WALNUT IMPROVEMENT PROGRAM 2005

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ABSTRACT

The goal of the Walnut Improvement Program is to provide new varieties 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 18,000 seedlings and selections in the program. Almost 10,000 are half sibs from selections at the Kearney selection block. Seven new selections were added from 1997 Tulare x mixed Chinese pollen crosses. Early harvest is a primary goal and several selections with Payne-time harvest dates are promising. These are 94-20-28, 94-20-35 and 95-11-14. We are seeking locations for field trials with them. Another that should go into field trials is 93-28-20 that has a beautiful nut and harvests two weeks before Chandler. Three 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.
- 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. This year our work focused on developing plants containing the crown gall silencing gene and finger printing the walnut breeding program selections with D. Golino.
- 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 have 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. Because these produce families a great deal larger than controlled crosses, 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 local farm advisors (Beede and Olson). 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 propagation report).

We attempted to initiate new somatic embryo cultures 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 two wingnut accessions (DPTE 1.09, DPTE10.01) 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.

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 six genotypes (RR1, RR4, J1, J8, J21, and J34) 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.

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 in progress.

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

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 varieties 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 collection.

RESULTS AND DISCUSSION

Cultivar breeding

Three new walnut cultivars (varieties) were released but wait formal patenting: 'Sexton', 'Gillet' and 'Forde'. These are characterized by high early yields, harvest dates before Chandler by 10-20 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 9 licensed nurseries and for two field trials with Joe Grant and Kathy Kelley (misses from 2004)

Currently we have 51 selections and our major focus is on getting earlier harvesting varieties. The most promising early varieties are 94-020-28, 94-020-35, and 95-011-14. Data on the selections are provided in Tables1-6. A description of each selection can be found at the end of this report. Seedlings under evaluation and pending evaluation are as follows:

Year	Crosses N	Original Seedlings N	Under evaluation N
1990	15	591	6
1991	18	493	11
1992	15	243	3
1993	14	116	4
1994	15	587	8
1995	15	758	32
1996	7	333	5
1997	13	611	55
1998	5	1759	1159
1999	1	993	774
2000	12	2503	997
2001	16	210	210
2002	5	1200	1200
2003	11	4608	4608
2004	7 hs**	6000	6000
2005	9 hs	3332	3332
Totals	178	24337	18404

** 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 932 seedlings under active evaluation. Attributes of the most commercially viable of the current backcross selections are listed in Table 7. Three backcross hypersensitive selections (92-16-1, 95-29-4, 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. They will be used to evaluate hypersensitivity after exposure to CLRV-infested pollen as well as commercial traits.

In 2001 we started a new testing block for final confirmation of hypersensitivity. Additional selections were added in 2002 -2005 and testing is in progress. We expect to add more material this year. Table 8 lists 90 selections of backcross material currently grafted for bark patch testing. Seven of these are reconfirmations of tolerant rootstock selections and two are reconfirmations of virus resistant BC2 selections previously bark tested. The remaining 80 items are the best BC2 and BC3 selections from crack-out. Twenty of these are likely virus resistant based on the DNA marker test. Sixty are likely tolerant based on the DNA test but were bark tested due to their superior nut and kernel quality.

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

DNA tests have indicated, and patch tests are now confirming, a number of BC3 generation trees suitable for use as parents.

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. Plants will be observed as they mature to determine if this line is hybrid.

Somatic embryos of a new potentially hybrid line were started this year from a cross using wingnut DPTE1.09. Microshoots of this line have also been developed from a germinated somatic embryo and will be rooted for testing. Additional seed was collected after shell hardening and placed refrigerated for stratification before culturing. A portion of this seed has also been planted in the greenhouse to see if it can be germinated directly.

Two wingnut tree selections identified by Joe Grant as potentially graft-compatible with walnut were established in culture this year from nodal cuttings and are beginning to multiply. These selections, designated North Zack and South Zack will be rooted and acclimated for field testing.

New technology for genetic improvement of walnut

Paradox shoot lines expressing genes for crown gall resistance and shown to be resistant to crown gall formation in laboratory testing (Dandekar report 2003) continue to be propagated. Plants of several of these clones were rooted, acclimatized, and grown in the greenhouse. Fifty-two fully dormant plants of the line MCG 42-1-1 (Table 2, Hackett report) will be tested to confirm resistance in the greenhouse by Dan Kluepfel. Additional trees now in the greenhouse and in the process of chilling (Table 1, Hackett report) will be grown in the nursery this year to size for future field trials

In addition, three new genotypes (J1, J21, and RR4) thought to be easier to culture and propagate than existing lines were used this year 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 for rooting. Preliminary testing of several lines in vitro material indicated that the gene is again effective. Rooting of shoots of all of these lines is in progress to develop plants for greenhouse and field testing. The following table summarizes the current numbers of acclimated plants of these available in the greenhouse.

Background genotype	#of independent lines	# plants with gene	# control plants
J1	18	84	18
J21	4	20	3
RR4	4	19	80

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, 22 plants from 10 of these genotypes have been fully acclimated in the greenhouse.

Microshoots derived from germinated embryos of very precocious Chinese germplasm, and which readily flower in vitro, are being used, in cooperation with researchers at Purdue University, to study genes and promoters that regulate flowering and to identify methods of regulating flower development in walnut.

Mature Chandler trees expressing the BT gene have shown good efficacy in tests conducted by the USDA. These trees in the field continue to be hedged to prevent flowering and are being held only to be available for future work if the political climate changes.

Transgenic lines expressing or silencing the polyphenol oxidase gene, thought to play a role in rootability and kernel traits, and FAD genes modifying oil composition, are being maintained for use in further studies.

A cross of Chandler x Idaho was made the last two years to generate a population for developing markers for marker-assisted breeding. The parents were chosen to give a seedling population segregating 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 this spring 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

Blocks of appropriate and diverse germplasm cannot be established on short notice. Genetically diverse and unsprayed blocks in particular are frequently unavailable elsewhere to researchers. Research materials requests supported by our program this year included husk fly, codling moth, aphid, aflatoxin, clonal propagation, molecular marker, and taxonomy research.