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Susceptibility of *Juglans* Species and Interspecific Hybrids to *Agrobacterium tumefaciens*

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Abstract. **Crown gall, caused by the common soil-borne bacterium *Agrobacterium tumefaciens*, can be an economic problem in walnut nurseries and production orchards in California. The principal rootstocks used for commercial walnut production in California are the native Northern California black walnut, *Juglans hindsii*, and "Paradox," which are interspecific hybrids between a black walnut, primarily *J. hindsii*, as the maternal parent, and *J. regia*, the English walnut, as the paternal parent. Recent evidence has shown that some commercial black walnut trees producing Paradox hybrid seedlings are actually hybrids between *J. hindsii* and two other North American black walnut species, *J. major* and *J. nigra*. Here, we document that there was a higher incidence of crown gall on Paradox (*J. hindsii* ×*J. regia*) than on *J. hindsii* in three sites with natural soil inoculum. Paradox seedlings (with a female parent that was primarily *J. hindsii* with some *J. nigra*) inoculated with *A. tumefaciens* on the roots during transplanting had a higher incidence of crown gall than either *J. hindsii* or *J. regia*. When stems were inoculated with *A. tumefaciens*, *J. hindsii* ×*J. regia* populations had significantly larger galls than either *J. hindsii* or *J. regia*. Similarly, in stem inoculations on four out of six Paradox genotypes with a hybrid black walnut maternal parent, the progeny produced significantly larger galls than either *J. hindsii* or *J. regia*. However, two Paradox populations from black walnut hybrids that contained *J. major*, *J. nigra*, and *J. hindsii* produced galls that were no different in size than in the black walnut species and *J. regia*. Results suggest that *J. regia* and black walnut species are less susceptible to crown gall than most Paradox populations.**

Juglans regia Jeps., the "English" or "Persian" walnut, is the scion for commercial walnut production in California (Forde and McGranahan, 1996). Rootstocks used for commercial walnut production in California may contain genetic material from several North American species of black walnut: *J. hindsii* (Jepson) ex R.E. Smith, the northern California black walnut (the most common); *J. californica* S. Watson, the southern California black walnut; *J. major* (Torrey) A. Heller, the Arizona black walnut; *J. nigra* L., the Eastern black walnut; and *J. microcarpa* Berlandier, the Texas black walnut (Potter et al., 2002). Although walnuts are monecious and self-fertile, many *Juglans* species can cross-pollinate with each other (Forde and McGranahan, 1996). In particular, different North American black walnut species cross with each other and with *J. regia* (McGranahan and Catlin, 1987; McKenna, unpublished). While black walnut rootstocks were most common in the first half of the 20th century in California, since the 1950s, hybrid rootstocks, known as "Paradox," from a black walnut female, primarily *J. hindsii*, and a *J. regia* pollinizer have become increasingly popular (Browne et al., 1977; McGranahan and Catlin, 1987).

During the past 20 years, increased planting of Paradox rootstock in California walnut orchards has been accompanied by an apparent increase in the incidence and severity of crown gall caused by the soil-borne bacterium *Agrobacterium tumefaciens*. In a survey conducted in 2000, 59 walnut growers in northern California estimated a 30% average incidence of crown gall on Paradox trees (n = 102 orchards) but only a 3% incidence on *J. hindsii* (n = 29 orchards) (Epstein, unpublished). The economic impact of crown gall is greatest in young orchards in which trees with large or numerous galls tend to be stunted and predisposed to other stresses (Sinclair et al., 1987). In the California grower survey, 94% of growers reported reduced profits from orchards on Paradox in which there was a crown gall incidence of 20% or more. Despite its susceptibility to crown gall, Paradox performs better than *J. hindsii* in a wider variety of soils and is either more resistant or tolerant to root lesion nematode *Pratylenchus vulnus* (Lowensbery et al., 1974), root and crown rot caused by *Phytophthora* sp. (Matheron and Mircetich, 1985), high water table, flooding, and zinc deficiency (McGranahan and Catlin, 1987).

Resistance to *A. tumefaciens* has been reported in other horticultural crops, particularly in grapes (Süle and Burr, 1998). However, crown gall on grapes is caused primarily by *A. vitis* (synonyms, *A. tumefaciens* biovar 3, *Rhizobium vitis*) (Young et al., 2001). Crown gall in walnut is caused by either *A. tumefaciens* (synonyms, *A. tumefaciens* biovar 1, *A. radiobacter*, *R. radiobacter*) or *A. rhizogenes* (synonyms, *A. tumefaciens* biovar 2, *R. rhizogenes*) (Moore and Canfield, 1996). In California, *A. tumefaciens* is the more common causal agent of crown gall on Paradox than *A. rhizogenes* (Kaur and Epstein, unpublished). A stem inoculation assay has been used to identify resistance to *A. tumefaciens* in roses (Martí et al., 1999; Reynders-Aloisi et al., 1998), *Prunus* spp. (Bliss et al., 1999), and grape (Ferreira and van Zyl, 1986; Stover et al., 1997; Süle et al., 1994). The present study was conducted to document the incidence of crown gall on Paradox and black walnut rootstocks in the field and to investigate the genetic variation of different walnut genotypes to infection by *A. tumefaciens*.

Material and Methods

Plant material and species identification. Seed and seedlings in all experiments were open-pollinated except for one clonal commercial Paradox variety 'Vlach', which was propagated by stem cuttings by the Burchell Nursery, Oakdale, Calif. The Walnut Improvement Program at the Univ. of California at Davis provided seed of the following sources: *J. hindsii* 'Rawlins'; *J. regia* 'Howard', 'Tulare', and 'Waterloo'; *J. hindsii* ×*J. regia* 'O'Farrell'; *J. californica* 'Pomology HQ'; and *J. californica* ×*J. nigra* 87-5. The USDA National Clonal Germplasm Repository at Davis, Calif., provided *J. californica* DJUG23.20 and *J. microcarpa* DJUG23.11. Commercially sold seed donated anonymously by 12 walnut nurseries are designated here by two letter codes. The commercial varieties include 21 Paradox and one black walnut hybrid. Here, "Paradox" indicates a cross between a female black walnut and a male *J. regia* in which the female parent always has a California black walnut lineage (either *J. hindsii or J. californica*) but may contain other species of black walnut too (Potter et al., 2002).

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The male parent was inferred by visual inspection. On seedlings, Paradox leaflets are intermediate in size between the narrow leaflets of the black walnuts and the broad leaflets of *J. regia* (IPGRI, 1994; McGranahan and Catlin, 1987). In orchard evaluations of mature rootstocks, Paradox hybrids were differentiated from black walnut by their bark. Paradox hybrids have smooth, gray-colored bark, similar to but darker than *J. regia*, while black walnuts have fissured, brown-colored bark (Manning, 1978).

For 31 of the 35 populations tested by stem inoculation, the genotype of the female parent was primarily inferred by DNA sequence analysis of the mother trees as reported previously (Potter et al., 2002). Briefly, Potter et al. (2002) identified unique DNA fingerprints for each of the North American black walnut species by sequence analysis of three regions of the maternally inherited chloroplast DNA (spacers between the following: *trn*D and *trn*T, *trn*T and *trn*L, and *trn*L and *trn*F) and two regions of the biparentally inherited nuclear DNA (ITS1 and ITS2 of the ribosomal DNA). The genotype of the maternal parent also was inferred by examining nut morphology of all populations tested, since nut morphology varies among the native North American walnut species (Manning, 1978; McGranahan and Catlin, 1987; Potter et al., 2002). Nut morphology is useful even when the maternal trees are themselves interspecific hybrids, because nut traits appear to be quantitatively inherited and a nut produced by a hybrid tree has a blend of characters from that tree's progenitor species. Consequently, in six instances in which sequence data indicated that the female parent was derived from a complex mixture of black species, nut morphology also was used to infer the maternal genotype. In addition, the maternal lineage of four commercial Paradox varieties that were not identified by DNA analysis was inferred solely by nut morphology.

Incidence of crown gall in nurseries and commercial orchards. In Aug. 1996, three nursery sites were fumigated with a mixture of 75 methyl bromide : 25 chloropicrin at 477 kg·ha^{-1}. The fumigant was injected 30 cm below the soil surface and the soil was covered with a plastic tarp. In Nov. 1996, nuts from 16 different *J. hindsii* trees were direct-seeded. The plantings from each seed-tree were essentially a completely randomized design of half-sibs, i.e., seeds with the same maternal parent, but with two types of pollen parents; the pollen parent was either a black walnut in a same species-cross or *J. regia*. After sprouting, Paradox and black walnut seedlings were identified by leaf characteristics as described above. One-year-old seedlings were dug in Jan. 1998 and rated as infected if at least one crown gall was visible. A total of 2,640 *J. hindsii* and 2,829 *J. hindsii* ×*J. regia* seedlings were evaluated.

In 2001, mature Paradox hybrid and *J. hindsii* black walnut rootstocks in two commercial walnut orchards near Rio Oso, Calif., were rated for the presence or absence of crown gall. Rootstocks for both orchards were planted by direct-seeding seeds from a *J. hindsii* tree that produced ≈ equal numbers of both Paradox hybrid (*J. hindsii* ×*J. regia*) and black walnut (*J. hindsii*) seedlings. Thus, both orchards can be considered as randomized designs of half-siblings that were either Paradox or black walnut types. Both orchards had fine sandy loam soil, were not fumigated, and were well maintained according to standard horticultural practices. The 32-year-old orchard, previously planted with peach trees, was planted in 1970 with seed from one female *J. hindsii* tree and contained 1121 trees (69% Paradox) grafted to *J. regia* 'Chico.' The 53-year-old orchard was planted in 1949 with seed from the same tree as the 32-year-old orchard and an additional *J. hindsii* tree. This field contained 649 trees (48% Paradox) grafted to *J. regia* 'Eureka'. For assessment of gall incidence, no soil was removed, and only galls at the crown or base of the trees were counted.

Agrobacterium inoculum. Three virulent *A. tumefaciens* (biovar-1) strains (18W-19C, 18W-7A, and 2516) were used. Strains 18W-19C and 18W-7A were isolated from walnut galls in 1997, and strain 2516 consistently produced large galls on walnut in previous research (M. Schroth, personal communication). Pure cultures were maintained at –70 °C in 15% glycerol. To prepare inoculum, each strain was streaked onto LB agar (Sambrook et al., 1989). Cultures were grown in the dark at 22 °C to 25 °C for 3 d for root inoculations and for 7 d for stem inoculations. Bacteria were collected from the agar plates with sterile phosphate-buffered saline (pH 7.2) (Sambrook et al., 1989) containing ≈1 mL·L^{-1} of Tween 20 surfactant (Curtis and Nam, 2001), added to prevent cells from agglutinating. Equal amounts of bacteria of each of the three strains were combined, and adjusted spectrophotometrically for a final concentration of ≈1.0×10^7 colony-forming units (cfu)/mL. Preliminary trials indicated that these three strains in combination, at this concentration, produced the most consistent and largest galls on walnut compared to each strain alone. For the stem inoculation experiments, inoculum concentration was determined at the beginning and end of the day by dilution plating; initial concentrations of *A. tumefaciens* cells ranged from 1.0×10^7 cfu/mL to 2.4×10^7 cfu/mL. On average, there was a 20% decrease in bacterial cfu by the end of each day. While in use in the field, inoculum was kept in the dark in an ice bath.

Root inoculation study. Open-pollinated *J. hindsii* 'Rawlins', *J. regia* 'Howard', and Paradox 'OZ' seed were planted in Fall 1998 in fumigated nursery soil at the Burchell Nursery, Oakdale, Calif. Seedlings with typical black, English, and Paradox leaf phenotypes were selected from the 'Rawlins', 'Howard', and 'OZ' seedlings, respectively. When 1-year-old seedlings were dug in Jan. 2000, no galled trees were found. After digging, roots and crowns were rinsed with a 10% commercial bleach solution, and then heeled into clean wood shavings. At the time of transplanting, a suspension of 1×10^7 cfu/mL was prepared as described above and sprayed onto the roots and crown until each plant was saturated. Trees were planted, without intentional wounding, in Mar. 2000 in Davis, Calif., as a randomized complete-block design with one treatment per block and 90 blocks. Trees were dug after dormancy in late Nov. 2000. After rinsing the soil from the roots, the location of every gall on each tree was classified as being on the crown, taproot, or a lateral root and the diameter of each gall was measured.

Stem inoculation study. Seedling populations were divided into thirds, and direct-seeded in fumigated soil at each of three nurseries. After sprouting, seedlings from each seed source were examined, the pollen parent was inferred by leaf characteristics, and trees with the appropriate black, English, or Paradox type were identified. To establish field plots for stem inoculations, dormant, gall-free, 1-year-old trees were planted bare-root in a randomized complete-block design with four blocks at the Univ. of California Kearney Agricultural Center, Parlier. Each population within each block consisted of a group of either six seedlings (two trees from each of three nurseries), or three seedlings (one tree from each of three nurseries). In 1998, trees were planted 6–7 Mar., spaced 0.6 m within the row and 4.6 m between rows. In 1999, the entire experiment was repeated with a new planting of 1-year-old seedlings on 15 Mar. with trees spaced 0.9 m apart within the row and 4.6 m between rows. After planting, the main stem was pruned 60 to 75 cm above the ground. During the growing season, three new current-year shoots were retained and other branches were removed.

Stems of trees were wounded and inoculated over the course of four consecutive days, 13–16 July 1998 and 20–23 July 1999, with inoculations of all trees in a single block on a single day. Inoculum from 7-day-old cultures was prepared daily as described above. Each tree was wounded with a dovetail carpentry saw fitted with a wooden stop set to cut to a maximum depth of 1.8 mm into the stem. Wounds were made at about equal intervals 6–10 cm apart on the north side of stems; five wounds were made on the 1-year-old stem (trunk) and five were made on a current-year stem. Immediately after wounding, ≈15 to 20 μL of inoculum was applied to saturate the wounds, which were then wrapped with Parafilm™ and covered with white plastic flagging tape. For control inoculations, in 1998, 24 *J. hindsii* seedlings and 16 *J. hindsii* ×*J. regia* seedlings were treated with sterile buffer on both current-year and 1-year wood, while in 1999, 23 *J. hindsii* ×*J. regia* hybrids were mock-inoculated only on current-year wood.

Gall expansion on the stems does not continue after trees become dormant. After the onset of dormancy, gall size was calculated by subtracting the stem diameter directly above or below the gall from the maximum gall diameter including the stem. Measurements were taken to the nearest 0.1 mm with a vernier caliper. To correlate the diameter measurement of gall size with biomass, 135 galls were measured, dried, and weighed.

Statistical analysis. The null hypothesis that crown gall incidence was independent of genotype was tested using chi-square for Paradox, *J. hindsii*, and *J. regia* seedlings grown in

infested soil or by root inoculation. Data on gall diameter of inoculated stems were analyzed by an analysis of variance (ANOVA) using SAS (SAS Inst., Cary, N.C.). Factors initially considered in the model were population, year, block, and age of wood. Year was removed from the model because year was not significant ($P = 0.42$). Therefore, the total eight blocks (four from each year) were classified as replicates. Age of wood was removed from the model because on individual trees, gall size on 1-year-old vs. current-year wood was correlated ($R^2 = 0.86$, $P = 0.001$, n = 296). Thus for each tree, the diameter of all 10 galls from both years' wood was averaged. Tukey's Studentized Range test ($P = 0.05$) was used to compare gall diameter among populations. When indicated, contrasts were used to compare gall diameter between taxonomic types, i.e., black, English, and Paradox hybrids. A paired *t* test was used to compare gall diameter of black and Paradox half-siblings of DJUG23.20 and 87-5. However, when errors were not normally distributed and a suitable transformation was not found, nonparametric tests were used. In particular, the Wilcoxin two-sample test was used to compare gall diameter of black and Paradox half-sib populations of 'Rawlins'.

Results

Incidence of crown gall in nurseries and commercial orchards. The 1-year-old Paradox seedlings grown in fumigated nursery soil had a significantly ($\chi^2 = 12.8$, $P = 0.0004$) higher incidence (0.9%) of crown gall than black walnut seedlings (0.2%). There was also significantly more crown gall on Paradox rootstock than on *J. hindsii* rootstock in two mature walnut orchards. In the 32-year-old orchard, 31% of the Paradox and 22% of the *J. hindsii* had crown gall ($\chi^2 = 8.6$, $P = 0.003$). In the 53-year-old orchard, 52% of the Paradox and 17% of the *J. hindsii* had crown gall ($\chi^2 = 86$, $P = 0.00001$). Thus, incidence was 1.5× and 3× greater on Paradox than on *J. hindsii* in the 32- and 53-year-old orchards, respectively.

Root inoculation study. Paradox 'OZ' seedling roots inoculated with *A. tumefaciens*, planted in soil, and observed 8 months later had a significantly ($\chi^2 = 9$, $P = 0.01$) higher incidence of crown gall than either *J. hindsii* or *J. regia* seedlings (Table 1). However, the difference between Paradox and the other rootstocks was only 17 or 19 percentage points. Many trees had galls on two or three tissue types, i.e., crown, taproot, and lateral roots. Paradox had more trees ($\chi^2 = 30$, $P = 0.001$) with galls on the lateral roots than *J. hindsii* or *J. regia* but gall incidence on either crown or taproots was statistically indistinguishable ($P > 0.05$) among rootstocks. There was no significant difference in gall size among rootstocks.

Stem inoculation study. Control plants inoculated with sterile buffer produced no galls, whereas galls resulted from ≈99% of our inoculations with *A. tumefaciens*. As a part of the stem inoculation study, half-sibling populations were analyzed from each of three female trees (Table 2). Paradox hybrid seedlings of *J. hindsii* 'Rawlins' produced significantly ($P =$

Table 1. Effect of rootstock on crown gall incidence in a field trial in which the roots of dormant 1-year-old seedlings were spray-inoculated with *A. tumefaciens* at transplanting.[z]

Rootstock type	Overall incidence of galled trees (%)	Crown gall incidence on specific tissues (%)			Avg gall diam (mm)
		Crown	Tap root	Lateral root	
J. hindsii	60	30	31	27	30
J. regia	62	22	24	35	23
Paradox OZ[y]	79**	28	30	65***	36

[z]**,***Indicates that means within a column differ significantly at $P = 0.01$ and 0.001, respectively. Incidence and gall size data were analyzed by chi-square and ANOVA, respectively. There were no significant differences between *J. hindsii* and *J. regia*.
[y]The female parent of Paradox 'OZ' was subsequently identified as a complex black walnut hybrid with primarily *J. hindsii* but some *J. nigra* lineage.

Table 2. Response of populations of seedlings of *Juglans* species and interspecific hybrids to stem inoculations with *A. tumefaciens*.

Walnut type[z]	Inferred maternal genotype[y]	Population[x]	Gall diam (mm)[w]
Black	*J. microcarpa*	DJUG23.11	6.3 a
	J. californica	DJUG23.20	8.4 abc
		Pom. HQ	11.5 bdefg
	J. hindsii	Rawlins[v]	9.7 abcd
	J. nigra ×*J. hindsii*	YZ[v]	11.0 abcdefg
	J. californica ×*J. nigra*	87-5	14.5 defghijk
English	*J. regia*	Tulare[v]	10.1 abcde
		Waterloo[v]	10.8 abcde
Paradox hybrids	*J. major* ×(*J. hindsii*, *J. nigra*)[t]	MX[v,u]	8.3 ab
	(*J. major* ×*J. hindsii*) ×*J. nigra*	AZ[v,u]	10.7 abcdef
	J. hindsii ×*J. regia*	O'Farrell	13.2 cdefghi
	J. californica	DJUG23.20	13.2 defghi
	J. californica ×*J. nigra*	87-5	15.2 fghijkl
	(*J. major*, *J. nigra*)[t] ×*J. hindsii*	CX[v,u]	16.1 hijklm
	J. hindsii ×(*J. hindsii*, *J. nigra*)[t]	LX[v,u]	14.0 defghij
		NX[v,u]	15.8 hijklm
		PX[v,u]	16.9 ijklmn
	J. hindsii and some *J. nigra*	OZ[v,s]	17.1 ijklmn
		HX[v,s]	17.3 ijklmn
	J. hindsii	Vlach[v,r]	14.8 defghij
		DX[v]	15.6 hijklm
		YX[v]	15.9 ijklm
		RZ[v]	16.9 ijklmn
		SZ[v]	16.9 ijklmn
		JX[v]	17.7 ijklmn
		CZ[v]	17.7 ijklmn
		ZZ[v]	18.0 jklmn
		WX[v]	18.1 jklmn
		Rawlins[v]	18.7 jklmn
		OX[v,s]	19.0 klmn
		KX[v,s]	19.2 klmn
		XZ[v]	19.3 klmn
		QZ[v]	19.4 lmn
		FX[v]	20.4 mn
		VX[v]	21.5 n

[z]The type identification is based on leaf morphology of the seedlings. Black and English seedlings have both parents of the same type. Paradox seedlings have a black walnut maternal parent and an English walnut paternal parent. Contrast analysis indicates that Paradox had significantly ($P \leq 0.001$) larger galls than either black or English walnut.
[y]The maternal genotype is primarily based on sequence analysis of chloroplast and nuclear DNA markers (Potter et al., 2002), except where footnotes indicate otherwise. Nut morphology was also used.
[x]Each population had the same female parent. Except for Vlach, which was clonally propagated, flowers were open-pollinated, but seedlings were separated into types based on leaf morphology.
[w]Average gall diameter of inoculated stems with four replicates in each of two years. Each replicate was the average of 10 inoculations per seedling (five on a 1-year-old stem and five on a current-year stem) on either three or six seedlings. An ANOVA for all populations was highly significant ($P \leq 0.0001$). Means followed by the same letter were not significantly different by Tukey's Studentized Range test ($P = 0.05$). Minimum significant difference was 4.8 mm.
[v]Commercial rootstocks in California.
[u]Nut morphology was critical in assignment of maternal genotype, in addition to nuclear and chloroplast DNA markers.
[t]*J. nigra* may have been either from the paternal or maternal grandparent.
[s]Maternal species was identified solely by nut morphology (Manning, 1978; Potter et al., 2002).
[r]A clonal Paradox rootstock derived from stem cuttings.

0.01) larger galls than their black walnut half-sibling counterparts did. Similarly, Paradox hybrid seedlings of *J. californica* DJUG23.20 produced significantly ($P = 0.02$) larger galls than their black walnut half-sibling seedlings. In contrast, the galls produced by Paradox and black walnut half-sib progeny of (*J. californica* ×*J. nigra*) 87-5 were statistically indistinguishable ($P > 0.05$). However, on average, black walnut seedlings from 87-5 produced the largest galls of all the black walnut populations assayed and were not significantly different from most Paradox.

Results of a multiple range test for all 35 populations are shown in Table 2. Galls on either of the two *J. regia* populations were not significantly different ($P > 0.05$) in size from those on *J. hindsii* 'Rawlins'. Galls on the black species *J. microcarpa* and *J. californica* also were not significantly different from *J. hindsii*. Contrast analysis (1 df) indicated that there were no significant differences in gall diameter between black walnut sp. and *J. regia* ($P = 0.47$) and highly significant differences ($P \leq 0.001$) in gall size between Paradox as a group and either black or *J. regia*.

Sixteen of the 24 commercial Paradox rootstocks assayed (Table 2) are *J. hindsii* ×*J. regia*. Among these 16 Paradox populations, there were slight significant differences in gall size, but none of the populations appeared to be notably different from the others. Table 2 lists six other Paradox genotypes that can be considered as "complex" hybrids since all have a maternal parent that contains another species in addition to *J. hindsii* or in one case, *J. californica*. All five commercial Paradox populations with an apparent maternal genotype of *J. hindsii* ×(*J. hindsii, J. nigra*) had a gall size that was not significantly different from those of *J. hindsii* ×*J. regia* Paradox. Two of the Paradox seedling populations with complex genotypes, MX [*J. major* ×(*J. hindsii* ×*J. nigra*)] and AZ [(*J. major* ×*J. hindsii*) ×*J. nigra*], developed significantly smaller galls than all other Paradox genotypes, and were not significantly different from black and English walnut (Table 2). Although both AZ and MX have a genetic background containing *J. major*, *J. nigra*, and *J. hindsii*, another Paradox population (CX) with a similar maternal lineage produced large galls.

The diameter of the gall was correlated with the biomass of the gall tissue ($R^2 = 0.76$, $P = 0.001$, n = 135). Thus, gall diameter was selected as an estimate of gall size. Several factors had no demonstrable effect on the assay: the year of the trial ($P = 0.42$); the size of the stem that was inoculated ($P = 0.66$, n = 15,185); and the annual growth rate of the seedling, i.e., the increase in trunk cross-sectional area. In general, galls were larger on current-year wood vs. 1-year wood by an average of 1.7 mm in 1998 and 1.3 mm in 1999. For each seedling, the size of galls on current-year wood was highly correlated ($R^2 = 0.86$) with the size of galls on 1-year wood. For *J. hindsii* ×*J. regia* Paradox and *J. hindsii* mock-inoculated trees, wound callus measured an average of 3.0 mm ± 1.5 SD for both current and 1-year-old wood in 1998, and 3.6 mm ± 1.2 SD in 1999.

Discussion

Our results indicate that under field conditions, Paradox (primarily *J. hindsii* ×*J. regia*) has a higher incidence of crown gall than black walnut (primarily *J. hindsii*). Similarly, in the stem inoculation experiments, most Paradox populations developed larger galls than black walnut species. In addition, in two of the three analyses of gall diameter of half-sibling populations of black vs. Paradox populations, the Paradox half-sibs developed significantly larger galls than their black walnut half sibs (Table 2).

Previously, literature on the comparative susceptibility of *Juglans* species to crown gall was limited to field observations (McGranahan and Catlin, 1987; McGranahan and Leslie, 1990). Field observations of *J. regia* were particularly limited. Although it was speculated that *J. regia* is as susceptible to crown gall as Paradox hybrids (McGranahan and Catlin, 1987), our data with root and stem inoculations indicate that *J. regia* has a similar response to *A. tumefaciens* as the black walnut species. This observation is important because there is renewed interest in planting new orchards on a *J. regia* rootstock. Overall, our data suggest that *J. regia* and the black walnut species are susceptible, and that most Paradox hybrids are highly susceptible to crown gall.

In the root-inoculated field trial, we detected a difference in gall incidence between the Paradox and the parental types. However, the difference was not as large as expected, based on our field observations of sites with "natural" inoculum and anecdotal reports from growers. In our root inoculation trial, crown gall incidence was relatively high (≥60%) on all rootstocks and it is possible that we would have seen greater differences between genotypes had we used less inoculum and consequently had a lower incidence of gall (Raio et al., 1997).

Whitham (1989) suggested that plant hybrids, in comparison to their parental species, respond to diseases and insect pests in one of four general patterns: additive, dominant, elevated susceptibility, and elevated resistance. Elevated hybrid susceptibility has been reported in other plant–pest systems (Fritz et al., 1994; Messina et al., 1996; Whitham, 1989). For example, in willows, the incidence of infection by the rust fungus *Melampsora*, and the density of six out of 11 herbivorous species (insects and mites) were significantly greater on hybrids than on either parent (Fritz et al., 1994). Based on the present study, Paradox hybrids exhibit elevated hybrid susceptibility to crown gall.

The variation in host response to *A. tumefaciens* may be caused by multiple factors. Beneddra et al. (1996) suggested that differences in susceptibility were related to differences in host sensitivity to cytokinin. Such differences would be detected in the stem inoculation assay if sensitivity in roots and stems were similar. However, the stem inoculation assay circumvents several stages in the development of *A. tumefaciens* on the host surface, such as adhesion to the host, development of a rhizoplane population, and movement to an infection site (Nam et al., 1997, 1999). Thus, there may be additional mechanisms of resistance in the field that are not detected by a stem assay. *Agrobacterium* generally enters plant cells via wounds and it is often assumed that pruning wounds are prime infection sites. In our root-inoculated field trial, very few of the galls formed at pruning cuts made during transplanting. Galls predominately formed at natural wound sites, i.e., at sites of secondary root emergence and on the below-ground region of the crowns in apparent association with either lateral roots or epicotyl buds.

Of the 16 *J. hindsii* ×*J. regia* genotypes tested, 15 were from seed and one was clonal. If there is substantial genetic variability for crown gall resistance in walnuts within a given seedling population, then the variation of gall size in seedling populations would be expected to be greater than the variation in clonal populations. We ranked the standard deviation in gall size of the 15 *J. hindsii* ×*J. regia* seedling populations from smallest to largest, and found that the clonal population 'Vlach' ranked fifth. A one-sided z-test of the SD of the 'Vlach' trees in comparison to the seedling populations showed no significant difference between them ($P = 0.17$). An analysis of the SD of a second Paradox clone 'Px1', which was only tested in 1999, also was not significantly different from seedling populations (data not shown). Furthermore, examination of scatter plots of the average gall size on each seedling from a given population did not indicate segregation of gall response into distinct categories, i.e., there was no indication of a bimodal or trimodal distribution. Although our comparison is limited, the data suggest that a significant portion of the variability in gall size is due to random error from the assay. Thus, for Paradox walnut, we found no evidence to support the hypothesis that there is genetic variability in response to crown gall within seedling populations as McGranahan and Leslie (1990) speculated. Consequently, screening for resistance to crown gall in different populations appears to be a better strategy than screening for more resistant individuals within a population.

One of the goals of our study was to determine if some of the Paradox populations used commercially in California were particularly susceptible. The data indicate that of the 16 *J. hindsii* ×*J. regia* Paradox populations tested, all are highly susceptible, and given the large number of multiple comparisons, probably equally so. Similarly, there were no significant differences in the susceptibility of the five presumed *J. hindsii* ×(*J. hindsii, J. nigra*) Paradox populations, and no evidence that they differed from the *J. hindsii* Paradox (Table 2). We only had one population per genotype of the other six Paradox genotypes derived from other black walnut hybrids. In agreement with anecdotal observations (McGranahan and Leslie, 1990), our results suggest that some "complex-Paradox" genotypes differ in their response to *A. tumefaciens*. Here, we identified two walnut rootstock varieties, both complex hybrids that contain *J. major, J. hindsii, and J. nigra* in their female parentage, that produce

Paradox seedling populations that appear to be as resistant as black walnut. Seedlings from these two varieties (MX and AZ) are currently being tested for field resistance and orchard performance.

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