# EVALUATION OF WILD *JUGLANS* SPECIES FOR CROWN GALL RESISTANCE

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

## INTRODUCTION

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 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*, 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.*** 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 0.5cm 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.

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.

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 1).

**Table 1.**

**Summary of germplasm material screened in 2005 and 2006**

| Species | No. of mother trees used as source (2005) | No. of mother trees used as source (2006) | Total Number of saplings screened (2005-2006) |
|---|---|---|---|
| **Black walnuts** | | | |
| *J. hindsii* | 76 | 30 | 440 |
| *J. nigra* | 5 | 3 | 32 |
| *J. microcarpa* | 20 | 5 | 57 |
| *J. major* | 9 | 13 | 98 |
| *J. californica* | 0 | 9 | 47 |
| **Butternuts** | | | |
| *J. ailantifolia* | 1 | 10 | 60 |
| *J. mandshurica* | 3 | 3 | 31 |
| *J. cathayensis* | 1 | 0 | 1 |
| **Others** | | | |
| *J. regia* | 0 | 4 | 20 |
| *J. sinensis* | 1 | 1 | 5 |
| *Pterocarya* | 0 | 5 | 29 |
| *Juglans hybrid* | 0 | 2 | 10 |
| **Total** | 116 | 85 | 830 |

**Table 2.**

**Summary table of results from 2005 screening experiments**

**(Observations at 3 and 12 months post inoculation)**

| Species | No. of mother trees producing CG resistant progeny, 3months post inoc | No. saplings showing CG resistance, 3months post inoc. | No. of mother trees showing CG resistance in saplings, 12 months post inoc. | No. of saplings showing CG resistance in saplings, 12 months post inoc. |
|---|---|---|---|---|
| **Black walnuts** | | | | |
| *J. hindsii* | 10 | 10 | 3 | 3 |
| *J. nigra* | 2 | 4 | 1 | 1 |
| *J. microcarpa* | 4 | 19 | 4 | 10 |
| *J. major* | 2 | 1 | 1 | 1 |
| **Butternuts** | | | | |
| *J. ailantifolia* | | 0 | | |
| *J. mandshurica* | | 0 | | |
| *J. cathayensis* | | 0 | | |
| **Others** | | | | |
| *J. sinensis* | | 1 | | |
| **Total** | 18 | 35 | 9 | 15 |

**Table 3. Summary of 2006 preliminary results**

| Species | No. of mother trees used as source | No. of mother trees producing CG resistant progeny | No. saplings showing CG resistance; 60 days post-inoculation |
|---|---|---|---|
| **Black walnuts** | | | |
| *J. hindsii* | 30 | 4 | 6 |
| *J. nigra* | 3 | 1 | 1 |
| *J. microcarpa* | 5 | 0 | 0 |
| *J. major* | 13 | 3 | 4 |
| *J. californica* | 9 | 3 | 3 |
| **Butternuts** | | | |
| *J. ailantifolia* | 10 | 8 | 18 |
| *J. mandshurica* | 3 | 3 | 10 |
| **Others** | | | |
| *J. regia* | 4 | 3 | 5 |
| *J. sinensis* | 1 | 0 | 0 |
| *Pterocarya* | 5 | 4 | 6 |
| *Juglans hybrid* | 2 | 1 | 1 |
| **Total** | 84 | 30 | 54 |

Results from the 2005 testing indicate that resistance is most common and durable in *J. microcarpa* (Table 2). The resultant phenotypes ranged from total resistance to delayed gall development after dormancy to immediate gall formation three week post inoculation. Unfortunatley, some of these promising accessions failed to germinate and could not be evaluated during the '06 cycle to confirm 2005 results. Further testing is needed in 2007 to confirm which mother trees are producing resistant progeny.

Preliminary 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 3). 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.

**Conclusion:** A simple and reproducible method for infecting trees with *Agrobacterium tumefaciens* to produce crown gall disease has been established. In our investigations we have found some variability in the rate of infection and growth of tumors on different saplings. Continued monitoring of resistant trees through a dormant cycle and into the next growing season is needed 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.