ROOTING OF SCOTS PINE FASCICULAR SHOOTS BY *AGROBACTERIUM RHIZOGENES*

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Received November 11, 1995; accepted March 29, 1996

ABSTRACT

The advantageous effect of *Agrobacterium rhizogenes* on root induction in Scots pine cuttings of fascicular bud origin was demonstrated. Two lots of cuttings, a spring and a late summer lot, were first incubated for 20 h in 0.5 mM IBA solution, then dipped in bacterial colonies of strains A4, A4(GUSint) or R1600, and then planted. In the summer lot the *Agrobacterium* treatment enhanced root formation, which was on an average 24.5% (±5.1), and 16.2% (±2.9) in IBA treatment alone. The genotypic variation in rooting ability was remarkable. In the best genotypes more than 80% of the fascicular shoots rooted, while the best results in the IBA treatment were around 30–40%. The chromosomal matrix of the A4Ri-plasmid seemed to affect the results, indicating that functions encoded by the bacterial chromosome are also involved. The agropine assays, the histochemical tests for ß-glucuronidase expression, PCR tests, and observations on root morphology show that the roots formed after *Agrobacterium* treatments are untransformed. When considering the practical applications of this rooting method, normal roots instead of transformed ones will be preferred due to the conflicting views on the release of foreign genes or chimeric plants into nature.

Key words: *Pinus sylvestris*, fascicular shoots, vegetative propagation, *Agrobacterium rhizogenes*, DNA transfer

INTRODUCTION

Vegetative propagation has many uses in forestry, such as conservation of genotypes, multiplication of genotypes for specific purposes, *e.g.* seed orchards, evaluation of genotypes through clonal testing, and capture of maximum genetic gain when used for regeneration in operational planting programs (ZOBEL & TALBERT 1984). Rooted cuttings are one of the most commonly used methods for vegetative propagation. Also some coniferous tree species, such as sugi (*Cryptomeria japonica* D. Don) in Japan, radiata pine (*Pinus radiata* D. Don) in Australia and New Zealand, and Norway spruce (*Picea abies* (L.) Karst.) in France, are propagated on a commercial scale by rooting cuttings (TALBERT *et al.* 1993).

Vegetative propagation of Scots pine (*Pinus sylvestris* L.) has been investigated by using either long shoots (BALLENGER & HUANG 1984, BOEIJINK & VAN BROEKHUIZEN 1974, MAYNARD & BASSUK 1987, MONTEUUIS & PAGES 1987, STRUVE & GERHOLD 1985) or needle fascicles as such (MONTEUUIS & PAGES 1987, YLI-VAKKURI & PELKONEN 1976), or after pruning the branches (BOEIJINK & VAN BROEKHUIZEN 1974, HILSON & DANCIK 1978) or by using chemical spraying treatment of stock plants (BORNMAN 1984, MAYNARD 1986, SALONEN 1990, WHITEHILL & SCHWABE 1975). In these studies the rooting percentages of cuttings have varied between 0 and 100%, depending on several factors, such as the stock plant age and growth regulator treatments. The success of rooting in Scots pine cuttings has been rather low, and so far this propagation method has not been applied to practical scale forestry.

Agrobacterium rhizogenes is a common soil inhabitant belonging to the *Rhizobiaceae* family. Phytopathogenic strains of *A. rhizogenes* cause hairy root formation in the infection sites in the host plants. Agrobacteria are able to transfer part of their Ri-plasmid DNA, the so called T-DNA, into plant cells, where it integrates into the plant genome. The disease symptoms are due to the expression of bacterial genes affecting the phytohormone balance in the plant cells at the infection site (CLARE 1990, GELVIN 1990, TEPFER 1989).

The root induction ability of *A. rhizogenes* has been used successfully for rooting cuttings of hybrid poplars (*Populus deltoides* × *nigra, P. nigra* × *maximowiczii*) in *in vitro*-conditions (CHAREST *et al.* 1992), apple cuttings *in vivo* and *in vitro* (LAMBERT & TEPFER 1991), and olive, apple, almond and pistachio shoots *in vitro* (RUGINI 1992). The root formation in these cases was caused by genetic transformation. There are also several

reports on other tree species, such as hazelnut (*Corylus avellana* L.) (BASSIL *et al.* 1991), tamarack (*Larix laricina* K.Koch), jack pine (*Pinus banksiana* Lamb.) and western white pine (*Pinus monticola* Dougl.) (MCAFEE *et al.* 1993), in which rooting of the cuttings or the *in vitro* shoots has been improved by using *A. rhizogenes*. In these cases, however, the increased rooting could not be ascribed to DNA transfer, but the presence of agrobacteria in the rooting medium appeared to be beneficial.

The aim of the present work was to investigate if genetic transformation by *A. rhizogenes* could be used for inducing root formation in Scots pine cuttings. This approach was compared with the commonly used growth regulator treatment, and a range of Scots pine genotypes were evaluated for their competence for *Agrobacterium* transformation.

MATERIAL AND METHODS

Production of fascicular shoots

The method used was based on cytokinin spraying treatments, which stimulate development of fascicular buds (KOSSUTH 1978, WHITEHILL & SCHWABE 1975). Two-year-old seedlings of Scots pine originating from controlled crossings of elite trees from southern Finland were used as stock plants. During the summer 1993 normally over-wintered seedlings were grown in the greenhouse under natural light, and they were treated eight times, twice a week, with foliar applications of 0.5 mM benzylaminopurine (BA, Sigma). BA dissolved in 1M NaOH was applied as an aqueous solution containing 0.004% (v/v) Tween 20 as a surfactant. Spraying started when the height growth of the leading shoots was almost finished, and new annual shoots were sprayed to run-off with a manual pump sprayer. Within 4–8 weeks development of fascicular buds could be seen in needle fascicles.

After a normal autumn the stock plants were subjected to another growing season between December 1993 and February 1994, by providing a 16-hour photoperiod (about 200µE $m^{-2}s^{-1}$) and by maintaining temperature at 22 °C in daytime and at 17 °C at nights. During this period the fascicular buds elongated into fascicular shoots (Fig.1). The plants were then given 8 weeks of short-day and cold treatment. The photoperiod was shortened to 7 hours and light intensity was half of the original. The temperature was gradually decreased from 22 °C to 3 °C, and for the last four weeks the seedlings were kept at 3 °C. Fascicular shoots, 2–5 cm of length, were taken from the stock plants in March, directly after the cold treatment (the spring lot) and in June, after the second growing season of the stock plants (the summer lot). Thus the cuttings in the summer lot had elongated for two growing seasons. The spring lot contained 698 cuttings from 12 different genotypes, the summer lot 596 cuttings from 22 different genotypes.

The dry matter content was estimated in the cuttings in the two lots. Fascicular shoots (5–9) were harvested, weighed and dried at 105 °C for 24 hours. The dry matter content, calculated as a percentage of fresh weight, was 32–35% for the cuttings in the spring lot and 26–35% for the ones in the summer lot.

Bacterial strains

Bacterial strains used for the rooting experiments were a wild-type *Agrobacterium rhizogenes* strain A4 (MOORE *et al.* 1979), a genetically engineered version of the A4 containing the ß-glucuronidase reporter gene with an intron, A4(GUSint) (VANCANNEYT *et al.* 1990), and an *A. tumefaciens* strain C58 carrying the Ri-plasmid A4b together with the extra copies of the virulence genes in the pTVK291 plasmid, called R1600 (PYTHOUD *et al.* 1987). The R1600 strain was cultured on Luria Broth (LB) medium (MILLER 1972), solidified with 1.5% agar, including the antibioticum kanamycin 50 $mg \cdot l^{-1}$, and the A4 strains were cultured on MYA medium (TEPFER & CASSE-DELBART 1987), also solidified with 1.5% agar, including kanamycin 50 $mg \cdot l^{-1}$ in the case of the strain A4(GUSint).

Rooting experiments

Two different lots of Scots pine fascicular shoots were treated with *Agrobacterium* strains carrying Ri-plasmids. In the spring lot one third of the cuttings were treated with the strain R1600, one third with the wild-type strain A4, and one third were controls, not treated with *Agrobacterium*. The number of cuttings of each genotype per treatment varied from 8 to 50. In the summer lot two thirds of the cuttings were treated with the strain A4(GUSint), and one third were kept as controls.The number of cuttings of each genotype was between 8 and 26 in the A4(GUSint) inoculations.

All the cuttings were first incubated for 20 hours in 0.5 mM indole-3-butyric acid (IBA, Fluka) solution, in the dark. After the growth regulator incubation, a 1 mm piece was excised from the base of the cuttings to provide a fresh wound for agrobacteria. The control cuttings were also excised as described, and planted in a soil mixture of horticultural peat, bark humus, and perlite (5:3:2). In the bacterial treatments the bases of the cuttings were dipped in bacterial colonies grown for three days on the appropriate agar medium including 100 µM acetosyringone. After bacterial inoculation the cuttings were planted in the same way as the controls.

Figure 1 Production of fascicular shoots in Scots pine. Cytokinin spraying of the two-year-old Scots pine seedling (A) causes the proliferation of the fascicular shoots (B), which have been used as cuttings in the present study

The planted cuttings were grown in greenhouse, in 7 cm diameter pots under continuous mist (the relative humidity being 90%), and during the rooting period the greenhouse benches were warmed from beneath to be approximately 5 °C warmer than the surrounding space, which was between 15–18 °C. After the rooting period (from 6 to 24 weeks) when the mist was turned off, the relative humidity was 60%, and the temperature in the greenhouse was kept above 15 °C. Root formation was examined 6, 10, 16, and 24 weeks after the treatments, and the rooted cuttings were transplanted into new containers. After transplantation the cuttings were fertilized with 0.2% commercial Superex fertilizer (Kekkilä) once a month during the growing season. In the first growing season, during and after the rooting period, the cutting plants of fascicular bud origin were sprayed once a week with a fungicide (0.1% Ronilan or 0.3% Tirama, Kemira), and in the second growing season the attacks of aphids were controlled three times by 0.1% Roxion (Kemira) sprays. The transplanted cutting plants were over-wintered for six months at 1–6 °C. At the beginning and at the end of the second growing season the elongation growth of the plants was measured. At the end of the season the growth habit and the root systems of the cutting plants were also examined.

Histochemical ß-glucuronidase test

Root tips (n = 82) from the cutting plants, rooted after the A4(GUSint) treatment, were tested histochemically for the expression of the ß-glucuronidase (GUS) gene as described by JEFFERSON (1987), with minor modifications: the staining solution contained 0.1 M sodium phosphate buffer (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0.1% Triton X-100. Samples were kept in test solution in the dark at 37 °C for 24 h, and the presence of GUS expressing cells or cell clusters was examined under a stereomicroscope.

Opine assay

Freshly formed roots of the one-year-old rooted cuttings (n = 150) were rinsed in tap water, and used for agropine assays, which were performed as described by PARSONS *et al.* (1986), with minor modifications. Agropine and mannopine standards for the assays were prepared according to the previously published protocol (PETIT *et al.* 1983).

PCR test for the presence of the GUSint sequence or remaining agrobacteria in root tissues

Genomic DNA was isolated from the roots of 76 plants originating from fascicular shoots and treated with A4(GUSint) as described by DOYLE and DOYLE (1990), with minor modifications according to ARONEN and HÄGGMAN (1995). Fresh roots were excised from the two-year-old plants, rinsed in tap water, and used as material for the DNA extraction.

Polymerase chain reactions (PCR) were performed to determine the presence of the intron containing ß-glucuronidase reporter gene introduced into the *A.*

rhizogenes strain A4(GUSint). Primers specific for the GUSint sequence, ACGTCCTGTAGAAACCCCAA and CCCGCTTCGAAACCAATGCC (BLAKE *et al.* 1991), which border a 1286 bp fragment of the GUSint gene (nucleotides 23–1120 according to JEFFERSON *et al.* 1986) were used for the reactions. The reaction mixture contained 1 µl of template DNA, 100 µM dNTP's, 150 pM primers, and 1 U of Dynazyme DNA polymerase (Finnzymes) in 50 µl of manufacturer's buffer. The reaction mixtures were heated at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 3 min, with a final extension step of 72 °C for 7 min in a DNA Thermal Cycler 480 (Perkin-Elmer, Cetus). Amplified samples were electrophoresed on a 2.0% agarose gel, stained with ethidium bromide, and examined under UV-light.

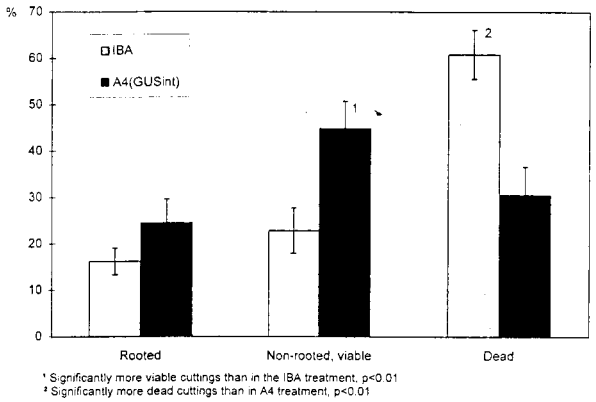

Figure 2 The effect of *Agrobacterium rhizogenes* strain A4(GUSint) on rooting and viability of Scots pine cuttings in the summer lot. Pooled data (SE) is based on 596 fascicular shoots treated with 0.5 mM indole-3-butyric acid (IBA) and agrobacteria

Microscopical observations

The anatomical changes in the base of fascicular shoots were examined in microscopical sections. The bases were excised six months after the auxin and *Agrobacterium* -treatment, fixed in FAA (formalin: acetic acid: 95% ethanol, 10:5:85, v/v/v), embedded in paraffin, and stained with safranin-fast green (GERLACH 1984).

Statistical analysis

Comparisons between treatments were carried out by analysis of variance. Means were compared either by Tukey's test or by Student-Newman-Keuls multiple-range test. For correlation analysis, the Pearson's product-moment correlation *r* was used.

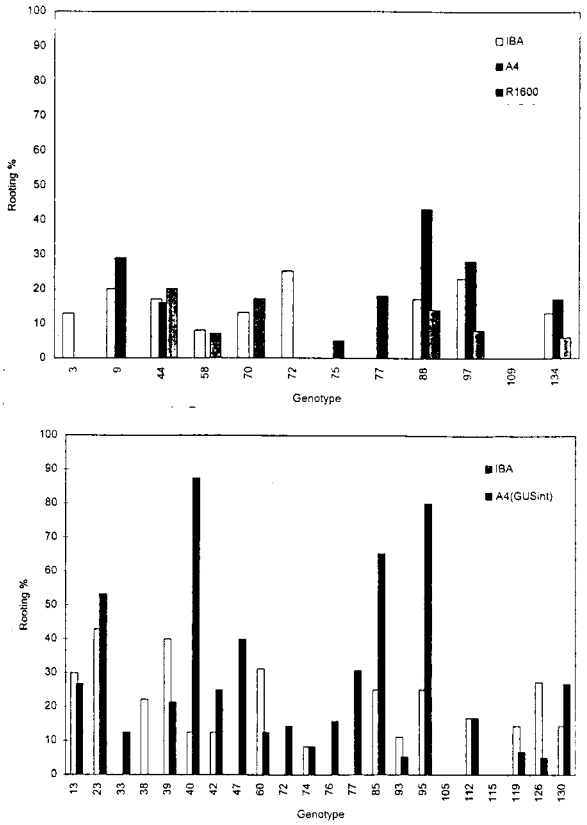

Figure 3 The effect of genotype on the rooting response with and without *Agrobacterium* treatment. Data of the genotypes included in the spring lot is presented in (A) (n=698) and data of the summer lot in (B) (n = 596)

RESULTS

Rooting of the fascicular shoots

In the spring lot, rooting percentages for the A4, R1600 and control treatments were 13.4 (±SE 4.1), 5.9 (±2.1), and 12.2 (±2.5), respectively. There were no significant differences between the means. In the summer lot, the treatment with *A. rhizogenes* strain A4(GUSint) enhanced root formation, which was on an average 24.5% (±5.1) in the bacterial treatment, and 16.2% (±2.9) in the control treatment. Also the amount of non-rooted, viable cuttings was doubled, 44.9% (±5.9), when compared with the control, 22.9% (±4.9) (Fig. 2). In both lots, genotypic variation was remarkable (Fig. 3), and no correlation could be found between the rooting percentages in bacterial and control treatments. In the best genotypes, more than 80% of the fascicular shoots rooted after A4(GUSint) treatment, while the best results achieved in the control treatment varied around 30–40% (Fig. 3B).

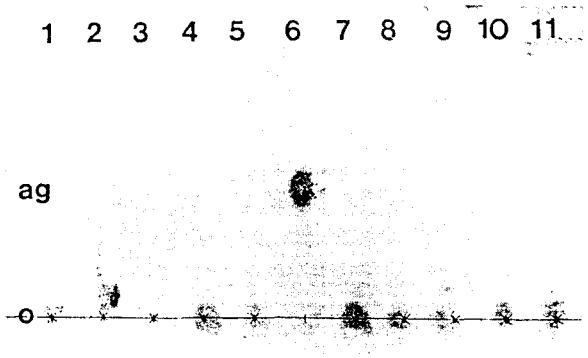

Figure 4 Agropine assay of the roots of the Scots pine cuttings. Lane 6: an agropine standard; lanes 1–5 and 7–11: root samples of ten cuttings; ag = agropine, o = origin of separation, in which neutral $AgNO_3$ positive compounds are visible

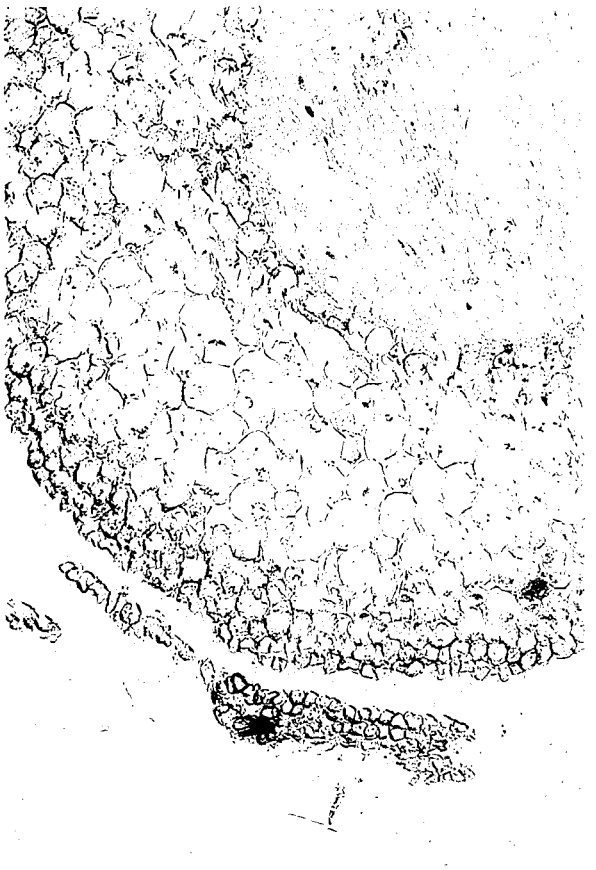

Figure 5 A cross section of a cutting root tested histochemically for β-glucuronidase expression. A few blue-stained cells were found in superficial cell layers detached from the root

Opine analyses and histochemical tests for reporter gene expression in the roots

The roots of one-year-old cutting plants were tested for the presence of agropine, but it could not be detected in any of the tested 150 roots (Fig. 4). When the root tips of the plants from the A4(GUSint) treatment were tested histochemically for the expression of ß-glucuronidase reporter gene, a few light-blue stained cells could be found in some samples. These cells were, however, not located in the inner part of the root tissue, but in the superficial cell layers often detached from the root (Fig. 5).

PCR test for the presence of GUSint sequence or remaining agrobacteria

Amplification of the GUSint fragment of 1286 bp could not be detected in any of the 76 DNA samples tested (Fig 6). Only some faint bands representing non-specific amplification products were seen in some of the samples. Positive controls using the *A. rhizogenes* strain A4(GUSint) as a template produced a strong and clear amplification product of expected size.

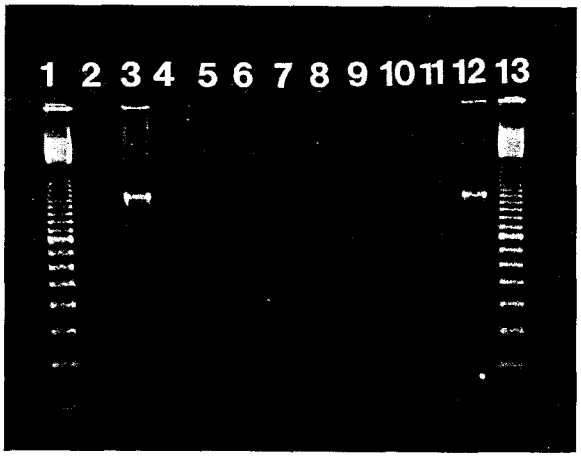

Figure 6 The PCR amplification of the GUSint fragment (1286 bp) by using the DNA samples isolated from the roots of Scots pine cuttings as a template. Lanes 1 and 13: a 100 bp ladder; lanes 3 and 12: a positive control using the DNA of the *A. rhizogenes* strain A4(GUSint) as a template; lane 2: a negative control using water as a template; and lanes 4–11: amplification results with the DNA of root samples of Scots pine cuttings.

Microscopical observations

Different kinds of anatomical changes were observed in the basal areas of the stems of the fascicular shoots after the rooting treatments. Some of the cuttings did not respond in any visible way (Fig.7A), and these cuttings usually died within a few months after treatment. Other cuttings did not form roots, but stayed green and viable through the whole first growing season, and were characterized either by callus formation (Fig.7B) and / or axillary bud proliferation (Fig.7C). In the rooted

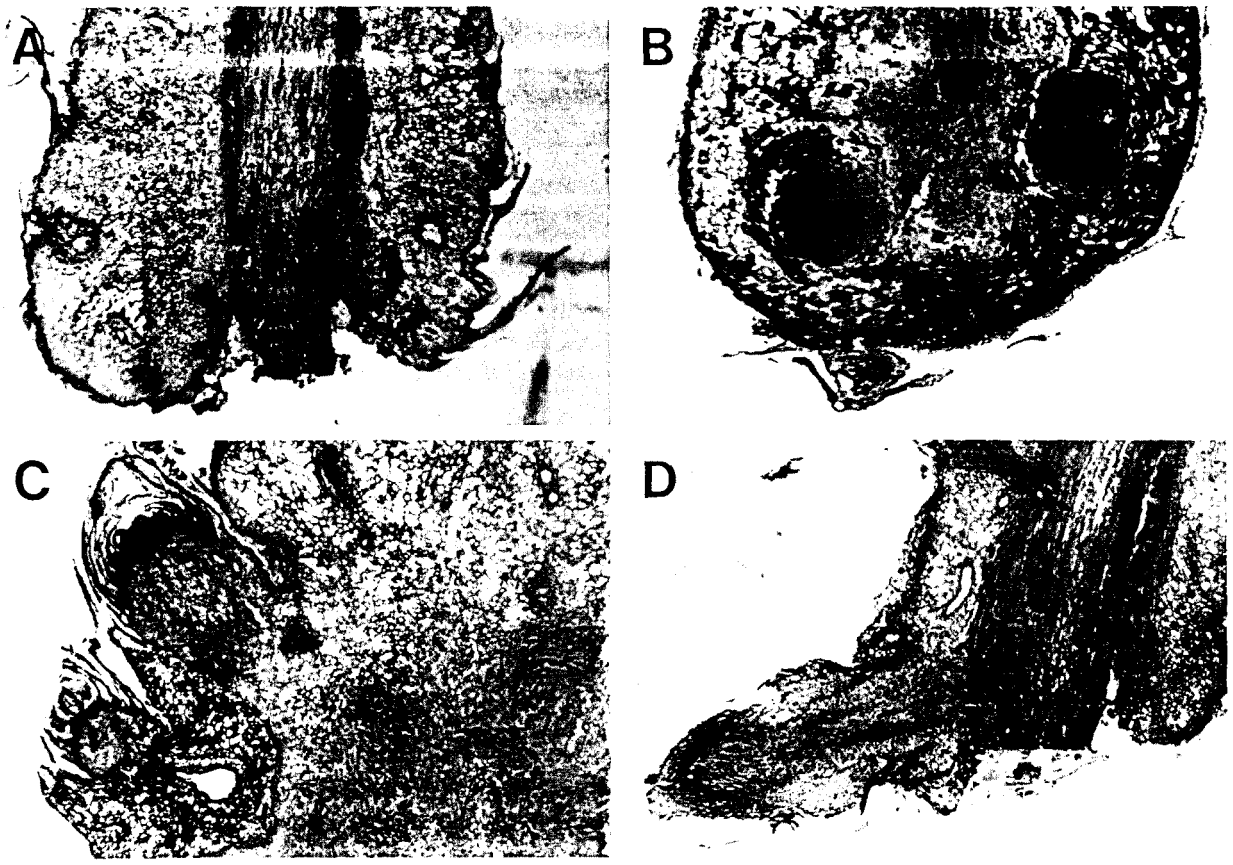

Figure 7 Anatomical changes in the basal areas of the cytokinin-induced fascicular shoots of Scots pine after *Agrobacterium rhizogenes* treatments. The cuttings without root formation could be divided into two categories: the ones with no visible changes in basal area (A), and the ones characterized by callus production (B) and / or axillary bud proliferation (C). In the rooted cuttings (D) usually formation of a single root was observed

cuttings (Fig.7D) proliferation of a single root was usually observed. Typical symptoms of hairy roots, such as numerous roots with short internodes and hairy characteristics, could not be detected.

Growth measurements

During the first growing season the elongation growth of the cutting plants was more or less retarded. The mean height of the rooted cuttings after the first cold period was 1.4 (± SE 0.07) cm. At the beginning of the second growing season the elongation growth of the cuttings was more pronounced (Fig. 8A), and at the end of the season the mean height of the cuttings was 6.3 (±0.25) cm. There were no differences in the growth rate between cuttings rooted after bacterial treatments and the control treatment. The growth habit and the root systems of the cuttings were also examined at the end of the second growing season. Approximately 40% of the cuttings were orthotropic, 51% were slightly plagiotrophic, and 9% were badly plagiotrophic, growing at an angle of 45 degrees or less to the horizontal plane (Fig. 8B). No significant differences could be seen in growth habit when comparing cuttings from the bacterial and control treatments. A majority of the cuttings had well developed root systems with one strong root and highly ramified lateral roots.

DISCUSSION

Rooting of Scots pine vegetative propagules has often proved difficult regardless of the type of the material used, *e.g.* long shoots (BALLENGER & HUANG 1984, BOEIJINK & VAN BROEKHUIZEN 1974, STRUVE & GERHOLD 1985), fascicular shoots (MAYNARD 1986), hypocotyl and epicotyl cuttings (GRÖNROOS & VON ARNOLD 1988, FLYGH *et al.* 1993), or adventitious shoots (HÄGGMAN *et al.* 1996). In the present study we demonstrate the positive effect of the *Agrobacterium rhizogenes* treatment on the rooting of fascicular shoots in Scots pine.

The ability of Scots pine to produce adventitious roots will diminish rapidly as the cutting donor ages: only 3.3% of cuttings rooted when the donor plant was 8 years old, compared with a rooting percentage of 43.4 for 3-year-old and 70% for 3.5-month-old seedlings

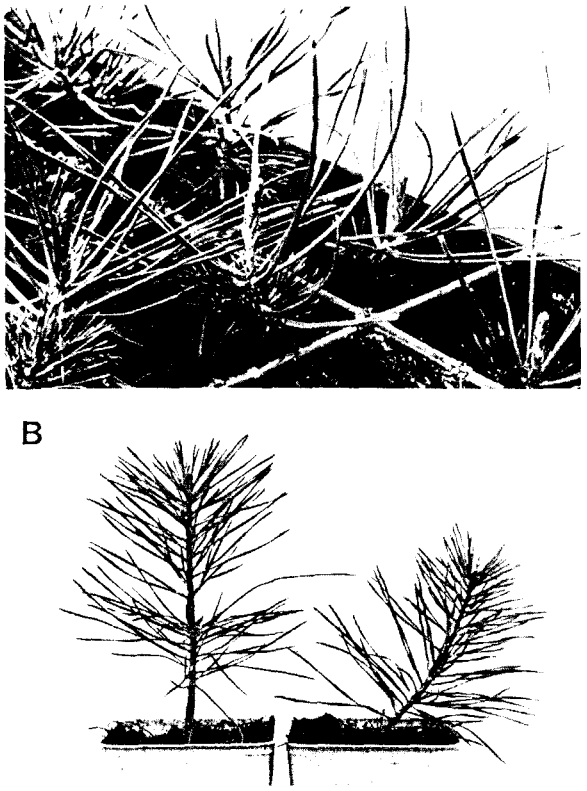

Figure 8 Rooted cuttings of fascicular bud origin in the greenhouse. Vigorous growth started in the beginning of the second growing season (A). Both orthotropic and plagiotropic growth habits were observed (B)

(MONTEUUIS & PAGES 1987). Fascicular shoots were chosen as vegetative propagules for the present study, because cytokinin spraying of the ortets makes it possible to produce high numbers of cuttings in young seedlings (SALONEN 1990). In addition to the age, the genotype of the donor plant has been reported to affect the rooting capacity of cuttings (BERG 1980, MONTEUUIS & PAGES 1987, SALONEN 1990). This phenomenon was also very remarkable in the present study.

External factors, such as conditioning and treatments of the ortets, and the excision time of the ramets can also have an important impact on root formation (reviewed by MONTEUUIS & BARNÉOUD 1991). In the present study, the summer lot cuttings rooted better than the spring lot, which may be due to differences in the developmental stage of the propagules. Fascicular shoots in the spring lot were comparable with the natural shoots at the beginning of the growing season, while the cuttings in the summer lot were collected at the time when their growth had already stopped. The best rooting results are generally obtained either during early spring or during early autumn (HANSEN & ERNSTSEN 1982, MONTEUUIS & PAGES 1987). On the other hand, the cuttings in the spring and summer lots in the present work were derived from different genotypes, which, of course, may also have an effect on rooting percentages.

Fascicular shoots responded to the bacterial and growth regulator treatments by producing either differentiated roots or undifferentiated callus tissue. In some cases, proliferation of axillary buds was also seen. This may be an after-effect of the cytokinin sprays of ortets. Over-stimulation with BA during *in vitro* culture is supposed to lead to abnormalities. Some plantlets of *Alnus glutinosa*, for example, produced an excessive number of buds low on the trunk a few months after they had been planted in the field (EVERS *et al.* 1988). Cuttings with callus formation around the base of the stem often survived for several months after planting. As seen in tissue culture, callus tissue is effective in taking water and nutrients from surrounding medium (*e.g.* AITCHISON *et al.* 1977). In the present study, callus proliferation has probably maintained the housekeeping metabolism of the cuttings and thus enhanced their survival.

The growth of the fascicular shoots during the first growing season was more or less retarded, probably due to root formation. This growth retardation may also be considered an after-effect of the growth regulator treatments of the stock plants. In loblolly pine (*Pinus taeda* L.), plantlets exposed *in vitro* to BA had a poor initial growth under greenhouse conditions compared to seedlings (TIMMIS & RITCHIE 1988). The plagiotrophic growth habit of some cutting plants could probably have been avoided by supporting the fascicular shoots with a stick in vertical position during the initial growth period.

The *Agrobacterium rhizogenes* strain A4 was chosen due to its good infection ability in several plant families (PORTER 1991), as shown also in woody plants (HUANG *et al.* 1993, LAMBERT & TEPFER 1992, PHELEP *et al.* 1991). The genetically engineered version of A4 containing the GUSint gene was included to enable easy monitoring of the transgene integration and expression in the roots of the cuttings. The *A. tumefaciens* strain R1600 carrying the A4Ri-plasmid, on the other hand, was used to study the effect of the chromosomal background on the root induction ability of the Ri-plasmid. When the results from the bacterial treatments in the spring experiment were examined, the strain A4 doubled root formation compared with the strain R1600. Since the Scots pine cuttings in the present study were derived from the same donors and all the environmental conditions as well as the Ri-plasmids were alike, the difference seems to be due to the chromosomal matrix.

The results of the agropine assays, the histochemical tests for ß-glucuronidase reporter gene expression, PCR tests, as well as the observations on the root morphology suggest that the roots formed in the Scots pine cuttings after the *Agrobacterium* treatments were

not stably transformed. Negative results in agropine assays do not necessarily indicate the absence of the whole T-DNA in root tissues, because TL- and TR-segments of T-DNA are transferred independently (TEPFER 1989). On the other hand, MAGNUSSEN *et al.* (1994) reported that the histochemical test for ß-glucuronidase expression was functional in the roots of *Pinus contorta* induced by *A. rhizogenes*. Negative results of the same histochemical test in the present study suggest that the roots are untransformed.

The enhancement in root induction caused by agrobacteria could, nevertheless, be due to the transient expression of the T-DNA genes in Scots pine cells. In chrysanthemum, a low efficiency gene transfer and transient expression of the TR-DNA auxin synthase genes after *A. rhizogenes* infection can mediate the induction of untransformed roots without stable integration of the T-DNA into the host plant genome (VAN WORDRAGEN *et al.* 1992). There are also reports on tree species, such as *Pinus monticola, P. banksiana* and *Larix laricina* (MCAFEE *et al.* 1993) and *Corylus avellana* (BASSIL *et al.* 1991), in which *A. rhizogenes* has improved rooting, but with no evidence of transformation. The other possibility is that a few cells have originally been transformed and are producing auxin due to the presence of the T-DNA genes, and normal roots can develop under the influence of transported auxin.

Agrobacteria may promote root induction, not only by transforming the host plant cells, but also by modifying the rhizosphere. LEYVAL & BERTHELIN (1989) have shown that interactions with rhizospheric micro-organisms are important for plant growth and nutrition. *Agrobacterium* sp. are acid-producing micro-organisms, which can increase the availability of soil mineral elements thus enhancing nutrient uptake and growth of the Scots pine root system (LEYVAL & BERTHELIN 1989). Besides affecting the nutrient balance in the rhizosphere, agrobacteria can secrete growth regulator substances. Both *A. tumefaciens* and *A. rhizogenes* strains have been shown to contain cytokinin synthase genes in their Ti- and Ri-plasmids, outside the T-DNA region (POWELL *et al.* 1988, REGIER *et al.* 1989). Moreover, KUTÁČEK and ROVENSKÁ (1991) have found that *A. tumefaciens* can synthesize auxin encoded by both the chromosomal and Ti-plasmid genes. In the present study, the chromosomal background of A4Ri-plasmid seemed to have an effect on rooting response. This observation suggests that the functions encoded by the chromosomal genes of agrobacteria are also involved.

The negative amplification results in the PCR tests indicate that the root tissues of the cutting plants do not contain the GUSint gene integrated into the host genome or remaining agrobacteria. After transforming the host plant, agrobacteria usually live in the intercellular spaces or on the surface of the transformed tissue, by using specific compounds, opines, secreted by transformed cells as a source of carbon, nitrogen and energy (AGRIOS 1988, CLARE 1990). DNA amplification by PCR is a very sensitive method for detecting micro-organisms in plant tissues (SCHAFF *et al.* 1992), and a remaining *A. rhizogenes* population in root tissues should have caused amplification of the GUSint fragment. It is, of course, still possible that root tissues contain agrobacteria, but without any selection pressure the strain A4(GUSint) may have lost the GUSint plasmid. As for practical application small numbers of remaining agrobacteria in the rhizosphere of the cutting plants are hardly hazardous, because agrobacteria do occur also in forest soils (BELL & RAMEY 1991).

The genotypic variation in the rooting ability of the Scots pine fascicular shoots was remarkable. Since the existence of the host-pathogen specificity between tree genotype and *Agrobacterium* strain has been demonstrated for other pine species, such as *Pinus taeda* (HUANG & TAUER 1994), and *P. radiata* (BERGMANN & STOMP 1994), the root induction in Scots pine might be improved further by testing different *A. rhizogenes* strains as rooting agents. Alternatively, agrobacteria could be genetically engineered to be more infectious, *e. g.* by incorporating a mutant gene leading to constitutive virulence gene induction, as suggested by HANSEN *et al.* (1994).

In the present study, we have shown the advantageous influence of *Agrobacterium rhizogenes* on root induction in Scots pine cuttings of fascicular bud origin, with no evidence of transformation. As for the practical applications of this rooting method, normal roots instead of transformed ones will be preferred, due to the conflicting views on the release of foreign genes or chimeric plants into nature. This is especially important in long-living forest trees, which grow in natural ecosystems in contrast to agricultural crops.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. Dr. L. Willmitzer for providing the GUSint plasmid and to Lic. For. J. Mikola for a critical reading of the manuscript. We also thank the personnel at Punkaharju and Haapastensyrjä for technical assistance. The research was supported by The Ministry of Agriculture and Forestry in Finland, and The Finnish Foundation for Research of Natural Resources.

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