WALNUT IMPROVEMENT PROGRAM 2006

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ABSTRACT

The goal of the Walnut Improvement Program is to provide new cultivars of walnut to the California walnut industry while developing new knowledge and maintaining a breeding population. We also work with collaborators to develop new rootstocks and propagate them. This year we have over 17 thousand seedlings and selections in the program. Almost 10,000 are half sibs from selections at the Kearney selection block. Early harvest is a primary goal and several selections with Payne-time harvest dates are promising. These are 91-77-6, 91-90-41, 94-19-85, 94-20-28, 94-20-35 and 95-11-14. We are seeking locations for field trials with them. Ten of our backcross selections for hypersensitivity to blackline are in field trials. Somatic embryos from a controlled cross between wingnut and walnut were initiated again this year. Plants initiated last year are being acclimatized and grown to verify parentage. Controlled crosses between Idaho and Chandler were made again this year to increase the population for developing a DNA map of traits of walnuts.

OBJECTIVES

The objectives of the Walnut Improvement Program are:

- to provide the California walnut industry with genetically superior walnut cultivars and rootstocks
- to develop knowledge that will increase the efficiency of walnut breeding
- to develop and maintain an array of traits available for breeding in the future

The program consists of several projects with specific objectives:

- The classical cultivar breeding project uses traditional methods to develop and release new cultivars that combine precocity (high early yield) and early harvesting with kernel quality, in-shell traits, and disease resistance.
- The backcross breeding project is designed to introduce resistance to blackline disease from the Northern California black walnut into a commercially acceptable English walnut cultivar.
- Rootstock improvement objectives include development of selections with genetic resistance to Phytophthora, nematodes, and crown-gall and are done in conjunction with the clonal rootstocks improvement project.
- New technologies that increase the efficiency of breeding and the scope of genetic material available for walnut improvement continue to be evaluated and adapted to walnut breeding as opportunities arise.
- Germplasm collections are maintained and augmented when possible for future breeding use and are available for other researchers.

PROCEDURES

Breeding program.

The procedures for the breeding program have changed as the advanced generation selections have matured and become available as parents. In 2004 and 2005 we collected nuts from the selected parents at the Kearney Agricultural Center to produce half sib families. In 2005 the following were the selected female parents: 90-31-10, 91-76-24, 91-90-41, 93-26-6, 94-19-45, 94-20-35, 95-7-6, 95-11-14, and 95-22-26. In 2006 we made controlled crosses instead of collecting open-pollinated (OP) seed because the family size required for OP seedlings is prohibitive. In all cases, they are close planted and any that appear to be terminal bearers or have any of the signs of inbreeding (dwarfs, extra lates, etc.) are culled at about age 3. If no nuts have been produced by age 5 (under good growing conditions) they are also cut down. Full evaluations are only done on precocious and laterally fruitful individuals. This is similar to the methods we used for the supplemental pollination families (see previous reports). Surviving seedlings are evaluated for phenology (leafing, flowering and harvest dates), precocity, lateral fruitfulness, estimated yield, blight incidence, and crack-out characteristics (shell shape, texture, thickness and strength, kernel weight, percent kernel, and kernel color, fill, plumpness and ease of removal in halves).

Data is evaluated at the annual crackout evaluation meeting that includes growers, processors, nurserymen, and farm advisors. Participants inspect kernel boxes and data sheets to identify possible selections. Data available includes current year field and crack-out data, performance data from past years, Diamond evaluations and computer-assisted selection. Team evaluations are followed by a general group discussion of each team's recommendations.

Promising individuals are repropagated into three selection blocks (Chico, Kearney and Davis) and grower trials where evaluations continue. The off-campus selection blocks are under the control of the Bill Olson (Chico) and the Kearney field staff. Grower field trials are an essential component of releasing a new cultivar. We have increased the number of field trials in the last few years. (See "Description of selections" in this report).

Backcross breeding for hypersensitivity to cherry leafroll virus.

The backcross breeding project is designed to introduce resistance to blackline disease from the Northern California black walnut into a commercially acceptable English walnut cultivar. Crosses are conducted using the same methods as in conventional cultivar breeding but the selection process is different. The first backcross cull is based on shell thickness and percent kernel; those exhibiting the black walnut shell characteristics are discarded. Those that are promising are tested by PCR for hypersensitivity to the cherry leafroll virus as reported in Walnut Research Reports (1998) and modified recently (see WRR 2003).

Marker selection has been improved but has a 10% chance of error. As potential parents and selections advance in the program, there is a need for more stringent testing for hypersensitivity. The screening method used is as described in previous papers: a selection is grafted on both black and English rootstock (two each); after the graft is established, bark from our CLRV-source trees is patched into the English rootstock or into the selection depending on the rootstock species. If the selection is hypersensitive it will survive on the black rootstock because the inoculum patch was rejected, and die (exhibiting a black line) on the inoculated English rootstock. Confirmed

hypersensitive, thin-shelled individuals with the best commercial traits are then used as parents for the next generation of backcrosses to an English walnut parent.

Rootstock improvement

Rootstock breeding is aimed at producing selections with genetic resistance to Phytophthora, nematodes, crown-gall, and environmental stress while retaining or enhancing the vigor of hybrid rootstock. The limiting factor in developing improved rootstocks had been the absence of a commercially viable clonal propagation method but this has been overcome for many rootstock selections (see Clonal Propagation report).

We attempted to initiate new somatic embryo cultures again this year from control-pollinated wingnut-walnut hybrids. This requires very early-season pollen. Gustine pollen was collected and used in mid-March to pollinate bagged flowers of three wingnut accessions (DPTE 1.09, DPTE10.01, DPTE 10.05) at the USDA Clonal Germplasm Repository in Winters. Seeds were collected in early May before shell hardening and were surface sterilized with 15% Clorox for 10 minutes. Zygotic embryos were excised and cultured in vitro on both DKW basal medium and DKW shoot medium.

New technology for genetic improvement of walnut

This part of the Walnut Improvement Program includes tissue culture, PCR, and isozyme analysis in support of genetic improvement as well as gene transfer and field-testing of transgenic plants. Current laboratory work includes micropropagation, use of DNA marker selection in backcrossing, and improvements in somatic embryogenesis.

In 2005 vector pDE00.0201, developed by Matt Escobar in the Dandekar lab, was used to insert crown gall resistance into additional rootstock genotypes expected to be more amenable to propagation than those previously employed. Somatic embryos of three genotypes (J1, J21 and RR4) which we had previously developed were used for this work. The vector, designed to silence the gall forming ipt and iaaM genes of wild-type Agrobacterium, were inserted into these genotypes using the somatic embryo transformation procedure we previously developed and have reported. Transformants were selected on 200 mg/L kanamycin medium and germinated to generate microshoot lines for rooting and field trials. Forty independent lines plus controls have been identified as transformed by marker gene expression and are being propagated for a field trial on campus to test the proof of concept. In addition lines have been produced for crown gall screening by Dan Kluepfel.

We continue to maintain somatic embryo and microshoot cultures of 12 genotypes exhibiting altered expression of shikimate dehydrogenase (SDH), an enzyme in the shikimate pathway that regulates gallic acid production. This gene is of interest for its effect on aflatoxin resistance. Rooting and acclimatization of these genotypes in the greenhouse is complete and they will be maintained in large pots in a lath house.

Transgenic trees in field trials or in large pots are now at bearing age and transgenic trees with the following genes continue to be observed and evaluated:

- Bt - insect resistance (inoculation with codling moth)
- FAD - altered oil composition to avoid rancidity.
- PPO - altered phenolic composition to improve rooting and kernel traits.

NOTE: Transgenic walnuts are only grown on campus under USDA guidelines and catkins and nuts are removed. They are grown for proof of concept experiments.

Germplasm resources

Germplasm collections are maintained and augmented when possible for future breeding use and are available for other researchers. Current collections at Wolfskill and Davis include a diversity of California cultivars, leading cultivars and selections from around the world, material with unusual traits, and germplasm of interest for rootstock development. It differs in emphasis, content, distribution policy, and cultural practices from the USDA Germplasm Repository collection.

Our major emphasis this year regarding germplasm was to determine the chilling requirements in *Juglans regia* germplasm. This was conducted under a Specific Cooperative Agreement between ARS and University of California, Davis. We used the methodology recommended by a recent workshop on standardizing methods for evaluating chilling requirements. Chilling was defined as hours under 45F. In early December, after the leaves had fallen and about 213 chill units had accumulated, 4 shoots, approximately one meter in length, were harvested from 12 genotypes of a diverse set of germplasm. After 200 hours of natural chilling another set was collected and so on until 1015 hours of chilling had accumulated. This was the maximum accumulated in 2005-2006. Items were chosen to represent terminal and lateral bearing habit, early, mid and late phenologies and reportedly low and high chill requirements. Shoots were placed at 22C under fluorescent lights (16h/8h) at 200-400µ mol m^{-2} s^{-1} in vessels of deionized tap water. Shoots were examined every several days and terminal bud break was recorded. The standard walnut descriptors were used to assess when bud break had occurred. The shoot bases were trimmed and water was changed every week.

RESULTS AND DISCUSSION

Cultivar breeding

Three new walnut cultivars (varieties) were patented in 2006: 'Sexton', 'Gillet' and 'Forde'. These are characterized by high early yields, harvest dates before Chandler by 5-20 days, low blight scores and large light-colored kernels. They are described in more detail in a separate report (2004). It is interesting to note that as young grafted trees the harvest date is later than on more mature trees. This fits with the observation that the phenology of young trees advances to earlier in the season as a tree matures. Scionwood of these new varieties was distributed to 13 licensed nurseries and two randomized complete block field trials are underway with Joe Grant and Kathy Kelley.

Currently we have 72 selections and our major focus is on getting earlier harvesting varieties. The most promising early selections are 91-077-6, 91-090-41, 94-019-85, 94-020-28, 94-020-35, and 95-011-14. Data on the selections are provided in Tables1-4. A description of each selection can be found at the end of this report. Seedlings under evaluation and selections are as follows:

Year	Crosses	Original Seedlings	Selections	Under Evaluation
	N	N	N	N
1990	15	591	8	8
1991	18	493	9	9
1992	15	243	5	8
1993	14	116	2	3
1994	15	587	8	14
1995	15	758	19	32
1996	7	333	1	2
1997	13	611	12	28
1998	5	1759	7	102
1999	1	993	1	9
2000	12	2503	-	542
2001	16	210	-	114
2002	5	1200	-	1200
2003	11	4608	-	4608
2004	7 hs**	6000	-	6000
2005	9 hs	3332	-	3332
2006	22	954	-	954
Totals	200	25291	72	10286

**hs denotes half sib families

Backcross breeding for hypersensitivity to cherry leafroll virus.

Backcross breeding to develop an English walnut with a hypersensitive response to the cherry leafroll virus is proceeding ahead of schedule. We continue to test backcross seedlings for both nut quality and virus resistance and currently have approximately 670 seedlings under active evaluation. Attributes of the most commercially viable of the current backcross selections are listed in Table 5. Three backcross hypersensitive selections (92-16-1, 95-29-4 (tolerant, see below), 97-27-55) have been propagated by Dave Wilson Nursery and have been established in a field trial with Janet Caprile in Contra Costa County. Bill Coates also has these selections as well as 93-45-1, 95-27-19, 95-27-38, 95-27-55, 96-17-12, 96-27-8, 97-27-24, 98-17-44. They will be used to evaluate hypersensitivity after exposure to CLRV-infested pollen as well as commercial traits. One selection 95-29-4 in both trials has tested hypersensitive in the DNA test and tolerant in the bark test.

In 2001 we started a new testing block for final confirmation of hypersensitivity by bark patch testing. Additional selections were added in 2002-2005 to a total of 81. Patches were checked for blackline formation this year and 18 tested hypersensitive, 49 were tolerant, and 14 were not yet ready to score. Only one selection that had tested hypersensitive by DNA appeared to be tolerant and one had inconclusive results.

A total of 55 additional backcross trees that have been identified as hypersensitive by DNA testing are being added to the patch test block to confirm the DNA results.

Rootstock improvement

A number of potential rootstock selections have been identified in the past and are maintained and micropropagated in the laboratory for confirmation testing and field trials (See Hackett et al. report). This material includes tolerant backcross selections (vigorous, CLRV tolerant), several Phytophthora survivors from growers' orchards, PDS selections for crown gall, nematode, and Phytophthora resistance.

Wingnut x walnut hybrid seed cultured in vitro at an early stage, while the shells were still soft, did not develop well again this year. Embryos were initiated on both basal medium and shoot medium, both of which work for walnut, but the embryos are much smaller than walnut and apparently require supplementary hormones for initial development if initiated at this early stage. The wingnut DPTE10.05 x (Gustine or UC86.011) embryo line (WNBxGRZ1) produced last year was germinated and microshoots were rooted and acclimated in the greenhouse. The plants are phenotypically hybrid and will be observed further in the greenhouse. An additional somatic embryo derived shoot line (DPTE 10.05 B) is phenotypically wingnut and is being propagated for use as a control in Phytophthora testing.

New technology for genetic improvement of walnut

Three new paradox genotypes (J1, J21, and RR4) thought to be easier to culture and propagate than existing lines were used to develop 40 new independent lines containing the construct for crown gall silencing. We used the same vector employed in the earlier lines to transform somatic embryos. Following selection of successful inserts on kanamycin medium and development of multiplying non-chimeric lines, embryos of each line were germinated. A total of 21 J1, 7 J21 and 12 RR4 shoot lines were developed and multiplied, along with controls, for rooting.

Preliminary testing of in vitro material indicated that the gene is effective. Thirty three of these lines were tested for crown gall resistance by infecting 20 or more 1 cm length stem pieces of each transgenic line and several control lines using a wild-type Agrobacterium strain (20W5A). An additional 20 or more segments of each line were infected with a disarmed Agrobacterium strain (EHA101).

Shoot pieces of controls exposed to the active Agrobacterium strain were extensively galled while shoot segments of twenty three of the transgenic lines showed no galling at all when exposed. Four transgenic lines exposed to 20W5A did show galling, indicating the inserted DNA was not effectively expressed. Another 6 showed ambiguous results, with either a few weak galls or some background callus, and will require further testing. Transgenic and control lines exposed to the disarmed Agrobacterium control showed no galling that could be distinguished from background callus.

All lines and controls were then propagated, rooted, and established as plants in the greenhouse for further testing. The non-expressing transgenic lines were included for use as controls and to confirm that laboratory results are indicative of field results.

Rooting and acclimatization of shoots of all of these lines was accomplished this year and the first set of plants has been tested by Kluepfel. A second set is being prepared for Janine Hasey for greenhouse screening, another for an on-campus field trial and a fourth set is being grafted to

determine whether there is transmission of the genes or gene products across the graft union.

Rooting and acclimatization in the greenhouse of genotypes exhibiting altered expression of shikimate dehydrogenase (SDH), an enzyme in the shikimate pathway that regulates gallic acid production, is in progress so they can be used to study gallic acid production in nuts and its role in insect and disease resistance. To date, 57 plants from 14 of these genotypes have been fully acclimated in the greenhouse and several flowered this year. They have been chilled and will be maintained in large pots in a lath house for further analysis of gene expression and observation of phenotype and gene efficacy.

Mature Chandler trees expressing the BT gene have shown good efficacy in tests conducted by the USDA and their field plot is in the process of being removed. The trees in the UC collection continue to be hedged to prevent flowering and are being held on campus to be available for future work if desired. Transgenic lines expressing or silencing the polyphenol oxidase gene, thought to play a role in rootability and kernel traits, FAD genes modifying oil composition, genes regulating gallic acid production for aflatoxin reduction, and genes regulating adventitious shoot and somatic embryo production are being maintained for use in further studies.

A cross of Chandler x Idaho was made during the last three years to generate a population for developing markers for marker-assisted breeding. The parents were chosen to develop a seedling population that segregates for as many important traits as possible (kernel color, phenology, lateral bearing, shell appearance, protogyny/protandry, insect resistance, blight). An additional cross may be needed this year to further increase the population size to 200. Trees from the first year of crossing were planted in the field in 2004 and will be evaluated for horticultural traits as they mature over the next several years. Additional trees from this year's cross have been germinated and will be planted in the spring. DNA from these trees will eventually be used to develop map of the traits in the walnut genome and to develop markers for more efficient selection in breeding.

Germplasm resources/Chilling

We continue to maintain a collection of in vitro germplasm for use by the Walnut Improvement Program, other cooperating researchers, and commercial labs and nurseries. We also maintain in vitro nematode population for use in nematode resistance research by the Dandekar lab and others.

We introduced several new items into in vitro culture this year in support of other programs. These included UZ229, a PDS selection identified by McKenry with possible nematode resistance and Hartley for use in Brevaria research by Kleupfel's lab.

Maintaining an in vitro germplasm collection is labor intensive and repetitive. In order to reduce costs and time committed to this activity we began to develop methods for cold storage of walnut shoots and somatic embryos.

Part of this work is in cooperation with the USDA-ARS cryopreservation facility at FT. Collins, CO. We sent in vitro shoot cultures and somatic embryos for them to use in improving our existing liquid nitrogen storage protocol and they will attempt to develop methods for cryopreservation of both in vitro and field-grown shoots.

In order to extend the time interval between transfers of cultures to fresh medium we began experiments to test methods of maintaining cultures in refrigeration. It appears that we can extend the transfer interval for shoot germplasm from the current 3 weeks to at least 3 months by cold storage under fluorescent lights for 2 months followed by 1 month at room temperature. A similar approach for somatic embryos is being tested. In addition it appears that somatic embryos can be successfully desiccated for germination by placing them in a refrigerator in empty Petri plates containing no medium. This can replace the standard method of drying over saturated salts and somatic embryos remain viable for at least several months when stored this way.

Chilling hours required to break bud in a varied set of germplasm accessions was studied. The maximum time an excised shoot could survive at 22C was 31days. All shoots showed a trend that as more chilling hours accumulated in the field fewer days at 22C were required to break bud (Fig. 1). The maximum chilling accumulated in the field was 1015 which might be too low for some genotypes but at this accumulation it took about 15 days at 22C to break bud even for the slowest. Genotypes grouped into 3 categories depending on how much chilling was required to result in bud break after 15 days at 22C. These categories closely correspond to their phenology in the field, i.e. early leafing genotypes required less chilling while later leafing genotypes required more

Genotype	Chilling hours required to break bud after 15 days at 22C
Early Ehrhardt	627
Payne	627
Placentia	627
Serr	827
Tulare	984
Hartley	984
Cascade	1015
Chandler	1015
Fernor	1015
Howard	1015
S. Franquette	1015
XXX Mayette	1015