Rhizoctonia - Scots pine interactions: detection, impact on seedling performance and host defence gene response

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Front cover picture: Containerized healthy Scots pine seedlings at Röykkä nursery (top left), tryphan blue stained Scots pine root colonised by BNR highlighting intracellular monilioid cells (top right) and the apical region of a Scots pine root infected with tryphan blue stained hyphae (bottom left) and sclerotia and hyphae on the root surface (photos Henrietta Grönberg).
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<td>AG</td>
<td>anastomosis group</td>
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<tr>
<td>AM</td>
<td>arbuscular mycorrhizal fungus</td>
</tr>
<tr>
<td>Sp-AMP</td>
<td>antimicrobial peptide gene</td>
</tr>
<tr>
<td>anamorph</td>
<td>the asexual form of fungi also known as the vegetative stage</td>
</tr>
<tr>
<td>avirulent</td>
<td>non-pathogenic</td>
</tr>
<tr>
<td>BNR</td>
<td>binucleate <em>Rhizoctonia</em></td>
</tr>
<tr>
<td>Ct</td>
<td>cycle threshold, the PCR cycle at which the fluorescence exceeds that of background</td>
</tr>
<tr>
<td>cDNA</td>
<td>DNA synthesized from mRNA with reverse transcriptase (contains no introns)</td>
</tr>
<tr>
<td>CHS</td>
<td>chalcone synthase</td>
</tr>
<tr>
<td>dhy-like</td>
<td>dehydrin-like protein</td>
</tr>
<tr>
<td>d.p.i.</td>
<td>days post inoculation</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid, the genetic material</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>PsGER1</td>
<td>germin-like protein</td>
</tr>
<tr>
<td>h.p.i.</td>
<td>hours post inoculation</td>
</tr>
<tr>
<td>HR</td>
<td>hypersensitive response</td>
</tr>
<tr>
<td>hypovirulent</td>
<td>from avirulent to low virulent strains, which show no disease symptoms in host plants</td>
</tr>
<tr>
<td>intron</td>
<td>non-coding sequences within coding genes in eukaryotic genome</td>
</tr>
<tr>
<td>ISR</td>
<td>induced systemic resistance</td>
</tr>
<tr>
<td>ITS</td>
<td>internal transcribed spacer. The non-coding sequence between coding areas of rDNA subunits</td>
</tr>
<tr>
<td>MCA</td>
<td>microbial control agents</td>
</tr>
<tr>
<td>MF</td>
<td>mycorrhizal fungi</td>
</tr>
<tr>
<td>MNR</td>
<td>multinucleate <em>Rhizoctonia</em></td>
</tr>
<tr>
<td>monilioid</td>
<td>hyphal structures typical for <em>Rhizoctonia</em> infection in host cell</td>
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<tr>
<td>NCBI</td>
<td>national center for biotechnology information</td>
</tr>
<tr>
<td>primer</td>
<td>short oligonucleotide sequence used for specific amplification of target DNA by PCR/qRT-PCR</td>
</tr>
<tr>
<td>probe</td>
<td>oligonucleotide sequence used in hybridizations with target DNA</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time PCR</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal DNA</td>
</tr>
<tr>
<td>r.l.c.</td>
<td>root length colonised</td>
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<tr>
<td><em>pal1</em></td>
<td>phenylalanine ammonia lyase</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td><em>Psyp1</em></td>
<td>peroxidase</td>
</tr>
<tr>
<td>RAPD</td>
<td>random amplified polymorphic DNA fragment</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>PR-proteins</td>
<td>pathogenesis related proteins</td>
</tr>
<tr>
<td><em>PsACRE</em></td>
<td>rapidly elicited defence-related gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCAR</td>
<td>sequence-characterized amplified region</td>
</tr>
<tr>
<td>sclerotia</td>
<td>structures formed by fungi typical to <em>Rhizoctonia</em></td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Sp</td>
<td>species (singular form)</td>
</tr>
<tr>
<td>Spp</td>
<td>species (plural form)</td>
</tr>
<tr>
<td>STS</td>
<td>stilbene synthase</td>
</tr>
<tr>
<td>teleomorph</td>
<td>the sexual form of fungi producing spores and sclerotia</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>the specific melting temperature, where respective double stranded DNA denatures to single strands</td>
</tr>
<tr>
<td>UNR</td>
<td>uninucleate <em>Rhizoctonia</em></td>
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Abstract

*Rhizoctonia* spp. are ubiquitous soil inhabiting fungi that enter into pathogenic or symbiotic associations with plants. In general *Rhizoctonia* spp. are regarded as plant pathogenic fungi and many cause root rot and other plant diseases which results in considerable economic losses both in agriculture and forestry. Many *Rhizoctonia* strains enter into symbiotic mycorrhizal associations with orchids and some hypovirulent strains are promising biocontrol candidates in preventing host plant infection by pathogenic *Rhizoctonia* strains.

This work focuses on uni- and binucleate *Rhizoctonia* (respectively UNR and BNR) strains belonging to the teleomorphic genus *Ceratobasidium*, but multinucleate *Rhizoctonia* (MNR) belonging to teleomorphic genus *Thanatephorus* and ectomycorrhizal fungal species, such as *Suillus bovinus*, were also included in DNA probe development work. Strain specific probes were developed to target rDNA ITS (internal transcribed spacer) sequences (ITS1, 5.8S and ITS2) and applied in Southern dot blot and liquid hybridization assays. Liquid hybridization was more sensitive and the size of the hybridized PCR products could be detected simultaneously, but the advantage in Southern hybridization was that sample DNA could be used without additional PCR amplification.

The impacts of four Finnish BNR *Ceratorhiza* sp. strains 251, 266, 268 and 269 were investigated on Scot pine (*Pinus sylvestris*) seedling growth, and the infection biology and infection levels were microscopically examined following trypan blue staining of infected roots. All BNR strains enhanced early seedling growth and affected the root architecture, while the infection levels remained low. The fungal infection was restricted to the outer cortical regions of long roots and typical monilioid cells detected with strain 268.

The interactions of pathogenic UNR *Ceratobasidium bicone* strain 1983-111/1N, and endophytic BNR *Ceratorhiza* sp. strain 268 were studied in single or dual inoculated Scots pine roots. The fungal infection levels and host defence-gene activity of nine transcripts [phenylalanine ammonia lyase (*pal1*), silbene synthase (*STS*), chalcone synthase (*CHS*), short-root specific peroxidase (*Psyp1*), antimicrobial peptide gene (*Sp-AMP*), rapidly elicited defence-related gene (*PsACRE*), germin-like protein (*PsGER1*), CuZn- superoxide dismutase (*SOD*), and dehydrin-like protein (*dhy-like*)] were measured from differentially treated and un-treated control roots by quantitative real time PCR (qRT-PCR). The infection level of pathogenic UNR was restricted in BNR- pre-inoculated Scots pine roots, while UNR was more competitive in simultaneous dual infection. The *STS* transcript was highly up-regulated in all treated roots, while *CHS*, *pal1*, and *Psyp1* transcripts were more moderately activated. No significant activity of *Sp-AMP*, *PsACRE*, *PsGER1*, *SOD*, or *dhy-like* transcripts were detected compared to control roots.

The integrated experiments presented, provide tools to assist in the future detection of these fungi in the environment and to understand the host infection biology and defence, and relationships between these interacting fungi in roots and soils. This study further confirms the complexity of the *Rhizoctonia* group both phylogenetically and in their infection biology and plant host specificity. The knowledge obtained could be applied in integrated forestry nursery management programmes.
Tiivistelmä (Abstract in Finnish)

**Rhizoctonia**-suvun sienet ovat maaperässä yleisesti esiintyviä sieniä, jotka voivat olla tautia aiheuttavia patogeeneja tai muodostaa hyödyllisiä symbiontisia vuorovaikutussuhteita kasvien kanssa. Yleensä **Rhizoctonia** spp. sienet ovat kasvipatogeeneja aiheuttaen mm. juurilahotautia niin metsäpuille kuin viljelykasveillekin. Useat **Rhizoctonia**-suvun sienet muodostavat orchidea-kasveille elintärkeitä symbiontisia sienijuuria (mykorritsia) ja jotkut tauteja aiheuttamattomista **Rhizoctonia**-kannoista voivat estää patogeenisten kantojen infektion tutkituilla kasveilla. Tässä työssä keskityttiin yksi- ja kaksitumaisiin **Rhizoctonia**-sieniin, jotka kuuluvat teleomorfin **Ceratobasidium**-sukuun, mutta DNA koettimet kehitettiin myös monitumaiselle **Rhizoctonia**-sienelle (teleomorfin **Thanatephorus**) ja nummitattu-ektomyrkkykoritäiselle (**Suillus bovinus**). Koettimet suunniteltiin ribosomaalisen DNA:n ”internal transcribed spacer” (ITS) -alueelle (ITS1, 5.8S ja ITS2) ja valmistettiin kahdella käsittelyllä: ”Southern dot blot”- ja ”Northern hybridisaation” etuna oli, että koettimen hybridisoinnissa voitiin käyttää suoraa DNAa, jolloin PCR-reaktiota ei tarvittu. Työssä tutkittiin suomalaisten kaksitumaisten **Rhizoctonia**-kantojen (**Ceratorhiza** sp.) 251, 266, 268 and 269 vaikutusta männyn (**Pinus sylvestris**) ja infektion taso määritettiin mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinelliä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja mikroskopialla trypaanisinellä värjättyjä juurijoitoja ja 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trypaanisinellä värjättyjä juurijoitoj
1. Introduction

1.1. **Rhizoctonia** taxonomy

The genus *Rhizoctonia* is a large, diverse and complex group of fungi (Sneh *et al.* 1996). In the identification of *Rhizoctonia* species, the teleomorph is the most important morphological character, but fruiting of *Rhizoctonia* species is difficult to induce under laboratory conditions. Therefore, characterization and species designation is mainly based on the vegetative characters of the anamorph that include mycelial colour, hyphal diameter, number of nuclei, length of cells, shape and size of monilioid cells and sclerotial size (Hietala 1998).

*Rhizoctonia* strains are designated as uni-, bi- or multi-nucleate (UNR, BNR and MNR, respectively), with respect to nuclear condition. The determination of nuclear condition is particularly informative in isolate characterization of vegetative hyphae. Where teleomorph data is available, *Rhizoctonia*-like anamorphs are distributed in several families: Platygloeaceae (genus *Helicobasidium*), Exidiaceae (*Sebacina*), Atheliaceae (*Athelia*), Tulasnellaceae (*Tulasnella*), Ceratobasidiaceae (*Thanatephorus*, *Ceratobasidium*), and Botryobasidiaceae (*Waitea*) (Hawksworth *et al.* 1995). Overall, the latter families with multinucleate (*Thanatephorus* and *Waitea*) and mostly binucleate (*Ceratobasidium*) anamorphs have received the most attention (see Sneh *et al.* 1996). Uninucleate *Rhizoctonia* strains, isolated from roots of conifer trees in Finland (Hietala *et al.* 1994) and Norway (Venn *et al.* 1986), belong to the *Ceratobasidium* family and are conspecific with a moss parasite *Ceratobasidium bicorne*, based on rDNA (internal spacer, ITS) sequence analysis (Hietala *et al.* 2001). More recently, a large number of fungi displaying *Rhizoctonia* anamorphs have been isolated from tropical orchids in Puerto Rico by Otero *et al.* (2002) who found that 66 of 108 examined were uninucleate and showed highly related anamorphic and ITS phylogenetic relationships with Finnish conifer pathogenic uninucleate *Rhizoctonia* (*Ceratobasidium bicorne*) (Hietala *et al.* 1994, Hietala *et al.* 2001).

Due to its broad host range and common pathogenic association with important agricultural crops, probably the most studied *Rhizoctonia* species is *Rhizoctonia solani* Kühn. The perfect state of *R. solani*, *Thanatephorus cucumeris*, belongs to the family *Ceratobasidiaceae* (order *Ceratobasidiales*) of class Basidiomycetes (Hawksworth *et al.* 1995). Based on hyphal interactions in pairing experiments, multinucleate *R. solani* has been grouped into different anastomosis groups (AGs) and represents a collection of closely related isolates grouped together based on their ability to anastomose with one another. In the so called “killing reaction” anastomosis results in death of anastomosing and adjacent cells, but no killing reaction occurs in self-anastomosis and in hyphal reactions between clonal isolates (Carling 1996).

Our understanding of *Rhizoctonia* genetic diversity and taxonomy has been greatly improved by this powerful tool, the anastomosis group concept (Vilgalys & Cubeta 1994). At least 12 anastomosis groups of *R. solani* are reported that include AG 1-11 and AG-B1 (Carling *et al.* 1994, Carling 1996).

The telemorph *Ceratobasidium* belongs to the family *Ceratobasidiaceae* (order *Ceratobasidiales*) of class Basidiomycetes (Hawksworth *et al.* 1995). The anamorphs of this genus are mainly uninucleate, except for the uninucleate *Ceratobasidium bicorne* Eriks. & Ryv. (Hietala *et al.* 1994, Hietala *et al.* 2001) mentioned earlier. The binucleate *Rhizoctonia* species are also grouped into different AGs. The genus is divided into 21 AGs (from AG-A to AG-S) (Sneh *et al.* 1991). *Ceratobasidium* species commonly cause root and foliar diseases (Kataria & Hoffmann 1988) but also forms symbiotic mycorrhizal associations with orchids (Andersen & Ramussen 1996).
Sen et al. (1999) proved by AG tests and rDNA-RFLP (restriction fragment length polymorphism) fingerprinting a high degree of intraspecific variation within BNR belonging to teleomorphs of Ceratobasidium cornigerum. These fungi include isolates able to enter into either mutualistic or pathogenic root association with susceptible host plants. In the anastomosis grouping analysis of different BNR strains, the Scots pine BNR isolates were found to be related to mycorrhizal BNR (anamorph Ceratorhiza spp.) endophytes of local Finnish and Canadian orchids. This was further confirmed in the ITS-RFLP analysis (Sen et al. 1999). Two of the Scots pine BNR isolates (268 and 251) fell into the AG-I grouping known to contain members that are plant pathogens (Ogoshi et al. 1983, Sneh et al. 1991). The AG-I tester isolate (AV-2) used, is a pathogen of Artemisia vulgaris var. indica (Ogoshi et al. 1979). The type anamorph of AG-I is R. fragariae (Ogoshi et al. 1983), which is known to cause the black root disease in strawberry (Wilhelm et al. 1972, Martin 1998). Interestingly, the Scots pine isolate 251 also anastomosed with two Canadian orchid isolates. Two other isolates (266 and 269) were not members of any of the other 20 described AGs (AG-A - AG-S) of binucleate Rhizoctonia (Sen et al. 1999).

1.2. Internal transcribed spacer (ITS) as a target marker for fungal identification

The ITS region occurs as a part of a multicyclop rDNA cistron in the fungal genome (I: Fig. 1), allowing PCR amplification also from degraded DNA samples. The ribosomal RNA genes are generally arranged as a tandemly repeated array with both variable and highly conserved regions (Gardes et al. 1991). Internal transcribed spacer regions (ITS 1 and ITS 2), between conserved rRNA genes (18 S, 5.8 S and 28 S subunits), are non-coding areas and are known to be quite variable within certain species and also tend to be highly variable between distantly related species and related genera (Bruns & Gardes 1993).

Universal primers for fungal PCR amplification of the ITS region (ITS1/ITS 4) were originally developed by White et al. (1990) (I: Fig.1). These primers allow fungal specific ITS amplification directly from conifer tree associated mycorrhizas as they discriminate against most conifer host DNA templates, for example Scots pine (Pinus sylvestris) (Timonen et al. 1997) and Norway spruce (Picea abies) (Erland 1995).

The ITS is the most well-studied DNA marker in fungi and there is an extensive ITS fungal sequence database (cf. Kõljalg et al. 2005). This enables multiple sequence alignments between numerous fungal ITS sequences for robust phylogenetic analyses. It also allows identification of target rDNA, both variable and conserved regions that could also represent suitable sites for molecular probe development. The ITS has been a target in several different molecular studies within Rhizoctonia species (see section 1.3) and molecular approaches have been very fruitful in answering basic questions concerning systematic relationships and genetic variation in Rhizoctonia spp. (Vilgalys & Cubeta 1994). The continued growth of Rhizoctonia ITS sequence data (Gonzalez et al. 2001) has enabled increasingly robust sequence comparisons and phylogenetic analyses that support the earlier anastomosis grouping in Rhizoctonia spp. classification (Sharon et al. 2008).

1.3. Rhizoctonia spp. identification and molecular detection

Because telemorph induction is a major problem within Rhizoctonia species, the characterization of isolates has been usually based on anamorphic characteristics. Since the vegetative characteristics of many Rhizoctonia species overlap, it is difficult to identify
these species (Hietala 1998). Anastomosis grouping based on hyphal fusion has been a powerful tool in recognising *Rhizoctonia* groups within species (Carling 1996), but molecular methods have improved *Rhizoctonia* isolate identification. Methods such as DNA/DNA hybridization (Kuninaga & Yokosawa 1984 and 1985), isozyme analysis (Liu *et al.* 1990), Restriction Fragment Length Polymorphism (RFLP) (Liu & Sinclair 1992), Randomly Amplified Polymorphic DNA (RAPD) analysis (Lilja *et al.* 1996, Leclerc-Potvin *et al.* 1999, Grosch *et al.* 2007) and sequence-characterized amplified region (SCAR) (Grosch *et al.* 2007) are specific tools used.

Another approach used, is PCR-based identification utilising strain specific rDNA-ITS region targeted primers (Salazar *et al.* 2000, Kasiamdari *et al.* 2002) or taxon-specific primers, as designed by Taylor and McCormick (2008) for basidiomycetous orchid mycorrhizas belonging families *Tulasnella* and *Thelephora-Tomentella* complex. Lees *et al.* (2002), on the other hand, applied ITS region for detection and identification of *Rhizoctonia solani* AG-3 in potato and soil by more sensitive qRT-PCR-method, which optimally permits quantification at sensitivity of one gene copy.

**1.4. *Rhizoctonia* spp. as economically important fungal pathogens of plants**

Many *Rhizoctonia* species cause economically important diseases on most of the world’s important crop plants, such as: cereals, cotton, sugar beet, potato, vegetables, field crops, turf grasses, ornamentals, fruit trees and forest trees (Sneh *et al.* 1996). The best known of these, the multinucleate *R. solani* Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk], causes losses averaging up to 20 % yearly in over 200 crops world-wide (Sneh *et al.* 1996). It causes damping-off disease on conifer trees and has been detected in forest nurseries in many countries including France (Perrin & Sampangi 1998) and Poland (Stepniowska-Jarosz *et al.* 2006).

In Finland, on the other hand, mainly uni- or binucleate *Rhizoctonia* strains have been isolated from diseased conifer seedlings, the most pathogenic strains being uninucleate (Lilja 1994, Hietala *et al.* 1994). Both in Finland and Norway, a uninucleate *Rhizoctonia* (*Ceratobasidium bicorne*) has been identified causing root dieback disease on Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) seedlings (Hietala *et al.* 1994; Hietala & Sen 1996, Hietala *et al.* 2001). These two tree species are economically the most important in Finland and thus form a major proportion of the different tree species grown in nurseries and delivered for planting. Nurseries are involved in cultivation of tree seedling stock (1-3 years) for reforestation. Seedlings can be cultivated as bare-root stock in open nursery beds or in containers made from plastic, paper or peat. In Finland, paper manufacture and wood industries are an important source of national income and *Rhizoctonia* species, that infects young conifer tree seedlings, may result in major economic losses. To date, no effective control treatment against these pathogenic fungi has emerged. The uninucleate *Rhizoctonia* attacks conifer roots from root tips and penetrates into the vascular cylinder (Hietala 1998). The infection results in the root dieback disease with symptoms such as needle discoloration, partial death of the root system and stunted seedling growth (Lilja 1996). Binucleate *Rhizoctonia* (BNR) are well known plant pathogens and have also been isolated from diseased and healthy looking Scots pine and Norway spruce seedlings (Lilja *et al.* 1992), but BNR isolates did not negatively influence seedling growth in nursery peat soil pathogenicity tests (Lilja 1994, Hietala 1995).
1.5. Binucleate *Rhizoctonia* as symbiotic fungi and bicontrol agents

Strains of *Ceratobasidium* spp. commonly exist in mycorrhizal associations with orchids (Sneh et al. 1996). These orchid associated *Rhizoctonia* strains are reported to form typical morphological structures in host tissues that have been identified as monilioid cells (Zelmer et al. 1996, Sharma et al. 2003).

BNR effects on plant growth promotion have been studied by Villajuan-Abgona et al. (1996) who observed that BNR strain W7 showed plant growth promotion in terms of significant increase in plant height (P = 0.01) and fresh weight (P = 0.05). Harris (1999) presented a study where BNR isolates increased *Capsicum* shoot and root dry weights. Chang & Chou (2007) showed both a binucleate *Rhizoctonia* spp. and multinucleate *Rhizoctonia* AG-6 of orchid mycorrhizal fungi (MF) stimulating the orchid *Anoectochilus formosanus* Hyata growth compared to non-mycorrhizal plants. Plant height, number of leaves, root length and fresh weights were enhanced by these fungi and they also showed higher enzymic activities of superoxide dismutase (SOD) in leaves, and acid and alkaline phosphatases in roots, together with higher contents of ascorbic acid, polyphenols, and flavonoids.

1.5.1. Biocontrol: principles, applications and mechanisms

According to a plant dictionary (Allaby 2006) biological control is defined as “human use of natural predators for the control of pests, weeds etc.” or the “total or partial destruction of pathogen populations by other organisms” (Agrios 1997). Biocontrol organisms are usually microbial control agents (MCA) namely bacteria, protozoa, fungi or viruses. Biopesticides, on the other hand, are “mass-produced, biologically based agents used for the control of plant pests” (Chandler et al. 2008), which can be divided to living organisms, naturally occurring substances, and genetically modified plants (Copping & Menn 2000). Biocontrol agents are mainly developed to prevent specific pests or pathogens from infecting the target plant. Examples include hypovirulent or avirulent strains, which infect the host without causing a disease. Protection is achieved either through direct biocontrol agent-mediated antagonism against the pathogen, through competition for infection sites, or the biocontrol agent may strengthen the host defence through induced resistance (Cardoso & Echandi 1987).

Due to its wide distribution and severity as an economically important pathogen of crop plants, many studies have concentrated on investigating promising biocontrols against *R. solani* Kühn. Both bacteria (Adesina et al. 2007, Yanqui et al. 2008, Scherwinski et al. 2008) and avirulent binucleate *Rhizoctonia* strains (e.g Escande & Echandi 1991, Villajuan-Abgona et al. 1996, Poromarto et al. 1998, Hwang & Benson 2003, Pascual et al. 2004) have shown promise as biocontrol agents against *R. solani* in different plants. BNR (*Ceratobasidium albatensis*) has been shown to control *R. solani* induced damping-off in *Pinus* spp. (Rubio et al. 2001 and pers. comm.). The authors suggested that the timing of the biocontrol inoculation is an important parameter. BNR protective effects against *R. solani* are usually obtained following BNR pre-inoculation before *R. solani* attack, either after short periods of preinoculation e.g. 24 h (Poromarto et al. 1998) or 48 h (Xue et al. 1998) and up to 7 or more days of pre-inoculation (Hwang & Benson 2003). Because the biocontrol effect is not activated during simultaneous infection, direct antagonism is probably not the mechanism involved (Herr 1995). Rather competition and induced resistance are suggested as being the controlling mechanism in these relationships (see 1.5.2. and 1.5.3.).
1.5.2. Competition as a potential mechanism for biocontrol

Competition signifies competition for nutrients or infection sites, or both. If the host plant cells are pre-occupied by an avirulent strain, the pathogen tends to be unable to infect same cells. Additionally, lack of nutrients may reduce the attraction of pathogens. Therefore indirect protection exists despite antimicrobial or defence related molecules being involved. Competition is suggested to be one possible biocontrol mechanism in _R. solani_ suppression in BNR treated plants (Cardoso & Echandi 1987, Herr 1995).

1.5.3. Induced resistance: another potential mechanism for biocontrol

Infection of plant with an avirulent strain or its secreted molecules might elicit a host defence reaction, which in subsequent challenge or infection is more rapidly and efficiently activated. This systemic acquired resistance (SAR) (Ryals _et al._ 1994) or induced systemic resistance (ISR) (Xue _et al._ 1998) can prevent further infection of plant host by potentially more severe pathogens. Thus, SAR/ISR can be exploited as a biocontrol mechanism. The SAR includes the activation of host defence genes and often also a plant hypersensitive response (HR). HR is expressed in plant tissues as self-induced localized cell death at the site of infection, which prevents the pathogen from further penetration into adjacent cells, and is associated with generation of activated oxygen radicals (Agrios 1997).

Many authors have suggested induced resistance being the mechanism of protection in BNR-preinoculated plants against pathogenic _R. solani_ (Cardoso & Echandi 1987, Xue _et al._ 1998, Porommarto _et al._ 1998, Hwang & Benson 2003) and several defence genes have been reported to be involved.

1.6. Defence-gene activity

1.6.1. Defence-genes in conifers

The arsenal of defence genes activated in coniferous trees and the timing of this activation depends on the specific elicitor and the pathogenicity of each strain. In Scots pine (_Pinus sylvestris_), for example, the transcriptional responses were differentially activated in mutualistic, saprotrophic or pathogenic fungi (Adomas _et al._ 2008), and during the early stage fewer genes were differentially expressed in pathogenic, as opposed to ectomycorrhizal or saprotrophic interaction. The authors suggested that as the host was unable to recognise the nature of the attack during the early stages of infection, this enabled the pathogen to overcome the host’s defence. In the later stages of infection, the host defence responded more strongly to interaction with the pathogen. Additionally, induction of defence genes in Norway spruce were more rapid in a pathogen resistant clone compared to less resistant one (Karlsson _et al._ 2007).

Pathogenesis related (PR) proteins are a diverse group of plant proteins possessing antimicrobial properties against invading fungal pathogen. Some of the best known are PR1 proteins, β-1,3-glucanases, chitinases, lysozymes, PR 4 proteins, thaumatinelike proteins, osmotinlike proteins, cysteine-rich proteins, glysine-rich proteins, proteinase inhibitors, proteinases, chitosanases, and peroxidases (Agrios 1997). Some of these have been reported as also being involved in the interactions between coniferous trees and different fungal strains.
Chitinases were an important example of differentially expressed genes in Scots pine (*Pinus sylvestris*) during the infections involving mutualistic, saprotrophic or pathogenic fungi (Adomas et al. 2008). These enzymes degrade the fungal cell wall and have, in many other studies, been reported to be involved in coniferous plants defence (Hietala et al. 2004, Jøhnk et al. 2005, Fossdal et al. 2007, Karlsson et al. 2007, Adomas et al. 2007). Peroxidases, on the other hand, are activated during both ectomycorrhizal (*Laccaria bicolor*) and pathogenic (*Heterobasidion annosum*) interaction in Scots pine (Adomas et al. 2008) and in *H. annosum* infection on Norway spruce (*Picea abies* (L.) Karst.) (Jøhnk et al. 2005, Karlsson et al. 2007). A class III peroxidase activity and thaumatin were activated constitutively (1, 5, and 15 d.p.i.) in *Pinus sylvestris* during *Heterobasidion annosum*- infection (Adomas et al. 2007) and related transcripts were also up regulated in saprotrophic (*Trichoderma aureoviride*) infection at 1 d.p.i. (Adomas et al. 2008). Other transcripts reported to be induced in *H. annosum*- Scots pine interaction includes for example glutathione-S-transferase and germin-like protein (Karlsson et al. 2007).

Another pathogen, a uninucleate *Rhizoctonia* (*Ceratobasidium bicorne*), induced 25 transcripts in Norway spruce, including oxidative-process-associated proteins, such as germin-like isoforms, peroxidase and a glutathione S-transferase (Jøhnk et al. 2005). Some of the sequences used in a related study (Fossdal et al. 2007) for primer design and many transcripts, including chitinases, and defensin, together with other previously mentioned enzymes, were activated in the same host by this pathogen. On Scots pine, an antimicrobial peptide gene *Sp-AMP* (Asiegbu et al. 2003) and a defence-related gene *PsACRE* (Li & Asiegbu 2004) were associated in *Heterobasidion annosum* infection. The antimicrobial gene was also induced by the saprotroph and symbiotic fungi at 1 d.p.i., but up-regulation continued only in the pathogenic interaction 5 and 15 d.p.i. (Adomas et al. 2008). This pathogen induced 179 differentially expressed sequence tags in a cDNA microarray study, the most abundant ones being enzymes involved in the phenylpropanoid pathway and defence-related proteins with antimicrobial properties (Adomas et al. 2007). Whether these genes are correspondingly up-regulated in Scots pine roots during the *Ceratobasidium bicorne* infection remains unknown.

### 1.6.2. Phenylpropanoid pathway

The amino acid L-Phenylalanine is a precursor for phenylalanine ammonia lyase (PAL) catalyzing the first reaction in a phenylpropanoid pathway leading to cinnamic acids. It consists of a C₆C₃ carbon skeleton, which by further hydroxylation and methylation yields simple phenylpropanoids such as, p-coumaric, caffeic, ferulic and sinapic acids (Gross 1985). Many other important and more complex secondary metabolite products are also produced in further steps of this pathway that include lignin, flavonoids, and stilbenes. The latter two are respectively synthesized by chalcone synthase (CHS) and stilbene synthase (STS), two closely-related polyketide synthases (PKS), in a condensation of p-coumaroyl-CoA/cinnamoyl-CoA with three molecules of malonyl-CoA (Hahlbrock & Grisebach 1979, Dixon & Paiva 1995). The STS in *Pinus sylvestris* is classified as pinosylvin synthase, since it prefers cinnamoyl-CoA to dihydrocinnamoyl-CoA as a substrate, but the substrate preference in expression studies *in vitro* is influenced by factor(s) co-existing in plant or bacterial extracts (Schannz et al. 1992). In *Pinus strobus* (Eastern white pine), on the other hand, both substrates are utilized and therefore two stilbene types - pinosylvin and dihydropriosylvin- exist (Raiber et al. 1995). The STS sequences of *P. strobus* clustered in their own subgenus (*STROBUS STS*) in the study of Kodan et al. (2001), while *Pinus densiflora* clustered together with *P. sylvestris* in another subgenus (*PINUS STS*) and all CHS sequences from these three *Pinus* species in their own CHS subgenus. Two *P. strobus* CHS sequences have been identified by Schröder et al. (1998), one being more closely related to *P. sylvestris* CHS, while the other using methylmalonyl-CoA in a condensation with c-methylated chalcones and suggested to have
a special role in the biosynthesis of secondary products. The latter type of CHS is not present in *P. sylvestris* (Schröder et al. 1998). The Scots pine defence-related activation of stilbenes is elicited by stress factors such as fungi, ozone, or mechanical stress (Lange et al. 1994, Fliegmann et al. 1992, Zinser et al. 1998, Chiron et al. 2000).

Many enzymes of the phenylpropanoid pathway are coded by multiple genes in many plants (Dixon et al. 2002). For example, PAL, is encoded by a multigene family in pine (*Pinus banksiana*) (Butland et al. 1998) and also has multiple isoforms in *Arabidopsis* (Cochrane et al. 2004). PAL defence-related activation together with many other defence-related transcripts, were recently shown in *Heterobasidium annosum* elicited conifers (Karlsson et al. 2007, Adomas et al. 2007, 2008) and uninucleate *Rhizoctonia* (*Ceratobasidium bicorne*)-infected Norway spruce, where the enzyme activation compared during the infection and drought stress (Fossdal et al. 2007).

1.7. Induction of host defence-gene activity by hypovirulent strains

Pre-inoculation of bean seedlings with non-pathogenic BNR have been reported to induce the expression of host defence enzymes e.g. peroxidases, and 1,3-β- glucanases, which positively correlated with induced systemic resistance (ISR) against *R. solani* and *Colletotrichum lindemuthianum* pathogens (Xue et al. 1998). Seedlings treated with BNR prior to infection elicited significantly more of these host defence enzymes compared to the diseased and control plants. Similar enzymes were also induced in soybeans pre-treated with *Pseudomonas aureofaciens* bacteria (Jung et al. 2007) and are also generally considered as being involved in plants induced resistance (Agrios 1997). Induced resistance was also suggested as being the mechanism in a study of Cardoso & Echandi (1987) who showed that BNR filtrates did not inhibit *R. solani* but BNR treated root exudates did such that the *R. solani* protective effect was retained in BNR pre-inoculated roots after surface sterilization.

Preinoculation of bean seedlings with an arbuscular mycorrhizal fungus (AM) *Glomus intraradices* Schenck and Smith, on the other hand, did not significantly prevent disease symptoms of *R. solani*, and no change in phenylalanine ammonia lyase, (PAL), chalcone synthase (CHS), or chalcone isomerase were observed. *R. solani* has been reported to induce a systemic increase of defence genes, regardless of the AM status of the plant (Guillon et al. 2002). Wen et al. (2005) found that the highest defence-gene activities of 1,3-β-D- glucanase, PAL, and CHS, were in *R. solani* infected beans, but were also expressed, though down-regulated in seedlings pretreated with non-pathogenic binucleate *Rhizoctonia* before *R. solani* infection. Cardinale et al. (2006) investigated the activities of biological defence markers PR1, laminarinase, and chitinase to follow the plant-mediated resistance of hypovirulent *R. solani* against *Botrytis cinerea* pathogen in tomato. Hypovirulent strain of *R. solani* boosted the *B. cinerea* induced enzyme activities and the authors suggested a systemic induced resistance being the nature of protection.
2. Aims of the study

The aim of this study was to investigate the infection biology and impact of pathogenic uninucleate *Rhizoctonia* (UNR) (*Ceratobasidium bicorne*) strains 1983-111/1N and 263, and endophytic binucleate *Rhizoctonia* (BNR) (*Ceratorhiza sp.*) strains 268, 251, 266 and 269 on Scots pine growth and host defence responses, and to detect these strains from infected roots. More specific goals in the respective studies were to:

- develop DNA probes targeted to ITS region of fungal rRNA to different *Rhizoctonia* groups (uni-, bi-, or multinucleate) and *Suillus bovinus* and apply probes in comparison of Southern- and liquid hybridization (Hendolin et al. 2000) against target DNA from relevant fungi (*Suillus, Rhizoctonia* and other plant root pathogenic fungi). Probes were designed to be used for strain-specific detection.
- investigate and to better understand fungal-host interactions - infection biology, morphology, and root and shoot growth responses - of Scots pine seedling infected with four binucleate *Rhizoctonia* strains (268, 251, 266 and 269).
- quantify Scots pine host defence-gene (*pal1, STS, CHS, Psyp1, Sp-AMP, PsGER1, PsACRE, SOD, dhy-like*) activities in roots during infection with the root pathogenic UNR strain 1983-111/1N and endophytic BNR strain 268, by infecting respective roots either separately on single-inoculations or as dual-inoculations in order to detect possible strain specific responses in Scots pine defence-reaction.
3. Materials and Methods

3.1. Strains and methods described in publications

This study targeted uni- and binucleate *Rhizoctonia* spp. strains (Table 1), but relevant fungi from the genus *Suillus* and *Rhizoctonia*, and other plant root pathogenic fungi were included in hybridizations (I: Table 1). All methods used in the three separate studies are briefly described in Table 2, except for methods relating to unpublished experiments, which are described separately.

*Table 1. Fungal Rhizoctonia spp. strains*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Telomorph</th>
<th>Nuclear status</th>
<th>Reference</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1983-111/IN</td>
<td><em>(Ceratobasidium bicorne)</em></td>
<td>UNR</td>
<td>Hietala <em>et al.</em> (1994)</td>
<td>III</td>
</tr>
<tr>
<td>263</td>
<td><em>(Ceratobasidium bicorne)</em></td>
<td>UNR</td>
<td>Hietala <em>et al.</em> (1994)</td>
<td>I</td>
</tr>
<tr>
<td>268</td>
<td><em>(Cerarhiza sp.)</em></td>
<td>BNR</td>
<td>Sen <em>et al.</em> (1999)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>251</td>
<td><em>(Cerarhiza sp.)</em></td>
<td>BNR</td>
<td>Sen <em>et al.</em> (1999)</td>
<td>I, II</td>
</tr>
<tr>
<td>266</td>
<td><em>(Cerarhiza sp.)</em></td>
<td>BNR</td>
<td>Sen <em>et al.</em> (1999)</td>
<td>I, II</td>
</tr>
<tr>
<td>269</td>
<td><em>(Cerarhiza sp.)</em></td>
<td>BNR</td>
<td>Sen <em>et al.</em> (1999)</td>
<td>I, II</td>
</tr>
</tbody>
</table>
Table 2. Methods used in three separate studies.

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
<th>study</th>
<th>Short description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seedling growth</td>
<td>Sen (2001), Heinonsalo et al. (2001)</td>
<td>II, III</td>
<td>pots: 20 h day length, &gt;230 µmol m⁻² s⁻¹, +20/12°C, cuttings in work II and seedlings in work III, 19 h day length, 170-300 µmol m⁻² s⁻¹, +19-24/15°C</td>
</tr>
<tr>
<td>Inoculation</td>
<td>Sen (2001)</td>
<td>II, III</td>
<td>II: over 30 days after or in the cuttings at the time of 14-day-old seedling removal, III: one week after or BNR in the experiment 2 at the time of 10-day-old seedling removal.</td>
</tr>
<tr>
<td>Fungal growth</td>
<td>Marx (1969), Modess, (1941), Sen et al. (1999), Murashige &amp; Skoog (1962)</td>
<td>I, II, III</td>
<td>+20°C, medias: Modified Melin Norkans (MMN), Hagem’s agar (HA), malt-extract agar (MA), potato-dextrose-agar (PDA), Murashige and Skoog nutrient salts medium (MS), Water agar (WA)</td>
</tr>
<tr>
<td>DNA extraction</td>
<td>Heinonsalo et al. (2001), Qiagen Product Manual</td>
<td>I, III</td>
<td>CTAB-method, DNeasy plant mini kit (Qiagen, Hilden, Germany)</td>
</tr>
<tr>
<td>RNA extraction</td>
<td>Qiagen Product Manual</td>
<td>III</td>
<td>Plant MiniRNeasy kit (Qiagen, Maryland, USA)</td>
</tr>
<tr>
<td>PCR</td>
<td>Heinonsalo et al. (2001)</td>
<td>I, III</td>
<td></td>
</tr>
<tr>
<td>Cloning</td>
<td>Kõljalg et al. (2002)</td>
<td>I</td>
<td>pGEM®-T easy vector (Promega, Madison, WI)</td>
</tr>
<tr>
<td>Sequencing</td>
<td></td>
<td>I</td>
<td>ALF sequencer (Amersham Biosciences, Chalfont) or ABI Prism 310 capillary sequencer (Applied Biosciences, Foster City, CA) Analysis: GCG Fragment Assembly System (Program Manual for the Wisconsin Package, Version 8, Wisconsin)</td>
</tr>
<tr>
<td>Sequence alignment</td>
<td>Higgins et al. (1992)</td>
<td>I</td>
<td>Clusteral method</td>
</tr>
<tr>
<td>Primer design</td>
<td></td>
<td>I, III</td>
<td>III: ABI Primer Express® (Applied Biosciences, Foster City, CA, USA)</td>
</tr>
<tr>
<td>Liquid- and Southern</td>
<td>DIG system’s user guide</td>
<td>I</td>
<td>DIG (Roche, Germany) or Biotin (Institute of Biotechnology, UH, Finland) labelled probes</td>
</tr>
<tr>
<td>hybridization</td>
<td>(Roche, Germany), Karcher (1991), Hendolin et al. (2000).</td>
<td></td>
<td></td>
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<tr>
<td>cDNA synthesis</td>
<td></td>
<td>III</td>
<td>Taqman Reverse Transcription Reagent, (Applied Biosystems, New Jersey, USA)</td>
</tr>
<tr>
<td>Tryphan blue staining</td>
<td>Koske &amp; Gemma (1989)</td>
<td>II, III</td>
<td>fungal root-infection visualization, III: 10 min. +60 °C in 2.5% KOH, rinsed twice with H₂O₂, neutralized in 1% HCl 1 h at RT, stained 10 min. +60 °C in tryphan blue.</td>
</tr>
<tr>
<td>Microscopy</td>
<td></td>
<td>II, III</td>
<td>II: Olympus BX50 (Olympus, Japan), III: Olympus BX50F-3 (Co., Hamburg, Germany) and Image-® PLUS software (version 4).</td>
</tr>
<tr>
<td>Statistics</td>
<td>Paffl (2001) (III)</td>
<td>II, III</td>
<td>II: one-way ANOVA and pair wise differences by Tukey’s HSD test, SPSS version 8.0 for MS Windows, III: GLM procedure, SAS (P &lt; 0.05), III: analysis of variance, GLM procedure of SAS (P&lt;0.05)</td>
</tr>
</tbody>
</table>
3.2. Phylogenetic analysis

A nearest neighbor tree based on ITS (ITS1-5,8S-ITS2) sequence (I) analysis was generated using the MEGA4 program (Tamura et al. 2007). Waitea circinata strain TGCC 17.1 (de la Cerda et al. 2007) was selected for outgroup in this analysis.
4. Results

4.1. ITS probe development for Southern and liquid hybridization

4.1.1. Primary ITS PCR fragments and sequence alignment

The size of the single fragments PCR products amplified with ITS 1 and ITS 2 primers from genomic DNA were between 644-703 base pairs (I). The Rhizoctonia solani HK1 PCR product was 703 bp.

As expected the 5.8 S was highly conserved among different Rhizoctonia strains and Suillus species. In the Suillus ITS sequence comparison the most variable region located in ITS 1 for the two S. bovinus strains (SBH1 and C3-1-M1) differed only by a single base pair in their ITS sequence (I). The beginning of this ITS 1 region was targeted for Suillus probe construction (I: Fig. 1). ITS 1 was also the most variable region identified in the Rhizoctonia ITS sequence comparison, but the similarly variable ITS 2 region was targeted for probe development, since suitable optimal primer sequences for PCR amplification were found in this region. The conserved 5.8 S ribosomal gene region was targeted for generation of a control ‘fungal’ probe (I: Fig. 1)

4.1.2. Phylogenetic analysis

The nearest neighbour phylogenetic analysis of the ITS sequences indicated that BNR AV-2 (AG-I) and 268 strains were closely related in the same Rhizoctonia cluster (Fig. 1.). Strains 251 and 266 were more closely related with each other than these strains to other binucleate Rhizoctonia strains. All binucleate Rhizoctonia strains (251, 266, AV-2, 268 and 269) were found in a general cluster and as was expected, the uninucleate 263 and multinucleate Rhizoctonia solani strains were clearly less related to the binucleate Rhizoctonia cluster.
4.1.3. Probe construction and application in Southern dot-blot- and liquid hybridization

Primer sequences designed to amplify particular probe sequence from ITS target sequence are presented (I: Table 3). The size of the PCR amplified probes were between 124 and 151 bp. PCR products were of the correct size, except for a few problematic samples. When control primers were used to amplify the control probe sequence and when \textit{Rhizoctonia solani} ITS insert in plasmid vector was used as a template, the PCR product occasionally included additional faint bands (data not shown). Amplification of \textit{Suillus} strain template with \textit{Suillus} primers also produced a faint smaller band, but DNA from the correct sized bands extracted from agarose gel and used in secondary PCR, produced mostly only the expected sized DNA fragment without additional bands.

The restriction analysis of amplified probes gave the expected size restriction fragments, except the \textit{Rhizoctonia solani} probe sequence amplified with \textit{Rhizoctonia} primers, where some additional bands were detected (data not shown).

The optimal hybridization temperature for oligonucleotide probes were about 15-30 °C lower than the $T_m$ while near the $T_m$ in PCR amplified probes (I: Table 2.). The estimated detection limit to DIG labeled probes in dot blotting were 0.1-0.01ng/µl (dilutions 1:10-1:100). Dot blot hybridizations with DIG-labelled probes targeted total DNA templates extracted from fungal pure cultures or from Scots pine (\textit{Pinus sylvestris}) roots and ITS-PCR amplified from this same DNA was used in biotin-probe hybridizations. A summary of dot blot hybridization results (I: Table 4) shows the biotin labeled oligonucleotide probes specificity over PCR amplified DIG-probes. For example, a probe (number 3) developed to strain 268, hybridized only with the respective strain, while the DIG-labelled probe (number 7) developed to the close relative AV-2 hybridized with all Finnish binucleate strains (I: Table 4). Biotin labelled \textit{S. bovinus} probe (number 2) also detected only strains of \textit{S. bovinus} species, while DIG-labelled probe (number 1) also detected \textit{S. granulatus}.

The same hybridization temperatures optimised for Southern hybridization also gave the best results in liquid hybridization, indicating the temperatures being dependent on probe sequences rather than method applied. Low temperature washing (30 or 35°C) occasionally affected the specificity with 268 or \textit{Suillus} probe (number 3 and 2 probes respectively in I: Table 2). The biotin labelled probe in combination with liquid hybridization delivered the highest detection specificity (I: Table 5 and Fig. 4).
4.2. Scots pine seedling growth and root architectural responses to binucleate *Rhizoctonia* inoculation

Compared to uninoculated controls, the shoot and root weights in the BNR-inoculated seedlings exhibited significant ($P=0.05$) differences at 87 d.p.i. (II). No strain specific differences were detected among the four Finnish BNR isolates (251, 266, 268 and 269). The root biomass and length increased, while the long root width decreased and the short root numbers (cm$^{-1}$ long root) remained of a similar magnitude as uninoculated seedlings.

By the second harvest (240 d.p.i.), the mean short root numbers increased significantly, but no differences in shoot biomass were detected compared to uninoculated controls. The root growth responses were also more attenuated. The root infection levels remained extremely low (<6% and 1% r.l.c. at 87 and 240 d.p.i., respectively).

4.3. Bi- and uninucleate *Rhizoctonia* infection biology: antagonism and microscopy studies

No antagonistic effects were detected between BNR 268 and UNR 1983-111/1N in a dual inoculation on water-agar Petri-dish (Fig. 3 a). Monilioid cells detected from the roots of tryphan blue stained Scots pine radicals infected with BNR strain 268 nine weeks p.i. (Fig. 2 a) (II: fig 2), and eight d.p.i. in the surface of UNR-infected roots (Fig 2. c) (III). No monilioid cells were developed in BNR infected roots during the monitored period in study III. In single inoculations, BNR and UNR colonization was detected in cortical cells throughout the entire root, but BNR colonized more intensively root cells from the basal region, while UNR colonized heavily the root tips and less intensively basal regions in a contrasting manner to BNR (Fig. 3 b, d). Both UNR colonized and uncolonized root tips were detected in dual-infected roots (Fig. 3 c, III).
Figure 2. a.) BNR moniloid cells b.) BNR septate hyphae (arrow), c.) UNR moniloid structures in the root surface and d.) UNR hyphae growing between root cells. Bars 1 µm.
4.4. Real time PCR

4.4.1. Quantification of fungal genomic DNA in infected roots

Fungal genomic DNA was measured as in Hietala et al. (2008) using strain-specific rDNA ITS-primers and GAPDH primers respectively for detection of fungal genomic DNA from infected roots and host genomic DNA (III: Table 1). The level of colonization was estimated from the fungal DNA/ (host DNA +fungal DNA) -ratio in each root sample.

4.4.1.1. Experiment 1.

The BNR infection in dual infected roots in experiment 1 remained low, but the difference in the BNR DNA content at 96 h.p.i between single and dual inoculation was not significant (p = 0.07). In the single inoculations, the mean DNA content of both UNR and BNR reached near 5% 96 h.p.i. (Fig. 4 a and b, III: Fig. 1). The contents of UNR
infection were similar both in single and dual inoculated roots (Fig. 4 b., III: Fig. 1 b). The BNR and UNR contents transiently decreased at 24 and 48 h.p.i., respectively.

![Image of fungal DNA contents](image)

**Figure 4.** The mean fungal DNA contents (%) of total (host and fungal DNA) in infected roots at inoculation (0) and 4, 24, 48, 96 and 192 h.p.i. DNA contents in single BNR (a) and UNR (b) infected roots, DNA of respective strains detected from dual- infected roots (+). In experiment 1, both fungi were inoculated simultaneously while in the experiment 2, BNR was inoculated one week earlier.

4.4.1.2. Experiment 2.

Experiment 2 was performed similarly to experiment 1, except that BNR was preinoculated one week earlier in respective roots in order to determine possible biocontrol effects of BNR against UNR, and prolonged inoculation times were used. In the BNR-preinoculated dual infected roots, the DNA content of UNR remained significantly (p = 0.02) lower 192 h.p.i. than in single inoculated roots (Fig. 4 b, III: Fig. 1 b). The overall BNR DNA contents were similar between single and dual infected roots throughout the experiment. (Fig. 4 a, III: Fig. 1 a).

4.4.2. Defence-gene transcript levels

The STS transcript was highly up-regulated in all treated roots compared to the control over the experimental period in both experiments (Fig. 5 a, and 6 a, III: Fig. 2 a). The highest activities were detected in UNR single infections. BNR and dual inoculated roots showed relatively similar STS transcript levels.
Figure 5. Defence-gene transcript activities in the experiment 1 (III). a.) STS, PAL, CHS, and PRX. b.) PAL, CHS, and PRX repeated in order visualize differences between the transcripts.

Figure 6. Defence-gene transcript activities in the experiment 2 (III). a.) STS, PAL, CHS, and PRX. b.) PAL, CHS, and PRX.

The mean PRX activity increased in all fungal treatments at 96 h.p.i. in experiment 2 (Fig. 6 b, and III: Fig. 2 b), but the standard deviation between replicates was extremely high, and only the activity in dual infected roots in experiment 1, 48 and 96 h.p.i., were significant ($P < 0.03$).

Significant up-regulation of PAL were detected in dual infection 96 h.p.i. in both experiments and 192 h.p.i. in the experiment 2. Single UNR infected roots showed significant up-regulation at all time points post 48 h.p.i.

The only significant CHS activity was detected in experiment 2 between dual inoculated roots and single UNR inoculated roots 96 (p =0.0075) and 192 h.p.i. (p = 0.0438).

AMP, RED, GLP, SOD, DEH transcripts were not significantly activated in any treatments or incubation times (data not shown.)
5. Discussion

5.1. *Rhizoctonia* spp. ITS sequences and phylogenetic analysis

The binucleate *Rhizoctonia* (BNR) strains used in this study have earlier been identified as anamorphic *Ceratorhiza* spp., which include binucleate *Rhizoctonia* that form mycorrhiza with orchids (Sen *et al.* 1999). A phylogenetic analysis of ITS sequences of BNR from this study and diverse binucleate *Rhizoctonia* (including members of all anastomosis groups) strictly confirms that our binucleate *Rhizoctonia* are the telemorphic species *Ceratobasidium cornigerum* (Gonzalez *et al.* unpublished data). This also now confirms the earlier anastomosis group/ RFLP study of Sen *et al.* (1999). The ITS-sequences of strains 268 and AV-2 (=AG-I) revealed 99% similarity (I). The uninucleate *Rhizoctonia* (UNR) strains 263 and 1983-111/1N, selected for this study, have been identified as *Ceratobasidium bicorne* based on rDNA (internal spacer, ITS) sequence analysis (Hietala *et al.* 2001).

In *Rhizoctonia* taxonomy, the faster and more precise ITS sequencing technique combined with phylogenetic analyses have now mostly replaced earlier anastomosis grouping methodology, nevertheless the AG grouping remain valuable tools in identification. Similarly, in agreement with a phylogenetic analysis of our strains (Gonzalez *et al.* unpublished data) many authors including Stepniewska-Jarosz *et al.* (2006) and Sharon *et al.* (2008) have more recently showed that the ITS sequence analyses distinguishes the isolates into separate clades according to their AG grouping. This confirms the value of this methodology and that the AG grouping has a clear genetic basis.

5.2. ITS as a target for *Rhizoctonia* spp. probe development

The ITS sequence within the rDNA cistron represents a suitable target for strain identification due to the presence of high sequence similarity between related strains and simultaneously sufficient differences between unrelated strains. We observed that the ITS-1 and ITS-2 regions offered relatively high sequence variation for >100 bp probe, and highly variable areas for shorter oligonucleotide probe development. Species/taxa specific probes specific for conifer root pathogenic and endophytic *Rhizoctonia* spp. and the ectomycorrhizal *Suillus bovinus* were successfully developed. PCR amplified (124-151 bp) probes and oligonucleotide (20-25 bp) probes were respectively developed for Southern and liquid hybridization (Hendolin *et al.* 2000). Oligonucleotide probes were also highly specific in Southern hybridization against ITS1/4 amplified-PCR samples.

The ITS sequences of uninucleate *Rhizoctonia* are highly conserved (Hietala *et al.* 2001), but more variable between binucleate *Rhizoctonia*. The 5.8 S ribosomal gene is known to be highly conserved among different *Rhizoctonia* species (Kuninaga *et al.* 1997, Hietala *et al.* 2001), but it also proved to be highly conserved in all fungi used in the sequence alignment. The non-coding ITS1 and ITS 2 regions were more variable, as could have been expected. Binucleate *Rhizoctonia* strains 268 and AV-2 (AG-I), which belongs to the same anastomosis group, were practically identical (99%) having only few nucleotide differences in ITS 1. Interestingly, two *Suillus bovinus* strains isolated from different forest areas in South and central Finland (Leppäkorpi and Hyytiälä), differed by a single nucleotide substitution; C and T at base position 123, respectively.

The additional PCR amplified bands obtained with control and *Suillus* primers can be explained by the fact that the ITS region occurs as a part of a multicopy rDNA cistron in the fungal genome so that ribosomal RNA genes are arranged as a tandemly repeated array (Gardes *et al.* 1991). This means that one ITS product obtained in PCR could include
several minor ITS sequence length variants. As such, a cloning step has to be included before sequencing, where only one DNA fragment is ligated to the vector and in this way overlapping signals can be avoided. However, additional bands with control primers appeared on only a single occasion and there might have been some contamination in the particular PCR reaction. *Suillus* primers regularly amplified additional bands in the primary PCR. Targeted sized PCR product was obtained after re-amplification of extracted DNA of the correct size.

We preferred the approach of direct fungal detection from root material, than using PCR amplified DNA, because it is faster and eliminate the amplification-related problems (errors or bias) that might occur during the PCR amplification. In this respect, >100 bp DIG labelled probes were targeted specifically against total DNA. All these longer probes were tested against DNA extracted from Scots pine roots. This plant serves as a host to both *Suillus bovinus* forming mycorrhiza and the uni- and binucleate *Rhizoctonia* strains in pathogenic and biotrophic interactions (Hietala & Sen 1996). Only the probe developed against uninnucleate *Rhizoctonia* strain 263 hybridized with Scots pine root DNA sample. While direct detection would shorten the identification process, the PCR based method used in liquid hybridization was found to be more sensitive and reliable than Southern hybridization, because the size of the hybridized PCR product is visualized on the computer screen simultaneously. Additionally, possible similarities between probe and non-target of fungal DNA sequences are eliminated through pre-amplification by PCR with ITS1 and ITS2 primers. These universal primers specifically amplify the ITS region from most fungi (Bruns & Gardes 1993) and in this way, for example bacterial or host plant DNA could have been excluded in hybridization test.

The *Suillus* probe was not, however, *Suillus bovinus* specific, since it hybridized with all other *Suillus* species except *Suillus luteus*. Further screening with additional *Suillus* strains and strains of other species should be carried out to determine the limits of probe specificity. Conversely, probes developed targeting specific binucleate *Rhizoctonia* strains were highly specific and thus of diagnostic value. The uninucleate 263 probe (number 8, I: Table 2) might need more stringent conditions or 1:100 dilutions should be used to avoid unspecific fungal or root binding.

Another alternative approach for fungal identification is to design specific primers for PCR detection of certain fungal species e.g. Salazar *et al.* (2000) and Taylor & McCormick (2008), but the possible contamination risk is one disadvantage in such a PCR-based procedure. Groppe *et al.* (1995), and Groppe & Boller (1997) have used microsatellite containing loci as a target in PCR detection for ecology and diversity studies of the plant associated fungus *Epichloë*. Buscot *et al.* (1996) used microsatellite-primed PCR in morels DNA polymorphism investigations. This is another alternative target in fungal DNA based studies, although ITS is a better DNA target for probe development. Microsatellite regions are stretches of tandem mono-, di-, tri-, and tetrancleotide repeats of various lengths and a wide range of repeat lengths can be present in a single population (Groppe & Boller 1997). The most sensitive method available is perhaps the quantitative real-time PCR (qRT-PCR), successfully used for *R. solani* AG-3 (Lees *et al.* 2002, Lehtonen *et al.* 2008) and AG-1 IA detection (Sayler *et al.* 2007). This method combined with TaqMan chemistry, allows also more reliable quantitative detection of the target fungi than the conventional PCR, since only a specific PCR product is detected.

Classical morphological methods are still of value, even though molecular methods have obvious advantages in species identification. Specific probes for direct detection of target sequences for example in soil, are almost impossible to obtain, since there are so many different organisms and different species in nature, with uncharacterized DNA and there is always a possibility that the developed probe give "false" positives in hybridizing to DNA regions from these other organism. However, the qRT-PCR with TaqMan probes, now allows more specific detection also from complicated soil samples, as showed by Lees *et al.* (2002) in comparison to qRT-PCR with conventional PCR. Nevertheless, in order to reduce errors, morphological characters of isolated fungi (where possible) and
lesions tissue analysis for example from *Rhizoctonia* infected plants should be the first step in species identification and detection with specific probes, in conventional hybridization methods or qRT-PCR associated, could be used to confirm fungal identity.

The developed *Rhizoctonia* probes (I) or the use of strain specific primers or probes in qRT-PCR would help us to detect these economically important strains both from nurseries and the forest site and probably greatly help improve our understanding of *Rhizoctonia* infection biology. These probes would also help further answer the question as to whether these different fungal groups or different strains of *Rhizoctonia* spp. commonly co-colonize the same root system (Hietala 1995).

### 5.3. Binucleate *Rhizoctonia* linked effects on Scots pine growth

BNR are typically associated with orchid species (Andersen & Rasmussen 1996, Zelmer *et al.* 1996, Sharma *et al.* 2003), but have also been reported to show beneficial effects on other plants. Growth inducing effects had earlier been reported in capsium (Harris 1999) and cucumber (Villajuan-Abgona *et al.* 1996), and now in Scots pine seedlings (II). Some BNR strains are pathogenic (Stepniewska-Jarosz *et al.* 2006) and this was originally thought to be the case with our Finnish binucleate *Ceratorhiza* spp. when isolated from diseased nursery Scots pine seedlings (Lilja *et al.* 1996). However, our BNR strains induced early (87 d.p.i.) Scots pine shoot and root growth, but in the second harvest (240 d.p.i.) the differences between inoculated and uninoculated seedlings were not significant. Instead, the short root number increased and was more numerous in BNR inoculated than uninoculated seedlings. The low root infection-rate, and morphology identified by tryphan blue staining, suggests that a true mycorrhizal relationship does not occur between host and fungus. In forest soil, Scots pine rapidly enters into mycorrhizal associations with several fungal species (Smith & Read 1997, Timonen *et al.* 1997), but typical ectomycorrhiza structures have, to our knowledge, not been reported in *Rhizoctonia* colonized conifer roots. Additionally BNR associated Scots pine seedlings showed yellow-green needles in N- and C- limited nursery soil (II), suggesting lack of nutrients, whereas in similar aged mycorrhizal seedlings associated with the true mycorrhizal fungus *Suillus bovinus*, the needles remained a healthy dark green in the same nursery soil (Sen 2001). These data indicate that early root induction was not related to improved nutrient uptake through a classical mycorrhizal association. However, BNR linked root growth could be explained through possible hormonal linked stimulation. Plant growth promoting hormonal signaling is crucial for development of plant-microbe interactions, and indole-acetic acid (IAA) has been detected in *R. solani* cultures (Furukawa *et al.* 1996); the amount of IAA was found to increased in *R. solani* rice suspension cultures (Furukawa & Syono 1998). In a related study, Kaparakis and Sen (2006) showed that similar Scots pine adventitious root inducing effects resulting from indol-3-butyric acid (IBA) exposure could be obtained in BNR inoculation experiments and, importantly, that BNR-linked root induction was even more efficient. Whether there are differences in hormonal communication between pathogenic or non-pathogenic *Rhizoctonia*-host interactions is not known. It is clear that further work is needed in order to understand the molecular and physiological mechanisms of hormonal interaction in these associations.

Hwang & Benson (2003) investigated the potential of BNR strains as biocontrol agents against *R. solani* attack in poinsettia cuttings, but they did not compare the rooting or growth promotion effects of BNR. Their work focused only on the biocontrol aspect, but it would be interesting to find out whether their BNR would stimulate the growth promoting effects as our results suggests from Scots pine cuttings (Kaparakis & Sen 2006). The typical monilioid cells present within the cells of the outer cortex, as visualized by tryphan blue staining, have been earlier reported in orchid mycorrhiza (Zelmer *et al.* 1996, Sharma *et al.* 2003) and uninucleate *Rhizoctonia* (Hietala 1997).
Seedlings infected by BNR produce more short roots, which in forest soils, would rapidly become colonized by mycorrhizal fungi during mycorrhiza development. In nursery soils these nonmycorrhizal short roots could represent sites for UNR entry. As Rhizoctonia spp. have been isolated from fine roots of Scots pine trees in Scottish forest stands (Steve Woodward, pers. comm.) and from S. bovinus mycorrhizas (Sen 2001), more work needs to be carried out using the developed probes in both nursery and forest sites.

5.4. Scots pine host defence responses to bi- and uninucleate Rhizoctonia and the interactions in co-inoculation

Preinoculation of binucleate Rhizoctonia (BNR) strain 268 decreased the infection level of pathogenic uninucleate Rhizoctonia (UNR) strain 1983-111/-1N in the Scots pine radicals. However, in in vitro confrontation assays on water agar it neither showed any antagonistic effects against UNR (Fig. 3a), nor prevented UNR entry into the root cells in vivo. The simultaneous coinoculation with UNR decreased the BNR infection level compared to BNR single inoculations, indicating UNR being more competitive in the infection process, despite the slower in vitro growth on WA plate. In BNR preinoculated roots, on the other hand, the UNR level was restricted compared to single UNR inoculations. This might be due to the root cells being precolonized with BNR. Surprisingly, both BNR and UNR infection levels were temporarily decreased during the infection process, respectively, 24 and 48 h.p.i., suggesting that the biochemical communication between fungus and host retards the infection.

Absolute quantification of UNR and BNR infection in this study was impossible, since data on the size of their respective genomes and ITS copy numbers is not available. However, our results show the relative variation in each treatment and at different time post inoculation. Regardless of potential differences in the ITS copy number between BNR and UNR, it would not alter the fact that the amount of UNR was restricted in BNR preinoculated roots, compared to simultaneous dual inoculation.

In a study by Cardoso & Echandi (1987), BNR also failed to show antagonistic interaction in dual cultures of bean (Phaseolus vulgaris L.) with R. solani. Filtrates alone from BNR cultures did not, but filtrates from BNR treated root exudates did inhibit R. solani hyphal growth and BNR pretreatment inhibited formation of R. solani infection cushions. Therefore, the authors suggested that BNR- induced metabolic response was the mechanism of R. solani suppression. In our study (III) the UNR infection specific DNA levels decreased similarly in BNR preinoculated roots, but the impact of host defence-gene activity is not clear.

In the BNR-preinoculated dual infected roots, lower PAL transcript activity was identified compared to simultaneous dual infection, although the transcript was significantly up-regulated in both experiments compared to control. Significant PAL activity was also obtained in single UNR infected roots in experiment 2 compared to control, but regardless of the respective identically accomplished treatments of single UNR infections in experiment 1, these later roots showed no significant PAL activation. Wen et al. (2005) detected increased PAL, CHS, and 1,3-β-D-glucanase activities in R. solani infected beans (Phaseolus vulgaris L.), while in BNR treated, and BNR -pretreated and R. solani-infected bean seedlings, the activity was lower. The preinoculation with the mycorrhizal fungus, Glomus intraradices Schenck and Smith, however, did not alter the bean PAL-, CHS-, chalcone isomerase-, and hydroxyproline-rich glycoprotein activities during R. solani infection (Guillon et al. 2002).

In our study, CHS was activated significantly only in dual infected roots in experiment 2, compared to UNR inoculated roots 96 and 192 h.p.i. Some activity, although not significant, was already detected at the time of inoculation (T=0). CHS gene-
activity is probably not solely induced in Scots pine defence reactions, since it has been shown to be also expressed in non-stressed plantlets (Fliegmann et al. 1992). In Norway spruce, on the other hand, Ophiostoma polonicum-infection elicited both CHS and STS activities, but in contrast to our results, the CHS was more stimulated (Bringolas et al. 1995). In our study, the STS activity was highly up-regulated during all treatments, being highest in UNR single inoculated roots. The STS responded also to stress in nicked Scots pine hypocotyls more efficiently than CHS (Fliegman et al. 1992). Our study showed relatively high variations in all transcript activities between replicates, indicating high degree of genetic variation between seedlings. Thus, the use of clonal material would be the solution in future experiments.

All three studied phenylpropanoid pathway involved enzymes, phenylalanine ammonia lyase, CHS, and STS, have long been known to be induced by different pathogens (Gehlert et al. 1990, Lange et al. 1994, Fliegmann et al. 1992, Zinser et al. 1998, Chiron et al. 2000, Yu et al. 2005). Adomas et al. (2007) reported that enzymes from the phenylpropanoid pathway, defence-related proteins with antimicrobial properties, and class III peroxidases, were the most abundant genes up-regulated during H. annosum infection in Pinus sylvestris. However, a Pinus sylvestris short root-specific peroxidase (Psyp1), which is also a class III peroxidase, is known to be down-regulated in ectomycorrhizas of the Scots pine- Suillus bovinus symbiosis (Tarkka et al. 2001). However, similar down-regulation of this enzyme, was not observed in our BNR treatments, regardless of their beneficial role in Scots pine roots (II). This might be due to the non-mycorrhizal role of this fungus in Scots pine. It would be interesting to determine the activation of Psyp1-related transcript in BNR-mycorrhizal associations in orchid plants. In our study, significant peroxidase activities detected only in the simultaneous dual infected roots suggests this infection type being more effective on Psyp1 activation than either of these strains separately. Rhizoctonia challenged peroxidases have been also detected from bean (Phaseolus vulgaris L.) and Norway spruce in several studies (Xue et al. 1998, Nagy et al. 2004, Johnk et al. 2005, Fossdal et al. 2007) and selected peroxidase genes most probably affect the results, since for example in a study of Nagy et al. (2004), 12 peroxidase isoforms are identified from the Norway spruce (Picea abies) roots. The same fungal strain also induced several other defence transcripts in Norway spruce, including phenylalanine ammonia lyase, and two germin-like proteins (Fossdal et al. 2007). In our experiment, however, the germin-like protein (Mathieu et al. unpublished) showed no significant up- or down-regulation compared to the control. Neither did the rest of the selected potential pathogen-induced transcripts: antimicrobial peptide gene Sp-AMP (Asiegbu et al. 2003), a defence related gene PsACRE (Li & Asiegbu 2004), CuZn- superoxide dismutase (Karpinski et al. 1992) or dehydrin-like protein (Pyhäjärvi et al. 2007). In addition to plant defence reactions during pathogen infection (Karlsson et al. 2007, Fossdal et al. 2007), the germin-like proteins are also involved in somatic and zygotic embryogenesis (Mathieu et al. 2006, Neutelings et al. 1998).

Herr (1995) suggested that the mechanisms of protection, by binucleate Rhizoctonia or hypovirulent strains of R. solani against pathogenic strains, are via competition and induced host resistance rather than direct antagonisms or mycoparatism. Our results support this hypothesis of competition, since BNR was not antagonistic against UNR, but restricted the UNR infection level in BNR-preinoculated roots. Analysis of a subset of defence-gene activities, however, showed no clear correlation in their activity and decreased UNR infection levels in BNR-preinoculated roots suggesting that they are not involved as inducing the host resistance in these interactions.

Plant defence reactions activated by the interaction of different micro-organisms are complicated and the enzyme activities are most probably differentially regulated in separate plant tissues and during different developmental stages. Therefore, the comparison of these different studies that involve a range of experimental designs is demanding. More detailed and extensive studies similar to Adomas et al. (2007 and 2008) are needed. Additionally, revealing the role of each metabolic pathway in particular host-
pathogen interaction and the regulation of the enzymes involved in the particular interactions, would increase our knowledge and the information could be used for future plant protection for example in agriculture or in forest nurseries.
6. Conclusions

The molecular detection of *Rhizoctonia* is essential in order to discriminate pathogenic and non-pathogenic strains, because vegetative characteristics often overlap. Rapid field diagnosis in agriculture or forest nurseries could provide targeted pathogen-control treatments before extensive problems arise and could reduce or eliminate the need for fungicides in an integrated pest management scenario. Our knowledge regarding host defence reactions, on the other hand, provides information for selection of disease resistant host genotypes as well as development of new more environmental friendly and more efficient antifungal agents.

This study further confirms the complexity of the *Rhizoctonia* group not only from a phylogenetical perspective, but also with regard to their infection biology and plant host specificity. Root infecting UNR and BNR strains, belonging to the teleomorph genus *Ceratobasidium*, respectively enter into pathogenic or beneficial associations in Scots pine. BNR strains revealed a propensity to early seedling growth enhancement and later the induction of short roots, which are vital for symbiotic mycorrhizal formation, in Scots pine. They also proved to enhance the rooting of hypocotyls cuttings in a related study (Kaparakis & Sen 2006).

The pathogenic UNR appeared to be more competitive in infection of Scots pine cells than endophytic BNRs, in co-inoculation. This supports the pathogenic role of UNR. The decrease in UNR infection level in BNR preinoculated roots indicates that beneficial microbes could be directly utilized as inoculants for example in tree seedling nursery soils, or used to supplement fertilizers in planting-process to benefit plant growth and productivity as such, but also induce the host response against oncoming fast spreading pathogenic strains. Nevertheless, neither *Suillus bovinus* (Sen 2001) nor BNR strains from this study entirely prevented UNR infection but both could be included in inoculation systems in Scots pine seedling production. *S. bovinus* improves the nutrient uptake, which supports improved host survival, while BNR strains stimulate the growth and rooting in the host. The later short-root inducing activity of BNR, on the other hand, could provide more entry points for increased beneficial ectomycorrhiza development in the nursery.

As UNR is not the only disease causing pathogen in nursery or forest soils, no single beneficial microbe will control all possible pathogens. Therefore, combinations of beneficial microbes could be introduced into the growth substrates of plants. However, little is known about the effects of these soil- or root-inhabiting beneficial microbes on each other *in vitro or in vivo* in nature and further work is needed to uncover such relationships. The combination of the BNR and *S. bovinus* and their impacts on the Scots pine defence and UNR infection, although complicated, would be very interesting topic for future investigation. Additionally more defence genes could be included as new candidate gene sequences become available in public databases. Another important future perspective that stems from this and earlier studies by our group members would be to initiate an applied programme under actual nursery condition.
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8. References


seedlings in response to inoculation with a nonpathogenic binucleate *Rhizoctonia* isolate. Phytopathology 95: 345-353.


