95-DG1

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# Almond Board of California

Annual Report - 1995

**Project Title:** Genetic Engineering of Nonpareil almond
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**Cooperating Personnel:** John Driver & Archie Tang, Dry Creek Laboratories, Modesto, CA

## Objectives:

1) To establish microbe-free *in vitro* germplasm of the almond variety Nonpareil, and the peach variety Ross;
2) to regenerate plants from tissue cultures; and
3) subsequently using *Agrobacterium* mediated methods to genetically transform these two varieties.

## Background and Rationale

Dry Creek Laboratories has an ongoing *Prunus* rootstock research program in collaboration with UC Davis Pomology. Within this program an inter-specific hybrid (Almond x Peach) rootstock, Hansen 536, has been studied for regeneration ability over a period of 18 months. *In vitro* established Hansen leaf materials have been cultured to regenerate shoots at a relatively high frequency (average 40%). Recently, transgenic shoots have been recovered from this hybrid's leaf materials after being genetically transformed using *Agrobacterium tumifaciens*, with a binary vector containing a gene conferring nematode resistance. Transformed plants are now ready to be established in the greenhouse and field.

Regeneration and transformation of clonal varieties of almond or peach (or hybrids) have not been reported before this time. Thus, this work has laid the foundation for almond and cling-peach variety regeneration/transformation methodology.

This project attempts to build on this foundation towards the efficient transformation and regeneration of well established and commercially important almond and cling-peach varieties. It is supported by matching funds from the Cling Peach Advisory Board, the Almond Board, and a USDA grant, as well as expertise, equipment and facilities at UC Davis. Transformation and regeneration work is performed at Dry Creek Laboratories, Modesto CA.

## Progress Summary:

Clonal plant materials of Nonpareil and Ross have been successfully propagated *in vitro*. Microbe-free leaf materials of these two plants have been used in tissue cultures for callus induction and shoot organogenesis attempts. To date, callus initiation followed by regeneration from Nonpareil leaf tissues have been achieved. Ross tissues have given rise to callus formation and regeneration attempts are now underway. Transgenic Nonpareil cells (callus) have been recovered following co-cultivation with *Agrobacterium*.

## MATERIALS and METHODS:

### 1. Germplasm establishment *in vitro*:

Orchard - grown, current season growth of Nonpareil and Ross were collected in May, 1995. Surface sterilization was done by treating washed shoots with 25% (v/v) commercial bleach for 25 minutes, followed by four rinses with sterile water. Short apical tips and single or double - node cuttings of disinfected shoots were cultured in proliferation medium consisting of MS salts (Murashige and Skoog 1962), NN vitamins (Nitsch and Nitsch 1969), 3% (w/v) sucrose, pH 5.7 (basal medium), plus 0.7% (w/v) phytagar as a gelling agent, also supplemented with 0.1 mg/1 IBA as growth regulators.

Shoots were incubated in 14 hour light (cool white at 40 $\mu$m/m2/s2) and 10 hour dark cycle at 24 +/1 2 C. Microbe-free shoots have been subcultured every 2 to 3 weeks onto fresh proliferation medium for multiple shoot formation. Rooting of shoot cuttings for both varieties occurred following treatment of single shoots (1.5 - 2.0 cm long) in root initiation medium (basal medium as previously described with the addition of 3.0 mg/1 IBA, deletion of BAP) in the dark for 7 to 10 days then transferred to hormone - free basal agar medium and moved into the light.

### 2. Tissues cultures, Callus induction and plant regeneration studies:

Well expanded, but young leaf materials from *in vitro* shoots were used for regeneration studies. Leaves were cut into 2-3 mm segments and were cultured on callus induction medium (consisting of basal agar medium supplemented with 1.0 mg/1 BA and 2.0, 4.0 mg/1 IBA, or IAA, or NAA). Cultures were incubated in constant dark conditions at 24 +/- 2 C. Leaf segments containing callus, were transferred to shoot organogenesis media (basal agar medium plus 0.5 mg/1 NAA and 2.0, 3.0 or 4.0 mg/1 BA with or without the addition of 1.0 mg/1 TDZ). Cultural conditions remained the same. Results were concluded in 4 to 8 weeks after transfer.

### 3. Genetic Transformation Studies:

*Agrobacterium* mediated transformation attempts have been made using the *Agrobacterium* strain EHA 101 containing binary plasmid pDU92.710 (containing the scorable marker GUS, the selectable marker NPTII, and CRY 1A Bt gene). This vector and plasmid was constructed by and supplied by A.M. Dandekar, Department of Pomology, University of California-Davis.

Growth and induction of *Agrobacterium* include growing the bacteria overnight in liquid bacterial growth medium. The bacterial suspension was diluted to a population of about $10^8$ cells/ml with a mixture of "523" medium and basal liquid medium (at a 1 to 1 ratio).

For co-cultivation of the Agrobacterium and Nonpareil leaf segments, leaf pieces were cut and inoculated with bacterial culture for 20 minutes, and then blotted dry on sterile filter paper. Explants are then co-cultivated in the dark @24 +/- 2C for 48 hours on solid callus inducing medium with filter paper bridges.

Co-cultivated leaf materials were then transferred to selection media consisting of basal agar medium supplemented with 1.0 mg/1 BA and 2.0 mg/1 NAA, as well as 200 mg/1 Cefotaxime to control bacterial growth and 40 or 50 mg/l Kanamycin monosulfate as selective agent. Medium without

Kanamycin was also included as a non-selective control. In 4 weeks leaf explants were subcultured onto fresh selection medium with continuous incubation in darkness for callus development. Leaf segments bearing callus were then transferred onto selective shoot regeneration media consisting of basal media above supplemented with 0.5 mg/1 NAA plus 2.0, 3.0 or 4.0 mg/ml BA, with or without 1.0 mg/l TDZ, as well as Cefotaxime and Kanamycin for transgenic shot regeneration attempt.

**RESULTS and DISCUSSION:**

Both Nonpareil and Ross were established from orchard grown materials for multiple shoot formation on proliferation medium. Subculture intervals are no longer than every 3 weeks. Rooting process is far better in Ross shoot cuttings (up to 60% with 3 to 6 roots initiated per shoot in 3 weeks) than in Nonpareil cuttings where only 1 to 3 roots induced at about 15% frequency. Increasing or decreasing the IBA level, shortening or prolonging the IBA treatment, with or without light exposure have not led to better rooting results in Nonpareil. Further investigation to improve rooting in Nonpareil or *in vitro* grafting may be necessary.

The callus induced from leaf segments of Nonpareil varied in frequency and mode, depending on the hormonal combinations. Among auxins tested NAA at 2.0 mg/l combined with 1.0 mg/1 BA produced most consistent callusing results. When these cells (still attached to the original leaf tissue) were transferred to shoot regeneration media containing 0.5 mg/1 NAA plus 3 or 4 mg/1 BA or 2 or 3 mg/BA along with 1 mg/1 TDZ adventitious shoot formation occurred 4 to 6 weeks after subculture. Regeneration frequency falls on an average of 14% (number of explants forming shoots over number of explants cultured). Number of shoots per regenerating explant is ranging from 1 to 3.

Experiments for determination of parameters for improvement of shoot regeneration in Nonpareil are underway. Regenerates have been isolated individually when they reached 2mm or longer in length. They are then propagated in proliferation medium and placed under lighted conditions. These adventitious shoots are growing normally like regular shoot tip cuttings.

For Ross, only callus has developed, as regeneration studies to date have not shown shoot formation.

*Agrobacterium* strain EHA101 with binary plasmid pDU92.710 infected Nonpareil leaf segments developed non-transgenic callus in Kanamycin free media. Occasional shoot formation have been observed on Kanamycin free media, but at a lower frequency relative to non co-cultivated materials. When 40 or 50 mg/l Kanamycin was present in the selection media, callus formation has been highly reduced. Some of these Kanamycin resistant calli have shown GUS positive reaction when subjected to X-GLUC treatment. However, no Kanamycin - resistant shoots have been recovered yet. As soon as transformed shots are recovered, they will be tested and confirmed by A.M. Dandekar using both Southern Blot and Western Blot methods.

The progress to date for Nonpareil and Ross supports the feasibility of gene transfer into these *Prunus* varieties.