1997 California Avocado Research Symposium pages 21-24
California Avocado Society and University of California, Riverside

Avocado Rootstock Development by Somatic Hybridization and Genetic Engineering

Continuing Project; Year 1 of 3

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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 with antifungal genes; 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, including *P. borbonia.* 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. 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 a 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 embryo maturation has been developed from these cultures.

Germination of somatic embryos resulting from the 'T362' + *P. borbonia* and 'T362' + *Nectandria* sp. somatic hybridizations has been accomplished. Plantlets, i.e., "somatic seedlings" from individual fusion events are currently being micropropagated. Axillary buds removed from the "somatic seedlings" have been induced to proliferate *in vitro* on plant growth medium supplemented with the cytokinin benzyladenine. At intervals, apical dominance has been restored, and the axillary buds within a culture are allowed to form shoots. Conditions for rooting of micropropagated shoots and acclimatization of plantlets is being carried out in a laboratory environment of ca. 25°C with high light intensity (ca. 160 umol m^{-2} s^{-1}) provided by cool white fluorescent tubes and in an atmosphere consisting exclusively of 20,000 ppm CO_2 in a nitrogen carrier (Tables 1 and 2. The morphology of putative somatic hybrid plantlets has been compared with non-fusion somatic embryo-derived plantlets (Figure 1). Their leaf size is significantly greater, their growth rate is more rapid and their internode length is significantly greater than the 'T362' parent. 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 vigorously.

Table 1. The effect of atmospheric CO_2 concentration on the growth variable and net photosynthetic rate of proliferating avocado shoot cultures after10 weeks in culture. Data across total nitrogen concentration were pooled for this analysis since their interaction with atmospheric CO_2 concentration was not significant ($p>0.05$)

Growth Variable	Atmospheric CO_2 concentration		P^z
	Elevated	Ambient	
	(Mean ± SE)	(Mean ± SE)	
Number of shoots<1 cm	4.41 ± 0.59	3.07 ± 0.44	0.0734
Number of shoots≥1 cm	6.41 ± 0.70	4.53 ± 0.42	0.0259
Total number of shoots	10.83 ± 0.98	7.60 ± 0.66	0.0090
Average shoot length	2.31 ± 0.22	2.70 ± 0.17	0.1562
Number small leaves(2-4 mm width)	15.52 ± 2.43	9.28 ± 0.95	0.0219
Number of expanding leaves (>4 mm width)	20.38 ± 1.80	12.20 ± 1.33	0.0006
Total number of leaves	35.90 ± 3.27	21.48 ± 2.12	0.0006
Net photosynthesis rate (μmol CO_2 cm^{-2} s^{-1})	11.13 ± 1.84	16.51 ± 1.60	0.0358
Dry matter content (%)	19.16 ± 2.82	15.08 ± 0.66	0.1790

[z] Mean separation by standard t-test

Table 2. Avocado plantlet development in two atmospheric CO_2 environments, 9 weeks after culture.

Growth variable	Atmospheric CO_2 concentration		
	Elevated	Ambient	
	(Mean ± SE)	(Mean ± SE)	P^z
Shoot length (cm)	4.13 ± 0.28	2.74 ± 0.21	0.0008
Number of leaves	12.75 ± 0.80	9.54 ± 1.67	0.1048
Leaf area total (cm^2)	13.76 ± 1.76	6.88 ± 1.01	0.0034
Average leaf area (cm^2)	1.09 ± 0.14	0.93 ± 0.18	0.4595
Number of root	6.0 ± 0.65	4.64 ± 0.62	0.1449
Average root length (cm)	4.74 ± 0.42	4.42 ± 0.54	0.6437
Root dry weight (g)	0.02 ± 0.00	0.01 ± 0.00	0.0062
Stem dry weight (g)	0.02 ± 0.00	0.01 ± 0.00	0.0148
Callus dry weight (g)	0.05 ± 0.01	0.03 ± 0.01	0.0150
Leaf dry weight (g)	0.07 ± 0.01	0.03 ± 0.00	0.0011
Net photosynthesis rate (μmol CO_2 cm^{-2} s^{-1})	19.34 ± 4.84	31.35 ± 7.24	0.1900
Dry matter content (%)	18.84 ± 3.65	16.94 ± 2.67	0.2849

[z] Mean separation by standard t-test

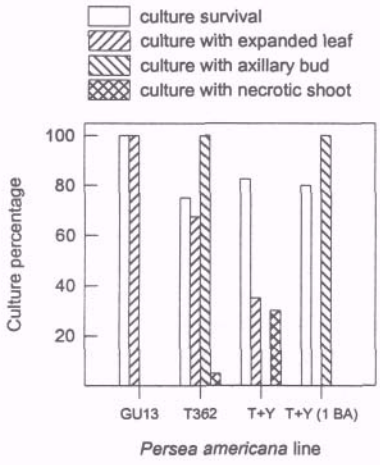
2. We would like to be able to modify existing avocado selections for a single horticultural trait, specifically resistance to PRR, by transferring a foreign resistance gene into an existing avocado rootstock or scion selection. In previous studies it was possible to genetically transform embryogenic 'T362' avocado cultures with a disarmed strain of genetically engineered *Agrobacterium tumefaciens* that contained a vector consisting of a that contained two bacterial genes: a selectable marker gene for kanamycin resistance, i.e., neophosphotransferase (NPT II) and a scorable marker gene for p-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 P-glucuronidase. The integration of NPT II and GUS into avocado somatic embryos was confirmed by Southern hybridization.

We have obtained the following antifungal genes, acidic p-l,3-glucanase isolated from *Nicotiana plumbaginifolia,* basic P-1,3-glucanase from *N. tabacum,* chitinase from *Zea mays* and *N. tabacum* and defensin from *Amaranthus caudatus.* Two strategies are being used for genetic transformation, a) The genes have been inserted into plasmid pKYLXSO containing the 35S promoter, NPT II and GUS, and the plasmid has been transferred to *Agrobacterium tumefaciens* by electroporation. This strategy would result in the constitutive expression of these genes in transformed avocado plants. At this time, chimeras that express the markers have been recovered, b) The other strategy involves the isolation of a root-specific promoter from avocado, and this 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. In conjunction with Dr. Miguel Gomez-Lim, we are currently 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'. Although our intention was to repeat this study with 'Duke 7' during the spring of 1996, we were unable to obtain material from trees in southern California due to low fruit set and other factors. We hope that this problem will not be limiting 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 USD A 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.

During the next year, pending their availability we will attempt to transform embryogenic nucellar cultures of 'Duke 7' and 'Thomas' with antifungal genes, i.e., glucanase and

defensin, that would confer a high level of resistance to PRR.



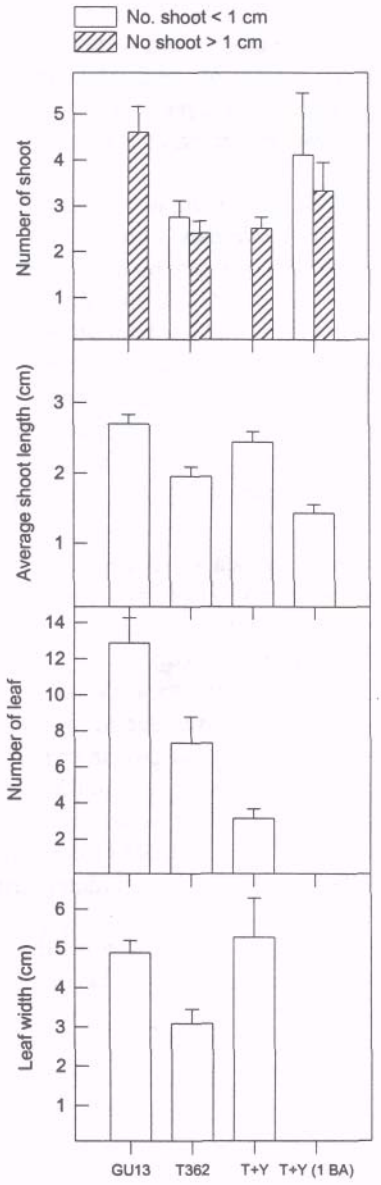


Figure 1. Growth responses of proliferating shot cultures from four avocado lines *in vitro* after 2 months. The growth medium for 'Gu13', 'T362' and 'T+Y'(1 BA) consisted of modified MS salts in which the nitrogen was supplied in the form of 3 NO_3^- : NH_4^+ at 40 mM, supplemented with 1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, 1 mg l^{-1} BA solidified with 7 g l^{-1} TC agar. For 'T+Y', the concentration of BA in the medium was reduced to 0.1 mg l^{-1}. The explants were nodal stem segments ca. 1–1.5 cm from previous cultures.