Workgroup/Department Walnut/Plant Pathology

University of California
Division of Agricultural Sciences
Project Plan/Research Grant Proposal

Project Year (May 1, 2008-April 30, 2009) **Anticipated Duration of Project** Yr 3 of 3

Project Leader D. A. Kluepfel **Location** USDA-ARS, Dept. of Plant Pathology

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Project Title Evaluation of wild *Juglans* species and Paradox root stock selections for crown gall resistance

Keywords Walnut, Crown Gall, *Agrobacterium tumefaciens*, Juglans germplasm, Paradox

Commodity Walnut **Relevant AES/CE Project No.**

Problem and its Significance:
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. Extensive formation of tumors around the crown of the tree can often stunt the tree and result in reduce vigor and yields. If left untreated, the tumors continue to grow and completely girdle the tree which contributes to premature death of the tree.

Currently, there are no effective control methods for crown gall disease. However, to reduce CG incidence in new orchards the following management practices are useful; use of clean planting material, proper pre and post cold storage handling of seedlings prior to planting, and carefully avoiding wounding of trees during cultural operations. Currently, Crown Gall Disease in mature orchards is managed using surgery to remove the gall and adjacent infected tissues. The resulting wound is flame "sterilized" followed by application of a bacteriocidal material to avoid further spread of the disease (UC Pest Management Guidelines).

Replanting in previously infested sites requires thorough decontamination of fields before planting with clean stock. Similar to soil-borne fungal and viral plant pathogens, soil-borne bacterial pathogens are difficult to control after infestation. Generally, 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. As a step towards development of CG resistant rootstocks, here we propose to identify and characterize walnut genotypes with moderate to high levels of field resistance/tolerance to *Agrobacterium tumefaciens*

Paradox rootstocks are widely used in CA walnut production. These rootstocks are usually interspecific hybrid between *J. hindsii* and *J. regia* (Howard, 1945), which are typically highly susceptible to *Agrobacterium tumefaciens*. Identification of a durable source of resistance to crown gall in the *Juglans* germplasm collection, that could be utilized directly or introgressed into a commercially viable rootstocks, would be of benefit to the U. C., Davis walnut rootstock breeding program and the walnut industry in California. The wild relatives of cultivated species are often a rich source of genes coding for such desirable traits as, resistance to insect and microbial pests and pathogens, and abiotic stresses. In keeping with these observations we hypothesize that wild relatives of *Juglans*, that are adapted to California walnut growing conditions, may be a potential source of Crown Gall resistance. 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.

In addition to our examination of the wild *Juglans* germplasm, we also propose to examine the currently available tissue culture derived Paradox plantlets which appear to exhibit a wide variation of *A. tumefaciens* susceptibility. Diversity of black walnut parentage in Paradox (Potter et al., 2002) may contribute to the variability in crown gall susceptibility observed in these rootstocks, since *J. californica, J. major* and *J. nigra* rather than *J. hindsii* have contributed to some of these hybrids . Given our ability to propagate numerous genetically identical individuals of any single genotype (e.g. Paradox) via in vitro tissue culture methods, we will be able to discriminate genotypes with varying degrees of susceptibility/tolerance/resistance to crown gall formation (Hackett et al. 2003).

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.

OBJECTIVES

1. 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.

2. Improve our plantlet based screening procedure for the detection of *Agrobacterium tumefaciens* resistant *Juglans spp* genotypes.

3. Conduct survey and greenhouse screen to examine the relative crown gall susceptibility as a function of rootstock genotype and plant propagation procedure.

PLAN AND PROCEDURES

Juglans Germplasm Screening: (Obj. 1 and 2)

Results from the prior year testing indicate that resistance is most common and durable in *J. microcarpa* (SeeTable 2 in 2005 annual report). The resultant phenotypes ranged from total resistance to delayed gall development after dormancy to immediate gall formation three week post inoculation. Further testing is needed in 2008 to confirm which mother trees are producing resistant progeny.

Nuts from the "resistant" accessions identified last year (2007), and additional accessions not previously screened, were collected, stratified, germinated and propagated in late summer. However, growth was limited and sufficient tree size and vigor required for reproducible crown gall screening was only just obtained prior to entering dormancy. Since *A. tumefaciens* inoculation is not advised at this time we allowed all 600 seedlings to enter dormancy. By mid Feburary (2008) these trees will be returned to the green house where they will be pushed and made ready for inoculation and crown gall screening by mid March

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. 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. Representatives from each of these accessions are represented in the group of 600 seedlings that will be screened in March. In addition, this past fall, nuts were collected from mother trees representing each of the accessions that exhibited partial to full resistance. These nuts will be germinated this spring and put into our screening procedure mid to late summer. By December 2008 we will have completed the screening of approximately 1200 individuals from accessions showing various degrees of crown gall resistance.

Inoculation, tree growth and evaluation: Open pollinated seeds collected from each of the black walnut and butternut accessions maintained at the Wolfskill Experimental Orchards in Winters, CA will be cold treated, germinated and grown under glasshouse conditions. Once the seedlings reach a trunk diameter of at least 0.5cm the crown of the tree will be inoculated with *A. tumefaciens* strain EC1. Depending on germination and growth rates, at least four trees from each accession will be screened in this test.

The inoculation procedure will consist of generating a "T-cut" 1-2mm deep at the crown in to which 1ml of a 10^7 cells/ml suspension of EC-1 will be introduced. After inoculation, the wound will be closed and wrapped with parafilm. (The *Agrobacterium* diversity study currently being

conducted in our lab will allow us to select a range of diverse virulent isolates for use in germplasm screening in the final two years of evaluation)

Standard cultural practices will be followed during the experiment and observations on tumor development will be recorded at bi-weekly intervals by noting first-appearance and then tumor size. Positive controls will consist of Paradox seedlings inoculated with EC1 as described above. Negative controls will consist of Paradox and a variety of accessions from the germplasm collection inoculated as described above with the exception that EC1 will be replaced with sterile water

To confirm any observed resistance, all saplings that show resistance, or "limited susceptibility", will be reinoculated 2 months after the original inoculation. These reinoculated plants will be handled as described above for the original inoculation series. In addition, trees which displayed resistance last year will be reinoculated. The following data will be generated; timed (i.e. first) appearance of tumors, tumor growth rates, and confirmation of CG resistance.

Production and testing of interspecific hybrids: In addition to naturally occurring interspecific hybrids among the half-sib progenies included in the study, a number of interspecific hybrids of known combinations involving the cultivated species, *J. regia* and other combinations that are known to show adaptation to California walnut growing conditions will be produced and included in the screening for CG resistance as described above. Controlled crosses were made during the 2006 flowering season and will be screened in 2007, with additional crosses to be made in 2007. We are particularly interested in testing hybrids involving *J. microcarpa,* the species which, in preliminary studies, has shown a relatively high level of resistance to CG as well as *Phytophthora* (Gale McGranahan personal communication). Selections that consistently show resistance to crown gall for three years will be clonally propagated and tested for resistance to *Phytophthora* root rot.
Note: The procedure described above is similar to that presented last year. We have found the technique to be an aggressive screen that will identify truly resistant selections. Consequently, we have decided not to change the screening technique to any significant degree.

Objective 3: A third objective has been added this year. Casual observations have indicated that the relative susceptibility to crown gall disease may vary as function of both plant genotype and propagation procedures (Janine Hasey personnal communication). To test this hypothesis an extensive state wide survey was conducted in the summer of 2007 by Janine Hasey in collaboration with D. Kluepfel. The results of this survey of replicated field plots across the state suggested that there does indeed exist as strong relation ship between plant propagation techniques and crown gall sensitivity. Here we propose to examine this relationship in more detail under greenhouse conditions. The following plant material will be screened using the procedures described above.

Plant Material	Source Person	Treatment #
Own-rooted Chandler (all California propagated)	Tom Burchell/ Walter Viss	1
Own-rooted Serr	Burchell/Lopez	2
Grafted roots, VX211	Wes	3
Rooted cuttings VX211	Wes	4
Seedling Chandler	Gale	5
Seedling Paradox (Trinta)	Dan/Gale	6
c- Pdx Vlach	Wes	7
c- Pdx VX211	Wes	8
c- Pdx RX1	Wes	9
c- Pdx AZ025	Wes	10
c-Pdx Wip3	Wes	11
c- Pdx PX1 (+ck)	Wes	12
c- Pdx Transgenic RR (-ck)	Chuck	13

These plants will be propagated as described above and inoculated with *A. tumefaciens* EC-1 at three different positions in the plant; 2-3 inches above the crown, at the crown, and on the first visible major root. After inoculation the plant will be handled as described above. At various times after inoculation the plants will be rated for the appearance of tumors. At the termination of the experiment the plants will harvested and the tumor tissue scanned using a flat bed scanner to determine tumor area and volume. These data will be statistically evaluated to quantify the relative level of *A. tumefaciens* susceptibility

Forthcoming years:

As stated last year, plants which appeared to be resistant to strain EC-1, will continue to be clonally propagated and tested against a diverse range of *A. tumefaciens* strains identified as being associated with walnut crown gall in California. It is very common for there to be substantial *A.tumefaciens* strain x genotype variability in other plant hosts.

ANTICIPATED OUTCOME

We anticipate the identification 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 vegetatively propagate.
In addition, we expect to identify Paradox germplasm selections with altered levels of Crown Gall susceptibility that will aid in the development of CG resistant rootsocks.

BUDGET JUSTIFICATION

In this proposal we are requesting a total of $22,000 to cover the cost of the following items: Cold storage of seeds, seed germination materials, pots, potting mixes, fertilizer, greenhouse care personnel (ie. watering and pest control), greenhouse heating and cooling, and technicians for inoculations and tumor evaluations.

LITERATURE CITED

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Potter, D., Gao, F.Y., Baggett, S., McKenna, J.R., and McGranahan, G.H. 2002. Defining the sources of Paradox: DNA sequence markers for North American walnut (Juglans L.) species and hybrids. Scientia-Horticulturae. 2002; 94(1/2): 157-170

PROJECT MANAGEMENT AND EVALUATION

Dr. Daniel Kluepfel will be responsible for growth of *Agrobacterium* inoculum, inoculation procedures, greenhouse growth and tree maintenance and assessment of tumor formation and development., and evaluation of genotype resistance to *Agrobacterium tumefaciens*. Dr.s Ed Stover, Malli Aradhya, andWes Hackett, will be responsible for the propagation, maintenance and selection of appropriate plant genotypes to be screened for Crown Gall resistance. In addition, Dr.'s Stover, Aradhya, Hackett, and Kluepfel will be involved in the evaluation and selection of Crown Gall resistant/tolerant phenotypes. Janine Hasey will be responsible for evaluation of the data generated while conducting the work described in objective 3.

BUDGET REQUEST

Budget Period: May 1, 2008 to April 30, 2009
Funding Source: Walnut Marketing Board and IAB

Item	**Amount ($)**
Greenhouse care personnel (ie. Seed processing, watering and pest control), technicians for inoculations and tumor measurements/evaluations.	$12,000
Supplies and Expenses: Cold storage/trt of seeds, seed germination materials, pots, potting mixes, fertilizer greenhouse heating and cooling, microbiological supplies,	$10,000
Total:	$22,000

Note: The total cost of this project is estimated to be $22,000. We are requesting $13,000 from the Walnut Marketing Board and $9,000 from the IAB to cover the total costs.

Signature of Principal Investigator: _____

Signature of Authorized Institution Rep,
USDA-ARS, Davis; Location Coordinator: _____

Signature of Authorized Institution Rep.,
University of California, Davis; Dept. Chair:_____