FAMILY AND CLONAL VARIATION IN SUSCEPTIBILITY OF *PINUS RADIATA* TO *AGROBACTERIUM TUMEFACIENS* IN RELATION TO *IN VITRO* SHOOT GROWTH RATE

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ABSTRACT

This work was carried out to examine the hypothesis that differences in *Agrobacterium tumefaciens* susceptibility observed among *Pinus radiata* D.Don clones and families after stem inoculation *in vitro* can be attributed to differences in growth rate at the time of inoculation. *Agrobacterium tumefaciens* strain 542 was used to inoculate *in vitro* shoots of 90 clones divided among 14 full-sib *P. radiata* families. The significant influence of host genotype at the clone and family levels on susceptibility of *P. radiata* to *A. tumefaciens* was confirmed, and further evidence was found that growth rate at time of inoculation is important. Significant correlations between growth rate at time of inoculation and gall frequency illustrated the tendency for families (r=0.72, $p<0.01$) and clones (r=0.58, p=0.01) with rapidly growing shoots to be more susceptible to the bacterium. Analysis of covariance, however, showed that genotype influenced susceptibility over and above its effect on growth rate, pointing to a genotypic component of resistance that is independent of growth rate.

Keywords: susceptibility; variation; genotype; growth rate; *in vitro*; *Agrobacterium tumefaciens*; *Pinus radiata.*

INTRODUCTION

Genetic engineering could provide a powerful complement to classical methods for the genetic improvement of pine species since traditional methods are slow because of the long generation times involved. However, the production of transformed pine trees has been elusive. While *Agrobacterium* systems provide the basis for the routine production of transgenic dicots (Bevan 1984; Comai *et al.* 1985) and monocots (Bytebier *et al.* 1987; Raineri *et al.* 1990), such a system has been developed only recently in a conifer (Huang *et al.* 1991).

Genotype and condition of the host plant tissue are critical factors for determining the success of an *Agrobacterium*-based transformation system. Previous investigations have illustrated the importance of host genotype to *Agrobacterium* susceptibility in conifers

(Smith 1939; Clapham & Ekberg 1986; Ellis *et al.* 1989; Morris *et al.* 1989; Stomp *et al.* 1990). It has also been pointed out that the host cell-cycle phase may be a common denominator in influencing gene transfer efficiency in several *Agrobacterium*-mediated systems (Potrykus 1991; Stomp 1991). Bergmann & Stomp (1992) found evidence that growth rate at the time of stem inoculation is associated with the susceptibility of *Pinus radiata* to *Agrobacterium tumefaciens*. This conclusion was based on the fact that the observed influence of clone on gall frequency after stem inoculation in the greenhouse could be at least partially explained by growth rate at the time of inoculation. This concept has not been investigated at the level of families, nor at the more closely defined level of clone-within-full-sib families in any pine species. Further, the influence of shoot growth rate on *Agrobacterium* infection has not been investigated with a large number of genotypes in an inoculation experiment under the uniform conditions provided by an *in vitro* environment.

A large-scale experiment was done to examine the influence of shoot genotype at the clone and family levels, and shoot growth rate at the time of inoculation, on susceptibility of *P. radiata* to *A. tumefaciens* in a well-controlled environment. This work was designed to determine whether, and to what degree, the differences in *A. tumefaciens* susceptibility observed among *P. radiata* clones and families after stem inoculation *in vitro* can be attributed to differences in growth rate.

METHODS

Forty families of control-pollinated seeds provided by the New Zealand Forest Research Institute were used to produce clonal shoots *in vitro*. Standard methods for adventitious shoot production from excised mature embryos were used (Aitken-Christie *et al.* 1988) for 40 embryos per family. Briefly, after seed pretreatment (Reilly & Washer 1977) embryos were aseptically excised and inverted so that cotyledons were half submerged in shoot-proliferation medium (LP5—Aitken-Christie *et al.* 1988). Cotyledons were removed from the embryo after 6 days and returned to the same medium. Three weeks later cotyledons proliferating meristematic tissue were transferred to shoot elongation medium (LP0—Aitken-Christie *et al.* 1988). As shoots elongated, they were transferred to fresh LP0 medium in 10-cm Magenta boxes. When shoots reached 4 cm in height they were placed eight to a box on fresh LP0 in preparation for inoculation. Cultures were in a growth chamber maintained at 23°C under 16-h photoperiod supplied by Gro-lux wide spectrum bulbs which provided 40 $\mu mol/m^2/s$.

The virulent *A. tumefaciens* strain 542 was chosen to inoculate *in vitro* shoots because Stomp *et al.* (1990) demonstrated that *P. radiata* is highly susceptible to this strain. Strain 542 and avirulent strain A136 (non-transforming control, Watson *et al.* 1975)) were maintained in 30% glycerol stock until 3 days prior to inoculation at which time they were cultured on 3.7% w/v potato-dextrose agar in the dark at 37°C. Inoculations were accomplished by dipping the tip of a No.11 scalpel blade into the *A. tumefaciens* culture and stabbing the stem of each shoot several times between 2 and 8 mm from the apex and deeply enough to penetrate the xylem tissue.

Only families which included at least five clones, each of which had to include at least 25 healthy shoots, were used in the study. Therefore, 14 of the original 40 families were used. From five to eight clones within each of these 14 families produced the necessary 25 shoots to be used in inoculations. Altogether 90 clones (an average of 6.4 from each of the 14

families) were used. Six shoots per clone were inoculated with avirulent strain A136 as controls, six shoots were used as wound-only controls, and an average of 19 shoots per clone were inoculated with virulent strain 542. Shoots were divided among three replicates which were inoculated 2 weeks apart and maintained on separate growth chamber shelves. Of the 2788 shoots used, 1080 were controls and 1708 were inoculated with *A. tumefaciens* strain 542.

Two shoot parameters were monitored during the experiment: growth and gall frequency. Shoot height was measured 4 weeks prior to inoculation and 4 weeks post-inoculation. From these figures, growth per week at time of inoculation was interpolated. Shoots were evaluated for the presence of galls 2, 4, 6, 8, 10, 12, and 14 weeks after inoculation. Data were statistically analysed using SAS (SAS Institute, Cary, NC). Three types of analysis were conducted using shoots inoculated with strain 542.

(1) Growth rate and gall frequency were each analysed using the data set with all 14 families, with individual shoots serving as basic data units, with the following sources: replicate, family, and clone-within-family effects, and replicate by family and replicate by clone-within-family interactions (PROC GLM). Family was treated as a fixed effect since the families can be recreated, and all other effects were considered random. Pearson correlation coefficients were determined for growth rate with gall frequency (PROC CORR), and regression equations were calculated for gall frequency on growth rate (PROC REG). In both types of analyses, means for families (N=14) and for clones (N=90) were used.

(2) Separate data sets consisting of one family each were used with the following sources to explain growth rate and gall frequency: replicate, clone, replicate by clone interaction (PROC GLM). Individual shoots served as the basic data units, and all effects were considered random. Means for clones within each of the 14 families (N=5 to 8) were used to determine Pearson correlation coefficients and regression equations using the two variables (PROC CORR).

(3) An analysis of covariance was done using growth rate at the time of inoculation as a covariate of gall frequency (PROC GLM). Because of the binomial nature of the observation of whether a gall formed or not, the data set used comprised means for individual culture boxes, i.e., replicate/clone subclass means (N=245 of the possible 270 prescribed). This analysis addressed a central question of the work: Once the influence of growth rate is removed, does genotype have an additional significant influence on *Agrobacterium* susceptibility? A full model was tested:

GallFrequency = Replicate Family Clone(Family) MeanGrowth MeanGrowth*Family MeanGrowth*Clone(Family)

Because the interactions, which provide a test of the heterogeneity of regression slopes, were not significant ($F_{13,244}$=0.97, p=0.49 for mean growth by family and $F_{73,244}$=1.18, p=0.24 for mean growth by clone-within-family), the model for gall frequency was then run without the interaction terms. In each case only family was considered as a fixed effect. Type III mean squares for family and clone-within-family from the covariate model (without interactions) were compared to those from the same model without mean growth as a covariate.

RESULTS

In vitro shoot growth rates at time of inoculation differed significantly among families ($p<0.001$) and clones-within-families ($p<0.001$) (Table 1). When separate analyses were done for each family, it was found that growth rates differed significantly ($p<0.01$) among clones within each of the 14 families (Table 1). Replicate was not a significant source of variation for shoot growth rate ($p=0.36$).

TABLE 1–Family means and spread of means for clones within each family for growth rate at time of inoculation and gall frequency 12 weeks post-inoculation.

Family (clones)	Growth rate (cm/week)		Gall frequency (%)		r†	b‡	p§
	Range	Mean	Range	Mean			
1(5)	0.71–1.36 **	0.94	0–10 *	2	0.94	0.16	0.02
11(8)	0.75–2.80 **	1.59	0–33	23	0.62	0.10	0.10
14(7)	0.66–1.20 **	0.90	10–32	22	0.38	0.14	0.40
15(7)	0.62–0.95 **	0.76	0–24	11	0.90	0.67	0.01
16(5)	0.67–1.35 **	0.97	0–35 *	14	1.00	0.53	0.00
18(5)	0.45–0.89 **	0.63	0–14	7	–0.66	–0.25	0.22
22(7)	0.38–0.89 **	0.56	0–19 **	5	0.72	0.33	0.07
27(8)	0.69–1.02 **	0.87	0–33	17	0.81	0.80	0.03
28(5)	0.75–2.68 **	1.37	8–68 **	34	0.59	0.18	0.30
34(5)	0.65–0.98 **	0.80	0–29	10	–0.55	–0.41	0.33
35(8)	0.68–1.61 **	0.88	0–50 **	16	0.84	0.46	0.01
36(7)	0.64–0.97 **	0.82	0–25	13	0.63	0.52	0.13
39(8)	0.98–3.55 **	1.95	10–38	27	0.50	0.05	0.21
40(5)	0.51–0.95 **	0.77	11–29	19	0.89	0.38	0.04
All(90)	0.38–3.55	1.01	0–68				

* Difference among clones within a family significant at $p\leq0.05$
** Difference among clones within a family significant at $p\leq0.01$
† Pearson correlation coefficient of growth rate at the time of inoculation with gall frequency.
‡ Slope of the regression among clones within families of gall frequency on growth rate at the time of inoculation.
§ Significance level of the correlation coefficient and regression slope.

Timing of gall formation and gall morphology was similar to that previously reported for *in vitro* shoots of *P. radiata* inoculated with *A. tumefaciens* strain 542 (Bergmann & Stomp 1992). No galls had formed within 2 weeks of inoculation, but there were many galls after week 6 (Fig. 1). Almost all galls became apparent between 4 and 10 weeks after inoculation, and no new galls appeared after 12 weeks post-inoculation (Fig. 1).

The gall frequency results presented are from 12 weeks after inoculation. Data from week 8 and week 10 observations were also analysed, but results therefrom did not differ from those obtained at 12 weeks. No galls or gall-like swellings appeared on wound-only or avirulent strain A136 control shoots. These results were consistent within each of the three replicates.

The influence of family on gall frequency was significant ($p<0.01$), as was the influence of clones-within-families ($p<0.01$) (Table 1). When separate analyses were done for each

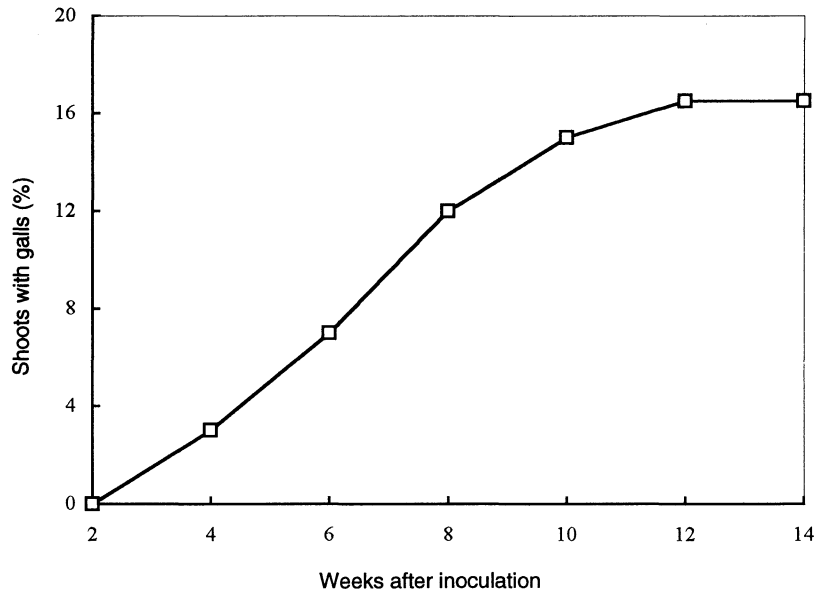

FIG. 1–Gall formation over time on *in vitro Pinus radiata* shoots after inoculation with *Agrobacterium tumefaciens* strain 542 (all families and clones combined).

family, it was found that gall frequency differed significantly ($p<0.01$) among clones within five of the 14 families (Table 1). These findings confirm previous observations that host genotype is an important factor in the susceptibility of *P. radiata* to *A. tumefaciens* (Bergmann & Stomp 1992).

An association was found between shoot growth rate at time of inoculation and gall frequency 12 weeks after inoculation. No association was found between shoot growth rate and the number of weeks between inoculation and gall appearance. When considering only shoots that were inoculated with the virulent strain 542, shoots that did not form galls had an average growth rate that was only 60% that of shoots which formed galls (0.92 *v.* 1.49 cm per week, respectively). This was the case even though non-inoculated controls, avirulent A136-inoculated controls, and 542-inoculated treatments (including all shoots whether or not they formed galls) exhibited the same mean shoot growth rates (1.05, 0.93, and 1.02 cm per week, respectively).

Families with faster growth rates tended to have higher gall frequencies at week 12 (Fig. 2). The Pearson correlation coefficient between these two traits ($r=0.72$) was significant ($p<0.01$) when whole family means were used (Fig. 2). Two families exhibited susceptibilities different from that expected given the apparent relationship found when growth rate was plotted against gall frequency using family means (No.28 and 1 in Fig. 2). In family No.28, gall frequency was higher than expected, while it was lower than expected for family No.1. We hypothesise that the susceptibility/resistance to *A. tumefaciens* of these families differed from the expected because of genetic influences unrelated to growth rate.

The Pearson correlation coefficient between gall frequency and growth rate and the slope for the regression of gall frequency on growth rate were also significant when means for the

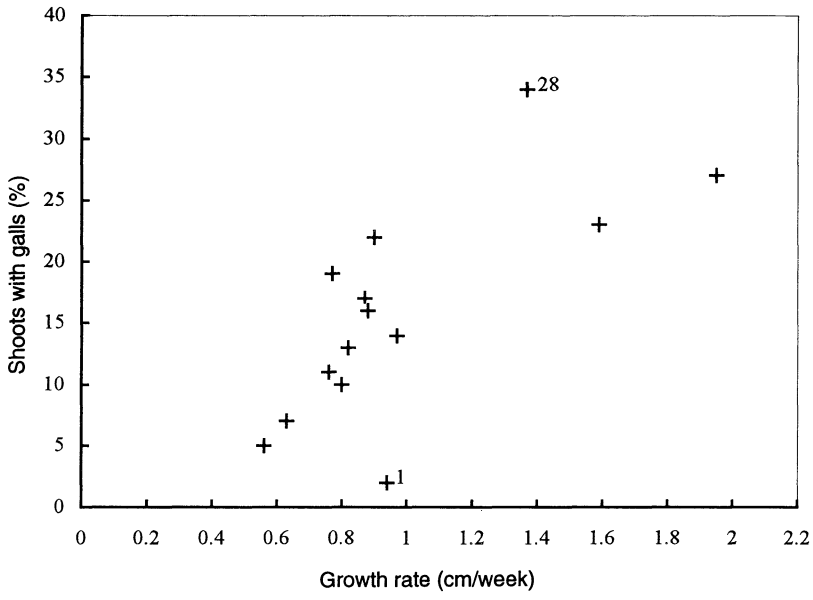

FIG. 2–Gall frequency *v.* growth rate at the time of inoculation, using family means. Galls observed 12 weeks after inoculation of *in vitro Pinus radiata* shoots with *Agrobacterium tumefaciens* strain 542. The Pearson correlation coefficient between the two traits is significant ($r = 0.72$, $p < 0.01$).

90 clones were used (r=0.58, b=0.14, p=0.01). When clone means were used in individual-family analyses, 12 of 14 families had positive correlations between growth rate and gall frequency at week 12 (Table 1). Of these, six had corresponding p-values of <0.05 (Table 1). Of the five families that exhibited significant differences among clones in gall frequency, four had positive growth rate to gall frequency correlations with significances of p<0.07. The fifth family, No.28, was unusual in its high gall frequency across clones.

Two families, No.18 and 34, had non-significant negative correlations between growth rate and gall frequency (Table 1). Several traits were common to these two families: (1) slow overall growth rate relative to all families (ranked 10 and 13 of the 14 families); (2) uniform growth rate among clones within the family as judged by the spread of clone means; (3) low overall gall frequency compared to all families (ranked 11 and 12 of the 14 families); and (4) lack of a significant difference among clones in gall frequency. These traits taken together did not permit a clear separation of clone performance for the two traits.

Gall frequency was analysed using growth rate at the time of inoculation as a covariate, with the aim of ascertaining the impact of genotype, over and above its influence on growth rate, on the susceptibility of *P. radiata* to *A. tumefaciens*. Genotype effects remained significant in the analysis of covariance, both family and clone-within-family (p=0.01 and p<0.01, respectively), indicating that the influence of genotype on gall frequency was more than simply the influence on shoot growth rate. The covariate analysis also showed that growth rate explained an additional portion of the variation in gall frequency, i.e., beyond family and clone-within-family effects. However, growth rate did not influence the effect

family and clone-within-family exerted on gall frequency. This was evidenced by the ratio of Type III mean squares from the model using growth rate as a covariate to the Type III mean squares from the same model leaving out the covariate. The ratio was not significant for family ($F_{13,13}$=1.62, p=0.20) or for clone-within-family ($F_{75,75}$,=1.21, p=0.21).

Given the large range in slopes for the regression of gall frequency on growth rate using clones within each of the 14 families, it was expected that the growth rate by family interaction would be significant in the covariance analysis of gall frequency. The non-significance of this interaction may be explained by the fact that most families were represented by a quite narrow range of growth rates so that sufficient power to detect an interaction was lacking. The high degree of uniformity within clones explains how it was possible to have relatively small yet significant differences in growth rate among clones in a family.

The amount of family and clone variation in growth rate is quite high. This may be a reflection of the fact that the uniform culture conditions used for shoot production were not optimal across genotypes—only 14 of the 40 families (35%) and 90 of the 560 clones within those 14 families (16%) produced large numbers of shoots for use in this study. A consequence of this may be the under-estimation of the relative contribution of inherent (as opposed to growth-rate mediated) resistances of genotypes to *A. tumefaciens*.

This work was done to determine whether there is a consistent relationship between growth rate at time of inoculation and gall frequency at the family and clone-within-family genotype levels. The data confirmed that there was a relationship between the two variables at the family and clone levels. Because growth rate is a function of genotype, complete separation of the effects of growth rate and genotype on gall frequency was not possible in this work.

The question remains as to whether susceptibility to *A. tumefaciens* can be increased by cultural techniques which induce a more rapid growth rate at the time of inoculation. To test this hypothesis, uniform genetic material induced to grow at varying rates must be used. This information would be valuable since improved gene transfer may occur in *Agrobacterium* inoculation/co-cultivation systems if the division rate of the target cells is increased.

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