INFECTION OF *PINUS RADIATA* WITH *AGROBACTERIUM RHIZOGENES* AND LONG-TERM GROWTH OF DETACHED HAIRY ROOTS *IN VITRO*

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ABSTRACT

Infection of *Pinus radiata* D.Don with *Agrobacterium rhizogenes* strains A4 and R1601 was demonstrated through greenhouse inoculation of seedlings and verification of opine production in gall tissue. Frequencies of seedlings exhibiting gall formation after epicotyl stem inoculation was 4% for strain A4 and 7% for strain R1601 (compared to 59% for the positive control inoculation with *A. tumefaciens* strain 542). Adventitious shoots of 22 *P. radiata* clones, micropropagated from seeds of the same bulk seedlot from which the seedlings had been grown, differed significantly in susceptibility to A4, R1601, and 542 when inoculated *in vitro*. *In vitro* shoots were more susceptible to both *A. rhizogenes* strains but less susceptible to *A. tumefaciens* 542 than seedlings inoculated in the greenhouse. The highest frequency of shoots forming a gall and/or hairy roots was observed in a different *P. radiata* clone for each *Agrobacterium* strain: 22% for A4, 44% for R1601, 58% for 542. *Agrobacterium rhizogenes* strain R1601 was superior to A4 for infection of *P. radiata* shoots grown *in vitro*. Gall production and hairy roots were observed in 11% and 1%, respectively, of *P. radiata* shoots 12 weeks after *in vitro* inoculation with *A. rhizogenes* strain R1601. Hormone autotrophic, bacteria-free cultures were established from stem segments that possessed tissue proliferating from *Agrobacterium* inoculation wound sites. Hairy roots formed directly from the wound site as early as 8 weeks after inoculation. Though opines were difficult to detect in callus tissues resulting from R1601 inoculations, the likelihood of detection was increased if the sample included roots. Cultures of detached hairy roots maintained on quarter-strength modified LePoivre medium reached an average length of 19.5 cm after 1 year and were shown to continue opine expression throughout that time. A greenhouse rooting experiment provided no evidence that rooting of *P. radiata* tissue culture shoots was improved by inoculation with *A. rhizogenes* R1601.

Keywords: susceptibility; *in vitro*; rooting; transformation; *Agrobacterium rhizogenes*; *Pinus radiata*.

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INTRODUCTION

The susceptibility of pine species to *Agrobacterium rhizogenes* may be important for genetic engineering and rooting enhancement of cuttings, applications that have been demonstrated in conifer species. The first published report describing use of *Agrobacterium* in a conifer to produce intact transformed plants was based on inoculation of larch hypocotyls with *A. rhizogenes* (Huang *et al.* 1991). Although a wild-type strain (containing pRi11325) was used in that work, transformed plants developed normally. McAfee *et al.* (1993) reported an enhancement of rooting of conifer cuttings using *A. rhizogenes* co-cultivation. In their work, tissue culture shoots of *Pinus monticola* D.Don co-cultivated with strains A4 (wild type) and R1600 (*A. tumefaciens* C58 with pRiA4b replacing the C58 Ti plasmid) rooted better than controls. When roots were removed from *Pinus banksiana* Lamb. and *Larix laricina* (Du Roi) K.Koch. seedlings, followed by co-cultivation of hypocotyls with *A. rhizogenes* A4, the number of roots and root length for re-rooted seedlings were greater on individuals that received *A. rhizogenes* treatment than on controls, although rooting frequency was no higher. However, rooting frequency was enhanced after treatment of *Pinus sylvestris* L. fascicular bud cuttings with *A. rhizogenes* strains A4 and R1600 (Aronen *et al.* 1996). Because increased rooting frequency or enhanced root system quality are of practical value in clonal forestry, experiments were conducted to demonstrate infection of *P. radiata* with *A. rhizogenes* and to determine if *A. rhizogenes* can be used to promote rooting of tissue-culture-produced shoots. An experiment was carried out to define the required conditions for long-term growth of hairy roots *in vitro*.

METHODS

Four experiments conducted with *Pinus radiata* focused on three objectives:

(1) To determine susceptibility to *Agrobacterium rhizogenes*;

(2) To establish *in vitro* hairy root cultures derived from *A. rhizogenes* stem inoculations; and

(3) To test the use of *A. rhizogenes* for enhancement of rooting of tissue culture shoots.

Agrobacterium Strains Used

Two *A. rhizogenes* strains were used—A4 because it has been shown to induce a response in *Pinus ponderosa* Dougl. ex Laws. and other conifers (Morris *et al.* 1989) and R1601 because it contains the hypervirulence of pTiBo542 (Hood *et al.* 1986) along with pRi4b (Pythoud *et al.* 1987). *Agrobacterium tumefaciens* strain 542 (Sciaky *et al.* 1978) was used throughout the experiments as a positive control because it is known to readily infect *P. radiata* and other pines (Bergmann & Stomp 1992; Stomp *et al.* 1990) and to serve as an indication of relative efficiency of the inoculation protocols used. *Agrobacterium tumefaciens* strain A136 (Watson *et al.* 1975) was used as an avirulent control.

Experiment I: Susceptibility of Greenhouse-grown Seedlings to *A. rhizogenes*

Pinus radiata bulk seed, from seedlot 102 in the *P. radiata* breeding programme of the New Zealand Forest Research Institute, was germinated and grown in the greenhouse. Germination medium consisted of 1 peat : 2 perlite : 2 vermiculite : 1 sand, and incandescent

lights providing 50 μmol/m^2/s were used to supplement natural light and provide a 24-hour photoperiod from October through March.

Three-week-old seedlings were inoculated by dipping the tip of a No. 11 scalpel blade into 3-day-old *Agrobacterium* cultures (on 3.9% w/v potato-dextrose agar) and stabbing seedling stems several times as close to the apex as possible. *Agrobacterium rhizogenes* strains A4 and R1601 and *A. tumefaciens* strain 542 were each used to inoculate 192 seedlings. The two control treatments, inoculation with *A. tumefaciens* strain A136 and wound-only, included 96 seedlings each. Seedlings were divided between two replicates in a balanced experiment design. Each replicate included three blocks, each block included four plots, and each plot included eight individuals per virulent *Agrobacterium* strain and four individuals per control treatment.

Seedlings were observed every 4 weeks for 24 weeks after inoculation to score individuals for gall formation and to monitor growth. A seedling was classified as reacting only if gall tissue from the inoculation site was positive for opine expression when extracted and electrophoresed following the procedures of Sederoff *et al.* (1986).

Experiment II: Susceptibility of *in vitro* Shoots to *A. rhizogenes*

Shoots from 22 clones were produced as per Bergmann & Stomp (1992) from seed of the same seedlot as used for Experiment I and were inoculated *in vitro* with *A. rhizogenes* strains A4 and R1601 and *A. tumefaciens* strains 542 and A136. The number of shoots for each treatment depended on the quantity available at the time of inoculation: between 16 and 56 shoots per clone were inoculated with strain A4, between 16 and 56 shoots per clone with strain R1601, between 16 and 24 shoots per clone with strain 542. As controls, nine shoots from each clone were inoculated with avirulent strain A136, and nine shoots from each clone were wounded without the application of bacterium. Shoots within each clone and treatment were split as evenly as possible among three replicates.

Shoots were observed every other week after inoculation for 12 weeks to score reacting individuals and to observe tissue growth at wound sites. A shoot was classified as reacting if roots, well-formed galls, or prolific callus emerged from the inoculation site.

Experiment III: *In vitro* Growth of Tissue Excised from Stem Inoculation Sites

Tissues proliferating from wound sites on *in vitro* shoots inoculated with *A. rhizogenes* or *A. tumefaciens* were cultured. The objective was to establish bacteria-free, hormone autotrophic, hairy root and callus cultures. Tissues from all 160 reacting shoots in Experiment II were used as they became ready.

Stem segments with tissues proliferating from wound sites were excised for subculture. In some instances a small shoot was present on the excised stem segment. Tissue arising from the three virulent *Agrobacterium* strains was distributed approximately evenly to the four media that were used: full-strength LP (Quoirin & LePoivre 1977, as modified by Aitken-Christie *et al.* 1988), quarter-strength LP, modified MS (Murashige & Skoog 1962) free of plant growth regulators, and modified MS with 0.75 mg benzylaminopurine/ℓ and 0.75 mg naphthaleneacetic acid/ℓ. The last medium was shown previously by the authors to support

in vitro P. radiata callus growth (unpubl. data). All media included 3% sucrose and 0.7 % bacto-agar (Difco Laboratories, Detroit, Michigan) and were adjusted to pH 5.7.

Forty cultures per medium were established and grown at 23°C under wide spectrum fluorescent lights providing 40 μmol/m^2/s and a 16-hour photoperiod. All cultures were transferred to fresh medium every 2 weeks. All tissue proliferating on the modified MS medium with growth regulators was transferred to modified MS medium devoid of growth regulators after 8 weeks. Media contained 250 mg timentin/ℓ (SmithKline Beecham Pharmaceuticals, Philadelphia, Pennsylvania) for 4 months to kill bacteria.

A subset of the hormone autotrophic cultures with no visible bacterial growth was sampled for opine expression after 6 months of culture. Thirty-three cultures initiated from *A. rhizogenes* strain R1601 inoculations were sampled, 22 callus and 11 callus with roots. Twenty-two callus cultures initiated from *A. tumefaciens* strain 542 inoculations were sampled. The small number of cultures established from inoculations with *A. rhizogenes* strain A4 did not permit subsampling at this stage. All tissues were tested for opine expression 1 year after culture initiation. Samples were extracted and electrophoresed following the procedures of Sederoff *et al.* (1986) to detect opines.

Experiment IV: Treatment of Tissue Culture Shoots with *A. rhizogenes* for Greenhouse Rooting

Shoots of clones produced *in vitro* for Experiment II were used in a trial to determine if treatment with *A. rhizogenes* strain R1601 would enhance rooting efficiency and/or root quality. Susceptibilities to strain R1601 ranging from 0% to 44%, as determined in Experiment II, were represented in the 16 clones that were used.

Six treatments were applied to clonal shoots. In each of two replicates, 28 shoots from each clone were treated with strain R1601, 14 or 16 shoots from each clone were treated with strain A136, and 14 or 16 shoots from each clone were used as a no-bacterium control. *Agrobacterium* inoculations were accomplished by dipping the tip of a No.11 scalpel blade into 2-day-old bacterium cultures and stabbing the base of each shoot four times, twice very close to the base and twice 5 to 10 mm above the base. Within each bacterium treatment, half of the shoots were treated with auxin and half were not. The auxin treatment was the standard protocol for rooting *P. radiata* tissue culture shoots, i.e., a 10-day *in vitro* exposure to medium containing 1.0% agar, 0.5 mg naphthaleneacetic acid/ℓ, and 1.0 mg indolebutyric acid/ℓ with a pH of 5.7. The shoots that did not receive auxin were placed on medium containing agar only. After 10 days, all shoots were placed under intermittent mist and 50% shade in the greenhouse in flats containing medium prepared as for seedlings in Experiment I. Temperatures in the greenhouse were 24±6°C during the day and 19±4°C at night.

Roots were counted and measured after a 10-week greenhouse rooting period. Shoots with roots that appeared to be of abnormal morphology were noted, and roots were analysed for opines following the procedures of Sederoff *et al.* (1986).

Statistical Analyses

Statistical analyses were performed using SAS (SAS Institute Inc., Cary, North Carolina). Chi-squared tests (PROC FREQ) were used to detect differences in frequency of gall

formation in Experiment I, gall or hairy root formation in Experiment II, and rooting in Experiment IV. In Experiment II, a data set composed of the three replicate means for each of the 110 clone/treatment combinations was used to determine the critical range to separate clones within a treatment according to Duncan's multiple range test (PROC GLM). Analysis of variance (PROC GLM) and Duncan's multiple range test were used to analyse root number and length.

RESULTS AND DISCUSSION

Experiment I: Susceptibility of Greenhouse-grown Seedlings to *A. rhizogenes*

Greenhouse-grown *P. radiata* seedlings exhibited limited susceptibility to both *A. rhizogenes* strains. Frequency of galls expressing opines 16 weeks after inoculation was as follows: 7% for A4, 4% for R1601, and 0% for A136 and wound-only controls, as against 59% for *A. tumefaciens* 542. All three virulent strains yielded gall formation frequencies that were different from the controls ($p < 0.01$). Gall formation frequency after inoculation with *A. tumefaciens* strain 542 was higher than with the two *A. rhizogenes* strains which were not different from one another at the 0.05 level. The high rate of gall formation on seedlings inoculated with *A. tumefaciens* strain 542 indicated that conditions were adequate for *Agrobacterium* infection.

Almost all galls first appeared at wound sites between 8 and 12 weeks after inoculation (Fig. 1a). *Agrobacterium rhizogenes* inoculation of greenhouse-grown seedlings resulted in the formation of knob-like swellings but not of roots or root-like structures. Strain 542 galls typically reached a diameter > 1.0 cm after 6 months but *A. rhizogenes* galls rarely exceeded 4.0 mm in the same time period. In several seedlings, a swelling at the site of an *A. rhizogenes* inoculation wound may have indicated infection by the bacterium, but the tissue did not proliferate enough to allow unambiguous opine detection. Seedling responses to *A. rhizogenes* inoculation were similar to those observed in *Pinus eldarica* Medwed. and *Pinus taeda* L. in previous work conducted by the authors (unpubl. data).

These findings show that *P. radiata* is susceptible to *A. rhizogenes* strains A4 and R1601. Susceptibility to *A. rhizogenes* has been demonstrated in other pine species, including *P. contorta* Loudon and *P. sylvestris* (Magnussen *et al.* 1994), *P. ponderosa* (Morris *et al.* 1989), and *P. nigra* Arn. (Mihaljevic *et al.* 1996). However, this is the first report of infection of *P. radiata*, and the results from this first experiment provided evidence to warrant further investigation of *P. radiata* inoculation with *A. rhizogenes*. The results further suggest that a small minority of genotypes may be susceptible and that the greenhouse conditions used may not be suitable for successful gall or hairy root growth from *A. rhizogenes* infections.

Experiment II: Susceptibility of *in vitro* Shoots to *A. rhizogenes*

In vitro P. radiata shoots were susceptible to *A. rhizogenes* as evidenced by the production of callus and/or hairy roots at wound sites within 12 weeks of inoculation (Table 1). None of the control shoots reacted to inoculation.

A fairly high frequency of dead shoots was observed 12 weeks after inoculation (Table 1). Wound-only and strain A136 control shoots showed that the inoculation process resulted in

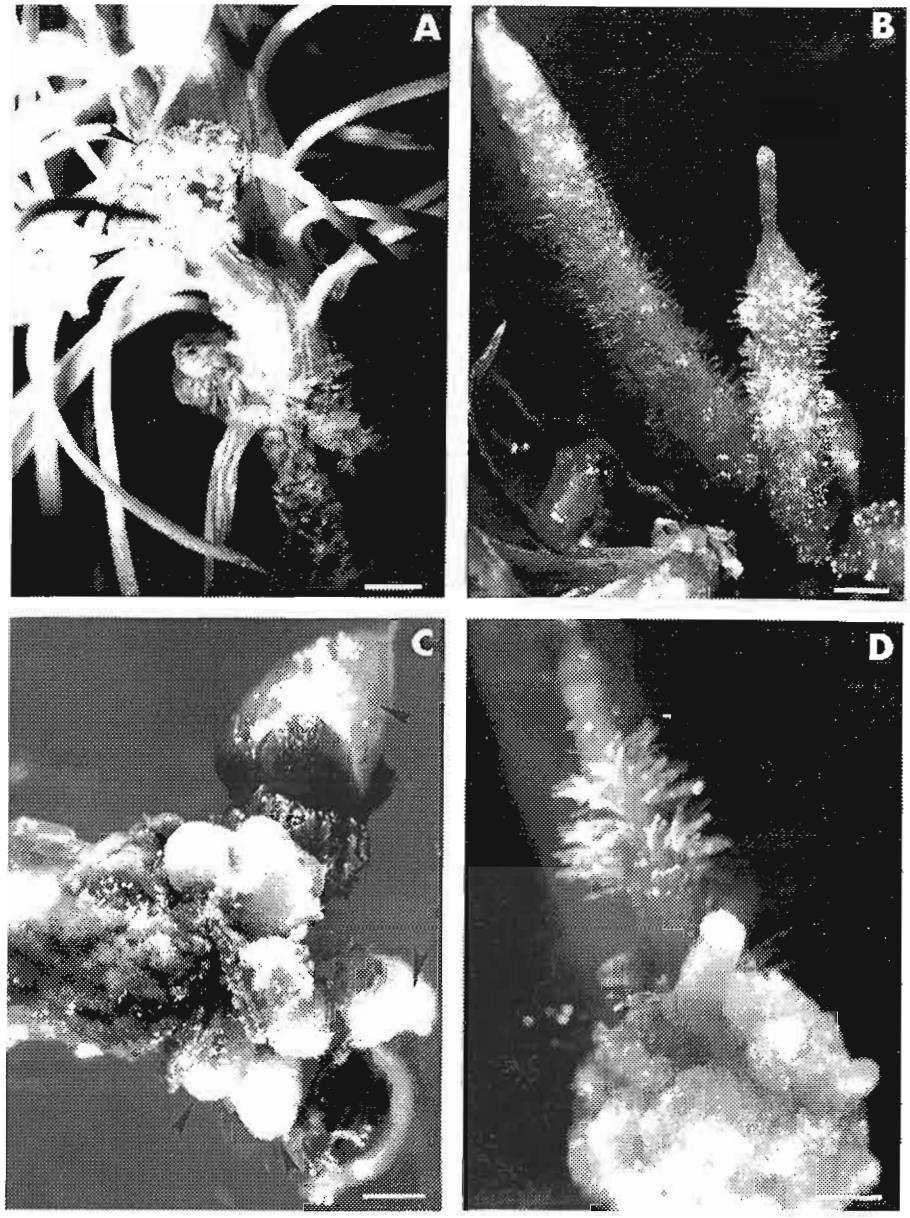

FIG. 1–Responses of *Pinus radiata* to inoculation with *Agrobacterium rhizogenes* strain R1601. **A**, newly formed gall (arrows) 8 weeks after greenhouse inoculation of a 3-week-old seedling (bar = 0.5 cm); **B**, hairy roots growing from inoculation site on a stem segment that was excised from a reacting tissue culture shoot after 8 weeks in culture (note the small shoot on the stem segment, bar = 0.5 cm); **C**, large roots (arrows) emerging from callus at an inoculation site 5 months after a shootless stem segment was excised and placed into culture (bar = 0.5 cm); **D**, hairy root emerging from a clump of callus 8 months after rootless callus from a wound site was placed on to medium devoid of growth regulators (bar = 0.25 cm).

TABLE 1–Frequency of dead, reacting, and root-forming *Pinus radiata* shoots 12 weeks after *in vitro* inoculation with *Agrobacterium*.

Treatment	Number* of shoots	Percentage shoots		
		Dead†	Reacting†‡	With roots§
A. rhizogenes A4	680	19 b	3 a	0
A. rhizogenes R1601	732	19 b	11 b	1
A. tumefaciens 542	392	14 b	23 c	0
A. tumefaciens A136	198	6 a	0 a	0
Wound-only control	198	4 a	0 a	0

* Across the 22 clones used.

† Values within the column followed by the same letter are not different at the 0.05 level according to Chi-squared test.

‡ A shoot was scored as "reacting" if roots, well-formed galls, or prolific callus emerged from the inoculation site.

§ Roots emerging directly from the inoculated stem.

approximately 5% shoot death. More shoots died after inoculation when a virulent bacterium was used, but the virulent strains did not differ from one another at the 0.05 significance level.

When calculated based on surviving shoots of all the clones combined, the frequency of reacting shoots (gall or hairy root production) was higher in those inoculated with *A. tumefaciens* strain 542 than those inoculated with either of the *A. rhizogenes* strains. Unlike Experiment I, *A. rhizogenes* strain R1601 resulted in greater frequency of reacting shoots than strain A4, whose reaction frequency was not significantly different from that of the controls.

The frequency of gall formation in response to inoculation with strain 542 was less for *in vitro* shoots in Experiment II (23%) than for greenhouse-grown seedlings in Experiment I (58%). This is consistent with previous work that included inoculation of *P. radiata* rooted shoots in the greenhouse and *in vitro* shoots of the same clones with strain 542 (Bergmann & Stomp 1992).

Reacting shoots inoculated with *A. tumefaciens* strain 542 produced friable, light green to yellow callus. Reacting shoots inoculated with *A. rhizogenes* A4 produced compact, spherical, dark brown callus. Reactions of shoots inoculated with *A. rhizogenes* strain R1601 were highly variable; occasionally compact brown callus formed, but green to light yellow friable callus was observed more frequently. Roots emerged directly from wound sites on stems inoculated with strain R1601 in four of the 593 surviving shoots.

Clones differed in susceptibility to each of the virulent strains (Table 2). While inoculation with each strain resulted in at least a few clones that yielded no reacting shoots, the highest frequencies of reacting shoots by clone were 22% for A4, 44% for R1601, and 58% for 542. No discernible pattern was detected regarding the susceptibility of clones to *A. rhizogenes* and/or *A. tumefaciens*. Examples were found of clones that were not susceptible to any strain (e.g., Clone 11), susceptible to all strains (e.g., Clone 15), susceptible to *A. rhizogenes* only (e.g., Clone 12), or susceptible to *A. tumefaciens* only (e.g., Clones 7 and 8). Five clones (6, 9, 20, 21, 106) were susceptible to R1601 but not susceptible to A4, but the reverse was not found. Three clones (14, 18, 24) had higher reaction frequencies after inoculation with A4 than with R1601, but the differences were not significant. These findings demonstrate that strain R1601 was more virulent than A4 across the clones tested.

TABLE 2–Frequency (%) of *in vitro* clonal *Pinus radiata* shoots reacting to inoculation with *Agrobacterium rhizogenes* (Ar) strains A4 or R1601 or *A. tumefaciens* (At) strain 542.

Pinus radiata clone*	*Agrobacterium* strain		
	Ar - A4	Ar - R1601	At - 542
2	0	7	10
3	7	7	8
5	0	3	7
6	0	15	17
7	0	0	20
8	0	0	21
9	0	16	0
10	0	0	18
11	0	0	0
12	8	27	0
14	22	9	29
15	18	22	50
16	0	0	19
18	20	14	27
19	8	8	20
20	0	22	40
21	0	44	36
22	5	6	58
23	0	7	8
24	11	6	22
26	10	14	25
106	0	17	52
Critical range†	8	7	12
Number of clones with ≥1 reacting shoot	9	17	19
Number of clones with ≥20% reacting shoots	2	4	12

* Clones in bold type are those that were used in the greenhouse rooting trial (Experiment IV).

† Critical ranges were calculated at the 0.05 level using all 22 means and are accurate when comparing the most disparate means. A comparison between clones with fewer intervening means will be conservative when this critical range is used.

Experiment III: *In vitro* Growth of Tissue Excised from Stem Inoculation Sites

After 6 months, 57 of the original 160 cultures were hormone autotrophic (Table 3), as judged by growth on medium devoid of growth regulators, and bacteria-free, as judged by visual inspection after 2 months of growth on medium without antibiotic. Thirty-four of these 57 cultures were grown on quarter-strength LP. All of the tissues placed on modified MS medium with growth regulators proliferated well during the first 2 months, but callus proliferation occurred from the cut surfaces of the stem segment rather than from the original tissue produced at the wound site. Callus growth stopped and cultures deteriorated rapidly once the growth regulators were removed. No roots were produced on medium containing growth regulators.

While four of the reacting R1601-inoculated shoots produced hairy roots directly from the stem within 12 weeks of inoculation, an additional 13 produced roots when cultured after

TABLE 3–Influence of culture medium on establishment of hormone autotrophic cultures of tissue resulting from *in vitro* inoculation of *Pinus radiata* shoots with *Agrobacterium rhizogenes* (Ar) strain A4 or R1601 or *A. tumefaciens* (At) strain 542.

Source tissue strain	Successful cultures* per cultures attempted (%) Culture medium†				
	Full LP	1/4 LP	MMS	MMS + PGRs	All
Ar - A4	3/5 (60)	5/5 (100)	0/4 (0)	0/5 (0)	8/19 (42)
Ar - R1601	7/17 (41)	15/17 (88)	3/17 (18)	0/17 (0)	25/68 (37)
At - 542	9/18 (50)	14/18 (78)	1/19 (5)	0/18 (0)	24/73 (33)
All	19/40 (48)	34/40 (85)	4/40 (10)	0/40 (0)	57/160 (36)

* Cultures that were hormone autotrophic and bacterium-free 6 months after culture initiation.
† See text for media definitions. Plant growth regulators were removed from MMS + PGRs after 2 months, and all cultures were assessed after 6 months.

excision. Eleven of these root cultures were among the 25 R1601-inoculated cultures which were bacteria-free. The production of hairy roots from 2.3% of the original strain R1601 inoculations was similar to the frequency of hairy root production observed by Magnussen *et al.* (1994). They had inoculated three conifers (*Picea abies* (L.) Karsten, *Pinus sylvestris*, *Pinus contorta*) with *A. rhizogenes* strain R1600 which contains the same root-inducing plasmid as strain R1601. None of the reacting A4-inoculated shoots produced hairy roots directly from the stem, but five produced roots in culture. Four of these root cultures were among the eight A4-inoculated cultures which were bacteria-free.

Almost all cultures with roots developed on quarter-strength LP. The timing of hairy root appearance varied greatly. Hairy roots formed directly from the wound site as early as 8 weeks after inoculation, and as late as 8 months after inoculation from a cultured clump of callus. It was most common for one or two roots to emerge from a site (Fig. 1b), but as many as seven roots emerged from all sides of a callus clump that resulted from inoculation with strain R1601 (Fig. 1c). The five root-forming cultures induced by strain A4 produced an average of 1.4 roots, and the 17 root-forming cultures induced by strain R1601 produced 3.6 roots on average.

The roots which developed in these cultures were called "hairy roots" with confidence because (1) they developed only on tissue proliferating from *A. rhizogenes* inoculation sites, (2) their gross morphology was distinct from normal roots, (3) they grew after detachment from stem tissue, and (4) they expressed opines 1 year after culture establishment. Developing hairy roots were much larger than typical *in vitro* adventitious P. *radiata* roots. A typical hairy root was 0.5 cm in diameter at 0.5 cm behind the tip (Fig. 1c). While elongating, roots would often maintain a thickness of 0.5 cm, and a dense covering of root hairs was common. Most hairy roots formed from tissue while attached to a stem segment that had a shoot, but some detached proliferating callus masses produced hairy roots (Fig. 1d).

Growth of individual hairy roots was maintained after excision from proliferating tissue in approximately 60% of the attempts. Detached hairy root growth was very slow, and roots had to be handled with great care. Eight hairy root cultures derived from strain R1601 inoculations and one from a strain A4 inoculation were maintained on quarter-strength LP for 1 year and grew to an average length of 19.5 cm.

After 6 months in culture, opine analyses were conducted on a subsample of hormone autotrophic tissue that developed after inoculations with strains R1601 and 542. Agropine and mannopine were expressed at high levels in all 22 samples of callus cultures resulting from strain 542 inoculations. However, opines were detected in only eight of the 22 samples (36%) of callus cultures resulting from inoculation with *A. rhizogenes* strain R1601. The frequency of opine detection increased to nine out of 11 (82%) if a sample originating from a strain R1601 inoculation included hairy roots in the sample rather than containing callus only. Root tissue from all nine of the long-term detached root cultures expressed high opine levels after 1 year.

Experiment IV: Treatment of Tissue Culture Shoots with *A. rhizogenes* for Greenhouse Rooting

The influence of *A. rhizogenes* inoculation on greenhouse rooting of *P. radiata* tissue culture shoots is shown in Table 4. Considering all shoots across auxin treatments and clones, the 35% rooting observed after shoot treatment with the virulent *A. rhizogenes* strain R1601 was significantly greater than the 25% obtained by treating shoots with the avirulent *A. tumefaciens* strain A136. However, treatment with either bacterium did not result in a rooting frequency different from the intermediate wound-only control treatment.

Treatment of shoots with *A. rhizogenes* R1601 did not increase the number of roots per rooted shoot or average root length. Number of roots per shoot may have been influenced negatively by the presence of bacteria, given that root number was slightly higher for wound-only control shoots than for *Agrobacterium*-treated shoots whether the virulent or avirulent strain was used. Root length did not vary among *Agrobacterium* and wound-only treatments.

The responses of the four clones which had been shown in Experiment II to be relatively highly susceptible to strain R1601 (Clones 12, 15, 20, and 21) were examined as a separate subset of the 16 clones used in this experiment. For none of these clones was a difference observed for rooting frequency, root number, or root length among the R1601, the avirulent strain A136, and the wound-only control shoots. While these four clones had a mean frequency of reacting shoots of 29% after *in vitro* stem inoculation with *A. rhizogenes* R1601

TABLE 4–Influence of *Agrobacterium rhizogenes* inoculation and auxin on rooting frequency, number of roots, and root length in *Pinus radiata* tissue culture shoots after 10 weeks in the greenhouse.

Treatment	Rooting percentage	Number of roots per rooted shoot	Mean root length (cm)
A. rhizogenes† 1601	35 a	2.1 ab	2.7 a
A. tumefaciens A136	25 b	2.0 b	2.9 a
Wound only	30 ab	2.5 a	2.5 a
With auxin	48 **	2.5 **	2.4 **
Without auxin	23	1.6	3.2

† Values for *A. rhizogenes*, *A. tumefaciens*, and wound only treatments within a column followed by the same letter are not different at the 0.05 level using the Chi-squared test for rooting frequency or Duncan's critical range test for root number and length.

** Indicates differences between auxin treatments at the 0.01 level.

in Experiment II, none of the 224 shoots from this set of clones treated with the same strain for rooting exhibited a response that could be interpreted as gall formation or hairy root production, and none of the sampled roots expressed opines.

Shoots treated with auxin had greater rooting frequency, greater number of roots per rooted shoot, and shorter roots than did shoots that did not receive auxin. The lack of a significant interaction between inoculation treatment and auxin treatment for rooting frequency, number of roots, and root length indicated that the influence of the two types of treatment was consistent with respect to one another.

Treatment with *A. rhizogenes* strain R1601 only (i.e., no auxin) was clearly inferior to the standard auxin treatment for rooting tissue culture shoots of *P. radiata* (i.e., no *Agrobacterium*) when considering rooting frequency (27% and 51%, respectively). This is in contrast to the improved rooting frequency after inoculation of *P. monticola* adventitious tissue culture shoots with *A. rhizogenes* strains A4 and R1000 observed by McAfee *et al.* (1993) and inoculation of *P. sylvestris* fascicular bud cuttings with *A. rhizogenes* strains A4 and R1600 observed by Aronen *et al.* (1996). Root number and root length did not differ between the virulent strain R1601 only and auxin only treatments. Thus, the benefit of obtaining a more robust root system was not achieved as it was when almond root systems were exposed to *A. rhizogenes* strain 232 (Strobel & Nachmias 1985). The reports of improved rooting frequency and root system size (McAfee *et al.* 1993; Strobel & Nachmias 1985) were based on relatively small sample sizes, and the benefit was attributed to the presence of the *Agrobacterium* in the rhizosphere rather than DNA transfer.

CONCLUSIONS

Taken together, the four experiments in this work showed that greenhouse-grown seedlings and *in vitro* tissue culture shoots of *P. radiata* can be infected with two strains of *A. rhizogenes*. The frequency of positive response to inoculation differed between the two inoculation systems and between the two *A. rhizogenes* strains. Susceptibility of *in vitro* inoculated tissue culture shoots to *A. rhizogenes* strains A4 and R1601 and *A. tumefaciens* strain 542 differed markedly among *P. radiata* clones. Hairy root and callus cultures that continued to express opines were grown for an extended period of time on medium devoid of plant growth regulators and antibiotic without the appearance of bacterium. This finding may be useful for basic research into transgene expression in whole conifer organs (i.e., roots) or gene expression patterns during rooting (e.g., Yibrah *et al.* 1996). Tissue culture shoots of *P. radiata* root readily, and so attention was focused more on the potential of producing more robust root systems on rooted individuals than on increasing the frequency of shoots that rooted. However, neither rooting frequency nor root system size was improved through *A. rhizogenes* inoculation of tissue culture shoots. This was true even when clones known to be susceptible to the *A. rhizogenes* strain were used.

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