their appressorium formation patterns on the *in vitro* leaves of the other *B. pubescens* clone, V5940, and the stomatal frequencies in appressorium formation with both rust isolates were also higher than with clone V5944.

In half of the combinations studied, the *B. pendula* rust formed more appressoria over stomata on the mature leaves than on the *in vitro* leaves. In experiment B (Fig. 3a) the *B. pendula* rust isolate had significantly more stomatal appressoria on the mature leaves than on the *in vitro* leaves of clone V5944. Conversely, the *B. pubescens* rust isolate formed more appressoria over stomata on the *in vitro* leaves than on the mature leaves (Fig. 3b). The *B. pubescens* rust isolate had significantly more stomatal appressoria on the *in vitro* leaves than on the mature leaves of the *B. pubescens* clone V5940 and the *B. pendula* clone E5398 in experiments A and B, respectively (Fig. 2b).

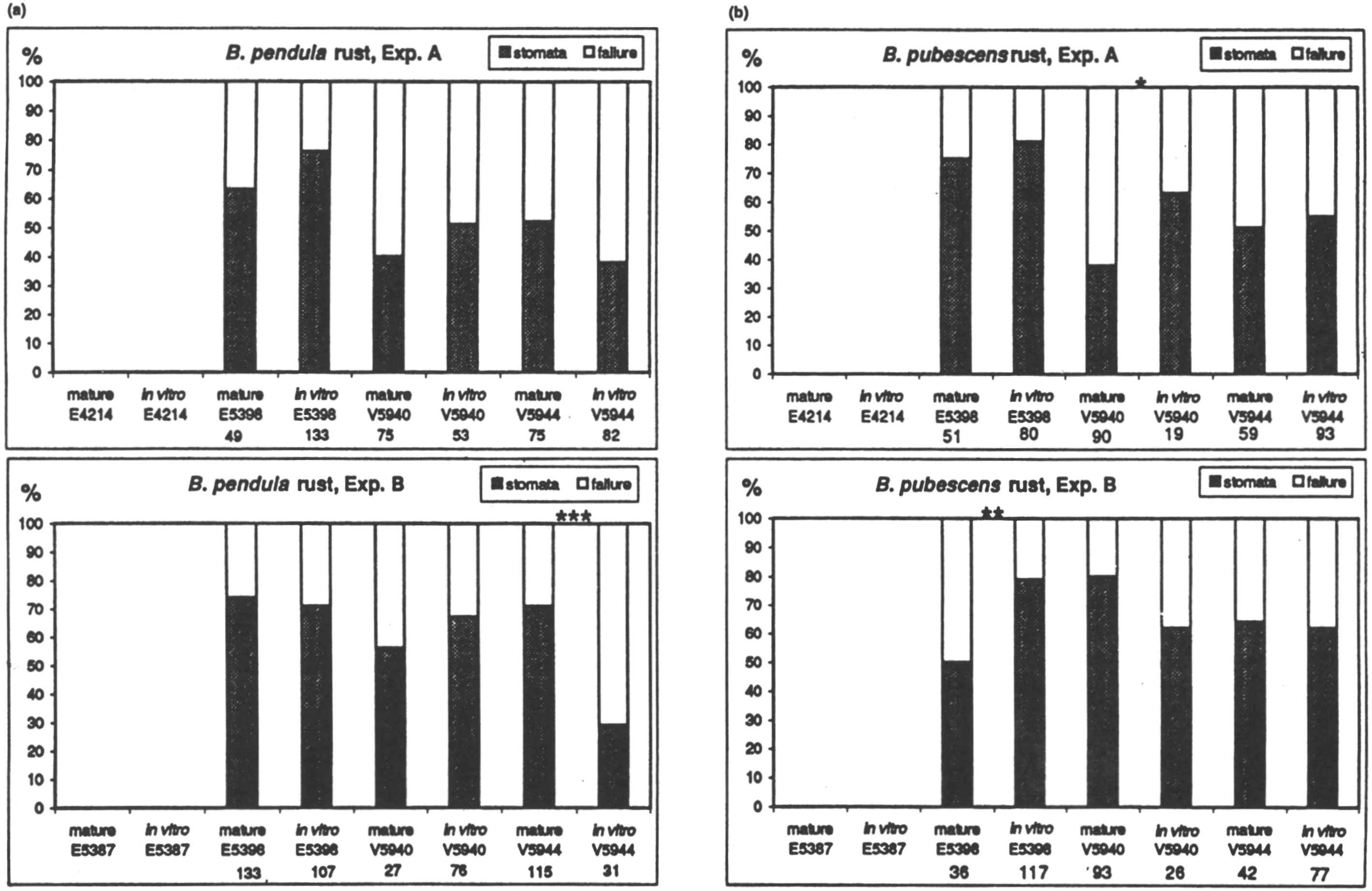
4 Discussion

The germ tubes of *M. betulinum* grew in a determined perpendicular pattern across the veins when germination took place on the leaf veins. The same type of directional growth of the urediniospores of *Puccinia graminis* f. sp. *tritici* on gramineous plants and those of *Puccinia helianthi* on the leaves of *Helianthus annuus* was first reported by Johnson (1934). No such regular pattern was observed between the veins in the epidermal area of *Helianthus* leaves; neither could we observe any signs of directional growth of germ tubes towards the stomata among the birch–rust interactions studied. However, in some host–rust interactions germ tube growth has been observed to occur towards stomata, e.g. in relation to the pH gradients in *Uromyces viciae-fabae* infections (Edwards and Bowling 1986) or in relation to the changes in volatile compounds, such as carbon dioxide near the stomata in *P. graminis* f. sp. *tritici* inoculations (Yirgou and Caldwell 1963).

With the aid of SEM techniques, remnants of some adhesive material on the leaf epidermis were visible, especially in the tips of the germ tubes of *M. betulinum* and, in some cases, on the surfaces of spore groups. Studies using low-temperature scanning electron microscopy (LTSEM) have shown that the secretion of a specific extracellular matrix (EM) also occurs with rust fungi. The matrix was released through the germ pores of *U. viciae-fabae*, thus also surrounding the emerging germ tubes and the tips of the tube (Beckett et al. 1990). Adhesion pads formed below the attached urediniospores of *U. viciae-fabae* had enzymatic activities towards the host cuticle layer (Deising et al. 1991).

A spore germling removal similar to this birch rust study with the *in vitro* leaves has been reported by Wynn and Staples (1981) using *Puccinia sorghi* urediniospores germinated on waxless corn leaves. An explanation for this may be the poor contact of urediniospores with the epidermal layer. Urediniospores are firmly attached onto hydrophobic surfaces, which, in plants, are supplied by cuticular waxes on the leaf epidermis. This cuticle formation is reduced on the leaves of microcultured *in vitro* plantlets (Ziv 1995). However, it is probable that most of the spore detachments in our experiments were artefacts, caused by the treatments required for the SEM. We believe that the only difference in handling the mature and *in vitro* leaf material, that is, the inoculation of the *in vitro* leaves as detached instead of intacts, has no effect on the results. According to Wynn (1976) appressoria formation over stomata did not vary between intact and excised bean leaves inoculated with bean rust.

In this study, both *B. pendula* and *B. pubescens* rust isolates could recognize and form appressoria on the mature leaves of both birch species, although *B. pubescens*, because of its tetraploidy, has bigger cells and stomata than diploid *B. pendula* (Kujala 1946). Abnormal



*** $G^2 = 18.22$ ($X^2_{0.001\,(1)} = 10.828$)

* $G^2 = 4.1$ ($X^2_{0.05\,(1)} = 3.841$)

** $G^2 = 10.44$ ($X^2_{0.01\,(1)} = 6.635$)

Fig. 3. Percentages of the appressoria formed over stomata or failures with the *Betula pendula* rust isolate (a) and *B. pubescens* rust isolate (b) of *B. pendula* and *B. pubescens* clones in *Melampsoridium betulinum* inoculation experiments A and B. Significant differences in appressorial ratios between the mature and *in vitro* leaves are indicated by an asterisk. The total number of appressoria is indicated separately below the columns for each leaf type–clone interaction

excessive growth and a more raised structure of the stomata of microcultured plantlets have been reported with woody plant species (Reuther 1988; Blanke and Belcher 1989). Moreover, in this study the stomata of the microcultured *in vitro* leaves differed from those of the mature leaves of birches. However, the *in vitro* leaves of birch were suitable for *M. betulinum* germination and pre-penetration. The appressoria formation was not disturbed on *in vitro* leaves and these leaves could provide even higher appressorial frequencies over stomata compared with the results obtained with mature birch leaves.

According to these inoculation experiments, it can be concluded that failures in locating appressoria over stomata is probably not the major factor in the incompatibility between *B. pubescens* leaves and the rust isolates derived from *B. pendula* (Poteri 1992). In these experiments failed appressoria did not dominate in the interactions between *B. pendula* rust and *B. pubescens* clones. Thus, the incompatibility of the rust isolates from *B. pendula* on the leaves of *B. pubescens* (Poteri 1992) is most probably caused by the processes in the post-penetration stage of the rust fungus.

Acknowledgements

The authors acknowledge the Department of Electron Microscopy at the University of Helsinki and Professor Timo Kurkela's comments on the manuscript. The language content was revised by Nancy Seidel. The study was funded by the Ministry of Agriculture and Forestry in Finland.

Résumé

Pré-pénétration des urédiospores de Melampsoridium betulinum sur des feuilles de Betula spp. obtenues en serre ou en culture in vitro

Dans deux expériences différentes, les urédiospores de *Melampsoridium betulinum* obtenues sur *Betula pendula* et sur *Betula pubescens*, germaient aussi bien sur des feuilles adultes de plants élevés en serre que sur des feuilles stériles de vitroplants, qui étaient des clones des mêmes génotypes de *B. pendula* et *B. pubescens*. Les urédiospores et les tubes germinatifs étaient plus facilement détachables des feuilles des vitroplants. Si la germination avait lieu sur une nervure, la croissance continuait perpendiculairement aux rides les rides de la nervure; dans les autres cas, aucune croissance orientée vers les stomates n'a été observée chez aucun des deux types de feuilles. Dans les deux expériences, les tubes germinatifs de l'isolat issu de *B. pendula* plaçaient mal leurs appressoria significativement plus souvent que ceux de *B. pubescens* sur les vitrofeuilles de *B. pubescens*, clone V5944. Sur les feuilles adultes de *B. pendula*, clone E4214, et de *B. pubescens*, clone V5940, il y avait aussi des différences significatives entre les deux isolats de rouille pour la position des appressorias, mais la réponse clonale n'était pas constante. Les résultats des inoculations suggèrent que l'inadaptation des isolats de *B. pendula* aux feuilles de *B. pubescens* n'est pas liée au taux significativement plus élevé d'échec de localisation des appressoria chez cette combinaison hôte-isolat.

Zusammenfassung

Verhalten der Uredosporen des Birkenrostes (Melampsoridium betulinum) vor der Infektion auf Blättern von verschiedenen Birkenarten (Betula spp.) im Gewächshaus und in vitro

In zwei unterschiedlichen Experimenten keimten die Uredosporen von *Melampsoridium betulinum* (Isolate von *Betula pendula* und *Betula pubescens*) auf ausgereiften Blättern von Gewächshauspflanzen sowie auf sterilen Blättern von *in vitro* vermehrten Pflanzen der gleichen Genotypen von *B. pendula* und *B. pubescens*. Die Uredosporen und ihre Keimhyphen hafteten auf der Oberfläche von *in vitro* gebildeten Blättern weniger stark. Falls eine Spore auf einer Blattader keimte, wuchs der Keimschlauch querzu dieser; ansonsten wurde, unabhängig vom Blattyp, kein gerichtetes Wachstum zu den Stomata beobachtet. In beiden Experimenten waren auf den *in vitro* gebildeten Blättern des *B. pubescens* Klons V5944 die Appressorien des Pilzisolates von *B. pendula* signifikant häufiger falsch plaziert als die des Pilzisolates aus *P. pubescens*. Auf ausgereiften Blättern *des B. pendula* -Klons E4214 und des *B*

pubescens -Klons V5940 wurden ebenfalls signifikante Unterschiede in der Lokalisation der Appressorien durch die beiden Rostisolate nachgewiesen, aber auf den klonierten Pflanzen *in vitro* waren die Ergebnisse widersprüchlich. Die Ergebnisse zeigen, dass die Inkompatibilität der Rostisolate von *B. pendula* mit Blättern von *B. pubescens* nicht mit der signifikant häufigeren falschen Plazierung der Appressorien in dieser Wirt-Rost-Kombination erklärt werden kann.

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V

Effect of *Betula pendula* clone and leaf age on *Melampsoridium betulinum* rust infection in a field trial

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Summary

Ten five-year-old *Betula pendula* clones were studied for their rust resistance in the field. Trees were treated by inoculating ten leaves in a shoot with *Melampsoridium betulinum* urediniospore suspension or spraying the leaves with water. The birch clones differed significantly in their resistance to *M. betulinum* leaf rust fungus and the clones also varied in their responses to the local rust strain and the inoculum rust strains. However, natural rust infections and inoculation treatment were positively correlated. The older leaves had fewer infections than the younger ones on the tip of the shoot in the control trees, but in the inoculation treatment no significant correlation was found between the leaf ages and rust infection. The factors behind the different leaf susceptibilities are discussed.

Key words: *Betula pendula* - Birch - Clones - *Melampsoridium betulinum* - Rust

1. Introduction

Leaf rust fungus, *Melampsoridium betulinum* (Fr.) Kleb, infects and colonizes the green leaves of a host plant. During this epidemic stage it is able to generate several urediniospore cycles before leaf fall at the end of the growing season. Weather conditions, such as temperature and moisture, control the rate of rust infections. For example, different temperature regimes may affect compatibility of rust races (Prakash and Heather 1986, Prakash and Thielges 1989), and external moisture, which especially condenses on the leaf surfaces in late summer, is essential for the urediniospore germination (Beckett et al. 1990, Hamelin et al. 1992). The genotypes of both pathogen and host plant also have a strong effect on rust infections (Flor 1956, Vanderplank 1984). Birch genotypes vary in their rust resistance when exposed to natural rust infections (Kechel and Böden 1984, Helander et al. 1997) and experimental rust inoculations using *Betula pendula* Roth clones have revealed that birch genotypes differ in their rust resistance both in leaf disc assays and field inoculations (Poteri 1992, Poteri and Rousi 1996).

The progress of the rust infection in a plant is also dependent on the age or ontogeny of the susceptible plant tissue. In agriculture, resistance to cereal rusts can be divided into seedling and matured plants resistance (Parlevliet and van Ommeren 1975). Among broad-leaved trees the effect of growth stages of shoots and leaves on the rust infections have been recognized, too. Upper leaves in the young shoots of *Populus* spp. were more susceptible to *Melampsora larici- populina* Kleb. infections than the leaves of the same phenological stage in the older shoots (Sharma et al. 1980). In addition, once the fungus has infected the host plant, the age of the colonized plant tissue is likely to negatively affect both uredinia and urediniospore production. For example, uredinia production by *M. medusae* Thüm. was significantly affected by leaf age among *P. deltoides* Bartr. clones (Coleman et al. 1987) and in oaks, *Quercus* spp., younger leaves produced more urediniospores in the inoculation experiments with *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Kuhlman 1987).

The aims of this study were 1) to evaluate variation in birch rust resistance among ten Finnish *B. pendula* clones under field conditions 2) to determine the effect of leaf age on the rust susceptibility of the birch clones and 3) to compare the responses of the birch clones to different birch rust isolates.

2. Material and methods

2.1. Field trial

Ten clones of three year old *B. pendula* plantlets were transplanted to a field in the Turku University Botanical Garden (60° 26' N, 22° 10' E) SW-Finland in June 1994. The clones were micropropagated (Jokinen et al. 1991) from mother trees growing in southern Finland (Table 1). By the time of this experiment the plants were five years old, 1.5 - 2.5 m in height and from here on we refer to them as trees. The trees were grouped into 15 randomized blocks of 20 trees (two trees of each clone; 300 trees together), with a c. 1.5 meter distance between trees. Within each of the 15 blocks one of the two trees from each clone was randomly assigned as control or rust inoculation treatment. During the hot summer days the field was irrigated and hay was cut between the trees.

The urediniospores for the inoculation were collected during the summer of 1996 from rusted *B. pendula* trees from two locations in Southern Finland, Jaala (61° 03' N, 26° 30' E) and Viikki (60°13' N, 25° 01' E). The spores were dried and stored in a desiccator at + 5 °C. For the inoculum equal amounts of both rust isolates were suspended in distilled water amended with Tween-20 (0.02 %). The spore concentration was adjusted high: 1.8×10^6 spores ml^{-1}. For control treatments distilled water with Tween-20 was used.

The inoculation was made in the evening of August 6, 1996. One shoot from each tree was chosen for treatment. To examine the effect of leaf age on rust infection, 10 leaves from the shoot tips were treated by spraying the abaxial surfaces of birch leaves with spore suspension (inoculation treatment) or Tween-20 water (control) until water was dripping. After the spraying, the shoots were enclosed in plastic bags until the next morning.

The rust infections were examined five weeks after the treatment which corresponds to approximately 2-3 urediniospore cycles in a natural environment. By the observation date both the colonization and sporulation abilities on different birch clones were possible to detect. Infections were scored as the cover percentage of uredinia on the abaxial side of the leaf. Reference leaves from which cover percentage (using 5 % intervals) of uredinia had been measured were used to standardize the estimates.

2.2. Statistical analyses

Analysis of variance was used to test if the rust inoculated trees had more infections than the control trees and whether the birch clones differ in their resistance to birch rust. Clone*treatment -interaction was added to the model to determine if there were differences in the treatment effects among the clones. Arcsin-transformation was used to normalize the data. Spearman's rank correlation was applied to test the effect of leaf age on rust infection level separately on both rust inoculated and naturally infected trees. All figures give the untransformed values. Statistical analysis were performed with the SAS Statistical Package (SAS Institute Inc. 1989).

3. Results and Discussion

Birch clones differed significantly in their rust resistance (Fig. 1, Table 2). Natural infection levels varied between 0.2 - 5.4 %, being lowest in clones A, B, C and F, which indicates their high resistance against local rust strains. Inoculation treatment increased infection levels to 6.0 and 25.3 % but the clones did not show similar resistance patterns to introduced rust strains compared to the resistance patterns to natural infection (Fig. 1). Only clones C and F clearly showed low infection levels when inoculated with the urediniospore suspension (Fig. 1). The ratio of the inoculation treatment to the natural infection varied from three (clone J) to 60 fold (clone A) leading to a significant interaction between host genotype and rust infection (Fig. 1, Table 2). Despite this interaction, the natural rust infections in the control trees correlated positively with the infections obtained in the inoculations when using clone specific means ($r_s = 0.6626$, $n = 10$, $p<0.0368$). This indicates differences in both natural and inoculated rust infection levels among clones but a similar tendency of susceptibility of each clone to both infection types. Clonal differences in resistance against different rust races may partly explain observed within clone variation against natural and inoculated rust infections. The variation in susceptibility between the birch clones may indicate genotypic differences in leaf physiology, e.g. in the photosynthesis activity (Pääkkönen et al. 1996) and leaf aging (Ridge et al. 1986).

To test the effect of leaf age on infection frequencies, infection rate was determined as the coverage of uredinia on the ten consecutive leaves from the shoot tip. There was a tendency towards negative correlations between the leaf ages and rust infections with both inoculated and naturally infected trees. However, among the inoculated trees only two clones had significantly fewer infections in the older leaves and moreover, a significant positive correlation between the leaf age and rust infection was found with the inoculated clone A (Table 3). Among the naturally infected trees three clones had significantly fewer infections in the older leaves (Table 3). The more diverse responses of the inoculated leaves was also evident as, with the pooled clonal means, the negative correlation between leaf ages and infection rates was significant only with the natural rust infections (Table 3). The infection rates within the studied ten leaves formed a bell-shaped curve with five of the ten inoculated clones (clones A, D, E, H and J) (Fig. 2b). With the control leaves only clone H had a clear bell-shaped pattern in the rust infections within the ten leaves. Higher resistance to leaf rust among the youngest and oldest leaves in a shoot has been reported with the leaf discs of *P. deltoides* inoculated with *M. medusae* (Coleman et al. 1987).

The variation in birch rust susceptibility between the ten studied leaves within an inoculated clone may reflect differences in the leaf photosynthesis activity and carbohydrate content as the growth and sporulation of rust fungi is highly dependent on the availability of free sugars in the plant tissue (Manners 1993, Mendgen 1981). Birch leaves in a shoot most probably behave like willow leaves which have their photosynthetic rate at a lower level in the aging and youngest leaves as compared to the fully expanded middle leaves of a shoot (Vapaavuori and Vuorinen 1989). In this study the birch leaves ranked as the oldest had already started their senescence process as the rust inoculation and scoring was performed at the end of the growing season, in August-September. Colonization of the poplar leaf by rust fungi can be restricted by inhibitory compounds and anatomical barriers (Siwecki et al. 1982). These compounds and barriers are more prevalent in the older leaves which is in accordance with the observed negative correlations between leaf age and infection rates with majority of the birch clones studied.

Experimental trees were subjected to at least three different geographical rust strains because, in addition to the local rust race, the treatment inoculum consisted of two geographical rust isolates which originated 150 and 250 km apart from the field location. Geographical rust isolates of *Melampsoridium* have differed in their pathogenicity to *B. pendula* clones in leaf assay screenings (Poteri and Ryynänen 1995). Thus, less variation and potentially stronger significances in rust resistance among birch clones might have been found in this field trial if only one rust isolate was used in the inoculum. Clone A had the most contrasting responses to the different rust strains. It was highly susceptible to the inoculation treatment, but very resistant to the local rust (Fig. 2). Clone A was also the only clone that had a significant positive correlation between leaf age and rust infection with the inoculated rust strains (Table 3). The introduced rust isolates may also have infected some of the controls, because

2-3 urediniospore generations had already been developed in the inoculated leaves by the observation date.

The rust susceptibility of the leaves in woody plants is a complex phenomenon which can be affected by several different factors. The infection rates of the leaves may depend on shoot age, not only on the age of individual leaves (Sharma et al. 1980). In addition, the genotypic adaptation of a tree to different environments affects the chemical composition and structure of leaves. This genotypic variation occurring between the host plants has importance that should also be taken into account in rust resistance studies of trees.

Acknowledgments

Mrs. Marja-Leena Lahtinen and Mr. Jouko Närhi assisted with the field work. The linguistic correction was made by Margot Stout Whiting. The study was funded by the Academy of Finland and The Finnish Konkordialiitto.

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Table 1. The origin of the Finnish *B. pendula* clones used for the *Melampsoridium* inoculation in a field trial in Turku 1996.

Code letter	ID Number	Provenance
A	V59562	Valkeakoski (-)
B	0154	Punkaharju x Punkaharju (61° 48' N, 29° 19' E x 61° 48' N, 29° 18' E)
C	V5818	Loppi x Rautalampi (60° 37' N, 24° 26' E x 62° 45' N, 26° 45' E)
D	V50080	Keuruu (62° 07' N, 24° 45' E)
E	V5920	Varkaus (62° 19' N, 27° 55' E)
F	V50079	Keuruu (62° 07' N, 24° 45' E)
G	K1659	Eno (62° 48' N, 30° 05' E)
H	K1898	Pielavesi (63° 18' N, 26° 47' E)
I	K1932	Pielavesi (63° 18' N, 26° 47' E)
J	E9661	Kihniö (62° 08' N, 23° 18' E)

Table 2. Results of analysis of variance on effects of clone, rust infection and clone*inoculation interaction on rust infection percentage of the birch leaves.

	df	MS	F	p
Block	14	0.0157	1.84	0.0331
Clone	9	0.1694	19.94	0.0001
Infection	1	4.5873	539.75	0.0001
Clone*inoculation	9	0.0391	4.60	0.0001

Table 3. Spearman rank correlation coeffients (r_s) between rust infections and leaf ages on ten birch clones. Differences are significant at the 5 % (*) and 1 % (**) level.

Clone (n)	A (10)	B (10)	C (10)	D (10)	E (10)	F (10)	G (10)	H (10)	I (10)	J (10)	Pooled (10)
Inoculation	0.6 *	-0.7939 **	-0.8328 **	-0.4316	-0.5030	-0.4620	-0.1879	-0.1152	-0.3576	-0.0909	-0.4667
Natural infection	-0.6278*	-0.7768**	-0.3119	-0.1094	-0.4802	-0.4137	-0.6121*	-0.5636	0.5106	0.0308	-0.7212*

LEGENDS FOR FIGURES

Fig. 1. Natural rust infection levels and *M. betulinum* inoculation effects on ten *B. pendula* clones.

Fig. 2. Classification of natural rust infection levels (a) and *M. betulinum* inoculation (b) effects on different leaf ages (1 = youngest, 10 = oldest) on ten *B. pendula* clones.

FIG. 1

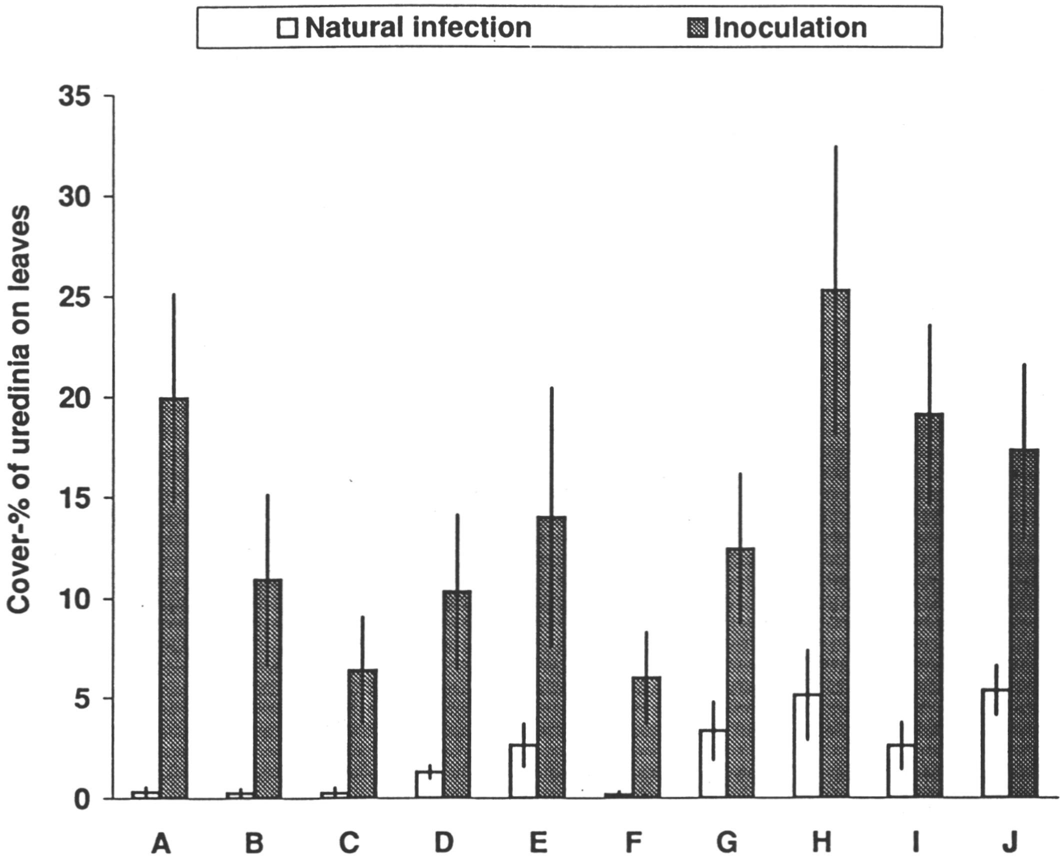

□ Natural infection
▨ Inoculation
Cover-% of uredinia on leaves
35
30
25
20
15
10
5
0
A
B
C
D
E
F
G
H
I
J

FIG. 2A

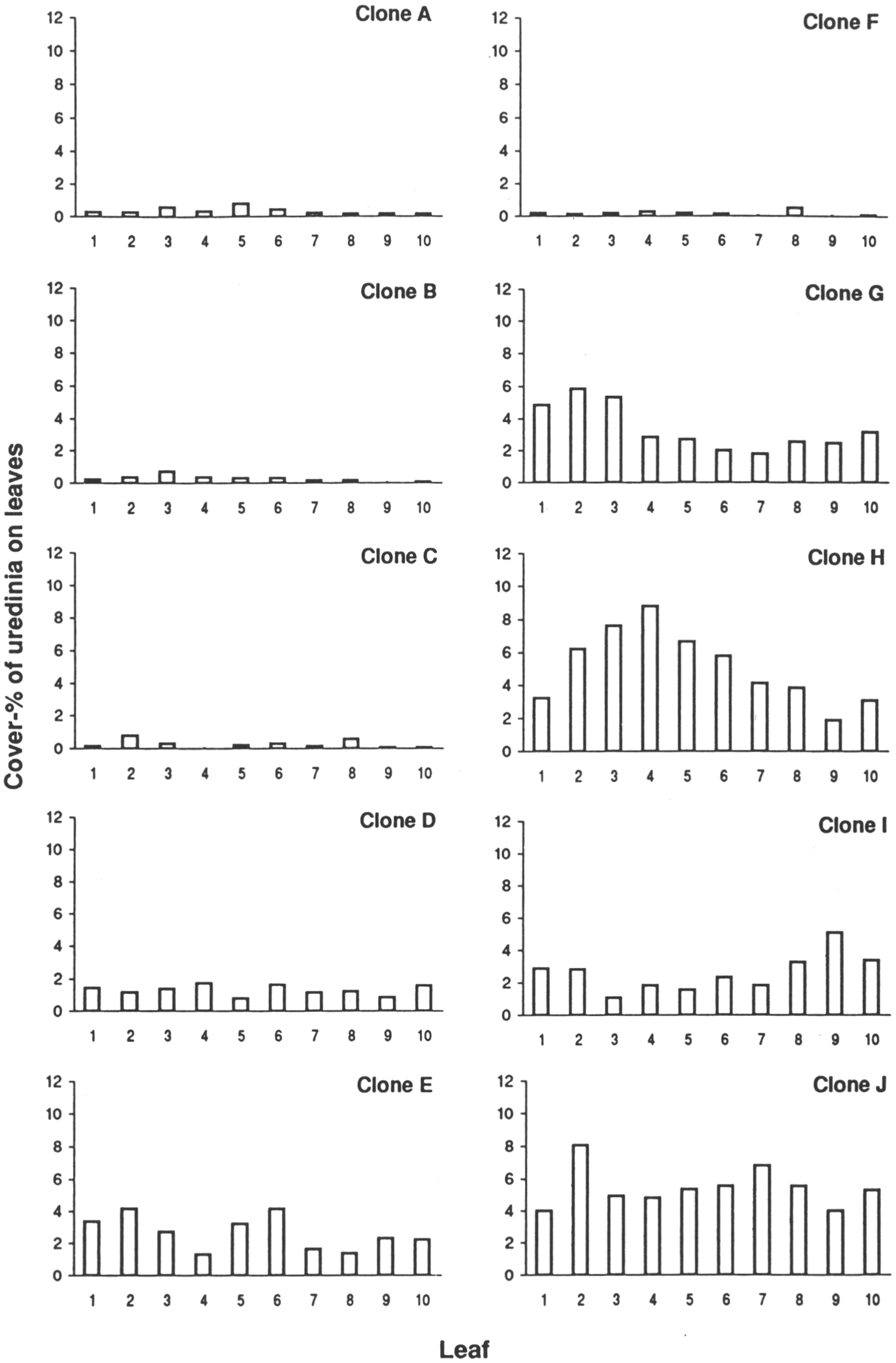
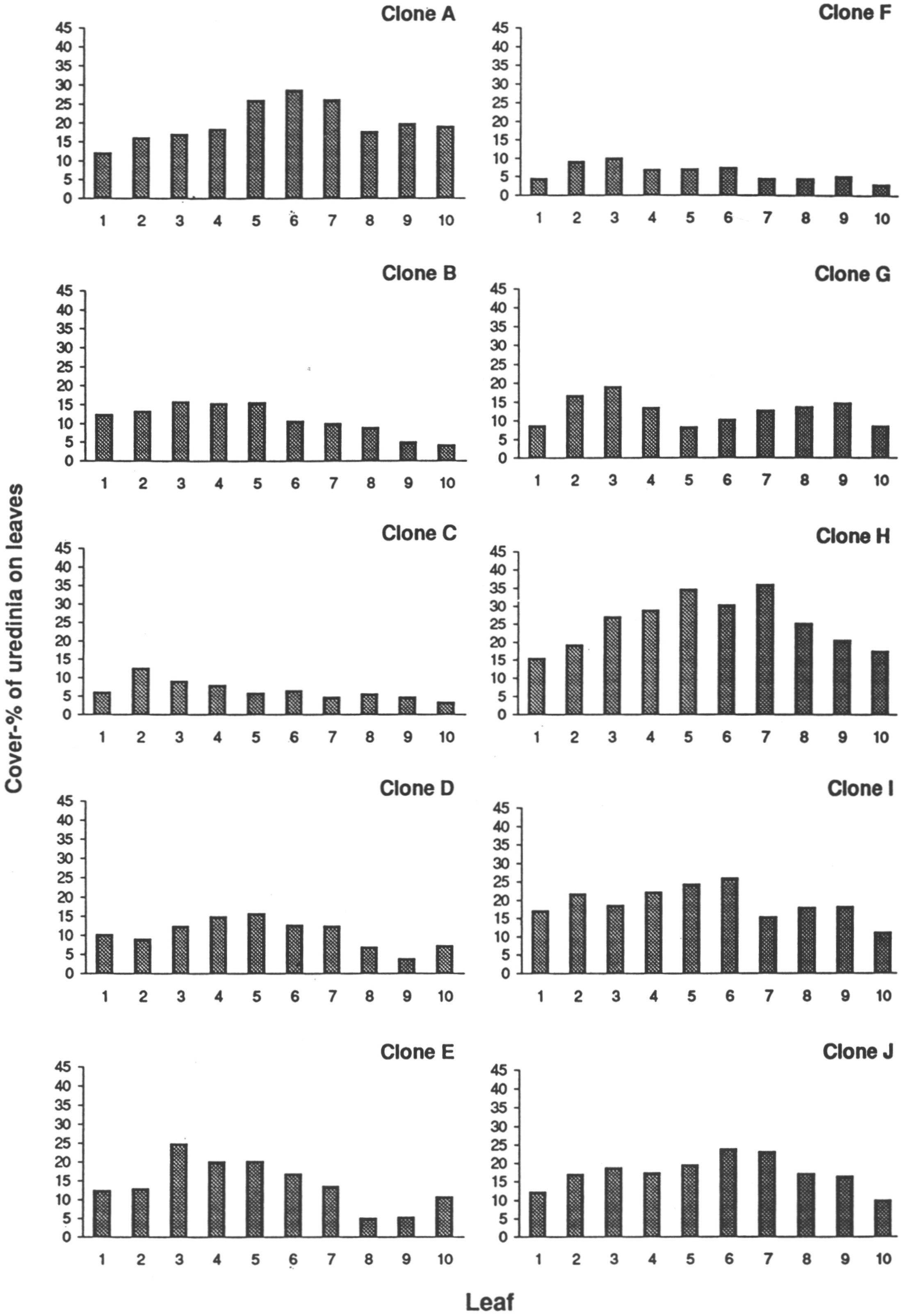

Cover-% of uredinia on leaves
Clone A
Clone B
Clone C
Clone D
Clone E
Clone F
Clone G
Clone H
Clone I
Clone J
0
2
4
6
8
10
12
1
2
3
4
5
6
7
8
9
10
Leaf

FIG. 2B


Clone A
Clone F
Clone B
Clone G
Clone C
Clone H
Clone D
Clone I
Clone E
Clone J
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Cover-% of uredinia on leaves
Leaf

ISBN 951-40-1630-0
ISSN 0358-4283