EVALUATION OF WILD *JUGLANS* SPECIES FOR CROWN GALL RESISTANCE

Ed Stover, Malendia Maccree, Malli Aradhya, Ali E. McClean, and Daniel A. Kluepfel

ABSTRACT

Crown Gall disease of walnut is caused by the ubiquitous soil-borne bacterium, *Agrobacterium tumefaciens,* which is able to transfer a specific piece of its own DNA into the genome of the plant host cell. The result of this genetic transformation is the autonomous undifferentiated massive growth of infected plant cells which generates the most obvious symptom of this disease, plant galls or tumors.

Paradox rootstocks are widely used 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 the soil 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, 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*, in addition to some of their hybrids with cultivated species. The Asian butternuts, *J. ailantifolia, J. mandshurica,* and *J. cathayensis*, which grow well in the germplasm collection, also could be used directly or in the development of Crown Gall resistant interspecific hybrids. 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 for crown gall resistance 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/tolerance 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 also will be shared with other pathologists and horticulturists for further evaluation for resistance to other diseases, especially *Phytophthora* and to test for their ability to propagate vegetatively.

PROCEDURES

***Seedling germination and inoculation*.** In each year, open pollinated seeds were collected from *Juglans* 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 0.5cm the crown of the trees were inoculated with *A. tumefaciens* strain EC1. Depending on germination and growth rates, 4-6 trees from each accession were screened. It should be noted that each seedling from each accession is a unique genotype and may even have a different pollen parent.

Seedlings were inoculated by generating a "T-cut" 1-2mm deep at the crown, in to which either 500ul or 300ul of a 10^7 cells/ml suspension of EC-1 was introduced by micropipet. After inoculation, the wound was closed and 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 recording tumor size. To confirm virulence of EC1, susceptible Paradox seedlings were inoculated with EC1 as described above. To assess typical wounding response in absence of the pathogen, Paradox seedlings and a variety of accessions from the germplasm collection were inoculated as described above with the exception that EC1 was replaced with sterile water.

Evaluation of inoculated saplings. Tumor formation was monitored at two week (indicates monthly in paragraph above) intervals following inoculation. Relative rates and trends in tumor initiation and formation in different germplasm assessions were noted and recorded. Tumor size was measured and recorded for each seedling at 60 days post-inoculation. Photos of each seedling were taken at various intervals following inoculation. Seedlings were monitored 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. Saplings which continue to show resistance or "limited susceptibility", will be reinoculated in subsequent growing seasons after the original inoculation. These reinoculated plants will be handled as described above for the original inoculation series.

RESULTS AND DISCUSSION

Objective: 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.

Material tested: As this project has progressed, we have gone from assessing a few plants of most species (the preliminary non-funded project), to collecting seed from every bearing accession in 2006, and in 2007 collected seed from every seedling tree in every accession. During the 2005 growing season, a total of 313 seedlings from 116 mother trees representing four species of black walnut (*J. hindsii, J. nigra, J. microcarpa*, and *J. major*); three of butternuts (*J. ailantifolia, J. mandshurica*, and *J. cathayensis*); and *J. sinensis* were tested for resistance to Crown Gall Disease (Table 1).

For the 2006 screening, additional germplasm from wingnut and butternut species were added to the study. A significant number of *J. microcarpa* accessions found promising from the 2005 study failed to germinate in 2006 and could not be investigated. During the 2006 season, a total of 468 seedlings from 85 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 of butternuts (*J. ailantifolia, J. mandshurica*, and *J. cathayensis*), a wingnut species (*Pterocarya stenoptera*), and a small number of intergenic hybrids were evaluated (Table 2).

For the anticipated screening in 2007, 496 plants produced from seed collected (in 2006) from 149 mother trees were germinated and grown. Adequate size was achieved too late in the season, and based on earlier results, the decision was made to overwinter the saplings before inoculation in the spring of 2008. In 2007 we collected seed from 600 mother trees, representing all bearing *Juglans* diversity in the NCGR except *J. regia* (only a few *J. regia* accessions were collected for reference). These seed are being stratified and will be germinated in spring of 2008 with inoculation likely in spring of 2009.

Crown gall development: Results from the 2005 testing indicated that resistance was most common and durable in *J. microcarpa* (Table 1). The resultant phenotypes ranged from total resistance to delayed gall development after dormancy to immediate gall formation three week post inoculation. There was some modest change between scoring of resistance at one and two years after inoculation, as a few saplings displayed delayed gall development.

Results from the 2006 screenings indicate that *J. ailantifolia* and *J. mandishurica* and *Pterocarya* accessions showed the greatest number of progenies with some level of resistance at 60 days post-inoculation (Table 2). Unfortunately, seed of some of these promising *J. microcarpa* accessions failed to germinate and could not be evaluated during the '06 cycle to confirm 2005 results. Nearly 50% of the progeny from *J. ailantifolia, J. madichurica, J regia, J. hindsii* and *Pterocarya* mother trees showed no tumor formation at 60 days post-inoculation. In the summer 2006 screen, *Pterocarya* species exhibited the highest degree of resistance to *A. tumefaciens* infection and subsequent tumor formation. Again, there was some change between scoring of resistance at an addition year after inoculation, as a few saplings displayed delayed gall development, some apparently resistant trees died, and especially in *J. ailantifolia* and *J. hindsii*, some plants grew vigorously and sloughed off galls. Further trials will determine whether these different forms of resistance and tolerance are reproducibly observed in the test genotypes.

Conclusion: A simple and reproducible method for infecting trees with *Agrobacterium tumefaciens* to produce crown gall disease has been established and is being vigorously used in assessing a broad range of plant material. In our investigations we have found tremendous variability in the rate of infection and growth of tumors on different saplings. Initial measurements and assessment of actively growing plants and tumors at 2-3 months post-inoculation serve as a useful indicator. However, there seems to be some benefit to re-assessing resistance after one growing season. Based on data from 2005 inoculations, there does not appear to be as much new information gained by monitoring plant for two growing seasons (2 years). Finally, our data suggest that several *Juglans* species and a single *Pterocarya* species exhibit resistance to the formation of crown gall after inoculation with *A. tumefaciens* strain EC1, which will be tested further for generalization to California *A. tumefaciens* strains isolated in walnut orchards.

Table 1. Summary table of results from 2005 screening experiments. Updated with observations at 12 and 24 months post-inoculation.

		1 year post-inoculation		2 years post-inoculation	
Species	Mother trees used as source	Mother trees showing CG resistance in saplings	Saplings showing CG resistance in saplings	Mother trees showing CG resistance in saplings	Saplings showing CG resistance in saplings
Black walnuts					
J. hindsii	76	3	3	2	2
J. nigra	5	1	1	0	0
J. microcarpa	20	4	10	4	6
J. major	9	1	1	1	1
Butternuts					
J. ailantifolia	1	0	0	0	0
J. mandshurica	3	0	0	0	0
J. cathayensis	1	0	0	0	0
Others					
J. sinensis	1	0	0	0	0
Total		9	15	7	9

Table 2. Summary table of results from 2006 screening experiments. Observations at 2 and 16 months post-inoculation.

		2 months post-inoculation (Preliminary results)		16 months post-inoculation	
Species	Mother trees used as source	Mother trees producing CG resistant progeny	Saplings showing CG resistance	Mother trees producing CG resistant progeny	Saplings showing CG resistance
Black walnuts					
J. hindsii	30	4	6	8	11
J. nigra	3	1	1	1	1
J. microcarpa	5	0	0	0	0
J. major	13	3	4	2	2
J. californica	9	3	3	1	1
Butternuts					
J. ailantifolia	10	8	18	9	36
J. mandshurica	3	3	10	2	5
Others					
J. regia	4	3	5	3	6
J. sinensis	1	0	0	0	0
Pterocarya	5	4	6	4	8
Juglans hybrid	2	1	1	2	4
Total	84	30	54	32	76