## SOMATIC EMBRYOGENESIS OF CLONAL CHANDLER

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### OBJECTIVES

Our procedure for introducing new genes into walnut through genetic engineering (McGranahan *et al.* 1990) requires rapidly multiplying somatic embryo cultures as a starting material. Historically, the embryo cultures used in our transformation experiments have been derived from immature zygotic embryos. While one or sometimes both parents of these embryo cultures are known, the horticultural quality of the resulting progeny is not. Thus, while genetic engineering has provided a valuable source of new genes for walnut, the resulting transgenic trees have had to be placed into the general breeding program for incorporation into new, commercially viable cultivars.

Much of the promise of genetic engineering lies in its potential for inserting one gene of interest into a known genetic background, leading to a specific change in an otherwise acceptable crop variety. It is therefore of interest to produce somatic embryo cultures from diploid tissues of elite walnut cultivars.

When cultured under suitable conditions, many plant organs have been induced to produce somatic embryos, including roots (Vuorela *et al.* 1993), cell suspensions (Mathur 1991), and anthers (Kiss *et al.* 1992). Previous work in our lab failed to produce somatic embryos from ovule tissue (Aly *et al.* 1990). The following report describes a successful method for inducing embryogenesis from cultured immature anthers of Chandler walnut.

### PROCEDURES

Immature walnut catkins, approximately 2.5 cm long, were harvested during late March and early April. At this stage, the florets have not begun to open, the anthers are yellow-green and translucent, and pollen has not yet begun to form. The catkins were placed in a plastic bag with a wet paper towel to prevent desiccation and chilled four days at 5°C.

The catkins were surface sterilized for 10 minutes in a solution of 10% bleach with a drop of dishwashing detergent to ensure wetting, and then rinsed thoroughly in several washes of sterile water. Seven florets were dissected on moist filter paper under a stereoscope, and the anthers (about 100 total) were placed on a small piece of moist filter paper. The paper was then dropped into a 125 ml Erlenmeyer flask with 20 ml of liquid DKW walnut medium containing various combinations of growth regulators as described below.

The cultures were kept at room temperature in the dark, with continuous shaking at 80 rpm. At weekly intervals, half of the medium in each flask was replaced with hormone-free DKW medium containing 500 mg/l of the antibiotic cefotaxime, to control bacterial contamination. This resulted in a gradual dilution of the hormones over a period of several weeks.

We tested three hormone combinations for their efficacy in inducing somatic embryogenesis in walnut anthers (Table 1). The hormone combinations in media A and B have been used to induce somatic embryogenesis in grape anthers (Rajasekaran and Mullins 1979, Stamp and Meredith 1988, respectively); medium C (in solid form) is used to induce somatic embryogenesis in walnut zygotic embryos (Aly 1992).

Anthers from the five walnut cultivars Serr, Vina, Sunland, Chandler, and Tulare were cultured in the three media, as described in Table 2.

## RESULTS AND CONCLUSIONS

During the first week, some anthers died and others showed swelling. Several flasks developed bacterial contamination, but the addition of cephotaxime to the culture medium proved sufficient to control the infection. The contamination did not appear to affect the health of the anthers.

After six weeks, only one Chandler culture, started on Medium A, was still alive. The anthers in this culture were heavily callused, with knobby lumps. This callus appeared to derive from the outside cell layers, since the anthers did not split open, as would be expected if the callus was derived from the developing pollen grains. To minimize the possibility of loss to contamination, the anthers were split between three flasks.

Seven weeks after the culture was started, it produced four well-formed, free-floating embryos with good cotyledons, and a number of globular embryo-like structures. The four embryos were removed and placed on solid medium with cephotaxime to prevent loss to any remaining bacteria, and to stimulate embryogenesis. None of the globular structures developed further into embryos.

Three of the four embryos placed on solid medium produced somatic embryos, and have since been multiplied into cultures suitable for genetic engineering.

All three somatic embryo cultures have the normal vigor associated with diploid embryos, as do the shoots derived from them. To confirm that the original three embryos were not haploids derived from pollen cells, chromosome counts were performed on the root tips of germinated embryos from each line. All three lines had the normal, diploid number of 32 chromosomes. (*The authors express thanks to Hamid Ahmadi, Department of Pomology, UC Davis, for chromosome counts.)*

There remains a small chance that these embryo lines were derived from pollen grains which underwent chromosome doubling, although one would expect reduced vigor under such circumstances. There is also a possibility that the tissue has undergone mutations in agronomically important traits due to the 2,4-D used to induce embryogenesis. Field evaluations will be necessary to confirm that the embryo lines have maintained the quality of the parent tissue. Embryos from all three lines have been germinated, and the shoots grafted onto seedling rootstock in preparation for such trails.

We are now proceeding to use these embryos in transformation experiments, using genes to confer resistance to fungal pathogens and blight.

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**Table 1. Hormones used in liquid anther culture media (all made with DKW)**

| hormone | concentration |
|---|---|
| Medium A | |
| benzyladenine (BA) | 0.23 mg/L |
| 2,4-dichlorophenoxyacetic acid (2,4-D) | 1.10 mg/L |
| Medium B | |
| benzyladenine (BA) | 0.40 mg/L |
| a-naphthoxyacetic acid (NOA) | 1.50 mg/L |
| Medium C | |
| benzyladenine (BA) | 1.00 mg/L |
| indolebutyric acid (IBA) | 0.01 mg/L |
| kinetin | 2.00 mg/L |
| l-glutamine | 250 mg/L |

**Table 2. Number of anther cultures started for each embryo induction medium.**

| Date | Cultivar | Medium A | Medium B | Medium C |
|---|---|---|---|---|
| 3-29 | Serr | 4 | 4 | 4 |
| 3-31 | Vina | 4 | 4 | 4 |
| 4-5 | Sunland | 4 | 2 | 0 |
| 4-7 | Chandler | 5[x] | 4 | 3 |
| 4-10 | Tulare | 7 | 5 | 0 |

[x] One yielded 3 embryogenic cultures