DEVELOPMENT OF DISEASE-RESISTANT WALNUT ROOTSTOCKS: INTEGRATION OF CONVENTIONAL AND GENOMIC APPROACHES (SCRI-match Year 2)

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ABSTRACT

Most commercially cultivated walnut trees, grown for nut production in California, are grafted on rootstocks with Paradox seedlings rootstock being the industry standard. However, Paradox is susceptible to four key soil borne pathogens and two emerging diseases: thousand cankers disease (TCD) and lethal Paradox canker (LPC). Combined, these diseases (new and old) cause an estimated 18% annual loss worth $191 million to the CA walnut industry. The availability of new pathogen-resistant rootstocks is critical for the viability of commercial walnut production and is ranked by the CA walnut industry as its top research priority. We are using an integrated approach that encompasses, disease resistance-screening, plant propagation, genomics, and conventional and novel breeding strategies to develop, characterize, and deploy clonal walnut rootstocks with improved resistance to the four major soilborne diseases caused by *Agrobacterium tumefaciens, Phytophthora* spp., *Pratylenchus vulnus,* and *Armillaria mellea*. To accomplish this task we are exploiting the Juglandaceae germplasm collection held at the USDA-ARS National Clonal Germplasm Repository in Davis, CA and the Missouri Horticulture and Agroforestry Research Center in New Franklin, MO. Together, these collections comprise the largest assemblage of wild *Juglans* spp. in North America. In this process we will identify genetic loci which confer resistance to these diseases and develop SNP markers for their selection in segregating populations and integration into commercial rootstocks. Emerging walnut rootstock diseases, such as TCD and LPC, also are being monitored by using an integrated outreach approach. Our project has made significant progress in each of its four objectives which are designed to provide the walnut industry with new disease-resistant walnut rootstocks.

OBJECTIVES

1. Identify and characterize Juglans germplasm resistant to key soil-borne pathogens.
2. Genetic, physical, and functional mapping of disease resistance genes and deployment of molecular markers for rapid screening of resistance.
4. Conduct extension efforts that:
   a. Deliver disease-resistant rootstocks to stakeholders.
   b. Assess emerging threats to walnut rootstocks.

(Objective numbering corresponds with SCRI grant objectives.)

SIGNIFICANT FINDINGS

Collected and cataloged >24,000 walnuts seeds (2013-2014) and propagated > 10,000 seedlings representing 7 *Juglans/Pterocarya* species and 56 accessions over the last two seasons.

Determined that some *J. microcarpa x J. regia and J. cathayensis x J. regia* clones provide resistance to *Phytophthora* which led to field validation and grower and nursery utilization.

Identified four *J. microcarpa*, two interspecific hybrids (*J. microcarpa x J. regia*) and one *J. cathayensis* mother tree which exhibit a high level of resistance to crown gall (CG), Phytophthora and/or lesion nematode. A selection of these putative disease resistance genotypes was delivered to a commercial nursery for micropropagation in preparation for commercial field trials to confirm disease resistance traits. Identification of putative disease resistant clones is the most significant early outcome from our project.

Micropropagated four of the putative disease-resistant clones on a commercial scale. These are now being cultivated in the field and will be June-budded this upcoming season.

Statistical analysis of the CG and Phytophthora screening results revealed two things; 1) the disease resistance phenotypes observed in our system are heritable; and, 2) some mother trees with in a given species are better than others for use in breeding for disease resistance.

Due to our success in identifying putative disease resistant clones, a cooperating commercial nursery has committed to in vitro propagation of 10 new "elite" clonal disease resistant rootstocks per year over the next three years of this project. Our team's ability to continue to develop novel clonal rootstocks is truly driving the walnut industry's approach to rootstock propagation and use across all CA walnut growing regions.

Screened thousands of progeny from over 330 *Juglans* MTs representing 12 species and >330 genotypes in the USDA-ARS NCGR. Two species, i.e. *J. microcarpa* and *J. cathayensis* repeatedly produce OP progeny resistant to *A. tumefaciens*, *Phytophthora* spp. or lesion nematode. These two species are the nucleus of our project and will be used in crossing experiments to identify and map the genetic loci mediating disease resistance.

Assembled large populations of black walnut and butternut seedlings in TN and MO which are now available for TCD testing this upcoming season.

Established new walnut rootstock website (http://www.rootstocks.net/) where we will be posting research results and extension activities supported by the USDA-NIFA-SCRI and the California Walnut Board.

Outreach/training: We gave 20 presentations at venues across the country and internationally. This included presentations to UC Farm Advisors, university faculty at scientific conferences, master gardeners, nursery operators, walnut growers, USDA APHIS regulators; undergraduate and graduate students, plant health professionals, and County Agriculture Commissioners. We also presented results at the International Walnut Symposium and in 3 posters at national scientific meetings, e.g. American Phytopathological Society, U.C. Agriculture and Natural Resources Statewide Conference. For details of our progress and outreach activities see http://www.rootstocks.net/.

PROCEDURES/APPROACH

Since the general approach we are taking in this rootstock screening-development project has not changed and is similar to what we described in last year's 2013 walnut research report, we have not repeated those comments here. Please see 2013 Walnut Research report pp. 79-80 for details of our approach.

RESULTS AND DISCUSSION

Obj. 1. Identify and characterize Juglans germplasm resistant to key soil-borne pathogens.

We continued to focus on assembly and propagation of half-sib open-pollinated (OP) progeny and full-sib progeny from selected mother trees (MTs) of *Juglans* spp. for disease resistance screening. We collected 22,000 seeds from *Juglans ailantifolia, J. cathayensis, J. hindsii, J. major, J. mandshurica, J. microcarpa* and *Pterocarya stenoptera* MTs. Approximately, ~50% of

these seeds germinated and were grown to size for disease resistance screening (see www.walnut.rootstocks.net). In addition we generated interspecific hybrids on 15 *Juglans* MTs. The MTs were chosen based on their ability to produce disease resistant OP progeny as revealed previously. The MT species used for interspecific hybridization included; 10 *J. microcarpa*, 2 *J. major*, and 3 *J. cathayensis* accessions. These crosses were generated using *J. regia* 'Serr' as the pollen parent. Embryos were excised from immature hybrid seed and micro-propagated. We produced approximately ~14,000 plants that entered our disease resistance screening.

Disease resistance screening: In 2013 we screened plants generated from OP seeds collected from MTs previously shown to produce disease resistant OP seedlings. Across the six *Juglans* species examined, we found approximately 11% of the OP progeny exhibited crown gall (*A. tumefaciens*) (CG) resistance at 4 months post inoculation. All CG-resistant seedlings were micropropagated and retested to confirm the resistance. Interestingly, *J. microcarpa* exhibited the most robust and durable CG resistance. Consequently, in 2014, we examined ~350 OP progeny from 22 *J. microcarpa* mother trees for CG resistance. Four months after inoculation 46 continued to exhibit CG resistance. We clonally propagated 11 of these CG-resistant *J. microcarpa* seedlings, from which 150 plantlets were propagated for Phytophthora (PHY) resistance screening. From this group, three elite clones have been micro-propagated at a commercial nursery for addition field testing.

We demonstrated the heritability of CG resistance and identified differences among MTs to produce CG-resistant progeny. We screened ~45 OP progeny/ mother tree representing 9 genotypes and three *Juglans* species for CG resistance. Analysis of variance revealed significant variation in mean CG ratings across the OP progenies of 9 MTs with *J. microcarpa* 31.10 showing a significantly greater CG resistance rating than other MTs. ANOVA revealed a statistically significant level of additive genetic variance among families with a narrow sense heritability of 12.9% (i.e., a strong indication of trait heritability in tree systems). These data will facilitate selection of MTs to generate mapping populations MPs, but also in developing strategies for mapping resistance.

We also evaluated resistance to *P. cinnamomi* in OP seedlings from 32 different MTs representing *J. ailantifolia, J. hindsii, J. major, and J. microcarpa*. Mean severity of root and crown rot differed significantly among seed families (i.e. progeny of individual MTs) ($P<0.0001$); for example severity root rot ranged from 28 to 93% among the seed families, and there were individual seedlings within progenies that developed little disease. Heritability analysis of *P. cinnamomi* resistance revealed a moderate level of additive genetic variance and narrow sense heritability of 16% (root rot).

Sixty-eight of the seedlings putatively resistant to *P. cinnamomi* were retained for micropropagation and resistance confirmation. We also evaluated resistance to *P. cinnamomi* and *P. citricola* in 54 clonal walnut rootstock selections from *J. hindsii* x *J. regia, J. microcarpa, J. californica* x *J. regia, J. microcarpa* x *J. regia, J. cathayensis* x *J. regia, and J. regia* x *J. cathayensis* MT or hybrids selected for putative resistance to the *Phytophthora* species in previous evaluations. Others were chosen for their resistance to CG or lesion nematode (*Pratylenchus vulnus*) (NEM). Due to their resistance to Phytophthora (PHY), two clones of *J. microcarpa* x *J. regia* 'Serr' and one clone of *J. cathayensis* x *J. regia* were released under test agreement for nursery propagation. We also conducted commercial orchard validation of resistance to *P. cinnamomi* in clone, RX1 (*J. microcarpa x J. regia*). Statistical analysis of the CG and PHY results revealed: heritable disease resistance to CG and PHY and superior levels of CG or PHY disease resistance in specific MTs.

Among the diverse interspecific hybrids we examined, RX1 was found to be among the most resistant and VX211 the most susceptible to Armillaria. In 2013-2014, we began evaluations for *Armillaria* resistance of wild species clones shown to exhibit CG and PHY resistance in 2013. This included four clones from the CG screening (*J. microcarpa* OP progeny) and three clones from the *Phytophthora* screening (two clones of *J. microcarpa x J. regia* 'Serr', and one clone of *J. cathayensis x J. regia*).

Interspecific hybrids RX032 and RX1 continue to exhibit resistance/tolerance to lesion nematode. In addition, 16 clones from *J. cathayensis* (OP seedlings) show tolerance to the lesion nematode at levels similar to RX032 and RX1. We identified 6 of 47 *J. cathayensis x J. regia* 'Serr' hybrids that were tolerant to lesion nematode.

Thousand Cankers Disease (TCD): TCD, caused by the pathogen *Geosmithia morbida* and vectored by the walnut twig beetle (WTB), is a newly emergent disease of walnut trees in California, but has been now confirmed in nine western and six eastern states in the U.S. We determined the landing rate of WTB in California, New Mexico, and Tennessee on cut branch sections from black walnut accessions (CA and MO) and on branches on live trees at the NCGR in CA. Results revealed a preference of the beetle for *J. californica, J. hindsii*, and *J. nigra* whereas *J. major* and *J. microcarpa* were among the least preferred hosts. This suggests that rootstocks of *J. microcarpa* may not be impacted by TCD due to reduced capacity of the vector to find the host.

Complementary to the studies on WTB selection behavior, host susceptibility to *G. morbida* was examined in the field (NCGR and UC-Davis) in 2011-13. Nine *Juglans* species, several hybrids for rootstock development, Paradox hybrid, and various *J. regia* cultivars were inoculated and evaluated. Paradox hybrids were the most susceptible to *G. morbida*, followed by *J. microcarpa*. Our team also discovered natural infections of TCD in the walnut family relative, Chinese wingnut, *Pterocarya stenoptera*.

TCD resistance screening in Missouri and Tennessee was directed toward accumulation of eastern-black walnut (*Juglans nigra*) germplasm from geographically and genetically diverse sources for screening and development of TCD-resistant black walnut cultivars for nut and wood production. In addition, collections of butternut, *Juglans cinerea*, trees were made.

The following germplasm was assembled:

- A plantation of black walnut grafts was established near Richmond, VA in a TCD quarantine zone;
- Black walnut provenance tests containing seedlings from 10 provenances were established in Tennessee, Virginia, and Pennsylvania;
- Production of OP black walnut progenies from 50 families produced from 2012 collection and 27 families planted from the 2013 collection;
- Bulked black walnut and butternut seedlings were planted as stock for field grafting;
- Seedlings from Forrest Keeling Nursery were planted in raised nursery beds for use as rootstock in grafting black walnut timbers and nut cultivars;
- Production of open-pollinated butternut progenies from 4 families produced from 2012 collection and 80 families planted from the 2013 collection.

TCD screening of these materials will commence in 2015.

Obj 2. Genetic, physical, and functional mapping of disease resistance genes and deployment of molecular markers for rapid screening of resistance.

At the 20-month mark in our SCRI project, we are poised to begin the genetic/genomic analysis and develop three reference genome sequences (i.e., for *J. regia*, *J. microcarpa*, and *J. cathayensis*) to be used in genotyping-by-sequencing analysis. The results from summer 2014 disease resistance screening of, 1) families of OP half-sib progeny and 2) progeny from interspecific crosses of *J. microcarpa* and *J. cathayensis* with *J. regia* 'Serr' pollen are currently being evaluated for molecular/marker analysis

Obj. 4. Conduct extension efforts that:

1) Deliver disease-resistant rootstocks to stakeholders: We prepared and extended a new rootstock bulletin which contained a glossary and performance summary to educate walnut stakeholders about rootstock terminology, new clonal rootstock technologies, and rootstock performance information (see www.walnut.rootstocks.net to view bulletin). We conducted surveys of 12 existing rootstock plots across the state that contained micropropagated clonal and seedling rootstocks of both commercially available and experimental stocks.

All the plots use seedling Paradox rootstock as a standard. Ten clonal walnut rootstocks that are being tested in various situations, i.e. fumigated and non-fumigated plots, areas with walnut blackline or *Phytophthora* diseases, etc., also were surveyed for CG. Clonal Paradox rootstocks had significantly lower CG incidence than the seedling walnut rootstocks at the Stanislaus County site. These important findings, and those from the other surveys, will be presented at grower meetings and through county newsletters.

2) Assess emerging threats to walnut rootstocks: We revised the protocol used for the 2013 surveys and incorporated an estimate of TCD severity. In addition to conducting more surveys in previously surveyed counties, we also conducted surveys in Colusa, Stanislaus and San Joaquin counties in 2013-14 totaling an additional 24 orchards. We have a statistically valid assessment of TCD status throughout most of the CA walnut growing regions. We also conducted detailed orchard surveys in two orchards that allow us to perform comprehensive spatial and pattern analysis of TCD incidence. We generated and maintain a collection of over 130 isolates of *G. morbida* from English walnut, *J. hindsii*, and *J. californica* and from walnut twig beetles trapped in funnel traps. Microsatellite markers, loop-mediated isothermal amplification, and real-time-PCR have been developed to facilitate forensic analyses of the pathogen in walnut orchards, improve detection of the pathogen in diseased samples, and estimate pathogen load on WTB. Population genetic studies of California isolates of *G. morbida* with microsatellite markers are underway.

For the emerging threat of lethal Paradox canker (LPC), we surveyed affected orchards in nine walnut-producing counties of California. Samples were collected from 19 healthy and 66 LPC-affected trees in 17 orchards. Disease incidence ranged from <1% to several percent. We conducted pathogenicity tests with several microorganisms that exhibited some association with the disease, but Koch's postulates have not been fulfilled for any isolate.

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