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Academic Dissertation in Forest Pathology

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Abstract

A disease referred to as root dieback has caused considerable losses in nursery production in Finland and Norway. Both containerized and bare-root seedlings of Scots pine and Norway spruce are affected by the disease. Wilting of young shoots, drooping tops, retarded height growth and discoloration of needles are the shoot symptoms observed in diseased seedlings in nurseries. Previous studies indicated that the disease could be of complex nature involving both *Rhizoctonia*, particularly uninucleate isolates, and *Pythium*. The aim of this thesis was to further characterize *Rhizoctonia* species associated with diseased seedlings.

In a case study, intensive isolations from roots of diseased Norway spruce seedlings showed that several *Rhizoctonia* species can be co-existing in the same root system. On the basis of cultural morphology, hyphal nuclear condition and anastomosis tests, the obtained isolates could be divided into 5 species: uninucleate *Rhizoctonia* sp. and four distinct binucleate *Rhizoctonia* species. One of these binucleate *Rhizoctonia* species anastomosed with the tester isolate of AG-I of genus *Ceratobasidium*. The remaining binucleate species could not be connected with any anastomosis group of this genus and further work applying various fruiting techniques is needed for their characterization. Under aseptic conditions binucleate *Rhizoctonia* infected only cortical cells in basal root regions; accordingly, the root growth of seedlings inoculated with binucleate *Rhizoctonia* was unaffected indicating that these species are not directly involved with the disease.

Hyphal and cultural morphology and anastomosis tests confirmed that the *Rhizoctonia* isolates considered most pathogenic in preceding studies, and originating both from Finland and Norway, represent the same species which is characterized by almost uniform cultural morphology, uninucleate nuclear condition and possession of a single anastomosis group. In RAPD-PCR analysis, the isolates showed over 75–80 % similarity coefficients further confirming the cultural observations. A new method involving the use of aseptically grown host seedlings was developed for fruiting. The method worked with all the tested hosts: Scots pine, Norway spruce and Siberian larch. On the basis of basidial characteristics, the uninucleate *Rhizoctonia* sp. belongs to genus *Ceratobasidium*, which has previously been regarded as a genus having only binucleate anamorphs. The basidial characteristics of the uninucleate *Rhizoctonia* sp. fit into the morphological species concept of *C. bicorne*, but further comparison (nuclear condition, hyphal anastomosis) was not possible since this species, described parasitizing a moss in Denmark, has apparently never been cultured. Considering the different nuclear condition, uniformity in cultural conditions and

possession of a teleomorph for which no anamorph state has been described, there is only one conclusion to draw; a new *Rhizoctonia* species should be described for these uninucleate isolates.

No differences were observed in the mode of infection on the three hosts. In the tip region, where protoxylem elements had not yet differentiated, proliferating hyphae formed aggregations on the root surface and within the intercellular spaces of the outer cortex. This gave rise to penetration hyphae that invaded the apical meristem and neighbouring vascular cylinder. Only actively growing roots were infected by the fungus; root exudates are suggested to induce the hyphal proliferation preceding the formation of penetration hyphae. Both under sterile and non-sterile conditions, the uninucleate *Rhizoctonia* sp. attacked particularly the tips of primary roots and long laterals. In pathogenicity tests, inoculation of pine and spruce seedlings with the uninucleate *Rhizoctonia* sp. resulted always in a considerably stunted and characteristic root system morphology whereas larch seedlings were less affected; possible differences in seasonal root growth patterns could account for this fact. It is possible that the broad shoot symptom list presented above may reflect differences in the causal pathogen. A systematic survey and comparison of the root system morphology and mycoflora of seedlings showing varying shoot symptoms is recommended to critically evaluate this proposal.

Contents

List of Publications

This thesis is based on the following articles, which in the text will be referred to by their Roman numerals.

I Hietala, A.M., Sen, R. & Lilja, A. 1994. Anamorphic and teleomorphic characteristics of a uninucleate *Rhizoctonia* sp. isolated from the roots of nursery grown conifer seedlings. Mycological Research 98:1044-1050.

II Hietala, A.M. 1995. Uni- and binucleate *Rhizoctonia* spp. coexisting in the roots of Norway spruce seedlings suffering from root dieback. European Journal of Forest Pathology 25:136-144.

III Lilja, A., Hietala, A.M. & Karjalainen, R. 1996. Identification of a uninucleate *Rhizoctonia* sp. by pathogenicity, hyphal anastomosis and RAPD analysis. Plant Pathology 45: 997-1006.

IV Hietala, A.M. 1997. The mode of infection of a pathogenic uninucleate *Rhizoctonia* sp. in conifer seedling roots. Canadian Journal of Forest Research 27: 471-480.

V Hietala, A.M. & Sen, R. 1996: *Rhizoctonia* associated with forest trees. In: B. Sneh, S. Jabaji-Hare, S. Neate & G. Dijst (eds.). *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology and disease control (pp. 351-358). Kluwer Academic Publishers, Dordrecht, Netherlands.

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1 Introduction

1.1. Root dieback disease in Nordic countries

1.1.1. Norway

The first serious root disease incidents on conifer seedlings in forest nurseries of the Nordic countries were observed in the 1970's in Norway (Sandvik 1985), where the disease had mainly affected containerized Norway spruce (*Picea abies* (L.) Karst.) seedlings. The term "root dieback" was adopted in association with shoot symptoms including wilting of young shoots, drooping tops, retarded height growth and discoloration of the foliage (Galaaen & Venn 1979). Root systems of diseased seedlings were partially or totally dead, characteristically very pigmented and had few, if any, non-pigmented root tips compared to healthy seedlings (Venn 1985). Losses have been considerable, resulting in a 4–5 % reduction in the production value of Norwegian forest nurseries, and the disease has been regarded as the most serious and persistent problem in production of containerized stock (Børja and Austara 1990).

Surveys for fungi associated with root dieback and subsequent pathogenicity experiments suggested that the disease could be of a complex nature involving several pathogens. In the first study (Galaaen and Venn 1979), *Pythium sylvaticum* Campbell & Hendrix was found to be prevalent on the roots of diseased 2-year-old Norway spruce seedlings, and based on a pathogenicity test using young germinants of Norway spruce under aseptic conditions, the fungus was suggested to be involved in the disease. Other associated fungi showing pathogenicity in that study included *Botrytis cinerea* Pers. ex Fr., *Trichoderma viride* Pers. ex Fr. and *Fusarium avenaceum* (Fr.) Sacc. Later Norwegian studies showed that *Pythium* spp. prevailed on the roots of diseased seedlings (Venn et al. 1986). In order to study the interaction of growth media, fungicides and pathogens associated with root dieback, Venn et al. (1986) set up extensive experiments where 10-week-old Norway spruce seedlings were inoculated with pathogens under nursery conditions. The pathogen candidates, a *Pythium* sp. and a *Rhizoctonia* sp., had been chosen on the basis of an unpublished experiment, where these two isolates had been highly pathogenic. Inoculations with the *Rhizoctonia* sp. resulted in considerably stunted shoot growth with symptoms of needle discolouration. Inoculations with the *Pythium* sp. showed a somewhat different effect: a small percentage of seedlings were killed whereas the surviving ones were only slightly stunted in shoot growth. Shoot wilting, drooping tops,

root-system morphology or the level of root damage were not reported for either pathogen (Venn et al. 1986).

1.1.2. Sweden

Death of roots of nursery-grown containerized conifer seedlings showing stunted shoot growth and needle discoloration has also interested researchers in Sweden (Unestam et al. 1989; Unestam and Beyer-Ericson 1990). Isolated fungi included *Pythium* spp., *Cylindrocarpon destructans* (Zins.) Scholten, *Alternaria alternata* (Fr.) Keissler, *Ulocladium atrum* Preuss and *Botrytis cinerea*. Unestam et al. (1989) chose *C. destructans* as a model pathogen to study it's behaviour in roots of stressed Scots pine (*Pinus sylvestris* L.) seedlings; low light conditions, anaerobic root environment and fungicide treatment were found to predispose seedlings to invasion by this pathogen.

1.1.3. Finland

In Finland, the first report of seedlings showing shoot wilting and drooping, needle discolouration and death of the root system is from a nursery in northern Finland (Jalkanen 1985). Later studies on a root disease of conifer seedlings in forest nurseries in southern and central Finland report a somewhat different list of symptoms: needle discolouration, stunted growth and partial or total root death (Lilja et al. 1992). In Finland, the term "root dieback" was adopted in association with the latter list of symptoms (Lilja et al. 1992). Both containerized and bare-rooted seedlings of Norway spruce and also Scots pine are being affected by the disease (Lilja et al. 1992). No estimates are available on the economical losses due to root damage of conifer seedlings in Finland, but based on the nursery inspectors' annual reports covering sampled nurseries, losses due to unspecified root rot can be very considerable in individual nurseries; in certain years in some nurseries, even 50 % of spruce or pine seedlings in inspected seedling lots have been discarded due to a disease classified as "root rot" (unpublished reports by the nursery inspectors of the Ministery of Agriculture and Forestry).

Compared to Norway and Sweden, the Finnish surveys of fungi associated with root dieback disease have been more intensive. The relatively high isolation frequencies of a uninucleate *Rhizoctonia* sp., and of *Pythium* spp. and *Phytophthora undulata* (H.E. Petersen) M.W. Dick from diseased seedlings and their pathogenicity in tests suggested that these fungi are involved in the root dieback disease (Lilja et al. 1992).

1.2. Characterization of *Rhizoctonia* isolates

Species of *Rhizoctonia* are ubiquitous soil inhabiting fungi. Many species are associated with worldwide diseases on important crop plants, such as cereals, cotton, sugarbeet and potato. Besides pathogens, the genus includes also saprophytes and orchid-associated mycorrhizal fungi (see Sneh et al. 1991). A lot of taxonomic confusion has been related to the genus over the years. The purpose of the following review is to critically evaluate the past and present approaches taken in classification of these species.

1.2.1. Morphological taxonomy: a historical review

The genus *Rhizoctonia* was established in 1815 by de Candolle to accommodate a non-sporulating root rotting fungus, *Rhizoctonia crocorum* (Pers.) DC. The basic characters of the genus set forth by de Candolle were quite liberal: formation of sclerotia with uniform texture and the association of hyphae with roots of living plants (the name *Rhizoctonia* originates from Greek and means "death of roots").

Like in other fungal genera, also in *Rhizoctonia* the taxonomy has been based on morphological characters and host species involved. In the absence of specific characteristics, nearly 100 species have been assigned to the genus (see Andersen & Stalpers 1994). Over the years, it became very clear that these *Rhizoctonia* species formed a heterogeneous group of fungi which were not closely related to each other. In addition, many of the species assigned to the genus did not even fit in the original frame set by de Candolle in 1815: some of these species had sclerotia which consisted of a rind and a medulla and some others were not associated with roots at all. It is perhaps a common misunderstanding among "non-Rhizoctonists" to regard *Rhizoctonia* species as fungi without a perfect state. *Rhizoctonia* species are not easily induced to fruit under laboratory conditions, and under natural conditions their inconspicuous fruitbodies are easily overlooked. During 1960's, observations on the indigenous or laboratory induced teleomorphs revealed that species designated as *Rhizoctonia* contained both ascomycetes and basidiomycetes (e.g. Warcup & Talbot 1966).

First observations on the nuclear condition of *Rhizoctonia* isolates were made over 70 years ago (Müller 1924). Parmeter at al. (1967) were the first to compare the nuclear condition of vegetative cells and the perfect state of *Rhizoctonia* isolates. They found that isolates having a *Thanatephorus cucumeris* (Frank) Donk perfect state always had multinucleate vegetative cells whereas other isolates with teleomorph characteristics of the genus *Ceratobasidium* were binucleate. This discovery that *Rhizoctonia* isolates can be divided into two groups, multinucleate or binucleate, based on nuclear condition of vegetative

cells became a corner stone in laying *Rhizoctonia* characterization on a more solid ground. Parmeter et al. (1967) also pointed out that the vegetative characteristics of these isolates are often indistinguishable if nuclear condition is not determined. This work casts a dark shadow over species identifications in earlier reports.

That work of Parmeter at al. (1967) resulted in a revision of taxonomic characteristics required for identification of the most studied *Rhizoctonia* species, *R. solani* Kühn (Parmeter and Whitney 1970). Later, these characteristics were expanded to cover the whole genus (Ogoshi 1975). As a result, the genus *Rhizoctonia* was now proposed to include basidiomycetous imperfect fungi having the following characteristics: (a) branching near the distal septum of cells in young, vegetative hyphae; (b) formation of a septum in the branch near the point of origin; (c) constriction of the branch; (d) dolipore septum; (e) sclerotium not differentiated into rind and medulla; (f) absence of clamp connections, conidia and rhizomorphs. Since species of *Ascomycota* have simple pores in septa without a dolipore structure, these criteria restrict the genus to *Basidiomycota*. This has resulted in the exclusion of ascomycetous species from the genus (e.g. Sneh et al. 1991). Sneh et al. (1991) and Andersen and Stalpers (1994) have published checklists for *Rhizoctonia* including information on the species excluded from the genus, taxa that are considered to be doubtful due to poor descriptions, and synonomy.

Of the morphological characters, the teleomorph is undoubtedly the most important single character, but due to the difficulty in fruiting these species under laboratory conditions, the isolate characterization is normally based on the vegetative stage. Vegetative characteristics such as mycelial colour, hyphal diameter, number of nuclei, length of cells, shape and size of monilioid cells and sclerotial size, have generally been used in the characterization of *Rhizoctonia* species. As pointed out by Andersen (1990), care is needed in the evaluation, as most of these characteristics vary considerably with temperature, light and composition of media. For example, hyphae have been reported to swell up to eight times their original diameter in a concentrated substrate (Burgeff 1936). The cell length, once thought to be of diagnostic value, was demonstrated in the measurements of Andersen (1990) to be of no value in characterizing taxa in *Rhizoctonia*. The dimensions of monilioid cells, criteria used by e.g. Saksena and Vaartaja (1961) when describing new species of *Rhizoctonia*, have been shown (Butler & Bracker 1970) to be of limited taxonomic value due to their high variability and dependence on the medium. On the basis of the structure of parenthesomes in dolipore septa, teleomorphic genera having *Rhizoctonia* anamorphs can be divided into two groups (Moore 1996). Determination of cell nuclear condition is a very useful first check when characterizing *Rhizoctonia* isolates; excluding this feature, *R. solani* and several other species belonging to genus *Ceratobasidium* are indistinguishable (Parmeter & Whitney 1970; Burbee et al. 1980). Lately, testing of anastomosis group and

the development of DNA based protocols have provided valuable tools for evaluation of traditional morphological taxonomy (see further text).

1.2.2. Morphological taxonomy vs. modern concept

1.2.2.1. Genus *Thanatephorus*

This genus contains several teleomorph taxa, but an anamorph state is known only for *Thanatephorus cucumeris* (anamorph *R. solani*) (see Sneh et al. 1991). Most of the teleomorphs with an unknown anamorph state are associated with orchids (see Andersen & Rasmussen 1996). Considering its broad host range, ubiquitous nature as a soil inhabitant and common association with some of the most important agricultural crops, it is only logical that of the species described as *Rhizoctonia*, *R. solani* has received the most attention. Again, the original description of the species (Kühn 1858) was very vague causing a lot of confusion. The work of Duggar (1915) considerably clarified the taxonomy of *R. solani*, but the final breakthrough came only after Parmeter et al. (1967) compared the nuclear condition and teleomorphs of *Rhizoctonia* isolates. As a result, the taxonomic criteria required for the identification of *R. solani* were modified to the presently accepted concept (Parmeter and Whitney 1970). The specific criteria include the multinucleate condition of vegetative cells, production of brown pigments in culture, usually rapid growth rate and hyphae greater than 5 μm in diameter. The perfect state of *R. solani*, *Thanatephorus cucumeris*, belongs to family *Ceratobasidiaceae (*order *Ceratobasidiales)* of class *Basidiomycetes* (Hawksworth et al. 1995) and it has the following key characteristics: barrel-shaped to subcylindrical metabasidia, not urniform nor constricted about the middle and little wider than the supporting hyphae. The stout, usually straight sterigmata vary in number per basidium (4(2-7)) and in length but are usually at least as long as the metabasidia when mature. Germination of basidiospore by repetition is common (Talbot 1965). Studies using transmission electron microscopy (TEM) have shown that the parenthesomes in the dolipore septum are perforate in *R. solani* (Moore 1996).

From the observations that this fungus causes numerous diseases on a very broad host range, its cultural variability on media, etc, *R. solani* has long been thought to contain many intraspecific groups and there have been many attempts to divide the species into rational groups, indeed. The first report on the occurrence of hyphal anastomosis reactions between *Rhizoctonia* isolates dates back several decades (Matsumoto et al. 1932). Since this pioneer work , several researchers have used hyphal anastomosis reactions as a basis for grouping *Rhizoctonia solani* isolates (e.g. Schultz 1936; Richter and Schneider 1953; Parmeter et al. 1969) but the meaning and usefulness of this kind of grouping remained unknown for a long period. Of the numerous approaches taken in the

classification of *R. solani* isolates, the method using hyphal anastomosis has by far been the most succesful.

Isolates of *Rhizoctonia* are assigned to anastomosis groups (AGs) by pairing them with tester strains and observing the hyphal interactions. Several systems have been used for pairing: a) water agar in Petri dishes (e.g. Parmeter et al. 1969); b) cellophane placed on agar media (e.g. Parmeter et al. 1969); c) agar-coated slides or cover slips (e.g. Tu et al. 1969); d) bare objective slides (Kronland and Stanghellini 1988). The interactions of overlapping mycelia are examined under a light microscope. The hyphal interactions can be divided into four categories (Carling 1996). The reaction type often described as "perfect fusion" involves cytoplasmic fusion between the opposing hyphae and does not result in cell death. This is a typical reaction in a self-pairing. Excluding pairings between isolates originating from the same field, perfect fusion is a very rare event in nonself pairings. The term "imperfect fusion" or "killing reaction" refers to a reaction where the anastomosing cells and adjacent cells die after the cell wall fusion and no cytoplasmic connection is produced. Imperfect fusion is the typical interaction type between closely related isolates that belong to the same anastomosis group. In the third hyphal interaction type, the anastomosis reaction is characterized by apparent cell wall contact between the opposing hyphae but only occasionally one or both anastomosing cells and adjacent cells die. This type of anastomosis can be observed between distantly related isolates of the same anastomosis group. If the opposing hyphae just grow past each other without interaction, the isolates do not belong to the same anastomosis group (a negative reaction). Using this categorization, most of the isolates of R. *solani* can be easily placed into a certain AG. However, the existence of bridging isolates can sometimes make the reading of anastomosis tests difficult; the bridging isolates are isolates that are able to anastomose with isolates from more than one AG. In intergroup anastomosis, the interaction type is usually the same as observed between distantly related isolates of the same anastomosis group (Carling 1996).

The genetic background of anastomosis behaviour in *R. solani* has not been studied, but a somatic incompatibility system has been offered as an explanation for the phenomenon (e.g. Anderson 1982). The genetic basis of somatic incompatibility is also poorly understood in other basidiomycetes, but like in *Heterobasidion annosum* (Fr.) Bref. (Hansen et al. 1993), the system could be controlled by several independent loci with probably multiple alleles at some loci (Adams 1996); for two heterokaryons to be somatically compatible, they would need to possess the same allele combination at these loci. Through meiosis and subsequent random mating of homokaryotic isolates, practically all heterokaryons, unless the nuclear pairs are closely related, will show differing allele combinations at these loci and are thus somatically incompatible. This would explain why the perfect fusion, if a somatic compatibility reaction, is so rare in anastomosis pairings between field isolates of *Rhizoctonia*. Following this reasoning, some researchers have used the anastomosis reactions as a tool

for mapping genotypes in a field and treated isolates producing a perfect fusion as a single clone (Ogoshi & Ui 1983; MacNish et al. 1993). Since related heterokaryons can show somatic compatibility, while still differing at other loci not regulating this feature, a term "vegetatively compatible population" has been proposed to describe *R. solani* isolates that produce the perfect fusion (MacNish & Carling 1995).

Presently, *Rhizoctonia solani* is divided into 12 anastomosis groups (AG 1 – AG11, AG BI) (Carling 1996). No isolates of AGs 1, 4, 5, 7, 9, or 10 are known to bridge with isolates of any other AG. At least some isolates of the remaining six AGs bridge with certain AGs (Carling 1996). Different AGs cannot be separated from each other on the basis of vegetative morphology. Excluding AG 4, all the AGs show identical teleomorph characteristics. Isolates belonging to this group average three sterigmata instead of the common four observed in other AGs (Ogoshi 1976). For this reason, AG 4 isolates have often (e.g. Sneh et al. 1991) been classified under another teleomorph, namely *Thanatephorus praticola* (Kotila) Flentje. In the most recent taxonomic review, *T. praticola* has not been recognized as a separate taxon (Stalpers & Andersen 1996).

Over the years, it became very clear that even the AG classification can be too general and most of the AGs have further been divided into subgroups, e.g. on the basis of colony morphology and pathogenicity anastomosis frequency with other members of the group, DNA homology and isozymes (see Sneh et al. 1991). Presently, there are still many geographic areas and host species where the information concerning the anastomosis grouping of associated isolates is lacking, but the available information indicates that the AGs and their subgroups show differences in host range or disease type. For example, isolates of AG 1 are mainly from the *Leguminaceae* and the *Graminaceae*, whereas most isolates of AG 3 are from the *Solanaceae* (Ogoshi 1987). As opposed to other groups, isolates of AGs 5, 6, 7, 9, 10 and BI are considered to be weakly pathogenic or non-pathogenic (Sneh et al. 1991).

The validity of anastomosis grouping and subgroup division has been examined in numerous studies using a variety of genetic and biochemical approaches. Supported by the results of studies based on DNA/DNA hybridization (Kuninaga & Yokosawa 1982ab; Kuninaga & Yokosawa 1983; Kuninaga & Yokosawa 1984ab; Kuninaga & Yokosawa 1985ab; Vilgalys 1988), restriction analysis of the ITS sequence (Liu & Sinclair 1993; Liu et al. 1993; Keijer et al. 1996) and isozymes (Liu et al. 1990; Liu & Sinclair 1992; MacNish & Sweetingham 1993), it has been concluded that not only are the AGs genetically different from each other but many of them can be broken into subgroups. Do the AGs or the subgroups represent independent biological species? The ultimate test would be a mating test and this would require homokaryotic isolates, but the difficulty in fruiting *Rhizoctonia* isolates under laboratory conditions has been a major obstacle. At least some AGs (subgroup 1C of AG 1 and AG 4) have isolates that belong to outcrossing populations

with a mating system controlled by a single locus with multiple alleles (Puhalla & Carter 1976; Adams & Butler 1982). Initial studies suggest that the mating system of AG 8 is also bipolar (Yang et al. 1992). In addition, several AGs (AG 1 through AG 4) are known to contain isolates that are capable of homokaryotic fruiting (see Adams 1996). Because of the general lack of information about mating or mating relationships in most AGs, the taxonomic position of the AG grouping remains open. However, supported by the accumulating evidence, it is logical to conclude that the AGs and the subgroups represent, if not biological species, at least diverging evolutionary units (Anderson 1982). Therefore, the convenient label *R. solani* cannot be regarded as a description sufficient by itself in studies and reports concerning this fungus; to be able to make valid comparisons between results obtained under variyng conditions (environment, host, etc.), information about the anastomosis and genetic relationships of *R. solani* isolates should be included in all studies. For this purpose, standardized tester strains representing different AGs of *R. solani* are available in culture collections (e.g. American Type Culture Collection).

1.2.2.2. Genus *Ceratobasidium*

Compared to *R. solani*, other *Rhizoctonia* species have received relatively little attention. Of these, the species belonging to genus *Ceratobasidium* are probably the most well-known. Morphological taxonomists consider this genus to be very closely related to the genus *Thanatephorus* (e.g. Stalpers & Andersen 1996). The teleomorph *Ceratobasidium* belongs to the family *Ceratobasidiaceae* (order *Ceratobasidiales*) of class *Basidiomycetes* (Hawksworth et al. 1995) with the major characteristics as follows: subglobose or obpyriform metabasidia which are 2 - 3 times the width of the supporting hyphae; sterigmata commonly four, sometimes fewer or more, about the same length as the metabasidia, sometimes forking; basidiospores germinate repetitively (Talbot 1965). Under TEM, the parenthesomes of the dolipore septum appear perforate like in *R. solani* (Moore 1996). In contrast to *R. solani*, the anamorphs of this genus have been regarded to be binucleate (Sneh et al. 1991). Excluding the nuclear condition, many of the anamorphs of *Ceratobasidium* are culturally indistinguishable from *R. solani*; they produce brown hyphal and sclerotial pigments in culture, have relatively wide hyphae (5 µm) and are able to grow rapidly (Parmeter & Whitney 1970; Burbee et al. 1980). In addition, this genus contains species that, instead of some shade of brown, have hyaline, white or yellow-white colonies that are thus easily separated from *R. solani*. Fourteen different *Ceratobasidium* species are recognized in the most recent taxonomical review (Stalpers & Andersen 1996); apparently, most of these species have never been cultured since an anamorph state is known only for five of these *Ceratobasidium* species (see Sneh et al. 1991). Many of the teleomorphs for which an anamorphic state is unknown are associated with orchids (see Andersen & Rasmussen 1996).

The anastomosis group concept has also been applied to this genus. Ogoshi et al. (1979) divided Japanese isolates having a *Ceratobasidium* fruiting state into 17 AGs (AG-A – AG-O). In USA, Burbee et al. (1980) applied anastomosis testing and divided the local strains into 7 anastomosis groups (CAG-1 – CAG-7). Later, the relation of these groupings was examined and they were combined since five of the anastomosis groups corresponded to each other (Ogoshi et al. 1983). Presently the genus is divided into 21 AGs (AG-A – AG-S) (see Sneh et al. 1991). Like *R. solani*, these fungi are associated with various diseases on numerous hosts. In addition, some of the AGs are regarded as non-pathogenic (see e.g. Sneh et al. 1991). Most of the AGs of *Ceratobasidium* have an anamorph that has not been identified at species level and is designated only as *Rhizoctonia* sp. In addition, nine taxonomic species are included in the genus (e.g. *R. endophytica* Saks. & Vaar., *R. callae* Saks. & Vaar., *R. fragariae* Husain & McKeen and *R. ramicola* Weber & Roberts) (Sneh et al. 1991). All four example species belong to anastomosis group AG-A (Ogoshi et al. 1983). Likewise, most of the AGs have a teleomorph designated as *Ceratobasidium* sp.; only 8 AGs have a teleomorph identified at the species level. It is also noteworthy that five different AGs have a teleomorph identified as *C. cornigerum* (Bourdot) Rogers (see e.g. Sneh et al. 1991). Using RFLP analysis of PCR-amplified rDNA, Cubeta et al. (1991) were able to separate 13 of the 21 AGs into distinct groups which were consistent with the anastomosis grouping. Examining isolates of 12 AGs with isozymes, Damaj et al. (1993) identified five different groups which corresponded well with those rDNA groups. Parmeter et al. (1967) were able to fruit single spore isolates of a *Ceratobasidium* sp. but this is practically all that is known about the sexuality of *Ceratobasidium* species. The four species designated as members of AG-A show differing cultural morphology (e.g. Sneh et al. 1991) and merit a status of distinct taxonomical species on this basis. The question whether these species show genetic differences has not been addressed in any study using genetic markers.

The facts that several AGs of *Ceratobasidium* are just designated as *Rhizoctonia* sp. with no specific morphological characteristics described and that some AGs share the same teleomorph (*Ceratobasidium cornigerum*) do not encourage the use of traditional morphological taxonomy in grouping binucleate *Rhizoctonia* isolates. Assessing the reports published on these fungi during the past 10–15 years, it seems clear that the traditional morphological characterization has been largely abandoned. Instead, after the determination of nuclear condition of isolates that morphologically fit into the genus concept, researchers go straight into anastomosis testing with tester strains of genera having a relevant nuclear condition. More information is clearly needed on the genetic relationships of the AGs of *Ceratobasidium*. In the mean time, encouraged by the observed genetic divergence among AGs of *R. solani*, it is also logical to treat the AGs as a basis for isolate characterization in this group when considering the morphological confusion related to these species.

1.2.2.3. Genus *Waitea*

This genus contains two *Rhizoctonia* species, *R. zeae* Voorhees and *R. oryzae* Ryker & Gooch. These species are associated with diseases on hosts like corn, rice and cereals (Sneh et al. 1991). Like *R. solani*, these species have a fast growth rate and relatively wide hyphae with multinucleate cells. In contrast, their sclerotia are either reddish (*R. zeae*) or salmon coloured (*R. oryzae*) and often covered with a gelatinous layer; neither of these characteristics can be observed in *R. solani* (Stalpers & Andersen 1996). The teleomorph of both anamorphs is *Waitea circinata* Warcup & Talbot. According to Hawksworth et al. (1995), this genus belongs to family *Botryobasidiaceae* of order *Tremellales*, whereas Moore (1996) regards it as a member of family *Ceratobasidiaceae* of order *Ceratobasidiales*. The key characteristics of the perfect state are: metabasidia suburniform; sterigmata small and horn-like, four in number, about one-fifth to one-quarter the length of the metabasidium, and non-repetitive basidiospores (Talbot 1965). Under TEM, the parenthesomes of the dolipore septum appear perforate like in *R. solani*. Isolates representing these two anamorphs do not anastomose with each other. The two anastomosis groups recognized for the anamorphs of *Waitea circinata* are designated as WAG-Z (*R. zeae* isolates) and WAG-O (*R. oryzae* isolates) (Sneh et al. 1991).

1.2.2.4. Genus *Tulasnella*

The genus *Tulasnella* belongs to family *Tulasnellaceae* of order *Tulasnellales* (Hawksworth et al. 1995). This genus is very rich in species, containing over 30 teleomorph taxa. The main characteristics of the teleomorph genus are: subglobose, pyriform or sphaeropedunculate metabasidia, often twice as wide as the supporting hyphae; sterigmata subglobose to broadly ellipsoid at the base; spores germinate by repetition (Stalpers & Andersen 1996). In contrast to the previous genera, the parenthesomes in the dolipore septum are imperforate or pauciperforate in this genus (Moore 1996). The described teleomorphs have been mostly associated with fallen trunks and branches of forest trees (e.g. Roberts 1992; 1993; 1994). In addition, some teleomorphs have been connected with orchids (e.g. Warcup & Talbot 1966; 1967; 1980). In contrast to the large number of teleomorphs, very few anamorphs have been described for these species. All the known anamorphs are associated with orchids (e.g. *R. repens* Bernard and *R. anaticula* Currah) (see e.g. Sneh et al. 1991). The most characteristic features of the vegetative state of these species include hyphae narrow in diameter (2 – 3.5 μm), binucleate cells and a very slow growth rate (3 mm/day) (Sneh et al. 1991).

1.2.2.5 Genus *Sebacina*

The teleomorph genus Sebacina belongs to the family *Exidiaceae* of order *Tremellales* (Hawksworth et al. 1995) and its most striking and characteristic single feature is the longitudinally septate metabasidia. Like in *Tulasnella*, the parenthesomes in the dolipore septum are imperforate or pauciperforate (Moore 1996). The anamorphs of *Sebacina* are very poorly known. A strain isolated from mycorrhizal rootlets of a conifer seedling and identified as *R. globularis* Saksena & Vaartaja (Saksena & Vaartaja 1960) (*R. globularis* is regarded as a *nomen ambiguum*, see Andersen & Stalpers 1994), has been fruited under laboratory conditions and it was identified as a *Sebacina* sp. (Warcup & Talbot 1966). Another verified case is orchid-associated (*Sebacina vermifera* Oberwinkler) (Warcup & Talbot 1967). The anamorphs of *Sebacina* resemble closely those of *Tulasnella*; they are slow growers, possibly binucleate in vegetative state (the nuclear condition has been reported only for the *R. globularis* isolate) and there is overlap in the hyphal diameter between these two genera (Currah et al. 1990; Sneh et al. 1991; Warcup & Talbot 1967).

1.2.3. *Rhizoctonia* taxonomy: conclusions and future prospects

Several basidiomycetous genera have anamorphs that fit into the present concept of *Rhizoctonia* but the difficulty in fruiting *Rhizoctonia* isolates under laboratory conditions has usually prevented the use of teleomorph characteristics in identification of the studied isolates. Therefore, it is understandable that the name *Rhizoctonia* has been almost exclusively used for these fungi, even if the perfect state was actually known. Since *Rhizoctonia* species do not form conidia, their vegetetative characteristics are quite limited in diagnostic features. As a result, many of the characteristics used in *Rhizoctonia* taxonomy overlap between different species.

The adoption of anastomosis grouping as a routine practice in *Rhizoctonia* studies has provided valuable information for evaluation of the taxonomic species concept. The application of genetic markers in association with anastomosis tests has further shown that the species identification based on vegetative characteristics of these fungi can be too general. As a result, the traditional taxonomy based on vegetative characteristics has largely been displaced by anastomosis tests. For example, isolates are now being characterized as "binucleate *Rhizoctonia* belonging to AG-E of genus *Ceratobasidium*" (see e.g. Runion & Kelley 1993). This tendency is very acceptable. Presently, the anastomosis groupings of *Rhizoctonia* isolates are based almost exclusively on Japanese and American populations and there are still large geographic areas and unexamined hosts where anastomosis testing has not been applied.

Therefore, new AGs are likely to arise even within the most well-known *Rhizoctonia* species, *R. solani*. This is also probably the reason why *Rhizoctonia* isolates failing to anastomose with testers of putative genera are continuously being reported. The only way to sort out this situation is to put increased efforts in fruiting these presently unassignable isolates. Sneh et al. (1991) have presented a summary of methods used for fruiting *Rhizoctonia* isolates.

The requirement for a dolipore septum in species acceptable as *Rhizoctonia* has created a nomenclatural problem; the type species of the genus, *R. crocorum* (teleomorph *Helicobasidium* (Desm.) has simple pores without the dolipore structure and would have to be excluded from the genus as proposed e.g. by Sneh et al. (1991). Unlike other teleomorphs of *Rhizoctonia*, the genus *Helicobasidium* does not belong to class *Basidiomycetes* but is included in class *Ustomycetes* (family *Platygloeaceae* of order *Platygloeales*) (Hawksworth et al. 1995). Taxonomically, it is not correct to exclude the type species from the genus and Moore (1987) has proposed that the name *Rhizoctonia* should be reserved for *R. crocorum* and related fungi which have septa with simple pores. He suggested that all the *Rhizoctonia* species possessing dolipore septa should be renamed according to their teleomorph genus. For example, anamorphs of genus *Thanatephorus* should be placed into genus *Moniliopsis* Ruhland. Because of the familiarity of plant pathologists with the name *Rhizoctonia*, this suggestion did not gain enthusiastic support. A compromise proposal has now been made to change the typification of *Rhizoctonia* by conservation and to adopt *R. solani* as the type species. This proposal has not yet been officially treated but its acceptance is anticipated (Stalpers and Andersen 1996). However, this proposal would restrict the name *Rhizoctonia* to anamorphs of the genus *Thanatephorus* and its acceptance will cause a nomenclatural chain reaction; the name *Rhizoctonia* in anamorphs of *Ceratobasidium*, *Waitea*, *Tulasnella* and *Sebacina* is proposed to be substituted with names *Ceratorhiza*, *Chrysorhiza*, *Epulorhiza* and *Opadorhiza*, respectively (Moore 1996). Considering the generic variability covered under the name *Rhizoctonia*, these nomenclatural proposals seem justified and hopefully will work to reduce the confusion that has surrounded these fungi ever since the genus *Rhizoctonia* was erected. On the other hand, what will those "*Rhizoctonia*" isolates unassignable in anastomosis tests and with an unknown perfect state be called?

2 Aims of the study

Under the heading "Root dieback disease in Nordic countries", I have tried to briefly summarize the knowledge available on the disease at the time I finished off my M.Sc. thesis (Hietala 1992). One of the encouraging results of that work was that the fungus found most pathogenic in Norwegian experiments (Venn et al. 1986), a *Rhizoctonia* sp., turned out to represent the same species found common and pathogenic in Finnish studies (Lilja et al. 1992). Even at that time, it was obvious that this *Rhizoctonia* species, besides being pathogenic, also had some interesting taxonomic characteristics that together could justify the following studies:

a) Characterization of *Rhizoctonia* species associated with root dieback
b) Mode of infection of associated *Rhizoctonia* spp.

The most comprehensive work on *Rhizoctonia* associated with trees was done almost 40 years ago (Saksena & Vaartaja 1960; 1961). Since that time the genus and species concepts of *Rhizoctonia* have evolved considerably. Therefore, substantial effort was required to determine the probable relationships of the isolates now under study. For this reason, the outcome of this literature review is included in the thesis (V).

3 Materials and methods

3.1. Characterization of *Rhizoctonia* isolates

The Finnish isolates used in the study were either obtained from the collections of Arja Lilja (Finnish Forest Research institute (I, III & IV) or isolated during the study process (II). In both cases, the isolates were obtained by placing root segments of nursery-grown conifer seedlings, surface-sterilized with 0.5 % NaOCl, onto distilled water agar (Lilja et al. 1992). The Norwegian reference isolates (I, III) were supplied from the collection of Kåre Venn (Norwegian Forest Research Institute).

To confirm that the isolates used in the studies (I, II) fit into the modern genus concept of *Rhizoctonia*, the isolates were examined for the criteria presented by Ogoshi (1975). Hyphal characteristics (growth pattern, hyphal diameter, septal structure and nuclear condition of cells) were examined after growth of the isolates on slides coated with low strength (1/8) potato dextrose agar (PDA). The septal condition was examined under phase contrast. Nuclei were stained with HCl-Giemsa according to the protocol of Wilson (1992) (I,II & III). Colony characteristics were determined after 21 d growth on PDA at 24°C (I) or 21°C (II). The growth rates of the isolates on PDA were examined using a temperature series (7, 14, 18, 21, 24, 28, 31°C) (I) or at a single temperature (21°C) (II).

To study hyphal anastomosis, isolates were paired on agar media by inoculating them at a distance of 2 cm. Pairings were incubated until the margins of opposing colonies overlapped; hyphal anastomosis reactions were examined under a light microscope (I, II, III). The standardized tester strains of genus *Ceratobasidium* (AG-A to AG-S), obtained from Akira Ogoshi (Hokkaido University, Japan), were used to assess the grouping of the studied isolates (I, II).

A new method was developed for fruiting isolates. The studied isolates were inoculated into a Petri-dish system containing three aseptically growing Scots pine (I, IV), Norway spruce (IV) or Siberian larch (IV) seedlings in distilled water. Petri-dishes were kept at room temperature with natural indirect lighting. Following the development of hymenia, samples were transferred to microscope slides for measurements of teleomorph characteristics.

The genetic similarity of uninucleate *Rhizoctonia* isolates was examined with RAPD markers (III). DNA was isolated from PDA grown cultures using the protocol described by Lee and Taylor (1990). Three randomly constructed primers (primer 91298: GGA CGA TTC G; primer 91299: CGA TTC GGC G;

primer 91300: CGA GGT TCG C) were used for amplification under conditions slightly modified from those of Williams et al. (1990). Amplifications were repeated twice for each isolate and only reproducible bands were scored. The statistical analysis of RAPD data, similarity coefficients (DICE) and clustering analysis was based on the NTSYS program (Rohlf 1989).

3.2. Pathogenicity of *Rhizoctonia* isolates

To study the pathogenicity and mode of infection of *Rhizoctonia* species isolated from nursery-grown Norway spruce seedlings displaying root dieback symptoms, 5-week-old aseptically growing Norway spruce seedlings were inoculated with isolates representing different anastomosis groups. Seedlings were incubated for four weeks and examined for root system morphology. The data were subjected to analysis of variance and Tukey's HSD test. To examine the infection characteristics, intact root systems were stained with trypan blue according to the protocol of Phillips and Hayman (1970) and examined under a microscope (II).

To study the pathogenicity of a collection of uninucleate *Rhizoctonia* isolates originating from Finland and Norway, ten-week-old Scots pine and Norway spruce seedlings were inoculated with the fungal isolates and incubated for 8 weeks under greenhouse conditions and examined for root system characteristics. In another experiment, one- and two-year old Scots pine seedlings were inoculated with the uninucleate isolate nr. 264 and incubated for 7 months under greenhouse conditions before examination of root system characteristics. The data from both experiments were subjected to analysis of variance and Duncan's multiple range test (III).

To further investigate pathogenicity of the uninucleate *Rhizoctonia* sp. and infection on observed hosts, 7-week-old seedlings of Scots pine, Norway spruce and Siberian larch were inoculated with three isolates originating from the respective hosts. After an incubation period of 5 weeks in a growth chamber, seedlings were transferred to a greenhouse and the experiment was harvested when the seedlings had reached the age of 7 months. Several root parameters were recorded for the seedlings both at the time of inoculation and harvesting. This data was subjected to analysis of variance and Tukey's HSD test. Fungal isolations and somatic compatibility tests were made to verify the presence of inoculated strains in the roots at the time of final harvesting. To screen the locality of infection, two entire root systems representing each treatment were stained according to the procedures described by Koske and Gemma (1989). In addition, all root systems harvested at the time of inoculation were similarly stained and examined under a microscope. Following this screening of infection and to examine the infection more closely, root pieces representing areas of interest were embedded in paraffin for microtome sectioning (IV).

4 Results and discussion

4.1. Binucleate *Rhizoctonia*: characteristics and role in the root dieback disease

The case study (II) showed that several *Rhizoctonia* spp. can be obtained from the roots of diseased seedlings; binucleate *Rhizoctonia* isolates were frequently coexisting with uninucleate *Rhizoctonia* isolates in the same root system. Before this, binucleate *Rhizoctonia* had been frequently isolated also from diseased seedlings and from seedlings considered healthy (Lilja et al. 1992) but not in association with uninucleate isolates.

On the basis of cultural morphology (II), these binucleate *Rhizoctonia* isolates could be divided into four morphological groups. These groups could be easily distinguished from the uninucleate *Rhizoctonia* sp. on cultural characteristics (colony colour, zonation and sclerotial characteristics). In hyphal diameter and growth rate, on the other hand, the binucleate isolates were in most cases not substantially different from the uninucleate isolates. Therefore, care should be taken when isolating *Rhizoctonia*; to discover the whole range of species associated with the studied host, a preliminary screening can be quickly done just by inoculating the isolates on a characteristic medium, e.g. PDA. To avoid potential mixed cultures, all isolates should be ideally obtained as single cell or hyphal isolations.

The morphological grouping corresponded well with the anastomosis behaviour of representative isolates; binucleate isolates anastomosed only with other isolates sharing the same morphological characters. A killing reaction was commonly observed in nonself pairings within a morphological group indicating that the isolates were closely related but genetically different. Based on similarity in morphological characteristics and anastomosis behaviour, these four binucleate groups can be regarded as four distinct species.

Of the four anastomosis groups found for binucleate Rhizoctonia (II), only one could be connected with AGs described for the genus *Ceratobasidium*; the isolates in question anastomosed with the culturally similar AG-I tester, producing the killing reaction, which indicates a close relationship (= common AG) between the Finnish isolates and the Japanese tester. The anamorph of AG-I is *R. fragariae* Husain & McKeen (Ogoshi et al. 1983). There are no other reports concerning AG-I or *R. fragariae* associated with conifer seedlings, as AG-I is normally associated with root rot in strawberry fields (Martin 1988). Since there was no indication of the teleomorph of the binucleate isolates

belonging to the remaining unassignable three groups, it may simply be that these isolates do not represent anamorphs of genus *Ceratobasidium*. For the moment, the other two teleomorph genera known for binucleate *Rhizoctonia*, *Tulasnella* and *Sebacina*, are culturally very poorly known and no AG grouping has been set up for them. On the other hand, the acknowledged AGs of genus *Ceratobasidium* are based on Japanese and American fungal populations. It is very likely that in the future many new AGs will be reported within this genus also. This will require increased efforts in fruiting the unassigned isolates, which are nowadays very frequently being reported due to the increased application of anastomosis testing in isolate characterization. Several methods have been described for fruiting *Rhizoctonia* isolates (see e.g. Sneh et al. 1991).

When comparing the effect of co-existing uni- and binucleate strains isolated from the same Norway spruce seedlings, the seedlings inoculated with uninucleate isolates showed generally considerably poorer root growth than the seedlings inoculated with the binucleate strains (II). Unlike uninucleate *Rhizoctonia* isolates, binucleate isolates seemed to have no effect on the seedling growth parameters. Observations on the hyphal behaviour of uni- and binucleate isolates in the roots were in agreement with the root system morphology. Compared to seedlings inoculated with uninucleate isolates, roots inoculated with binucleate isolates had relatively few surface hyphae, except in the older parts of the main root. In this region, binucleate isolates commonly infected cortical cells with intensely stained monilioid hyphae filling the entire cell. This type of infection was rarely observed in the roots inoculated with uninucleate isolates. Unlike binucleate isolates, uninucleate isolates colonized particularly the root tips. Saksena and Vaartaja (1961) found that several *Rhizoctonia* species infected cortical cells of some conifer seedlings with monilioid cells in a poorly developed root system. However, the authors do not mention whether the root tips or the vascular cylinder were infected in these seedlings. In addition to conifers, the infection of root cortical cells with monilioid hyphae of *Rhizoctonia* has been reported in numerous studies on various hosts. The high intensity in staining of monilioid hyphae in contrast to undifferentiated hyphae reflects differences in the cell wall thickness. Sclerotia, the resting stage of *Rhizoctonia* fungi, are commonly composed of monilioid hyphae (see *e.g.* Sherwood 1970) and monilioid hyphae within host cells probably act as sclerotium-like dispersal and survival units for *Rhizoctonia* (e.g. Ferris et al. 1984).

These results from the pathogenicity test (II) are in agreement with other studies made in Finland (Lilja et al. 1992; Lilja 1994). The fact that the AG concept has not been applied in all studies concerning binucleate *Rhizoctonia* does not allow direct comparisons between different studies. Anyway, it can be concluded that the studied binucleate isolates (II) are not directly involved in the actual root dieback disease. Concentration on uninucleate *Rhizoctonia* in later studies was thus justified. Whether the co-existed binucleate *Rhizoctonia* had already colonized roots when the seedlings were healthy or followed after

infection by uninucleate *Rhizoctonia* sp. remains an open question. Most of the studies on *Rhizoctonia* in relation to trees report these fungi as damping-off pathogens (V). All the performed Finnish studies are based on pathogenicity in older seedlings (Lilja et al. 1992; Lilja 1994; II) and do not necessarily implicate non-pathogenicity in young seedlings at damping-off age. In addition, the binucleate isolates used in the third paper as reference isolates did show moderate pathogenicity, so the results obtained in the second study should not be generalized concerning all binucleate species or different genotypes.

4.2. Uninucleate *Rhizoctonia* isolates

4.2.1. Isolate characterization

All six cultures examined (I) showed the general characteristics of *Rhizoctonia*. Basally constricted hyphae arose at acute angles behind the apices of the advancing hyphae and at right angles in the older hyphal regions. A dolipore septum was always observed near the point of origin of the branch. The sclerotia were not differentiated into a rind and medulla and no conidia, rhizomorphs or clamp connections were observed. Hyphal cells were predominantly uninucleate except for one isolate which contained a relatively high percentage (11 %) of binucleate tip and subapical cells. Culturally, the isolates were almost identical on PDA: distinctive, small, hazel-coloured regions within the otherwise buff-coloured surface hyphae gave cultures a spotted appearance. Surface-located and submerged, fulvous to umber coloured sclerotia were readily formed.

Isolates fitting the above cultural description were confirmed to be predominantly uninucleate also in the later studies (II, III). In the massive literature on *Rhizoctonia*, there are very few papers reporting a uninucleate nuclear condition. Uninucleate *Rhizoctonia* isolates have been isolated from roots of winter wheat (Hall 1986). However, based on considerable differences in hyphal and colony morphology and in growth rate, these isolates are very unlikely to be related to the uninucleate isolates obtained from the roots of conifer seedlings. Burbee et al. (1980) found an isolate of *R. quercus* Cast. and an isolate of *R. alpina* Cast. to be uninucleate. Ogoshi et al. (1983) examined the same isolates and could not confirm that result; they found the *R. alpina* isolate to be binucleate and could not say whether *R. quercus* was binucleate or not. The isolates examined in those two studies originated in the 1930's (Castellani 1934). We have also examined these two isolates and they both contain sectors; certain areas have constantly uninucleate cells whereas others are invariably binucleate (Hietala & Sen, unpublished observations). This could explain the inconsistency between the earlier studies, resulting possibly from genetic changes occurred during the long storage in culture collections.

The six studied isolates had very similar growth rates (I). Maximum growth

for two isolates was obtained at 21°C and for the others at 24°C. At these temperatures, the radial growth rates of the isolates were around 8 mm/24 h. In this respect, these isolates can be regarded as fast growing, not substantially differing from many isolates of R. *solani* (e.g. Mordue et al. 1989). The diameter of subapical hyphae of uninucleate isolates ranged between 5 and 8 μm (I, II), resembling *R. solani* also in this respect (e.g Parmeter & Whitney 1970).

All the uninucleate isolates anastomosed with each other. In contrast to the perfect fusion observed in self pairings, the killing reaction was commonly formed in nonself pairings (I, II, III). In nonself pairings, perfect fusions were observed only between three isolates, which all originated from the same nursery (III). Even in these pairing combinations the killing reaction was frequently observed. The genetics of anastomosis reaction types are not known in these fungi but the killing reaction is usually regarded as a somatic incompatibility reaction, whereas perfect fusion is interpreted as a somatic compatibility reaction (Anderson 1982). Perfect fusions have been very rare in nonself pairings in other studies except between some isolates originating from a common field; in these cases, perfect fusion has been interpreted as a sign of clonality (Ogoshi & Ui 1983; MacNish et al. 1993). Therefore, it seems logical to interpret the killing reaction commonly observed in nonself pairings of the uninucleate isolates as a sign of genetic difference. No positive anastomosis reactions were observed when the isolates were paired against the *R. alpina* and *R. quercus* isolates indicating that the species are not related (I).

In the RAPD analysis (III), the uninucleate *Rhizoctonia* isolates showed high similarity within the group, over 75–80 % similarity coefficients in a dendrogram analysis and differed drastically from the binucleate reference isolates used in the study. The Norwegian isolates included did not group together closely, but showed more similarity with certain Finnish isolates. In addition, the Finnish uninucleate isolates did not group together according to their geographic origin.

Using the novel method employing Scots pine seedlings (I), all isolates excluding one (isolate 256) could be fruited. In a further experiment (IV), representative isolates (including isolate 256) fruited in the presence of all the hosts observed for this uninucleate *Rhizoctonia*. No fruiting was observed in either experiment when the seedlings were absent from the system. On the basis of basidial characteristics (I), these isolates can be placed into genus *Ceratobasidium* (Talbot 1965). The ratio between the widths of metabasidia and their supporting hyphae was generally over two, which is a major feature defining the genus. The observed development of basidia on basal hyphae or on short side branches and the repetitive germination of basidiospores also connect the uninucleate isolates to this teleomorph genus. In the anastomosis testing, the chosen two isolates did not anastomose with testers of any of the 21 AGs described for the genus. Compared to other members of this genus, the nuclear condition of these isolates is very unusual; the genus *Ceratobasidium* has been regarded to contain only binucleate *Rhizoctonia* (e.g. Parmeter & Whitney 1970;

Ogoshi et al. 1983; Sneh et al. 1991). The teleomorph characteristics of the uninucleate isolates resemble very closely those of *C. bicorne* Erikss. & Ryvarden, described from Danish field material, parasitic on the moss *Polytrichum attenuatum* (Brid.) (Eriksson & Ryvarden 1973). There are very few observations on *C. bicorne*. Besides the sample on which the species description is based, there are only two additional specimens of the teleomorph; one was found on a *Polytrichum* moss and the other one on bark of living Norway spruce in Bayern, Germany (Luschka 1993). The characteristics of basidia and basidiospores of *C. bicorne* are easily distinguished from other *Ceratobasidium* species (see e.g. Stalpers & Andersen 1996). The teleomorph characteristics of the uninucleate isolates fit well into the taxonomic (= morphological) species concept of *C. bicorne*. Further comparison (vegetative morphology and cytomorphology, anastomosis interaction) between the uninucleate *Rhizoctonia* isolates and the *C. bicorne* observed under natural conditions was not possible, since *C. bicorne* has apparently never been cultured.

Based on vegetative and teleomorph characteristics, it can be concluded that the uninucleate *Rhizoctonia* isolates represent a single species. This species seems to be very common in Finnish forest nurseries around the country (Lilja 1994). From Norway, the other country reporting root dieback of Norway spruce, there are no data on the occurrence of the species. The aberrant nuclear condition makes this *Rhizoctonia* species very easy to distinguish from other *Rhizoctonia* species and, it raises the question whether the present root dieback disease is the first time the vegetative stage has been observed. The literature on *Rhizoctonia* associated with trees (V) may provide some answer. First, it is evident that the genus *Rhizoctonia* has received relatively little attention among pathologists and mycologists dealing with trees. It is also true that even today papers are being published without sufficient characterization of the isolates in question. For example, there are many recent papers on tree diseases, where the pathogen has been identified as *R. solani* without presenting any criteria for this judgement. As shown in several studies (e.g. Parmeter et al. 1967; Burbee et al. 1980), unless nuclear or teleomorph condition is known, *R. solani* and many binucleate *Rhizoctonia* are practically indistinguishable. The vegetatitive characteristics of the present uninucleate *Rhizoctonia* sp. (production of brown pigments, relatively wide hyphae and fast growth rate) are also similar to those of *R. solani* (see e.g. Ogoshi 1975) and there is thus clearly room for error. Therefore, one can only present worthless speculations about whether or not the uninucleate *Rhizoctonia* sp. has been observed before or is associated with roots of conifer seedlings only in Finland and Norway.

4.2.2. Pathogenicity

Under non-sterile conditions, inoculations with uninucleate strains considerably reduced the root growth of both Scots pine and Norway spruce seedlings, but did not usually result in death of seedlings (III). In both Scots pine and Norway spruce significant reductions (Duncan's multiple range test, $p = 0.05$) in parameters related to main root length, lateral root length and root dry weight were observed for practically for all the seventeen uninucleate isolates tested. Clear reduction was also shown in the shoot dry weight of both hosts, but the difference to the control seedlings was statistically significant only in Norway spruce seedlings. There seemed to be no connection between the original host and isolate pathogenicity; all the isolates were approximately equally pathogenic in both hosts, when comparing the individual root parameter values of seedlings inoculated with each isolate against the mean value for all isolates within each host. Similar growth reduction patterns were also observed in the other experiment (III), where one- and two-year-old Scots pine seedlings were inoculated with an isolate originating from this host. None of the seedlings were killed during this experiment.

The root staining methods developed for studying infection by VA mycorrhizal fungi proved to be very useful, not only for locating *Rhizoctonia* on the roots, but also for studying the mode of infection. The characteristic branching pattern of *Rhizoctonia* hyphae makes them easy to trace in a non-sterile system. No substantial difference was observed in the resolution of the two protocols (II, Phillips & Hayman 1970; IV, Koske & Gemma 1989), but the latter protocol is preferrable since it does not involve the use of toxic lactophenol. Under aseptic conditions, Norway spruce seedlings inoculated with uninucleate *Rhizoctonia* isolates displayed root systems with significantly stunted main and lateral roots (Tukey's HSD, $p = 0.05$). Staining of roots without sectioning showed hyphal proliferation at the root tips and suggested that the fungus penetrated the vascular cylinder via the root tips (II).

The observations of the second study (II) are in agreement with the results obtained under non-sterile conditions for all three hosts (IV). The uninucleate *Rhizoctonia* isolates reduced the lateral and main root growth of all host species (Fig. 1) although the reduction in Siberian larch was not always significant (Tukey's HSD, $p = 0.01$). No host specificity was observed; seedlings inoculated with isolates originating from different hosts did not show substantially differing root or shoot parameters. For the three hosts, the only statistically significant reduction in shoot parameters (length and dry weight) was observed in Scots pine. The shoots of inoculated Norway spruce seedlings were clearly although statistically insignificantly reduced in growth whereas inoculated Siberian larch seedlings showed equal shoot growth to the uninoculated control seedlings. Combining microtome sectioning in the root analysis (IV) confirmed the observations made on the stained unsectioned roots (II, IV); in the root tips, the

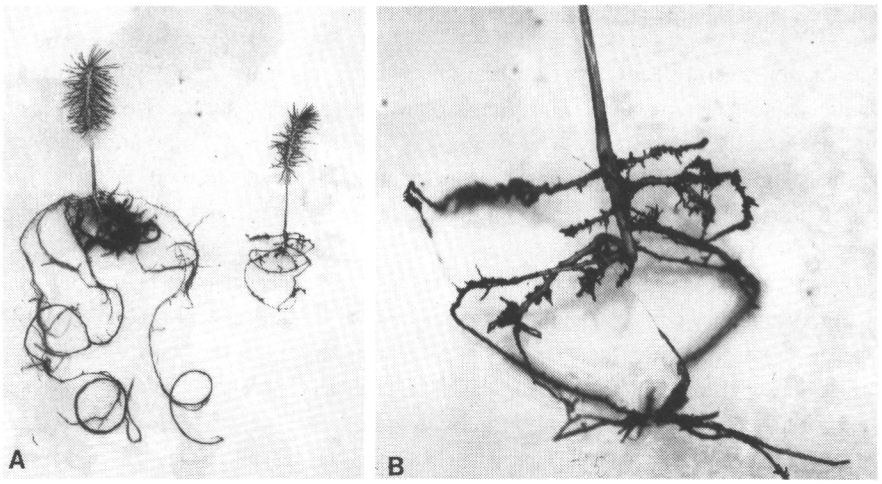

Fig. 1. Root system characteristics of Norway spruce inoculated with the uninucleate *Rhizoctonia* sp. (IV). 1A: An uninoculated control seedling (left) and a seedling inoculated (right) at the age of 7 weeks. Seedlings were harvested at the age of 7 months. 1B: A close-up of the inoculated seedling in Fig. 1A. The root tips of long roots are commonly missing in infected seedlings and the remaining root tips are heavily pigmented and arrested in growth giving the root system a stunted appearance.

uninucleate *Rhizoctonia* sp. penetrated into the vascular cylinder. The prepenetration stage was characterized by formation of hyphal aggregates on the root surface giving rise to penetration hyphae. *Rhizoctonia solani* has been shown to form similar hyphal aggregations, termed infection cushions, on several plants (e.g. Dodman & Flentje 1970). The now observed aggregations on conifer roots were commonly hundreds of µm long and there seemed to be a close connection between the formation of penetration hyphae and the state of root growth. No penetration hyphae were observed on such roots, which on the basis of a present metacutization layer, were regarded to be in dormancy. In addition, penetration hyphae were seldom formed in actively growing roots in the region were first protoxylem elements had already differentiated.

Considerable hyphal proliferation was observed when isolates were grown under membrane-isolated host roots. From these observations, it is concluded that the uninucleate *Rhizoctonia* sp. infects actively growing roots and root exudates probably provide energy for the luxurious hyphal proliferation preceding the formation of penetration hyphae. Components of root exudates of some conifer seedlings have been shown to stimulate hyphal growth of *Rhizoctonia* (Agnihotri & Vaartaja 1969) and sporangium germination of *Pythium* (Agnihotri & Vaartaja 1967). Moreover, there is considerable evidence from other hosts that plant exudates are essential for the formation of infection cushions of *R. solani* (e.g. Dodman & Flentje 1970; Armentrout et al. 1987).

At the time of inoculation, both Norway spruce and Siberian larch had a high percentage of dormant roots. Unfortunately, possible differences in seasonal root growth pattern between different hosts were not examined by sequential harvesting. Possible differences in growth patterns could partially account for the fact that larch seedlings were less affected by the pathogen, since no differences were observed in the actual infection sites in different hosts. Presently, there is very limited data about seasonal growth patterns of fine roots in tree seedlings (Wilcox 1954: *Abies procera* Rehd.; Wilcox 1968: *Pinus resinosa* Ait.; Johnson-Flanagan & Owens 1985: *Picea glauca* (Moench) Voss) and further research in this area should be interesting from several angles, not only from a pathologist's point of view.

4.2.3. Conclusions

It would be unwise to assume that the uninucleate *Rhizoctonia* isolates and the *C. bicorne* teleomorphs found in natural conditions share the same vegetative characteristics (e.g. uninucleate nuclear condition), belong to a common anastomosis group and further, represent a single biological species. This was the reasoning behind the rather cautious taxonomic conclusion in paper I: "in conclusion, this uninucleate fungus does fit into *Ceratobasidium* but because of the unusual nuclear condition we would prefer to describe it as a uninucleate *Rhizoctonia* sp. having a *Ceratobasidium* fruiting stage". Ideally, a taxonomic species should represent a single biological species. However, there is ample evidence that this would not be the case with many *Rhizoctonia* related taxa. *Thanatephorus cucumeris*, *Ceratobasidium cornigerum* and *Waitea circinata* are probably species complexes, considering their subdivision into anastomosis groups. In this light, the taxonomic conclusion of paper I has elements of an understatement. There are no reasons why these uninucleate *Rhizoctonia* isolates could not be treated as anamorphs of a morphologically identical teleomorph, *C. bicorne*. Future will tell whether this uninucleate *Rhizoctonia* sp. and *C. bicorne* as described by Eriksson and Ryvarden (1973) are truly conspecific.

Hyphal anastomosis data would indicate that the uninucleate isolates represent distinct but closely related genotypes and that the species includes a single anastomosis group. RAPD analysis does not support any further division among these isolates. It appears that the dual nomenclature for these fungi (anamorph vs. teleomorph) will be maintained in the future because of the difficulties in fruiting *Rhizoctonia* isolates. Considering the uninucleate nuclear condition, uniformity in cultural characteristics, high genetic similarity and possession of a teleomorph for which no anamorph state has been described, there is only one conclusion to draw; a new *Rhizoctonia* (or *Ceratorhiza*) species could and should be described for these uninucleate isolates. In the mean time, these uninucleate isolates can be referred to with the description "uninucleate *Rhizoctonia* sp. (or

Ceratorhiza sp.) having a *Ceratobasidium bicorne* perfect state".

Nuclear condition has been treated as a key character in distinguishing anamorphs of the related two teleomorph genera, *Ceratobasidium* and *Thanatephorus*. Before the present study, only binucleate *Rhizoctonia* spp. have been known to possess a *Ceratobasidium* fruiting stage. Is there now a need for a taxonomic rearrangement due to the aberrant nuclear condition? The fact is that presently the nuclear condition is still unknown for several *Ceratobasidium* species and there is no quarantee that they will show the familiar binucleate nuclear condition. In addition, practically nothing is known about the sexuality of binucleate *Rhizoctonia* and the key question is: are they heterokaryotic and thus heterothallic? If they are, uninucleate isolates could represent homothallic clones or species. Therefore, an unusual nuclear condition, in itself, is most certainly not a sound basis for any taxonomic rearrangements. Phylogenetic data would be desperately needed to address this kind of questions. This approach would also be most welcomed in order to critically evaluate the validity of several *Rhizoctonia* taxa (e.g. anamorphs that belong to AG-A of genus *Ceratobasidium*).

Lilja (1994) has first presented a hypothesis that root dieback may be a disease of successive infections. Primary infection by uninucleate *Rhizoctonia* results in a high moisture content in the growth substrate and wet conditions favour zoosporic fungi like *Pythium*. This is a sound conclusion, and it is striking that after the work of Venn at al. (1986) Norwegian studies on root dieback disease have concentrated on *Pythium* regarding these fungi as major pathogens and practically ignoring *Rhizoctonia* (Børja 1995). Seedlings inoculated with uninucleate *Rhizoctonia* sp. appear to show very characteristic root system morphology (II; IV). Unfortunately, available information on root system morphology of diseased nursery seedlings (Galaaen & Venn 1979; Venn 1985; Lilja et al. 1992; II) and seedlings inoculated with different pathogens (Venn et al. 1986; Lilja et al. 1992; Lilja 1994) lacks sufficient details for definitive conclusions. However, I would claim that in pathogenicity tests (II, III, IV), the resulting root system morphology corresponds well with the one observed in those seedlings, from which I have isolated this species.

Wilting of young shoots, hanging tops, discolouration of needles, retarded height growth and partial or total death of root systems are the symptoms related to the root dieback disease in Norway (Venn et al. 1986). In Finland, studies have concentrated on seedlings showing needle discolouration, stunted growth and partial or total root death (Lilja et al. 1992; Lilja 1994; II) but, as reported by Jalkanen (1985), we do have seedlings also showing wilting and hanging tops. Although the present study was focused on stunted nursery seedlings showing no shoot wilting or hanging tops, it raises a question whether the broad symptom list presented by Venn et al. (1986) actually reflects differences in the causative agent. I have occasionally inspected nursery seedlings showing shoot wilting and top hanging; in these particular seedlings, the root system has been

totally dead but structurally normal (*i.e.* lateral roots and main roots have not appeared stunted in growth). In addition, these roots invariably hosted *Pythium* spp. but no *Rhizoctonia*. A systematic survey and comparison of the root system characteristics and mycoflora of seedlings showing either wilting and hanging tops or shoot stunting would be needed to critically evaluate this proposal. This will require increased collaboration between nursery inspectors and pathologists, as nursery managers often tend to keep a low profile on their disease incidences.

In Norway, root dieback losses are reported to have decreased following a) removal of old sand beds, b) implementation of container sanitation between crops and c) development of appropriate fungicide and irrigation regimes (e.g. Børja & Austara 1990). The results of Venn et al. (1986) implicated supporting sand beds act as an inoculum source for *Rhizoctonia* associated with root dieback. Most *Rhizoctonia* spp. probably survive in soil between annual crops particularly as sclerotia formed in colonized plant debris This is evidently the case with the uninucleate *Rhizoctonia* sp. also, which is characterized by abundant sclerotial production under cultural conditions (I). Several edaphic factors may influence survival of sclerotia; e.g. survival of *R. solani* in moist soil is considered to be lower than in dry soil. Under dry conditions, the sclerotia have remained viable several years (see Sherwood 1970). Therefore, the measures a) and b) can be recommended as a control procedure in a *Rhizoctonia* disease case also. However, ecologically (e.g. moisture requirement) and structurally (e.g. cell wall composition choice of fungicides) *Rhizoctonia* and *Pythium* show striking differences and will require different approaches.

Sorting out the raised etiological questions will give us a more complete picture of the root dieback disease and would allow consideration of sensible target measures to control the primary pathogen in question in each particular case.

4.3. Ongoing research

Development of DNA-based methods allow testing the crucial question whether the now studied uninucleate isolates and *C. bicorne* are truly conspecific. Preliminary results based on ITS-PCR (primers ITS1-F and ITS4-B, Gardes & Bruns 1993) combined with RFLP indicate at least a close relationship between them; the restriction patterns of the ITS-region of the uninucleate isolates and a *C. bicorne* herbarium specimen (on *Polytrichum* moss found in Luschka's (1993) survey in a forest in Bayern, Germany) are the same and differ from binucleate *Rhizoctonia* isolates and tested herbarium samples of other *Ceratobasidium* species found in Finland (Hietala, Sen & Hantula, unpublished results).

When sibling single-spore isolates of this uninucleate *Rhizoctonia* species are paired with each other, no mating reactions (e.g. tuft formation) have been observed and a killing reaction is commonly formed in a majority of pairings.

Single-spore isolates can also be fruited and the killing reaction is common in sib-pairings of the second generation, too (Hietala, Hantula, Korhonen & Sen, unpublished results). Therefore, it is clear that this fungus is homothallic and being uninucleate, the observed somatic incompatibility (= killing reaction) in pairings between sibling single-spore isolates makes the species a very interesting model organism for future research. We started this "freetime project" already back in 1994 and it is not due to lack of efforts or tools that we can presently provide no explanation for this highly interesting phenomenon.

The high genetic homogeneity of the uninucleate *Rhizoctonia* sp. (III) could implicate a narrow source population, resulting e.g. from seedling exchange between different nurseries. Analysing mitochondrial genes could sort out this question. Field isolates derived from different nurseries invariably produce the killing reaction when paired. In addition, several genotypes, based on observed killing reaction in pairings, can be found in a single nursery (III). These observations do not exclude the possibility of a common origin since killing reactions are commonly formed in pairings between sibling single-spore isolates. However, they would implicate that the fungus is fruiting in the nurseries or nearby. Under natural conditions, fruit bodies of *Rhizoctonia* have been recorded on a wide variety of hosts, including some broadleaved trees, and on surrounding soils. In fields, the fruit bodies of *Rhizoctonia* usually develop at relatively high temperature (20°C) and moisture conditions (90 RH) at the lower side of infected leaves but also on the surface of the lower stem (see e.g. Naito 1996). Compared to hyphal growth, airborne basidiospores can disseminate rapidly over long distances which could be of importance in the development of disease epidemics. It is not uncommon to isolate uni- and binucleate *Rhizoctonia* spp. from the lower stems of seedlings suffering from root dieback (Hietala, unpublished observations) and this possible fruiting place is worth further look. In addition, *Polytrichum* mosses are quite common in Finnish forests and also in our forest nurseries and an investigation of their mycoflora will form another logical investigation line.

On the basis of anastomosis groups, most of the binucleate isolates studied in the second paper could not be assigned to known anastomosis groups of genus *Ceratobasidium*. Further work using various fruiting techniques is needed to place these isolates and to see whether *Ceratobasidium* testers were actually valid. The method developed for uninucleate *Rhizoctonia* sp. (I) does not seem to work for binucleate *Rhizoctonia* associated with root dieback of seedlings (Hietala, unpublished results). In pathogenicity tests of Lilja (1994), some binucleate *Rhizoctonia* seemed to promote seedling growth. This is quite interesting, since on the basis of anastomosis affinity and common ITS-RFLP patterns, certain binucleate isolates derived from roots of nursery-grown conifer seedlings would seem to be related to orchid-associated *Rhizoctonia* (Hietala, Zelmer & Sen, unpublished results). Orchids represent an example of hosts for which the anastomosis groups of associated *Rhizoctonia* have not been tested.

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Anamorphic and teleomorphic characteristics of a uninucleate *Rhizoctonia* sp. isolated from the roots of nursery grown conifer seedlings

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Vegetative, anastomosis, fruiting and basidial characteristics were analysed in one Norwegian and five Finnish *Rhizoctonia* isolates from the roots of various nursery grown conifer seedlings. The isolates displayed common hyphal and colony morphology that also confirmed their designation as a *Rhizoctonia* sp. Hyphal cells were predominantly uninucleate except for one isolate which contained a relatively high number (11%) of binucleate tip and subapical cells. All isolates had similar temperature dependent growth rates and formed a single anastomosis group within which killing reactions were detected in opposing fusion cells of all non self pairings.

Fruiting was induced in all but one Finnish isolate using a novel method involving axenic liquid culture of the fungi in the presence of Scots pine (*Pinus sylvestris*) seedlings. Basidial and basidiospore dimensions indicate that the isolates represent a single *Ceratobasidium* sp. although two selected isolates showed no hyphal anastomosis reactions with binucleate testers of the different *Ceratobasidium* anastomosis groups (AG-A to AG-S). The identified teleomorph closely resembles *C. bicorne* but further confirmation was not possible because this fungus is only available as herbarium material.

Rhizoctonia DC. was established in 1815 by de Candolle to accommodate a non-sporulating root rotting fungus, *Rhizoctonia crocorum* DC.: Fr. However, the subsequent description of numerous heterogenic *Rhizoctonia* species prompted a major revision of the taxonomic criteria required for the identification of *R. solani* Kühn that were later expanded to cover the whole genus (Parmeter & Whitney, 1970; Ogoshi, 1975). Accepted vegetative features included the requirements of hyphae with dolipore septa, basally constricted branching near the distal septum of cells, absence of clamp connections, conidia and rhizomorphs and sclerotia with undifferentiated structure.

On the basis of the known teleomorphs the genus is limited to the sub-division Basidiomycotina; class Hymenomycetes (sub-class Holobasidiomycetidae or Phragmobasidiomycetidae) (Sneh, Burpee & Ogoshi, 1991). The four main genera represented are *Thanatephorus* Donk (includes the teleomorph of e.g. *R. solani*), *Waitea* Warcup & P. H. B. Talbot (e.g. *R. zeae* Voorhees), *Tulasnella* J. Schröt. (e.g. *R. repens* Bernard) and *Ceratobasidium* Rogers (e.g. *R. endophytica* var. *endophytica* H. K. Saksena & Vaartaja and *R. cerealis* E. P. Hoeven). A major taxonomic feature of the anamorphs that separates the former and latter two genera is the respective presence of multinucleate and binucleate cells in young vegetative hyphae (Sneh *et al.*, 1991). The multinucleate *R. solani* (*T. cucumeris* (A. B. Frank) Donk and *T. praticola* (Kotila) Flentje and binucleate *Rhizoctonia* spp. (*Ceratobasidium* spp.) have been respectively further sub-divided into 11 and 21 anastomosis groups (AG) based on affinity and fusion of interacting hyphae of paired cultures (Sneh *et al.*, 1991 and references therein).

Isolates of two species, *R. quercus* Cast. and *R. alpina* Cast., were found to contain uninucleate hyphae (Burpee *et al.*, 1980) but this could not be confirmed by Ogoshi *et al.* (1983) who regarded the latter fungus to be binucleate. The anamorph of an authenticated uninucleate *Rhizoctonia* sp. from the roots of winter wheat has been described (Hall, 1986).

Many *Rhizoctonia* species are economically important plant pathogens in agriculture and thus receive considerable attention but less is known about these fungi and their effect on forestry production, e.g. in forest tree nurseries. It has been known for a long time that damping-off in nursery conifer seedlings can be caused by *R. solani* (Vaartaja & Cram, 1956; Saksena & Vaartaja, 1961). More recently in Georgia, U.S.A., Huang & Kuhlman (1990) showed that *R. solani* and a binucleate *Rhizoctonia* sp., representing anastomosis groups AG-4 and CAG-3, respectively, were able to induce damping-off symptoms in nursery Slash pine (*Pinus elliottii* Engelm. var. *elliottii*) seedlings. A binucleate *Rhizoctonia* sp. (CAG-3) was also identified causing seedling blight of longleaf pine (*P. palustris* Mill.) in Florida (English, Ploetz & Barnard, 1986). Ten different *Rhizoctonia* species causing root rot in pine (*P. sylvestris* L. and *P. resinosa* Ait.) seedlings in Canadian nurseries included *R. callae* Cast., *R. globularis* H. K. Saksena & Vaartaja and *R. endophytica* var. *endophytica* (Saksena & Vaartaja, 1961). All have since been confirmed to be binucleate *Rhizoctonia* species and the latter is an anamorph of *Ceratobasidium cornigerum* (Bourdot) Rogers (Sneh *et al.*, 1991).

In the Nordic countries, a binucleate *Rhizoctonia* sp. has been identified killing the needles of Norway spruce (*Picea*

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Table 1. *Rhizoctonia* isolates from the roots of nursery grown conifer seedlings

Isolate number	Location	Coordinates	Host	Seedling type	Year	Source
250	Lapinlahti, Finland	63° 21′ N, 27° 24′ E	*Picea abies*	Containerized	1992	A. Lilja
256	Jomala, Finland	60° 09′ N, 19° 57′ E	*Larix sibirica*	Containerized	1991	A. Lilja
260	Lapinlahti, Finland	63° 21′ N, 27° 24′ E	*Pinus sylvestris*	Bare-rooted	1989	A. Lilja
263	Lapinlahti, Finland	63° 21′ N, 27° 24′ E	*Pinus sylvestris*	Bare-rooted	1987	A. Lilja
264	Lapinlahti, Finland	63° 21′ N, 27° 24′ E	*Pinus sylvestris*	Bare-rooted	1989	A. Lilja
83-111/1N[a]	Namsos, Norway	64° 29′ N, 11° 29′ E	*Picea abies*	Containerized	1983	K. Venn[b]

[a] Venn *et al.* (1986).
[b] Norwegian Forest Research Institute.

abies (L.) Karst.) seedlings (Roll-Hansen & Roll-Hansen, 1968) and, more recently, an uncharacterized *Rhizoctonia* sp. was also found to cause root dieback in seedlings of the same tree species in Norwegian nurseries (Venn, Sandvik & Langerud, 1986). A nursery survey of fungi present in the roots of both bare-rooted and containerized grown conifer (*P. sylvestris* and *P. abies*) seedlings in Finland included a uninucleate *Rhizoctonia*-like fungus that was found to be an aggressive root pathogen of Scots pine (*P. sylvestris*) in pathogenicity tests (Lilja *et al.*, 1992).

The aim of this work was to further characterize the uninucleate *Rhizoctonia* sp., which has been commonly isolated from roots of Finnish nursery grown conifer seedlings, together with the isolate from *P. abies* in Norway (Venn *et al.*, 1986).

MATERIALS AND METHODS

Fungal isolates

Information on the geographic origin of the isolates studied, host species and seedling type, year and source are given in Table 1. The Finnish isolates were originally isolated on distilled water agar (DWA) (1%) from surface sterilized root pieces as described by Lilja *et al.* (1992).

Anamorphic characteristics

Hyphal characteristics (growth pattern, hyphal diameter, septal structure and the number of nuclei per cell) were examined after growth of the isolates on microscope slides coated with low strength (1/8) potato dextrose agar (PDA) (4·88 g PDA and 13·12 g agar (Difco laboratories, USA) l^{-1} H_2O) that were maintained in a moist atmosphere for 48 h at 24 °C. All the following hyphal and basidial dimensions were measured using a light microscope equipped with a stage and eyepiece micrometer. The diameter of 15 subapical cells of main runner hyphae were randomly measured from four colonies of each isolate at 400 × magnification. The septal condition of hyphae was examined under phase contrast at 1000 × magnification. Nuclei were stained with HCl-Giemsa following the fixing and staining procedures described by Wilson (1992), and numbers in tip and subapical cell pairs (100 cells each) were counted at 400 × magnification.

For characterization of general cultural features, petri-dishes containing PDA were centrally inoculated by transferring a 5 × 5 mm block from the margin of an actively growing colony and incubated in the dark at 24°. Colony colour and the distribution size, shape, colour and structure of sclerotia for each isolate were regularly examined over a period of 21 d. The colours were designated using the mycological colour chart of Rayner (1970).

Growth rates

Five replicate PDA plates, similarly inoculated (as above) with each isolate were incubated in the dark at 7, 14, 18, 21, 24, 28 and 31°. The colony diameter was measured at 24 h intervals along two right angled axes.

Hyphal anastomosis

Hyphal anastomosis was microscopically examined on the surface of distilled water agar (DWA) (15 g agar l^{-1}) in petri-dishes to determine anastomosis groupings (Parmeter, Sherwood & Platt, 1969) and on microscope slides coated with 1/8 PDA for detailed identification of the type of hyphal fusion reaction (Matsumoto, Yamamoto & Hirane, 1932; Yokoyama, Ogoshi & Ui, 1983; Yokoyama & Ogoshi, 1986). Isolates were paired in all combinations at a distance of 2 cm by inoculating the agar surface using a modified Pasteur pipette (Korhonen & Hintikka, 1980) producing small cylindrical inoculum plugs (2 mm × 1 mm diam.). Pairings were incubated at 21° until the margins of opposing colonies began to overlap. Hyphal anastomosis on DWA was directly observed through the petri-dishes at 100 × and confirmed at 400 × magnification. The frequency of perfect and imperfect hyphal fusions on 1/8 PDA was recorded from a total of 75 contact points, where in each case two opposing hypha either fused, crossed or grew in a juxtapositioned manner (Sneh *et al.*, 1991), using phase contrast at 400 × magnification. All pairing combinations were made on two separate occasions.

Two isolates, 263 and 264, were also paired in all combinations on DWA with the binucleate *Rhizoctonia* (*Ceratobasidium* spp.) tester isolates (AG-A to AG-S, deposited in the ATCC) (Sneh *et al.*, 1991), *R. alpina* (CBS 309.35) and *R. quercus* (CBS 313.35) to identify any anastomosis reactions.

Teleomorphic characteristics

A new method utilizing living Scots pine seedlings to induce the perfect state was developed. Sterile Scots pine seedlings

were prepared by first imbibing seeds in distilled water at 5° for 36 h and then washing in a Tween-80 solution (3 drops per 100 ml distilled water) for 5 min. After three separate rinses in distilled water the seeds were treated with hydrogen peroxide (30%, v/v) for 15 min and then washed in three changes of sterile distilled water. The seeds were plated on 1·2% water agar, 15 to 20 seeds per petri-dish, and incubated inverted at 21° in dark for 14 d. Three sterile seedlings were then aseptically transferred to each petri-dish containing 20 ml sterile distilled water. A 5 × 5 mm block from the margin of a 3-d-old colony of an isolate grown on PDA at 21° was transferred to the petri-dish which was then incubated on a laboratory bench with natural indirect lighting. Between 3 and 5 replicate petri-dish culture systems per isolate were prepared in separate experiments over the whole year.

Following the development of hymenia on the water and seedling surface, determined visually and at 40 × magnification, samples were transferred to microscope slides and squash preparates made for measurements of basidial and basidiospore dimensions.

RESULTS

Anamorphic characteristics

All cultures showed the general *Rhizoctonia* hyphal characteristics. Basally constricted branches arose at acute angles behind the apices of the advancing hyphae and at right angles in the older hyphal regions. A dolipore septum was always formed near the point of origin of the branch. The mean width of subapical cells of the main runner hyphae ranged from 6·3 to 7·2 μm (Table 2). All the counted tip and subapical cells of isolates 250, 264 and 83-111/1N were uninucleate (Fig. 1A). The percentages of binucleate (tip:subapical) cells in 256, 263 and 260 were 4:4, 1:3 and 11:11%, respectively. In the latter isolate, hyphae containing many consecutive binucleate cells originating from uninucleate hyphae (Fig. 1B) were mainly restricted to small areas in the central parts of the colony.

The isolates showed almost uniform cultural morphology on PDA. Buff coloured, velvety-looking, young colonies grew in a radial manner and no zonation was observed. Hazel coloured spots first appeared on the central surface of the colony within 5 days and later appeared over the whole surface area to give a spotted appearance. Some of these very characteristic pigmented spots continued to spread covering an area of several mm^2 after an incubation period of 21 d. Within 14 d the first submerged, usually rounded sclerotia appeared. The individual submerged, but occasionally surface

Table 2. Subapical cell widths of main runner hyphae[a] and dimensions of monilioid cells of sclerotia[b] (μm)

Isolate	Hyphal width ± S.D.	Monilioid cell	
		Length	Width
250	6·6 ± 0·56 (5·4–7·7)	16–29	13–20
256	6·4 ± 0·54 (5·4 ± 7·8)	16–30	11–18
260	7·2 ± 0·51 (5·8–8·4)	18–35	11–19
263	6·7 ± 0·63 (5·2–7·8)	18–32	11–20
264	6·5 ± 0·60 (4·8–7·7)	17–34	11–18
83-111/1N	6·3 ± 0·57 (5·0–7·5)	14–32	10–17

[a] 60 measurements (15 measurements from four separate colonies).
[b] 60 measurements.

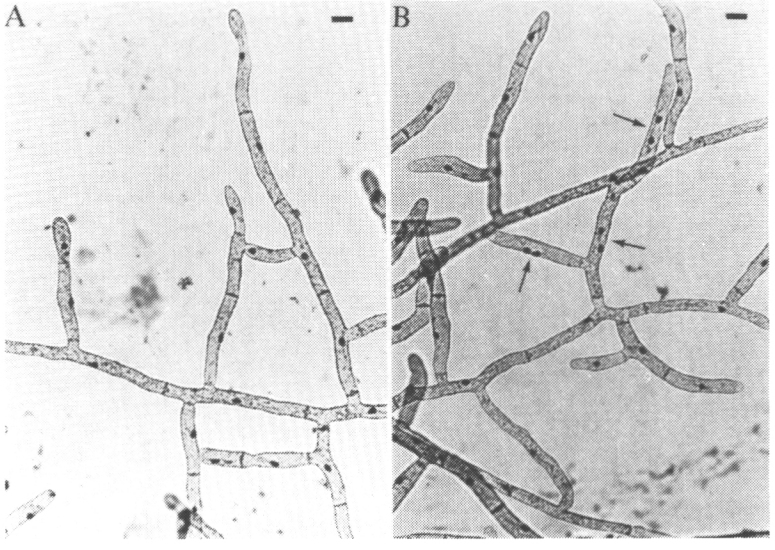

Fig. 1. Nuclei of isolate 260 stained with HCl-Giemsa. Hyphae with (A) only uninucleate cells and (B) a uninucleate hypha giving rise to a sidebranch with binucleate cells (arrowed). Bars = 10 μm.

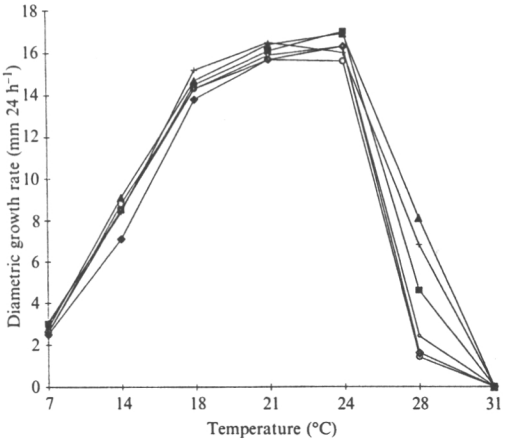

Fig. 2. The effect of temperature on the growth of different isolates on potato dextrose agar. ■—■, 250; ▲—▲, 256; ◍—◍, 260; ◇—◇, 263; o—o, 264; +—+, 83-111/1N.

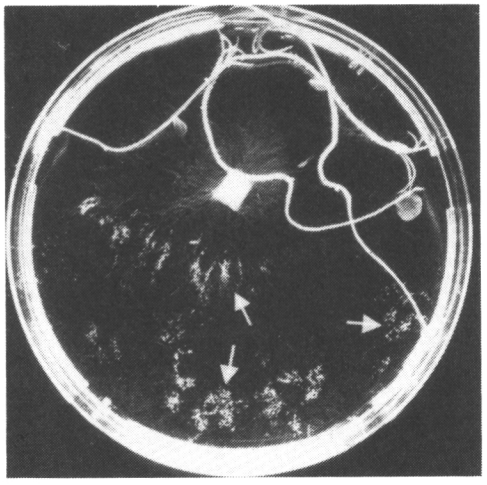

Fig. 3. Petri-dish/Scots pine fruiting system. Note white hymenial clusters (arrowed) of isolate 260 on the water surface after incubation of two weeks.

located, sclerotia were fulvous to umber in colour with diameters up to 900 μm. These submerged sclerotia tended to aggregate to form several mm large cauliflower-like structures after 21 d or longer incubation periods. The sclerotia were constructed of doliform to subglobose monilioid cells that were not organised into a rind and medulla. The dimensions of monilioid cells are presented in Table 2.

Growth rates

All isolates had very similar growth rates up to 24° on PDA, at 28° it was possible to separate some of the isolates (Fig. 2). Maximum growth rates of two isolates, 260 and 83-111/1N, were at 21° and the others at 24° and all were unable to grow at 31°.

Hyphal anastomosis

The hyphae of all the uninucleate isolates anastomosed with each other both on DWA and thin films of 1/8 PDA. In self pairings on the latter agar, opposing hyphae often showed varying degrees of hyphal attraction which was followed by cell wall contact either between tips, a tip and a hyphal side wall or via bridging between adjacent side walls. Following contact a positive anastomosis reaction resulted in a perfect fusion which involved the loss of cell walls at the fusion junction and a maintenance to cytoplasmic continuity which occurred in 95–100% of the fused cells in all pairings. In non self pairings, similar hyphal pre-contact interactions were followed by imperfect fusions identified by a characteristic rapid sequence of events: cell wall dissolution, cytoplasmic granulation, a loss of cell turgor as detected by a narrowing of cell diameter and finally a complete vacuolation of between one and three cells on either side of the fusion junction, a phenomenon termed the killing reaction (Yokojama & Ogoshi, 1986). In most pairings, over 98% of the fusion cells were killed following anastomosis. The overall anastomosis fusion frequencies of self and non self pairings were in the range 49–57 and 45–71%, respectively.

No hyphal fusions were recorded in pairings of 263 and 264 and either the binucleate *Rhizoctonia* isolates representing the known anastomosis groups of *Ceratobasidium* spp. (AG-A to AG-S) or *R. alpina* and *R. quercus*.

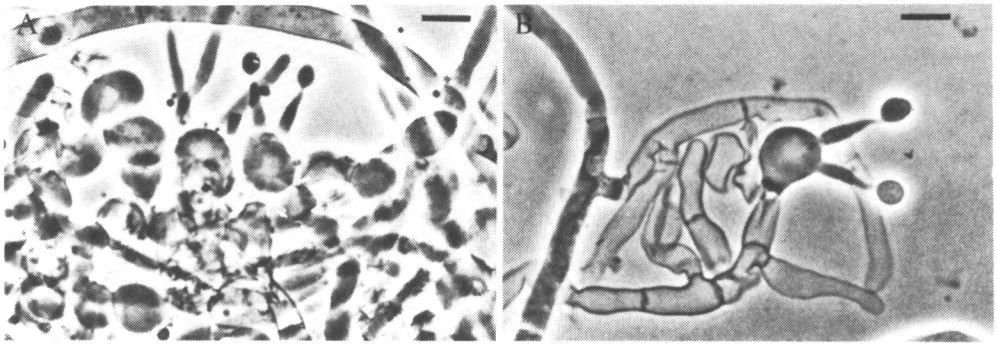

Fig. 4. Typical basidia of the uninucleate *Rhizoctonia* sp. under phase contrast; isolates (A) 264 and (B) 83-111/1N. Bars = 10 μm.

Table 3. Basidial[a] and basidispore[b] dimensions (μm) of the uninucleate *Rhizoctonia* isolates

					Sterigma							
	Metabasidium						Number				Basidiospore	
Isolate	Length	Width	Basal width	Width/b.w.	Length	Width	1	2	3	4	Length	Width
250	13·4 (10·7–14·9)	10·8 (9·3–12·1)	5·0 (3·4–6·2)	2·2 (1·6–3·1)	13·9 (10·0–25·8)	2·7 (1·8–3·8)	7	12	1		10·5 (9·6–13·4)	6·8 (5·3–7·6)
260	14·0 (12·0–16·4)	11·1 (10·0–12·3)	5·0 (3·4–6·7)	2·3 (1·8–3·1)	14·6 (9·4–44·0)	2·6 (2·0–3·6)	2	18			11·4 (9·6–13·4)	7·0 (5·7–8·6)
263	13·9 (12·0–16·3)	11·3 (10·0–13·0)	4·9 (3·9–7·7)	2·3 (1·7–2·9)	14·2 (7·0–20·3)	3·0 (1·9–4·8)	6	8	4	2	11·6 (9·6–14·3)	6·9 (5·3–8·4)
264	13·6 (11·1–17·0)	11·1 (8·7–13·4)	5·0 (3·1–6·8)	2·4 (1·6–3·6)	15·9 (8·7–27·2)	2·9 (1·8–4·4)	4	13	3		11·1 (8·6–14·8)	6·9 (5·1–8·6)
83-111/1N	15·7 (13·1–19·9)	12·0 (10·0–14·0)	5·2 (2·8–7·9)	2·5 (1·5–4·6)	16·8 (9·5–28·6)	3·2 (2·2–4·6)	1	14	5		11·6 (9·3–14·3)	6·8 (5·1–7·9)

[a] Measurements from 20 basidia.
[b] Measurements from 40 basidiospores.

Teleomorphic characteristics

All the isolates, except 256, could be repeatedly fruited with the new method between April and August but not at other times of the year. The diurnal day and night room temperature fluctuations during the spring and summer varied between 21 and 30° but remained at a temperature of 20–22° during the autumn and winter. White coloured hymenium usually developed on the water surface near the margins of the petri-dish but occasionally more centrally (Fig. 3) and also on the seedling surface. Basidia appeared within 8 to 15 d arising directly from basal hyphae or short side branches and the shape of metabasidia varied from obovate to subglobose (Fig. 4A and B). The basidial and basidiospore dimensions are given in Table 3. The most frequent number of normally stout sterigmata was two and occasionally an adventitious septum was formed in the central or apical regions of the sterigmata. Branched sterigmata were also infrequently observed and in very rare cases unbranched sterigmata lacking basidiospores grew hundreds of μm long below the water surface, maintaining their characteristic width and forming regularly spaced adventitious septa. The basidiospores were ovoid to ellipsoid and germinated on the water surface by direct germ-tube formation or in a minority of cases following repetition.

DISCUSSION

It is clear, from the anamorphic and teleomorphic data presented, that the isolates described represent a *Rhizoctonia* species. We have further examined the nuclear condition of over 30 isolates of similar cultural morphology, originating from conifer seedling roots taken from nurseries in Finland and Norway and they all contain predominantly uninucleate hyphae as described (unpublished data). It was not possible to compare our isolates with the uninucleate *Rhizoctonia* species described by Hall (1986) as the isolates were not deposited in a culture collection (G. Hall, pers. comm.). However, from his descriptions of the anamorph it is clear that these two species are not the same. No positive anastomosis reactions were detected in pairings of either 263 or 264 with the morphologically dissimilar isolates of *R. alpina* and *R. quercus* which had earlier been identified as being uninculeate by Burpee *et al.* (1980).

In the data presented, only isolate 260 had binucleate cell numbers that were clearly higher than the expected frequencies explainable by random mitotic divisions (Tu, Kimbrough & Aldrich, 1977). The origin of occasional series of binucleate cells amongst the prevailing uninucleate cells in hyphae restricted to the central areas of the colony is not clear. Contamination of cultures can be ruled out as both nuclear types exist in the same hypha as shown in Fig. 1B. The phenomenon does seem to be a general feature of our isolates as it has been observed to occur sporadically on various occasions under the same cultural conditions. Similar unpredictable changes may also explain the differences in nuclear condition reported for *R. alpina* and *R. quercus* (Burpee *et al.*, 1980; Ogoshi *et al.*, 1983). We have also observed consecutive binucleate cells in hyphae of a colony grown out of a single uninucleate tip cell. Whether the two nuclei are identical, being brought together either by self anastomosis or as a result of loss of septal synchronization, or, more interestingly, are dissimilar possibly resulting from a reduction division requires further detailed genetic investigation.

The very similar growth rates of all the isolates studied suggest that they are quite closely related, although at 28° it was possible to separate 260, 263 and 264 from the others. The uniformity of isolates was also further confirmed by the identification of a single anastomosis group. Within this group it is clear that the isolates represent different genotypes as non self anastomoses always resulted in the appearance of a killing reaction in neighbouring cells on either side of the point of hyphal fusion.

Unsuccessful attempts to obtain the perfect state, made using the nutrient step-down (Adams & Butler, 1983*a*, *b*) and antibiotic induction (Kangatharalingham & Carson, 1988) methods, prompted the development of the described method incorporating living seedlings of Scots pine. As no fruiting was observed on the water surface in the absence of seedlings, it is likely that the presence of plant material provides the fungus with a limited nutrient resource that may also actively induce fruiting through production of plant specific metabolites or breakdown products. During the autumn and winter months the centrally heated room temperatures remained very stable and thus the lack of a diurnal temperature gradient and inadequate natural lighting conditions may have contributed to the inhibition of fruiting. The method was reliable enough during spring and summer but the short window for fruiting is rather inconvenient and therefore requires further development. It was not possible to induce basidia of isolate 256, which had originally been isolated from larch (*Larix sibirica* Ledeb.), in the petri-dish system containing *P. sylvestris* although 83-111/1N and 250 from *P. abies* did fruit regularly. It may be the case that fruiting of 256 is host

specific but further work including other larch isolates is needed to confirm this.

On the basis of basidial characteristics, these isolates can be placed in *Ceratobasidium* (Talbot, 1965). The ratio between the widths of metabasidia and their supporting hyphae was generally over two, which is a major feature defining the genus. The development of basidia on basal hyphae or on short side branches and the repetitive germination of basidiospores are also typical of *Ceratobasidium* species. In contrast, there was a lack of affinity between two representative uninucleate isolates (263 and 264) and tester isolates of known *Ceratobasidium* anastomosis groups AG-A to AG-S. The differing cultural morphology of these testers also indicates that they were not related to this uninucleate fungus. There is no description of similar anamorphs that could be related to these Nordic isolates in the anamorph keys of *Ceratobasidium* species summarized by Sneh *et al.* (1991).

The teleomorph of our uninucleate isolates does seem to resemble that of *C. bicorne* J. Erikss. & Ryvarden, described from Danish field material, parasitic on the moss *Polytrichum attenuatum* (Brid.) (*Polytrichastrum formosum* (Hedw.) G. L. Sm.) (Eriksson & Ryvarden, 1973). The basidia of *C. bicorne* are obovate to subglobose (15–20 × 8–10 μm) with two (in one case three) large and stout sterigmata (length, 12–18 μm; basal width, 3 μm). Basidiospores were narrowly ovoid to narrowly ellipsoid or subcylindrical (13–16 × 6–8 μm). Since *C. bicorne* is known only from the type location, the basidial dimensions given above are not necessarily completely representative in terms of the intraspecific variation of this species, but allowing for this variation in the uninucleate isolates they could well be *C. bicorne* (L. Ryvarden, pers. comm.). For confirmation it would be necessary to isolate this fungus and obtain information on the nuclear condition, cultural characteristics and anastomosis reaction with the uninucleate isolates.

The sexuality of the described uninucleate *Rhizoctonia* is at present being further investigated. The presence of predominantly uninucleate cells in all these, and many other isolates, would suggest that they are monokaryons of heterokaryotic bi- or multinucleate fungi. However, no morphological evidence of heterokaryon formation (e.g. aerial tufted mycelium) has yet been detected between paired isolates or within and between their single spore progeny although evidence of vegetative incompatibility, as indicated by strong demarcation zones, between paired progeny and their parents, is common (unpublished data). The fact that these isolates can be frequently isolated from conifer roots only as uninucleate hyphae does not support the monokaryon hypothesis either. Therefore it is possible that the killing reaction between uninucleate *Rhizoctonia* isolates is a sign of somatic incompatibility between the vegetative stage of this fungus, analogous to the one between *R. solani* field isolates (Ogoshi, 1987; Adams, 1988). It has also been possible to induce fruiting of single basidiospore progeny using the described system, as has been achieved in a *Ceratobasidium* sp. (Parmeter, Whitney & Platt, 1967). This may suggest primary homothallism but further analysis of the genetic condition of single basidiospore derived cultures using isozyme or DNA/RFLP markers are needed (see Adams, 1988).

These uninucleate *Rhizoctonia* appear to be important pathogens in the Nordic countries, causing root die-back in nursery seedlings of a range of conifer species, and thus information on the host range, mode of infection, population genetics and sexuality of these fungi are a major priority.

In conclusion, this uninucleate fungus does fit into *Ceratobasidium* but because of the unusual nuclear condition of the field isolates we would prefer to describe it as a uninucleate *Rhizoctonia* sp. having a *Ceratobasidium* fruiting stage.

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II

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Uni- and binucleate *Rhizoctonia* spp. co-existing on the roots of Norway-spruce seedlings suffering from root dieback

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Summary

Rhizoctonia fungi were isolated from the roots of 2-year-old nursery-grown Norway-spruce seedlings displaying root-dieback symptoms. The most frequently isolated species, a uninucleate *Rhizoctonia* sp., was found to co-exist with binucleate *Rhizoctonia* in the same root system of several seedlings. All the uninucleate isolates anastomosed with each other forming a single anastomosis group with common cultural characteristics. Binucleate *Rhizoctonia* isolates were divided into several, morphologically dissimilar anastomosis groups (AG-I, *R.* spp.). In a pathogenicity test under sterile conditions, isolates belonging to the uninucleate *Rhizoctonia* sp. infected all root regions, particularly the root tips, resulting in a stunted root-system morphology, as was also observed in the isolation material.

Binucleate *Rhizoctonia* spp. colonized only basal root regions, occasionally infecting cortical cells with monilioid hyphae, and had no effect on root growth.

1 Introduction

Since the first description of a *Rhizoctonia* species, *R. crocorum* (Pers.) DC: Fr., nearly 100 further *Rhizoctonia* species have been described (PARMETER and WHITNEY 1970). The genus encompasses a heterogeneous group of fungi with diverse relationships. PARMETER and WHITNEY (1970), and later OGOSHI (1975), therefore established criteria which restrict the genus *Rhizoctonia* to imperfect states of basidiomycetes.

Several basidiomycete genera, e.g. *Thanatephorus* and *Ceratobasidium*, possess the *Rhizoctonia* vegetative state. Since induction of fruiting of *Rhizoctonia* species in culture is often difficult, the identification of *Rhizoctonia* isolates has traditionally been based on anamorphic characteristics. However, the vegetative characteristics of some *Rhizoctonia* species overlap considerably, which leads to difficulties in species identification. The discovery of differences in the nuclear condition of field isolates of *R. solani* (multinucleate, genus *Thanatephorus*) and some *Rhizoctonia* anamorphs closely resembling them (binucleate, genus *Ceratobasidium*) has reduced the confusion frequently associated with these fungi (PARMETER and WHITNEY 1970), and the introduction of anastomosis groupings, together with cytomorphology of hyphae and morphology of cultures, has placed the characterization of *Rhizoctonia* isolates on a more solid footing. Today, *R. solani* isolates are assigned to anastomosis (incompatibility) groups based on affinities for hyphal fusion with members of designated anastomosis groups (AG-1 — AG-10 and AG-BI). In this way, *Rhizoctonia* species belonging to the genus *Ceratobasidium* have been grouped into 21 anastomosis groups (AG-A — AG-S; SNEH et al. 1991).

In Norway, root dieback of Norway spruce (*Picea abies* (L.) Karst.) seedlings has caused considerable losses in nursery production (VENN et al. 1986). The symptoms of the disease are needle discolouration, partial or total death of the root system, and stunted growth. A similar disease has occurred on Norway-spruce and Scots-pine (*Pinus sylvestris* L.) seedlings in Finnish forest nurseries (LILJA et al. 1992). A *Rhizoctonia* sp. has been found to be

involved with the disease in Norway and Finland (VENN et al. 1986; LILJA et al. 1992; LILJA 1994). Anastomosis pairing has confirmed that these Norwegian and Finnish isolates causing root dieback represent the same uninucleate *Rhizoctonia* sp., which, on the basis of basidial morphology, belongs to genus *Ceratobasidium* (HIETALA et al. 1994). Previous reports on the pathogenicity of *Rhizoctonia* spp. associated with root dieback of conifer seedlings (VENN et al. 1986; LILJA et al. 1992; LILJA 1994) have not included information on the vegetative characteristics, anastomosis groups, or mode of infection of the *Rhizoctonia* isolates studied. The aim of this study was to obtain information on the identity, pathogenicity and mode of infection of *Rhizoctonia* spp. isolated from Norway-spruce seedlings displaying root-dieback symptoms.

2 Material and methods

2.1 Isolates

Containerized 2-year-old Norway-spruce seedlings suffering from root dieback were collected from a nursery in southern Finland (Lamppi: 61°39′ N, 22°44′ E) in autumn 1992. Fungal isolations were obtained from the roots of 47 diseased seedlings as follows: 3-mm long pieces were cut from different parts of the root system displaying root-rot symptoms, washed under tap water, surface sterilized for 1 min in 0.5% sodium hypochlorite, rinsed three times with sterile distilled water, and transferred onto potato-dextrose agar (PDA) or 1.2% water agar. Plates were incubated at room temperature and examined daily. Mycelia typical of the form-genus *Rhizoctonia* were transferred onto PDA and grown for 21 days at 21 °C in darkness. A total of 145 *Rhizoctonia* isolates were obtained from 40 seedlings. They were grouped according to their cultural morphology on PDA and by screening of anastomosis reactions. Representative isolates from each group were chosen for further characterization. Since some of the original cultures contained sectors from which two *Rhizoctonia* types could be isolated, the representative isolates were purified by isolating a tip from a primary hypha (KORHONEN and HINTIKKA 1980).

Two strains, previously isolated from nursery-grown Norway-spruce seedlings, designated 249 (Kerimäki: 61°52′ N, 29°03′ E) and 250 (Lapinlahti: 63°21′ N, 27°24′ E), were also included in further studies. Isolate 250 was included in a paper describing a uninucleate *Rhizoctonia* sp. (HIETALA et al. 1994). The isolates used in this study are listed in Table 1.

2.2 Vegetative characteristics

For establishment of hyphal characteristics, isolates were grown on 1/8 PDA (4.88 g PDA and 13.12 g agar/l H_2O) coated slides at 21 °C for 48 h (HIETALA et al. 1994). The diameter of 15 subapical cells of the main runner hyphae were examined from four colonies at 1000 × magnification under a light microscope for each of the investigated isolates. The septal structure was examined under phase contrast. Nuclei were stained according to the procedures described by WILSON (1992). Colonial characteristics were determined from five replicate PDA plates of each isolate, which had been centrally inoculated and incubated at 21 °C in darkness for 21 days. The colours were designated according to RAYNER'S (1970) mycological colour chart. The colony diameter was measured every 24 h along two axes at right angles.

2.3 Hyphal anastomosis

Hyphal anastomosis was microscopically examined on 2% water agar to determine anastomosis groupings. Isolates were paired in all combinations at a distance of 1.5 cm by

Table 1. Isolate characteristics and the effects of inoculation with different isolates on the root growth of Norway-spruce seedlings. Uni- and binucleate isolate pairs B3-L/B3-J, T8-A/T8-B and B11-K/B11-L were originally isolated from the same seedlings

Isolate characteristics				Root-growth indices after incubation				
Strain no	Morphological[1] group	Nuclear condition	Anastomosis group	Total root length (cm)	Main-root length (cm)	Lateral-root length (cm)	Longest lateral root (cm)	No. of root tips
B3-L	1	uninucleate	UAG[2]	9.8^{c4}	7.0^{c}	2.7^{bc}	1.1^{cd}	9.9^{a}
T8-A	1	uninucleate	UAG[2]	9.1^{c}	6.0^{c}	3.1^{bc}	0.4^{d}	11.6^{a}
B11-K	1	uninucleate	UAG[2]	8.7^{c}	6.8^{c}	1.8^{c}	0.4^{d}	10.0^{a}
249	1	uninucleate	UAG[2]	13.5^{bc}	7.0^{c}	6.5^{abc}	1.6^{bcd}	18.2^{a}
250	1	uninucleate	UAG[2]	13.3^{bc}	7.9^{c}	5.4^{abc}	1.1^{cd}	15.9^{a}
mean				10.9^{B5}	6.9^{B}	3.9^{B}	0.9^{B}	13.1^{B}
T8-B	2	binucleacte	AG-I	24.2^{a}	11.9^{abc}	12.3^{a}	4.3^{abc}	20.2^{a}
T5-Y	2	binucleate	AG-I	27.3^{a}	14.5^{ab}	12.9^{a}	5.2^{ab}	20.5^{a}
B3-J	3	binucleate	?[3]	22.6^{ab}	11.7^{abc}	10.9^{ab}	3.8^{abcd}	20.5^{a}
B7-I	3	binucleate	?	28.0^{a}	16.8^{a}	11.1^{ab}	5.4^{a}	15.8^{a}
T1-C	4	binucleate	?	24.1^{a}	14.1^{ab}	10.0^{abc}	4.1^{abc}	18.7^{a}
B11-L	5	binucleate	?	22.2^{ab}	10.5^{bc}	12.0^{a}	3.9^{abcd}	20.2^{a}
mean				24.7^{A}	13.3^{A}	11.5^{A}	4.5^{A}	19.3^{A}
control				26.1^{aA}	14.1^{abA}	11.9^{aA}	4.4^{abA}	20.3^{aA}

[1] Morphological grouping is based on colonial characteristics on PDA after 21 days growth in darkness at 21 °C
[2] Anastomosis group of a uninucleate *Rhizoctonia* sp.. Tester strain was isolate 250 (HIETALA et al. 1994)
[3] Isolates anastomose with Finnish strains belonging to the same morphological group, but not with the tester strains (AG-A–AG-S) of genus *Ceratobasidium*
[4] Values are the means of 20 (control) or 10 (fungal inoculation) replicates. Means followed by the same lower-case letter in each column are not significantly ($p = 0.05$) different from each other using Tukey's HSD test
[5] Treatments are grouped on the basis of isolate nuclear condition. Means followed by the same upper-case letter in each column are not significantly ($p = 0.01$) different from each other using Tukey's HSD test

inoculating the agar using a modified Pasteur pipette (KORHONEN and HINTIKKA 1980). Pairings were incubated at 21 °C until the margins of opposing colonies began to overlap. Hyphal anastomosis was scanned at 100 × and confirmed at 400 × under a light microscope. Binucleate isolates were also paired with binucleate *Rhizoctonia* (*Ceratobasidium* spp.) tester isolates (AG-A to AG-S, deposited in the ATCC; SNEH et al. 1991).

2.4 Pathogenicity

The pathogenicity of isolates was tested under sterile conditions. Norway-spruce seeds were surface sterilized with 30% H_2O_2 as described by HIETALA et al. (1994). Sterilized seeds were plated on 1.2% water agar and incubated at 21 °C in the dark until germination. Germinated seedlings were transferred to 55 ml test tubes containing 20 ml fertilized peat (ST400 Finnpeat, Satoturve Oy, Finland) commonly used in forest nurseries. The peat had been sterilized by gamma irradiation (5 Mrad, 72 h) and moistened to a level of 40% (w/v). Tubes were sealed with sterile cotton plugs, weighed, and transferred to a green house, where the test was performed between June and August under natural lighting. During the test, the moisture of the peat was kept constant by weighing and watering each tube with sterile distilled water three times a week. The seedlings were inoculated at an age of 5 weeks with a 5 × 5 mm block taken from the margin of an actively growing PDA colony; 10 seedlings were inoculated with each isolate. As a control, 20 seedlings were inoculated with a sterile 5 × 5 mm PDA block. Seedlings were harvested at an age of 9 weeks, their roots were washed clean under tap water, and each root system was photocopied. The length of the roots was measured with a map measurer and the number of root tips was counted. Four root systems from each treatment were stained with trypan blue according to the procedures described by PHILLIPS and HAYMAN (1970). Stained root systems were stored in lactic acid at room temperature in darkness and examined for surface hyphae and infection with a light microscope at 40–400 × magnification. The analysis of variance and Tukey's HSD test were used for statistical analysis.

3 Results

3.1 Vegetative characteristics and hyphal anastomosis

All examined isolates showed *Rhizoctonia* characteristics. Basally constricted hyphal branches arose near the distal septum of cells. A dolipore septum was formed near the point of origin of the branch. No rhizomorphs, clamp connections, or conidia, except for monilioid cells, were observed. The sclerotia were constructed of monilioid cells that were not organized into a rind and medulla.

The isolates could be grouped into five groups on the basis of cultural morphology and anastomosis reactions (Table 1). Of the 145 isolates, 73 (obtained from 32 seedlings) had uninucleate cells and common cultural characteristics, and the remaining 72 isolates (obtained from 25 seedlings) were quite evenly distributed within four binucleate, morphologically dissimilar groups. A total of 18 seedlings hosted both a uni- and a binucleate *Rhizoctonia* group; all the four binucleate groups were represented in these seedlings. In one seedling, isolates belonging to two binucleate groups could be found together with a uninucleate *Rhizoctonia*. When the seedling hosted more than one *Rhizoctonia* type, they were almost always isolated from different root segments.

Isolates B3-L (code: seedling — isolation), T8-A, B11-K, 249 and 250 were uninucleate, with mean hyphal diameters of 5.7–6.3 μm (range of individual measurements: 5–8 μm) and a diametric growth rate of 13.5–15.0 mm/24 h. Culturally, the isolates were practically identical to the reference isolate 250: distinctive, small, hazel-coloured regions within the otherwise buff-coloured surface hyphae gave cultures a spotted appearance (HIETALA et al.

1994). Surface-located and submerged, and fulvous to umber-coloured sclerotia were readily formed. All isolates anastomosed with each other producing a killing reaction, as described by YOKOYAMA and OGOSHI (1986) for *R. solani* isolates. Isolates did not anastomose with the binucleate Finnish isolates tested.

Isolates T5-Y and T8-B were binucleate, with a hyphal diameter of 5.8–6.3 μm (range 5–7 μm) and a growth rate of 13.1–15.4 mm/24 h. Isolates had white-to-cream-coloured surface hyphae with strong zonation. Isolate T8-B also had aerial tufts of monilioid hyphae. These two isolates anastomosed with each other, and with the culturally similar AG-I (tester isolate ATCC 76143), producing a killing reaction.

Isolates B3-J and B7-I were binucleate, with a hyphal diameter of 5.2–5.4 μm (range 4–6 μm) and a growth rate of 11.8–13.2 mm/24 h. Surface and aerial mycelium were buff coloured and both isolates formed a few embedded, isabelline-coloured sclerotia. Isolate B7-I also readily formed buff-to-isabelline-coloured surface sclerotia. Isolates anastomosed with each other producing a killing reaction. Isolates did not anastomose with any of the tester strains of the genus *Ceratobasidium*.

Isolate T1-C was binucleate, with a hyphal diameter of 5.1 μm (range 4–6 μm) and a growth rate of 12.6 mm/24 h. Culturally, the isolate was quite different from other examined groups; the buff-to-rosy-buff-coloured surface mycelium was strong and continuous in the centre of the colony. Surface sclerotia were primrose; embedded sclerotia almost orange. T1-C did not anastomose with any of the Finnish isolates belonging to another morphological group, nor with any of the tester isolates.

Isolate B11-L was binucleate, with a hyphal diameter of 5.6 μm (range 5–7 μm) and a growth rate of 14.2 mm/24 h. The velvet-like, white-to-buff-coloured surface mycelium was centrally zonated. No sclerotia or aerial tufts of monilioid cells were formed. B11-L did not anastomose with any of the Finnish isolates belonging to another morphological group, nor with any of the tester isolates.

3.2 Pathogenicity test

Staining of sampled root systems showed the presence of *Rhizoctonia* hyphae on all inoculated roots. However, the seedlings survived in all treatments during the experiment.

Roots inoculated with uninucleate isolates were strongly pigmented and clearly stunted. The effect of inoculations on root growth is shown in Table 1. The roots inoculated with binucleate isolates were not statistically different from the control in any root terms. Seedlings inoculated with a uninucleate isolate clearly showed poorer root growth than the control seedlings and those inoculated with binucleate strains, and they differed statistically from the control seedlings in terms of total root length and length of the main root. When comparing the effect of uni- and binucleate strains isolated from the same seedling, the seedlings inoculated with uninucleate strains showed generally poorer root growth than the seedlings inoculated with the binucleate strains. This was most clearly shown in total root length; all the treatments inoculated with a uninucleate isolate differed statistically from the treatment inoculated with the corresponding binucleate isolate. When seedlings were grouped on the basis of the nuclear condition of the inoculated fungus, the uninucleate group was significantly different from the control and binucleate group in all root indices.

The uninucleate isolates colonized the root surface along the whole root system and practically all root tips were infected by continuous mass of aggregations of short, branched hyphae and appressoria-like structures, often several hundred μm long (Fig. 1a, b). Similar aggregates, although considerably smaller, were also formed abundantly on other root areas infecting the cortex (Fig. 1c). Focussing through the tissues to the centre of the root suggested that, in the root tips, uninucleate isolates grow in the vascular cylinder (Fig. 1d).

In seedlings inoculated with binucleate isolates, roots were commonly free of hyphae up to several mm behind the root tip. Compared to treatments inoculated with uninucleate

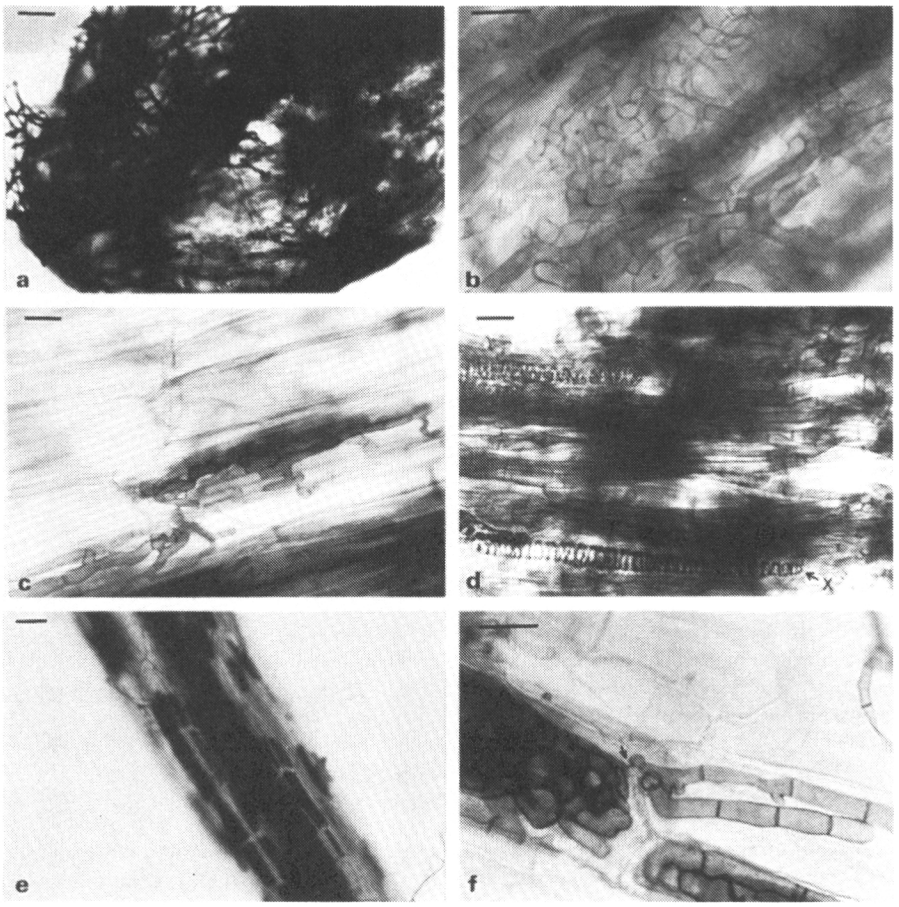

Fig. 1. Root infection by isolates T8-A (uninucleate *Rhizoctonia* sp., a–d) and T8-B (AG-I, e, f) that were originally isolated from the same root system; a. A lateral root tip typically covered with hyphae (bar 50 μm); b. Hyphal aggregates on the surface of a lateral root tip (bar 20 μm); c. Hyphae within cortical cells in the main root (bar 20 μm); d. Internal hyphae as seen in the root tip near the apical meristem when focussing through the tissues to the vascular cylinder (x ° xylem, bar 20 μm); e. An overall view of a region near the base of the main root. The cortical cells filled with monilioid hyphae are heavily stained (bar 100 μm); f. A close-up picture of cortical cells seen in e. Note hyphal growth through plant cell wall (arrowed, bar 20 μm)

isolates, roots inoculated with binucleate *Rhizoctonia* had relatively little surface hyphae, except in the older parts of the main root. In this region, binucleate isolates, excluding isolate T1-C, occasionally infected cortical cells with intensely stained monilioid hyphae filling the entire cell. In certain regions of roots inoculated with isolates T5-Y and T8-B, whole areas could be found with this kind of infection (Fig. 1e). Within the cortex, the hyphae seemed to spread to neighbouring cells through cell walls (Fig. 1f). Similar infection of cortex cells by monilioid hyphae was observed in one sample of the uninucleate isolate B11-K. In the seedlings inoculated with the binucleate isolate T1-C, there was little surface mycelium in basal root areas without any clear infection.

4 Discussion

On the basis of vegetative characteristics and hyphal anastomosis, the uninucleate isolates belong to a *Rhizoctonia* sp. characterized by HIETALA et al. (1994). The data presented here and in a previous report (HIETALA et al. 1994) suggest the presence of a single anastomosis group within this morphologically very homogeneous species. There are no reports about this species outside the Nordic countries. Excluding the nuclear condition, the anamorphic characteristics of this uninucleate *Rhizoctonia* sp. closely resemble those of *R. solani* (PARMETER and WHITNEY 1970), which emphasizes the importance of the determination of nuclear condition in *Rhizoctonia* studies.

Binucleate isolates T5-Y and T8-B belong to anastomosis group AG-I of genus *Ceratobasidium*. The anamorphic state of AG-I is *R. fragariae* (SNEH et al. 1991). There are no previous reports concerning AG-I or *R. fragariae* associated with conifer seedlings, as AG-I is normally associated with root rot in strawberry fields (MARTIN 1988). In Finland, isolates belonging to AG-I seem to be rather common in the roots of nursery-grown Scots pine and Norway spruce seedlings (R. SEN and A. M. HIETALA unpubl. data). Negative results in the anastomosis pairings between T5-Y, T8-A, and the other Finnish isolates (T1-C, B3-J, B7-I, B11-L) indicate that they represent different species, as their differing cultural morphology would also suggest. There are two alternative explanations for the negative result in the anastomosis test between the latter isolates and the tester strains of the genus *Ceratobasidium*; either these Finnish isolates do not belong to this genus, or they represent new anastomosis groups. Further studies applying different fruiting techniques are needed to solve this question.

The staining method employed proved to be convenient for screening entire root systems for surface hyphae and internal infection, especially in the cortical regions. In a detailed histopathological study, this method would be useful in pointing out areas of interest for microtome sectioning and further analysis of infection. In roots inoculated with binucleate *Rhizoctonia* spp., the surface hyphae and infection were restricted to basal root regions, while isolates belonging to the uninucleate *Rhizoctonia* sp. also infected root tips, which resulted in the stunted morphology of the root system observed in the isolation material. Results from the pathogenicity test are in agreement with previous work done in Finland. When testing the pathogenicity of *Rhizoctonia* isolates obtained from nursery-grown conifer seedlings suffering from root dieback, LILJA et al. (1992) and LILJA (1994) found that only uninucleate *Rhizoctonia* isolates affected root growth of the test plants, Scots pine and Norway spruce, while binucleate and multinucleate *Rhizoctonia* isolates were non-pathogenic. Unfortunately, the anastomosis groups of these bi- and multinucleate *Rhizoctonia* isolates were not examined, which makes direct comparison with this study difficult.

Many isolates, especially the binucleate ones, formed monilioid hyphae within the cells of the root cortex in this study. *R. fragariae*, the anamorph of AG-I (SNEH et al. 1991), is associated with black root rot in strawberries and infects cortical cells with monilioid hyphae causing sloughing of the cortex and death of roots (RIBEIRO and BLACK 1971; WILHELM et al. 1972). Longer incubation during the pathogenicity test is possibly needed to show whether the now characterized binucleate *Rhizoctonia* spp. have a similar effect on the root growth of Norway spruce. However, recent results from work with Scots pine seedlings (R. SEN and A. M. HIETALA unpubl. data) suggest that several binucleate *Rhizoctonia* spp., including isolates belonging to AG-I, promote seedling growth, as also observed by LILJA (1994) for uncharacterized binucleate *Rhizoctonia* spp. associated with Norway-spruce seedlings.

There are also other reports concerning the infection of cortical cells with monilioid hyphae by *Rhizoctonia* spp.. SAKSENA and VAARTAJA (1961) reported that several *Rhizoctonia* species (*R. endophytica* var. *endophytica* Saks. & Vaar., *R. globularis* Saks. & Vaar., *R. repens* Bernard and *R. callae* Cast.) infected all root parts of conifer seedlings with

monilioid hyphae in a poorly developed root system. According to SNEH et al. (1991), *R. endophytica* var. *endophytica* belongs to anastomosis group AG-A, and *R. globularis* to AG-C of genus *Ceratobasidium. R. repens* is binucleate, has narrow hyphae (2–3.5 μm) and belongs to the genus *Tulasnella. R. callae* is also binucleate, however, its teleomorph has not been determined (SNEH et al. 1991).

There are few reports concerning the co-existence of *Rhizoctonia* spp. on the same plant. YAMAMOTO and ARAGAKI (1982) suspected that, when studying damping-off of papaya, the causative agent, *R. solani* (AG-4), was overlooked in the random selection of hyphae, and a co-existing avirulent binucleate *Rhizoctonia* sp. was subcultured. These now characterized *Rhizoctonia* spp. have such similar hyphal diameters and growth rates, that this possibility of their being overlooked is a real one if only a small proportion of emerging hyphae are subcultured at the isolation stage. When studying co-existing *Rhizoctonia* species, the isolates should be obtained as single hyphal isolations to avoid potential mixed cultures. Future studies will show how common the co-existence of *Rhizoctonia* spp. is on conifers and, with dual inoculations for comparison, the nature of this relationship could be clarified.

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Résumé

Co-existence de Rhizoctonia spp. uni- et binucléés dans les racines de semis d'épicéas communs présentant un dépérissement racinaire

Des *Rhizoctonia* ont été isolés des racines de semis d'épicéa commun âgés de 2 ans en pépinière, qui montraient des symptômes de dépérissement. L'espèce la plus fréquemment isolée, un *Rhizoctonia* sp. uninucléé, a été trouvée en coexistence avec des *Rhizoctonia* binucléés dans le système racinaire de plusieurs semis. Tous les isolats uninucléés s'anastamosaient entre eux, formant un seul groupe d'anastomose avec les mêmes caractères culturaux. Les isolats binucléés ont été divisés en plusieurs groupes d'anastomose, morphologiquement distincts (AG-I, *R.*spp). Dans un test de pouvoir pathogène en conditions stériles, les isolats du groupe uninucléé infectaient toutes les parties de racines et en particulier les extrémités, conduisant à une morphologie chétive du système racinaire, déjà observée sur le matériel lors des isolements. Les *Rhizoctonia* binucléés ne colonisaient que les parties basales des racines, infectant occasionnellement les cellules corticales avec des hyphes monilioides, mais sans effet sur la croissance racinaire.

Zusammenfassung

Gemeinsames Vorkommen von uni- und binucleaten Rhizoctonia-Arten in geschädigten Wurzeln von Picea abies-Sämlingen

Aus Wurzeln von 2-jährigen Fichtensämlingen, die in einer Baumschule wuchsen und Symptome von Wurzelsterben zeigten, wurden *Rhizoctonia*-Arten isoliert. Die am häufigsten isolierte Art, eine uninucleate *Rhizoctonia* sp., kam bei mehreren Sämlingen gleichzeitig mit binucleaten *Rhizoctonia*-Arten im gleichen Wurzelsystem vor. Alle uninucleaten Isolate anastomosierten miteinander und bildeten eine gemeinsame Anastomosierungsgruppe mit gleicher Kulturcharakteristik. Die binucleaten *Rhizoctonia*-Isolate waren in mehrere morphologisch verschiedene Anastomosierungsgruppen unterteilt (AG-I, *Rhizoctonia* spp.). In einem Pathogenitätstest unter sterilen Bedingungen infizierten die uninucleaten *Rhizoctonia*-Isolate alle Bereiche des Wurzelsystems, insbesondere jedoch die Wurzelspitzen. Dies führte zu einer Stauchung des Wurzelsystems, wie sie auch an dem Material beobachtet wurde, von dem die Pilze isoliert worden waren. Binucleate *Rhizoctonia*-Arten besiedelten nur die

basalen Teile der Wurzel; sie infizierten gelegentlich Cortexzellen mit monilioiden Hyphen, hatten aber keine Auswirkungen auf das Wurzelwachstum.

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III

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Identification of a uninucleate *Rhizoctonia* sp. by pathogenicity, hyphal anastomosis and RAPD analysis

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Finnish and Norwegian uninucleate *Rhizoctonia* sp. isolates, originating from roots of nursery grown conifer seedlings suffering from root dieback, and having *Ceratobasidium* perfect state, were tested for pathogenicity and genetic relatedness. All tested isolates of this pathogen considerably reduced the root system development of Scots pine and Norway spruce seedlings resulting in death or stunted growth. The uninucleate isolates anastomosed readily with each other producing a killing reaction. In a RAPD-PCR analysis, the uninucleate isolates had different banding patterns from our reference isolates, two Finnish binucleate isolates (AG-I and *R.* sp.) and standard tester isolates of genus *Ceratobasidium* representing anastomosis groups AG-A, AG-C, AG-E, AG-G and AG-I. UPGMA analysis clustered the uninucleate isolates together at a greater similarity than 75% while the binucleate isolates formed distinct clusters and were 10–25% similar to the uninucleate *Rhizoctonia* sp. Hyphal anastomosis and DNA data suggest that the uninucleate *Rhizoctonia* sp. is an homogeneous group and distinct from the tested binucleate *Rhizoctonia* isolates.

INTRODUCTION

Root dieback of nursery grown conifer seedlings has caused considerable economic losses in Norway and Finland. Surveys for fungi present in diseased roots and subsequent pathogenicity trials indicate that root dieback is a complex involving both *Rhizoctonia* and *Pythium* spp. (Venn *et al.*, 1986; Lilja *et al.*, 1992). In Finland, the most pathogenic *Rhizoctonia* species is uninucleate (Lilja *et al.*, 1992; Lilja, 1994) and, on the basis of cultural morphology and hyphal anastomosis it represents the same *Rhizoctonia* sp. studied in Norway by Venn *et al.* (1986; Hietala *et al.*, 1994). Uninucleate *Rhizoctonia* sp. can be fruited under laboratory conditions and belongs to the genus *Ceratobasidium* (Hietala *et al.*, 1994). All previously known anamorphs of *Ceratobasidium* have binucleate hyphal cells. In addition, anastomosis tests indicated that this species is not related to the known anastomosis groups (AG-A–AG-S) of *Ceratobasidium* (Hietala *et al.*, 1994).

Besides the uninucleate *Rhizoctonia* sp., binucleate *Rhizoctonia* spp. have also been isolated from roots of conifer seedlings showing root dieback symptoms in Finland. The tested isolates representing binucleate *Rhizoctonia* spp. have not been shown to be pathogenic (Lilja *et al.*, 1992; Lilja, 1994; Hietala, 1995). These binucleate *Rhizoctonia* spp. show considerable morphological variation and can be divided into several anastomosis groups (AG-I, R. spp.) (Hietala, 1995). Anastomosis groups AG-A (Ogoshi *et al.*, 1983) and AG-E (= CAG-3) (English *et al.*, 1986; Huang & Kuhlman, 1990; Runion & Kelly, 1993) have been identified for isolates related to other diseases of conifer seedlings.

The classification of *Rhizoctonia* spp. is based on hyphal and cultural morphology, nuclear condition, hyphal anastomosis and morphology of teleomorphs (Sneh *et al.*, 1991). The perfect stage of *Rhizoctonia* is often difficult to obtain and the presence of bridging isolates has led to the search for biochemical tools for better taxonomic resolution (Mordue *et al.*, 1989; Vilgalys & Cubeta, 1994). *Rhizoctonia* strains have been identified by electrophoresis of soluble proteins (Reynolds *et al.*, 1983) and by isozymes (Sweetingham *et al.*, 1986; Cruickshank, 1990; Damaj *et al.*, 1993; Masuhara & Neate, 1994). DNA/DNA hybridization analysis has been

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Table 1 Identities and sources of *Rhizoctonia* spp. isolates

Country	Nursery	Location	Date	Tree species	Code of isolate
Finland	Taavetti	60°55′N, 27°33′E	17.05.91	*Picea abies*	298
Finland	Mellanå	62°13′N, 21°39′E	15.10.90	*Pinus sylvestris*	290
Finland	Mellanå	62°13′N, 21°39′E	15.05.91	*Pinus sylvestris*	297
Finland	Imari	66°29′N, 25°32′E	15.08.91	*Pinus sylvestris*	257
Finland	Jomala	60°09′N, 19°57′E	29.08.91	*Larix sibirica*	256
Finland	Ahlainen	61°39′N, 22°44′E	27.09.87	*Pinus sylvestris*	261
Finland	Lapinlahti	63°21′N, 27°24′E	23.10.87	*Pinus sylvestris*	263
Finland	Lapinlahti	63°21′N, 27°24′E	05.05.89	*Pinus sylvestris*	264
Finland	Lapinlahti	63°21′N, 27°24′E	11.10.89	*Pinus sylvestris*	260
Finland	Lapinlahti	63°21′N, 27°24′E	14.08.92	*Picea abies*	250
Finland	Metsätyllilä	61°25′N, 25°53′E	05.05.89	*Pinus sylvestris*	268
Finland	Metsätyllilä	61°25′N, 25°53′E	28.09.93	*Picea abies*	248
Finland	Metsätyllilä	61°25′N, 25°53′E	10.10.95	*Abies koreana*	245
Finland	Suonenjoki	62°39′N, 27°04′E	23.05.94	*Pinus sylvestris*	246
Finland	Syrjälä	61°53′N, 29°10′E	23.07.93	*Picea abies*	249
Finland	Ukonniemi	61°12′N, 28°15′E	05.07.91	*Pinus sylvestris*	255
Norway	Gvarv	59°23′N, 09°11′E	27.03.87	*Picea abies*	87-691/3
Norway	Kvatningen	64°29′N, 11°29′E	12.09.83	*Picea abies*	83-111/1N
Norway	Prestebakke	58°55′N, 11°40′E	25.03.85	*Picea abies*	85-387/Na

successfully used to show that multinucleate and binucleate *Rhizoctonia* spp. are unrelated (Vilgalys, 1988; Vilgalys & Cubeta, 1994). Restriction analysis of amplified PCR products (Cubeta *et al.*, 1991) has been used to study the genetic relationships among groups of binucleate *Rhizoctonia*, and their results supported anastomosis grouping.

Randomly Amplified Polymorphic DNA (RAPD) markers have been used for genetic mapping (Welsh & McClelland, 1990; Williams *et al.*, 1990), population variation studies (Hadrys *et al.*, 1992) and taxonomic problems (Demeke *et al.*, 1992; Heun *et al.*, 1994). RAPD markers have been widely applied to measure genetic variation with *Fusarium* spp. (Manulis *et al.*, 1994; Yli-Mattila *et al.*, 1996) and *R. solani* (Duncan *et al.*, 1993). The reliability and reproducibility of RAPD fingerprinting in standard reaction conditions was recently demonstrated (Tommerup *et al.*, 1995). In this study we investigated the similarity of isolates representing the recently characterized uninucleate *Rhizoctonia* sp. by pathogenicity tests, nuclear condition, hyphal anastomosis and RAPD markers.

MATERIALS AND METHODS

Rhizoctonia spp. isolates

Seventeen Finnish and Norwegian uninucleate *Rhizoctonia* isolates isolated from roots of nursery-grown conifer seedlings showing root dieback symptoms were used in this study (Table 1). Using basidial dimensions and hyphal anastomosis, six isolates (five Finnish: 250, 256, 260, 263, 264; one Norwegian: 83-111/1N) were characterized previously as an anamorph of the genus *Ceratobasidium*, not related to the known anastomosis groups AG-A–AG-S (Hietala *et al.*, 1994). In addition, nine uninucleate Finnish and two Norwegian *Rhizoctonia* sp. isolates were examined in further studies. Two Finnish binucleate *Rhizoctonia* spp. isolates, 268 and 245, were included as reference isolates. Standard tester strains (see Sneh *et al.*, 1991) of selected anastomosis groups of the genus *Ceratobasidium* (AG-A, AG-C, AG-E, AG-G and AG-I), supplied by Akira Ogoshi (Hokkaido University), were also included as reference isolates in RAPD analysis.

Pathogenicity tests

Ten-week-old seedlings

In the first experiment, 10-week-old Scots pine and Norway spruce seedlings growing in fertilized (N 15%, P 5%, K 15% plus micronutrients, 1 kg/m^3), low-humified sphagnum peat (Finn peat M6) were inoculated with each of the 17

uninucleate isolates and with the two Finnish binucleate isolates. The experimental conditions and design were as described by Lilja (1994), with the exception that the number of replicates per treatment was six. The controls were inoculated with pure agar blocks. The seedlings were harvested after an incubation period of 8 weeks. Root systems were carefully washed under tap water and the main root length and the lengths of the three longest lateral roots and shoot and root dry weights were measured. Before the determination of the dry weight of the roots, 1-mm segments were cut from the main roots of one randomly chosen living seedling representing each treatment and from all dead seedlings. Fungal isolation was done from these segments as described before (Lilja *et al.*, 1992) to assess the presence of the inoculated fungus.

One- and 2-year-old seedlings

In the second experiment, 1- and 2-year-old Scots pine seedlings were transplanted into 0·8 L pots containing the same kind of peat as described before. Uninucleate *Rhizoctonia* isolate 264 was grown on cellophane on water agar (12 g/L, Bacto agar, Difco) for 1 week, and two 3·5 cm^2 pieces of the mycelium on cellophane were buried 9 cm deep on both sides of the seedling 2 cm from the edge of the pot. There was one 1-year-old and one 2-year-old seedling in each pot. Twenty inoculated and 20 uninoculated pots were arranged randomly in a greenhouse, where the temperature was 20°C and day length 16 h. The seedlings were watered so that the peat in the pots dried between waterings, and they were fertilized (0·1% Suprex 5: N 11%, P 4%, K 25% plus micronutrients) every second week. The dry weight of the root system and the length of the main root were measured after the test period of 7 months. The data from both experiments were analysed using ANOVA and the significance of the mean differences in the first pathogenicity test was determined with Duncan's multiple range test using BMDP statistical software (Anonymous, 1990).

Nuclear condition and hyphal anastomosis

The nuclear condition of all Finnish and Norwegian isolates was examined after growing the isolates on microscope slides coated with low strength (1/8) potato dextrose agar (4·88 g/L PDA, Difco and 13·12 g/L Bacto agar) that were maintained in a moist atmosphere at 24°C for 48 h (Hietala *et al.*, 1994). The nuclei were stained with HCl–Giemsa following the fixing and staining procedures described by Wilson (1992), and nuclear numbers in tip and subapical cell pairs (100 cells each) were counted at 400× magnification.

Hyphal anastomosis was examined in Petri dishes containing water agar (15 g/L Bacto agar) amended with malt extract (1 g/L). Uninucleate *Rhizoctonia* isolates were paired against each other in all combinations at a distance of 2 cm by inoculating the agar surface using a modified Pasteur pipette (Korhonen & Hintikka, 1980). The two Finnish binucleate isolates were similarly paired against each other and the included tester isolates of *Ceratobasidium*. Pairings were incubated at 21°C until the margins of opposed colonies overlapped. Hyphal anastomosis was scanned at 100× and confirmed at 400× magnification using light microscopy.

RAPD analysis

DNA isolation

Isolates were cultured for 5–7 days at 21°C on PDA (39 g/L) with a sterile cellophane membrane on the surface. The mycelium was scraped off and ground to fine powder under liquid N_2 with a mortar and pestle. DNA was purified essentially as described by Lee & Taylor (1990), but including additional phenol extraction and RNAse treatments (Karjalainen & Kammiovirta, 1994). The genomic DNA pellet was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8·0), and stored at −20°C until used.

DNA amplification

Amplification conditions were modified from those of Williams *et al.* (1990) in the following way: 50 μL reaction volume was used, including 25 ng of genomic DNA, 200 μM of each dNTP, 200 nM of primer, and 5 μL 10 × polymerase buffer (0·1 M Tris–HCl, pH 8·3, 1 mg/mL BSA, 0·5 M KCl, 10–50 mM $MgCl_2$) to give a final Mg concentration in the PCR mixture ranging from 1·0 to 5·0 mM according to suggestions by D. Howland, G. Arnan and R. Oliver from the University of East Anglia, UK, and 1 U of DynaZyme polymerase (Finnzymes Co, Finland). Premixtures (without DNA) of each reaction were made to minimize the risk of contamination. The reaction was overlaid with sterile paraffin oil (two drops). Amplification was

Table 2 The effects of inoculation with different *Rhizoctonia* isolates on the root growth of Scots pine seedlings after 2 months' incubation

Code of isolate	Nuclear condition	Percentage of dead seedlings	Main root length (cm) mean±SE	Lateral root length (cm) mean±SE	Dry weight of roots (mg) mean±SE	Dry weight of shoots (mg) mean±SE
Control		0	60·8 ± 5·6	32·0 ± 2·3	278·0 ± 32·7	559·3 ± 61·9
245	Binucleate	5·6 ± 5·6	49·7 ± 4·2	21·5 ± 1·3[a]	234·5 ± 25·9	590·0 ± 54·5
268	Binucleate	16·7 ± 11·4	43·8 ± 4·8[a]	18·5 ± 3·1[a]	205·2 ± 55·3	583·5 ± 122·1
Mean±SE		11·1 ± 6·3	46·7 ± 3·2	20·0 ± 1·7	219·8 ± 29·4	586·7 ± 63·7
246	Uninucleate	11·0 ± 7·0	15·7 ± 2·3[a]	5·5 ± 0·4[a]	44·0 ± 8·5[a]	250·0 ± 41·6[a]
248	Uninucleate	11·1 ± 11·1	29·5 ± 4·2[a]	13·7 ± 1·9[a]	118·8 ± 25·9[a]	463·8 ± 50·4
249	Uninucleate	5·6 ± 5·6	35·2 ± 1·8[a]	14·0 ± 1·9[a]	123·5 ± 18·9[a]	400·8 ± 56·4
250	Uninucleate	0	38·2 ± 4·2[a]	18·0 ± 2·4[a]	170·8 ± 22·0[a]	572·3 ± 54·2
255	Uninucleate	11·1 ± 7·0	32·5 ± 4·0[a]	13·0 ± 1·8[a]	116·5 ± 23·1[a]	433·2 ± 37·2
256	Uninucleate	5.5 ± 5·6	31·7 ± 3·4[a]	12·2 ± 1·3[a]	125·0 ± 17·8[a]	434·7 ± 52·6
257	Uninucleate	0	24·2 ± 2·5[a]	10·3 ± 0·9[a]	90·7 ± 9.6[a]	412·5 ± 49·8
260	Uninucleate	5·6 ± 5·6	26·2 ± 4·3[a]	10·5 ± 2·2[a]	84·8 ± 16·6[a]	366·0 ± 56·9
261	Uninucleate	0	28·5 ± 8·3[a]	9·7 ± 2·2[a]	102·5 ± 31·6[a]	384·0 ± 68·7
263	Uninucleate	0	32·2 ± 2·5[a]	11·2 ± 1·0[a]	105·2 ± 11·7[a]	435·0 ± 30·0
264	Uninucleate	5·6 ± 5·6	30·7 ± 4·5[a]	12·8 ± 1·9[a]	125·2 ± 36·5[a]	372·2 ± 73·5
290	Uninucleate	11·1 ± 7·0	22·7 ± 3·3[a]	10·0 ± 1·1[a]	97·5 ± 16·9[a]	487·2 ± 53·7
297	Uninucleate	16·6 ± 7.0	22·5 ± 1·9[a]	10·8 ± 1·1[a]	98·7 ± 17·7[a]	344·0 ± 18·3[a]
298	Uninucleate	11·1 ± 7.0	27·8 ± 3·5[a]	10·5 ± 1·5[a]	98·8 ± 27·7[a]	430·0 ± 103·5
83-111/1N	Uninucleate	0	37·2 ± 5·6[a]	11·7 ± 1·6[a]	136·3 ± 19·3[a]	464·8 ± 43·1
85-387/Na	Uninucleate	16·6 ± 7·4	34·8 ± 3·5[a]	12·8 ± 1·3[a]	126·3 ± 10·8[a]	502·7 ± 20·4
87-691/3	Uninucleate	0	48·0 ± 4·4[a]	19·0 ± 1·8[a]	227·5 ± 22·2	611·5 ± 32·6
Mean±SE		6·5 ± 1·4	30·4 ± 1·1	12·1 ± 0·5	117·2 ± 5·9	433·2 ± 14·4
F			6·40	10·64	5·50	2·52
P			0·001	0·001	0·001	0·002

[a] Means differ significantly ($P = 0·05$) from control using Duncan's multiple range test.

performed in an MJ Research Programmable Thermal Controller programmed for 40 cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. Amplification products were electrophoresed in 1·4% agarose gels with 1 × TBE (0·089 M Tris–borate, 0·002 M EDTA) and stained with ethidium bromide. Amplifications were repeated twice for each isolate and only reproducible bands were scored.

The nucleotide sequences of primers were generated randomly using the Applied Biosystems DNA synthesizer (Model 381A) at the Biotechnology Institute of the University of Helsinki. Seven primers were tested, and the three best ones (91298: GGA CGA TTC G; 91299: CGA TTC GGC G; 91300: CGA GGT TCG C) were selected for further study. Primers 91299 and 91300 have been used in previous studies for identifying strains of *Heterobasidion annosum* (Fabritius & Karjalainen, 1993; Karjalainen & Kammiovirta, 1994) and *Fusarium avenaceum* (Yli-Mattila *et al.*, 1996). The statistical analysis of RAPD data, similarity coefficients (DICE) and clustering analysis was based on the NTSYS program (Rohlf, 1989).

RESULTS

Pathogenicity

In the first pathogenicity experiment 0–16·6% and 5·6–16·7% of Scots pine seedlings and 0–22·2% and 0–5·6% of Norway spruce seedlings were killed, 2 months after inoculation with uninucleate and binucleate *Rhizoctonia*, respectively (Tables 2 and 3). All control seedlings were alive (Tables 2 and 3). In general, both uni- and binucleate *Rhizoctonia* decreased the growth of Scots pine and Norway spruce seedlings, but the

Table 3 The effects of inoculation with different *Rhizoctonia* isolates on the root growth of Norway spruce seedlings after 2 months' incubation

Code of isolate	Nuclear condition	Percentage of dead seedlings	Main root length (cm) mean±SE	Lateral root length (cm) mean±SE	Dry weight of roots (mg) mean±SE	Dry weight of shoots (mg) mean±SE
Control		0	21·2 ± 1·4	12·5 ± 0·9	34·7 ± 3·4	78·0 ± 6·2
245	Binucleate	5·6 ± 5·6	14.3 ± 1·1[a]	8·2 ± 0·7[a]	22·0 ± 1·3[a]	54·7 ± 4·3[a]
268	Binucleate	0	17·8 ± 1·2	9·3 ± 1·2[a]	20·5 ± 2·9[a]	53·3 ± 5·9[a]
Mean±SE		2·8 ± 2·8	16·1 ± 0·9	8·7 ± 0·7	21·2 ± 1·5	54·0 ± 3·5
246	Uninucleate	0	12·8 ± 1·0[a]	4·0 ± 0·4[a]	19·5 ± 2·1[a]	48·0 ± 4·9[a]
248	Uninucleate	0	14·6 ± 1·7[a]	5·2 ± 0·4[a]	20·2 ± 4·4[a]	45·3 ± 8·3[a]
249	Uninucleate	0	14·8 ± 1·1[a]	6·0 ± 0·5[a]	22·3 ± 3·3[a]	59·3 ± 7·3
250	Uninucleate	11·1 ± 7·0	15·8 ± 1·5[a]	6·7 ± 0·6[a]	21·7 ± 3·2[a]	48·7 ± 7·1[a]
255	Uninucleate	5·6 ± 5·6	13·1 ± 1·8[a]	6·0 ± 0·8[a]	16·5 ± 3·4[a]	44·3 ± 6·9[a]
256	Uninucleate	22·2 ± 7·0	12·8 ± 1·8[a]	3·8 ± 0·5[a]	14·0 ± 3·5[a]	41·7 ± 10·6[a]
257	Uninucleate	0	13·7 ± 1·4[a]	4·1 ± 0·6[a]	15·5 ± 3·0[a]	40·3 ± 6·4[a]
260	Uninucleate	11·1 ± 7·0	13·0 ± 0·7[a]	4·5 ± 0·6[a]	15·8 ± 3·9[a]	41·3 ± 10·9[a]
261	Uninucleate	5·6 ± 5·6	13·5 ± 1·5[a]	5·3 ± 0·8[a]	22·5 ± 3·1[a]	55·5 ± 4·9[a]
263	Uninucleate	11·1 ± 7·0	18·3 ± 2·7	5·5 ± 0·9[a]	19·0 ± 3·1[a]	41·8 ± 5·3[a]
264	Uninucleate	5·6 ± 5·6	15·8 ± 2·2[a]	7·2 ± 1·3[a]	25·0 ± 2·9[a]	43·5 ± 7·3[a]
290	Uninucleate	5·6 ± 5·6	13·0 ± 1·7[a]	4·0 ± 0·9[a]	17·3 ± 3·8[a]	50·7 ± 7·8[a]
297	Uninucleate	0	11·8 ± 1·0[a]	3·7 ± 0·3[a]	13·7 ± 1·5[a]	38·3 ± 1·9[a]
298	Uninucleate	5·6 ± 5·6	15·3 ± 2·1[a]	5·2 ± 0·8[a]	17·0 ± 2·0[a]	37·6 ± 5·5[a]
83-111/1N	Uninucleate	11·1 ± 7·0	17·0 ± 1·4	5·2 ± 1·0[a]	17·1 ± 2·9[a]	45·7 ± 5·3[a]
85-387/Na	Uninucleate	5·6 ± 5·6	15·5 ± 0·9[a]	5·1 ± 0·4[a]	18·0 ± 1·5[a]	49·8 ± 3·7[a]
87-691/3	Uninucleate	5·6 ± 5·6	16·6 ± 1·0	7·0 ± 1·1[a]	23·1 ± 3·6[a]	54·1 ± 10·3[a]
Mean±SE		5·2 ± 1·4	14·6 ± 0·4	5·2 ± 0·2	18·7 ± 0·8	46·2 ± 1·7
F			2·18	7·34	2·37	1·79
P			0·006	0·001	0·003	0·03

[a] Means differ significantly ($P = 0·05$) from control using Duncan's multiple range test.

uninucleate isolates were more virulent based on all measured seedling parameters. The effect of *Rhizoctonia* inoculation was most clearly shown in the root parameters, particularly in the lateral root length. All *Rhizoctonia* isolates, despite the nuclear number or isolate, significantly reduced ($P < 0·05$) the lateral root length of both tree species (Tables 2 and 3). The main root length and the dry weight of whole root system of both tree species were also decreased, but the difference was not statistically significant in all cases (Tables 2 and 3). The shoot growth of inoculated Norway spruce seedlings was reduced compared to controls; shoot dry weights were significantly lower ($P < 0·05$) in all treatments excluding uninucleate isolate 249 (Table 3), while on Scots pine only two uninucleate *Rhizoctonia* isolates, 246 and 297, significantly decreased the shoot dry weights ($P < 0.05$). Re-isolation of *Rhizoctonia* from inoculated seedlings was successful in most cases.

In the second pathogenicity test, all 1-year-old and 2-year-old seedlings inoculated with uninucleate isolate 264 were alive after the 7-month test period. Inoculation significantly decreased the main root length and root dry weight of both 1-year-old and 2-year-old seedlings. The main root length of 1-year-old seedlings was 16·41 ± 0·75 cm, while the control value was 44·10 ± 7·09 cm ($F = 18·13$, $P = 0·0004$). The root dry weight of the same seedlings was 186 ± 24 mg for treatment and 289 ± 55 mg for control ($F = 3·20$, $P = 0·08$). The root parameters for inoculated 2-year-old seedlings were 19·90 ± 2·32 cm and 326 ± 44 mg while the control values were 56·77 ± 8·29 cm and 478 ± 39 mg. The decreases both in the main root length ($F = 20·10$, $P = 0·0003$) and root dry

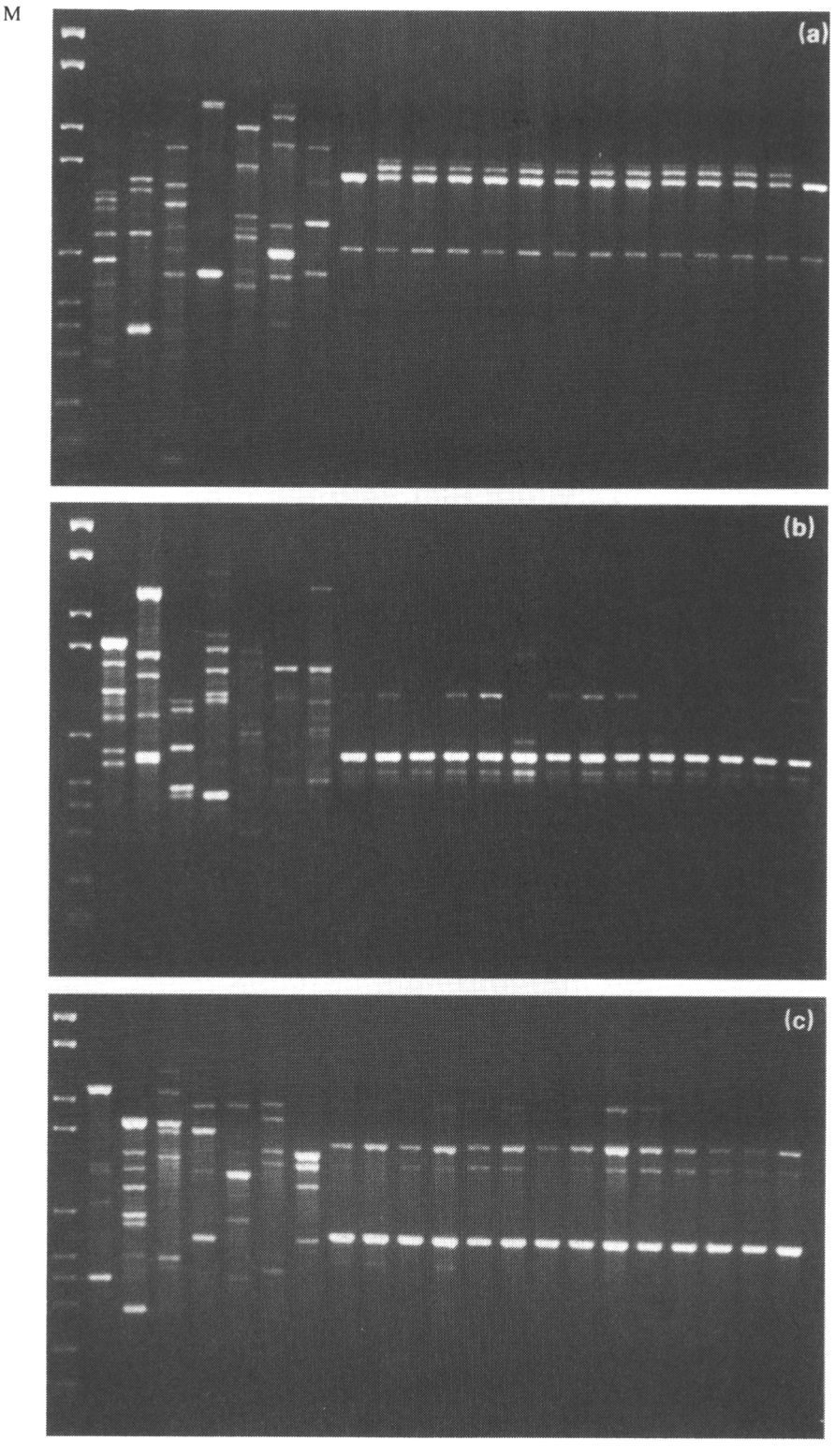

Fig. 1 (a) Primer 91298; (b) primer 91299; (c) primer 91300.

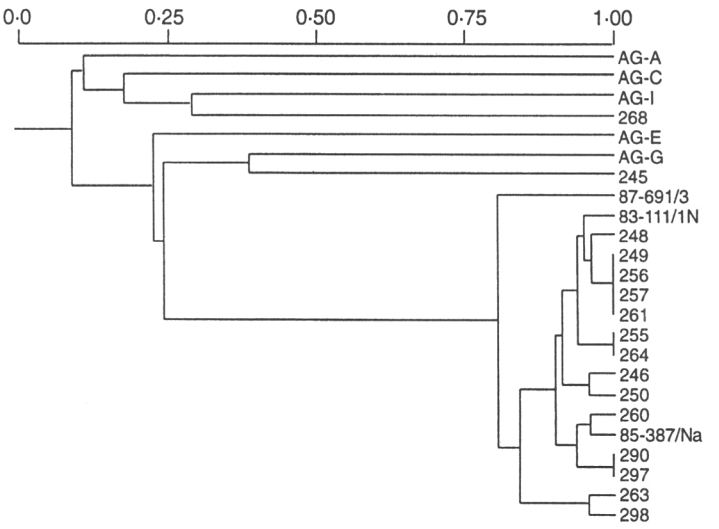

Fig. 2 UPGMA dendrogram of relationships among *Rhizoctonia* isolates based on similarity (DICE) coefficients using SAHN computer program (NTSYS-pc, Rohlf, 1989). Uninucleate isolates form a separate group from the other tester strains.

weight ($F = 6{\cdot}32$, $P = 0{\cdot}02$) were statistically significant.

Nuclear condition and hyphal anastomosis

Isolates identified previously as uninucleate *Rhizoctonia* sp. possessed predominantly uninucleate hyphal tips. All the counted tip/subapical cell pairs of isolates 246 and 250 were uninucleate. Fourteen isolates had 94–99% of uninucleate tip/subapical cell pairs. Isolate 87-691/3 had the lowest number of uninucleate tip/subapical cell pairs, 87%. The total number of observed cell pairs for these 17 isolates was 1700; of this amount, 56 cell pairs were not uninucleate. The types and frequencies of the aberrant nuclear condition in tip/subapical cell pairs were uninucleate/binucleate (8), binucleate/uninucleate (11) and binucleate/binucleate (37). The reference isolate 268 had 98% binucleate/binucleate and 2% trinucleate/binucleate tip/subapical cell pairs. The other reference, isolate 245, had 96% binucleate/binucleate, 2% trinucleate/binucleate, 1% trinucleate/trinucleate and 1% tetranucleate/binucleate tip/subapical cell pairs.

All uninucleate *Rhizoctonia* isolates anastomosed readily with each other; several anastomosis points were commonly detected in a single microscopic field. In self pairings, anastomosed cells fused together forming living bridges between the opposed colonies. In non-self pairings, hyphal anastomosis resulted in a killing reaction: after the cell wall fusion, between one and three cells died on either anastomosing hypha. In non-self pairings, living bridges between the opposed colonies were observed only when isolates 260, 263 and 264 were paired against each other, but even in these three pairing combinations the killing reaction was observed frequently. The binucleate isolate 268 anastomosed with the tester isolate of AG-I, producing a killing reaction, but the anastomosis frequency

Fig 1 Analysis of uninucleate *Rhizoctonia* isolates by RAPD-PCR method. a, b and c: lane 1 = molecular weight markers (MW, Boehringer IV, Germany) are 2176, 1766, 1230, 1033, 653, 517, 473, 394, 298, 234, 220 and 154 bp. (a) Primer 91298, lanes 2–8, binucleate reference isolates = AG-A, AG-C, AG-E, AG-G, AG-I, 245, 268; lanes 9–22, uninucleate isolates 87-691/3, 83-111/1N, 246, 248, 249, 250, 255, 256, 257, 260, 261, 263, 264 and 85-387/Na. (b and c) Primers 91299 and 91300; lanes 2–8, binucleate reference isolates = AG-A, AG-C, AG-I, 268, AG-E, AG-G, 245; lanes 9–22, uninucleate isolates = 87-691/3, 83-111/1N, 246, 248, 249, 250, 255, 256, 257, 260, 261, 263, 264 and 85-387/Na.

was lower than that observed among uninucleate isolates. The other binucleate isolate, 245, did not anastomose with isolate 268 or with the tester isolates of *Ceratobasidium*.

RAPD analysis

RAPD-PCR analysis using three primers indicated that all uninucleate isolates had different RAPD-DNA profiles compared with the Finnish binucleate isolates and tester isolates (Fig. 1a–c). Amplification of DNAs by the primers 298 and 300 revealed three common bands for almost all uninucleate isolates (size of the fragments, 298: 650–1100 base pairs (bp), 300: 500–950 bp). One dominant band (*c.* 650 bp) was typical of all uninucleate isolates produced by the primer 299. Generally, uninucleate isolates showed relatively homogenous DNA profiles compared with the high variability found in Finnish binucleate and *Ceratobasidium* anastomosis tester isolates (Fig. 1a–c).

The uninucleate *Rhizoctonia* sp. showed high similarity within the group, over 75–80% similarity coefficients in a dendrogram analysis (Fig. 2). UPGMA clustering (Fig. 2) indicates that uninucleate *Rhizoctonia* isolates are different at the DNA level from Finnish binucleate isolates and *Ceratobasidium* anastomosis testers which were only about 10–25% similar to the uninucleate isolates. The similarity of the Japanese tester strain of AG-I and the Finnish isolate 268 anastomosing with this group was about 28%.

DISCUSSION

The data suggest that uninucleate *Rhizoctonia* sp., causing root stunting of Scots pine and Norway spruce seedlings, forms a genetically homogenous group that is distinct from the binucleate Finnish *Rhizoctonia* isolates, and *Ceratobasidium* anastomosis tester isolates employed in this study.

All tested *Rhizoctonia* isolates decreased the lateral root length of both Scots pine and Norway spruce. The pathogenicity of uninucleate *Rhizoctonia* sp. has been shown in previous studies, but all Finnish binucleate isolates tested before have been non-pathogenic (Lilja *et al.*, 1992; Lilja, 1994; Hietala, 1995). However, the two binucleate isolates tested here decreased the length of laterals. The most comprehensive studies related to *Rhizoctonia* associated to root diseases of conifer seedlings were carried out by Saksena & Vaartaja (1960, 1961). Later studies have shown that the species studied by them were multi- or binucleate *Rhizoctonia* spp. (see e.g. Sneh *et al.*, 1991); one binucleate species causing stunted root growth of pine seedlings, *R. endophytica* var. *endophytica* Saks. & Vaar., was confirmed to belong to the AG-A of genus *Ceratobasidium* (Ogoshi *et al.*, 1983). The phenomenon that older seedlings infected with uninucleate *Rhizoctonia* sp. can survive but become stunted because of decreased root mass (Lilja, 1994; Hietala, 1995) was obvious also in this study, especially with Norway spruce seedlings (Table 3). The root systems of Scots pine seedlings infected at the age of 1 or 2 years were also decreased after the 7-month incubation period.

Anastomosis tests indicated that there is a single anastomosis group within the uninucleate *Rhizoctonia* sp. This is in agreement with earlier observations that culturally the species is extremely homogeneous (Hietala *et al.*, 1994; Hietala, 1995). In *R. solani*, the killing reaction is regarded as a somatic incompatibility reaction (see e.g. Sneh *et al.*, 1991); based on this interpretation, all the uninucleate isolates represent different genotypes. In addition to the killing reaction, a low frequency of perfect fusions was observed in the pairing combinations between isolates 260, 263 and 264. At present, the genetic background of the killing reaction in this species is not known but possibly these three isolates, originating from the same nursery, are more closely related than the other isolates.

Nuclear staining confirmed that the fungus is predominantly uninucleate. As previously shown (Hietala *et al.*, 1994), binucleate cells can co-exist with prevailing uninucleate cells in the same mycelium. The high frequency of binucleate/binucleate cell pairs in the isolate 87-691/3, as observed by Hietala *et al.* (1994) for the isolate 260 (11/11%), gives further evidence that these binucleate cells do not represent random mitotic events prior to cell division. However, the explanation behind this phenomenon remains unknown.

Results from the anastomosis test and RAPD analysis are in agreement: uninucleate isolates had rather uniform RAPD-PCR profiles in all tested primers. In contrast, the banding patterns of binucleate isolates were very heterogeneous with all primers. The low degree of genetic similarity between uni- and binucleate isolates was at a similar level to that often found between different species of several other fungi (Smith &

Anderson, 1989; Taylor & Natwig, 1989; Maclean *et al.*, 1993).

Geographic isolation may contribute to divergency and subsequent dissimilarity. Low similarities were observed in a RAPD analysis by Duncan *et al.* (1993) for some *R. solani* isolates obtained from various geographic locations and representing the same AGs; correspondingly, there was a relatively low similarity between the Finnish AG-I isolate and the Japanese tester strain.

The high homogeneity of the uninucleate *Rhizoctonia* sp. may reflect that the isolates have not been geographically separated for a time long enough to lead to significant divergence. Alternatively high genetic homogeneity within the uninucleate *Rhizoctonia* sp. may be an implication of a narrow source population. A small, homogenic source population may be a consequence of seedling exchange between nurseries. Spruce seedlings have also been imported from Norway, where pathogenic uninucleate *Rhizoctonia* sp. was first reported (Venn *et al.*, 1986). The influence of pathogen migration on reducing genetic variability has been previously found in a *Phytophthora infestans* population (Goodwin *et al.*, 1994) where contaminated seed tubers were an important source of pathogen spread.

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IV

The mode of infection of a pathogenic uninucleate *Rhizoctonia* sp. in conifer seedling roots

Ari M. Hietala

Abstract: The mode of infection of a uninucleate *Rhizoctonia* sp., a root-dieback pathogen in Finnish and Norwegian conifer nurseries, was studied in seedlings of *Picea abies* (L.) Karst., *Pinus sylvestris* L. and *Larix sibirica* Ledeb. growing under nonsterile conditions. In all hosts, the pathogen attacked growing tips of primary roots and long laterals. After a 5-month incubation period, the root systems of inoculated pine and spruce were heavily stunted, whereas larch seedlings were less affected. In the tip region where protoxylem elements had not yet differentiated, proliferating hyphae formed aggregations on the root surface and within the intercellular spaces of the outer cortex. This gave rise to penetration hyphae that invaded the apical meristem and neighbouring vascular cylinder. On the root surface, at basal root regions, hyphal growth was sparse and the infection was characterized by formation of monilioid hyphae within cortical cells. The hyphae spread intracellularly towards the basal root regions within the vascular cylinder. Considerable hyphal proliferation was observed when the pathogen was grown under membrane-isolated host roots. It is suggested that root exudates induce hyphal proliferation preceding the formation of penetration hyphae. All isolates were induced to fruit in the presence of all hosts.

Résumé : Le mode d'infection d'une espèce uninucléée de *Rhizoctonia*, responsable d'un dépérissement racinaire dans les pépinières de conifères en Finlande et en Norvège, a été étudié chez des semis de *Picea abies* (L.) Karst., de *Pinus sylvestris* L. et de *Larix sibirica* Ledeb. croissant en conditions non stériles. Chez tous les hôtes, le champignon pathogène attaquait l'apex des racines primaires et des racines latérales longues. Après une période d'incubation de 5 mois, le système racinaire du pin et de l'épinette était très rabougri tandis que les semis de mélèze étaient moins affectés. Dans la région de l'apex, où les éléments de protoxylème ne s'étaient pas encore différenciés, les hyphes proliféraient et formaient des amoncellements à la surface de la racine et dans les espaces intercellulaires de la partie externe du cortex. Cela entraînait la formation d'hyphes capables de pénétrer la racine et qui envahissaient le méristème apical et le cylindre vasculaire avoisinant. À la surface des racines, dans la zone située à la base de celles-ci, les hyphes étaient plus clairsemés et l'infection était caractérisée par la formation d'hyphes monilioïdes dans les cellules corticales. Les hyphes progressaient vers la base de la racine dans les cellules du cylindre vasculaire. Une prolifération considérable des hyphes a été observée lorsque le champignon pathogène était cultivé sur les racines d'un hôte isolées par une membrane. Les résultats suggèrent que des exudats racinaires induisent la prolifération des hyphes préalablement à la formation des hyphes capables de pénétrer la racine. Tous les isolats ont fructifié en présence de tous les hôtes.
[Traduit par la Rédaction]

Introduction

Root dieback, a root disease of nursery-grown conifer seedlings, is a considerable problem in forest nurseries of Norway and Finland (Venn et al. 1986; Lilja et al. 1992). Containerized and bare-root seedlings of both Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.) are affected by the disease. The visual symptoms of the disease, wilting of shoots, retarded height growth, discolouration of the needles, and partial or total death of the root system, often appear after midsummer on first-year seedlings or occasionally later during the second growing season.

Surveys for associated fungi and subsequent pathogenicity tests indicated that this disease was a complex involving both *Rhizoctonia* and *Pythium* spp. (Venn et al. 1986; Lilja et al. 1992; Lilja 1994). In Finland, the most pathogenic *Rhizoctonia* species was characterized by a uninucleate nuclear condition (Lilja et al. 1992). Hietala et al. (1994) showed that these uninucleate Finnish isolates represent the same species as the *Rhizoctonia* studied in Norway (Venn et al. 1986), based on cultural morphology, nuclear condition, hyphal anastomosis, and teleomorph characteristics. This species has also been isolated from diseased seedlings of Siberian larch (*Larix sibirica* Ledeb.) in one Finnish forest nursery (Lilja 1994). In pathogenicity tests, the uninucleate *Rhizoctonia* sp. causes damping off in very young seedlings, while older seedlings usually survive but show stunted shoot and root growth (Venn et al. 1986; Lilja et al. 1992). In a preliminary study with Norway spruce seedlings under axenic conditions (Hietala 1995), the pathogen attacked particularly the root tips, and based on a method involving staining of whole roots without sectioning, it was proposed that the pathogen could further penetrate the vascular cylinder.

Traditionally, *Rhizoctonia* species have been divided into binucleate and multinucleate species (Sneh et al. 1991). The former are represented, for example, by anamorphs of *Ceratobasidium* and the latter by *R. solani*, the anamorph of *Thanatephorus cucumeris* (Frank) Donk. A number of *Rhizoctonia* species have been reported in association with various tree

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seedling diseases. However, all these, including those involved in the comprehensive studies of Saksena and Vaartaja (1960, 1961), fall into the bi- or multi-nucleate groups (see Hietala and Sen 1996). In the massive literature on *Rhizoctonia*, there is only one additional reference to uninucleate *Rhizoctonia*; these isolates were obtained from the roots of winter wheat (Hall 1986). That species is not related to the *Rhizoctonia* causing root dieback on conifer seedlings, based on morphogical characteristics (Hietala et al. 1994). The perfect state of this conifer pathogen has not been observed in the nurseries. However, the characteristics of the induced teleomorph (Hietala et al. 1994) fit into the taxonomic species concept of *Ceratobasidium bicorne* Eriksson & Ryv., described from field material parasitizing a moss in Denmark (Eriksson and Ryvarden 1973). Further comparisons (e.g., analysis of the nuclear condition, anastomosis test) with the uninucleate *Rhizoctonia* sp. were not possible, since *C. bicorne* has apparently never been cultured (see Sneh et al. 1991). Previously, the genus *Ceratobasidium* has been considered to include only binucleate *Rhizoctonia* spp. (Ogoshi et al. 1983; Sneh et al. 1991).

The objective of the present study was to further investigate the infection and effect of the pathogen on the root system morphology of the observed hosts, under nonsterile conditions and beyond the most susceptible stage of damping off. The fruiting of the uninucleate *Rhizoctonia* sp. in the presence of the different hosts was also investigated.

Materials and methods

Pathogenicity test

At the end of January, germlings of Norway spruce, Scots pine, and Siberian larch were transferred to 2-dL pots filled with fertilized peat (Vapo XL) commonly used for containerized seedlings in Finnish forest nurseries. No further fertilization was applied during the experiment. The seedlings, one per pot, were grown in a growth cabinet (16-h day, 320 $\mu E \cdot m^{-2} \cdot s^{-1}$, day temperature 23°C, night temperature 16°C, 85% relative humidity). During the experiment, the moisture of the peat was kept at 45% (w/v) by weighing and watering the pots every second day.

Three isolates, representing the recently characterized uninucleate *Rhizoctonia* species (Hietala et al. 1994), were used for inoculation when the seedlings were 7 weeks old. These originated from container-grown diseased roots of Norway spruce (isolate 248, geographic origin 61°25′N, 25°53′E), Scots pine (isolate 255, 61°12′N, 28°15′E), and Siberian larch (isolate 256, 60°09′N, 19°57′E). Prior to inoculation, the isolates were grown for 4 days at 21°C in darkness in Petri dishes containing potato dextrose agar (PDA) covered with a cellophane membrane. The colony was divided into three equal sectors, each sector being used to inoculate one seedling of each tree species. Colonial sectors were peeled off the membrane and buried 3 cm deep in the peat, close to the margin of the pot. Fifteen seedlings of each tree species were inoculated with each isolate; uninoculated seedlings served as the control. At the inoculation time ($T = 0$), 15 seedlings of each tree species were harvested to assess the condition of the root system. At the beginning of May, the seedlings were transferred to a greenhouse under natural lighting and they were harvested in August at the age of 7 months ($T = 1$).

Root characterization followed the terminology of Sutton (1980). Shoot parameters (length, dry weight) and root parameters (fresh weight, length of the primary root, length of the five longest first-order lateral roots, number of first-order lateral roots over 5 mm in length, number of second-order lateral roots in the oldest (= first) first-order long lateral, and the number of further proliferating roots among these second-order laterals) were measured for all seedlings, including those seedlings harvested at $T = 0$. The data were subjected to analysis of variance and Tukey's honestly significant difference (HSD) test ($p = 0.01$).

Fungal isolations were made from root tips of four randomly chosen seedlings representing each treatment, including the control. The root was surface sterilized with sodium hypochlorite before isolation (Hietala 1995). The isolates obtained were tested for somatic compatibility against the original, cold-room-stored cultures by inoculating isolates 1 cm apart in a Petri dish containing PDA. Petri dishes were incubated at 21°C in darkness for 2 weeks until examination. After the measurements and fungal isolation, roots representing each treatment were stored in FAA (5 parts 37% formaldehyde, 5 parts acetic acid, and 90 parts 50% ethanol) for paraffin sectioning. The remainder of the roots were stored at –20°C.

To screen the locality of infection, two entire root systems representing each treatment were cleared in KOH, bleached with alkaline H_2O_2, and stained with trypan blue following exactly the procedures described by Koske and Gemma (1989). In addition, all root systems of seedlings harvested at $T = 0$ were similarly stained. Stained root systems were studied with a light microscope at 100× to 400× magnifications. Following this screening of infection, FAA-stored root samples representing infection areas were dehydrated in a butanol–paraffin series and embedded in paraffin for sectioning. The paraffin was dissolved from the sections (5–10 μm) in a Histo-Clear (National Diagnostics, U.S.A.) – alcohol series followed by staining in an aqueous solution of Toluidine blue O (0.025%) and mounting in Euparal (Asco Laboratories, England).

Membrane experiment

To study hyphal growth in the close vicinity of roots without physical contact, Petri dishes containing water agar (12 $g \cdot L^{-1}$) were inoculated with fungal isolates. A sterile cellophane membrane was placed on top of the agar, and one aseptically grown 3-week-old Norway spruce, Scots pine, or Siberian larch seedling was transferred onto the membrane. The root was then covered with an additional membrane, and aluminium foil was wrapped around the Petri dishes to shadow the roots. The seedlings were sequentially harvested during a growth period of 10 days under constant light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$). Hyphal growth was examined with a light microscope at 100× to 400× magnifications.

Fruiting experiment

The method developed by Hietala et al. (1994) was used to induce fruiting of the studied isolates. Surface sterilization and germination of seeds were performed as described in that study and three 14-day-old seedlings of Norway spruce, Scots pine, or Siberian larch were transferred to a Petri dish containing sterile distilled water. Seedlings were inoculated with a 6 × 6 mm block taken from the margin of an actively growing colony on PDA. Eighteen seedlings (in six Petri dishes) of each tree species were inoculated with each isolate, and the Petri dishes were kept on a laboratory bench with natural indirect lighting during May. Petri dishes containing only distilled water were similarly inoculated with each isolate as a control.

Results

The condition of root systems at the time of inoculation ($T = 0$)

At the age of 7 weeks, all seedlings had a well-developed root system with several first-order laterals, and the majority of all host seedlings had grown a few second-order laterals. The root systems of larch and pine seedlings were more advanced than those of spruce on the basis of all root parameters (Table 1). Staining of the root systems harvested at $T = 0$ showed no *Rhizoctonia*-like hyphae on the roots.

Table 1. The effect of inoculation with uninucleate *Rhizoctonia* sp. on the shoot and root growth indices of different hosts.

			Shoot parameters		Root parameters					
									First 1st-order long lateral	
Host	Treatment*	*T*	Length, cm	Dry wt., g	Fresh wt., g	Length of the primary root, cm	Length of 1st-order long laterals, cm†	No. of 1st-order laterals >5 mm in length	No. of 2nd-order laterals‡	No. of proliferating 2nd-order laterals‡
P. abies	Control	0	4.3*a*	0.015*a*	0.05*a*	11.1*a*	2.5*a*	7.9*a*	1.9*a*	
	Control	1	9.0*b*	0.249*b*	1.57*b*	41.7*c*	18.7*c*	23.7*c*	28.2*b*	12.9*a*
	248	1	7.7*ab*	0.209*ab*	0.70*a*	24.3*b*	8.3*b*	17.6*bc*	19.8*b*	5.1*a*
	255	1	7.3*ab*	0.150*ab*	0.43*a*	19.1*ab*	5.4*ab*	14.5*ab*	20.6*b*	5.9*a*
	256	1	8.6*b*	0.236*b*	0.67*a*	17.3*ab*	5.8*ab*	15.9*b*	20.9*b*	6.1*a*
P. sylvestris	Control	0	3.9*a*	0.015*a*	0.06*a*	14.9*a*	3.3*a*	8.1*a*	6.0*a*	
	Control	1	10.3*c*	0.718*c*	2.91*c*	100.5*c*	25.1*c*	44.1*c*	25.3*c*	14.6*b*
	248	1	6.7*b*	0.520*b*	1.41*b*	51.4*b*	12.3*b*	24.7*b*	14.9*b*	6.2*a*
	255	1	6.9*b*	0.377*b*	0.94*b*	43.6*ab*	8.6*ab*	20.5*ab*	16.4*b*	9.1*ab*
	256	1	6.5*b*	0.494*b*	1.52*b*	64.0*b*	12.2*b*	28.9*b*	20.7*bc*	9.5*ab*
L. sibirica	Control	0	4.3*a*	0.013*a*	0.09*a*	17.1*a*	3.9*a*	8.9*a*	4.7*a*	
	Control	1	20.1*b*	0.516*b*	1.98*b*	54.9*b*	12.1*c*	18.4*b*	14.4*b*	7.6*a*
	248	1	19.5*b*	0.417*b*	1.65*b*	33.0*ab*	9.4*bc*	16.1*b*	13.1*b*	8.0*a*
	255	1	18.6*b*	0.450*b*	1.69*b*	39.1*ab*	7.8*b*	17.1*b*	17.6*b*	7.4*a*
	256	1	20.7*b*	0.525*b*	1.84*b*	26.4*a*	8.5*bc*	17.4*b*	14.4*b*	7.6*a*

Note: Seedlings were inoculated at the age of 7 weeks ($T = 0$) and harvested after 5 months incubation ($T = 1$). Values ($n = 15$) followed by the same letter do not differ from each other (Tukey's HSD test, $p = 0.01$).

*The strains shown in bold were originally isolated from the respective host.

†Values represent the average length of the five longest first-order long laterals.

‡Parameters were recorded at a standard region (i.e., first 6 cm at the base of the long lateral).

The primary root of all larch, pine, and spruce seedlings was actively growing at the time of inoculation, possessing a pointed tip with a well-developed root cap and showing no signs of a metacutization layer (see Wilcox 1954). The first protoxylem elements were observed 1500–3500 μm behind the apical initials. Primary roots possessed the largest distance between the apical initials and the first xylem elements within the root system of all seedlings.

In six Norway spruce seedlings, 10–50% of the long laterals (>5 mm in length) had a rounded root apex with a structure identified as a metacutization layer, indicating ceased growth and dormancy. Similarly, 10–90% of long laterals of six Siberian larch seedlings had a rounded root apex and a metacutization layer (Fig. 1). In the remaining spruce and larch seedlings and in all 15 pine seedlings, all the long laterals had a pointed apex with a well-developed root cap and showed no signs of a metacutization layer. These were regarded as actively growing roots (Fig. 2). In the dormant long laterals of spruce and larch, the protoxylem elements differentiated usually 150–500 μm behind the apical initials, whereas in the growing long laterals of spruce, larch, and pine the protoxylem elements differentiated 700–2500 μm behind the apical initials.

Similarly, the short laterals (<5 mm) of all host species could be divided into growing and dormant roots. In short laterals with a metacutization layer, the protoxylem commonly differentiated 100–200 μm behind the apical meristem. In the growing short laterals, protoxylem differentiation was observed 200–500 μm behind the meristem. In pine and spruce, some of the growing short laterals, unlike the growing long laterals, had a rounded apex with a poorly developed root cap. These roots were regarded as short roots, whereas all the growing short laterals of larch had a pointed apex with a well-developed root cap. At this time, no mycorrhizal roots were observed on any tree species.

Root system morphology and infection

Rhizoctonia could be isolated from all inoculated seedlings, but not from the control seedlings. The *Rhizoctonia* isolates obtained from the inoculated seedlings showed somatic compatibility against the original, cold-room-stored culture. A somatic incompatibility reaction, formation of a demarcation line between the paired colonies, was produced when an obtained isolate was paired against the other two strains of *Rhizoctonia* used in this study.

Visually, the root growth of spruce and pine seedlings was notably affected by the inoculation. The primary roots and many of the first-order long laterals were commonly broken with the tip missing, the remaining root tips were heavily pigmented, and the root system seemed considerably stunted. In inoculated larch seedlings, root tips of the primary roots and first-order long laterals, particularly at the basal part of the root system, were occasionally missing. In contrast with pine and spruce, no visually striking differences were observed in pigmentation or in the root system size compared with the control seedlings. Needle discolouration symptoms were not observed in any host species.

Significant decreases (Tukey's HSD, $p = 0.01$) were observed among the root parameters of all inoculated trees (Table 1). No major differences were detected between seedlings inoculated with different isolates. The only significant decreases in the shoot length and dry weight were found for the inoculated seedlings of pine. In pine, the inoculated treatments were significantly different from the control at $T = 1$ in all measured root parameters, excluding the two related to second-order laterals. In spruce, the inoculated seedlings were different from the control at $T = 1$ in terms of root fresh weight, length of the primary root, length of the five longest first-order laterals, and with one exception, also in the number of first-order laterals exceeding 5 mm in length. In pine and spruce, there was a clear, although in most cases not significant, decrease in the root parameters relating to second-order laterals. In larch, a decrease in the root growth was evident in length of the primary root and in length of the five longest first-order laterals, but the difference to the control at $T = 1$ was not statistically significant in most cases.

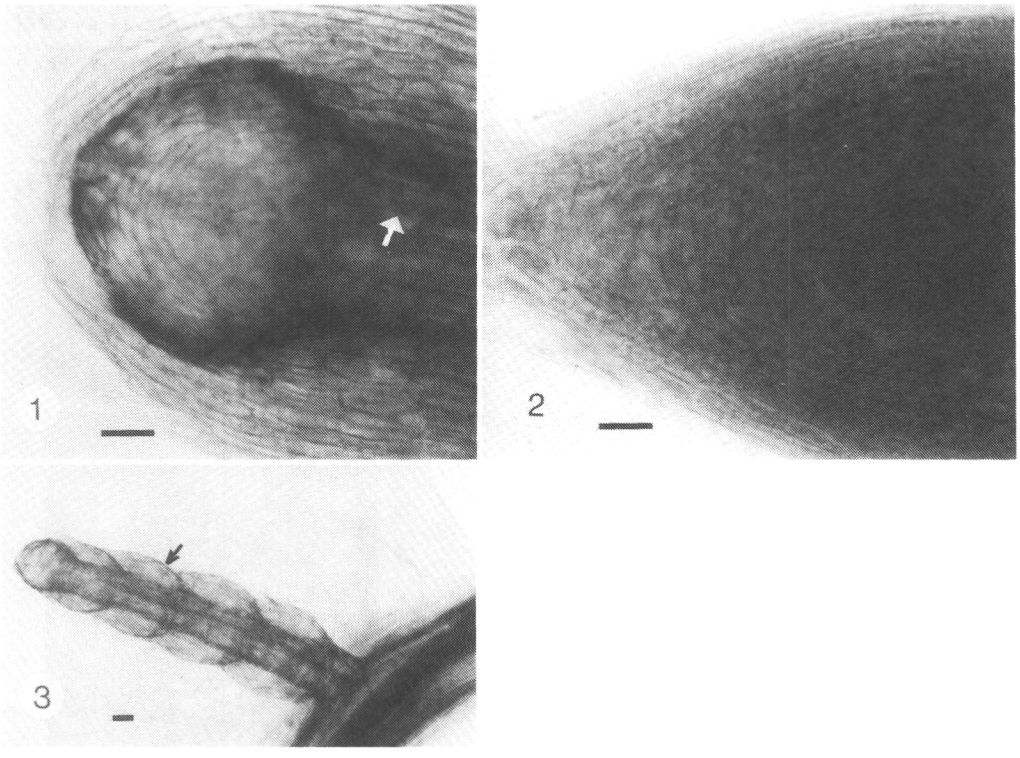
All the stained root systems of inoculated pine, spruce, and larch seedlings had relatively wide hyphae (4–8 μm) (see Hietala et al. 1994). These could be easily identified as *Rhizoctonia* on the basis of the growth characteristics (branching near the distal septum of cells, formation of a septum in the branch near the point of origin, constriction of the branches, and absence of clamp connections, see Ogoshi 1975). *Rhizoctonia*-like hyphae were not detected on the roots of uninoculated control seedlings; the observed fungi had narrow hyphae (1–3 μm) with different growth characteristics and often clamp connections.

At the time of final harvesting, many pine and spruce seedlings in all treatments had some mycorrhizal root tips. In general though, including the control seedlings, the level of mycorrhizal infection was low and mycorrhiza were localized. The associated mycorrhizal fungi were characterized by narrow (2–3 μm), clamped hyphae. No mycorrhizal roots were observed in larch seedlings. Several collars indicating previous metacutization layers were commonly observed on the non-mycorrhizal short roots of pine and spruce (Fig. 3).

No differences were observed in the external growth of *Rhizoctonia* hyphae nor in the locality of infection areas on the roots of different hosts. On the root surface, excluding the tip region where protoxylem had not yet differentiated, the growth of *Rhizoctonia* hyphae was commonly sparse. Cortical cells were frequently infected by a short hyphal branch either directly or via an appressorium-like swelling. Occasionally, the fungus filled cortical cells with monilioid hyphae (Fig. 4), but the frequency of this type of infection was low and the phenomenon was not observed in every stained root system. No *Rhizoctonia* hyphae were observed at the root collar region.

In the tips of growing roots, aggregations of short-celled hyphae were commonly observed on the root surface (Fig. 5) and within the intercellular spaces of the outer cortex in the region where protoxylem elements had not yet differentiated. Further spread to the vascular cylinder by penetration hyphae, initiated underneath these hyphal aggregations, was both inter- and intra-cellular (Figs. 6–7). Penetration hyphae were most frequently formed at the apical initials and 100–300 μm behind them (Fig. 8). After this region, the frequency of penetration hyphae decreased gradually, and penetration hyphae were seldom observed after the protoxylem differentiation. At the apex of the root, penetration hyphae were commonly orientated towards the apical meristem (Fig. 9). After reaching the vascular cylinder, the *Rhizoctonia* hyphae turned to grow towards the base of the root (Fig. 10).

Figs. 1–3. Unsectioned roots of uninoculated control seedlings cleared with KOH, bleached with alkaline H_2O_2, and stained with trypan blue according to Koske and Gemma (1989). Scale bar = 40 μm. Fig. 1. A dark metacutization layer at the apex of a dormant long lateral of Siberian larch. Note the differentiation of protoxylem elements (arrow) close to the apical meristem. Fig. 2. The apex of an actively growing long lateral of Siberian larch. Fig. 3. A dormant non-mycorrhizal short root of Norway spruce with several basal collars (arrow) indicating previous metacutization layers.



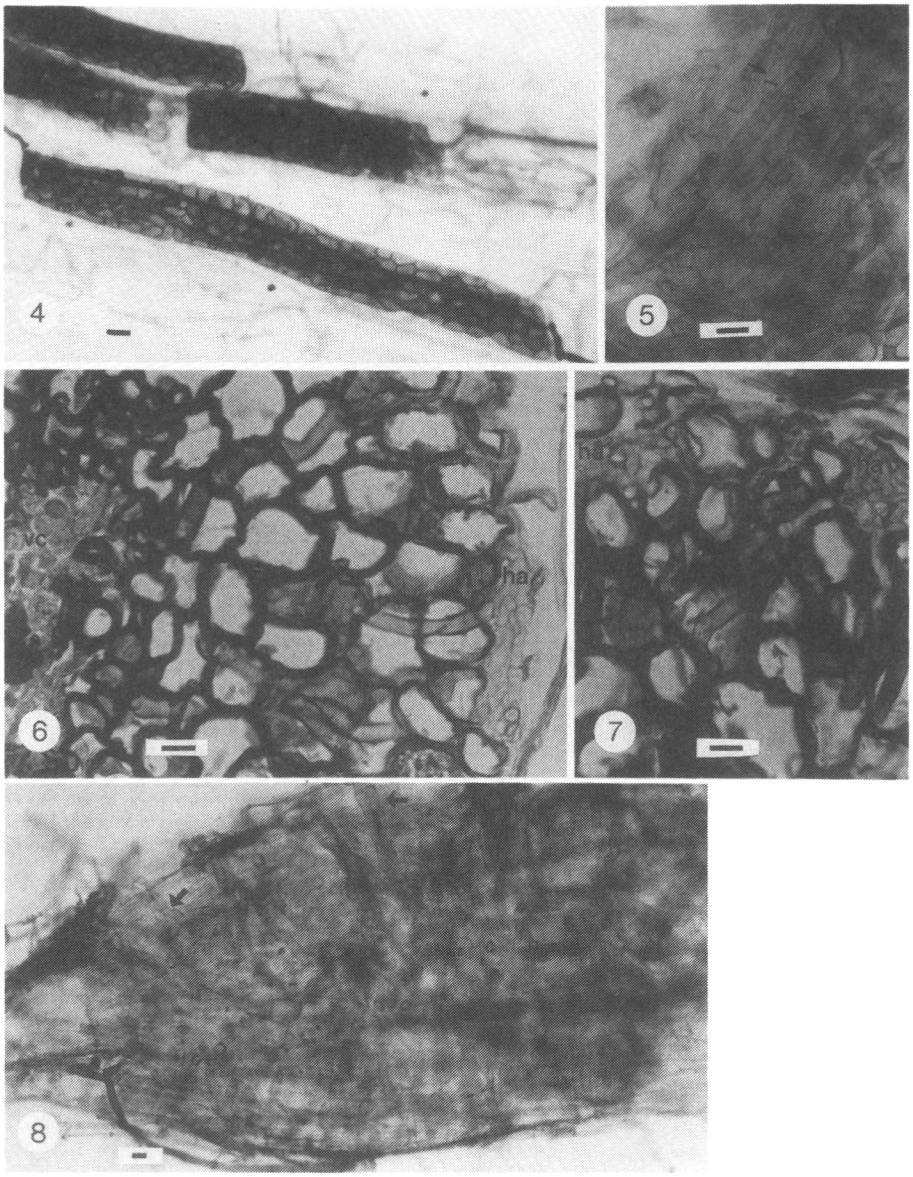
In all stained root systems of pine and spruce, the majority of the first-order long laterals, including the primary root, were infected. In roots where the root tip was missing, protoxylem elements with intracellular *Rhizoctonia* hyphae were frequently protruding at the breakage point (Fig. 11). Young long laterals, probably adventitious roots, were commonly observed immediately behind the breakage point in all host species and in most cases, had been infected.

In the most stunted root systems, several long laterals, excluding first-order roots, were free of *Rhizoctonia* hyphae and infection in the root tip region. The majority of the uninfected long laterals of all seedlings were dormant, whereas none of the infected long laterals had formed a metacutization layer. Occasionally, there was a small amount of surface hyphae at the apex of a dormant long lateral, but the hyphal growth was relatively sparse and no penetration hyphae were formed. No surface hyphae were observed on the root tips of the uninfected, growing long laterals. Compared with primary roots and long laterals, short roots were rarely infected by *Rhizoctonia* even in those root systems of pine and spruce with no mycorrhizal root tips. The vast majority of short roots were free of *Rhizoctonia* hyphae and a metacutization layer had been formed, and as in control seedlings, several collars indicating previous metacutization layers were commonly observed.

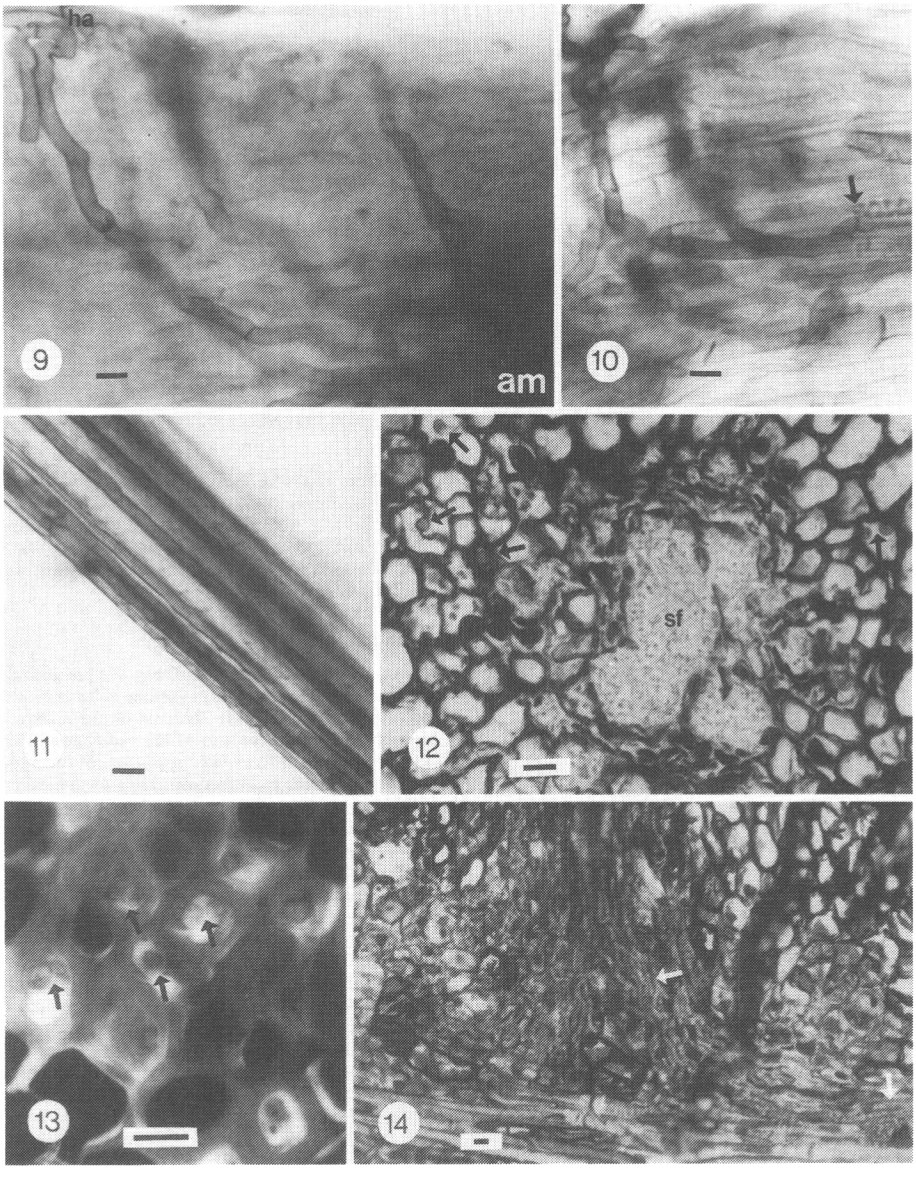
Besides short roots, long laterals of pine and spruce were also occasionally mycorrhizal. In inoculated pine and spruce seedlings, dual infection of a common long lateral root tip by *Rhizoctonia* and a mycorrhizal fungus was occasionally observed. The area with *Rhizoctonia* penetration hyphae was followed by a Hartig net region.

Compared with the stained root systems of pine and spruce, the infection level in inoculated larch seedlings was variable. In three of the six stained root systems, several laterals, particularly first-order roots, had been infected. In the other three seedlings, regardless of the origin of the inoculated isolate, the infection of root tips was very localized, restricted to a few

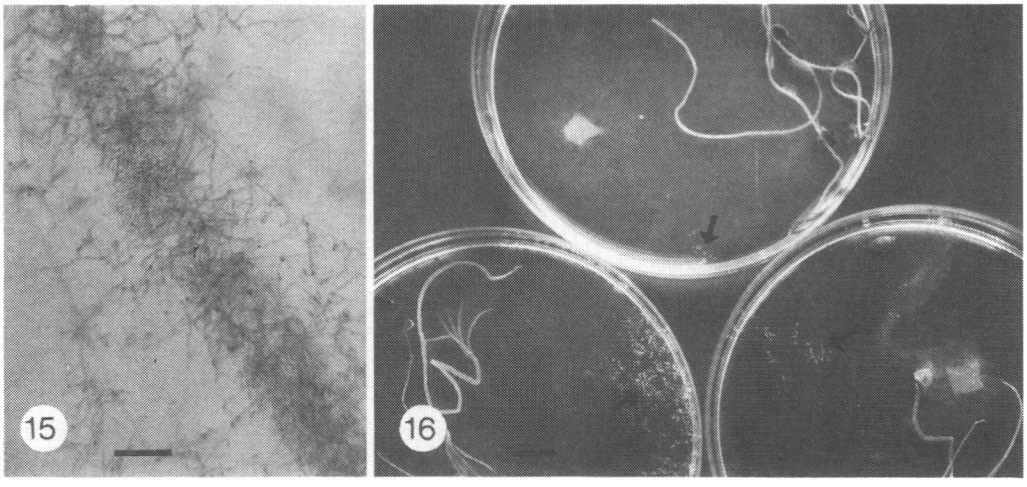
Figs. 4–14. Infection of roots by uninucleate *Rhizoctonia* sp. Unless otherwise stated, the infection characteristics are demonstrated on Norway spruce; no major differences were observed in the mode of infection on different hosts. Figures 4–5 and 8–11 represent observations on unsectioned roots treated according to the protocol of Koske and Gemma (1989). The rest of the figures (6–7 and 12–14) represent paraffin sections. The scale bar = 10 μm. Fig. 4. Monilioid hyphae within cortical cells at the base of a long lateral. Fig. 5. Hyphal proliferation on the root surface at the apex of a long lateral. Note the bidirectional growth pattern (arrows), probably a result of hyphal anastomosis. Fig. 6. A hyphal aggregation (*ha*) under the epidermis at the apex of a long lateral and intracellular penetration towards the vascular cylinder (*vc*) filled with *Rhizoctonia* hyphae. Fig. 7. Intercellular hyphal aggregations (*ha*) within the cortex close to the apex of a long lateral. Note intercellular



penetration under the hyphal aggregations (arrows). Fig. 8. Multiple penetration hyphae (examples shown with arrows) at the apex of a long lateral. Fig. 9. A hyphal aggregation (*ha*) on the root surface and penetration towards the apical meristem (*am*). Fig. 10. A penetration hypha and basipetal growth within the vascular cylinder. Note the differentiation of the protoxylem (arrow). Fig. 11. Intracellular *Rhizoctonia* hyphae within protoxylem sticking out at a breakage point of a long lateral (Siberian larch). Fig. 12. A typically macerated vascular cylinder of an infected long lateral (Scots pine) filled with narrow hyphae of secondary fungi (*sf*). *Rhizoctonia* hyphae are indicated with arrows.
Fig. 13. *Rhizoctonia* hyphae (arrows) within the metaxylem of a first-order long lateral. Fig.14. Spread of *Rhizoctonia* hyphae (arrows) within the vascular cylinder of a young long lateral to the mother root.



Figs. 15–16. Hyphal growth of the uninucleate *Rhizoctonia* sp. under the membrane-isolated roots of Norway spruce and fruiting of the fungus in the presence of the host seedlings. Fig. 15. Proliferation of hyphae under the membrane-isolated root in a region where root growth has occurred (bar = 100 μm). Fig. 16. Hymenial clusters (arrows) on the water surface in the presence of Norway spruce (top), Siberian larch (right), and Scots pine (left) seedlings (bar = 10 mm).



neighbouring laterals in a common mother root. In general, very few surface hyphae of *Rhizoctonia* were observed at the basal root regions in these root systems.

No differences were observed in the hyphal growth within the vascular cylinder of different seedlings. The apical meristem and the neighbouring vascular cylinder were usually heavily macerated and were often colonized by secondary fungi with very thin hyphae (1–2 μm) (Fig. 12). *Rhizoctonia* hyphae spread intracellularly within the vascular cylinder, particularly in the xylem elements and associated vascular parenchyma (Figs. 11–13). Hyphal growth was usually restricted close to the original infection site. At the breakage point of a first-order long lateral, where a relatively short (<1 cm) long lateral had been infected, *Rhizoctonia* hyphae occasionally spread within the vascular cylinder to the parent root (Fig. 14).

Hyphal growth under the membrane-isolated root

Hyphal growth was greatly stimulated under the growing root tips of all host species. First initials of hyphal proliferation were observed 48 h after the root was introduced to the system. After 7 days incubation, abundant aggregations had formed under the root in the region between the original and immediate position of the grown root apex (Fig. 15). Increased hyphal proliferation was also observed near the root collar. The hyphae were not able to penetrate through the membrane during the 10-day incubation period.

Fruiting

In the fruiting experiment, all the seedlings died within a few days of inoculation. All isolates produced the basidial stage in the presence of each host, but not in control Petri dishes without seedlings. Fruiting occurred between 7 and 14 days after inoculation. The most abundant hymenial production was observed, regardless of the inoculated strain, in the presence of Scots pine (Fig. 16). On pine the fruiting frequencies of the isolates 248, 255, and 256 were four, two, and one, respectively. On spruce, these frequencies were five, two, two and on larch, three, three, and five, respectively.

Discussion

Somatic compatibility tests confirmed the presence of the inoculated strains on the roots at the time of harvesting. No *Rhizoctonia* like hyphae were observed on the roots of stained control seedlings at the end of the experiment. This is not surprising, since *Rhizoctonia* spp. have not commonly been isolated from peat and other soil-free growth media. The results of Venn et al. (1986) implicated the supporting sand beds as an inoculum source for *Rhizoctonia* related to root dieback in container production.

No differences were observed in the root system characteristics of seedlings inoculated with different isolates, and there were no differences in the mode of infection on different seedlings. The apical region of primary roots and long laterals was the primary penetration route into the vascular cylinder, similar to that found by Farquhar and Peterson (1989) in *Fusarium oxysporum* f.sp. *pini* on growing primary roots of young red pine (*Pinus resinosa* Ait.) seedlings. In growing conifer roots, the endodermis close to the apical initials is still at the primary, nonsuberized stage (e.g., Wilcox 1954; Leshem 1974; Johnson-Flanagan and Owens 1985; Warmbrodt and Eschrich 1985; Kottke and Oberwinkler 1990). Cessation of root elongation results in the formation of a so-called metacutization layer, where lignified and suberized cells connect the subsurface cells of the root apex to the suberized endodermis

(e.g., Wilcox 1954). The isolated root cap and cortical cells die, which causes rounding of the root tip (Johnson-Flanagan and Owens 1985). At the time of inoculation, both larch and spruce seedlings had long laterals that, based on the presence of a metacutization layer, were in dormancy. At the time of final harvesting, the majority of the uninfected long laterals of all host species were in dormancy. No infection structures had been formed when there were surface hyphae of *Rhizoctonia* at the apex of a dormant root. In contrast, no signs of a metacutization layer were observed in the infected root tips. These observations suggest that only actively growing roots are infected by this pathogen.

The prepenetration stage of the uninucleate *Rhizoctonia* sp. was characterized by formation of hyphal aggregates giving rise to penetration hyphae. *Rhizoctonia solani* has been shown to form similar hyphal aggregations, termed infection cushions, on several plants (e.g., Dodman and Flentje 1970). In addition, hyphal growth was notably stimulated under the membrane-isolated root tips. It is, therefore, hypothesized, that exudates from the growing root tips stimulate hyphal growth of this *Rhizoctonia* species and provide energy for the luxurious growth preceding the formation of penetration hyphae. Components of the root exudates of some conifer seedlings have been shown to stimulate hyphal growth of *Rhizoctonia* (Agnihotri and Vaartaja 1969) and sporangium germination of *Pythium* (Agnihotri and Vaartaja 1967). Moreover, there is considerable evidence from other hosts that plant exudates are essential for the formation of infection cushions of *R. solani* (e.g., Dodman and Flentje 1970; Armentrout et al. 1987).

It should be noted that compared with primary roots and long laterals, short roots were seldom infected by the pathogen. In primary roots and long laterals, the likelihood of being infected should be multiple, since they will encounter pathogen propagules and hyphae within large medium volumes. Short roots, on the other hand, are restricted in growth and thus confined to a particular space. Spruce and pine seedlings showed considerably stunted root systems, while larch seedlings seemed less affected. However, the first-order long laterals of uninoculated larch seedlings showed considerably poorer growth capacity than those of spruce and pine, which undoubtly partially contributes to the relatively small difference in growth between the inoculated and control seedlings of larch. Unlike pine and spruce, considerable differences were observed in the level of infection between individual larch seedlings. Wilcox (1954) found that individual long laterals of noble fir (*Abies procera* Rehd.) had several growth periods during the growing season, a single growth period of individual roots ranging from 2 to 6 weeks. Similar periodicity has been shown for roots of *Picea glauca* (Moench) Voss (Johnson-Flanagan and Owens 1985) and *Pinus resinosa* (Wilcox 1968). At the time of inoculation, some of the larch seedlings had a high percentage of dormant first-order long laterals. Unfortunately, the root growth patterns of different hosts and individual seedlings were not followed by sequential harvestings after the inoculation. If only actively growing roots stimulate hyphal growth, the presence, frequency, and longevity of root dormancy at the time of inoculation could have a considerable effect on the infection potential and the multiplying rate of the inoculum present in the system.

At the basal parts of the roots, hyphal growth of the uninucleate *Rhizoctonia* sp. was sparse and cortical cells were occasionally colonized by monilioid hyphae. Saksena and Vaartaja (1961) showed that several binucleate *Rhizoctonia* species (see Sneh et al. 1991) associated with conifer seedlings were able to cause stunting of root systems. The infection was characterized by massive production of monilioid hyphae within cortical cells, but the authors do not mention whether the root tips or the vascular cylinder were infected by these fungi. Hietala (1995) examined the mode of infection of several binucleate *Rhizoctonia* (AG-I, *R* spp.) coexisting with the present uninucleate *Rhizoctonia* sp. in the roots of Norway spruce seedlings suffering from root dieback. The binucleate *Rhizoctonia* spp. infected cortical cells with monilioid hyphae, at basal root regions only and had no effect on the root growth. Ferris et al. (1984) suggested that monilioid hyphae within host cells probably act as sclerotium-like dispersal and survival units for *Rhizoctonia*. Over time, cortical cells will be sloughed off, the intracellular monilioid hyphae will be liberated into the growth medium, and if promoted by a nutritional stimulus (e.g., root exudates), they will germinate.

In a previous study where the present uninucleate *Rhizoctonia* species was initially characterized (Hietala et al. 1994), isolate 256 did not fruit on the used test plant, Scots pine. The success in fruiting this isolate on all hosts in the present study indicates that the process may be very sensitive. When single basidiospore isolates of a strain are paired against each other, a somatic incompatibility reaction is commonly produced. Single basidiospore isolates of this species can also be fruited, without mating, and the somatic incompatibility reaction is common among the second-generation offspring (A.M. Hietala, K. Korhonen and R. Sen, unpublished). This indicates that this species is homothallic. Several genotypes, based on the somatic incompatibility reaction, have been found in a single forest nursery (A.M. Hietala, unpublished), which could imply that the pathogen fruits here. Genetically, this uninucleate *Rhizoctonia* sp. is very homogeneous (Lilja et al. 1996), which would be a natural result of inbreeding.

The possible interaction between *Pythium* and *Rhizoctonia* in root dieback disease has not been investigated. Excluding studies related to seedlings at the damping-off stage (e.g., Børja et al. 1995), the mode of infection of *Pythium* spp. associated to root dieback disease of conifer seedlings has not been studied, but as observed for this *Rhizoctonia* species, growing root tips are also regarded as the main target of *Pythium* spp. (Hendrix and Campbell 1973; Endo and Colt 1974). During the present study, the moisture of the growth medium was artificially kept at a constant level, but it was obvious that inoculated seedlings of pine and spruce needed gradually less watering than the control seedlings. Under nursery conditions, such a drastic reduction in the root biomass would result in moisture stress promoting fungal spread with zoospores. Further studies on the coexistence of these fungi can be justified.

Acknowledgments

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V

V.11 *RHIZOCTONIA* ASSOCIATED WITH FOREST TREES

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I. INTRODUCTION

Many *Rhizoctonia* species are economically important plant pathogens in agriculture and thus receive considerable attention. However, relatively little is known about these fungi and their effects on trees in forestry production. There are well documented cases of *Rhizoctonia*-linked diseases of tree seedlings cultivated in forest nurseries although information concerning their interactions with forest trees in natural ecosystems is limited and fragmentary.

The early descriptions of *Rhizoctonia* spp. associated with tree species were mainly based on a few anamorphic traits. More recently, a re-evaluation of these species using presently accepted criteria (Parmeter and Whitney, 1970; Ogoshi, 1975) has resulted in the exclusion of many taxa from the genus *Rhizoctonia* (Andersen and Stalpers, 1994).

In this review, the conventions of recently published checklists for *Rhizoctonia* are followed (Sneh *et al.*, 1991; Andersen and Stalpers, 1994). Where possible, the present taxonomic position of the earlier described species will be indicated.

II. *RHIZOCTONIA* IN FOREST TREE NURSERIES

In a comprehensive nursery study, Saksena and Vaartaja (1961) grouped *Rhizoctonia* species into three classes that caused (a) root and hypocotyl rot and damping-off, (b) varying root disorders with intracellular chlamydospores (= monilioid hyphae) infections and (c) no apparent disease symptoms following root penetration. Later reports also confirmed their role in shoot diseases of trees.

A. *Rhizoctonia* as a damping-off agent

Damping-off, either as pre- or post-emergence is a common disease of seeds, germlings and young seedlings of many plants including woody species. It has long been known that anamorphs attributed to *Rhizoctonia solani* Kühn (multinucleate, see Parmeter and Whitney, 1970) are a causal agent of damping-off in conifer seedlings (Wiant, 1929). World-wide, the seeds and young seedlings of a number of conifer and broad-leaved species (e.g. *Pinus*, *Picea*, *Larix*, *Ulmus*, *Eucalyptus* spp.) are known to be attacked (Hartley, 1921; Roth and Riker, 1943; Wright, 1944; Vaartaja and Cram, 1956; Saksena *and* Vaartaja 1960, 1961; Vaartaja *et al.*, 1961; Vaartaja, 1967; Gomez-Nava, 1967; Sharma *et al.*, 1984; Perrin and Sampangi, 1986). Another multinucleate species, *R. endophytica* var. *filicata* Saks. and Vaar (= *R. zeae* Voorhees) (see Sneh *et al.*, 1991), was also reported as a damping-off pathogen on conifer seedlings (Saksena and Vaartaja 1961; Vaartaja, 1967)

B. Sneh et al. (eds.),
Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Constrol, 351–358.

The anastomosis groups (AGs) (see Chapter I.A2) of *R. solani* associated with trees have only been determined in a few studies. The most common, AG 4, has been isolated from *Pinus banksiana* Lamb. and *Picea glauca* Voss (Anderson, 1982), nursery soils (Camporota and Perrin, 1994) and the soil amendment, pine bark mulch (Huang and Kuhlman, 1990). Isolates from nursery soils, representing AGs 4 (the most common group), 5 and 1-2, but not AG 2-2, were very aggressive damping-off pathogens on *Pinus nigra* Arnold and the AG 4 isolate from pine bark mulch caused damping-off on *Pinus elliottii* Engelm. var. *elliottii*. Which AGs were involved in the early studies of Saksena and Vaartaja (1961)? The most common species, *Ceratobasidium praticola* (Kotila) Olive, causing damping-off, is presently included in the *Thanatephorus cucumeris* complex (and suggested to be *T. praticola*, Sneh *et al.*, 1991), despite sterigmatic characteristics previously regarded as specific, and now forms AG 4 of *R. solani* (Ogoshi, 1975). In examining the AG affinities of many earlier studied isolates, Parmeter *et al.* (1969) confirmed that three *C. praticola* isolates and one *R. solani* isolate (Saksena and Vaartaja, 1961), *R. solani* var. *cedri deodarae* (Castellani, 1934b) and five North American conifer isolates from different sources were all representatives of AG 4. This highlights the common occurrence of AG 4 on trees and seedlings.

Binucleate *Rhizoctonia* spp. causing damping-off in conifer seedlings include *R. callae* Cast. (Saksena and Vaartaja, 1961), *R. endophytica* var. *endophytica* Saks. and Vaar. (= AG-A, see Ogoshi *et al.*, 1983) (Saksena and Vaartaja, 1961; Gomez-Nava, 1967), and a *Rhizoctonia* sp. AG-E (=CAG 3, see Ogoshi *et al.*, 1983) (Huang and Kuhlman 1990).

The damping-off pathogens, *Rhizoctonia, Pythium* and *Fusarium* are ubiquitous inhabitants in forest nursery soils (e.g. Perrin and Sampangi 1986) and reports indicate that seedlings grown directly in seed beds are more affected by damping-off than containerized seedlings that are normally cultivated in soil-free growth media. The activities of these pathogens are strongly dependent on prevailing soil conditions, e.g. pH, temperature, moisture etc. (Roth and Riker 1943; Camporota and Perrin 1994). Soil fumigation and seed treatments with fungicides have been recommended in nursery manuals as measures for broad-spectrum control. Potential damping-off pathogens, including *R. solani* (AG 4), may also be introduced into soils via organic amendments (Huang and Kuhlman, 1990). Huang and Kuhlman (1991) subsequently formulated a mixture of organic and inorganic materials which stimulated antagonistic fungi and reduced the populations of *R. solani* and *Pythium aphanidermatum* (Edson) Fitzp. enabling effective control of damping-off both in fumigated and non-fumigated soils.

B. *Rhizoctonia* as a root pathogen of older seedlings

Descriptions of *Rhizoctonia* spp. causing root damage in older conifer seedlings are mainly those of Saksena and Vaartaja (1960, 1961). Fungi were isolated from various conifer species displaying damping-off symptoms, mycorrhizal rootlets or apparently non-diseased seedlings. Isolates of *R. solani*, *R. endophytica* var *endophytica* (= AG-A), *R. endophytica* var. *filicata* (= *R. zeae*) and *R. callae*, causing damping-off, and *R. repens* Bernard (binucleate, see Sneh *et al.* 1991) also induced stunted shoot and root growth in seedlings of *Pinus sylvestris* L., *P. resinosa* Ait., *Ulmus americana* L., *Caragana arborescens* Lam. and *Thuja occidentalis* L. A distinguishing characteristic of *R. endophytica* var. *endophytica*, *R. callae* and *R. repens* was the production of monilioid hyphae in root cortical cells of all test plants.

However, the results obtained from these tests conducted under sterile conditions were only considered indicative of the potential pathogenicity of these isolates (Saksena and Vaartaja, 1961).

More recently, root dieback of both containerized and bare-rooted Norway spruce [*Picea abies* L. (Karst.)] and Scots pine (*P. sylvestris*) seedlings has been a considerable problem in Nordic nurseries (Venn *et al.*, 1986; Lilja *et al.*, 1992). The visual symptoms: wilting of young shoots, hanging tops, retarded height growth, discoloration of the foliage and partial or total death of the root system often appear after midsummer, on first year seedlings or occasionally later during the second growing season. Surveys for fungi present in the diseased roots and pathogenicity testing suggest that root dieback is a complex phenomenon involving both *Rhizoctonia* spp. and *Pythium* spp. (Venn *et al.*, 1986; Lilja *et al.*, 1992). Pathogenicity experiments performed under nursery conditions implicated the supporting sand beds as an inoculum source for *Rhizoctonia* spp. in container seedling production (Venn *et al.*, 1986).

Based on conserved anamorphic and induced teleomorphic characteristics, the *Rhizoctonia* sp. causing damage on nursery tree stocks in Norway and Finland represents a single species possessing uninucleate cells (Hietala *et al.*, 1994; see preface). Basidial characteristics refer it to the teleomorph genus *Ceratobasidium*, possibly *C. bicorne* Erikss. and Ryv., which is exceptional since all the known anamorphs of *Ceratobasidium* species are binucleate (see Sneh *et al.*, 1991). However, there was no affinity with the other *Ceratobasidium* AG testers, and similar analysis with *C. bicorne*, only available as herbarium material, was not possible (Hietala *et al.*, 1994). In pathogenicity tests with Norway spruce (Hietala, 1995) and Scots pine (Sen and Hietala, unpublished), uninucleate *Rhizoctonia* isolates attacked all parts of the root system, although the root tips were most heavily infected resulting in a stunted root system morphology (see preface). Cortical cells infected with monilioid cells were occasionally observed.

In the Finnish surveys, non-pathogenic binucleate *Rhizoctonia* spp. were also isolated from the roots of similarly diseased and healthy-looking conifer seedlings (Lilja *et al.*, 1992; Lilja 1994). In a case study, binucleate *Rhizoctonia* spp. were found to co-exist with the uninucleate *Rhizoctonia* sp. in the same root system and were divided into four anastomosis groups including AG-I of the genus *Ceratobasidium* and three anastomosis groups not related to known anastomosis groups of *Ceratobasidium* (Hietala, 1995). In pathogenicity tests on Norway Spruce (Hietala, 1995) and Scots pine (Sen and Hietala, unpublished) seedlings, isolates representing these groups infected only basal root regions and cortical cells filled with monilioid hyphae were commonly observed. Although the infection had no effect on root growth of Norway spruce seedlings, isolates representing AG-I and two other AGs significantly increased shoot and root growth of Scots pine seedlings. A non-orchid mycorrhizal relationship could be hypothesized with the latter conifer species (see chapters V.14 and VI. B4) although fungal infection levels were low (<20 % of root length infected).

C. Other *Rhizoctonia* associated with tree roots

Castellani (1934b) described three species, *R. pini-insignis* Cast., *R. quercus* Cast. and *R. fraxini* Cast., from tree roots. The former two were respectively isolated from nursery grown *Pinus insignis* Dougl. (= *Pinus radiata* D. Don) and *Quercus*

pedunculata Ehrh. and the latter from apparently naturally growing *Fraxinus excelsior* L. Burpee *et al.* (1980) found *R. fraxini* and *R. pini-insignis* to be binucleate and *R. quercus* uninucleate although Ogoshi *et al.* (1983) could not confirm a uninucleate condition in the latter species. Unfortunately, the pathogenicity of these species was not tested on the respective hosts (Castellani 1934a).

D. *Rhizoctonia* associated with shoot diseases

In India, *R. solani* has been identified as the causal agent of web blight in a number of nursery-grown broad-leaved trees including *Eucalyptus*, *Acacia* and *Albizia* species (Sharma *et al.*, 1984; Sharma and Sankaran 1984; Sankaran *et al.*, 1986; Mehrotra, 1990). The symptoms vary depending on the host, but a common feature is the premature defoliation of mycelium covered diseased leaves of young plants (Mehrotra, 1990). Isolates of *R. solani* obtained from the different hosts could be separated into three morphological groups, but cross inoculations of different hosts indicated no host specificity (Mehrotra, 1990). Another shoot disease caused by *R. solani* was reported in the same study; top flagging of khasi pine (*Pinus kesiya* Royle ex Gordon). Diseased nursery seedlings, 15 - 25 cm in height, displayed withered tops which later drooped and turn ash-colored. Infected needles were loosely bound with hyphae and the stems were also attacked. In both these shoot diseases, the respective host trees were most susceptible to *R. solani* over the wet season when the prevailing humid conditions enabled foliage colonization from soil inoculum sources via the stems (Mehrotra, 1990). Certain weed species were also highly susceptible to the disease and were suspected of being an additional source of infection. Disease control measures involve immediate removal of affected seedlings, weeding and a reduction in sowing densities.

Needles of four- to five-year-old Norway spruce seedlings have been reported to be killed by a binucleate *Rhizoctonia* sp. in Norwegian forest nurseries (Roll-Hansen and Roll-Hansen, 1968). A binucleate *Rhizoctonia* sp. AG-E (=CAG 3), was shown to be associated with foliar blight of nursery grown longleaf pine (*P. palustris* Mill., English *et al.*, 1986) and loblolly pine (*P. taeda* L., Runion and Kelly, 1993) in USA. On longleaf pine, the symptoms include necrosis of needles and terminal buds which are especially severe in sandy soils where sand accumulates around the needle bases and terminal buds. The sand possibly acts as an inoculum source and provides warm and humid conditions that apparently favour disease development. Affected needles of loblolly pine are characteristically bound in a mycelial webbing, turn grey and eventually drop-off. The severity of disease generally increases in seedbeds following crown closure which allow vegetative spread of the fungus via shoot contact (Runion and Kelly, 1993). Fungicide application does to some extent control the disease (Runion *et al.*, 1994). Prior to sowing, seedbed soils are routinely fumigated with methyl bromide (English *et al.*, 1986).

III. *RHIZOCTONIA* IN FOREST HABITATS

Limited observations on the occurrence of the perfect states of *Rhizoctonia* confirm that the genus does thrive in forest habitats. Eriksson and Ryvarden (1973) reported the occurrence of *Ceratobasidium cornigerum* (Bourdot) Rogers on a variety of substrates and particularly the fresh bark of detached branches of both conifer and deciduous trees. Other *Ceratobasidium* species observed on bark of trees include *C.*

pseudocornigerum M.P. Christ. (Eriksson and Ryvarden, 1973; Breitenbach and Kränzlin, 1986; Kotiranta and Saarenoksa, 1990) and *C. stridii* Erikss. and Ryv. (Eriksson and Ryvarden, 1973). The green needles of standing pines and the upper soil litter layer were implicated as a source of the orchid endophyte, *R. goodyera-repentis* Costantin and Dufour (teleomorph, *C. cornigerum*), in baiting experiments using the compatible orchid host (Downie, 1943). Understory plants have been found to host *T. cucumeris* (Poldmaa *et al.* 1982; Kotiranta and Saarenoksa, 1993) and *C. bicorne* (Eriksson and Ryvarden (1973).

A. *Rhizoctonia* spp. as disease agents in forests

The nuts of beech (*Fagus sylvatica* L.) have been reported to be attacked by *R. solani* in France (Perrin and Muller, 1979) and Germany (Dubbel, 1989). In Denmark, beechnuts are often similarly affected by *Rhizoctonia* (J. Koch, personal communication) and the isolates appear to be binucleate (Koch and Hietala, unpublished). The soilborne nature of *R. solani* was demonstrated in comparisons of seed from the forest floor with those collected from nets suspended above the ground (Dubbel, 1989). The disease is characterized by a decay of the cotyledon bearing tissue within the seed resulting in reduced germination or death after cotyledon emergence (Perrin and Muller, 1979). Higher disease incidence is related to increased pH and the level of soil organic material but soil cultivation before the mast is a good disease control strategy. It is recommended that beechnuts are rapidly harvested and where possible incubated before storage at 34 - 37°C at 10 % relative humidity for 24 hours. This procedure significantly reduced disease incidence (Perrin and Muller, 1979).

Surface sterilized seeds of Norfolk Island pine *Araucaria heterophylla* (Salisbury) Franco, growing in Egypt, were found to harbor *Rhizoctonia solani* while still within cones on the tree (El-Lakany *et al.*, 1981). The fungus was isolated from the endosperm and embryo tissues and was also implicated as the principle seedborne pathogen causing pre- and post emergence damping-off in this and two other *Araucaria* species (Kamara *et al.*, 1981).

In two tropical Indian forests, *R. solani* has been identified as the causative agent of a root rot disease on seedlings of *Phyllanthus emblica* L., *Quercus serrata* Thunb. and *Citrus* sp. (Chakravarty and Mishra, 1982). Disease symptoms, on mainly one to six-month-old seedlings, included either wilting or, in severe cases, stem collapse due to rotting of tissues at the base of the seedlings. The high temperatures and rainfall prevalent between May and August coincided with the highest disease incidence.

IV. Future prospects

A range of *Rhizoctonia* species are reported to be associated with trees both in nurseries and forest habitats. However, there is still a clear need for further identification of the putative *R. solani* anamorph. This was highlighted by Parmeter *et al.* (1967) who observed that mycelial and cultural characteristics of some *Rhizoctonia* species with a *Ceratobasidium* perfect state resemble those of *R. solani* so closely that only numbers of nuclei per cell or the perfect state distinguish them. Burpee *et al.* (1980) also stated that "many of the binucleate isolates in AGs-E -F and -R (=CAGs 3, 4 and 5) were morphologically indistinguishable from isolates of *R. solani*". Yet, in the majority of papers reviewed the observed anamorph was assigned to *R. solani* although neither the teleomorph nor the nuclear condition of cells were described. As induction of the teleomorph is problematic in many species, the nuclear condition is a

minimum diagnostic characteristic that can easily be determined using a number of nuclear stains (see Sneh *et al.*, 1991).

Few studies have been reported on a number of *R. solani* anastomosis groups (see Chapter I.A2), particularly AG 4, that are associated with nursery tree seedlings. The AGs represent independent genetic units that often show different host preferences and levels of pathogenicity (see Chapter II.1). As the AG testers of *R. solani* and also *Ceratobasidium* are standardized and available from culture collections (e.g. American Type Culture Collection), the affinities of unknown multi- and bi-nucleate *Rhizoctonia* isolates associated with all plants, including trees, can be readily determined. This is of particular importance in enabling valid comparisons of the increasing data on the effects of *Rhizoctonia* spp. in nursery tree production and in natural forest ecosystems.

It is clear that *Rhizoctonia* spp. are associated with forest trees but are mainly pathogens of young seedlings in forest nurseries. Increasing global pressure for re-forestation will require expanded nursery seedling production where conventional chemical disease control practices have often proved ineffective. Due to their broad-spectrum toxicity, the use of many of the pesticides and fumigants e.g. benomyl and methyl bromide is being phased-out leading the way to the development of biological control solutions. For the successful application of this alternative control strategy further detailed information is needed on the *Rhizoctonia* spp. involved in seedling diseases, their alternative host preferences (e.g. weed species) and interactions with other pathogens (e.g. *Pythium* and *Fusarium* spp.) and beneficial micro-organisms (e.g. *Trichoderma* spp. and mycorrhizal fungi, see chapters VI.B2, VI.B4 and VI.B7). Re-forestation of former agricultural land also may present additional problems relating to the potential broad host-range of pathogenic *Rhizoctonia* species.

These challenges will require increased effort and co-operation between researchers in fields such as *Rhizoctonia* taxonomy and genetics, plant/tree physiology and pathology, agronomy, silviculture, molecular biology and biotechnology.

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