

Susceptibility of Intra- and Inter-Specific Hybrid Poplars to *Agrobacterium tumefaciens* Strain C58

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ABSTRACT

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Susceptibility of seedlings from 95 intra- and inter-specific hybrid *Populus* families and 27 F₂ families of *Populus deltoides* to *Agrobacterium tumefaciens* strain C58 was evaluated. Controlled crosses were made between *P. deltoides* (females) and *P. maximowiczii*, *P. balsamifera*, *P. nigra*, and *P. deltoides* (males). At least one family from each parental species combination formed crown galls after inoculation with strain C58. Control plants, inoculated with sterile bacterial medium only, did not form galls. Gall tissue proliferated new gall tissue in vitro in the absence of phytohormones and produced nopaline. A second experiment, using 112 seedlings from six families, indicated that familial differences in

susceptibility to crown gall were attributable to the female parent. Results demonstrated that, although *A. tumefaciens* strain C58 infected 27 of 95 hybrid poplar families derived from several parent species, susceptibility within one subpopulation could be attenuated by maternally inherited host genotype. Thus, although strain C58 may be used as the basis of vector construction for the transformation of many poplars, testing of individual hybrids and use of alternative bacterial strains may be necessary. Hybrid families of contrasting susceptibility may be useful in the study of the biochemical control of *Agrobacterium* virulence genes by tree species.

Additional keywords: breeding, crown gall, host range, in vitro, nopaline.

The soil bacterium *Agrobacterium tumefaciens* causes crown gall disease by transferring bacterial plasmid DNA to plants (5). Some poplars and poplar hybrids from several taxonomic sections are within the host range of *Agrobacterium*. For example, *Agrobacterium* has been used to genetically transform various poplars (*Populus* sp.) with marker (1,14,18) or commercially useful genes (11). In addition, several investigators have induced *Agrobacterium* crown galls on poplars by inoculating trees in vivo (8,9,13,20).

However, previous studies provided little systematic information about host-mediated constraints to the transformation of poplars with *Agrobacterium*. For example, it is not known if any individual strains of *Agrobacterium* can transform genetically diverse populations of poplars. Host range information is important because resistance of certain host genotypes may necessitate use of alternative *Agrobacterium* strains or may even preclude *Agrobacterium*-mediated genetic transformation altogether. Host range data do not exist for hybrids between *Populus deltoides* Bartr. and other Aegeiros or Tacamahaca section poplars. Such hybrids have much potential for use in intensive poplar culture (12) and are prime targets for genetic engineering (18).

The current study was conducted to test the hypothesis that a genetically diverse population of intra- and inter-specific hybrid *Populus* seedlings derived from a large controlled-cross-breeding experiment differed in susceptibility to *A. tumefaciens* strain C58. A second objective was to test the hypothesis that susceptibility was influenced by intra-specific genetic variation in one or both parents.

MATERIALS AND METHODS

Plant material. Controlled crosses between *P. deltoides* (females) and *P. maximowiczii* Henry, *P. balsamifera* L., *P. nigra* L., and *P. deltoides* (males) were made in April 1987 at Rosemount, MN (Table 1). Female trees and males of *P. deltoides* were 15-year-old interprovenance polycross progenies growing in

a field test. Pollen of *P. nigra* was collected from trees growing in an adjacent 30-year-old provenance test. Pollen of *P. balsamifera* was collected from wild trees in northern Wisconsin. Pollen of *P. maximowiczii* was a gift from Shigeru Chiba, Oji Institute for Forest Tree Improvement, Oji Paper Co., Ltd., Hokkaido, Japan. In addition, 27 F₂ families of *P. deltoides* of various provenance ancestries were produced.

Seedlings were grown in the greenhouse (25 C days, 20 C nights, 18-hr photoperiod) from June to October 1987, at which time growth cessation and dormancy were induced by lowering greenhouse temperature to 4 C and reducing fertilization. Dormant seedlings were pruned to a uniform height of 10 cm in March 1988 and reflushed in a warm greenhouse (25 C, 18-hr photoperiod).

Bacterial strain. *A. tumefaciens* strain C58, a nopaline-producing strain supplied by Joanne Fillatti, Calgene, Inc., Davis, CA, was used in all inoculations. Strain C58, in modified form as a binary vector, has been used successfully in transformation research on hybrid poplar NC-5339 (*P. grandidentata* Michx. × *P. alba* L. 'Crandon') (11,19). C58 cultures were maintained on AB minimal medium (23) with transfer to fresh medium every 30 days. Five milliliters of MG/L broth (4) in a 25 × 150 mm test tube was inoculated with a single bacterial colony on the day before seedling inoculations. The liquid cultures were grown overnight at 30 C and 200 rpm in an incubated orbital shaker, then diluted to a concentration of 2 × 10⁸ bacteria/ml based on culture absorbance at 550 nm.

Inoculations. *Experiment 1.* Seedlings from each hybrid and F₂ family of *P. deltoides* were inoculated with *A. tumefaciens* strain C58 after the most dominant shoot reached a length of about 15 cm; stems were pierced with a No. 3 (0.5-mm diameter) insect mounting pin that had been dipped in either the bacterial culture (six seedlings per family) or sterile MG/L broth (six seedlings per family). Stems were pierced twice: first through the internode subtending the first 2-cm-long leaf, then through the next lower internode. Gall formation was evaluated after 60 days.

Experiment 2. Twenty seedlings from five families and 12 seedlings from a sixth were prepared for inoculations as described above. The six families formed a 2 × 3 (*P. deltoides* [D4 and D5] × *P. balsamifera* [B1, B2, and B3]) factorial subpopulation of the larger Experiment 1 crossing design (Table 1). All seedlings

TABLE 1. Crossing scheme used to produce hybrid poplar families for inoculation trials*

Females												Males						Females												Males					
Set 1	M1	M2	M3	B1	B2	B3	N1	D11	D12	D15		Set 1	M4	M5	M6	B4	B5	B6	N1	N2	D13	D14		Set 1	M4	M5	M6	B4	B5	B6	N1	N2	D13	D14	
D1	101(2)*	102	103	104(1)	105	106	107			110		D6	111	112	113	114	115	116	117	118	119	120		D7	211(2)	212	213(2)	214(1)	215	216	217	218	219(2)	220	
D2	201(1)*	202(1)	203(1)		205(1)*	206	207(3)	208	209			D8	311(3)	312(1)*	313(1)	314	315(1)	316	317	318	319	320(1)													
D3	301	302	303(1)*	304	305(1)	306	307	308	309	310																									
D4	401	402	403	404	405	406	407	408	409(1)			D9	411	412	413	414	415	416	417	418	419	420													
D5	501	502	503(2)	504(4)	505(8)*	506(1)*	507	508	509(1)	510		D10	511	512(2)*	513	514	515	516(1)	517(1)	518	519	520													

* Females (D1–D10) were all *Populus deltoides*. Males were *P. maximowiczii* (M1–M6), *P. balsamifera* (B1–B6), *P. nigra* (N1 and N2), and *P. deltoides* (D11–D15). Blank cells indicate failed crosses. Number of crown galls produced per six twice-inoculated seedlings in Experiment 1 are shown in parentheses. Galls from seedlings in families marked with an asterisk proliferated new gall tissue in vitro in the absence of phytohormones and produced nopaline. The subpopulation of families inoculated in Experiment 2 are indicated by the box.

were inoculated with strain C58 as described above except that four internodal stem positions below the first 2-cm-long leaf were pierced instead of two. Galls were counted after 60 days and weighed after excision and drying at 50 C for 72 hr.

Data were analyzed by analysis of variance according to the following model:

$$X_{ijk} = \mu + F_i + M_j + FM_{ij} + \epsilon_{ijk} \quad (1)$$

where X_{ijk} was the observed number of galls per tree or total gall weight per tree; μ was the population mean; F_i was the effect of the female parent; M_j was the effect of the male parent; FM_{ij} was the effect of the female \times male interaction; and ϵ_{ijk} was experimental error. Gall number and gall weight were transformed as $\sqrt{X + 1}$ to reduce heterogeneity of error variance.

In vitro culture. All galls were excised from one seedling in each of eight families (Table 1) inoculated in Experiment 1. The then gall-free seedlings were retained for use as controls in subsequent nopaline assays. Galls were surface sterilized in 10% commercial bleach (v/v) for 3 min, then washed three times in sterile water. Surface-sterilized galls were sectioned aseptically into disks 3–5 mm thick and plated on solidified (0.7% Bacto agar w/v) Murashige-Skoog medium that contained 20 mg/L of glucose, 300 mg/L of carbenicillin, but no phytohormones. Cultures were maintained at 25 C and 25–30 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (GE Cool White F20T12-CW and F40-CW). Proliferating tissue was subcultured to the same medium at 30-day intervals.

Nopaline assays. A 5- to 10-mm piece of tissue from each of eight in vitro tissue cultures was homogenized (VerTishear, The Virtis Co., Gardiner, NY) for 5 sec in 3 ml of 80% ethanol at 5 C. In addition, about 3–5 cm of gall-free stem apex, with leaves, from the eight gall-producing seedlings used for in vitro cultures, was minced with scissors, placed immediately in 3 ml of 80% ethanol at 5 C, and homogenized. Homogenates were transferred to 1.5-ml Eppendorf tubes, centrifuged at approximately 2,000 g for 5 min, and kept at 5 C until electrophoretic separation. Paper electrophoresis and visualization of nopaline were done according to the methods of Otten and Schilperoort (17). Nopaline assays were done twice, using tissue from the second and fifth culture passages.

RESULTS AND DISCUSSION

Experiment 1. Crown galls (Fig. 1) formed on seedlings from 27 of the 95 hybrid families (Table 1) inoculated with *A. tumefaciens* strain C58 in Experiment 1. No galls formed on seedlings that were inoculated with sterile MG/L broth only. Gall-forming families produced a mean of 1.8 ± 0.3 galls per 12 inoculation sites (six trees and two inoculations per tree). Families 504 (D5 \times B1) and 505 (D5 \times B2) produced the most galls (four and eight, respectively). Crown galls formed on at least one family from each parental species combination in the crossing scheme. In addition, galls were produced on nine of 27 F_2 families of *P. deltoides* tested. No pattern emerged relating frequency of crown gall formation to the provenance ancestries of the F_2 families.

All eight gall cultures (Table 1) proliferated tissue on phytohormone-free medium over 10 subcultures from May 1988 to

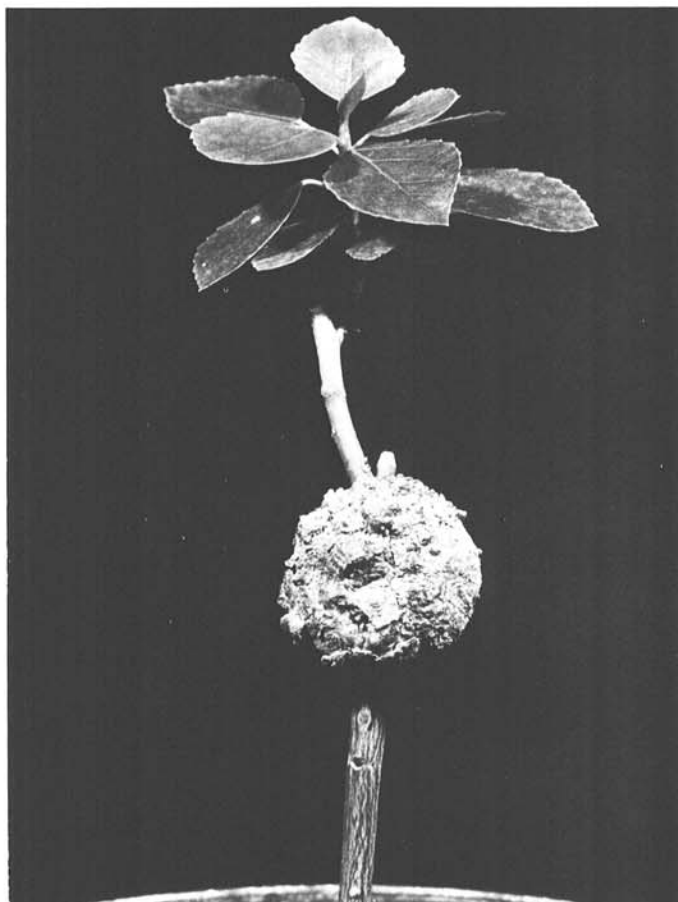


Fig. 1. Typical crown gall symptoms on an *Agrobacterium tumefaciens* strain C58-inoculated seedling of family No. 305, *Populus deltoides* \times *P. balsamifera*.

March 1989. Tissue derived from crown galls on the family 101 seedling was initially watery, was brown or gray in color, and grew very rapidly (Fig. 2). Tissue from the remaining seven seedlings was initially hard and green and grew more slowly than tissue from the family 101 seedling (Fig. 2). Nopaline was detected in aqueous ethanol extracts from all eight cultures at both sampling times. Nopaline, however, never was detected in any of the uninfected stem and leaf tissues taken from the original gall-free seedlings.

A broad range of intra- and inter-specific poplar hybrids was susceptible to *A. tumefaciens* strain C58 in Experiment 1 (Table 1). Insertion and expression of wild-type T-DNA was suggested by three lines of evidence. First, none of the control seedlings inoculated with sterile MG/L broth produced galls. Thus, gall formation was most probably due to interaction of the host plant with *A. tumefaciens* strain C58 and not due to wound healing, interaction with bacterial medium, or infection by a local pathogen. Second, other studies have shown that growth of crown

gall-derived tissue in vitro on phytohormone-free medium (Fig. 2) depends on plant expression of auxin (22) and cytokinin (2) synthesis genes introduced on bacterial T-DNA. Lastly, nopaline synthesis was demonstrated in gall-derived tissue from all eight genotypes tested (Table 1). Nopaline synthesis by gall-derived tissue requires host expression of the nopaline synthase gene also introduced on bacterial T-DNA (16). Furthermore, gall-free stem and leaf tissue from the eight gall-forming seedlings had no detectable nopaline content. Thus, production of opines by untransformed plants (6), which may yield false evidence of transformation, does not appear to have happened in the current study.

Resistance of hybrids of *P. nigra* × *P. maximowiczii* to crown gall induction by *A. tumefaciens* strain A208 has been reported (9). Yet hybrids between *P. deltoides* (female) and both aforementioned species (males) were susceptible to strain C58 in the current study. Thus, it is clear that germ plasm of *P. nigra* and *P. maximowiczii* does not present an insurmountable barrier to *Agrobacterium*-mediated transformation. Also, the overall gall-forming frequency previously achieved (9) was higher than achieved in the present study. Possible explanations include inherent bacterial strain differences (C58 vs. A208), inoculation protocol (piercing stem with dipped needle vs. inoculation with 10 μ l of liquid culture), and microenvironment (unwrapped vs. wrapped inoculation site).

Experiment 2. Six families from the subpopulation selected from Table 1 differed significantly in number of galls formed per tree and total gall dry weight per tree (Table 2). No significant variation in gall number or weight could be attributed to a female × male interaction or to differences among male parents. For example, apparent differences in number of galls per tree among families descended from female D5 (Fig. 3), which would be attributable to male effects, were not statistically significant. However, variation in mean number of galls per tree and total gall dry weight per tree was attributable to differences between the two female parents (Table 2). The effect of female parent was most apparent in combination with male B2 of *P. balsamifera*

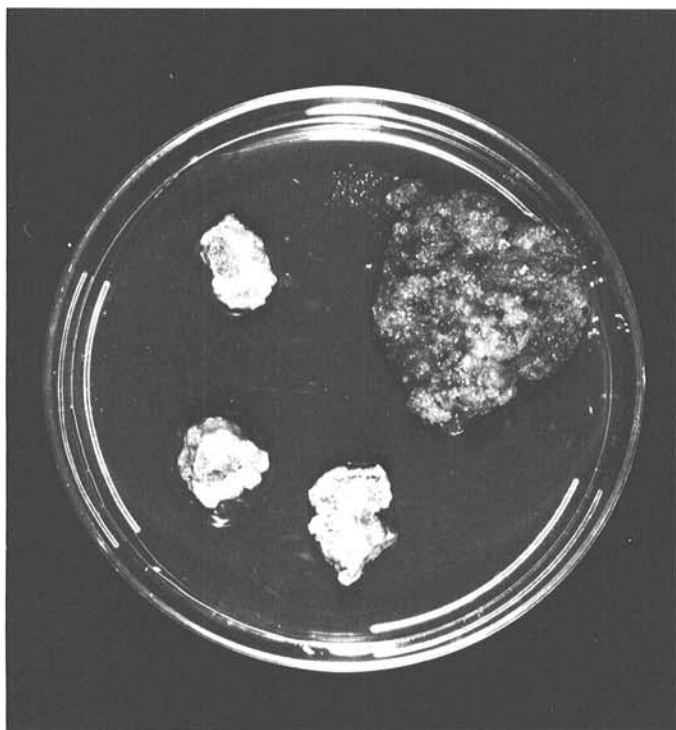


Fig. 2. Tissue grown in vitro from crown galls on seedlings of families (beginning in upper right and proceeding counterclockwise) 101, 312, 512 (*Populus deltoides* × *P. maximowiczii*), and 506 (*Populus deltoides* × *P. balsamifera*). Tissue derived from the gall-infected seedling in family 101 had a markedly different morphology compared with tissue from other gall-infected seedlings.

(Fig. 3), in spite of the lack of a significant female × male interaction. Offspring of male B2 and female D4 produced no crown galls in Experiment 2, whereas offspring of male B2 and female D5 had the highest mean number of galls per tree (Fig. 3A) and total tree gall weight of any family (Fig. 3B). Seedlings from the latter family, No. 505, also formed the most galls in Experiment 1.

Susceptibility of poplar seedlings to crown gall depended on parental (maternal) genotype in the subpopulation of families inoculated in Experiment 2 (Fig. 3, Table 2). Thus, whereas results

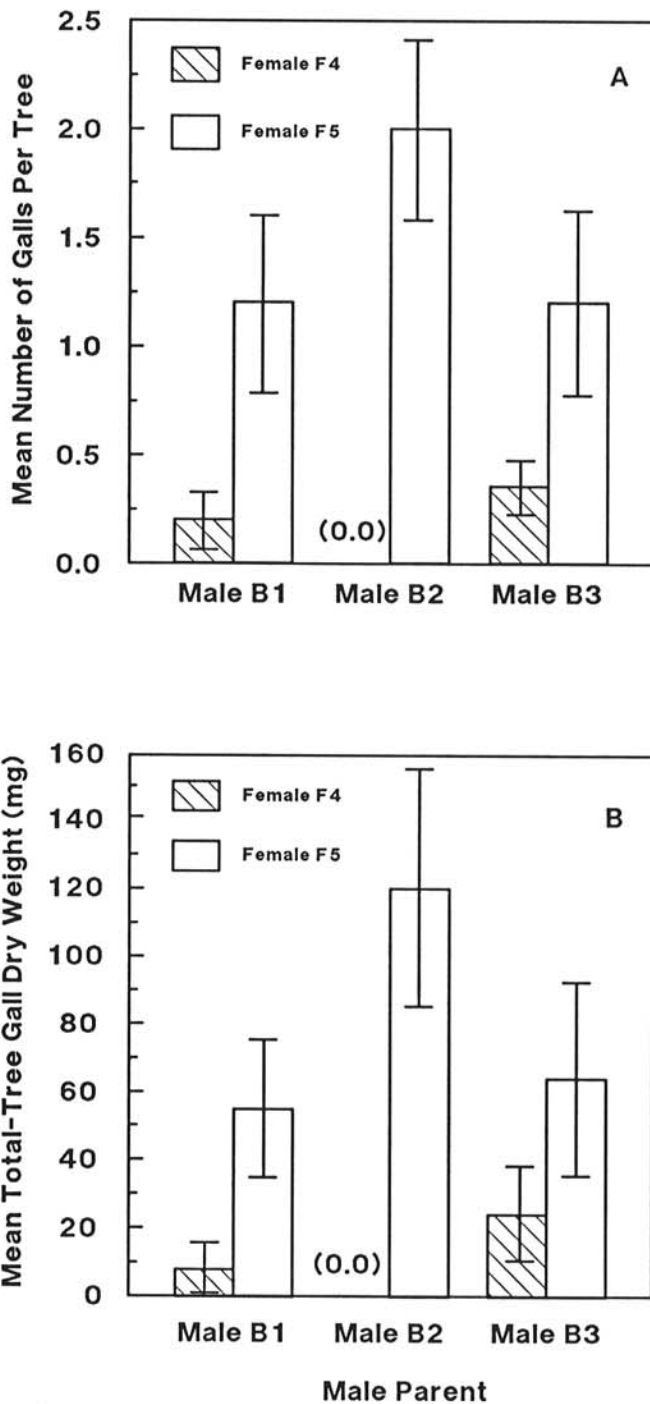


Fig. 3. A, Mean number of galls per tree, and B, mean total-tree gall dry weight \pm standard error of the mean in six hybrid poplar families in Experiment 2. Analyses of variance of transformed data $\sqrt{X + 1}$ indicated that familial differences in both variables were attributable to the effect of female parent and not to differences in male parent or to a female × male interaction. Progeny of female D4 and male B5 formed no galls.

TABLE 2. Analyses of variance of total gall weight per tree and number of galls per tree (four inoculation sites) on seedlings from six full-sib families in Experiment 2^a

Source	Degrees of freedom	Number of galls per tree		Gall weight per tree	
		MS	f	MS	f
Families	5	10.825	7.03**	0.035	3.52**
Female	1	48.000	31.18**	0.130	13.41**
Male	2	0.672	0.44 ns	0.007	0.71 ns
Female × Male	2	2.390	1.55 ns	0.016	1.66 ns
Error	106	1.540		0.010	

^a Data were transformed as $\sqrt{X+1}$ before analysis. ** = mean square significant ($P < 0.01$). ns = mean square not significant ($P > 0.05$).

of Experiment 1 indicated that *A. tumefaciens* strain C58 was pathogenic on hybrid poplars in general, the selected subpopulation examined in Experiment 2 indicated that susceptibility may be attenuated by intra-specific variation in the female parent. Results of the present study increase knowledge of differences in crown gall susceptibility between species and cultivars of poplars because the small clonal populations used in past research (8,9) provided only limited insight into possible modes of inheritance. Results of Experiment 2 also have practical significance because they imply that genetic selection for modified host susceptibility or strain pathogenicity may be necessary to achieve *Agrobacterium*-mediated genetic transformation of some hybrid poplars. Results should be interpreted cautiously when only one or a few poplar clones are used in genetic transformation studies. Caution also is warranted when only one strain of *A. tumefaciens* is used, such as in the current study.

Overall, two future areas of research are needed. First, the biochemical basis for familial differences as described above must be elucidated. Of possible mechanisms (15), virulence gene activation by plant wound substances merits initial attention because of its well-defined biochemistry and genetics (3,7,21) and because of the enhancing effect of tobacco feeder layers on plant leaf disk transformation (10). A second need is to determine the mechanism of inheritance of susceptibility to *Agrobacterium*. Female parent-related differences in susceptibility could have resulted from nuclear inheritance if the male parents were genetically invariant for the trait. Differences also could have arisen from cytoplasmic inheritance. Classical tests of cytoplasmic inheritance would be difficult, however, because poplars are predominantly dioecious, making reciprocal crosses difficult to perform.

The current study demonstrated that many intra- and inter-specific poplar hybrids derived from several parent species were susceptible to *A. tumefaciens* strain C58. However, within at least one parental species combination, maternally inherited host genotype strongly affected susceptibility. Thus, although strain C58 may be used as the basis of vector construction for the transformation of many poplars, testing of individual hybrids and use of alternative bacterial strains may be necessary. Hybrid families of contrasting susceptibility may be useful in the study of the biochemical control of *vir* genes by tree species.

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