

Avocado Rootstock Development by Somatic Hybridization and Genetic Engineering

Continuing Project; Year 3 of 5

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Benefit to the Industry

This project has been undertaken in order to develop a new generation of avocado rootstocks with a high level of resistance to *Phytophthora* root rot (PRR) and with other good horticultural characteristics.

Objectives

There are 3 major objectives: 1) somatic hybridization of avocado with PRR-resistant *Persea borbonia*, other wild *Persea* species and *Nectandria* sp. by means of protoplast fusion; 2) genetic transformation of existing avocado rootstocks; 3) developing an efficient *in vitro* protocol for propagating existing and newly developed rootstocks. All of these objectives are dependent upon the availability of an efficient system for regenerating avocado from cell and tissue cultures, i.e., somatic embryogenesis together with standard micropropagation.

Summary

1. High levels of resistance to PRR have been identified in some of the wild relatives of avocado in the subgenus *Eriodaphne* and in other genera. Unfortunately, *P. borbonia* is sexually and graft-incompatible with avocado. This research was undertaken to force the hybridization (by protoplast fusion) of the two species in order to produce a

potentially useful rootstock for avocado. This approach is dependent upon the ability to isolate viable protoplasts enzymatically from embryogenic avocado suspension cultures, to re-establish embryogenic cultures from the protoplasts and to regenerate somatic embryos and plantlets (Figures 1 - 3). We have successfully carried out this procedure with embryogenic cultures of genotypes 'T362', 'Esther', 'Thomas' and 'M25864', and have achieved an average plating efficiency of ca. 24%. Seedlings of *P. borbonia*, *P. pachypoda*, *P. cinerascens* and *Nectandria* sp. have been germinated under sterile conditions and their leaves have been used as sources of mesophyll protoplasts. Protoplasts from leaves of the wild species and from embryogenic cultures of either 'T362' or 'Thomas' have been cocultured in plant growth medium containing polyethylene glycol in order to stimulate their fusion. Putative somatic hybrid cells have been identified and cloned. Embryogenic cultures have been recovered from the putative hybrid cells and somatic embryos have been recovered.

Germination of somatic embryos resulting from the 'T362' + *P. borbonia* and 'T362' + *Nectandria* sp. somatic hybridizations has been accomplished (Figure 4). The fusions occur at very low frequency, and plantlets, i.e., "somatic seedlings" from individual fusion events are currently being micropropagated. Conditions for transfer of the first rooted plantlets to soil are currently being addressed in order to assure high frequency of survival. The rate of survival after transfer is still low; however, plants have been established and are growing.

2. We would like to be able to modify existing avocado selections for a single horticultural trait, specifically resistance to PRR, by transferring a foreign resistance gene into an existing avocado rootstock or scion selection. In previous studies it was possible to genetically transform embryogenic 'T362' avocado cultures with a disarmed strain of genetically engineered *Agrobacterium tumefaciens* that contained a vector with two bacterial genes: a selectable marker gene for kanamycin resistance, i.e., neophosphotransferase (NPT II) and a scorable marker gene for (3-glucuronidase (GUS). The genes were transferred to and were expressed in avocado embryogenic cultures. Putatively transformed cultures were identified that could grow on medium containing high levels of kanamycin, and somatic embryos were later regenerated from these cultures that tested positive for the presence of β -glucuronidase. The integration of NPT II and GUS into avocado somatic embryos was confirmed by Southern hybridization.

Phytophthora cinnamomi is sensitive to the presence of the enzyme glucanase, which degrades the hyphal walls of the fungus. We have engineered plasmid pKYLXSO containing the 35S promoter, NPT II, GUS and the following genes: acidic β -1,3-glucanase isolated from *Nicotiana plumbaginifolia*, basic β -1,3-glucanase from *N. tabacum*, chitinase from *Zea mays* and *N. tabacum*, defensin from *Amaranthus caudatus* and the gene that confers resistance to glyphosate. The plasmids have been transferred to *Agrobacterium tumefaciens* by electroporation. This strategy would result in the constitutive expression of these genes in avocado cells following infection by *A. tumefaciens*. At this time, chimeras that express the markers + glucanase or defensin have been recovered. Solid transformants of avocado containing the genes for chitinase (Figure 5) and glyphosate resistance have been obtained, and cultures are undergoing maturation. In addition, in cooperation with Dr. Miguel Gomez-Lim, we are attempting to

isolate a root-specific promoter from avocado, that would be utilized instead of the 35S promoter. According to this approach, the fungal genes would then only be expressed in the roots of avocado. Currently we are working on the isolation of a root-specific promoters that are homologues of two root-specific promoters from rice, ZRP3 and ZRP4, and two root-specific promoters from maize, RCc2 and RCc3.

3. Micropropagating avocado rootstocks. Many tropical and subtropical fruits, e.g., *Citrus* and mango, reproduce vegetatively by the formation of polyembryonic seeds. The clonal embryos and seedlings develop from the nucellus, a tissue located within the integumental layers of the seed. We have demonstrated that it is possible to stimulate the formation of somatic embryos from the extracted nucellus of the following avocado selections: 'T362', 'Lamb', 'Hass' and 'Thomas'. We have not had access to 'Duke 7' during early fruit set; we hope that this problem will be resolved this year. Conditions for the efficient growth of embryogenic cultures and for the recovery of large numbers of somatic embryos have been determined; however, a significant proportion of nucellar somatic embryos do not develop functional shoot meristems. In order to address this problem, we are expanding effort this year in conjunction with Dr. Raymond Schnell, curator of tropical fruit and geneticist of the USDA ARS in Miami, who is interested in the application of this technique for removing the pathogen that causes sun blotch disease from clonal selections in the national avocado germplasm repository. A graduate student Ms Z. Viloría, who is supported by the Venezuelan government will be addressing this problem.

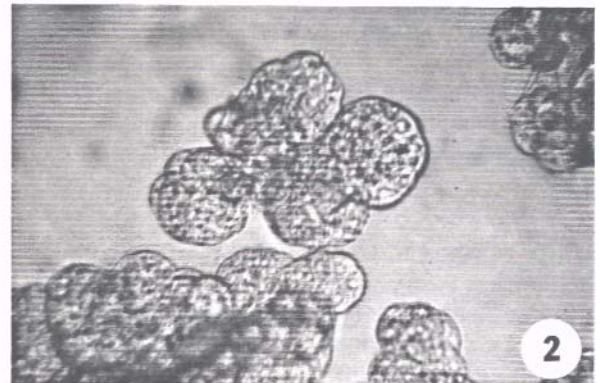
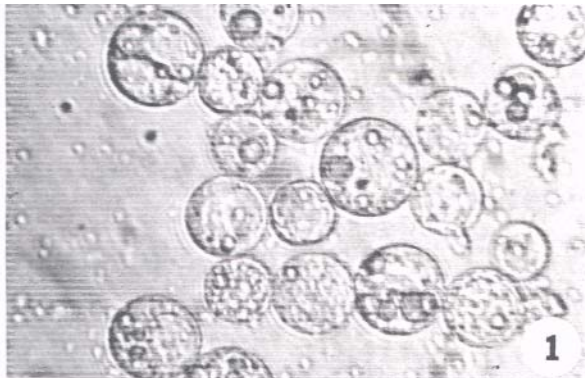


Figure 1. Embryogenic avocado protoplasts

Figure 2. Microcolonies developing from single protoplasts

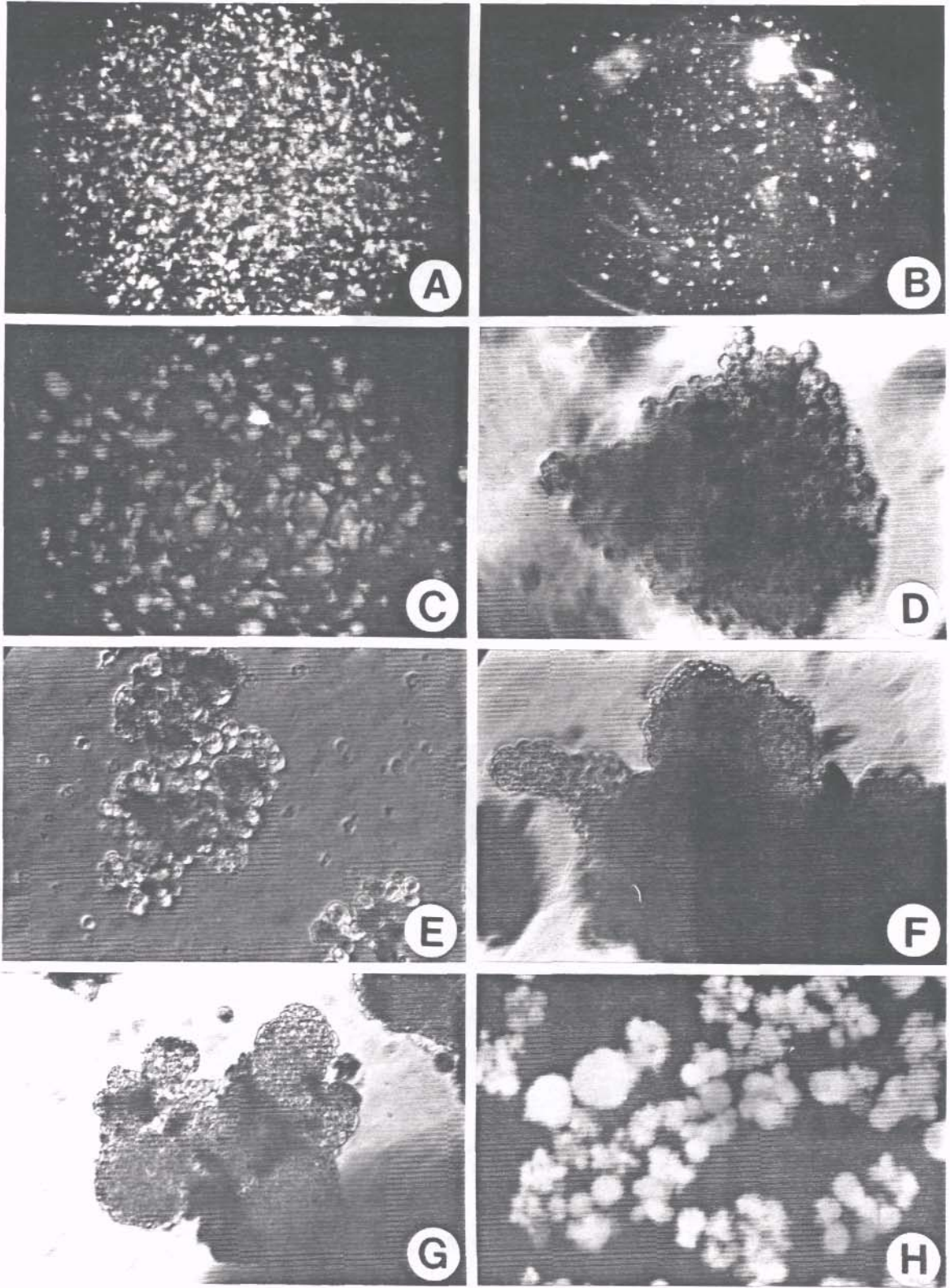


Figure 3 (A-H). Sequence of development from single protoplast-derived microcolonies (A) to globular stage somatic embryos (H)

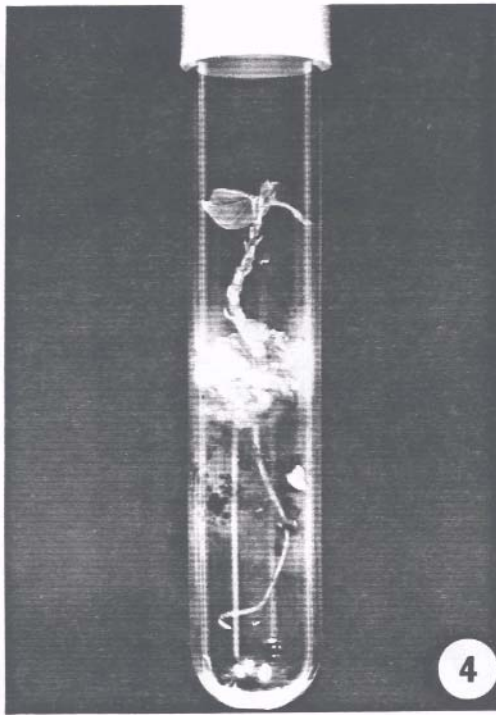


Figure 4. Recovery of avocado plant from protoplast fusion between avocado and *Nectandra*.

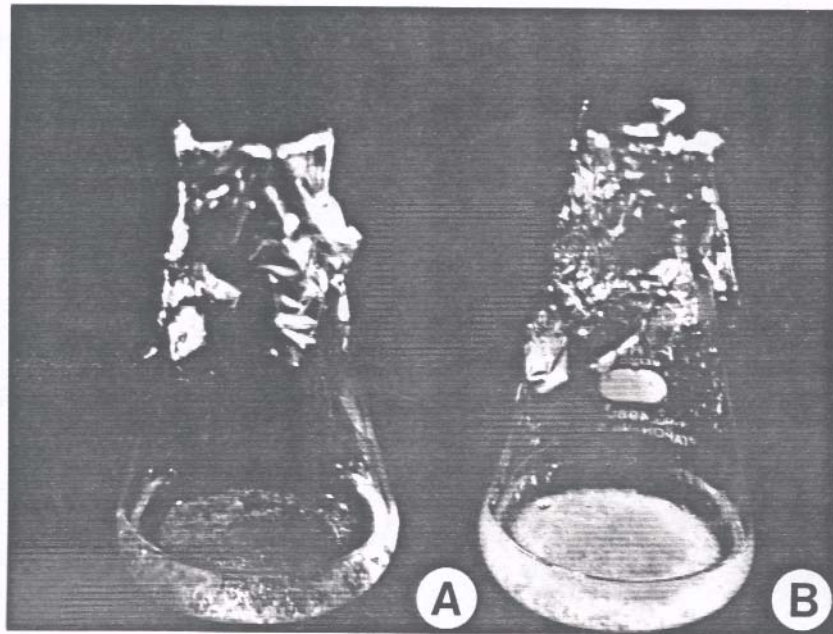


Figure 5 A & B. Recovery of transformed embryogenic avocado cultures with the chitinase gene. A. nontransformed control in selection medium; B. Transformed embryogenic culture.