EVALUATION OF WILD *JUGLANS* SPECIES FOR CROWN GALL RESISTANCE

Daniel Kluepfel, Malli Aradhya, Malendia Maccree, Jeff Moersfelder, Ali McClean, and Wes Hackett

INTRODUCTION

Paradox is the most widely used rootstock in CA walnut production. These rootstocks are usually interspecific hybrids between *J. hindsii* and *J. regia* (Howard, 1945), which are typically highly susceptible to *Agrobacterium tumefaciens*. Extensive formation of tumors around the crown of the tree can often stunt the tree and result in reduced vigor and yields. If left untreated, tumors continue to grow and completely girdle the tree which contributes to premature death of the tree. Currently, crown gall disease in mature orchards is managed using surgery to remove the gall and adjacent infected tissues.

However, durable host resistance is the preferred form of resistance to all soil borne plant pathogens. This is especially important for crown gall disease given the fact that *Agrobacterium* spp are found in all the walnut growing regions of California examined.

The wild relatives of cultivated species are often a rich source of genes coding for such desirable traits as, resistance to insect pests and microbial pathogens, and abiotic stresses. Identification of a durable source of resistance to crown gall in the *Juglans* germplasm collection, that could be utilized directly or introgressed into commercially viable rootstocks, is likely to be an effective strategy for controlling crown gall disease in walnut.

The walnut germplasm collection at the National Clonal Germplasm Repository, USDA-ARS in Davis, CA represents a wide range of intra- and interspecific diversity for some of the black walnuts and butternuts that are adapted to California conditions. The potentially useful black walnut species include *J. hindsii, J. nigra, J. microcarpa, J. major*, and some of their hybrids with cultivated species. Other members of the germplasm repository collection that could be used to develop crown gall resistant rootstock include the Asian butternuts, *J. ailantifolia*, *J. mandshurica*, and *J. cathayensis*. In addition, wingnuts belonging to the genus *Pterocarya* have shown interesting disease resistance characteristics that need to be exploited.
Although wild species have contributed to walnut rootstock development programs, the range of genetic variation for crown gall resistance within and between these wild species has never been examined. It is anticipated that a systematic evaluation of the *Juglans* germplasm will unravel a hitherto unknown source of resistance/tolerance to crown gall disease and other plant pathogens.

As a step towards development of crown gall resistant rootstocks, here we report on the identification of *Juglans* species exhibiting resistance to infection by *A. tumefaciens* EC1. Once identified, these novel sources of *Agrobacterium* resistance can be exploited in the ongoing U.C. Davis Walnut root stock breeding program to help reduce the incidence of crown gall in both nursery and production fields.

OBJECTIVE

Identify and characterize a novel source of crown gall (CG) resistance in the *Juglans* germplasm collection maintained in the USDA/ARS National Clonal Germplasm Repository in Davis, CA.

Anticipated Outcome

We anticipate the identification of a new source of crown gall resistance which will be useful in the development of crown gall resistant rootstocks in the UC Davis walnut breeding program. The germplasm thus identified will be shared with other pathologists and horticulturists to evaluate resistance to other diseases and to test their ability to propagate vegetatively.

PROCEDURES

***Seedling germination and inoculation*.** Open pollinated seeds were collected from each of the black walnut and butternut accessions maintained at the Wolfskill Experimental Orchards in Winters, CA. Seeds were cold treated, germinated and grown under glasshouse conditions. Once the seedlings reach a trunk diameter of at least 0.5 cm the crown of the trees were inoculated with *A. tumefaciens* strain EC1. Depending on germination and growth rates, 6-9 trees from each accession were screened.

Seedlings were inoculated by generating a "T-cut" into the cambium layer and opening up the outer layers of bark to which 1/2ml of a 10^9 cells/ml suspension of EC-1 was introduced. After inoculation, the wound was wrapped with parafilm. Standard cultural practices were followed during the experiment and observations on tumor development were recorded at monthly intervals by noting first-appearance and then percent girdling (Gall rating: 1=no tumors, 2 = $\leq$ 25% of trunk circumference galled, 3 = 25-50% trunk circum. galled, 4 = 50-100% trunk circum galled).

CG susceptible Paradox seedlings inoculated as described above, served as positive controls. To assess the wounding response in the absence of the pathogen, Paradox seedlings and a variety of accessions from the germplasm collection were inoculated as described above with sterile water.

Evaluation of inoculated saplings. Tumor formation was monitored at two week intervals following inoculation. Relative rates and trends in tumor initiation and formation in different germplasm accessions were noted and recorded. Percent girdling of the stem was assessed and recorded for each seedling at 60 days post-inoculation. Photos of representative seedlings were taken at various intervals following inoculation. Seedlings were observed for three to six months after inoculation to monitor for late-forming or slow growing tumors.

To confirm the durability of observed resistance throughout a natural growing cycle, previously inoculated saplings were cold-treated and allowed to go dormant. After emerging from dormancy, saplings were monitored for tumor formation during a second growing season. A select group of saplings which continued to show resistance or "limited susceptibility" after two or more growing seasons, were propagated and retested as described above.

Seedlings that showed 25% or less of the stem girdled at 60 days post inoculation were designated as a source of potential resistant germplasm and retained for further study. Mother trees associated with the seedlings showing resistance in 2006 and 2007 were identified and targeted for seed collection in 2008.

Evaluation of relative virulence in Agrobacterium isolates obtained from California walnut orchards. Ten isolates representative of the genetic diversity of *A. tumefaciens* found in CA walnut growing regions were examined for their relative virulence on walnut seedlings. Two additional laboratory *A. tumefaciens* strains, EC-1 and C58, were used as positive controls on susceptible Paradox clones. Negative controls, used to differentiate early gall formation from the wound healing response, consisted of water inoculated Paradox clones. Five trees per *A. tumefaciens* strain were inoculated and scored as described above.

Evaluation of virulence caused by varied densities of Agrobacterium culture. Young clonal propagants of a susceptible Paradox background were inoculated and scored as described above using three concentrations of *Agrobacterium* cell suspension:10^3 cells/ml, 10^6 cells/ml, and 10^9 cells/ml. Clonal propagants of the following Paradox selections were tested in groups of either 3 or 5 plants per treatment: Vlach, VX211, AZ025, RX1 and AX3.

RESULTS AND DISCUSSION

O**bjective:** Identification of a novel source of crown gall (CG) resistance in the *Juglans* germplasm collection maintained in the USDA/ARS National Clonal Germplasm Repository in Davis, CA.

During the 2008 NCGR germplasm screening season, we examined a total of 1,100 seedlings for their resistance to *A. tumefaciens.* This consisted of seedlings from 242 mother trees representing the English walnut (*J. regia*), and its conspecific taxon, *J. sinensis*, five species of black walnut (*J. hindsii*, *J. nigra*, *J. microcarpa*, *J. californica* and *J. major*), three species of butternuts (*J. ailantifolia*, *J. mandshurica*, and *J. cathayensis*), a wingnut species (*Pterocarya stenoptera*) (Table 1).

Table 1. *Juglans* Germplasm Screened. In order to be retained in the screening program, a given accession must exhibit galling on 25%, or less, of the tree circumference 60 days post-inoculation.

2008 Seedlings Screened in 2008	Tested	Number Retained	% Retained
Juglans species	8	6	
Mother trees represented	99	27	27.27%
Individual trees	608	87	14.31%
Juglans species	Trees Tested	Trees Retained	% Retained
J. ailantifolia	150	18	12.00%
J. californica	7	3	42.86%
J. cathyensis	5	0	0.00%
J. hindsii	261	38	14.56%
J. major	96	9	9.38%
J. mandshurica	60	10	16.67%
J. nigra	29	7	24.14%
J. sinensis	1	0	0.00%

2007 Seedlings Screened in 2008	Tested	Retained	% Retained
Juglans species	7	6	
Mother trees represented	143	64	45%
Individual trees	436	92	21%
Juglans species	Trees Tested	Trees Retained	% Retained
J. ailantifolia	85	31	36%
J. californica	3	0	0%
J. cathyensis	6	1	17%
J. hindsii	18	2	11%
J. major	298	47	16%
J.microcarpa	8	6	75%
J. regia	17	5	29%
J. sinensis	1	0	0%

In the 2008 screening, additional germplasm from wingnut and butternut species were added to the study. A significant number of *J. ailantifolia, J. californica J. hindsii, J. major J. mandshurica,* and *J. nigra*. accessions exhibited $\leq$ 25% crown gall formation at the inoculation site. In addition, our 2008 screening of germplasm collected in 2007 also revealed potential *A. tumefaciens* resistance in the following *Juglans* species; *J. ailantifolia, J. hindsii, J. major.* New *Juglans* species exhibiting crown gall resistance include; *J. regia, J. microcarpa, and J. cathyensis*.

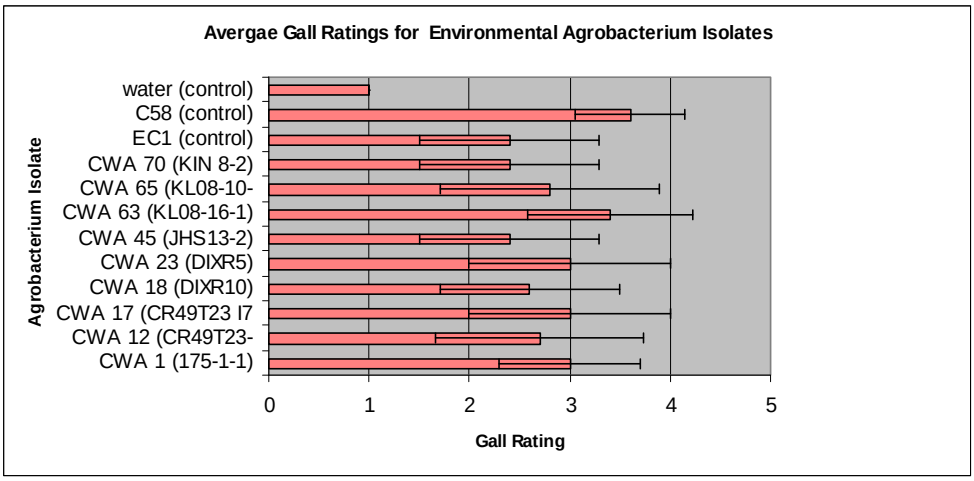
Our results illustrate that resistance is most common and durable in *J. microcarpa, J. major, J. mandshurica, and Pteracaya sp.*(Table 2). The resultant phenotypes ranged from total resistance to delayed gall development after dormancy to immediate gall formation three week post inoculation. The results from our experiments examining rooted cuttings, taken from the seedlings exhibiting CG resistance, suggest that crown gall resistance is a genetically stable trait. These crown gall resistant genotypes will now be examined for potential use in the UC Davis walnut breeding program.

Table 2: Summary of Germplasm Material Screened 2005-2008.

							Retested Rooted Cuttings		
Genus	Species	Accessions	Seedling Years Tested	# Trees Tested	# Trees Retained @ 60 days	% Trees Retained @ 60 Days	Year	# Trees Retested	# Trees Retained
Juglans	Californica	12	3	55	5	9%	0	0	0
Juglans	Hindsii	105	4	645	53	8%	2008	6	0
Juglans	Major	80	4	505	63	12%	2008	8	4
Juglans	Microcarpa	22	3	85	13	15%	2008	7	6
Juglans	Nigra	8	3	56	8	14%	0	0	0
Juglans	Regia	15	2	34	15	44%	2008	2	0
Juglans	Ailantifolia	64	4	291	89	31%	2008	5	0
Juglans	Cathayensis	2	3	12	1	8%	0	0	0
Juglans	Mandshurica	11	3	74	18	24%	2008	1	1
Juglans	Sinensis	2	4	7	0	0%	0	0	0
Juglans	Hybrid	2	1	10	4	40%	0	0	0
Pteracarya	Pteracarya	5	1	29	13	45%	2008	15	9
Totals (all trees)	12	328	4	1803	282	16%	1	44	20

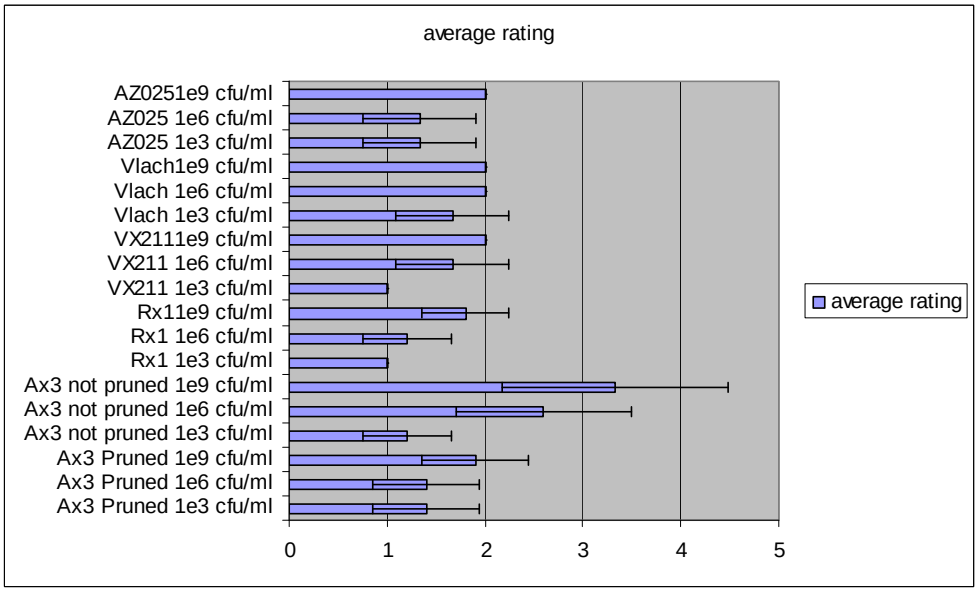
Ten genetically diverse California *A. tumefaciens* isolates each exhibited a similar level of virulence when tested on a common host genotype (Fig.1). Statistically, each of the isolates were no more, or less, aggressive than our bench mark strain EC1.

Fig. 1. Virulence of genetically diverse *A. tumefaciens* environmental isolates. All the bacterial concentrations and inoculation procedures were carried out as described above. Gall rating: no tumors=1, ≤25% of trunk circumference galled=2, 25-50% trunk circum. galled=3, 50-100% trunk circum galled=4.



The initial inoculum density of *A. tumefaciens* had a significant impact on tumor formation on susceptible Paradox selections. At populations of 10^3 CFU/ml we observed a significant decrease in tumor formation as compared to an initial inoculum of 10^9 CFU/ml (Fig. 2). Interestingly, the influence of inoculum density was reduced if the trees had been aggressively pruned prior to our standard tree-crown (lower stem) inoculations.

Fig. 2. Effect of inoculum density on tumor formation. Five Paradox genotypes (AZ025, Vlach, VX211, RX11, AX3) were inoculated as described above using 10^9, 10^6 or 10^3 *A. tumefaciens* cells/ml. Tumor formation evaluation was performed as described above using the same rating scheme as described in Fig 1.



CONCLUSIONS

Crown gall resistant walnut genotypes have been identified using a simple, high through-put and reproducible quantitative method for infecting trees with *Agrobacterium tumefaciens*. Our assay also has uncovered variability in the rate of tumor formation among different host genotypes and found that continued monitoring of putatively resistant trees through a dormant cycle was required to confirm resistance. Finally, our preliminary data suggests that several *Juglans* species and a single *Pterocarya* species exhibit resistance to the formation of crown gall after inoculation with *A. tumefaciens* strain EC1. In addition, approximately 50% of the rooted cuttings taken from these CG resistant selections continue to exhibit high levels of CG resistance under extremely stringent screening conditions.