A mycobacterium isolated from tissue cultures of mature *Pinus sylvestris* interferes with growth of Scots pine seedlings

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Summary  We isolated a rapidly growing, pigment-producing mycobacterium from senescent tissue cultures derived from mature Scots pine (*Pinus sylvestris* L.). The bacterium was found in three senescent suspension cultures and in a senescent protoplast culture. Growth of Scots pine cells had ceased in all of these cultures. Exogenous contamination was eliminated by rigorous surface sterilization of the buds with hypochlorite before aseptic removal of the bud scales. Based on biochemical and physiological properties and DNA sequence comparisons, the isolated mycobacterium did not belong to any known species. Its sequence most closely resembled those of *Mycobacterium obuense* (97%) and *M. aichiense* (96%). Tissue browning was frequently observed in callus or suspension culture of Scots pine. Because the effect of the mycobacterium on growth of undifferentiated tissues that were browning was difficult to evaluate, we applied the bacterium to Scots pine seeds in aseptic conditions. Seedlings grown in the presence of the mycobacterium had shorter hypocotyls than control seedlings and seedlings cocultivated with a *Pseudomonas* strain known to be harmless to plants. However, hypocotyl growth of seedlings cocultivated with another mycobacterium, *M. chlorophenolicum*, was similar to that observed in the presence of the isolated mycobacterium. Phenylalanine ammonia-lyase activity of seedlings cocultivated with the mycobacterium isolate was significantly higher than that of control seedlings or seedlings cocultivated with *M. chlorophenolicum* or *Pseudomonas fluorescens*. We believe that this is the first report of the isolation of mycobacteria from tissue cultures of a tree. Our finding that the mycobacterium may interfere with the growth of Scots pine in vitro warrants further study.

Keywords: bud, callus, phenylalanine ammonia-lyase, tissue browning.

Introduction

Numerous species of bacteria have been detected on or in plants, and the bacteria may be harmless, beneficial or deleterious for plants (Holland and Polacco 1994). It is also known that plant tissue cultures, which should be aseptic, often have contaminants of unknown origin and that these contaminants may reduce the regenerative capacity of plant cells (Holland and Polacco 1994, Cassells 1997). Bacteria isolated from plants may also interfere with seed germination and subsequent growth of the plant. When Gram-negative bacteria isolated from roots of sugar beet (*Beta vulgaris* L.) were reintroduced to the roots of the host plant, they caused reduced seed germination, root distortions, root lesions, reduced root elongation, increased root infection by root-colonizing fungi, and significantly reduced plant growth (Suslow and Schroth 1982).

Mycobacteria are abundant in soil and water, and they may also occur in the intestinal flora of humans and animals. Sphagnum moss, sawdust and wood shavings have been identified as substrates for the proliferation of numerous mycobacteria (Kazda et al. 1979). There is one previous report on the occurrence of a mycobacterium in plant tissue cultures (Taber et al. 1991). *Mycobacterium scrofulaceum* was found to be responsible for significant commercial losses in micropropagated ornamentals. There are no reports on the occurrence of mycobacteria in tissues of growing trees or in tissue cultures of trees.

In common with other pine species, Scots pine (*Pinus sylvestris* L.) is known for its recalcitrance in tissue culture. In addition, tissue cultures derived from buds of mature trees suffer from browning (Lindfors et al. 1990, Laukkanen et al. 1997), which reduces growth and regenerative capacity of callus (Laukkanen et al. 1999a). Browning of Scots pine callus results from oxidation of phenolic substances (Laukkanen et al. 1999b).

We describe the isolation of a *Mycobacterium* sp. from senescent tissue cultures of Scots pine. Growth and phenylalanine ammonia-lyase activity of Scots pine seedlings cocultivated with the mycobacterium isolate were compared with those of uncontaminated controls and seedlings cocultivated with *Pseudomonas fluorescens*, a strain known to be harmless to plants, and with *Mycobacterium chlorophenolicum*, which is able to degrade phenolic compounds (Häggblom et al. 1994).
Materials and methods

Plant tissue cultures

Buds were collected from 10- to 25-year-old Scots pine trees growing in stands around Oulu, northern Finland. The buds were surface-sterilized in 6% calcium hypochlorite, and callus cultures were started from shoot tips as described by Hohtola (1988). Suspension cultures were initiated by transferring callus clumps to agitated liquid growth medium (LGM-1) containing modified MS salts (Murashige and Skoog 1962, Hohtola 1988) and cultured at room temperature. The capacity of suspension cultures to produce callus was monitored by plating suspended cells in drops on a solid growth medium (SGM-1) supplemented with 0.6% Phytagel (Sigma Chemical Co., St. Louis, MO). Protoplasts were prepared and cultured according to Hohtola and Kvist (1991).

Scots pine seeds were allowed to imbibe sterile water over night before surface sterilization with 4% calcium hypochlorite for 20 min. Surface-sterilized seeds were germinated on solid growth medium (SGM-2) composed of the same modified MS salts as LGM-1, except that (NH₄)₂HPO₄ was used instead of KNO₃, potassium was added as K₂HPO₄, and callus cultures were started from shoot tips as described by Hohtola (1988). Suspension cultures were initiated by transferring callus clumps to agitated liquid growth medium (LGM-1) containing modified MS salts (Murashige and Skoog 1962, Hohtola 1988) and cultured at room temperature. The capacity of suspension cultures to produce callus was monitored by plating suspended cells in drops on a solid growth medium (SGM-1) supplemented with 0.6% Phytagel (Sigma Chemical Co., St. Louis, MO). Protoplasts were prepared and cultured according to Hohtola and Kvist (1991).

Identification of the mycobacterium by DNA methods

A loopful of bacterial colonies was suspended in 1 ml of 0.9% NaCl, inactivated at 80 °C for 10 min and collected by centrifugation. The pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and glass beads (diameter < 106 µm; Sigma, St. Louis, MO) were added. The suspension was mixed for 2 min and centrifuged, and 10 µl of the supernatant was used in PCR amplifications.

Amplification of a 423-bp fragment of the gene encoding a 32-kD protein that is specific for mycobacteria was performed as described by Soini et al. (1994). The oligonucleotide primers used have been shown to be specific for the genus Mycobacterium (Soini et al. 1992). To allow for immobilization of the PCR product, one of the primers used in the amplification reaction was biotinylated.

Amplification of a 1500-bp fragment of 16S rDNA was performed as described by Saiki et al. (1989). The total reaction volume was 50 µl, containing 5 µl of the sample DNA, 2 units of Dynazyme DNA polymerase (Finnzymes Oy, Espoo, Finland), 10 pmol of primers (one of the primers was biotinylated) and 5 µl of 10 x PCR buffer (Finnzymes Oy). The PCR steps (94 °C for 60 s, 55 °C for 30 s, 72 °C for 60 s) were repeated 35 times with a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT).

The biotinylated PCR products were captured and purified with streptavidin-coated Dynabeads according to the manufacturer’s instructions (Dynabeads M-280 Streptavidin, Dynal AS, Oslo, Norway). The immobilized DNA was denatured with 0.1 M NaOH, washed and suspended in 20 µl of water. The resulting single-stranded DNA was used as template in the sequencing reactions.

Sequence of the amplified part of the 16S rDNA was done with the Taq DyeDeoxy Termination Cycle sequencing Kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The sequencing products were resolved with a 373A Sequencer (PE Applied Biosystems). The nucleotide sequences were analyzed with the Sequence Editor program (PE Applied Biosystems). Sequence comparison to a reference database was done with a locally developed identification program that used a recursive longest match selection for optimal alignment of the compared sequences (unpublished algorithm; E. Eerola, Department of Medical Microbiology, University of Turku). The reference database consisted of 3827 sequences retrieved and combined from GenBank (Benson et al. 1993), EMBL (Rice et al. 1993) and the ribosomal database project (Larsen et al. 1993).

Phenylalanine ammonia-lyase activity

Phenylalanine ammonia-lyase (PAL) activities were determined by conversion of L-(2,6,–H³)-phenylalanine to radioactive cinnamic acid according to Lee et al. (1992). Hypocotyls (1 g) were removed from the infected and control seedlings after 14 days of culture, and proteins were extracted and assayed.
for PAL activity. Radioactivity of cinnamic acid products was measured with a scintillation spectrometer. The protein concentration in the extracts was determined by the Bradford assay (Bradford 1976). Enzymatic activity was expressed as pkat mg⁻¹ protein.

Statistical analysis
The differences between treatments and controls were determined by the Mann-Whitney U-test.

Results

Isolation and identification of the mycobacterium
Suspension cultures derived from Scots pine callus turned brown and the cells ceased to grow after 3 months of culture. A few drops of the suspension culture fluids were plated on solid culture medium and incubated at ambient temperature. Yellow bacterial colonies became visible on the surface of the medium after 3 to 4 days of incubation. Similar colonies were found three times in senescent suspension cultures and once in a senescent protoplast culture. The colonies were initially pale lemon-yellow, but later changed to deep orange-yellow. The color of the colonies led us to suspect the presence of mycobacteria in the suspension cultures.

As is typical for mycobacteria, the isolated bacterium stained only weakly by the Gram method, but stained well by the Kinyon and auramin staining methods. Morphologically, the bacterium was a small rod with a slight tendency to branch.

The biochemical and physiological properties of the microorganism are shown in Table 1. The bacterium closely resembled other rapidly growing, pigment-producing mycobacteria. However, the biochemical and physiological properties did not completely match those of any known mycobacterium.

Amplification of a 423-bp fragment of the gene encoding the 32-kD protein specific for mycobacteria yielded positive results from the isolated bacterium. A segment of 16S rDNA of the bacterium was amplified and sequenced. Although the sequence did not match any sequence in the databases, it closely resembled those of *M. obuense* (97%) and *M. aichiense* (96%) (Figure 1).

Effect of the mycobacterium on Scots pine seedling growth
After 14 days of culture, the hypocotyls of the seedlings growing on media containing the isolated mycobacterium or *M. chlorophenolicum* were significantly shorter than those of the controls (Table 2). Hypocotyls were also more contorted in appearance in the media containing the mycobacterial species than in the control medium. Generally, the roots turned upward from the surface of the growth medium in the presence of mycobacteria. The presence of the isolated mycobacterium also reduced the length of the roots (Table 2).

The pH of the culture media was 5.7 at the onset of the incubations. After 2 weeks of incubation, the pH of the medium containing the isolated mycobacterium had decreased significantly more than that of the control medium (3.5 versus 5.5), whereas the pH in the presence of *M. chlorophenolicum* had decreased less (3.9 versus 5.5). The effect of low pH on the growth of the seedlings was therefore tested. Growth of seedlings on a medium adjusted to a pH of 3.5 resulted in a reduction in the lengths of the hypocotyls and roots, but did not

### Table 1. Microscopic and biochemical properties of a *Mycobacterium* isolated from Scots pine tissue cultures.

<table>
<thead>
<tr>
<th>Laboratory tests</th>
<th>Properties of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigmentation</td>
<td>Yellow color in light and dark</td>
</tr>
<tr>
<td>Growth rate</td>
<td>Rapid</td>
</tr>
<tr>
<td>20 °C</td>
<td>+ at 3–4 days; +++ at 14 days¹</td>
</tr>
<tr>
<td>30 °C</td>
<td>+ at 3–4 days; +++ at 14 days</td>
</tr>
<tr>
<td>35 °C</td>
<td>Negative</td>
</tr>
<tr>
<td>Acid fastness</td>
<td>Auramin positive, Kinyon positive</td>
</tr>
<tr>
<td>Niacin</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Negative</td>
</tr>
<tr>
<td>Catalase 68 °C</td>
<td>Positive</td>
</tr>
<tr>
<td>Catalase, semiquantitative</td>
<td>25 mm</td>
</tr>
<tr>
<td>Tween 80 hydrolysion</td>
<td>Positive/-negative</td>
</tr>
<tr>
<td>Tellurite reduction</td>
<td>Positive at 4 h; negative at 12 h</td>
</tr>
<tr>
<td>Urease</td>
<td>Negative at 3 days; positive (weak) at 12 days</td>
</tr>
<tr>
<td>Arylsulphatase</td>
<td>Negative</td>
</tr>
<tr>
<td>Iron uptake</td>
<td>Negative</td>
</tr>
<tr>
<td>McConkey agar</td>
<td>Negative at 5 days; negative at 13 days</td>
</tr>
</tbody>
</table>

¹ Symbols: + = some cell divisions, +++ = many cell divisions.
increase the contortion of seedlings (Table 2).

The PAL activities were markedly higher in seedlings co-cultivated with the isolated unknown mycobacterium than in seedlings cocultivated with *M. chlorophenolicum*, or *Pseudomonas fluorescens*, or cultivated on the control medium (Table 2).

### Discussion

We isolated a rapidly growing, pigment-producing mycobacterium from senescent tissue cultures derived from mature Scots pine. Based on biochemical and physiological properties and DNA sequence comparisons, the isolated mycobacterium did not belong to any known species. Its sequence most closely resembled those of *Mycobacterium aichiense* (96%) and *M. obuense* (97%). The bacterium had a deleterious effect on the growth of Scots pine seedlings.

The origin of the mycobacterium in the Scots pine tissue cultures is not known. We believe that the bacterium was inside the explant and not introduced during the preparation process. The surfaces of the buds were carefully sterilized and the explants were prepared aseptically. Cultures of several other plant tissues were carried out concurrently with the Scots pine tissue cultures, but no evidence of the presence of mycobacteria was seen in these other tissues. Tap water was found to be free from mycobacteria (data not shown). We suggest that the mycobacterium was carried in the xylem fluid to the buds. Bacteria have also been found in non-distressed wood of living aspen, pine and alder, where they were probably transported in xylem fluid (Bacon and Mead 1971). It is probable that mycobacteria present in soil are transported by water into the xylem fluid through surface lesions in roots. Scots pine is able to grow on acid soil under conditions that favor growth of mycobacteria (Iivanainen et al. 1993). Mycobacterial resistance to acid pH is attributable to the high concentrations of mycolic acid in the cell wall, which make the cells wax-like and markedly hydrophobic (Schlegel 1993).

The presence of bacteria in plant tissue cultures without any obvious signs of contamination is not uncommon (Holland and Polacco 1994, Leifert et al. 1994). It is also known that endophytic bacteria and fungi often escape detection *in vivo* and *in vitro* (Holland and Polacco 1994, Cassells 1997, Ewald et al. 1997). We isolated the mycobacterium from four separate cultures initiated several months apart. It appears that the mycobacterium is able to proliferate only if growth and production of phenolic substances in Scots pine tissue cultures have ceased. Further studies are needed to determine how frequently Scots pine tissue cultures are contaminated by mycobacteria.

There are suggestions that covert microbial contaminants might alter the capacity of plant cells to regenerate and grow in culture (Holland and Polacco 1994). We obtained circumstantial evidence that mycobacteria may be harmful to Scots pine cultures *in vitro*. In the presence of the mycobacterium, the hypocotyls and roots of seedlings were shorter than in the controls. Also, the hypocotyls were more contorted in seedlings grown in the presence of either mycobacterium or a pseudomonad than those of controls. On the other hand, the roots grew even longer in the presence of the pseudomonad.

The PAL activity was higher in seedlings cocultivated with the isolated mycobacterium than in control seedlings. These findings imply that the reduced growth of seedlings is a consequence of a stress reaction induced by the mycobacterium. Campbell and Ellis (1992) also obtained evidence that microbial components may increase PAL activity in pine tissue cultures. In contrast, PAL activity in seedlings cocultivated with *M. chlorophenolicum* was lower than in control seedlings, perhaps indicating that the ability of *M. chlorophenolicum* to degrade phenolic compounds (Häggbom et al. 1994) affected the PAL activity of the seedlings.

A low pH may interfere with the growth of Scots pine seed-
lings cocultured with the mycobacterium. Production of acidic compounds by the mycobacterium could cause acidification of the growth medium. On the other hand, Scots pine seedlings may produce acidic phenolic compounds as a result of increased PAL activity (Dixon and Palva 1995). At the beginning of germination, the roots often grew upward, which may be a consequence of one-sided cell enlargement activated by lowered pH, according to the acid growth theory (Moore 1989). Acidity may also alter nutrient availability and thereby cause disturbances in plant development (Smith and Krikorian 1990). Because the pH 3.5 medium alone resulted in a marked reduction in root length, but caused much less contortion than was observed in seedlings cocultured with the mycobacterium, we conclude that, in addition to lowering the pH, the mycobacterium used some other means to interfere with seedling growth.

To our knowledge, this is the first report of a Mycobacterium sp. in the tissue culture of a tree. We suggest that the bacterium originated from soil and was carried by xylem fluid into the buds of Scots pine. The reduced growth and increased PAL activity of Scots pine seedlings cocultivated with the mycobacterium imply that it may be harmful for Scots pine cultures in vitro. Further experiments are needed to determine how common mycobacteria are in the tissues of trees. Additional studies on the effects of mycobacteria on the growth of trees are also warranted.

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References


